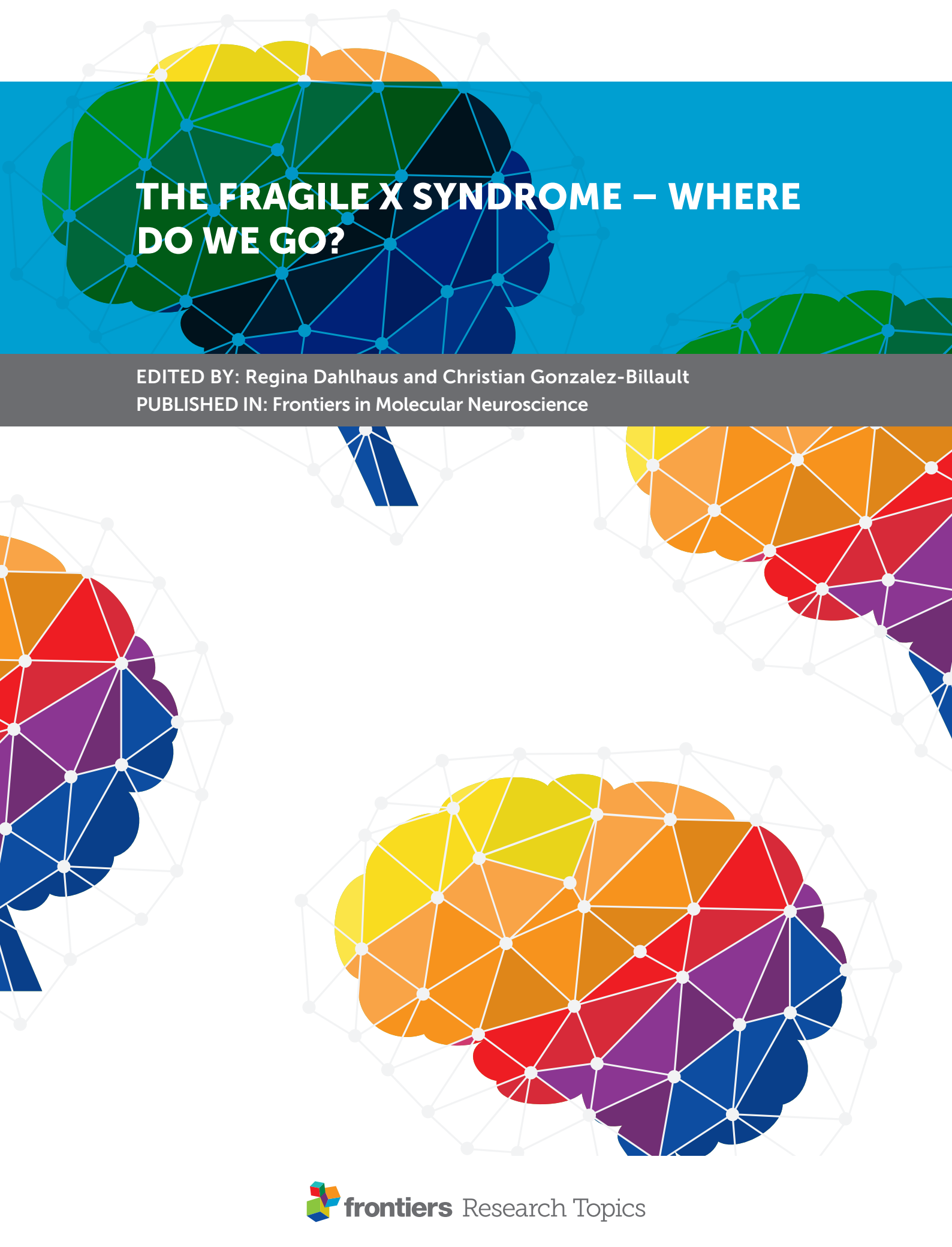


The background of the entire page features a stylized brain composed of various colored segments (yellow, orange, red, purple, blue, green) arranged in a circular pattern. Overlaid on this brain is a network of white lines connecting small white dots, representing neural connections. The top half of the image has a solid blue background, while the bottom half is white.

THE FRAGILE X SYNDROME – WHERE DO WE GO?

EDITED BY: Regina Dahlhaus and Christian Gonzalez-Billault
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THE FRAGILE X SYNDROME – WHERE DO WE GO?

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Commentary: Depletion of the Fragile X Mental Retardation Protein in Embryonic Stem Cells Alters the Kinetics of Neurogenesis

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Keywords: APP, FMRP, fragile X syndrome, secretases, neurogenesis

A commentary on

Depletion of the Fragile X Mental Retardation Protein in Embryonic Stem Cells Alters the Kinetics of Neurogenesis

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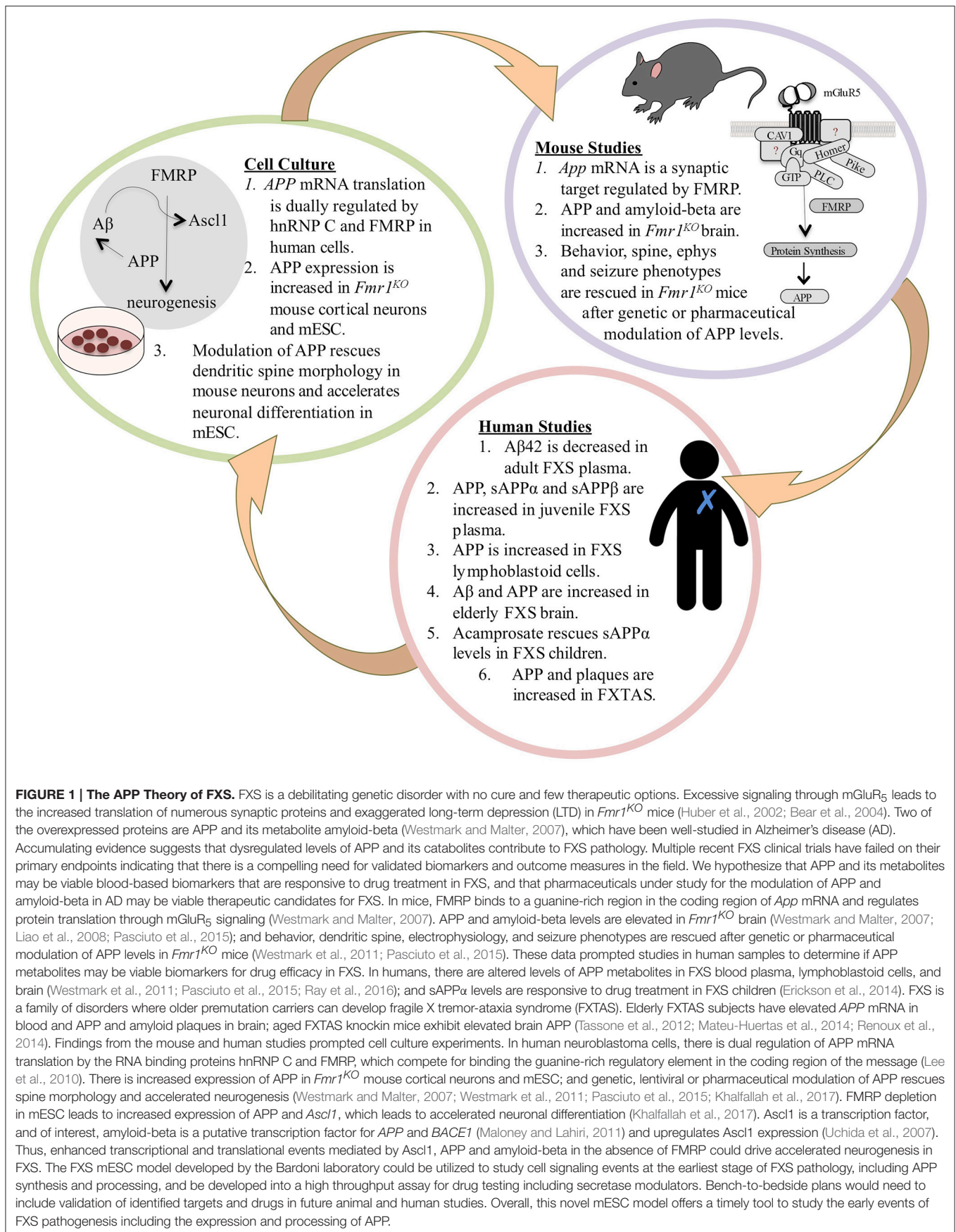
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Fragile X syndrome (FXS) is a neurodevelopmental disorder characterized by cognitive impairment, attention deficit, hyperactivity, anxiety, unstable mood, autistic behaviors, language delay, and seizures (Hagerman et al., 2010). This X-linked chromosome disorder is the most common known cause of autism with 30% of boys meeting full autism criteria (Harris et al., 2008). In the majority of cases, FXS is caused by a trinucleotide repeat expansion (CGG) in the *FMR1* gene, which causes loss of expression of fragile X mental retardation protein (FMRP) (Santoro et al., 2011). FMRP is an RNA binding protein that plays a critical role in synaptic protein synthesis. There are medications for managing symptoms of FXS, but there are no disease specific therapies and no cure.

