# Learning and behavioral deficits associated with the absence of the fragile X mental retardation protein: what a fly and mouse model can teach us

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The Fragile X syndrome (FXS) is the most frequent form of inherited mental disability and is considered a monogenic cause of autism spectrum disorder. FXS is caused by a triplet expansion that inhibits the expression of the *FMRI* gene. The gene product, the Fragile X Mental Retardation Protein (FMRP), regulates mRNA metabolism in brain and nonneuronal cells. During brain development, FMRP controls the expression of key molecules involved in receptor signaling, cytoskeleton remodeling, protein synthesis and, ultimately, spine morphology. Symptoms associated with FXS include neurodevelopmental delay, cognitive impairment, anxiety, hyperactivity, and autistic-like behavior. Twenty years ago the first *Fmr1* KO mouse to study FXS was generated, and several years later other key models including the mutant *Drosophila melanogaster*, *dFmr1*, have further helped the understanding of the cellular and molecular causes behind this complex syndrome. Here, we review to which extent these biological models are affected by the absence of FMRP, pointing out the similarities with the observed human dysfunction. Additionally, we discuss several potential treatments under study in animal models that are able to partially revert some of the FXS abnormalities.

## The Fragile X syndrome: clinical features

Complex cognitive brain functions are affected in patients with learning and intellectual disabilities (IDs), adult onset dementias such as Alzheimer disease, aging-dependent dementias and the various amnesias. A large body of evidence suggests that local protein synthesis regulates memory formation and cognition, two processes that rely on activity-dependent synaptic plasticity (Kang and Schuman 1996; Antion et al. 2008; Kelleher and Bear 2008; Wang et al. 2009; Ho et al. 2011; Redondo and Morris 2011; Jung et al. 2012). Dysregulation of such mechanisms contribute to spine dysmorphogenesis and a variety of other neuropathological conditions (Bagni and Greenough 2005; Sala and Segal 2014). Synaptic inputs dictate the time, place, and amount of protein synthesis necessary for individual synapses: events often impaired in individuals with IDs.

Fragile X syndrome (FXS) is the most frequent monogenic cause of inheritable mental disability (Bagni and Greenough 2005). The syndrome's name refers to a cytogenetic marker on the X chromosome at Xq27.3, a "fragile site" in which the chromatin fails to condense properly during mitosis (Hecht and Sutherland 1985). The brittle point, denoted as FRAXA (FRAgile site, X chromosome, A site), is evident in ∼50% of the metaphase chromosomes of virtually all clinically affected humans (Fu et al. 1991; Bagni et al. 2012).

FXS has a greater incidence in males (one in 4000 males and one in 6000 females; Mandel and Biancalana 2004; Bagni et al. 2012), because the X chromosome with the fragile X site is more often inactivated compared with the nonaffected X. FXS leads to a spectrum of physical, intellectual, emotional, and behavioral characteristics ranging from mild to severe. Symptoms include, among others, developmental and behavioral deficits, attention deficits, autistic behaviors, aggression, anxiety and hyperactivity, sleep disorders, epileptic seizures, hypersensitivity to sensory stimulation including noise and touch, and deficits in social-personal skills. Physical features displayed by affected individuals include flat feet, flexible joints, and low muscle tone, large forehead or ears with a prominent jaw, long face, soft skin, strabismus, and enlarged testes (Berry-Kravis 2014; Jacquemont et al. 2014). Boys with FXS are usually diagnosed within the first 3 yr of life, when they experience delays in speech development and social interactions. Hypotonia, hand flapping, poor eye contact, and autistic-like behavior can also be observed at this age (Cordeiro et al. 2011; Bagni et al. 2012). As girls have fewer symptoms, they tend to be diagnosed much later, often in their early teens, when a high percentage exhibit ovarian failure (Sherman 2000). The ID which is the hallmark of this condition is milder or absent in females.

Recently exome sequencing of samples from patients diagnosed with schizophrenia (SCZ) (Fromer et al. 2014; Purcell et al. 2014) or autism (AD) (Iossifov et al. 2012; Waltes et al. 2014) identified several mutations in genes targeted by FMRP (for review, see Fernandez et al. 2013). In addition, proteins interacting with FMRP such as the cytoplasmic interactor CYFIP1 were

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shown to be associated with AD (Waltes et al. 2014) and SCZ (Fromer et al. 2014; Purcell et al. 2014). Furthermore, our laboratory has recently identified (De Rubeis et al. 2013) several proteins encoded by genes involved in SCZ, ASD, and ID that are associated with CYFIP1 in the mouse model. This strongly suggests that FMRP expression needs to be tightly controlled, because any alteration of the FMRP pathway might lead to multiple dysregulated processes that probably underlie these conditions and syndromes.

## From genetic causes to model systems for FXS

In most cases FXS is the result of loss or functional silencing of the *FMR1* gene due to methylation of the CGG triplet expansion in the 5′ untranslated region of the gene (Pieretti et al. 1991; Verheij et al. 1993). Additional, but less frequent, cases have been reported with point mutations in the *FMR1* gene (De Boulle et al. 1993; Lugenbeel et al. 1995; Wang et al. 1997) leading to classic features of FXS. The gene encodes for the Fragile Mental Retardation protein (FMRP), an RNA-binding protein with four RNA-binding domains affecting different aspects of mRNA metabolism including transport, stability, and translation (Bagni et al. 2012; Doyle and Kiebler 2012; Wang et al. 2012; Darnell and Klann 2013; Hornberg and Holt 2013; Rajan 2014). The absence of FMRP leads to a deregulated protein translation resulting in excessive accumulation of certain proteins and reduction of others (for review, see Bagni et al. 2012). Furthermore, FMRP appears to be involved in developmental decisions at the level of neurite extension, guidance, and branching (for review, see Doll and Broadie 2014). Golgi studies of human postmortem brain from patients with FXS, as well as from the *Fmr1* KO mouse brains, revealed that the absence of FMRP leads to longer and thinner dendritic spines compared with normal. These "filopodia-like shape" resemble immature spines (for review, see Bagni and Greenough 2005). Notably, dysgenesis of dendritic spines is a feature shared by several ID disorders (for review, see Fiala et al. 2002; Penzes et al. 2011; De Rubeis et al. 2012).

In the *Fmr1* KO mice, the absence of FMRP leads to an increased long-term depression (LTD; Huber et al. 2002), dependent on the metabotropic glutamate receptor 5 (mGluR5), which is protein synthesis independent (Nosyreva and Huber 2006). Based on these findings, Bear et al. proposed the "mGluR theory of FXS" (Bear et al. 2004), postulating that alterations of the mGluR pathway-dependent plasticity contribute to FXS pathophysiology. Accordingly, a broad range of affected phenotypes observed in animal models to study FXS have been rescued by pharmacological and genetic reduction of mGluR5 activity as well as by targeting downstream signaling pathways (discussed below and for review, see Krueger and Bear 2011; Bagni et al. 2012). However, in addition to the mGluR5 signaling, other receptors and key molecules have been implicated in the development and manifestation of this disability, such as the ionotropic glutamate receptors (NMDA and AMPA), GABAergic receptors (GABA A and B), and muscarinic acetylcholine receptors (for review, see Sethna et al. 2013). The majority of the drugs developed to correct the behavioral phenotypes observed in patients with
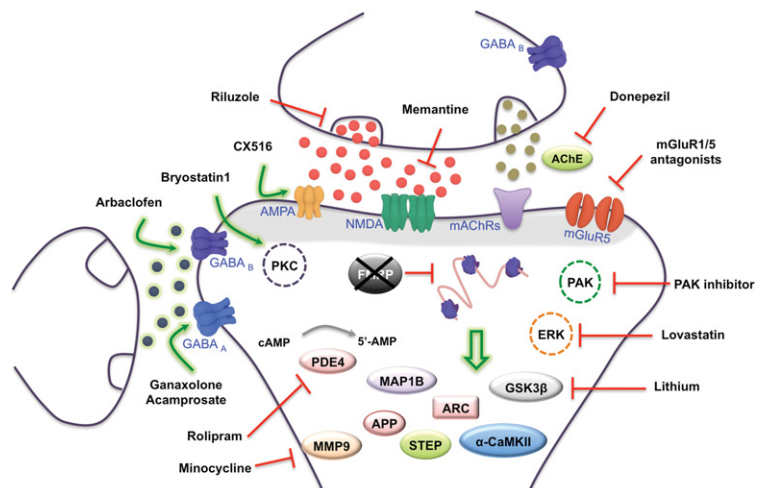


