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Modeling fragile X syndrome in the *Fmr1* knockout mouse

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Summary **Fragile X Syndrome (FXS) is a commonly inherited form of intellectual disability and one of the leading genetic causes for autism spectrum disorder. Clinical symptoms of FXS can include impaired cognition, anxiety, hyperactivity, social phobia, and repetitive behaviors. FXS is caused by a CGG repeat mutation which expands a region on the X chromosome containing the *FMR1* gene. In FXS, a full mutation (> 200 repeats) leads to hypermethylation of *FMR1*, an epigenetic mechanism that effectively silences *FMR1* gene expression and reduces levels of the *FMR1* gene product, fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein that is important for the regulation of protein expression. In an effort to further understand how loss of *FMR1* and FMRP contribute to FXS symptomology, several FXS animal models have been created. The most well characterized rodent model is the *Fmr1* knockout (KO) mouse, which lacks FMRP protein due to a disruption in its *Fmr1* gene. Here, we review the behavioral phenotyping of the *Fmr1* KO mouse to date, and discuss the clinical relevance of this mouse model to the human FXS condition. While much remains to be learned about FXS, the *Fmr1* KO mouse is a valuable tool for understanding the repercussions of functional loss of FMRP and assessing the efficacy of pharmacological compounds in ameliorating the molecular and behavioral phenotypes relevant to FXS.**

Keywords: Fragile X Syndrome, *Fmr1* knockout mouse, behavior, phenotyping, anxiety, social behaviors, cognition, attention

1. Introduction

Fragile X Syndrome (FXS) is one of the most commonly inherited forms of intellectual disability and monogenic causes of autism spectrum disorder (ASD) (*1,2*). Prevalence estimates for FXS are approximately 1:4,000 males (*3,4*) and 1:8,000 females (*5*), although a recent epidemiological meta-analysis reports FXS prevalence to be lower (1:7,143 males and 1:11,111 females) (*6*). This neurodevelopmental disorder is caused by a CGG repeat mutation on chromosome Xq27.3 (*7*), expanding the 5'-non-coding region of the fragile X mental retardation 1 (*FMR1*) gene. The *FMR1* gene encodes the fragile X mental retardation protein (FMRP) which regulates protein expression *via* its interaction with mRNA (*8*), associating with up to 4% of mRNA in the mammalian brain (*9,10*). The full mutation (> 200 CGG repeats) leads to hypermethylation of the *FMR1* promoter, an epigenetic mechanism which transcriptionally silences *FMR1* and reduces FMRP levels (*11*). FMRP is widely expressed throughout the body, but is enriched in neurons and testes (*12-14*). FMRP's binding targets include several synaptic proteins crucial for neurotransmission and structure (*15,16*), including postsynaptic density-95 (PSD-95), AMPA receptor subunits GluR1 and GluR2, and microtubule-associated protein 1b (MAP1b) (*17-22*), and further, binds to its own *Fmr1* mRNA (*23-25*). Through its association with target mRNAs, FMRP is thought to assist in the localization, transport, stabilization and translational regulation of the mRNA for these proteins (*10,16,26-29*). Loss of FMRP is also associated with elevated mTOR signaling (*30*), which is vital to cellular growth, energy metabolism and protein synthesis (*31*).

Due to the X-linked nature of its inheritance, FXS phenotypes are heterogeneous and vary considerably

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between males and females (*32,33*). In general, females typically display milder symptoms than males due to compensation by the second non-affected X chromosome (*34*). Common characteristics of individuals with FXS include intellectual impairment, increased anxiety, hyperarousal to stimuli and unusual physical features (*e.g.*, an elongated face, flat feet and hyperextendable finger joints) (*35*). In individuals carrying the full mutation, the severity of the physical and behavioral phenotypes correlates with lower levels of FMRP (*36*). To be noted, there are limitations in FMRP quantification, as many techniques utilize immunohistochemistry to label peripheral white blood cells (*37,38*) or hair roots (*39,40*) with monoclonal antibodies to indirectly measure FMRP levels. These methods cannot quantify FMRP protein levels, which is essential for understanding how the degree of FMRP loss relates to FXS clinical phenotypes. Development of additional detection methods, such as quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (*41*), time-resolved Förster's resonance energy transfer immunoassay (*42*) and semi-quantitative western blot protein analysis (*43*), has provided additional tools for the detection and quantification of FMRP protein levels, allowing for further investigation of the relationship between FMRP and FXS phenotypes.