The main challenges confronting the FXS field include early diagnosis, validating outcome measures for clinical trials, and identifying viable treatment targets. In terms of early diagnosis, population wide screening is feasible. Recent advances in *FMR1* allele analysis allow rapid and inexpensive assessment of CGG repeat size, the number of AGG interruptions, and methylation status from blood or saliva samples (Hayward et al., 2016). However, a FXS genetic test is not included in the newborn screening (NBS) panel in the United States due to ethical debates regarding screening for genetic disorders where no therapeutic intervention exists and contemporaneous identification of premutation carriers. Proponents of FXS NBS argue that screening is needed for early detection and intervention (Tassone, 2014). Numerous disease mechanism-based drugs are in clinical trials for FXS and early intervention may be required for better therapeutic efficacy. Preliminary results indicate that babies with *FMR1* premutations exhibit an altered developmental trajectory on measures of nonverbal communication and hyperresponsivity to sensory experiences (Wheeler et al., 2016). Thus, early diagnosis could benefit both full and premutation carriers.

With regard to the urgent need to validate outcome measures for FXS clinical trials, recent trials failed on primary endpoints (Berry-Kravis et al., 2013, 2016). Soluble amyloid precursor protein alpha (sAPP α) is elevated in plasma of autistic children and can be detected in human umbilical cord blood supporting feasibility of this APP metabolite as an early diagnostic autism biomarker (Sokol et al., 2006; Bailey et al., 2008). APP metabolites are also altered in FXS (Westmark et al., 2016), and a recent trial with acamprosate indicates that sAPP α is responsive to drug treatment (Erickson et al., 2014).



Concerning identification of viable treatment targets, excessive signaling through metabotropic glutamate receptor 5 (mGluR₅) leads to increased translation of numerous synaptic proteins and altered plasticity in FXS (Bear et al., 2004). Many of these proteins are under investigation as potential FXS drug targets. Of relevance herein, two overexpressed proteins are APP and amyloid-beta (Westmark and Malter, 2007), which have been well-studied in Alzheimer's disease (AD). Accumulating evidence suggests that dysregulated levels of APP metabolites contribute to FXS pathology (Figure 1), supporting the hypothesis that pharmaceuticals under study for modulation of APP and amyloid-beta in AD may be viable therapeutic strategies for FXS (Westmark et al., 2013; Pasciuto et al., 2015).

In their *Stem Cells* article, Khalfallah and colleagues report the development of a mouse embryonic stem cell (mESC)-based FXS disease model. Specifically, they generated an isogenic stable cell line by targeted knockdown of the *Fmr1* gene with a short hairpin (sh)RNA, and used this model to demonstrate that decreased expression of FMRP triggers accelerated differentiation of neurons and elevated expression of the transcription factor *Ascl1*/Mash1 and the membrane glycoprotein APP (Khalfallah et al., 2017). *Fmr1* mRNA and protein levels were specifically and significantly reduced in *shFmr1* mESC compared to control cells while expression of FMRP homologs was unaffected. *Fmr1* knockdown did not alter mESC morphology or proliferation; however, there was premature generation of neural progenitors as evidenced by a rosette-like morphology at 4 days *in vitro* and altered expression of neuro-specific markers. The authors confirmed that neurogenesis was accelerated *in vivo* in *Fmr1*^{KO} mouse embryonic brain. They further showed that accelerated neurogenesis in the *shFmr1* mESC model was rescued by genetic introduction of the human *FMR1* gene or pharmacological treatment with BACE-1 inhibitor LY2811376.

Neurogenesis is the process through which neurons are generated from neural stem and progenitor cells. Both FMRP and APP have evolutionarily conserved roles in regulating embryonic and adult neurogenesis (Hayashi et al., 1994; Ohsawa et al., 1999; Caille et al., 2004; Callan et al., 2010; Luo et al., 2010; Demars et al., 2011; Nicolas and Hassan, 2014; Wang et al., 2014, 2016; Faulkner et al., 2015; Halevy et al., 2015). FMRP regulates the translation of both *Ascl1* (Fahling et al., 2009) and APP (Westmark and Malter, 2007; Lee et al., 2010). *Ascl1* is involved in the transcriptional regulation of genes associated with all major steps of neurogenesis (Castro et al., 2011). APP expression, trafficking, and processing are dynamically regulated during neuronal differentiation (Bergstrom et al.,

2016; Ramaker et al., 2016). The APP metabolite amyloid-beta upregulates expression of *Ascl1* (Uchida et al., 2007) and drives the differentiation of progenitor cells toward a neuronal lineage (Calafiore et al., 2006). Thus, defective crosstalk among *Ascl1* and APP metabolites in the absence of FMRP likely contributes to accelerated neurogenesis in FXS. Early interventions targeted at normalizing this signaling pathway could promote normal brain development.

The *shFmr1* mESC model developed by the Bardoni laboratory overcomes the inherent ethical and scientific problems associated with human *FMR1* embryonic stem cells (hESC), most of which carry the methylated and silenced full mutation and/or are mosaic in CGG-repeat length and exhibit residual FMRP expression. In essence, this elegant work addresses the three main challenges of the FXS field by: (1) developing a *Fmr1* knockdown stem cell model that allows study of the earliest events of neurogenesis to support NBS and early intervention; (2) identifying a role for APP in the kinetics of neurogenesis, which supports the development of APP metabolites as potential FXS biomarkers; and (3) demonstrating rescue of *shFmr1* mESC morphology with a BACE-1 inhibitor thus promoting study of APP and secretases as therapeutic targets for FXS.

Khalfallah and colleagues contribute a vital piece to the FXS puzzle in describing development of a mESC model that allows study of early molecular events underlying disease development and provides a new platform for preclinical drug testing. Substantial data is provided validating the morphological and molecular characteristics of the *shFmr1* mESC as well as demonstrating rescue of phenotypes by re-introduction of FMRP or by targeting APP processing via inhibition of BACE-1. Future experiments could examine expression of various APP metabolites on neurogenesis, compare BACE-1 and mGluR₅ inhibitors, confirm FXS signaling pathways in this early disease-stage model, and transfect plasmids carrying varying length CGG repeats in the human *FMR1* gene to mimic the repeat expansion aspect of the disorder.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Altered Developmental Expression of the Astrocyte-Secreted Factors Hevin and SPARC in the Fragile X Mouse Model

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Astrocyte dysfunction has been indicated in many neurodevelopmental disorders, including Fragile X Syndrome (FXS). FXS is caused by a deficiency in fragile X mental retardation protein (FMRP). FMRP regulates the translation of numerous mRNAs and its loss disturbs the composition of proteins important for dendritic spine and synapse development. Here, we investigated whether the astrocyte-derived factors hevin and SPARC, known to regulate excitatory synapse development, have altered expression in FXS. Specifically, we analyzed the expression of these factors in wild-type (WT) mice and in *fragile X mental retardation 1* (*Fmr1*) knock-out (KO) mice that lack FMRP expression. Samples were collected from the developing cortex and hippocampus (regions of dendritic spine abnormalities in FXS) of *Fmr1* KO and WT pups. Hevin and SPARC showed altered expression patterns in *Fmr1* KO mice compared to WT, in a brain-region specific manner. In cortical tissue, we found a transient increase in the level of hevin in postnatal day (P)14 *Fmr1* KO mice, compared to WT. Additionally, there were modest decreases in *Fmr1* KO cortical levels of SPARC at P7 and P14. In the hippocampus, hevin expression was much lower in P7 *Fmr1* KO mice than in WT. At P14, hippocampal hevin levels were similar between genotypes, and by P21 *Fmr1* KO hevin expression surpassed WT levels. These findings imply aberrant astrocyte signaling in FXS and suggest that the altered expression of hevin and SPARC contributes to abnormal synaptic development in FXS.