**Figure 1.** Molecular signaling altered in Fragile X syndrome. In the absence of FMRP protein translation is enhanced and several proteins encoded by FMRP targets such as APP, STEP, MAP1B, ARC, CaMKIIα, and others are up-regulated. In addition, many receptors are deregulated in FXS. Therefore, to normalize the observed behavioral phenotypes, several drugs have been used—in fly, animal models, and affected patients—to correct or revert some of the abnormalities. Among them: mGluR5 antagonists (MPEP, Fenobam, AFQ056, STX107, Acamprosate, RO491752, CTEP, LY341495, MPPG, and MTPG), agonists of the GABAergic pathway (Asbaclofen, Ganaxolene, and Acamprosate) and AMPAR signaling (CX516), antagonist of the cholinergic pathway (Donepezil), among others. Studies using fly and animal models have shown that targeting specific molecules/pathways corrected some defects and this information may be used to develop nontoxic drugs (denoted by the dashed line), such as ERK, PAK, and PKC inhibitors.

FXS target these receptors or downstream effectors as shown in Figure 1.

The ideal animal model to study FXS, or any other human disease, should re-create most of the complex deficits observed in the human condition, including cellular, physiological, and behavioral alterations. Intraspecies characteristics and differences in genetic background contribute to genetic disease variability and therefore make the generation of a biological model a difficult task; nevertheless, following the discovery of *FMR1* gene in 1991 (Pieretti et al. 1991; Verkerk et al. 1991) the first animal model to study FXS was generated. In this case, a Neo cassette was inserted into exon 5 of the *Fmr1* coding region of the mouse ortholog (Bakker 1994). This biological model can partially mimic human FXS pathology inasmuch as it leads to several phenotypes similar to the ones observed in patients. The use of this mouse model has enormously advanced the FXS field. The subsequent capitalizing of the power and advanced genetics of *Drosophila melanogaster*, a fruit fly model for FXS, further contributed to the understanding of FXS (Zhang et al. 2001; Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002; Lee et al. 2003; Xu et al. 2004).

In this review, we discuss some of the most relevant studies that enhanced the understanding of the behavioral deficits observed in patients with FXS, using the *Fmr1* KO mouse and the *dFmr1* mutant fruit fly as a model system. Ultimately, we briefly introduce some strategies aiming at rescuing the FXS phenotypes that hopefully will open new routes into more direct disease-targeted therapies.

## The Mouse model for FXS

### *Fmr1* KO mice show anxiety-like behavioral phenotype

Because FXS patients are often described as hyperactive and under hyperarousal (Berry-Kravis 2014), the *Fmr1* KO mice have been evaluated in anxiety-like and exploratory tasks. In the "light–

dark compartment" assay, shown to be sensitive to anxiolytic drugs (Rogoz and Skuza 2011), the choice between the interest to explore novel environments and the aversion to brightly lit compartments is investigated. In this test, the *Fmr1* KO mice were shown to present lower anxiety-like behaviors (Peier et al. 2000; Veeraragavan et al. 2012), as observed by a decreased transition between the light/dark compartments. The "open-field" test is commonly used to observe general exploratory activity. In this case, the time spent in the center, the numbers of crossings, and velocity are all used to monitor activity, which was found to be increased in *Fmr1* KO mice compared with control animals. Because *Fmr1* KO mice are hyperactive (Peier et al. 2000; Chen and Toth 2001; Yan et al. 2004; Restivo et al. 2005; Olmos-Serrano et al. 2011), they are usually reported as less anxious in the open-field arena. We should keep in mind that anxiety measured in this assay is highly influenced by locomotor activity (Peier et al. 2000; Spencer et al. 2005). However, an increase in anxiety-like behaviors (Restivo et al. 2005) in the open-field has also been reported for the *Fmr1* KO mice. The "elevated-plus maze (EPM)" is usually the preferred test to investigate anxiety-like phenotypes comparing the time spent in open or closed arms. Similarly to the above-mentioned discrepancies, the *Fmr1* KO showed both a decrease (Yuskaitis et al. 2010) and an increase (Bilousova et al. 2009) in anxiety during the EPM task when compared with control animals. Considering that FXS patients show anxiety when faced with certain social situations, it is crucial to dissociate social and nonsocial anxiety in the mouse model of FXS (Liu and Smith 2009). Further support towards increased anxiety of the *Fmr1* KO mice came from the "mirrored test chamber" where a reflection of the mouse image is interpreted by the test mouse as another animal. In this context, the *Fmr1* KO mice exhibited increased anxiety-like behaviors, measured by their greater avoidance of the central mirrored chamber (Spencer et al. 2005). Importantly, the reintroduction of human FMRP in the *Fmr1* KO background alleviates deficits in anxiety and exploration (Peier et al. 2000), highlighting the role of FMRP for normal behavior.

Although the *Fmr1* KO mouse model was fundamental for the identification of the cellular and molecular pathways affected in FXS, we believe that the study of the anxiety-like behaviors resulted in more difficult and sometimes with opposite outcomes possibly because in rodents anxiety is difficult to interpret due to multiple factors that interfere with the experimental settings.

## *Fmr1* KO mice show cognitive impairments

In addition to the anxiety aspect, the *Fmr1* KO mouse has been used to model the intellectual disability of patients with FXS (Berry-Kravis 2014) with variable results, possibly due to differences in protocols, environment, and genetic background (see Table 1).

### Spatial learning

The Morris water maze (MWM) task is commonly used to evaluate hippocampal-dependent spatial learning in rodents. The latency to find an escape platform from a pool of opaque water decreases with the number of training trials. This test, initially developed by Richard Morris (Morris et al. 1982), is used as a readout for learning. *Fmr1* KO mice do not show differences in latency compared with controls (Bakker 1994; Kooy et al. 1996; Paradee et al. 1999; Peier et al. 2000; Yan et al. 2004), suggesting that spatial learning is not affected by the absence of FMRP. Memory consolidation and retrieval are not affected either; both *Fmr1* KO and WT mice spent more time in the target quadrant (Kooy et al. 1996; Paradee et al. 1999; Yan et al. 2004), indicating that both groups learned and remembered the initial position of the escape plat-

form. To further understand the role of FMRP in spatial learning and memory, the performance of *Fmr1* KO mice was also analyzed in other mazes. In the Barnes maze (BM), rodents are trained to find an escape hole based on distal cues, and contrary to what has been found in the MWM, in the BM the *Fmr1* KO mice show significant differences in retrieval and memory consolidation compared with controls (Yan et al. 2004). In addition, recent evidence (Guo et al. 2012) indicated that *Fmr1* KO mice, when studied in the radial arm maze apparatus, perform worse compared with wild-type littermates, as measured by the decreased ability of *Fmr1* KO mice to preserve spatial information after food reward.