Animal models of FXS have been developed in various species, such as the Drosophila fruit fly, zebrafish, mouse, and rat (*44-48*). Much effort has focused on the characterization of mouse models of FXS, in particular the *Fmr1* knockout (KO) mouse. The *Fmr1* KO mouse was created and initially characterized by the Dutch-Belgian Fragile X Consortium (*48*). The first *Fmr1* KO mice were generated using embryonic stem cells and C57BL/6J (B6) wildtype mice, a commonly used inbred mouse strain. A targeting vector containing a disrupted *Fmr1* DNA sequence with an insertion in exon 5 (the knockout allele) was inserted into embryonic stem cells and transferred into pseudo-pregnant female mice. These founder mice yielded offspring that were crossed with B6 mice to generate experimental animals. *Fmr1* KO mice harboring this mutation did not produce FMRP protein, but did possess detectable levels of *Fmr1* mRNA (*49*). Subsequently, these mice were bred into different background strains, such as the FVB inbred mouse strain. Since its initial description in 1994, many labs continue to use *Fmr1* KO mice to further understand the outcomes of functional FMRP loss in mice, and how it relates to FXS clinical symptoms. The goal of this review is to outline the progress to date, and discuss which areas will benefit from future research.

2. The *Fmr1* KO mouse

2.1. *Physiology of the Fmr1 KO Mouse*

Males with FXS tend to possess certain dysmorphic features, such as prominent ears, narrow face, loose joints, smooth skin and macroorchidism (enlarged testes) (*35,50*). The presence of macroorchidism is due to the loss of FMRP, which is highly expressed in the testes (*13*). *Fmr1* KO mice have significantly heavier testes than wildtype controls, but normal structural morphology (*48,51*). This is likely due to an increase in the proliferative activity of Sertoli cells found in the seminiferous tubules, which increases the number of germs cells in the testicles, and therefore, their weight (*51*). Other physical features, such as core temperature and body weight, and neurological reflexes did not differ between genotypes, suggesting otherwise normal gross physical and neural development (*48,52*). The presence of enlarged testes mirrors the macroorchidism found in male individuals with FXS, and therefore lends face validity to the *Fmr1* KO mouse model in this aspect of the clinical disorder.

2.2. *Dendritic spine morphology and neurotransmission*

FMRP is an RNA-binding protein that is enriched in neurons, particularly in the cell body, dendrites and postsynaptic spines (*14,28,53,54*). Dendritic spines, small protrusions along neuronal dendrites, are sites of excitatory synaptic input, which contain receptors and signaling molecules that are essential for synaptic neurotransmission (*55*). Postmortem analysis of human cortical tissue revealed that individuals with FXS have an increased density of dendritic spines relative to controls, with a majority of spines appearing elongated and immature (*56-63*). Directly analogous deficits in spine number and morphology have been found in *Fmr1* KO mice bred onto both B6 and FVB genetic backgrounds (*64-67*), providing additional face validity to the *Fmr1* KO mouse model. Developmental analysis of the barrel cortex of young (1 week old) *Fmr1* KO mice revealed an increase in spine density and length in mutant mice compared to controls, which was not present at 4 weeks of age (*65*). This absence of spine abnormalities at 4 weeks of age was also detected in the developing somatosensory cortex of *Fmr1* KO mice by the Greenough laboratory (*63*). In addition, in the same study, adult *Fmr1* KO mice exhibited increased density of immature, thin spines compared to controls (*63*). Therefore, there may be a period of synaptic development during which dendritic spine morphology briefly normalizes in the absence of FMRP, but is not sustained. In other brain regions, similar structural deficits in dendritic spines were seen at older ages of *Fmr1* KO mice. For example, *Fmr1* KO mice possess greater densities of elongated spines in the visual cortex at 16 weeks of age compared to wildtype controls (*66*). These data suggest that FMRP expression is necessary for the development of normal dendritic spine morphology, and that the loss of FMRP negatively impacts the physical structure of the synapse.

As a negative regulator of mRNA translation, FMRP influences protein synthesis and can therefore affect the synaptic components located in dendritic spines. Long term potentiation (LTP) and depression (LTD) are the long lasting enhancement and reduction, respectively, of signal transduction between two neuronal synapses (*68,69*). These activity-dependent cellular events rely on translational regulation of synaptic proteins in order to rapidly respond to synaptic activity and maintain cognitive function. Analyses of LTP and LTD, which are considered to represent electrophysiological correlates of learning and memory (*69*), have revealed abnormalities in the neurotransmission of mice lacking the *Fmr1* gene. LTD, which is dependent on protein synthesis and metabotropic glutamate receptor (mGluR) activation, was enhanced in *Fmr1* KO hippocampus and hippocampal neuron cultures (*70-72*). LTP, along with decreased AMPA receptor surface expression and selective increases in NMDA receptor subunit protein expression, was impaired in *Fmr1* KO mice (*17,21,71,73,74*), although these findings are inconsistent (*17,21,61,70,74,75*). *Fmr1* KO2 mice, another *Fmr1* null mouse model that lacks both FMRP protein and *Fmr1* RNA due to deletion of the *Fmr1* promoter and first exon (*76*), also displays abnormal synaptic plasticity. In the *Fmr1* KO2 hippocampus, a lower ratio of AMPA to NMDA receptors was detected early in development compared to wildtype controls (*77*). The upregulation of NMDA receptors in the *Fmr1* KO2 hippocampus resulted in increased NMDA receptor-dependent LTP. These data demonstrate that lack of *Fmr1* produces alterations in normal synaptic activity, which likely contributes to the FXS phenotype. Given the importance of FMRP for the regulation of proteins integral to synaptic function, it is unsurprising that loss of FMRP results in abnormalities in the structure and functionality of neuronal synapses.

