Deciphering Discord: How *Drosophila* research has enhanced our understanding of the importance of FMRP in different spatial and temporal contexts

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Abstract:

Fragile X Syndrome (FXS) is the most common heritable form of intellectual impairment as well as the leading monogenetic cause of autism. In addition to its canonical definition as a neurodevelopmental disease, recent findings in the clinic suggest that FXS is a systemic disorder that is characterized by a variety of heterogeneous phenotypes. Efforts to study FXS pathogenesis have been aided by the development and characterization of animal models of the disease. Research efforts in *Drosophila melanogaster* have revealed key insights into the mechanistic underpinnings of FXS. While much remains unknown, it is increasingly apparent that FXS involves a myriad of spatial and temporally specific alterations in cellular function. Consequently, the literature is filled with numerous discordant findings. Researchers and clinicians alike must be cognizant of this dissonance, as it will likely be important for the design of preclinical studies to assess the efficacy of therapeutic strategies to improve the lives of FXS patients.

Introduction:

Fragile X syndrome (FXS) is the most prevalent heritable form of intellectual disability and a leading genetic cause of autism. In addition to cognitive and social impairments, Fragile X patients present with behavioral symptoms including sleep disorders, attention deficit hyperactivity disorder (ADHD), and motor stereotypies. Several irregular physical features have also been observed in FXS patients such as altered facial structure, prominent ears, high-arched palate, hyperflexible joints, and macro-orchidism in post-pubertal males (Kidd et al., 2014). In a highly informative and comprehensive review, Kidd et al. (2014) discuss the current status of the clinical literature with regard to the prevalence of medical problems observed in FXS patients. Of note, FXS patients experience a spectrum of medical problems that likely occur due to connective tissue defects. These conditions include otitis media, ocular disorders, gastrointestinal disturbances, and growth problems (Kidd et al., 2014). The vast heterogeneity of phenotypes observed in affected individuals has made it difficult to precisely ascertain comorbid disorders in FXS. Discordant findings in clinical studies emphasize the variability of data obtained from human patients and suggest that selection bias and large differences in study design and data collection methods make it difficult to predict which of the associated medical problems physicians are most likely to encounter when treating FXS patients. Given the myriad of phenotypes that have been documented in the clinical literature, it has become increasingly apparent that FXS is a systemic disorder. Despite this evidence, the majority of research has been focused on the neurological aspects of FXS and many of the systemic phenotypes have been ignored. Consequently, a large gap has arisen between the observations in the clinic and the discoveries at the bench.

At the molecular level, FXS is caused by loss-of-function of the *Fragile X Mental Retardation 1* (*Fmr1*) gene. Typically this loss-of-function occurs due to an expansion of a trinucleotide repeat in the 5' untranslated region (UTR) of the *Fmr1* gene (Verkerk et al., 1991). In affected individuals, this expansion contains over 200 repeats, which results in hypermethylation of the locus and subsequent transcriptional silencing of the gene (Fu et al., 1991; Pieretti et al., 1991). As such, this pathogenic mutation eliminates the expression of the product of the *Fmr1* gene, termed Fragile X Mental Retardation Protein (FMRP). Many studies have demonstrated that FMRP is an RNA binding protein that is enriched in the nervous system (Ashley et al., 1993; Brown et al., 1998; Chen et al., 2003; Darnell et al., 2005; Siomi et al., 1993). It is estimated that FMRP binds to and regulates over 800 distinct mRNA targets (Darnell et al., 2011). Although FMRP is primarily thought to act as a translational repressor, it has been shown to promote translation in some cases (Bechara et al., 2009; Krueger et al., 2011; Monzo et al., 2006). Additionally, FMRP participates in several other pathways that are known to modulate gene expression. For example, it has been shown that FMRP stabilizes and assists the transport of mRNAs within the cell (Dictenberg et al., 2008; Zalfa et al., 2007). It has also been found to interact with components of the RNA interference, small interfering RNA, and microRNA pathways (Caudy et al.,

2002; Ishizuka et al., 2002; Jin et al., 2004). Taking the large number of putative mRNA targets along with evidence that FMRP is implicated in multiple aspects of gene regulation, it appears that while FXS is monogenic in nature, *Fmr1* has pleiotropic effects that likely underlie the diversity of phenotypes observed in FXS patients.

Since the etiology of FXS is well characterized, researchers have been able to generate valuable animal models of the disease. These animal models are highly relevant on a translational level and facilitate an increased understanding of FXS pathogenesis because they allow researchers to control for genetic and environmental heterogeneity and thereby circumvent many of the limitations of human studies. With the advent of a wealth of genetic tools, *Drosophila melanogaster* has emerged as a highly tractable model for the study of FXS (Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Zhang et al., 2001). The *Drosophila* genome contains a single gene, *dfmr1*, whose product shares both sequence identity and biochemical properties with its mammalian ortholog (Wan et al., 2000). Much like its mammalian counterpart, dFMRP is an RNA-binding protein that has been shown to bind to select mRNA transcripts and repress their translation (Reeve et al., 2005; Zhang et al., 2001). Moreover, it has been demonstrated that the RNA binding affinity of dFMRP is highly conserved, which suggests that the RNA-binding profile of dFMRP may greatly resemble the list of mRNA targets bound by FMRP (Brown et al., 2001; Darnell et al., 2009; Darnell et al., 2011; Miyashiro et al., 2003). While only a handful of mRNA targets of dFMRP have been identified in *Drosophila*, the targets that have been validated encode proteins that function in cytoskeleton maintenance and larval crawling behavior (Lee et al., 2003; Reeve et al., 2005; Xu et al., 2004; Zhang et al., 2001). The products of these mRNA targets have functional properties that are very similar to those identified in the murine model of FXS (Castets et al., 2005; Lu et al., 2004). In addition to these genetic and functional similarities, flies that harbor loss-of-function mutations in the *dfmr1* gene recapitulate many of the characteristics of FXS (Table 1). Specifically, *dfmr1* null flies show defects in memory, social behavior, circadian rhythmicity, and sleep (Bolduc et al., 2008; Bushey et al., 2009; Dockendorff et al., 2002; Inoue et al., 2002; McBride et al., 2005; Morales et al., 2002). Further, *dfmr1* null flies exhibit defects in neural architecture that are reminiscent of the dendritic spine defects seen in both human patients and murine models of FXS (Comery et al., 1997; Dockendorff et al., 2002; Lee et al., 2003; Michel et al., 2004; Morales et al., 2002; Zhang et al., 2001). These robust correlates to mammalian phenotypes render the *Drosophila* model an ideal paradigm to study the cellular and molecular mechanisms involved in FXS pathogenesis and to ascertain the efficacy of novel therapeutic approaches.