### Associative learning

In the "passive avoidance" task, animals learn to avoid a location (dark compartment) in which they had previously received an unpleasant stimulus (for example as a footshock). While control mice take more time or even refuse to enter the dark compartment, because they associate it with the shock of their paws, the *Fmr1* KO mice show a range of behavioral responses. It has been reported that the *Fmr1* KO mice showed a deficit in associative learning (Yuskaitis et al. 2010; Michalon et al. 2012), but also normal performance (Bakker 1994; Dolen et al. 2007; Veeraragavan et al. 2012; Udagawa et al. 2013). However, memory extinction was exaggerated in the *Fmr1* KO mice (Dolen et al. 2007; Michalon et al. 2012), as observed by shorter latencies to enter the dark compartment. In the "five-choice serial reaction time" task, an assay to measure visuospatial attention and impulsivity, mice are required to detect a random light in one of five holes and to respond with a nose-poke in the correct spatial location to receive a food reward. *Fmr1* KO mice needed significantly more time to complete the task, as observed by the increased number of errors per trial during the training period (Krueger et al. 2011); however, they were able to complete the task. Additionally, mutant mice performed normally in the "olfactory discrimination task" (Mineur et al. 2002; Yan et al. 2004; Moon et al. 2008; Guo et al. 2011), suggesting that working memory (which correlates with IQ in humans) is not affected.

Associative learning dependent on hippocampus and amygdala (Phillips and LeDoux 1992) is usually monitored via "cued and contextual fear conditioning." In this task the conditioned and nonaversive stimulus occurs in association with a harmful unconditioned stimulus. As a result of this pairing, the conditioned stimulus acquires the aversive properties of the unconditioned stimulus, inducing freezing as a readout of a defensive behavior (Curzon et al. 2009). When *Fmr1* KO mice were tested in both cued (tone) and contextual (environment) fear conditioning, they were found to freeze less than the control littermates indicating memory deficits (Paradee et al. 1999; Hayashi et al. 2007; Guo et al. 2011, 2012; Olmos-Serrano et al. 2011). However, other groups using this test did not report memory deficits in the *Fmr1* KO compared with control (Peier et al. 2000; Van Dam et al. 2000). The diverse outcome could stem from differences in the protocols used and/or to the influence of the strain used. Interestingly, one group reported a decreased freezing in cued fear conditioning, when the same tone is presented in an altered environment (different context) from the initial chamber where the training took place (Olmos-Serrano et al. 2011), suggesting that the amygdala-dependent learning is mostly impaired. These findings are consistent with an intact hippocampal function/s in the KO mouse, consistent with the electrophysiological results indicating normal long-term potentiation (LTP) of hippocampal Schaffer Collateral fibers after tetanic (Paradee et al. 1999) or high frequency stimulation (Godfraind et al. 1996).

**Table 1.** Behavioral abnormalities observed in the Fmr1 KO mice

| Behavior phenotypes | Fmr1 KO genetic background | Behavioral task | Observed phenotype | Parameter used | References |
|---|---|---|---|---|---|
| Anxiety | FVB/129; C57BL/6 | Open-field | Decreased | Ratio time spent center/periphery | Peier et al. (2000); Chen and Toth (2001); Liu et al. (2011); Westmark et al. (2011); Ronesi et al. (2012); Udagawa et al. (2013) |
| | C57BL/6 | Mirrored chamber | Increased | Crosses center/periphery | Restivo et al. (2005) |
| | | Elevated-plus maze | Increased | Avoidance of central chamber | Spencer et al. (2005) |
| | | | Decreased | Time spent in open arms | Yuskaitis et al. (2010) |
| | FVB | | Increased | Ratio time spent in open/closed arms | Bilousova et al. (2009) |
| Hyperactivity | C57BL/6 | Light–dark box | Decreased | Transitions light/dark | Peier et al. (2000); Veeraragavan et al. (2012) |
| | Hybrid FVB/NJ C57BL/6 | Elevated-plus maze | Normal | Ratio time spent in open/closed arms | Yan et al. (2004) |
| | Mouse C57BL/6j; Mouse FVB | Open field | Hyperactivity | Distance traveled; number of crosses; velocity | Peier et al. (2000); Restivo et al. (2005); Min et al. (2009); Yuskaitis et al. (2010); Liu et al. (2011); Olmos-Serrano et al. (2011); Michalon et al. (2012); Dolan, et al. (2013) |
| **Learning and memory** | | | | | |
| Associative learning | C57BL/6 | Passive avoidance | Normal | Latency to enter dark compartment | The Dutch–Belgian Fragile X Consortium (1994); Dolen et al. (2007) |
| | | | Decreased | | Yuskaitis et al. (2010); Michalon et al. (2012); Veeraragavan et al. (2012); Udagawa et al. (2013) |
| | C57BL/6j; FVB | Context and cued fear | Decreased | Freezing | Paradee et al. (1999); Hayashi et al. (2007); Peier et al. (2000); Van Dam et al. (2000); Olmos-Serrano et al. (2011); Guo et al. (2011, 2012) |
| | C57BL/6j | | Normal | | |
| Spatial learning | C57BL/6j; FVB/NJ C57BL/6j hybrid | Morris water maze | Normal | Latency to platform; time on quadrant | The Dutch–Belgian Fragile X Consortium (1994); Kooy et al. (1996); Paradee et al. (1999); Peier et al. (2000); Yan et al. (2004) |
| | Hybrid FVB/NJ C57BL/6j | Barnes maze | Decreased | Time on the target hole | Yan et al. (2004) |
| Working memory | C57BL/6; FVB | Novel task discrimination | Decreased | Time spent in proximity of the object | Ventura et al. (2004); Bhattacharya et al. (2012); Busquets-Garcia et al. (2013) |
| | FVB; C57BL/6j hybrid | Olfactory discrimination | Normal | | Yan et al. (2004) |
| | | Radial maze | | | Yan et al. (2004); Mineur et al. (2002); Moon et al. (2008); Guo et al. (2011) |
| | C57BL/6 | Y/T-maze | Decreased | Number of errors | Yan et al. (2004); Guo et al. (2012) |
| | C57BL/6; FVB | Reverse MWM | Normal | Alternation | Bilousova et al. (2009); Udagawa et al. (2013) |
| Cognitive inflexibility | C57BL/6; FVB | Reverse MWM and Y-maze | Decreased | Time in target quadrant; Latency to platform | Paradee et al. (1999); Yan et al. (2004); The Dutch–Belgian Fragile X Consortium (1994); Kooy et al. (1996); D'Hooge et al. (1997); Bhattacharya et al. (2012); Krueger et al. (2011) |
| | | Five-choice serial reaction time task | Decreased | Number of errors | |
| Social preference and novelty | C57BL/6 | Social preference | Normal | Time spent with Stranger 1 | Mines et al. (2010); Bhattacharya et al. (2012) |
| | | Social novelty/interaction | Decreased | Time spent with Stranger 2 | Mines et al. (2010); Bhattacharya et al. (2012) |
| Sensory gating | FVB | | Increased inhibition | | Chen and Toth (2001); Nielsen et al. (2002); Frankland et al. (2004); de Vrij et al. (2008); Baker et al. (2010); Olmos-Serrano et al. (2011); Veeraragavan et al. (2012) |
| | FVB/NJ C57BL/6j hybrid | Prepulse inhibition of acoustic startle | Normal | Body flinches | Peier et al. (2000); Yan et al. (2004); Spencer et al. (2006); Thomas et al. (2012); Renoux et al. (2014) |
| | | | Decreased inhibition | | |
| Seizures susceptibility | FVB/NJ; C57BJ/6j; FVB/NJ C57BL/6j hybrid | Audiogenic seizures | Increased | Seizures-like behavior | The Dutch–Belgian Fragile X Consortium (1994); Musumeci et al. (2000); Peier et al. (2000); Chen and Toth (2001); Yan et al. (2004, 2005); Min et al. (2009); Pacey et al. (2009); Osterweil et al. (2010); Liu et al. (2011); Westmark et al. (2011); Goebel-Goody et al. (2012); Michalon et al. (2012); Ronesi et al. (2012); Thomas et al. (2012); Veeraragavan et al. (2012); Busquets-Garcia et al. (2013); Dolan et al. (2013); Osterweil et al. (2013); Udagawa et al. (2013) |
| Communication deficits | FVB/129 FVB | Ultrasonic vocalization | Decreased | Specific features of vocalizations | Rotschafer et al. (2012) |
| | | | Increased | Number of calls of pups | Lai et al. (2014) |
| Circadian rhythms | C57BJ/6j | Locomotor activity | Increased | Ambulatory counts | Baker et al. (2010) |
| | C57BJ/6j | | Decreased | Wheel running | Zhang et al. (2008) |