2.3. *Seizure and stimuli hypersensitivity*

Approximately 10-20% of individuals with FXS with full mutations exhibit childhood seizures (*78-81*). Seizures associated with FXS are infrequent, are often partial, and are typically controlled with medications (*82,83*). *Fmr1* KO mice have not been reported to display spontaneous seizures, but are more susceptible to audiogenic seizures, induced by exposure to a 125 decibel, high-intensity siren (*48,81,84-93*). Audiogenic seizure vulnerability in *Fmr1* KO mice may reflect seizure susceptibly in FXS, although audiogenic seizure severity in *Fmr1* KO mice varied in degree depending on age and background strain (86,91,94,95).

Individuals with FXS report hyperarousal and heightened sensitivity to sensory stimuli (*7*). For example, subjects with FXS had stronger and more frequent responses and reduced habituation to sensory stimulations (*e.g.*, olfactory, auditory, visual, tactile, and vestibular stimuli) as measured by electrodermal responses (*96*). Electrophysiological recordings in the auditory cortex demonstrated enhanced responses to auditory tones in *Fmr1* KO mice, indicating that auditory neurons of *Fmr1* KO mice are hyper-responsive to stimuli (*97*). These data are consistent with the increased responses to pure tones seen in individuals with FXS (*98,99*).

Prepulse inhibition (PPI), a measure of sensorimotor gating, occurs when a weak pre-stimulus attenuates the response to a sudden strong stimulus (pulse) within 100 milliseconds (*100,101*). Deficits in PPI have been noted in FXS, correlating with other clinical FXS features, such as IQ severity and attention (*102-104*). Studies of *Fmr1* KO mice have yielded mixed results. The majority of studies indicate *Fmr1* KO mice exhibit enhanced PPI and reduced startle (*89,90,105-107*); this is a significant effect but in the opposite direction to the results in human FXS. In contrast, others report impaired PPI in *Fmr1* KO mice (*108*), increased startle responses to low intensity auditory stimuli (*109*), or minimal or no PPI differences between genotypes (*49,91,109,110*). As has been previously discussed, *Fmr1* KO behavior phenotypes are influenced by genetic background (*89,107*). Explanations for the divergent findings on PPI in *Fmr1* mice reported by different laboratories include use of different murine genetic backgrounds and differences in testing protocols (*111*). Of greater concern are the contrasting phenotypes between the majority of PPI studies in the *Fmr1* KO mouse and FXS human studies. These data suggest that while certain aspects of FXS are recapitulated in the *Fmr1* KO mouse, other clinical features are not reproduced.

2.4. *Attention and hyperactivity*

Individuals with FXS are hyperactive and have difficulties with attention and impulse control (*35,112-115*). Subjects with FXS performed better than learning disabled controls on selective attention, but the subjects with FXS had deficits similar to the learning disabled controls in sustained attention and working memory (*116*). Further, studies have found that FXS confers more drastic attentional deficits as task difficulty increases, such that individuals with FXS have more difficulty inhibiting/switching responses (*117*). In light of clinical FXS symptomology (*i.e.*, its comorbidity with ADHD), *Fmr1* KO mice were evaluated in the five-choice serial reaction time task, considered the gold standard task for attention and impulsivity in rodents (*118*). Although *Fmr1* KO mice were impaired in select phases of a visual-spatial discrimination task, they did not differ from wildtype controls in the five-choice serial reaction time task (*119,120*). Specifically, Krueger and colleagues found that *Fmr1* KO mice took longer to reach criterion during the

second phase of training (> 50% correct of > 15 trials for 2 consecutive days), when nose-pokes in a signaled nose-poke hole were correct and non-signaled nose-pokes were incorrect, but this effect did not replicate in subsequent studies (*121*). Sidorov and colleagues instead demonstrated augmented extinction of nose-poke responses in *Fmr1* KO mice. In another series of attention tasks, *Fmr1* KO mice had impaired inhibitory control, exhibiting a higher rate of premature responses than wildtype mice (*122*). This was associated with changes in task contingencies, suggesting inhibitory control in *Fmr1* KO mice may be affected by stress or novelty. Additionally, *Fmr1* KO performance was disrupted by olfactory distracters, with mutant mice making more inaccurate responses during distracter presentations (*122*). A consistent behavioral finding in *Fmr1* KO mice is their increased locomotor activity compared to wildtype controls in the open field test (*48,52,89,90,123-130*). It is important to note that the robust hyperactivity phenotype seen in *Fmr1* KO mice could be a confounding factor for the assessment of sustained attention, given that the general activity of mutant mice may interfere with task engagement.