Several recently published reviews elegantly summarize the current understanding of the molecular pathways that are altered in FXS and the corresponding molecular targets that have been identified for therapeutic intervention (Doll and Broadie, 2014; Lozano et al., 2014; Santos et al., 2014; Scharf et al., 2014). **In this review, we discuss the diverse functions of dFMRP in neuronal circuits, behavioral output pathways, and the response to cellular stress.** Recent work in the *Drosophila* model of FXS suggests that dFMRP plays an important role in the modulation of intracellular processes that are important for cellular stability and maintenance. Multiple lines of evidence suggest that dFMRP acts in a highly cell type-, temporal-, and pathway-specific manner. These findings, coupled with observations in murine models of FXS, provide a compelling case that future progress depends upon increased understanding of the spatiotemporal requirements for FMRP.

Neuronal Circuits:

Neural Stem Cells

The process of neurogenesis is critical for proper assembly of neural circuitry and the establishment of accurate brain connectivity. This intricate, highly orchestrated process is driven by the proliferation of stem cells and progenitor cells, which subsequently differentiate into neurons and glia. It

is essential to achieve the correct balance between neurons and glia, as an incorrect ratio of neurons to glia promotes aberrant wiring of the brain. Importantly, *Drosophila* neuroblasts (NBs) follow general stem cell principles and employ conserved molecular pathways (Callan et al., 2010; Callan and Zarnescu, 2011). As such, NBs have emerged as a valuable model to study stem cell proliferation and differentiation during development. Callan et al. (2010) utilized the wealth of genetic tools available in *Drosophila* to elucidate the role of dFMRP during early neurogenesis *in vivo*. The results of their loss of function studies and clonal analyses in larval NBs suggest that dFMRP is required during early stages of development before synaptogenesis occurs. Loss of dFMRP was found to increase the proliferative capacity of larval NBs and promote the generation of supernumerary neurons that survive into adulthood. Moreover, these studies revealed that larval NBs lacking dFMRP exit quiescence prematurely and proliferate sooner than their wild-type counterparts. However, this enhanced early proliferation is followed by a deceleration of proliferation later in development. This important discovery provided the first evidence that dFMRP modulates proliferation in larval NBs by regulating the time at which neural progenitors exit quiescence. In a subsequent publication, Callan et al. (2012) expanded upon these findings and utilized tissue specific RNA interference to elucidate the spatiotemporal role of dFMRP during larval brain development. Their observations suggest that there is heterogeneity in the temporal requirement for dFMRP such that dFMRP regulation is required during distinct developmental windows in different cell types. Specifically, dFMRP appears to be required first in NBs and subsequently in glia to control exit from quiescence. Importantly, this is the first study to assert that dFMRP functions to regulate NB reactivation in glial cells *in vivo*. To address the mechanism by which dFMRP regulates the timing of NB reactivation, Callan et al. (2012) monitored components of the insulin signaling pathway, as this pathway has been shown to modulate neurogenesis (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Callan et al. (2012) showed that elevations of pAkt in the larval brain are concurrent with the developmental window during which dFMRP is required in glial cells. They also demonstrated that *dfmr1* genetically interacts with *dFoxO*, a key transcriptional regulator of insulin signaling. Together, these results suggest that the interplay between dFMRP and the insulin signaling pathway modulates NB reactivation *in vivo*. The complexity of the results from these *Drosophila* studies provide strong evidence that dFMRP plays distinct, cell type-specific roles during different developmental time points *in vivo*. This conclusion emphasizes the need for members of the field to be cognizant of the spatial and temporal context in which studies are conducted to gain a better understanding of the molecular underpinnings of FXS disease pathology.

Consistent with observations in *Drosophila*, studies in mice have shown that FMRP regulates the timing and proliferation of neural stem and progenitor cells and that it is required to generate appropriate numbers of neurons and glia (Guo et al., 2011; Luo et al., 2010; Saffary and Xie, 2011). Much like *Drosophila* larval NBs, one such study used a combination of *in vitro* and *in vivo* approaches to assess the effect of FMRP deficiency on adult neurogenesis in the mammalian hippocampus (Luo et al., 2010). The results from experiments conducted in cultured explants of mouse adult neural progenitor cells (aNPCs) and *in vivo* monitoring of endogenous mouse aNPCs suggest that proliferation is augmented in *Fmr1* knockout aNPCs. Moreover, loss of FMRP alters aNPC fate specification such that *Fmr1* knockout aNPCs exhibit decreased neuronal differentiation and increased glial differentiation. Additional experiments revealed that FMRP regulates the expression of proteins involved in cell cycle progression as well as GSK3 β , a key component of the Wnt signaling pathway. Based on these and other mechanistic findings, Luo et al. (2010) hypothesize that *Fmr1* deficiency results in dysregulation of the Wnt signaling pathway and thereby alters the proliferative capacity and fate specification of mouse aNPCs. Moreover, loss-of-function studies in the neocortex of mouse embryos suggest that FMRP suppresses the transition from stem-cell like radial glial cells (RGCs) to intermediate progenitor cells (IPCs) in a time dependent manner (Saffary and Xie, 2011). This result is consistent with the observation that dFMRP is important in the early stages of neurogenesis in *Drosophila* (Callan et al., 2010). However,

the results in Luo et al. (2010) indicate that loss of *Fmr1* depletes the population of RGCs, which is the opposite of what is seen in *Drosophila* larval NBs and mouse aNPCs (Callan et al., 2010). This discrepancy is likely due to differences in neural stem cell proliferation in different tissues at different times.

From a behavioral standpoint, ablation of FMRP and the consequent alterations in hippocampal neurogenesis result in impaired performance in two different hippocampus-dependent learning paradigms. However, selective restoration of FMRP in aNSCs rescues the cellular and behavioral phenotypes that occur in *Fmr1* knockout mice (Guo et al., 2011). It is possible that GSK3 β hyperactivity modulates these phenotypes, as pharmacological treatment with a highly specific GSK3 β inhibitor ameliorates aberrant cell fate specification, enhances the integration of newborn neurons into circuits, and rescues impairments in hippocampus-dependent learning in *Fmr1* KO mice (Guo et al., 2012).