Keywords: astrocyte, development, Fragile X syndrome, hevin, SPARC, synapse

INTRODUCTION

Fragile X syndrome (FXS), the most common inherited, single-gene cause of autism spectrum disorders (ASD) and cognitive impairment (reviewed in Lubs et al., 2012; Wang et al., 2012), is a neurodevelopmental disorder characterized by a deficiency in the fragile X mental retardation protein (FMRP; reviewed in Bhakar et al., 2012). Individuals with FXS can

Abbreviations: ASD, autism spectrum disorders; GFAP, glial fibrillary acidic protein; *Fmr1*, fragile X mental retardation 1; FMRP, fragile X mental retardation protein; FXS, Fragile X syndrome; KO, knock-out; MACS, magnetic-activated cell sorting; P, postnatal day; RGC, retinal ganglion cell; TBS-T, Tris-buffered saline solution with Tween-20; WT, wild-type.

exhibit mild to severe cognitive impairment, autistic behaviors, attention deficits, susceptibility to seizures, hypersensitivity to sensory stimuli, disrupted sleep, as well as an assortment neurobiological abnormalities (Comery et al., 1997; Nimchinsky et al., 2001; Beckel-Mitchener and Greenough, 2004; Kronk et al., 2010; Marco et al., 2011). Numerous studies examining the altered neurobiology in FXS have focused on the changes at the level of dendritic spines, the primary site for excitatory connections between neurons (Ivanov et al., 2009). The absence of FMRP in FXS has been associated with altered synapse structure, number and function (reviewed in Pfeiffer and Huber, 2009). Studies of FXS in humans or animal models have described a significant increase in the number of dendritic spines associated with FXS, with a greater proportion of immature spine phenotypes (i.e., long, thin, tortuous dendritic spines; Comery et al., 1997; Irwin et al., 2001; Nimchinsky et al., 2001). Under normal conditions, FMRP is expressed in neurons (Sidorov et al., 2013), oligodendrocyte precursor cells (Wang et al., 2004), and astrocyte cell lineages (Pacey and Doering, 2007) where it influences synaptic development through its ability to bind, transport, and regulate the local translation of several mRNAs corresponding to synaptic proteins (reviewed in Bhakar et al., 2012).

Recently, astrocytes have emerged in the literature as important regulators of synapse development and have been shown to promote both synapse formation and maturation (reviewed in Allen, 2013; Chung et al., 2015). For example, astrocyte-secreted factors, such as hevin (also known as synaptic cleft-1 or SPARC-like 1) and SPARC, have been implicated in governing the formation of excitatory synapses within the brain (Kucukdereli et al., 2011; Risher et al., 2014; Singh et al., 2016). In cultured retinal ganglion cells (RGCs) with enhanced expression of the matricellular protein hevin, a known target of FMRP (Darnell et al., 2011), there was a significant increase in synapse number (Kucukdereli et al., 2011). Likewise, the prevention of hevin expression in knock-out (KO) mice models causes a decrease in RGC-collicular synapses *in vivo*. Alternatively, Kucukdereli et al. (2011) demonstrated that in contrast to hevin, SPARC negatively regulates the formation of excitatory synapses by inhibiting the synaptogenic function of hevin, revealing an antagonistic relationship between these two factors. More recently, hevin has been shown to function as a trans-synaptic linker between presynaptic neurexin-1 α and post-synaptic-1B (Singh et al., 2016). In this way, hevin assists in the formation of synapses expressing this particular neurexin and neuroligin pair, a category that includes both thalamocortical synapses and RGC-collicular synapses.

Given the respective roles known for hevin and SPARC in synapse development, aberrant expression of these astrocyte-secreted factors could account for the abnormal development and maturation of excitatory synapses in FXS. Here, we compared the developmental (postnatal day [P]7–P21) expression of hevin and SPARC in wild-type (WT) mice and mice that do not express FMRP (*Fragile X mental retardation 1* [*Fmr1*] KO; Bakker et al., 1994) across two brain regions with high levels of FMRP (cortex and hippocampus; Bakker et al., 2000). Additionally, we examined WT levels of FMRP during the same postnatal

period (P7–P21) as well as thalamocortical synapse number in co-cultures containing either WT neurons and WT astrocytes or WT neurons and KO astrocytes. Importantly, our findings demonstrated that the expression of hevin and SPARC is dysregulated in both cortical and hippocampal regions with FXS. Thus, it is likely that astrocyte-mediated mechanisms significantly contribute to the neurobiological deficits associated with FXS.

MATERIALS AND METHODS

Animals

WT and *Fmr1* KO mice (FVB.129P2[B6]-*Fmr1*^{tm1Cgr}) were housed and bred in the McMaster University Central Animal Facility. All experiments and animal-handling procedures followed the guidelines set by the Canadian Council on Animal Care and were approved by the McMaster Animal Research Ethics Board (AUP 13-12-49).

Genotyping

The tails from eight randomly selected pups from a pool of pups at ages P7, P14 or P21 (4 pups from each genotype, WT and *Fmr1* KO) were collected and the genotypes of the mice were confirmed for each group via PCR (data not shown). Segments of tails 0.5–1 cm in length were each combined with 100 μ l of Extraction Solution (catalog#: E7526; Sigma-Aldrich) and 25 μ l of Tissue Preparation Solution (catalog#: T3073; Sigma-Aldrich). Samples were incubated for 10 min at 55°C and then for 3 min at 95°C. Following these incubations, 100 μ l of Neutralization Solution B (catalog#: N3910; Sigma-Aldrich) was added to each sample. To perform PCR, REDExtract-N-Amp PCR Reaction Mix (catalog#: R4775; Sigma-Aldrich) was added to each sample along with the following primers (with final primer concentrations of approximately 1 μ M): CAC GAG ACT AGT GAG ACG TG (mutant forward; primer oIMR2060; Jackson Laboratory, Bar Harbor, ME, USA), TGT GAT AGA ATA TGC AGC ATG TGA (WT forward; primer oIMR6734; Jackson Laboratory), CTT CTG GCA CCT CCA GCT T (common; primer oIMR6735; Jackson Laboratory). Following PCR, the amplified DNA samples were run through a 2% agarose gel. Gels were imaged using SYBR Safe DNA Gel Stain (Invitrogen) and a ChemiDoc Imaging System (Bio-Rad).

Cortical and Hippocampal Tissue Isolation for Western Blotting

WT and *Fmr1* KO male pups were decapitated at the age of P7, P14 and P21 and whole brains were extracted. Extracted brains were immediately placed into ice-cold, sterile, 0.01 M PBS and cortical and hippocampal tissue was dissected from each brain. Samples were immediately placed into separate microcentrifuge tubes, snap-frozen on dry ice, and stored at -80°C . Each sample of cortical or hippocampal tissue consisted of tissue from a single hemisphere.

Samples intended for hevin or FMRP analysis were mechanically homogenized on ice in lysis buffer (0.05 M Tris [pH 7.5], 0.5% Tween-20, 10 mM EDTA, Roche ULTRA protease

inhibitor tablet, Roche PhosSTOP phosphatase inhibitor tablet). Homogenates were left on ice for 15 min and then centrifuged at $2350\times g$ for 10 min at 4°C . Samples intended for SPARC analysis were mechanically homogenized on ice in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% Deoxycholic Acid, 0.1% SDS, 50 mM Tris [pH 8.0], Roche ULTRA protease inhibitor tablet, Roche PhosSTOP phosphatase inhibitor tablet). Homogenates were left on ice for 1 h and then centrifuged at $16,000\times g$ for 15 min at 4°C . The protein concentration of each supernatant was determined by a DC protein assay (Bio-Rad, Mississauga, ON, Canada). Samples were aliquoted and stored at -80°C .