Finally, the *Fmr1* KO mice were studied to explore a possible behavioral inflexibility, a feature often associated to patients with ASD (Geurts et al. 2009). The test allows the monitoring of a performance of "reverse learning" after a task has been acquired. This behavior requires intact cortical function (Dalley et al. 2004). The *Fmr1* KO mice, tested using the reverse MWM or reverse T/Y-maze, needed more time to learn the new position of the platform (Bakker 1994; Kooy et al. 1996; D'Hooge et al. 1997; Bhattacharya et al. 2012), suggesting that acquisition of the new spatial information is impaired in the absence of FMRP (Krueger et al. 2011). Other studies did not reach a similar conclusion (Paradee et al. 1999; Yan et al. 2004). Of note, cellular and molecular findings support a role of FMRP in the prefrontal cortex whose intact function/s is/are required for cognitive flexibility. Indeed, the expression of synaptic proteins, such as glutamate receptors, PSD95 and Arc is reduced in the prefrontal cortex of adult *Fmr1* KO (Krueger et al. 2011) in accordance with the increased density of longer (and possibly immature) spines (Liu et al. 2011). Finally, the *Fmr1* KO mice have a decreased neuronal activity in the prefrontal cortex, as observed by a reduction of c-fos positive neurons (Krueger et al. 2011).

## *Fmr1* KO mice show impaired social interaction and communication

Patients with FXS do not show major deficits in social interactions, although they show high levels of anxiety (for review, see Gross et al. 2012). However, problems with social communication are observed in patients with FXS and autistic features (Berry-Kravis 2014). In rodents, a test that mimics social preference and novelty in humans is the "three-chambered apparatus," in which the target mouse is given the choice between exploring a cage containing a stranger mouse or an empty one. In the second phase, the test mouse can choose between the familiar mouse and a new (i.e., a second) stranger mouse. The numbers of approaches and the time spent in proximity with each mouse are scored (Moy et al. 2004). While the *Fmr1* KO mice do not show any deficit in social preference (Mines et al. 2010; Bhattacharya et al. 2012), they are impaired in social novelty discrimination, because they do not show any preference for the unfamiliar stranger mouse (Mines et al. 2010; Bhattacharya et al. 2012). In addition, *Fmr1* KO males display reduced social interaction with novel females (Mineur et al. 2006) as well as impaired social dominance with unfamiliar mice (Spencer et al. 2005). Such deficits may be explained by the enhanced anxiety aspect, as seen by increased rearing and digging behavior the *Fmr1* KO mice exhibited in the presence of another mouse (McNaughton et al. 2008; Mines et al. 2010; Liu et al. 2011). Impaired preference for unfamiliar mice may indicate lack of interest in novelty and/or impairment to discriminate between familiar and novel mice. Indeed, when tested in the "novel object recognition" task, the *Fmr1* KO mice fail to recognize the novel object (Ventura et al. 2004; Bhattacharya et al. 2012; Busquets-Garcia et al. 2013). These findings suggest that discrimination deficits are not only observed in social tasks.

Because problems in communication and speech are often reported in patients with FXS (for review, see Bagni et al. 2012; Hagerman et al. 2012), communication in the *Fmr1* KO mice was also investigated by evaluating the pattern of "ultrasonic vocalizations (USV)." Pairing *Fmr1* KO male with a female showed deficits in communication as observed by the reduced number of calls per second (Rotschafer et al. 2012), although no apparent difference in mating behavior was observed. After maternal separation, the type and duration of USV emitted by the *Fmr1* KO pups (8 d old pups) were different from the control littermates with no apparent changes in the total number of calls (Roy et al. 2012). Recently, Lai et al. reported an increased number of USVs in youn-

ger (P7) *Fmr1* KO pups (Lai et al. 2014) suggesting that the FMRP function on communication is age-dependent.

## *Fmr1* KO mice show deficits in sensory gating and audiogenic seizures

Abnormal sensory inhibition may reflect a deficit in processing and prioritizing incoming information, a feature of schizophrenic patients (Braff et al. 1978; Siegel et al. 1984; Brockhaus-Dumke et al. 2008; Hammer et al. 2013; Rihs et al. 2013). Treatments with antipsychotic drugs improve those deficits in rats and humans (Curzon et al. 1994; Sanchez-Morla et al. 2009; Suryavanshi et al. 2014). It is known that patients with FXS are hyperarousal in situations of excessive stimulation and habituate poorly to sensory stimuli (Berry-Kravis 2014). "Prepulse inhibition (PPI)" is used to evaluate the ability of human and rodents to filter irrelevant information in their surroundings. Therefore, *Fmr1* KO mice and control littermates were tested in PPI in an acoustic startle task to investigate possible sensory gating deficits. In this task a weak stimulus, such as a tone, inhibits the subsequent response to a stronger, louder stimulus, if presented within 100 msec (Nielsen et al. 2002). Presently, how the *Fmr1* KO mouse processes incoming information is still unclear, because either no differences (Peier et al. 2000; Yan et al. 2004; Spencer et al. 2006; Thomas et al. 2012) or enhanced startle responses were shown (Chen and Toth 2001; Nielsen et al. 2002; Frankland et al. 2004; de Vrij et al. 2008; Baker et al. 2010; Olmos-Serrano et al. 2011; Veeraragavan et al. 2012) compared with control animals. However, in one study (Frankland et al. 2004), *Fmr1* KO mice showed an excessive reaction to auditory stimuli, similar to humans (Renoux et al. 2014). Despite the reported discrepancies, it is clear that FMRP plays a role in sensorimotor function/s underlying the altered sensitivity to sensory stimulation observed in both patients and mouse models.

Susceptibility to audiogenic seizures is one of the most robust features of FXS and indeed ~20% of patients with FXS experience epileptic episodes (Musumeci et al. 1999; Berry-Kravis 2002). In accordance with the human data, *Fmr1* KO mice display enhanced susceptibility to audiogenic seizures (Bakker 1994; Musumeci et al. 2000; Peier et al. 2000; Chen and Toth 2001; Yan et al. 2004, 2005; Min et al. 2009; Liu et al. 2011; Goebel-Goody et al. 2012; Thomas et al. 2012; Veeraragavan et al. 2012; Osterweil et al. 2013; Udagawa et al. 2013). Although the severity of the phenotype may be affected by the genetic background (Table 1; Yan et al. 2004), susceptibility to audiogenic seizures is the most consistent behavioral phenotype and is usually implemented to screen for potential drugs to ameliorate FXS.