Despite the differences inherent to each model system, it is clear that findings in the literature converge on a common notion that the role of FMRP in neurogenesis is diverse and tightly regulated at different time points and in different cellular populations. There appears to be a shared theme of overproliferation and differentiation defects in neural stem and progenitor cells in the absence of FMRP (Callan et al., 2010; Guo et al., 2011; Luo et al., 2010; Saffary and Xie, 2011). The observation that FMRP regulates neurogenesis in aNPCs by controlling the production of neurons and glia underscores the important role that FMRP plays in the establishment of proper neural circuitry and accurate connectivity. The findings by Guo et al. (2011) that loss of FMRP in aNPCs results in learning deficits that can be rescued by restoration of FMRP in this cell population provide further support for the hypothesis that loss of FMRP can result in aberrant connections that are detrimental to behavioral and cognitive output. From a therapeutic perspective, recent findings in *Drosophila* and mice have uncovered novel targets that have the potential to benefit patients and have identified critical time points during which intervention might be most efficacious.

Cytoskeleton and neural architecture

FMRP plays an important role in the establishment and maintenance of intracellular microtubule and actin scaffolds. Previous reports have demonstrated that FMRP represses the translation of microtubule associated protein 1B (MAP1B) in both flies and mice (Lu et al., 2004; Zhang et al., 2001). Additionally, FMRP has been shown to associate with components of kinesin and dynein to regulate mRNA trafficking along microtubules (Dicthenberg et al., 2008; Estes et al., 2008; Ling et al., 2004). More recently, *dfmr1* was shown to genetically interact with the *spastin* gene, which encodes a microtubule severing protein (Yao et al., 2011). Under normal conditions, *spastin* promotes the proper assembly of neuronal microtubules to ensure appropriate synaptic growth (Trotta et al., 2004). The interaction between *dfmr1* and *spastin* is essential because it enables migration of mitochondria along microtubules (Yao et al., 2011). Outside of the nervous system, dFMRP facilitates the organization of microtubules in peripheral tissues. As such, loss of *dfmr1* expression in the germline alters the configuration of microtubules in spermatozoa and likely contributes to the infertility observed in *dfmr1* null males (Zhang et al., 2004).

Analogous to what is observed in the case of microtubule organization, normal *dfmr1* expression is also critical for maintenance of the actin cytoskeleton. It has long been known that dFMRP binds to and represses the translation of *chickadee*, which is the *Drosophila* homolog of *profilin* (Reeve et al., 2005). This finding is particularly intriguing because the protein encoded by *chickadee* binds to actin. In a recent study, *dfmr1* was also shown to interact with *cheerio*, the *Drosophila* homolog of *filamin A* (Bolduc et al., 2010a). Since *cheerio* is important for remodeling of the actin cytoskeleton, the results from the studies conducted by Reeve et al. (2005) and Bolduc et al. (2010a) suggest that actin dynamics are altered in *dfmr1* null flies. These molecular changes in actin dynamics appear to affect behavior and cognition, as flies that are heterozygous for loss-of-function mutations in *cheerio* and *dfmr1* have impaired olfactory long-term memory (Bolduc et al., 2010a). To complement this behavioral data,

Bolduc et al. (2010a) present molecular evidence that *cheerio* expression is lower in *dfmr1* null flies than in wild-type counterparts after training in a memory paradigm. The observed changes in the maintenance of the actin cytoskeleton in the *Drosophila* model of FXS are also present in murine models of the disease, as *Fmr1* knockout mice exhibit impaired phosphorylation of p-21-activated kinase (PAK) and subsequent delayed stabilization of newly formed actin filaments in response to theta burst afferent stimulation (Chen et al., 2010). These results are relevant from a therapeutic standpoint because genetic and pharmacological inhibition of PAK has been shown to mitigate audiogenic seizures and hyperactivity in *Fmr1* knockout mice (Dolan et al., 2013; Hayashi et al., 2007). Together, these discoveries suggest that improper regulation of the actin cytoskeleton is likely one mechanism by which the absence of FMRP results in aberrant behavior and cognition.

Along with cytoskeletal defects, *dfmr1* null flies also have aberrations in neural architecture. In the central nervous system (CNS), overelaborated branching has been observed in the small ventral lateral neurons (sLNvs), which constitute the component of the central circadian clock that drives circadian rhythms in free-running conditions in *Drosophila* (Dockendorff et al., 2002; Gatto and Broadie, 2009; Morales et al., 2002). Additionally, the architecture of the Mushroom Bodies (MBs), which constitute the primary learning and memory center of the fly brain, cross over the midline and exhibit varying levels of fusion (Michel et al., 2004; Reeve et al., 2005).

To expand upon these behavioral and neuroanatomical findings, subsequent studies utilized a variety of genetic and pharmacological manipulations to demonstrate that inhibition of matrix metalloproteinase (MMP), an enzyme that is important for remodeling of the extracellular matrix, rescues the observed morphological defects in the sLNvs and MBs (Siller and Broadie, 2011). Since *dfmr1* null flies also exhibit architectural defects in the neuromuscular junction (NMJ) (Zhang et al., 2001), Siller and Broadie (2011) expanded upon their findings in the CNS and examined the effect of treatment with minocycline, an MMP inhibitor, on peripheral motor structural changes. While this manipulation did ameliorate structural alterations in NMJs, the NMJ phenotype was less sensitive to minocycline dosage than the alterations in the CNS. Together these results indicate that central and peripheral neurons respond differently to drug treatment (Siller and Broadie, 2011). Similarly, studies in *Fmr1* knockout mice indicate that *mmp9* mRNA co-localizes with FMRP both at the synapse and in dendrites (Janusz et al., 2013). The observed co-localization likely has functional relevance, as treatment with minocycline or genetic reduction of MMP9 rescues dendritic spine defects and aberrant behavior in *Fmr1* knockout mice (Bilousova et al., 2009; Sidhu et al., 2014). Compellingly, treatment of FXS patients with minocycline showed some improvements in both mood and anxiety, indicating that the positive effects of minocycline observed in the fly and mouse models may translate to the clinic (Leigh et al., 2013; Paribello et al., 2010).

Clearly the study of disruptions in the neural architecture of the sLNvs, MBs and NMJs has yielded promising insight into the ways in which the loss of dFMRP affects neuron morphology. However, the role of dFMRP extends beyond regulation of neural branching. Specifically, a novel phenotype has been identified in a subset of the pigment dispersing factor (PDF)-positive neurons located in the tritocerebrum, known as the PDF-TRI cells. While these neurons normally undergo apoptosis within two days of eclosion (Gatto and Broadie, 2011; Renn et al., 1999), experimental results indicate that the PDF-TRI cells fail to properly undergo apoptosis in *dfmr1* null flies (Gatto and Broadie, 2011). Further, another class of neurons entitled the crustacean cardioactive peptide (CCAP) peptidergic neurons exhibit delayed clearance in *dfmr1* null flies (Gatto and Broadie, 2011). Thus, these findings highlight a critical role for dFMRP in the spatiotemporal regulation of apoptosis in the CNS. Although the functional implications of the ectopic persistence of these neurons are yet to be determined, these findings highlight another mechanism by which loss of dFMRP disrupts neural architecture. These results suggest that further exploration of the mechanism by which FMRP influences synaptic architecture and

the resultant functional consequences that occur in the absence of FMRP may reveal promising, novel targets for therapeutic intervention.