Cortical Astrocyte Isolation via Magnetic-Activated Cell Sorting (MACS) for Western Blotting

WT and *Fmr1* KO pups at age P14 were decapitated, whole brains were extracted, placed in ice-cold, calcium and magnesium-free Hanks buffered saline solution (CMF-HBSS), and cortical tissue was isolated from each brain. Each collected sample consisted of tissue from 2.5 cortices. Tissue and CMF-HBSS were transferred to collection tubes containing 8 mL CMF-HBSS and subsequently treated with 1.5 mL DNase (Gold Biotechnology, St. Louis, MO, USA) and 1.5 mL 2.5% trypsin (Life Technologies, Carlsbad, CA, USA). Cell suspensions were then incubated for 5 min at 37°C , after which they were triturated using a 10 mL serological pipette (Falcon, Durham, NC, USA). Cell suspensions were incubated again for 5 min at 37°C and then triturated using a 5 mL serological pipette (Falcon). The cell suspensions were then passed through a $70\text{ }\mu\text{L}$ cell strainer and centrifuged at $150\times g$ for 5 min. Cells were re-suspended in 1800 mL of PBS (pH 7.4) containing 0.5% BSA.

In order to remove myelin debris from each sample, cell suspensions were first magnetically labeled via 15-min incubation at 4°C with $200\text{ }\mu\text{L}$ of Myelin Removal Beads II (catalog#: 130-096-731; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then washed with 18 mL of PBS with 0.5% BSA and centrifuged at $150\times g$ for 10 min. Cells were then re-suspended in $2000\text{ }\mu\text{L}$ of PBS with 0.5% BSA and passed through a MACS MS column (Miltenyi Biotec) that was mounted within the magnetic field of a MACS separator (Miltenyi Biotec). The negative fraction from each cell suspension, containing unlabeled cells, was collected for the subsequent isolation of astrocytes using an Anti-Astrocyte Cell Surface Antigen-2 (ACSA-2) Microbead Kit (catalog#: 130-097-678; Miltenyi Biotec). Of note, a maximum of 1×10^7 cells/sample were used for the next steps of the astrocyte isolation protocol.

Cell suspensions lacking myelin debris were next centrifuged at $150\times g$ for 10 min and re-suspended in $80\text{ }\mu\text{L}$ of PBS with 0.5% BSA with an additional $10\text{ }\mu\text{L}$ of Fc receptor Blocking Reagent (catalog#: 130-097-678; Miltenyi Biotec). Cell suspensions were incubated at 4°C for 10 min. Following this incubation, $10\text{ }\mu\text{L}$ of Anti-ACSA-2 Microbeads (catalog#: 130-097-678; Miltenyi Biotec) were added to each sample and incubated again at 4°C for 15 min. Cells were then washed with 2 mL of PBS with 0.5% BSA and centrifuged at $150\times g$ for 10 min. The pellet was re-suspended in $500\text{ }\mu\text{L}$ of PBS with 0.5% BSA and the

cell suspension was then passed through a MACS MS column mounted within the magnetic field of a MACS separator. The positive fraction from each sample, containing magnetically-labeled cells, was collected and centrifuged at $150\times g$ for 10 min. The supernatant was removed and the cells were immediately flash frozen using isopentane and stored at -80°C . Cells were later homogenized in lysis buffer (0.05 M Tris [pH 7.5], 0.5% Tween-20, 10 mM EDTA, Roche ULTRA protease inhibitor tablet, Roche PhosSTOP phosphatase inhibitor tablet) and the protein concentration of each sample was determined by a DC protein assay (Bio-Rad). The homogenized samples were then aliquoted and stored at -80°C .

Primary Cortical Astrocyte Cultures

Isolation and establishment of cortical astrocytes was carried out according to a protocol previously described by our laboratory (Jacobs and Doering, 2009). Cortical astrocytes were isolated from four WT or *Fmr1* KO pups at P1 or P2 and grown in T75 tissue culture flasks in minimum essential media (Invitrogen, Carlsbad, CA, USA) supplemented with 6% glucose and 10% horse serum (Invitrogen). Cultures were maintained for approximately 1 week at 37°C and 5% CO_2 . Cells were then removed from the T75 tissue culture flasks and re-plated onto coverslips coated with Poly-L-Lysine (Sigma-Aldrich, St. Louis, MO, USA; 1 mg/mL) and laminin (Invitrogen; 0.1 mg/mL) at a density of 5000 cells per coverslip. Cells were maintained on coverslips for 2 days *in vitro* for subsequent immunocytochemical processing or for astrocyte-neuron co-culture and subsequent immunocytochemical processing.

Cortical and Thalamic Neuron and Cortical Astrocyte Co-Cultures with MACS

WT and *Fmr1* KO cortical astrocytes were plated onto coverslips coated with Poly-L-Lysine (Sigma-Aldrich; 1 mg/mL) and laminin (Invitrogen; 0.1 mg/mL) at a density of 5000 cells per coverslip and maintained for 2 days *in vitro* in minimal essential media (Invitrogen) supplemented with 6% glucose (Sigma-Aldrich) and 10% horse serum (Invitrogen). After 2 days this media was switched to neural maintenance media (NMM) composed of minimal essential media (Invitrogen) supplemented with 6% glucose (Sigma-Aldrich), 1% N2 supplement (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). The following day, cortical and thalamic tissue was isolated from 5–6 WT pups aged P1 or P2. Cortical and thalamic tissue was dissociated using a neural tissue dissociation kit (catalog#: 130-092-628; Miltenyi Biotec). Following dissociation, cortical and thalamic cells were re-suspended in $80\text{ }\mu\text{L}$ of PBS with Mg^{2+} and Ca^{2+} and 0.5% BSA. Cells suspensions were then incubated with a biotin-antibody cocktail (catalog#: 130-098-754; Miltenyi Biotec). Cell suspensions were then washed with PBS with Mg^{2+} and Ca^{2+} and 0.5% BSA and centrifuged for $200\times g$ for 10 min. Cells were re-suspended in $80\text{ }\mu\text{L}$ of PBS with Mg^{2+} and Ca^{2+} and 0.5% BSA and magnetically labeled with anti-biotin microbeads (catalog#: 130-098-754; Miltenyi Biotec) that would label non-neuronal cells within the suspension. These cell suspensions were then passed twice through a MACS MS column (Miltenyi Biotec) that was mounted within a magnetic field (MACS

separator, Miltenyi Biotec). The negative fraction from each suspension, containing unlabeled cells, was collected and plated at a density of 10,000 cells per well with the previously plated astrocytes (**Figure 3**). Each neuronal suspension from one litter was always split and plated onto one independent WT astrocyte culture and one independent *Fmr1* KO culture in order to compare growth and synaptic development in a paired manner. This process was repeated across four independent experiments. Co-cultures were maintained in NMM for 14 days at 37°C and 5% CO₂ and then processed for immunocytochemical analysis.

Immunocytochemistry

Immunocytochemistry was carried out with primary cortical astrocyte cultures following a protocol previously described by Cheng et al. (2016). The following antibodies were used: rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; catalog#: Z0334; Dako, Burlington, ON, Canada), chicken anti-GFAP (1:2000; catalog#: CH22102; Neuromics, Minneapolis, MN, USA) rabbit anti-hevin antibody (1:100; catalog#: bs-6110R; Bioss, Woburn, MA, USA), goat anti-SPARC antibody (10 µg/mL; catalog#: AF942; R&D Systems, Minneapolis, MN, USA). Cells were then incubated in secondary antibodies (in 0.01M PBS) for 3 h at room temperature. These included donkey anti-rabbit Alexa Fluor 568 (1:200; catalog#: A10042; Invitrogen), donkey anti-goat FITC (1:100; catalog#: 705-095-147; Jackson, West Grove, PA, USA), donkey anti-chicken FITC (1:100; catalog#: 703-095-155; Jackson). Coverslips were mounted onto slides using ProLong Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (Life Technologies, Carlsbad, CA, USA). Two independent cultures ($n = 2$) and a total of 50 cells were examined per genotype. Images were acquired using a Zeiss AxioImager M2 (Zeiss, Oberkochen, Germany) microscope.

In addition, astrocyte and neuron co-cultures were processed in the same manner in order to identify co-localized VGlut2⁺ pre-synaptic and PSD95⁺ post-synaptic puncta. The following primary and secondary antibodies were used: rabbit anti-vesicular glutamate transporter 2 (VGlut2; 1:500; catalog#: 135 403; Synaptic Systems, Göttingen, Germany), mouse anti-post-synaptic density protein 95 (PSD95; 1:100; catalog#: MAB1596; Millipore), rabbit anti-GFAP (1:500; catalog#: Z0334; Dako, Burlington, ON, Canada), chicken anti-microtubule associated protein 2 (MAP2; 1:1000; catalog#: CH22103; Neuromics, Minneapolis, MN, USA), goat anti-rabbit FITC (1:100; catalog#: 111-095-144; Jackson ImmunoResearch), donkey anti-mouse Alexa Fluor 594 (1:1500; catalog#: A-21203; Invitrogen), donkey anti-rabbit Alexa Fluor 568 (1:200; catalog#: A10042; Invitrogen), donkey anti-chicken FITC (1:100; catalog#: 703-095-155; Jackson ImmunoResearch). Eight independent co-cultures ($n = 4$) were examined per paired condition. Co-cultures were plated on 24 well plates and wells were randomly selected for analysis (minimum 5 wells to a maximum of 17 wells were used for each n). Wells were discarded for analysis based on astrocyte density, only coverslips with astrocytes 70%–80% confluent were used to reduce variability among the conditions. The synapse counts were averaged across the wells to produce the value for each n .