Sleep problems are a common feature of patients with FXS (Berry-Kravis 2014), and in the mouse model FMRP has been suggested to regulate circadian rhythmicity as measured by locomotor analysis. In complete darkness, FMRP was found to regulate the length of circadian period (Zhang et al. 2008) since shorter activity periods of wheel running were observed in the *Fmr1* KO mice compared with controls. Interestingly, FMRP affects circadian rhythmicity differently in females and males since ambulatory activity during the light phase was enhanced only in the *Fmr1* KO females (Baker et al. 2010) and no changes were reported in males.

# The fly model for FXS

In contrast to the two *FMR1*-related genes found in the mammalian genome (i.e., *FXR1P* and *FXR2P*), in *Drosophila* there is only one *FMR1* homolog referred to as *dFmr1*, or more rarely as *dfxr*. Sequence comparison of the human and fly genes shows a high level of similarity between the functional regions of the proteins, with a total 56% similarity and 35% identity (Zhang et al. 2001;

Gao 2002). The protein encoded by the *dFmr1* (dFMRP) presents an analogous structure and shows similar RNA-binding properties to mammalian FMRP (Wan et al. 2000). Immunohistochemical analysis showed that dFMRP is continuously expressed during early embryogenesis, with strong expression in the mesoderm, the lobes of the brain and the abdominal (ventral) ganglia in larval and adult stages (Wan et al. 2000; Zhang et al. 2001; Dockendorff et al. 2002). Additional tissues where dFMRP is detected are the embryonic wing discs, testes, and ovaries (Zhang et al. 2001; Zarnescu et al. 2005). Like the homologous protein in mammals, dFMRP is highly expressed in the cytoplasm of neurons; low levels have been detected in glia cells (Wan et al. 2000; Zhang et al. 2001; Morales et al. 2002). To characterize the physiological functions of dFMRP, several loss-of-functions mutations have been generated in the *dFmr1* gene (Zhang et al. 2001; Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002; Lee et al. 2003).

Lack of a functional *dFmr1* gene results in many phenotypes reminiscent of those observed in the human condition, thus *Drosophila* is an appropriate and simple genetic model to study molecular, cellular, and behavioral features associated with the syndrome. At the cellular level, loss of *dFmr1* results in neuron overgrowth, over-branching, and abnormalities in synapse formation, both at the peripheral nervous system (PNS) and the central nervous system (CNS), including the relatively mild defect of aberrant midline crossing of the Mushroom bodies (MB) β lobes (Morales et al. 2002; Michel et al. 2004; Pan et al. 2004; McBride et al. 2005).

### dFmr1 mutant flies have abnormal circadian rhythms

As mentioned above, patients with FXS have sleep problems reflecting abnormal circadian behavior (Hagerman et al. 1996; Gould et al. 2000; Berry-Kravis 2014). "Circadian rhythm" studies in *dFmr1* mutant flies, using the Trikinetics system (http://www .trikinetics.com/), revealed normal circadian rhythms but interrupted sleep and arrhythmic locomotor activity under constant darkness (Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002; Sekine et al. 2008). These behavioral defects are not due to motor dysfunctions because *dFmr1* mutant flies did not show differences in the total activity in constant darkness when compared with the control (Dockendorff et al. 2002). These results are in agreement with the clinical observations that patients with FXS have attention deficit hyperactivity disorder (ADHD) (Hagerman 1997; Torrioli et al. 2008). Analysis of the circadian molecular clock revealed that cycling of the two key elements of the circadian clock PER and TIM proteins and their respective mRNA levels are not affected in *dFmr1* mutants (Dockendorff et al. 2002; Inoue et al. 2002; Morales et al. 2002; Helfrich-Forster 2005; Chang 2006; Sehgal et al. 2007). However, the eclosion rhythm, which is controlled by the circadian system, is affected in *dFmr1* mutant flies and the circadian oscillation of cAMP response element binding protein (CREB), a known output of the circadian clock in *Drosophila* (Belvin et al. 1999), is dramatically affected in *dFmr1* mutant flies (Dockendorff et al. 2002; Morales et al. 2002). These data suggest that *dFmr1* mutants present an abnormal circadian output pathway. It is well established that a molecular clock mechanism in the small ventro-lateral neurons (sLNs) of the brain controls the rest–activity rhythms (Helfrich-Forster 2005; Chang 2006; Nitabach et al. 2006). Notably, several laboratories have observed overextended and overgrowth axons in the sLNs (Dockendorff et al. 2002; Morales et al. 2002; Reeve et al. 2005; Gatto and Broadie 2008; Sekine et al. 2008; Gatto and Broadie 2009). These findings suggest that the axonal defects in sLNs might underlie the observed deficits in circadian behaviors. Interestingly, Gatto and Broadie found that in the sLNs dFMRP has a very restricted function in late brain develop-

ment at complete synaptogenesis. Genetic expression of dFMRP in the *dFmr1* mutant background restored the synaptic defects observed in sLNs only when expressed at a late brain developmental stage and failed to do so at early developmental stages or in the adult (Gatto and Broadie 2009). Additional evidence of dFMRP function in the regulation of circadian rhythm derives from its interaction with the RNA-binding protein, LARK. LARK is a known clock output factor, and when overexpressed causes arrhythmic locomotor activity. Accordingly, the double heterozygous mutant for *dFmr1* and *Lark* revealed improvements in these defects (Sofola et al. 2008). FMRP and its cytoplasmic interactor CYFIP1, also known as specific RAC-1 activated protein (SRA-1), (Kobayashi et al. 1998; Schenck et al. 2003) control in an activity-dependent manner actin remodeling and local protein synthesis (Napoli et al. 2008; Galy et al. 2011; De Rubeis et al. 2013; Zhao et al. 2013). These findings suggest that the disorganization of the FMRP-CYFIP complex may result in the abnormal synaptic arborization and the defective sLNs observed in d*Frm1* mutant flies. In addition to the arrhythmic activity, a prolonged "sleep phase" has been observed in *dFmr1* mutant flies. The MBs, functionally analogous to the mammalian hippocampus, are essential for different types of behavior, such as sleep, associative learning, and memory (Davis 1993; Roman and Davis 2001; Joiner et al. 2006). The MBs contain the neuronal population required to regulate normal sleep activity; overexpression of *dFmr1* in the MBs reduced the prolonged sleep episodes of mutant flies (Bushey et al. 2009). In another recent study, *dFmr1* mutants showed a deeper night-like sleep phenotype during the day, which was regulated by two molecules, dFMRP and cAMP (van Alphen et al. 2013).

### dFmr1 mutant flies show impairment in social interaction

The majority of neuropsychiatric and neurodevelopmental disorders entail defects in social behavior. The "courtship paradigm" in the fruit fly is used to probe potential social impairments upon *dFmr1* loss in the fly. Courtship in *Drosophila* is based on visual, auditory and pheromonal cues and is used to assess behavioral interactions between male and female flies (Hall 1994; Greenspan and Ferveur 2000; Dockendorff et al. 2002; Yamamoto and Koganezawa 2013). The courtship repertoire is composed of four basic phases starting with orientation of a male towards a female and culminating with the final step of copulation. *dFmr1* mutant males spend less time in active courtship in comparison with control flies and do not proceed to more advanced stages of courtship such as copulation. Therefore, *dFmr1* mutant flies present shorter courtship duration in comparison with control flies. This behavior is consistent with lack or loss of interest by the mutant flies, a common characteristic of patients with FXS and ADHD (Dockendorff et al. 2002).