Inhibitory Circuit

At the circuit level, multiple studies have shown that the excitatory/inhibitory (E/I) balance is perturbed in FXS neuronal networks (Cea-Del Rio and Huntsman, 2014; Doll and Broadie, 2014; Gatto and Broadie, 2010). Since the correct E/I balance is critical for proper maintenance of neuronal networks, several groups have postulated that E/I imbalance in the circuitry of CNS might underlie many of the observed deficits in FXS. Recent work in the *Drosophila* model system has broadened our understanding of how inhibitory and excitatory neural circuits contribute to FXS disease pathogenesis.

One factor that has been implicated in circuit dysfunction in FXS is altered inhibitory GABAergic neurotransmission (Cea-Del Rio and Huntsman, 2014; Doll and Broadie, 2014; Paluszkiwicz et al., 2011). While the theory that hypoinhibition is a causal factor in FXS has garnered much appreciation, the field has been stymied by seemingly contradictory results regarding alterations observed in GABAergic signaling in animal models of FXS. Fortunately, recent advances in *Drosophila* genetics have rendered the fly model of FXS a particularly useful tool to assay GABAergic circuitry.

Drosophila researchers pioneered the notion that GABA has therapeutic potential for FXS following the result of a high throughput chemical screen to identify compounds that rescued excess dietary glutamate-induced lethality in *dfmr1* null flies. Strikingly, in addition to preventing lethality, pharmacological treatment with GABA or GABA reuptake inhibitors was able to rescue neuroanatomical defects, excess *futsch* translation, and abnormal naïve courtship behavior in *dfmr1* null flies (Chang et al., 2008). Despite these promising initial observations, recent research suggests that pharmacological augmentation of GABAergic signaling alone is not sufficient to restore MB-dependent olfactory learning deficits (Gatto et al., 2014). Close examination of GABAergic circuitry in the MB identified several molecular changes that arise in the absence of dFMRP (Gatto et al., 2014). Specifically, the *dfmr1* null flies have reduced expression of GABA_A receptor subunits as well as decreased levels of the rate-limiting enzyme involved in GABA synthesis, glutamic acid decarboxylase (GAD). From a functional standpoint, Gatto et al. (2014) found that calcium signaling and kinetics in response to acute depolarization are altered in the inhibitory neurons of the MB in *dfmr1* null flies. Moreover, the GABAergic neurons that innervate the MB exhibit aberrant architectural development. Interestingly, the developmental trajectory of these neurons is defined by underdevelopment at earlier stages that is subsequently followed by overelaboration. Gatto et al. (2014) noted that they observe undergrowth in these inhibitory MB cells at the same time that overgrowth has been reported in excitatory MB cells (Siller and Broadie, 2011). Based on this contrast, Gatto et al. (2014) postulate that dFMRP may play different roles in inhibitory versus excitatory MB cell populations. This possibility suggests that dFMRP function is exquisitely cell-type specific.

The notion that GABAergic circuit function in the CNS may not be uniform has also been noted in murine models of FXS. At the molecular level, there appear to be brain region specific alterations in the levels of GABA receptor subunits as well as in enzymes involved in GABA synthesis, transport, and catabolism in *Fmr1* knockout mice (Adusei et al., 2010; D'Hulst et al., 2009; El Idrissi et al., 2005; Olmos-Serrano et al., 2010; Paluszkiwicz et al., 2011). These changes include an apparent decrease in the levels of GAD and vesicular GABA in the amygdala of *Fmr1* knockout mice (Olmos-Serrano et al., 2010). In contrast, increases in the levels of GAD and the β -subunit of the GABA receptor have been observed in the cortex, hippocampus, brainstem, and diencephalon of *Fmr1* knockout mice (El Idrissi et al., 2005). In addition to these regional differences, GABA_A receptor subunits and GABA enzymes demonstrate extremely dynamic and complex changes in their expression profiles at different times during

development in *Fmr1* knockout mice (Adusei et al., 2010). From a behavioral standpoint, treatment with GABA_A receptor agonists has been shown to decrease the incidence of audiogenic seizures in *Fmr1* knockout mice (Darnell and Klann, 2013; Heulens et al., 2012). Similarly, treatment with arbaclofen, a GABA_B receptor agonist, was shown to correct exaggerated protein synthesis, reduce susceptibility to audiogenic seizures, and normalize repetitive behavior in the murine model of FXS (Darnell and Klann, 2013; Henderson et al., 2012). However, it has been shown that while pharmacological administration of a GABA_A receptor agonist ameliorates excitability deficits in the amygdala, hyperactivity, and deficits in pre-pulse inhibition, this manipulation exacerbates cued fear and startle responses (Olmos-Serrano et al., 2011). Given these findings it appears that from a therapeutic perspective GABA receptor agonists are potentially advantageous in some contexts, but that they are by no means a panacea. Additional studies must be conducted to ascertain the precise spatiotemporal role of FMRP in GABAergic circuitry in order to identify whether this is a viable intervention strategy and, if so, which patients are the best candidates for treatment with these compounds.

Excitatory Circuit

Much like the extensive literature on inhibitory circuits, it is widely appreciated that FMRP is important in excitatory circuits. It is primarily thought that FMRP modulates excitatory circuits through its relationship with metabotropic glutamate receptors (mGluRs). A prevalent hypothesis in the field suggests that the mGluR pathway is hyperactive in FXS (Bear et al., 2004). Consistent with this theory, it has been shown that inhibition of the mGluR pathway rescues behavioral and anatomical phenotypes in both fly and mouse models of FXS (Dolen et al., 2007; McBride et al., 2005; Michalon et al., 2012; Yan et al., 2005).