2.5. *Repetitive behaviors*

Perseveration and repetitive behaviors, such as hand flapping, are associated with the full mutation in FXS (*33,35,131,132*). In the five-choice serial reaction time task, *Fmr1* KO mice demonstrated heightened perseveration and responding during novel rule acquisition, which normalized with training (*119*). *Fmr1* KO mice also exhibited higher levels of self-grooming, a repetitive behavior, than wildtype controls (*89,133*). Additionally, *Fmr1* KO mice buried more marbles in the marble burying test (*93,107,124*), a measure of repetitive behavior (*134*). However, marble burying was not significantly different between genotypes in some studies (*91,110,135*). Genotype differences in marble burying in *Fmr1* KO mice appear to be dependent on background strain (*107*). Overall, these data suggest that *Fmr1* KO mice show signs of repetitive behaviors, which parallels FXS clinical features.

2.6. *Anxiety*

Anxiety is one of the core behavioral features of FXS, in both children and adults (*35,132,136*). The evaluation of anxiety-related behaviors in *Fmr1* KO mice has generated inconsistent results, ranging from less anxiety-like scores in *Fmr1* mutant mice to no genotype differences to increased anxiety-like scores on several tasks. The elevated plus-maze is an anxiety-related task that utilizes a mouse's preference for dark spaces by evaluating the amount of time and entries made into dark, enclosed arms as compared to open arm runways (*137,138*). *Fmr1* KO mice spent significantly more time in the open arms and less time in the closed arms, but also traveled more throughout the maze, which may indicate higher general locomotion (*52,84,129,130*). In the zero-maze, *Fmr1* KO mice spent more time in the open quadrants (*130,139*). In the open field, the time or distance spent in the center of the open arena is sometimes considered an indicator for anxiety-related behavior, since wildtype mice prefer to remain in the perimeter when introduced to a novel environment. *Fmr1* KO mice spent a greater portion of their distance traveled in the center area of the open field compared to wildtype control mice (*49,52,123,129*). Together, these publications indicated a profile of lower anxiety-related behaviors in *Fmr1* KO mice, which is contrary to the FXS clinical phenotype. In contrast, others have shown that *Fmr1* KO mice exhibited increased anxiety-like responses in the mirrored chamber task (*123*), avoidance of the center of the open field (*128*) and reduced open arm time in the elevated plus-maze (*140*). In the light↔dark exploration test, an anxiety-related task in which a subject mouse typically spends more time in a dark chamber than a well-lit chamber (*141*), and in which number of transitions between compartments is increased by anxiolytic drug treatments (*142*), *Fmr1* KO mice made more transitions between the chambers (*90,107*), but did not differ from wildtype mice in time spent in the light chamber. In some studies, no genotype differences were detected in *Fmr1* KO mice as compared to wildtype littermates in the elevated plus-maze (*49,109,127*), in light↔dark exploration (*107*), or on center time in the open field (*91,93,135*). These differing results could potentially be explained by differences in testing and housing conditions, genetic background, and age at testing, as these factors can influence performance on conflict tests in mice (*143*). Given the sensitive nature of anxiety-related assays, it is imperative that similar testing protocols are used across labs to determine the robustness of the *Fmr1* KO genotype on anxiety-related phenotypes.

2.7. *Sociability and social communication*

Along with increased anxiety, individuals with FXS are often diagnosed with social phobia and avoidance (*35,132,144,145*). In the three-chambered sociability task, a subject mouse is evaluated for its exploration of a novel social stimulus (*e.g.*, novel mouse) versus a novel object stimulus (*146*). Wildtype mice will preferentially explore a novel mouse when given the choice between a novel mouse and a novel object with no social valence. Results using the three-chambered social approach with *Fmr1* KO mice to evaluate their sociability vary in the literature. For example, several groups report that *Fmr1* KO mice have normal sociability, preferring to explore the novel mouse over the novel object (*89,130,133,139*). Similarly, direct