FXS patients present an increased rate of cognitive impairments with age (Galvez and Greenough 2005; Larson et al. 2005). Therefore, social interactions such as naïve courtship were studied in aged *dFmr1* mutant flies, but no further differences were reported in comparison to young flies (Choi et al. 2010). Additional studies using *dFmr1* transgenic flies expressing only proteins with mutations in the KH domains (I244N or I307N) revealed reduced naïve courtship activity, suggesting that this effect is likely due to the lack of dFMRP's ability to bind a specific subset of mRNAs (Banerjee et al. 2007). Furthermore, it was shown that *dFmr1* mutant flies exhibit decreased locomotor activity and exploratory performance and reduced conspecific interactions compared with control flies (Bolduc et al. 2010), presenting additional evidence of defects in social interactions. These findings further support the use of *dFmr1* mutant flies to address questions about the affected neuronal circuitry and molecular pathways controlling these behaviors.

### Learning and memory in *dFmr1* mutant flies

Two main behavioral paradigms have been described to investigate learning and memory in the *Drosophila* model of FXS, courtship, and olfactory conditioning (McBride et al. 2005; Bolduc et al. 2008; Coffee et al. 2010, 2012; Kanellopoulos et al. 2012; Gatto et al. 2014). In 2005, using the "courtship-conditioning paradigm," McBride et al. investigated learning in *dFmr1* mutant flies for the first time. In courtship conditioning a naïve male alters his courtship efforts towards any female after experiencing rejection by an unreceptive female: this constitutes a learning paradigm in flies (Hall 1994). *dFmr1* mutant flies were found to have normal learning during the training phase of conditioned courtship but impairments in the immediate recall and short-term memory phase (McBride et al. 2005). Learning and memory were further assessed through the "olfactory classical conditioning paradigm," which couples aversive olfactory stimuli (conditioned stimulus) with electric shock (unconditioned stimulus) (Tully 1984; Tully and Quinn 1985). Using this paradigm, it has been shown that *dFmr1* mutant flies exhibit a robust associative learning deficit in comparison with wild-type flies (McBride et al. 2005; Bolduc et al. 2008; Coffee et al. 2010, 2012; Gatto et al. 2014). Similarly to the mouse model, *dFmr1* homozygous mutants also have deficits in long-term memory. It has been proposed that protein synthesis through the miRNA pathway involving Staufen and AGO1 may be affected in those flies (Bolduc et al. 2008). These findings might explain the long-term memory deficits. In addition, heterozygous loss of function of both *dFmr1* and *cheerio*, an ortholog of filamin A, revealed long-term memory deficits (Bolduc et al. 2010). These data suggest that microRNA patways acting on filamin A mRNA and/or other processes might be responsible for these behavioral phenotypes. Finally, a recent study by Kanellopoulos et al. showed that d*Fmr1* mutant heterozygotes exhibited a robust learning deficit under sensitive conditions of limited US/CS stimulus pairings (Moressis et al. 2009) and verified that under the typically used extended training conditions (Bolduc et al. 2008) these flies appear normal. In this study, using genetic and pharmacological tools, it was found that the effects of increased mGluR levels upon dFMRP reduction are linked to reduced cAMP, which in turn likely results in the associative learning and memory deficits (Kanellopoulos et al. 2012).

The *Drosophila* MBs are the main part of the brain involved in the two learning and memory paradigms, such as conditioned courtship and classical conditioning (Davis 1993; McBride et al. 1999; Pascual and Preat 2001). Several studies revealed that *dFmr1* mutant flies have MBs developmental defects. Specifically, the β-lobe axons of the MBs do not terminate in the midline, leading to the formation of fused lobes (Michel et al. 2004; Pan et al. 2004; McBride et al. 2005; Bolduc et al. 2008; Chang et al. 2008; Coffee et al. 2010, 2012; Gatto et al. 2014). Absence of dFMRP causes over-elaboration and overgrowth of axons of the MB neurons (Pan et al. 2004; Tessier and Broadie 2008) suggesting that the structural abnormalities of the MBs may explain the learning and memory deficits of the *dFmr1* mutant flies.

### Pharmacological and genetic rescue of behavioral deficits observed in the mouse and fly models for FXS

In this section, we review some of the strategies used with the *Fmr1* KO mouse and the fly mutant model to ameliorate the FXS phenotypes (Table 2). Due to the initial discovery, i.e., increased mGluRs signaling in FXS (Huber et al. 2002) many of the therapeutic strategies are based on targeting of key dysregulated molecules of the mGluR pathway and downstream players regulating protein translation (www.clinicaltrial.org).

Over the past 10 years, additional receptor pathways have been shown to be affected in FXS, including the GABAergic, NMDA, TrkB, and cannabinoid systems (for review, see Bagni et al. 2012), and specific molecules have been used to ameliorate those pathways. Furthermore, since FMRP plays a central role as translational repressor (for review, see Bagni et al. 2012; Darnell and Klann 2013), a few other approaches have been developed to tackle the increased protein synthesis.

A hallmark of FXS is the increased susceptibility to seizures, and administration of several drugs targeting the mGluR pathway such as MPEP (Yan et al. 2005; Westmark et al. 2011; Thomas et al. 2012) and Fenobam (Westmark et al. 2011) were able to ameliorate these deficits. Similar effects were observed by targeting specific molecules which are dysregulated in the *Fmr1* KO mouse (for review, see Bagni et al. 2012) with in vivo administration of compounds such as lithium (Min et al. 2009; Liu et al. 2011), GSK-3β inhibitors (Min et al. 2009), mTOR inhibitors (Busquets-Garcia et al. 2013), and MEK1/2-ERK1/2 inhibitors (Osterweil et al. 2010). The long-lasting mGluR5 inhibitor, CTEP, was also shown to correct elevated protein synthesis, hypersensitivity to sensory stimuli and hyperactivity in the *Fmr1* KO mice (Michalon et al. 2012). In addition, the mGluR5 inhibitors showed an effect on learning and memory deficits (Michalon et al. 2012; Vinueza Veloz et al. 2012).

Genetic and pharmacological manipulations reducing the mGluRs activity were able to restore many phenotypes observed in d*Fmr1* mutant flies. Using the naïve courtship and memory recall assays, McBride et al. (2005) showed that dFMRP mutant flies fed with mGlu5R antagonists (MPEP, MPPG, MTPG, and LY341495) exhibit reversed social and learning behaviors. Some of these affected behaviors could only be reestablished if the drug was administered during development (McBride et al. 2005). In addition, administration of MPEP could restore the following characteristics of the *dFmr1* mutant flies: the structural defects observed in the MBs (McBride et al. 2005; Pan et al. 2008), the impaired axon arborization (Pan et al. 2008), the increased embryonic lethality on glutamate enriched food (Chang et al. 2008), the social learning deficits (Tauber et al. 2011), and short- and long-term olfactory memory deficits (Bolduc et al. 2008; Kanellopoulos et al. 2012). In contrast, MPEP failed to restore the circadian deficits of the *dFmr1* mutant flies (McBride et al. 2005). Kanellopoulos et al. demonstrated that the administration of MPEP and/or the PDE inhibitor Rolipram, which increases cAMP levels, restores the olfactory associative learning and memory deficits of *dFmr1* heterozygous mutants. In addition, genetic abrogation of mGluRs in *dFmr1* mutant background or the double-mutant *dFmr1* and *dnc (PDE4)* could also restore cAMP levels and the corresponding behavioral phenotypes.