Lately, the focus of the field has shifted towards the study of several other signaling pathways that are dysregulated in FXS. Many recent studies have sought to assess the interplay between these pathways, particularly the way in which molecules that participate in multiple signaling pathways contribute to the observed behavioral and cognitive deficits. One such study in the *Drosophila* model of FXS indicates that while mGluR antagonists rescue an age-onset defect in a social learning paradigm, this deficit was also rescued by lithium, a known direct and indirect inhibitor of Glycogen Synthase Kinase-3 (GSK-3) (Choi et al., 2010). Lithium was previously identified as a compound of interest when it was shown to rescue short-term memory deficits in *dfmr1* null flies (McBride et al., 2005). Subsequent studies in murine models of FXS provide additional evidence that GSK-3 is hyperactive in the disease state and suggest that the observed dysregulation of GSK-3 is likely caused by decreased levels of inhibitory phosphorylation at a conserved residue (Yuskaitis et al., 2010). A recent study that probed the interaction between the mGluR pathway and the GSK-3 pathway found that inhibition of mGluR5 resulted in increased inhibitory phosphorylation of GSK-3 (Yuskaitis et al., 2010). This finding suggests that the observed excess in glutamatergic signaling might be linked to GSK-3 hyperactivity. Notably, another group found that treatment of *Fmr1* knockout mice with highly specific pharmacological inhibitors of GSK-3 normalized multiple phenotypes that appear to be resistant to pharmacological inhibition of the mGluR pathway (Franklin et al., 2014). The dichotomy between the observations in Yuskaitis et al. (2010) and Franklin et al. (2014) indicates that the mGluR and GSK-3 pathways collaborate in some, but not all situations to promote normal behavior.

The field must remain cognizant that mGluR inhibitors do not rescue all of the phenotypes observed in animal models of FXS. Studies in *dfmr1* null flies suggest that pharmacological treatment with mGluR inhibitors does not ameliorate irregular grooming patterns, arrhythmic circadian behavior, and increased sleep (Bushey et al., 2009; McBride et al., 2005; Tauber et al., 2011). Similarly, it has been shown that inhibition of the mGluR pathway does not rescue macro-orchidism in the murine model of FXS (Dolen et al., 2007). Taken together, these results indicate that while increased activity of the mGluR pathway is involved in FXS, this disease is not solely a product of mGluR hyperactivation. Instead

the dysregulation of and complex interactions between multiple signaling pathways underlie FXS pathogenesis.

Peripheral Motor Output Pathways

While much emphasis has been placed on understanding the role of FMRP in the CNS, many studies have demonstrated that FMRP is also important in peripheral motor output pathways. In *Drosophila*, the larval NMJ has been established as a highly tractable, well-defined system for the study of synaptic structure and function in a peripheral motor context. It is widely appreciated that loss of dFMRP results in robust NMJ phenotypes including synaptic overgrowth and altered neurotransmission (Zhang et al., 2001). However, the precise mechanistic underpinnings of these defects remain largely unknown. Recently, research in *dfmr1* null flies has greatly expanded our understanding of how dFMRP involvement in post-transcriptional gene regulation as well as in cascades that govern trans-synaptic signaling is critical for proper synaptic structure and function (Bhogal et al., 2011; Friedman et al., 2013; Xu et al., 2013).

In addition to its predicted function as a translational regulator, FMRP has also been shown to modulate other aspects of RNA biology (Bhogal et al., 2011; Xu et al., 2013). An elegant series of biochemical and genetic experiments revealed that dFMRP acts in the RNA editing system to regulate synaptic architecture (Bhogal et al., 2011). Specifically, Bhogal et al. (2011) demonstrated that dFMRP and *Drosophila* adenosine deaminase acting on RNA (dADAR) biochemically interact. To probe the physiological relevance of this interaction *in vivo*, genetic epistasis experiments were conducted in flies with loss-of-function mutations in both *dfmr1* and *dAdar*. Since the phenotypes associated with each single mutant are distinct, analysis of double mutant flies allowed Bhogal et al. (2011) to ascertain that dFMRP acts upstream of dADAR in a common pathway responsible for the establishment of NMJ morphology. Examination of editing in several candidate transcripts revealed that RNA editing was in fact altered in *dfmr1* null flies, indicating that the interaction between dFMRP and dADAR has functional consequences (Bhogal et al., 2011). Further work will be necessary to determine if RNA editing is similarly affected in *Fmr1* knockout mice and human patients (Bassell, 2011). Importantly, this study was the first to identify an interaction between a disease-associated protein and a protein involved in Adenosine-to-Inosine RNA editing.

Similarly, dFMRP has been shown to biochemically and genetically interact with *Drosophila* topoisomerase 3 β (dTop3 β) and *Drosophila* Tudor domain-containing protein 3 (dTDRD3) in a complex that is conserved in mammals (Xu et al., 2013). Like dFMRP, both dTop3 β and dTDRD3 associate with mRNA (Linder et al., 2008; Xu et al., 2013). To evaluate the function of the resultant protein complex *in vivo*, Xu et al. (2013) examined phenotypes generated in the *Drosophila* eye and NMJ in response to manipulation of the corresponding genes. Based on the results of genetic epistasis experiments, Xu et al. (2013) concluded that *dTop3 β* and *dfmr1* functionally antagonize each other to regulate eye development and NMJ formation. Notably, this result is reminiscent of the genetic interaction that Bhogal et al. (2011) observed between *dfmr1* and *dAdar*. In contrast, the other member of this complex, dTDRD3, appeared to have a positive effect on dFMRP function in the *Drosophila* eye. The opposing roles that dTop3 β and dTDRD3 play with respect to eye development suggest that these proteins may have an antagonistic relationship in this context. Together, these findings reveal that the interaction between dFMRP, dTop3 β , and dTDRD3 is incredibly cell-type specific. In addition to cell-type specificity, (Xu et al., 2013) demonstrated that the functional relationship between dFMRP and dTop3 β is distinct at the level of gene regulation. Contrary to the observed negative interaction between dFMRP and dTop3 β in eye development and overall NMJ architecture, these proteins were shown to operate coordinately to promote the expression of a common target, *ptk2*, in NMJs. As such, it is possible that dFMRP may antagonize dTop3 β function on certain mRNAs and facilitate its action on other mRNAs to regulate gene expression at the synapse. To further illustrate the importance of Top3 β in synapse

formation, Xu et al. (2013) cultured and stained primary cortical neurons from Top3 β knockout mice and showed that synapse formation is altered in these animals. The discovery of these novel roles for FMRP in synaptogenesis greatly expands our understanding of FMRP function. However, the evidence for seemingly contradictory functions of FMRP in different contexts leaves the field with more questions than answers. It has become increasingly evident that to gain a better grasp of FXS pathogenesis the field needs to delve deeper into the mechanistic underpinnings of the divergent actions of FMRP.