Western Blotting

Cortical and hippocampal samples containing 30 µg (homogenized whole tissue) and P14 cortical astrocyte samples containing 10 µg (isolated astrocytes) of protein were combined with 2× Laemmli Sample Buffer (Bio-Rad). Samples were heated for 5 min at 95°C, centrifuged briefly, and immediately loaded onto a gradient 4%–15% precast polyacrylamide stain-free gel (Bio-Rad) for electrophoresis. Gels intended for hevin or SPARC analysis contained age-matched WT and *Fmr1* KO samples isolated from either the whole-cortex or whole-hippocampus, and cortical astrocytes. A total of $n = 8$ samples/group were run to examine whole-cortical and -hippocampal levels of hevin and SPARC for each time-point (P7, P14 and P21) and genotype (WT and *Fmr1* KO), while a total of $n = 4$ samples/group were run to examine P14 cortical astrocyte-derived levels of hevin for each genotype (WT and *Fmr1* KO). Gels intended for FMRP analysis contained WT samples isolated from either the whole-cortex or whole-hippocampus at each time-point (P7, P14 and P21), with a total of $n = 4$ –8 samples/group. Following electrophoresis,

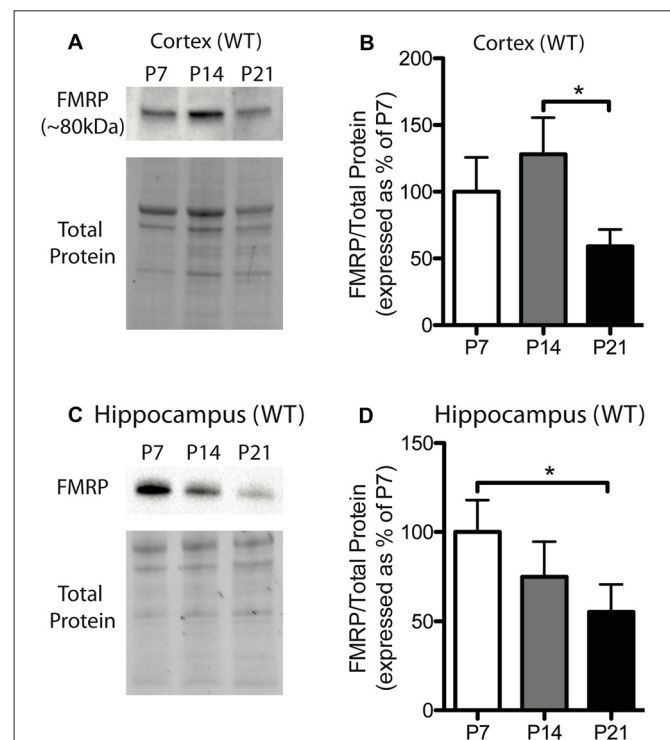


FIGURE 1 | Fragile X mental retardation protein (FMRP) expression is developmentally regulated in the cortex and hippocampus. **(A)** A representative Western blot showing FMRP (~80 kDa) in wild-type (WT) cortical samples (30 µg of protein per lane) from postnatal day (P) 7, P14 and P21 mice, as well as the total protein within each lane. **(B)** FMRP expression in the cortex of WT mice at P7 (white; $n = 8$), P14 (gray; $n = 4$), and P21 (black; $n = 8$). Bands representing FMRP were normalized against the total protein within the same lane on the membrane and a cross gel control, and then expressed as a percentage of P7 FMRP. **(C)** A representative Western blot showing FMRP expression in WT hippocampal samples (30 µg of protein per lane) from P7, P14 and P21 mice, as well as the total protein within each lane. **(D)** FMRP expression in the hippocampus of WT mice at P7 (white; $n = 6$), P14 (gray; $n = 6$), and P21 (black; $n = 6$). Statistical differences were denoted with a single asterisk, $P < 0.05$.

gels were activated with UV light (302 nm) for visualization of total protein (1 min) and the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were imaged for total loaded protein using a ChemiDoc Imaging System (Bio-Rad, Mississauga, ON, Canada), after which they were incubated for 1 h at room temperature in a 5% non-fat milk solution in Tris-buffered saline solution with Tween-20 (TBS-T). Membranes were then incubated overnight at 4°C in either anti-hevin antibody (host rabbit; 1:500; catalog#: bs-6110R; Bioss) or anti-FMRP (host rabbit; 1:1000; catalog#: 4317; Cell Signalling Technology, Danvers, MA, USA) in 5% non-fat milk/TBS-T or in anti-SPARC antibody (host goat; 0.4 µg/mL; catalog#: AF942; R&D Systems) in 2% bovine serum albumin/TBS-T. Antibodies against hevin, SPARC, and FMRP recognized bands at ~130 kDa (**Figure 2B**), ~37 kDa (**Figure 3B**), and ~80 kDa (**Figure 1A**) respectively. These bands representing hevin, SPARC, and FMRP were absent in negative controls incubated with only secondary antibody or an absence of primary antibody against either hevin, SPARC, or FMRP (**Figures 2B, 3C**). Following the incubation in primary antibody,

membranes were washed in TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody against either rabbit (1:5000; catalog#: NA934-1ML; GE Healthcare Life Sciences, Mississauga, ON, Canada) or goat (1:5000; catalog#: sc-2020; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% non-fat milk/TBS-T for hevin detection, or in TBS-T for SPARC detection, for 1 h at room temperature. Membranes were washed again in TBS-T and developed using enhanced chemiluminescence developer solutions (Bio-Rad). Membranes were scanned using a ChemiDoc Imaging System (Bio-Rad). Densitometry measurements were conducted using Image Lab Software 5.2 (Bio-Rad). Each band corresponding to either hevin (~130 kDa), SPARC (~37 kDa), or FMRP (~80 kDa) was first normalized to total protein within the same lane, and then, if necessary, to a cross gel control. These values were then expressed as a relative percentage of the average densitometry value obtained from the age-matched WT samples.

Synaptic Puncta Analysis

Images were obtained using a Zeiss AxioImager M2 (Zeiss, Oberkochen, Germany) microscope with Zeiss Zen Blue Imaging