These improvements using the fly model for FXS are similar to those observed in the mouse model indicating that the behavioral plasticity deficits could be attributed to a large degree to exaggerated responses to glutamate.

Because these findings pointed out the central role of the mGluR and downstream signaling pathway/s, a few compounds targeting the mGluR activity have been used in clinical trials. RO4917523 (Roche Pharmaceuticals) and STX107 (Seaside Therapeutics) are in Phase 2 trials. The outcome of a clinical trial with Fenobam showed an improvement of 20% in the PPI (Berry-Kravis et al. 2009), although the mGluR5 antagonist AFQ056 (Novartis) was very recently discontinued due to the lack of positive outcome measurements (Busquets-Garcia et al. 2014; Gomez-Mancilla et al. 2014).

As previously mentioned, animal and fly models for FXS express certain subunits of the GABA receptors at lower level compared with WT (for review, see D'Hulst and Kooy 2007). This defect suggests an enhancement of excitatory signaling, consistent

**Table 2.** Behavioral abnormalities observed in the mouse and fly models for FXS and restored upon drug treatment

| Ameliorated phenotypes | FXS model | Compound | Cellular target | References |
|---|---|---|---|---|
| Learning and memory | Fly | Puromycin, cycloheximide | Protein synthesis inhibitors | Bolduc et al. (2008) |
| | Fly | MPEP | mGluR5 antagonist | McBride et al. (2005); Bolduc et al. (2008); Tauber et al. (2011); Kanellopoulos et al. (2012) |
| | Mouse | CTEP | Long-lasting mGluR5 inhibitor | Michalon et al. (2012) |
| | Mouse | Taurine | GABA A agonist | El Idrissi et al. (2009) |
| | Fly | Rolipram | PDE4 inhibitor | Kanellopoulos et al. (2012) |
| | Mouse | Lithium, SB216763 | GSK-3 inhibitor | Yuskaitis et al. (2010); Guo et al. (2012) |
| | Mouse | Bryostatin-1 | PKC activator | Sun et al. (2014) |
| | Mouse | Fenobam | mGluR5 antagonist | Vinueza Veloz et al. (2012) |
| Anxiety | Mouse | Minocycline | Metalloproteinase-9 inhibitor | Bilousova et al. (2009) |
| | Mouse | Lithium, SB216763 | GSK-3 inhibitor | Liu et al. (2011); Guo et al. (2012) |
| Susceptibility to seizures | Mouse | MPEP | mGluR5 antagonist | Yan et al. (2005); Westmark et al. (2011); Thomas et al. (2012) |
| | Mouse | Fenobam | mGluR5 antagonist | Vinueza Veloz et al. (2012) |
| | Mouse | CTEP | Long-lasting mGluR5 inhibitor | Michalon et al. (2012) |
| | Mouse | Lithium | GSK-3 inhibitor | Min et al. (2009); Liu et al. (2011) |
| | Mouse | Rimonabant, AM630 | CBR1-CBR2 agonist and antagonists | Busquets-Garcia et al. (2013) |
| | Mouse | Temsirolimus | mTOR inhibitor | Busquets-Garcia et al. (2013) |
| | Mouse | SL327 | MEK1/2-ERK1/2 inhibitors | Osterweil et al. (2010) |
| | Mouse | Rapamycin | mTOR inhibitor | Osterweil et al. (2010) |
| | Mouse | Lovastatin | Reduces Ras-ERK1/2 activation | Osterweil et al. (2013) |
| | Mouse | FRAX486 | PAK inhibitor | Dolan et al. (2013) |
| | Mouse | Baclofen | GABA B agonist | Pacey et al. (2009) |
| Hyperactivity | Mouse | CTEP | Long-lasting mGluR5 inhibitor | Michalon et al. (2012) |
| | Mouse | FRAX486 | PAK inhibitor | Dolan et al. (2013) |
| | Mouse | THIP | GABA A agonist | Olmos-Serrano et al. (2011) |
| Sensory gating | Mouse | THIP | GABA A agonist | Olmos-Serrano et al. (2011) |
| | Mouse | FRAX486 | PAK inhibitor | Dolan et al. (2013) |
| Social interactions/ courtship activity | Fly | GABA | GABA A and B agonist | Chang et al. (2008) |
| | Fly | MPEP | mGluR5 antagonist | McBride et al. (2005) |
| | Fly; Mouse | Lithium | GSK-3 inhibitor | McBride et al. (2005); Mines et al. (2010) |
| Repetitive behaviors | Mouse | FRAX486 | PAK inhibitor | Dolan et al. (2013) |

with the high susceptibility to seizures observed in patients with the syndrome (Hagerman et al. 2012) confirmed in the mouse model. In accordance with these findings, treatment with GABA agonists normalized hyperactivity and sensory gating defects (Pacey et al. 2009; Olmos-Serrano et al. 2011) and significantly improved conditioned learning (El Idrissi et al. 2009) in *Fmr1* KO mice. Similarly, treatment with GABA also restored the courtship defects of mutant flies (Chang et al. 2008). Taking advantage of the fly model, a large screening was performed using 2000 chemical compounds (Chang et al. 2008). This study has led to the identification of a few molecules, implicated in the GABAergic pathway, able to restore the courtship phenotype (Chang et al. 2008). In a more recent study, GABAergic modulation after feeding *dFmr1* mutant flies with GABA or Nipecotic Acid (a GABA reuptake inhibitor) failed to restore the associative learning deficits, suggesting that the GABAergic signaling alterations in the *dFmr1* mutant flies do not appear to underlie their associative learning deficits (Gatto et al. 2014). The alterations in the GABAergic system led to the development of clinical trials with compounds able to restore the GABA receptor signaling. For example, patients with FXS given Arbaclofen (STX209), an agonist of GABA B receptor, showed an improvement in social behavior (Berry-Kravis et al. 2012). Ganaxolone and Acamprosate, agonists of GABA A receptor, are also in clinical trials (for review, see Hagerman et al. 2014).

In addition to targeting the receptors, other strategies have envisioned the use of compounds able to target the dysregulated proteins in the absence of FMRP. For example, GSK-3β activity is increased in *Fmr1* KO mice (Min et al. 2009; Guo et al. 2012), and because lithium acts as a nonspecific inhibitor of GSK-3β (Min et al. 2009; Guo et al. 2012), it has been used in both mouse and fly models for FXS. Lithium administration improved performance in several deficient behavioral tasks in the *Fmr1* KO mouse such as open-field (Min et al. 2009; Yuskaitis et al. 2010; Liu et al. 2011), passive avoidance memory (Yuskaitis et al. 2010), and social preference (Mines et al. 2010). Lithium had also a positive effect on courtship and MBs structural abnormalities in the fly model (McBride et al. 2005). However, some symptoms reappeared when the treatment was discontinued (King et al. 2013). Finally, a specific inhibitor of GSK-3β was shown to normalize hippocampus-dependent learning deficits in *Fmr1* KO mice (Guo et al. 2012), highlighting a role for GSK-3β dysfunction in FXS pathology. While lithium is a common mood stabilizer that seems to improve the anxiety and hyperactivity features of patients with FXS, no additional clinical trials have been envisioned so far (Hagerman et al. 2014).