One approach that addresses the nuances of FMRP function is a shift in focus from cell-autonomous to non-cell autonomous roles of FMRP. Several studies have elucidated both pre- and post-synaptic requirements for FMRP (Gatto and Broadie, 2008; Schreiner et al., 2012). As such, examination of the extracellular synaptomatrix and the relevant trans-synaptic signaling pathways in *dfmr1* null flies has provided a broader view of how FMRP acts on both sides of the synaptic cleft to impact neural architecture (Friedman et al., 2013). Several lines of evidence suggest that dysregulation of a class of membrane-bound proteins that bind ligands proximal to the cell surface to modulate downstream signaling pathways, collectively entitled heparin sulfate proteoglycans (HSPGs), might underlie the aberrant synaptogenesis present in FXS. Central to this hypothesis, FMRP has been shown to bind to HSPG mRNA directly (Darnell et al., 2011). To test whether the absence of FMRP impacts HSPG levels and consequentially alters downstream trans-synaptic signaling, Friedman et al. (2013) conducted a series of experiments to evaluate the effect of genetic manipulation of these factors on NMJ structure and function. First, Friedman et al. (2013) demonstrated that the synaptic HSPG co-receptors, Dally-like protein (Dlp) and Syndecan (Sdc), are upregulated in the NMJs of *dfmr1* null flies. Next, they established that in the absence of dFMRP, trans-synaptic signaling was depressed in two parallel anterograde pathways that are regulated by the presynaptic ligands Wingless (Wg) and Jelly belly (Jeb). The altered abundance of Wg and Jeb in *dfmr1* null flies resulted in reduced downstream signaling in postsynaptic muscle nuclei. In contrast, retrograde BMP signaling was not impaired in *dfmr1* null flies. Taken together, these results suggest that dFMRP specifically alters anterograde trans-synaptic signaling pathways. To validate the functional consequences of HSPG co-receptor overexpression on synaptic morphology and function, Friedman et al. (2013) genetically reduced HSPG co-receptor expression in the *dfmr1* null background. As expected, correction of HSPG co-receptor overabundance was sufficient to normalize synaptic overelaboration and mitigate elevations of neurotransmission in *dfmr1* null NMJs. Moreover, this genetic manipulation restored the observed reductions in Wg and Jeb signaling to wild-type levels. These findings suggest a model in which dFMRP negatively regulates HSPG co-receptor levels during synaptogenesis at the *Drosophila* NMJ. FMRP then functions in a non-cell autonomous manner to modulate anterograde trans-synaptic signaling pathways and thereby ensure proper synaptic morphology and function. While these findings in the *Drosophila* model of FXS provide the first mechanistic evidence that aberrant trans-synaptic signaling is a causal factor in FXS pathogenesis, the functions of the extracellular synaptomatrix in structural and functional maturation of synapses are conserved in mammals (Friedman et al., 2013). Thus, it will be important to determine whether these promising observations in the *Drosophila* model of FXS can be replicated in mammalian FXS models, as further investigation of trans-synaptic signaling might have great translational relevance and provide clinicians with additional therapeutic strategies to treat FXS.

Behavioral Output Pathways

Social Interaction and Memory

In accordance with the array of neuronal aberrations that occur in the absence of dFMRP, *dfmr1* null flies exhibit abnormal behavior and cognition. Importantly, the behavioral and cognitive deficits observed in the *Drosophila* model of FXS are reminiscent of the symptoms seen in human patients (Bolduc et al., 2008; Dockendorff et al., 2002; McBride et al., 2005). While at a superficial level it seems

that alterations in neural circuitry are causative of the observed behavioral and cognitive defects, evidence suggests that this view is oversimplified. Rather, there are instances in which therapeutic interventions remedy behavioral but not neuroanatomical phenotypes in *dfmr1* null flies (McBride et al., 2005).

One behavior that is altered in *dfmr1* null flies is courtship. An innate social program exists in *Drosophila* in which male flies court female flies using a stereotypical sequence of behaviors (Greenspan and Ferveur, 2000). When a female fly is receptive to the advances of a male fly, the male typically remains engaged in the courtship ritual until copulation is achieved. In contrast, *dfmr1* null males quickly lose interest in social interaction with females in this courtship paradigm. As a result, *dfmr1* null flies exhibit an overall decrease courtship levels and fail to proceed to the latter, more advanced stages of the courtship ritual (Dockendorff et al., 2002). Consistent with these observations, novel findings presented in Bolduc et al. (2010b), indicate that *dfmr1* null flies have an additional deficit in social interaction. Specifically, the authors found that *dfmr1* null flies in a chamber are less likely to interact with conspecifics placed in an adjacent chamber than their wild-type counterparts (Bolduc et al., 2010b).

In addition to the observed social deficits, *dfmr1* null flies exhibit memory impairments as assessed using both odor-based classical and courtship-based conditioning paradigms. The basic premise of odor-based classical conditioning is that adult flies are exposed to two odors, one of which is paired with an electrical shock. After a training period, flies are queried for their ability to remember which of the two odors was associated with the aversive stimulus during the training phase (Tully and Quinn, 1985). In courtship-based testing, adult males are paired with an unreceptive female during training and are subsequently tested for a reduction in courtship amount when paired with a receptive female. It is thought that the suppression of courtship amount during the testing phase indicates that the male fly remembers the negative experience with an unreceptive female during training (Siegel and Hall, 1979). The length of time between the training and testing phases varies based upon the type of memory that is being assessed. Many studies that employed these memory assays found that *dfmr1* null flies exhibit deficits in immediate recall-, short-term-, and long-term memory (Banerjee et al., 2010; McBride et al., 2005). However, it should be noted that some manipulations that were found to ameliorate the observed memory deficits did not normalize defects in MB architecture (McBride et al., 2005). Further, it appears that the requirement for *dfmr1* expression varies in subsets of neurons that comprise the MB. Specifically, concurrent *dfmr1* expression in the α , β , and γ lobes of the MB in a genetic background that is otherwise *dfmr1* null is sufficient to rescue olfactory learning defects. In contrast, restoration of *dfmr1* expression in the γ lobes of the MB alone or in the ellipsoid body alone is not sufficient to rescue the observed olfactory learning defect (Kanellopoulos et al., 2012). Similarly, genetic knock down of *dfmr1* expression in the α and β lobes of the MB was detrimental to learning, whereas genetic knock down *dfmr1* expression in the γ lobes of the MB or ellipsoid body had no negative effect on learning (Kanellopoulos et al., 2012). Thus it is likely that multiple cell type-specific requirements for *dfmr1* expression converge to regulate behavioral and cognitive output.