social interactions with freely moving juvenile mice of the same sex, or in adult male subjects interacting with estrus females, were reported as normal (*89,147*) or even enhanced, as evidence by greater sniffing duration and interaction time of a partner mouse by *Fmr1* KO mice (*123,148*). In contrast, other research suggests that the sociability of *Fmr1* KO mice is abnormal, such that mutants do not exhibit a preference for a novel mouse over an object (*126*) and have reduced sniffing duration of the novel mouse compared to wildtype mice (*133*). Furthermore, additional studies demonstrate *Fmr1* KO mice spent less time engaging in affiliative behaviors, such as nose-to-nose sniffing, nose-to-anogenital sniffing and crawling over or under the partner's body during social interaction with a female mouse (*89*). Social scores appeared to be dependent on the background strain into which the *Fmr1* mutation had been bred (*107,149*). Although individuals with FXS are described as having social interaction deficits and social phobia, it has been suggested that these social deficits are due to hyperarousal and heightened anxiety rather than a lack of social understanding (*i.e.*, the "Fragile X handshake" in which an initial gesture, such as brief eye contact or social remark, is paired with active gaze avoidance (*150,151*)). The rodent models described here may differentially account for these factors.

Children with FXS are delayed in their language development, but this is associated with other cognitive delays (*152-154*). Rodent pup ultrasonic vocalizations are considered to be biologically meaningful (*155,156*), as they are emitted in young pups during stressful situations (*157*) and elicit retrieval behaviors by the parents. Adult male mice and rats emit ultrasonic vocalizations during interaction with females and in response to urine from estrus females (*158*). Studies focusing on ultrasonic vocalizations of *Fmr1* KO mice have been inconsistent in their findings. While there are reports of increases (*107*) or no differences in the number of calls of *Fmr1* mutant and wildtype mice (*89*), other labs observe a significant reduction in vocalizations in *Fmr1* KO mice (*124,147*), including call-type specific deficits (*159*). Together, data suggest that while *Fmr1* KO mice exhibit some aspects of normal sociability, they exhibit some abnormalities in social behavior and communication.

2.8. *Cognitive deficits*

A majority of individuals with FXS exhibit intellectual impairment, which can range from mild to severe. IQ scores decrease over time, which is likely a result of delayed development in individuals with FXS (*160,161*). Novel approaches to intelligence testing have found that traditional IQ tests can be modified to reveal subtle differences within this select population (*162*). Starting with the Dutch-Belgium Fragile X Consortium, many researchers have conducted thorough characterizations of *Fmr1* KO mice to compare their phenotypes to the intellectual disabilities displayed by individuals with FXS. One cognitive test conducted very early on in the development of the *Fmr1* KO mouse model was passive avoidance, a task that utilizes association of a footshock with a dark chamber to assess memory for the aversive event. Passive avoidance learning relies on the dorsal hippocampus (*163*) but also requires the amygdala (*164*). Dependence of passive avoidance performance on the dorsal hippocampus and amygdala would predict that animals deficient in the function of either or both of these brain regions would be impaired in this task, but the data are mixed. While amygdala volumes are not generally affected in subjects with FXS, affected individuals with FXS have difficulty with emotion regulation. A recent study revealed that individuals with FXS demonstrated less activation of the amygdala while viewing fearful faces than neurotypical subjects (*165*). Passive avoidance learning was not altered in *Fmr1* KO mice in some studies (*48,93,135,166*) but was disrupted in others (*90-92,129,167,168*). Interestingly, passive avoidance extinction may occur more rapidly in *Fmr1* KO mice (*92,166*), which is consistent with augmented extinction in *Fmr1* KO mice in other assays (*121*). It may be that cognitive deficits combined with augmented fear responses are working in opposition, explaining some of the disparate results in fear-associated tasks such as passive avoidance.

Fear conditioning studies were used to further elucidate whether other specific cognitive domains are disrupted in *Fmr1* KO mice. Fear conditioning can be parsed out into several distinct subtypes that rely on the amygdala, hippocampus, and prefrontal cortex to different extents. Contextual fear conditioning requires both the amygdala and hippocampus, while delay-cued fear conditioning requires the amygdala but not the hippocampus (*169-172*). Contextual and delay-cued fear conditioning can be acquired during the same training session and assessed in independent settings to reveal hippocampus-dependent and hippocampus-independent memory effects, respectively. In amydgala-dependent delay-cued fear conditioning, a deficit was reported in *Fmr1* KO mice (*75,90*), but other studies did not observe this effect (*173-175*). In hippocampus-dependent contextual fear conditioning, one report indicated a deficit (*75*) and another identified a context-discrimination deficit (*176*); other studies did not detect genotype differences in contextual fear conditioning in *Fmr1* KO mice (*52,173,175*). Trace-cued fear conditioning requires hippocampus and prefrontal cortex (*177,178*) and may or may not be independent of the amygdala (*179,180*). Trace fear conditioning, a more difficult task in which the tone and shock are not simultaneous during training, indicated that *Fmr1* KO mice may have deficits (*74*) but others showed that

Fmr1 KO mice appeared equal or superior to wildtype mice in the acquisition of trace fear conditioning (*106*).