The intra-signaling cascade regulating protein synthesis, spine shaping, and synaptic plasticity, such as mTOR (Busquets-Garcia et al. 2013), ERK (Osterweil et al. 2010, 2013), and PKC (Weiler et al. 2004), are also impaired in FXS. Consistent with this finding, a potent PKC activator (bryostatin-1) was reported to restore cellular, molecular, and behavioral phenotypes in the *Fmr1* KO mice. In addition, the phosphorylation levels of GSK-3β, affected in FXS, were also restored (Sun et al. 2014).

Finally FMRP links local protein synthesis to actin remodeling (Schenck et al. 2003; De Rubeis et al. 2013) via its cytoplasmic interactor CYFIP1 (Napoli et al. 2008). Additional contacts with the cytoskeleton occur via the adenomatous polyposis coli (APC) tumor suppressor (Mili et al. 2008) and p21-activated kinase (PAK1) (Hayashi et al. 2007). PAK1 has been shown to antagonize the role of FMRP by reducing both spine number and the proportion of longer and thinner dendrites (Hayashi et al. 2004). Consistently, genetic manipulation or drug treatment leading to PAK1 activity reduction (Hayashi et al. 2007; Dolan et al. 2013) correct hyperactivity (Hayashi et al. 2007; Dolan et al. 2013) and repetitive behaviors as well as reduce the frequency of seizures (Dolan et al. 2013) of mutant mice.

En masse, in the absence of FMRP, many molecules that are members of second messenger pathways including mTOR, PKC, ERK1/2, GSK-3β, cAMP, and PAK1 are dysregulated. Because these pathways converge on the translational initiation factor eIF4E, changes in their activity might explain the increased protein synthesis observed in FXS. In support of these findings, the use of protein synthesis inhibitors appears to ameliorate some of the cellular and molecular defects. For example, cycloheximide and puromycin, two inhibitors of general protein synthesis, restored long-term memory deficits of *dFmr1* mutant flies (Bolduc et al. 2008). Additionally, minocycline, thought to exert its antimicrobial effect by the inhibition of protein synthesis (Garrido-Mesa et al. 2013), restores the anxiety-like phenotype (Bilousova et al. 2009) in the *Fmr1* KO mice leading to long-term improvements after drug withdrawal (Dansie et al. 2013). Intracellular minocycline has been shown to inhibit metalloproteinase-9 (MMP-9) involved in synaptic plasticity and dendritic structure (Michaluk et al. 2011; Janusz et al. 2013). Accordingly, MMP-9 activity is enhanced in hippocampi of *Fmr1* KO mice and restored to control levels after minocycline treatment (Bilousova et al. 2009). Treatment of *dFmr1* null animals with minocycline restored the abnormal synaptic morphology observed in three different neuronal areas: the neuromuscular junction (NMJ), the clock neurons, and the Kenyon cells of MBs (Siller and Broadie 2011). Finally, minocycline administration in patients with FXS was shown to improve attention deficits, language use, and communication skills (Paribello et al. 2010; Utari et al. 2010; Leigh et al. 2013).

Besides investigations of drug effects and potential treatments, several groups have explored genetic rescue approaches of FXS deficits. Expression of several key molecules implicated in FXS pathology has been modulated to reestablish some of the phenotypes characterizing FXS.

The increased receptor activity in FXS was first observed upon genetic reduction of mGluR5 expression (Dolen et al. 2007), or modulation of mGluR5-Homer interaction (Ronesi et al. 2012). In both genetic rescues the authors observe a normalization of the enhanced susceptibility to auditory stimuli displayed by *Fmr1* KO mice. Recently, double-mutant *Fmr1* KO mice with reduced expression of endocannabinoid receptor 1 (CBR1) were shown to revert to normalcy with respect to several phenotypic deficits, such as cognitive impairment and audiogenic seizure susceptibility (Busquets-Garcia et al. 2013). Interestingly, the authors of this study show that treatment with inhibitors of the endocannabinoid system is also effective, both acutely and chronically, in normalizing altered spine morphology and several behavioral phenotypes.

Three up-regulated molecules involved in the intracellular signaling and cytoskeleton remodeling, such as amyloid Precursor Protein (APP), striatal enriched protein tyrosine phosphatase (STEP), and microtubule associated Protein 1 (MAP1b), are encoded by known FMRP target mRNAs (for review, see Bagni et al. 2012). The genetic cross of the *APP* KO with the *Fmr1* KO lead to amelioration of cellular and behavioral FXS effects (Westmark et al. 2011). Reduction of STEP (Goebel-Goody et al. 2012), a target of FMRP, corrects some behavioral phenotypes of *Fmr1* KO mice. Furthermore, the doubly mutant *dfmr1* and *futsch* (a *Map1b* homolog and target of FMRP) in flies could rescue the synaptic defects in the neuromuscular junction and in the eye, but failed to rescue the locomotor activity (Zhang et al. 2001).

Finally, the modulation of proteins involved in protein synthesis seems to counteract the absence of FMRP. For instance, one of the RNA-binding proteins colocalizing with FMRP in dendrites is the cytoplasmic polyadenylation element-binding protein 1 (CPEB1) (Ferrari et al. 2007), which regulates polyadenylation and mRNA metabolism. Double *Fmr1* and *CPEB1* KO restored the enhanced susceptibility to seizures of *Fmr1* KO and working memory deficits (Udagawa et al. 2013). Furthermore, in agreement with increased protein translation in *Fmr1* KO mice, genetic reduction of S6K1, a key translation initiation and elongation factor, corrected deficits in cortical-dependent tasks, such as novel object recognition and inflexibility, while reduction of S6K1 was not sufficient to prevent hyperactivity (Bhattacharya et al. 2012). These findings suggest that multiple key proteins may regulate brain function in a tissue and developmental-specific manner ultimately causing the complex FXS symptomatology. Further investigations are required to understand the molecular events underlying all these successful different genetic rescues.

## Conclusions

Over the past few years, intense research on FXS has led to the identification of several hundreds of putative FMRP mRNA targets (for review, see Fernandez et al. 2013). This large number of FMRP mRNA targets might explain the extensive and heterogeneous behavioral deficits in FXS, suggesting that FMRP loss influences different circuitries and causes alterations in various receptor pathways. Although, it is very well established that FMRP loss alters the glutamatergic signaling (mGluR theory), many other molecular pathways such as BDNF, mTOR, ERK1/2, cAMP, and PKC cascades are affected. In addition, different neuronal circuits, such as the GABAergic, cholinergic, dopaminergic, and serotonergic systems, are modified by FMRP loss.

Although the genetic cause leading to FXS is the absence of a single gene, the *FMR1*, the wide spectrum of disabilities and heterogeneity of clinical and cognitive features among patients with FXS renders the cure of the disease difficult. The combination of pharmaceutical treatments targeting different molecules altered in FXS might be the key for the amelioration of FXS deficits. On the other hand, the use of fly and mouse models has been of utmost importance. Both models re-create the cellular and molecular alterations caused by the absence of a functional FMRP, suggesting that the pathophysiology of FXS is evolutionarily conserved between these species.

Furthermore, as discussed above, loss of FMRP leads to several behavioral deficits in both mice and flies. Taking advantage of such deficits, several drug therapies have been tested and developed using these models. While, due to the diversity of the phenotypes and genetic heterogeneity observed in FXS patients, the clinical impairments are only partially ameliorated, it is remarkable that the responses to treatment in flies and mice are so well conserved. These findings strongly suggest that how these treatments manifest behaviorally depends on the complexity of the nervous system in different species.

Because a single animal model cannot always fully re-create the FXS behavioral phenotypes, the search for new and targeted therapies should also be focused on the development of assays to properly evaluate the effect of a specific drug on more than one model and in the context of different genetic backgrounds.

# Acknowledgments

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