Studies of behavior and cognition in the *Drosophila* model of FXS have also allowed for the identification of signaling molecules that are dysregulated in the disease state. The results of these studies are highly relevant at the translational level, as they uncover novel target for therapeutic intervention. One molecule that has recently been implicated in FXS pathogenesis is cyclic AMP (cAMP). Multiple lines of evidences indicate that cAMP levels are decreased in the absence of dFMRP and that that the circadian oscillation of cAMP response element binding protein (CREB) is dampened in *dfmr1* null flies (Dockendorff et al., 2002; Kelley et al., 2007). These pioneering studies prompted exploration of the therapeutic potential of pharmacological augmentation of cAMP levels. As predicted, experiments in both the *Drosophila* and murine models of FXS demonstrated that treatment with Rolipram, a Phosphodiesterase-4 (PDE-4) inhibitor, pharmacologically restored cAMP levels and rescued deficits in associative learning, memory, and mGluR-dependent long term depression (Choi et al., 2015;

Kanellopoulos,etal.,2012).

Circadian Rhythms and Sleep:

Although *dfmr1* null flies display normal entrainment and circadian behavior in the presence of light cues, they are unable to maintain a free-running circadian rhythm in complete darkness (Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002). Despite this robust behavioral phenotype, the circadian clock in the CNS appears to function normally in *dfmr1* null flies. Thus it is likely that the observed behavior results from a defect in the circadian output pathway (Dockendorff et al., 2002; Inoue et al., 2002). With the advent of increasingly nuanced genetic tools and high-resolution technology, researchers have begun to elucidate the mechanism by which circadian behavior is altered in *dfmr1* null flies.

Consistent with the observed arrhythmic behavior in the absence of light cues, circadian gene expression is altered in *dfmr1* null flies (Xu et al., 2012). In addition to differences in mRNA levels, the expression and processing of several miRNAs appear to be altered in a circadian manner in *dfmr1* null flies (Xu et al., 2012). This finding is particularly interesting because it suggests that the interaction between dFMRP and the miRNA pathway functionally impacts circadian regulation of gene expression. In addition to defective circadian behavior, evidence suggests that *dfmr1* null flies have altered sleep profiles. Specifically, *dfmr1* null flies have been shown to sleep longer than their wild-type counterparts (Bushey et al., 2009). The observed increase in total sleep appears to be largely due to a higher frequency of sleep episodes and occurs both in the presence and absence of light: dark cues, indicating that it is mechanistically distinct from the circadian phenotype (Bushey et al., 2009). Moreover, *dfmr1* null flies exhibit augmented resistance to arousal (Bushey et al., 2009; van Alphen et al., 2013). At the molecular level, sleep deprivation results in increased levels of dFMRP (Bushey et al., 2009). Structurally, dFMRP has been shown to regulate the pruning of synaptic branches in response to sleep (Bushey et al., 2011). These results indicate that proper *dfmr1* expression plays a critical role in maintaining sleep levels and sleep-dependent synaptic homeostasis.

Notably, the symptoms reported in children with FXS appear to contradict the sleep phenotype observed in *dfmr1* null flies. For example, caregiver reports indicate that FXS patients have difficulty falling and asleep have frequent episodes of arousal during the night (Gould et al., 2000; Kronk et al., 2010; Kronk et al., 2009). In contrast, it has been reported that FXS patients have relatively increased levels melatonin, a sleep-promoting hormone, throughout the circadian period. (Gould et al., 2000). It is possible that these seemingly incompatible phenotypes can be explained by other medical problems such as sleep apnea, which appears to co-occur with sleeping difficulties in FXS patients (Kronk et al., 2010; Kronk et al., 2009). Such contradictory results emphasize the limitations of subjective, self-reported phenotypic measures and the urgent need for the discovery of biomarkers, which would provide tools for more objective assessments. Moreover, they highlight the necessity for more comprehensive studies of the FXS population to determine the prevalence of such disturbances.

Response to Stress

DNA Damage Response

Recently, it has been shown that dFMRP plays a role in the cellular response to DNA damage. Phenotypic evidence suggests that *dfmr1* null flies are more susceptible to irradiation or mutagen-induced genotoxic stress than their wild-type counterparts. Moreover, in the absence of dFMRP, larval brain cells fail to arrest at the G2/M checkpoint in response to DNA damage. It is possible that this improper response is due in part to increased levels of cyclin B in *dfmr1* null flies (Liu et al., 2012). Further evidence that dFMRP functions in the nuclear response to DNA damage comes from a recent study that demonstrated that damage induced by UV irradiation and pharmacological treatment with mutagenic compounds leads to increased nuclear localization of dFMRP and a greater amount of

chromatin bound by dFMRP (Zhang et al., 2014). Consistent with these findings, *dfmr1* null flies have little to no phosphorylation of H2AV, which is the *Drosophila* form of the mammalian histone variant H2AX. Loss of H2AV phosphorylation is particularly deleterious because when double-stranded breaks are induced in DNA, H2AV is activated by phosphorylation and proceeds to mediate the cellular response to DNA damage (Zhang et al., 2014). Much like what is observed in *Drosophila*, FMRP binds to chromatin and regulates H2AX phosphorylation in response to DNA in mammalian models (Alpatov et al., 2014). Interestingly, the prevalence of cancer appears to be decreased in FXS patients (Schultz-Pedersen et al., 2001; Sund et al., 2009). Conversely, increased *Fmr1* expression has been correlated with increased tumor metastasis (Luca et al., 2013). Since experimental evidence suggests that FMRP plays a role in the regulation of the DNA damage response and cancer susceptibility, these recently discovered functions of FMRP merit further exploration.

Stress Granules

Several studies in mice have demonstrated that FMRP recruits mRNA into stress granules (SGs), which are foci for translational repression in response to cellular stress (Kim et al., 2006; Mazroui et al., 2002). However, these studies did not assess whether FMRP is critical for the formation of SGs. This question was addressed in a recent study in a *Drosophila* cell based model. The authors found that much like its mammalian ortholog, dFMRP is also recruited into SGs upon heat shock or other forms of cellular stress (Gareau et al., 2013). While it appears that dFMRP is dispensable for SG formation, Gareau et al. (2013) revealed that dFMRP shuttles in and out of SGs and identified the motifs in dFMRP that are important for these shuttling dynamics. This finding is exciting, as this is the first report of a protein that shuttles between RNA localized in SGs and the cytosol.