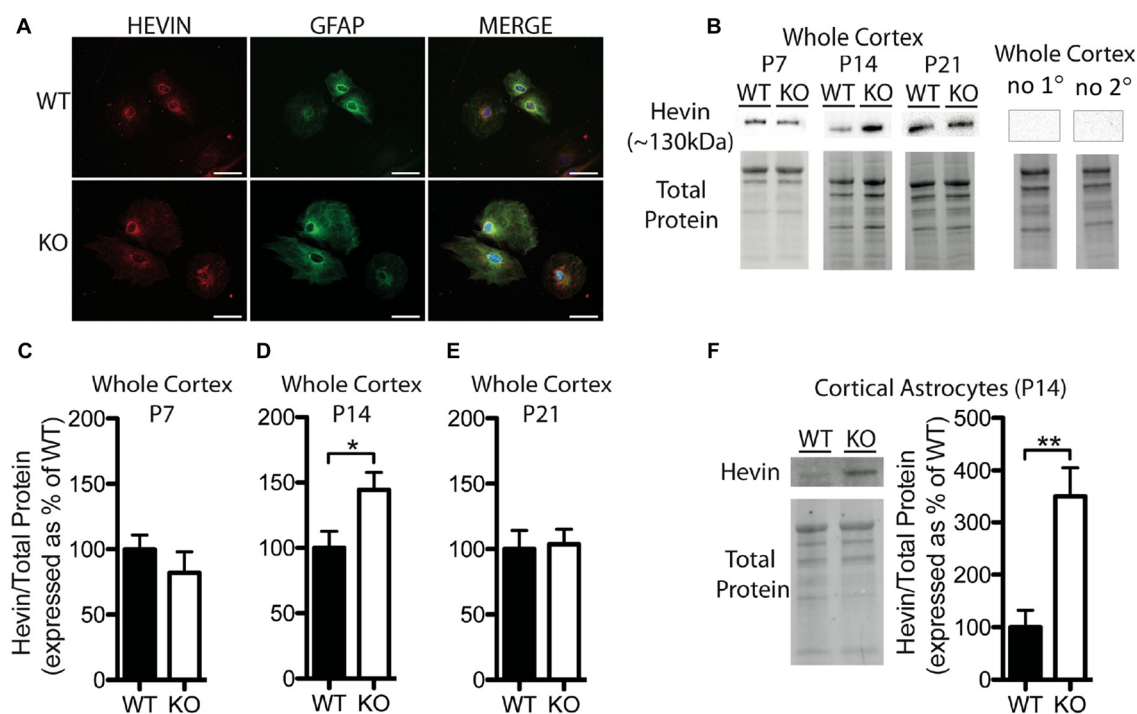


FIGURE 2 | Hevin expression is altered at postnatal day (P) 14 in the cortex of *Fmr1* knock-out (KO) mice. **(A)** Cultured cortical astrocytes co-labeled with anti-glial fibrillary acidic protein (GFAP; green) and anti-hevin (red) after 2 days *in vitro*. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Images were obtained using a 40x objective with a Zeiss AxioImager M2. Scale bars = 50 µm. **(B)** Representative western blots showing hevin (~130 kDa) in cortical samples (30 µg of protein per lane) from P7, P14 and P21 WT and *Fmr1* KO mice, as well as the corresponding total protein within each lane. Negative controls that were run using P14 WT whole cortical tissue with either no primary antibody or no secondary antibody are shown. **(C–E)** Hevin expression in the cortex of WT (black; *n* = 8) and *Fmr1* KO (white; *n* = 8) mice at P7, P14 and P21. Bands representing hevin were normalized against the total protein within the same lane on the membrane, and were then expressed as a percent of the average level of hevin in the WT group. **(F)** Hevin expression in cortical astrocytes isolated from P14 WT (black; *n* = 4) and *Fmr1* KO (white; *n* = 4) mice. Immediately to the left of the graph is shown a representative Western blot with bands corresponding to hevin from P14 WT and *Fmr1* KO cortical astrocyte samples (10 µg of protein per lane), as well as the corresponding total protein. Statistical differences were denoted with either a single asterisk, *P* < 0.05, or a double asterisks, *P* < 0.01.

Software. SynapCountJ, a custom written plug-in for ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to identify co-localized puncta. Thalamocortical synapse candidates were identified by the co-localization of presynaptic VGlut2⁺ and postsynaptic PSD95⁺ puncta. Cortical neurons were imaged, while thalamic neurons were avoided by the presence of intense VGlut2⁺ staining within the cell body. Low frequency background was removed from both the red and green channels of each image using the ImageJ rolling ball background subtraction algorithm. The dendrites of a neuron were traced using the ImageJ plugin NeuronJ. The coordinates of these tracings were uploaded into SynapCountJ along with the corresponding red and green channel images. The number of colocalized puncta was measured for each tracing and normalized to the tracing length.

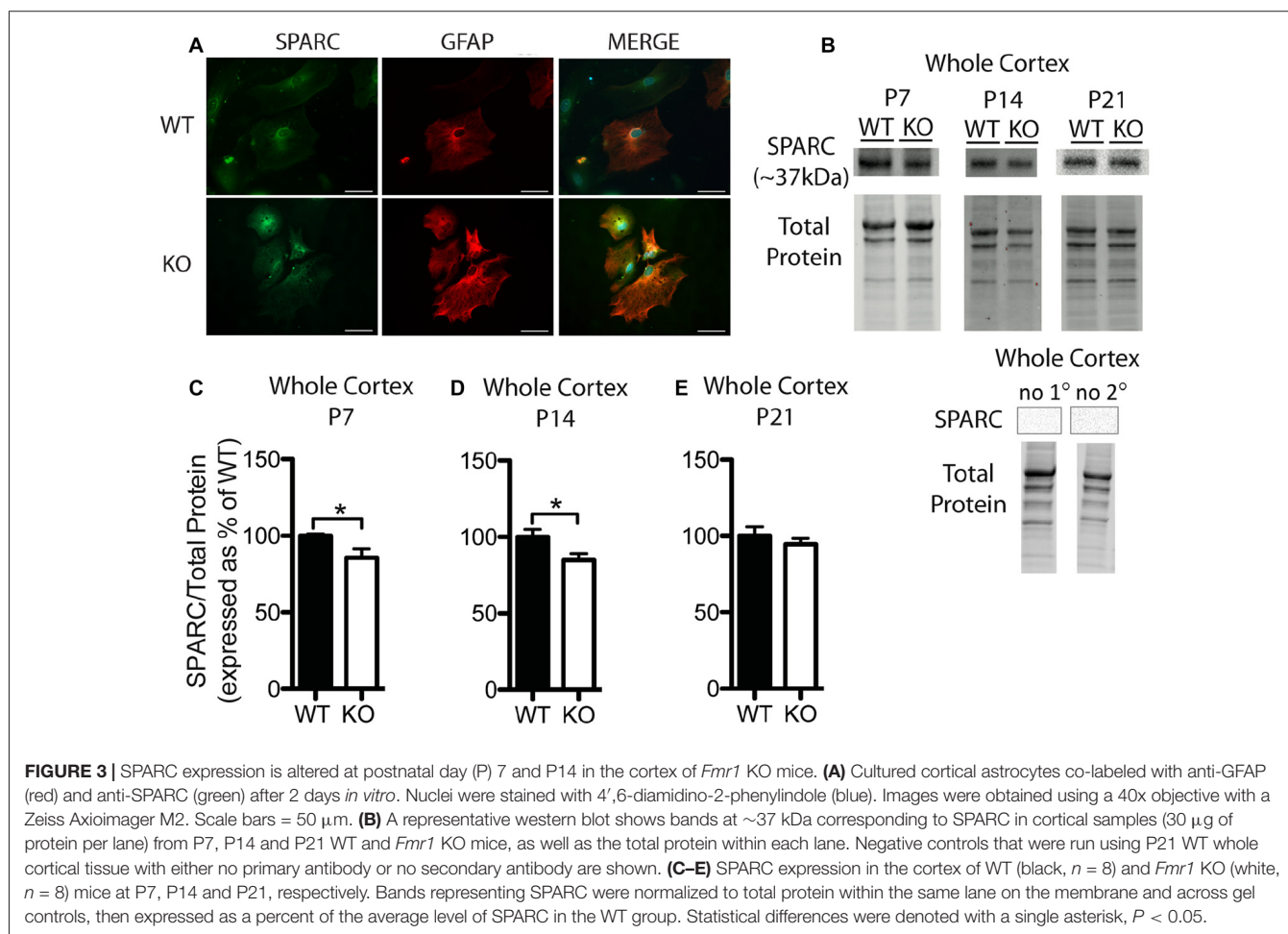
Statistical Analyses

Statistical analysis was conducted using GraphPad Prism Software 5.0 (GraphPad Software Inc., San Diego, CA, USA). Unpaired, two-tailed *t*-tests were used to identify significant differences in hevin and SPARC expression between WT and KO groups, using Welch's correction when required. Significant differences in FMRP expression between the examined

time-points were determined by pairwise comparisons using the nonparametric Mann-Whitney test. Paired, two-tailed *t*-tests were used to identify significant differences in thalamocortical synapse number between co-cultures containing WT and co-cultures containing KO astrocytes. All results are shown as mean $\pm$ SEM. Probability values <0.05 were considered statistically significant.

RESULTS

In this study, we investigated *in vivo* levels of hevin and SPARC in cortical and hippocampal brain regions of WT and *Fmr1* KO mice at ages P7, P14 and P21. Importantly, these factors are secreted by astrocytes and are important for synapse development and maturation. In FXS, dendritic spine morphology is distorted within the hippocampus and cortex (Irwin et al., 2001; Antar et al., 2006; Cruz-Martín et al., 2010), indicating abnormal development of excitatory connections with in these brain regions. We hypothesized that levels of astrocyte-derived hevin and/or SPARC may be altered in *Fmr1* KO mice and may underlie aberrant astrocyte signaling in the FXS brain. Indeed, we found that protein levels of hevin and SPARC were different in *Fmr1* KO mice compared to WT mice. While the



distribution of both proteins in astrocytes maintained for 2 days *in vitro* appears consistent across the two genotypes, the overall dysregulation of these factors in *Fmr1* KO mice suggests likely contributes to the altered neurobiology in FXS.