The hippocampus is larger in individuals with FXS (*181,182*) and functional deficits in the hippocampal domain in subjects with FXS (*183,184*) would suggest that any fear task requiring the hippocampus would show a deficit. The FXS association with larger hippocampal volumes (*182*) and/or subjectively assessed hippocampal morphology differences in affected individuals (*185*) may or may not relate to deficits in hippocampal-dependent memory. Further, while individuals with FXS have normal amygdala and prefrontal cortex volumes, they have altered behavioral responses to tasks requiring the amygdala (*165*), frontal lobe (*186*) and prefrontal cortex (*187*). This may represent another instance in which behavioral tasks that require functional circuits (*i.e.*, the limbic system) may lead to variable results when multiple neural substrates within that system are affected (*i.e.*, prefrontal cortex, amygdala, and hippocampus).

Decades of research characterizing the cognitive abilities of individuals with FXS predict that deficits in a FXS mouse model should occur in short-term (visual) memory, visual-spatial abilities, sequential information processing, executive function and attention (*188-191*). The Morris water maze, a hippocampus-mediated task, was used to evaluate *Fmr1* KO visual-spatial abilities to determine whether subject mice could locate a submerged platform using spatial cues (*48*). The study did reveal subtle genotype differences, such that *Fmr1* KO performance was significantly worse in reversal (*i.e.*, a change in platform location) than wildtype littermates, specifically during the first trials after location-switching. This may indicate difficulty in changing reinforcement contingencies. Interestingly, however, there were no performance differences in the probe trial when the platform was removed, suggesting no impairment in visual-spatial memory. Kooy and colleagues (*192*) added additional animals (22 KO and 17 wildtype mice) to the original Consortium study (14 KO and 11 wildtype mice) and pooled these results. The larger sample sizes revealed similar results on Morris water maze reversal, with the additional finding of a genotype effect during the initial spatial memory acquisition. However, no significant probe trial differences were observed, indicating that while there are some differences in Morris water maze performance, they may not be functionally relevant to the FXS condition. Despite the *Fmr1* KO deficit occurring in reversal trials, a similar reversal learning task conducted in an E-shaped maze revealed no such genotype difference. However, while *Fmr1* KO mice did not show a persistent perseveration phenotype across cognitive modalities (*i.e.*, impaired reversal in Morris water maze, but not E-shaped maze (*192*)), a cross-shaped maze replicated the Morris water maze acquisition deficit (*173,175*). These acquisition deficits have been replicated (*106*), but not consistently (*75,174*). Similarly, deficits in reversal learning in *Fmr1* KO mice were replicated in some studies (*106,193*), but not all (*75*). Based on the variable results across laboratories, the spatial learning deficits identified in earlier studies may require very specific conditions in order to reproduce these results. In the majority of published studies, however, probe trial analyses revealed no differences between *Fmr1* KO and wildtype mice, indicating limited and selective deficits in spatial learning and memory (*48,75,174,192,193*). However, some probe trial differences have been observed in *Fmr1* KO mice (*106*). Some researchers have observed task-specific impairments in spatial cognition rather than global impairments (*183,184*), although global cognitive impairments in individuals with FXS have also been reported (*160-162*). The mild deficits in spatial learning and memory observed in *Fmr1* KO mice may support the idea of task-specific cognitive deficits and not global dysfunction.

The mixed results in cognitive assays to date has initiated a debate as to whether the *Fmr1* KO mouse is a sufficient model of FXS in humans, since the primary symptom of intellectual impairment is not prominent in the mutant mouse model. In an effort to find cognitive tasks with more ethological relevance, recent studies have included novel object recognition as well as spatial and temporal order object recognition tasks. Novel object recognition, which is typically conducted as a short-term memory task, relies on rodents' natural tendency to investigate novelty. A mouse is placed into an arena with two identical copies of an object, where their species-typical response is to explore and investigate the objects. After a certain interval, subject mice are returned to the arena with one familiar object and a novel object. If the mouse recognizes the previously seen object, it preferentially investigates the novel object. *Fmr1* KO mice have a deficit in this task (*194,195*), but as with the previously discussed cognitive domains, this impairment has not always been replicated (*49*). A recent study identified hippocampus-dependent spatial object recognition deficits in *Fmr1* KO mice (*195*), such that *Fmr1* mutant mice did not preferentially explore an object when it was moved to a new location.