Conclusion:

Although disheartening, recent studies highlight the notion that dFMRP functions in an exquisitely cell type- and temporal-specific manner. Therefore it is likely that apparent contradictions in the literature reflect the heterogeneity of dFMRP function in different spatiotemporal contexts. Nevertheless, several exciting new discoveries in *Drosophila* and murine models of FXS have exposed nuances in FMRP activity. In a broad sense, studies in the CNS of *dfmr1* null flies revealed that dFMRP is involved in processes from NB reactivation to the proper establishment and maintenance of the cytoskeleton to the regulation of inhibitory and excitatory circuits. Moreover, dFMRP was observed to play a role in RNA editing and trans-synaptic signaling in larval NMJs. From a behavioral and cognitive standpoint, recent studies of the *Drosophila* model of FXS allowed for the discovery of novel therapeutic targets and lead to the identification of specific cellular populations that contribute to the observed memory, circadian, and sleep deficits. dFMRP was also found to be involved in the DNA damage response and the response to cellular stressors. The potential link between FMRP and cancer susceptibility is particularly intriguing at a translational level. Viewing the current literature through a broader lens is useful both for re-evaluation of existing data as well as for exploring the non-neuronal, systemic aspects of FXS. Future experiments in the *dfmr1* null flies would be particularly advantageous because the vast array of genetic manipulations available in *Drosophila* allow for expedient determination of the precise spatial and temporal requirements for dFMRP. These experiments would enable researchers to ascertain critical time points for interventions as well as explore the influence of variability in genetic background on treatment efficacy with a much greater degree of statistical power than can be achieved in clinical studies.

Ultimately, it is an exciting time in FXS research because the field is at an ideological crossroads. The etiology of FXS and the hallmark symptoms observed in human patients are well characterized, however, there is still much to be learned with regard to the molecular underpinnings of FXS. This incomplete understanding of the precise mechanisms that contribute to disease pathogenesis has

precluded the identification of effective therapeutic approaches to improve the lives of affected individuals. The most salient example of this problem is the recent finding from two independent clinical trials that pharmacological treatment with mGluR antagonists failed to provide therapeutic benefit to FXS patients. Moving forward, there are several ways in which more stringent preclinical research can improve the likelihood of future success. One important contribution would be the identification of biomarkers that clinicians could use to assess which patients would be the best candidates for treatment with certain therapeutic approaches. The discovery of biomarkers would also provide objective, quantitative measures of treatment efficacy, as clinical trials are currently limited to subjective assessments. Additionally, once a therapeutic target has been identified, there should be preclinical efforts to determine whether there are any interactions between the therapeutic candidate and the combination of medications prescribed to FXS patients. It is possible that the clinical trials with mGluR antagonists were unable to detect a therapeutic effect because other medications in the patients' treatment regimen modulated or impaired the activity of the mGluR antagonists used in the studies. This can be easily addressed at the preclinical stage in *Drosophila* or mice by administering a combination of the medications currently used to treat FXS patients along with a promising therapeutic target such as mGluR inhibitors in several different genetic backgrounds to determine whether this intervention is able to ameliorate the observed defects in a context that more closely reflects the patient population. Given the widespread phenotypes and medical problems found to be associated with FXS, it may not be realistic to expect a panacea. However, the identification of relevant biomarkers along with discovery of more ubiquitous therapeutic targets that reduce the number of medications necessary to improve the lives of affected individuals constitute profound and achievable goals for the field.

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Phenotype	Drosophila	Mouse	Human
Cognitive Impairment	<p>Impaired immediate recall and short-term memory in courtship conditioning paradigm (McBride et al., 2005)</p> <p>Impaired long-term memory in courtship conditioning paradigm (Banerjee et al., 2010)</p> <p>Impaired immediate recall and long-term memory in olfactory conditioning assay (Bolduc et al., 2008)</p>	<p>Impaired spatial learning (Guo et al., 2012; Yan et al., 2004)</p> <p>Impaired passive avoidance (Ding et al., 2014; Michalon et al., 2012; Yuskaitis et al., 2010)</p> <p>Deficits in cued and contextual fear conditioning (Ding et al., 2014; Guo et al., 2012; Olmos-Serrano et al., 2011)</p> <p>Reversal learning deficit (Bakker et al., 1994; Perchiacca et al., 2012)</p> <p>Impaired novel object recognition (King and Jope, 2013; Perchiacca et al., 2012)</p>	<p>Intellectual disability (Hall et al., 2008; Skinner et al., 2005)</p> <p>Working memory impairment (Baker et al., 2011; Cornish et al., 2001)</p>
Alterations in Circadian Behavior and Sleep	<p>Arrhythmic circadian activity and defective clock output (Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002)</p> <p>Increased sleep that is more resistant to arousal (Bushey et al., 2009)</p>	<p>Length of circadian period shortened (Zhang et al., 2008)</p>	<p>Difficulty with falling asleep and sleep maintenance (Gould et al., 2000; Kronk et al., 2010; Kronk et al., 2009)</p> <p>Snoring and obstructive sleep apnea (Kronk et al., 2009)</p>
Abnormal social and repetitive behavior	<p>Failure of males to maintain interest in courtship (Dockendorff et al., 2002)</p> <p>Reduced social interaction (Bolduc et al., 2010b)</p> <p>Excessive grooming (Tauber et al., 2011)</p>	<p>Increased aversion to mirror (Spencer et al., 2005)</p> <p>Failure to distinguish novel social stimulus (Mines et al., 2010; Perchiacca et al., 2012; Pietropaolo et al., 2011)</p> <p>Males interact less with novel females (Pietropaolo et al., 2011)</p> <p>Altered ultrasonic vocalizations in pups and adults (Lai et al., 2014; Rotschafer et al., 2012)</p> <p>Increased self-grooming (McNaughton et al., 2008; Pietropaolo et al., 2011)</p> <p>Increased marble burying (Spencer et al., 2011; Veeraragavan et al., 2011)</p>	<p>High incidence of social anxiety (Cordeiro et al., 2011; Hall et al., 2009)</p> <p>Delayed language development (Finestack and Abbeduto, 2010; Roberts et al., 2001)</p> <p>Repetitive and self-injurious behavior (Symons et al., 2003; Wolff et al., 2013)</p>
Neuronal Architecture Defects	<p>Synaptic overgrowth at neuromuscular junction (Zhang et al., 2001)</p> <p>Lateral neurons show abnormal branching (Dockendorff et al., 2002; Morales et al., 2002)</p> <p>Branching and axon guidance defects in dorsal neurons (Morales et al., 2002)</p> <p>Cross-over of mushroom body β-lobes (Michel et al., 2004; Reeve et al., 2005)</p>	<p>Increased dendritic spine density and length (Comery et al., 1997; Irwin et al., 2002; Nimchinsky et al., 2001)</p>	<p>Increased spine density with elongated spines (Hinton et al., 1991; Irwin et al., 2001; Wisniewski et al., 1991)</p>

Table 1. Comparison of phenotypes in *Drosophila* and mouse models of fragile X with symptoms seen in human patients.

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