FMRP Expression in the Cortex and Hippocampus of WT Mice Is Developmentally Regulated

FMRP is capable of regulating the translation of many mRNAs to their corresponding proteins and can thus influence the protein milieu within the brain. Here, we assessed the developmental expression of FMRP in WT mice. Previously, hevin was identified as an mRNA target of FMRP, and thus, understanding the expression pattern of FMRP in developing WT mice may be important for understanding hevin expression patterns in *Fmr1* KO mice. FMRP in WT mice showed differential expression between time-points in both the cortex and hippocampus. FMRP expression in the cortex of WT mice was greatest at P14, and then, by P21, declined to a level less than that expressed at P7. Pairwise comparisons between time-points showed that FMRP expression at P14 was significantly greater than P21 in the cortex (P14 $128.1 \pm 27.50\%$ of P7; P21 $59.13 \pm 12.59\%$ of P7; $n = 4-8/\text{group}$; $P < 0.05$; **Figures 1A,B**). FMRP expression in the hippocampus was greatest at P7, and

significantly higher than levels at P21 (P14 $74.83 \pm 19.77\%$ of P7; P21 $55.34 \pm 13.23\%$ of P7; $n = 6/\text{group}$; $P < 0.05$; **Figures 1C,D**).

Hevin and SPARC Protein Levels Are Altered in the Cortex of *Fmr1* KO Mice

Hevin was highly expressed in primary cortical astrocytes cultured from both WT and *Fmr1* KO P1 or 2 pups, and showed a similar distribution pattern between the groups following 2 days *in vitro* ($n = 2$, 50 cells/group; **Figure 2A**). Western blotting revealed a difference between WT and *Fmr1* KO groups in hevin expression in cortical tissue by P14. The P14 *Fmr1* KO group showed significantly higher hevin levels than the WT group (*Fmr1* KO $144.50 \pm 13.36\%$ of WT; $n = 8/\text{group}$; $P < 0.05$; **Figures 2B,D**). Interestingly, there were no differences between WT and *Fmr1* KO groups at either P7 (*Fmr1* KO $81.92 \pm 16.35\%$ of WT; $n = 8/\text{group}$; **Figure 2C**) or P21 (*Fmr1* KO $103.80 \pm 11.33\%$ of WT; $n = 8/\text{group}$; **Figure 2E**) in the cortex. In order to verify that the difference observed between WT and *Fmr1* KO groups in cortical hevin levels at P14 could be attributed more specifically to differences in levels of astrocyte-derived hevin, we conducted a MACS separation to isolate astrocytes from other cell types within the cortex of both WT and *Fmr1* KO P14 mice. Consistent with our findings from whole cortical

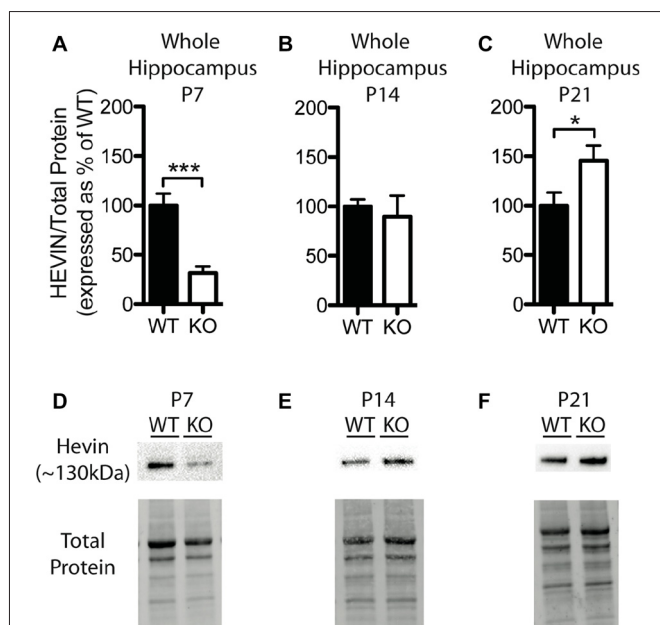


FIGURE 4 | Hevin expression is altered at postnatal day (P) 7 and P21 in the hippocampus of *Fmr1* KO mice. **(A–C)** Hevin expression, determined via Western blotting, in the hippocampus of WT (black; $n = 8$) and *Fmr1* KO (white; $n = 8$) mice at P7, P14 and P21, respectively. Bands representing hevin were normalized against the total protein within the same lane on the membrane and cross gel controls, then expressed as a percent of the average level of hevin in the WT group. **(D–F)** Representative western blots show hevin (~130 kDa) in hippocampal samples (30 μg of protein per lane) from WT and *Fmr1* KO mice at P7, P14 and P21, as well as the total protein within each lane. Statistical differences were denoted with either a single asterisk, $P < 0.05$, or a triple asterisks, $P < 0.0005$.

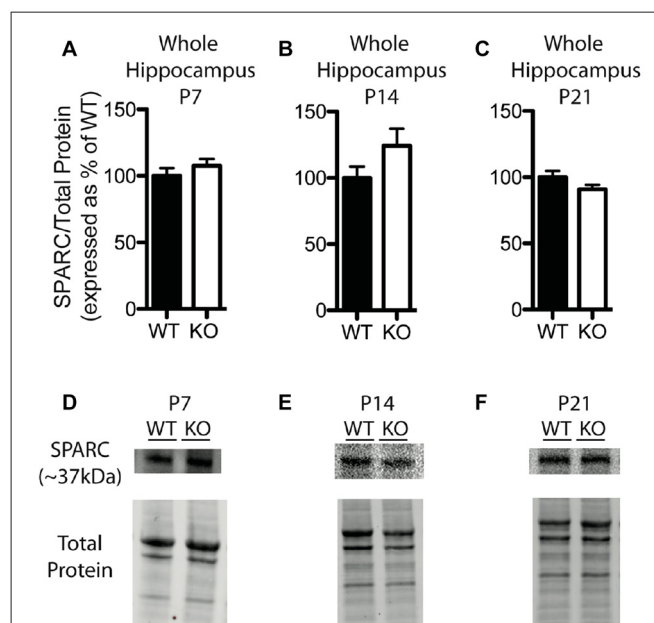


FIGURE 5 | SPARC expression is not significantly altered in the hippocampus of *Fmr1* KO mice. **(A–C)** SPARC expression, determined via Western blotting, in the hippocampus of WT (black; $n = 8$) and *Fmr1* KO (white; $n = 8$) mice at postnatal day (P) 7, P14 and P21, respectively. Bands representing SPARC were normalized against the total protein within the same lane on the membrane, and were then expressed as a percent of the average level of SPARC in the WT group. **(D–F)** Representative western blots with bands at ~37 kDa corresponding to SPARC in hippocampal samples (30 μg of protein per lane) from WT and *Fmr1* KO mice at P7, P14 and P21, as well as the total protein within each lane.