Working memory deficits have been suggested as being a core feature of FXS (*196*). In several human clinical studies, individuals with FXS had low performance on specific working memory tasks under low-control conditions (*i.e.*, verbal and visual-spatial (*116,185,197,198*), or visual-spatial alone (*199*)). A recent study identified working memory deficits under high-control conditions (*i.e.*, a dual task request; for example, selective word recall only when a stimulus with particular properties was presented) in individuals with FXS that were specific to another component of working memory, central executive functioning

(*200*). Further, while central executive processing was impaired in individuals with FXS, both verbal and visual-spatial working memory modalities were intact. While these studies and others (*183,184*) suggest that human cognition deficits in FXS are task-specific and not global in nature, additional research has revealed impairments in all components of working memory in FXS (*i.e.*, visual-spatial sketchpad, central executive, and phonological loop) (*198*). Similarly, a study in young boys with FXS revealed working memory deficits regardless of task complexity and modality (*196*). The differing results on specific versus general working memory deficits in FXS may be due to task-specific differences (*e.g.*, the type of stimuli used), as individuals with FXS have more accurate recall with familiar stimuli rather than abstract material (*189*). In rodents, working memory tasks, such as olfactory working memory and radial arm maze, can rely heavily on other brain regions (*i.e.*, olfactory bulb or hippocampus, respectively). In several tasks, including the radial arm maze, *Fmr1* KO mice did not show robust working memory deficits (*49*), although others have identified a working memory impairment in *Fmr1* KO mice in a serial reversal version of the Morris water maze (*106*). It is possible that the olfactory bulb and hippocampus in *Fmr1* KO mice are compensating for deficiencies in working memory in some of these tasks. Therefore, identification of a behavioral task that is less reliant on other brain regions is necessary to determine if *Fmr1* KO mice exhibit a reliable working memory impairment, as this would add further face validity to the model.

3. Conclusions

The development of FXS animal models has furthered our understanding of several molecular and synaptic deficits underlying FXS, including abnormal dendritic spine morphology, protein dysregulation and neurotransmission. In addition, animal models provide an opportunity to evaluate novel drug targets to ameliorate FXS symptoms. Indeed, gene therapy (*124*) and pharmacological compounds such as minocycline (*147,201*), mGluR5 antagonists (*202*), arbaclofen (*203*), ganaxolone (*84*), lovastatin (*204*) and lithium (*195,205*) have shown efficacy in ameliorating some of the phenotypes detected in *Fmr1* KO mice. Thorough evaluation of the *Fmr1* KO mouse on numerous genetic backgrounds across a multitude of labs indicates that several phenotypes, such as neuronal morphology and hyperactivity, are robust and consistent across studies. In contrast, several aspects of cognition, anxiety and social phenotypes of *Fmr1* KO mice are highly variable across published reports (Table 1). Additionally, many reported *Fmr1* KO phenotypes are in direct opposition to the clinical FXS phenotype, such as a lack of robust cognitive impairments, enhanced prepulse inhibition and reduced anxiety in the mouse model. The *Fmr1* KO mouse was generated by genetically modifying the *Fmr1* DNA sequence to reduce FMRP protein levels. This is contrast to the human FXS condition, which is generally caused by expansion of the *FMR1* gene region and subsequent promoter hypermethylation, although there are rare instances of FXS being due to point mutations and partial or complete deletion of the *FMR1* gene (*206-208*). Given that FXS clinical symptomology is associated with lower levels of FMRP, one would expect that complete disruption of *Fmr1* and resulting loss of FMRP would recapitulate the most severe clinical phenotypes of FXS. However, this is not the case for the *Fmr1* KO mouse model, which may limit its utility. The mechanistic differences between the mouse model and the human genotype underlying loss of FMRP, *i.e.* deletion and expansion, respectively, could be a contributing factor to the phenotypic differences seen between *Fmr1* KO mice and individuals with FXS. Therefore, in order to more fully recapitulate the clinical features of FXS, such as severe intellectual disability and social anxiety, it will be important to explore other mechanisms associated with FXS in combination, such as CGG expansion and hypermethylation of the *Fmr1* gene, as well as loss of FMRP protein.

It is possible that the variance seen in the *Fmr1* KO phenotype reflects the range of FXS clinical symptoms, rather than being due to subtle differences in methodology or genetic background influence alone. The variability in the strength and direction of phenotypic differences observed in the *Fmr1* KO mouse may at first seem unsettling and worthy of discarding the model altogether. However, the heterogeneity of FXS is such that affected individuals exhibit a range of cognitive impairments, with affected males presenting with mild to severe cognitive symptoms (*162,209*). This poses a challenge for FXS animal models, but it also might be considered a strength. If the *Fmr1* KO model is expected to primarily encompass only the most severe symptoms of FXS, then more is expected of the model than exists in the human syndrome. Instead, if the model is looked at through a clinician's lens, one would expect a heterogeneous population with a portion of the animals showing severe impairments with others displaying mild to moderate effects or none at all. Indeed, it is a challenge to think of how variable FXS symptomology in both the human syndrome and the animal model can be leveraged toward the identification of successful treatments for individuals with FXS. Despite these challenges, pharmacological interventions using the *Fmr1* KO mouse have demonstrated predictive validity for this model, as results from several drug studies in *Fmr1* KO mice parallel findings from human FXS open-label treatment trials (*e.g.* minocycline (*210*) and lithium (*211*)). As research of the molecular and behavioral dysfunction in

Table 1. Summary of behavioral and cognitive phenotypes of *Fmr1* knockout mice (↓ = decrease; ↑ = increase; ↔ = no change)

Domain	Fragile X Syndrome Clinical Phenotype	Rodent Assay	*Fmr1* Knockout Mouse		References
			Direction	Phenotype	
Cognition	Intellectual disability; working memory deficits	Passive avoidance	↓	Impaired performance; augmented extinction	*90-92,129,166-168*
			↔	No genotype differences	*48,93,135,166*
		Fear conditioning	↓	Deficits in delay-cued and contextua fear conditioning; deficits in trace fear conditioning	*74, 75, 90, 176*
			↔	No genotype differences	*52,106,173-175*
		Morris water maze	↓	Impaired performance during acquisition and/or reversal	*48,106,192,193*
			↔	No genotype differences	*49,75,174*
		Maze learning	↓	Impaired acquisition of a cross-shaped maze	*173,175*
			↔	No genotype differences in radial arm maze	*49*
		Reversal task	↔	No genotype differences in E-shaped maze	*192*
		Novel object recognition	↓	No preference for novel object	*194,195*
			↔	No genotype differences	*49*
Anxiety	Increased anxiety	Elevated plus-maze and zero-maze	↑	Reduced open arm time	*140*
			↓	Increased open arm and open quadrant time	*52,84,129,130,139*
			↔	No genotype differences	*49,109,127*
		Light↔dark exploration test	↓	Increased transitions	*90,107*
			↔	No genotype differences	*107*
		Center area of open field	↑	Avoidance of center area	*128*
			↓	More distance traveled in the center area	*49,52,123,129*
			↔	No genotype differences	*91,93,135*
		Mirrored chamber task	↑	Increased anxiety responses	*123*
Communication	Delayed language development	Ultrasonic vocalizations	↓	Reduction in vocalizations	*124,147,159*
			↑	Increased vocalizations	*107*
			↔	No genotype differences	*89*
Social	Social phobia and avoidance	Three-chambered sociability task	↓	No preference for novel mouse; social preference with reduced sniffing of novel mouse compared to wildtype mice	*126,133*
			↔	No genotype differences	*89,130,133,139*
		Direct social interactions with juvenile or with estrus female mice	↓	Reduction in affiliative behaviors	*89*
			↑	Greater sniffing duration and interaction time with partner mouse	*123,148*
			↔	No genotype differences	*89,147*
General Activity	Hyperactivity	Open field	↑	Increased locomotor activity	*48,52,89,90,123-130*

(To continue)

Table 1. Summary of behavioral and cognitive phenotypes of *Fmr1* knockout mice (↓ = decrease; ↑ = increase; ↔ = no change) (continued)

Domain	Fragile X Syndrome Clinical Phenotype	Rodent Assay	*Fmr1* Knockout Mouse		References
			Direction	Phenotype	
Attention and Impulse Control	Deficits in attention, particularly as difficulty increases; difficulty in response inhibition and rule switching	Visual-spatial discrimination task	↓	Longer to reach criterion; augmented extinction	*120, 121*
		Attentional task with odor distractors	↓	Impaired inhibitory control, with a higher rate of immature responses associated with rule changes	*122*
		Five-choice serial reaction time task	↔	No differences	*119, 120*
Repetitive Behaviors	Perseveration and repetitive behaviors	Five-choice serial reaction time task	↑	Increased perseveration and responding during novel rule acquisition	*119*
		Cage observations	↑	Higher levels of self-grooming	*89, 133*
		Marble burying	↑	Higher number of buried marbles	*93, 107, 124*
Stimuli Sensitivity	Hyperarousal and heightened sensitivity to sensory stimuli	*In vivo* single unit extracellular electrophysiology	↑	Enhanced responses to auditory tone	*97*
		Auditory startle response	↑	Increased startle response to low intensity auditory stimuli	*109*
Sensorimotor Gating	Reduced prepulse inhibition	Prepulse inhibition	↓	Impaired prepulse inhibition	*108*
			↑	Enhanced prepulse inhibition; reduced startle response	*89, 90, 93, 105-107*
			↔	Minimal or no differences in prepulse inhibition	*49, 91, 109, 110*

the *Fmr1* KO model accumulates, our understanding of how these molecular differences translate into observed behavioral dysfunction will continue to increase, providing a platform for the future identification of targeted FXS treatments.

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