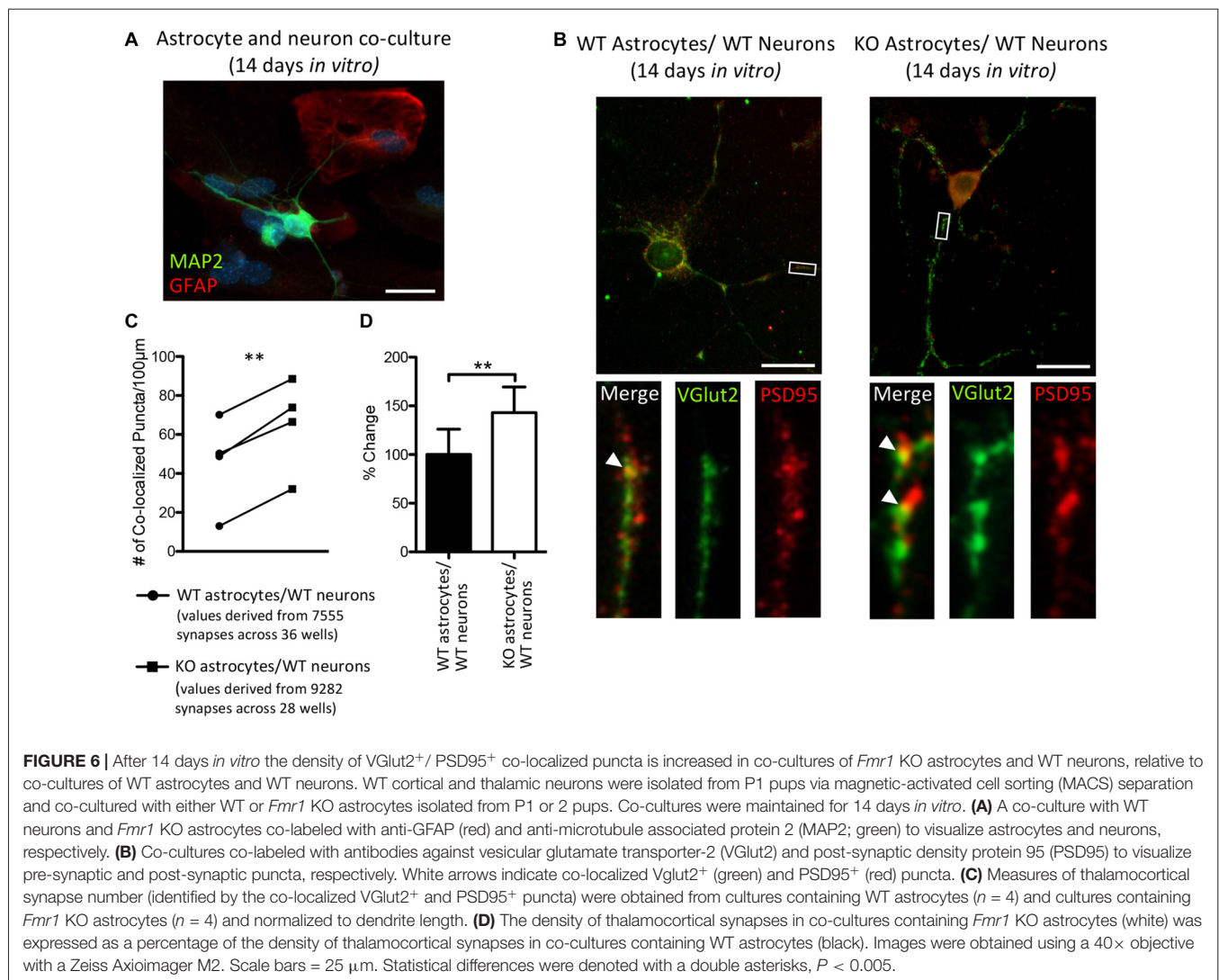
P14 tissue, hevin was expressed at higher levels in *Fmr1* KO P14 cortical astrocytes than in WT P14 cortical astrocytes (*Fmr1* KO $349.80 \pm 55.78\%$ of WT; $n = 4/\text{group}$; $P < 0.01$; **Figure 2F**).

In cultured cortical astrocytes derived from P1 or 2 pups, SPARC was similarly expressed between genotypes following 2 days *in vitro* ($n = 2$, 50 cells/group; **Figure 3A**). Representative Western blots showing SPARC (~37 kDa) from WT and *Fmr1* KO cortical samples collected at P7, P14 and P21 are shown in **Figure 3B**. Again, differences between groups were evident in Western blots from the different developmental time-points. In the cortex, at P7 and P14, the *Fmr1* KO group had slightly lower SPARC levels than the WT group (approximately 15% reduction at both time points; $n = 8/\text{group}$; $P < 0.05$ for both comparisons; **Figures 3C,D**). There was no significant difference between WT and *Fmr1* KO groups at P21 (*Fmr1* KO $94.65 \pm 3.87\%$ of WT; $n = 8/\text{group}$; **Figure 3E**). Thus, cortical levels of hevin and SPARC displayed differences between WT and *Fmr1* KO groups at differential developmental

time-points, suggesting that altered expression of these factors during certain developmental windows contribute to aberrant synapse development in FXS.

Hevin Protein Levels, but Not SPARC Levels, Are Altered in the Hippocampus of *Fmr1* KO Mice

Levels of hevin in the hippocampus differed between WT and *Fmr1* KO mice; however, these alterations were notably distinct from those in the cortex. At P7, the *Fmr1* KO group showed significantly lower hevin levels than the WT group ($31.41 \pm 6.86\%$ of WT; $P < 0.0005$; $n = 8/\text{group}$; **Figures 4A,D**). At P14 there was no significant difference in hevin levels between *Fmr1* KO and WT groups (*Fmr1* KO $89.80 \pm 21.03\%$ of WT; $n = 8/\text{group}$; **Figures 4B,E**), and at P21, the *Fmr1* KO group had significantly higher hevin levels than the WT group (*Fmr1* KO $145.70 \pm 15.17\%$ of WT; $n = 8/\text{group}$; $P < 0.05$; **Figures 4C,F**).



In contrast to our findings with hevin expression, there were no significant differences in hippocampal SPARC levels between WT and *Fmr1* KO mice at P7 (*Fmr1* KO $107.60 \pm 4.99\%$ of WT; $n = 8/\text{group}$; **Figure 5A**), P14 (*Fmr1* KO $124.10 \pm 12.94\%$ of WT; $n = 8/\text{group}$; **Figure 5B**), or P21 (*Fmr1* KO $90.86 \pm 3.26\%$ of WT; $n = 8/\text{group}$; **Figure 5C**). Representative Western blots showing SPARC from WT and *Fmr1* KO P7, P14 and P21 hippocampal samples are shown in **Figures 5D–F**, respectively.

The Number of VGlut2⁺/PSD95⁺ Co-Localized Puncta of WT Neurons Was Increased When Plated with *Fmr1* KO Astrocytes, Compared to Those Plated with WT Astrocytes

Thalamic and intracortical axonal projections that contact dendritic spines make up the majority of excitatory synapses in the cortex, and these two inputs can be distinguished by their VGlut2 or vesicular glutamate transporter-1 (VGlut1) contents, respectively (Fremeau et al., 2001; Kaneko and Fujiyama, 2002; Graziano et al., 2008). Hevin is necessary for the formation of thalamocortical excitatory synapses (Risher et al., 2014; Singh et al., 2016) and we found an increase in the cortical protein expression of hevin in P14 *Fmr1* KO mice, relative to WT mice. Therefore, we sought to determine whether a difference in the number of thalamocortical synapses would result in when WT thalamic and cortical neurons were co-cultured with either WT astrocytes or KO astrocytes (**Figure 6A**). Excitatory thalamocortical synaptic candidates were identified by the colocalization of VGlut2⁺ and PSD95⁺ puncta (**Figure 6B**). In co-cultures maintained for 14 days *in vitro* there was a 43.2% increase in the density of thalamocortical synapses when WT neurons were grown with *Fmr1* KO astrocytes (65.23 ± 11.97) relative to those grown with WT astrocytes (45.56 ± 11.88 ; $t_{(3)} = 10.37$, $P < 0.005$; **Figures 6C,D**).

DISCUSSION

The first few weeks of postnatal development are a time of vigorous growth, maturation, pruning, or elimination of synapses. These events must occur in a highly concerted fashion in order to establish proper synaptic connections and neuronal circuitry. Alterations in the development of synaptic structures are a hallmark of FXS (Comery et al., 1997; Irwin et al., 2000, 2001; Nimchinsky et al., 2001). Importantly, the various abnormal synapse phenotypes reported in the literature seem to be highly dependent upon the stage of development and brain region studied. Astrocytes play a significant role in the regulation of synaptic development and astrocyte dysfunction has recently been linked to neurodevelopmental disorders, such as FXS (reviewed in Sloan and Barres, 2014). Previous research from our laboratory has shown that dendrite and synapse abnormalities in cultured hippocampal neurons derived from the *Fmr1* KO mouse can be prevented by either co-culturing with WT astrocytes (Jacobs and Doering, 2010) or culturing

with media conditioned by WT astrocytes (Cheng et al., 2016). Additionally, an astrocyte-specific lack of FMRP *in vivo* results in synaptic deficits within the cortex (Higashimori et al., 2016). Together, these findings suggest that aberrant astrocyte-signaling occurs in the absence of FMRP and underscore the importance of proper astrocyte-neuron interactions in the developing brain.

In this study, we examined the expression of the astrocyte-secreted factors hevin and SPARC, both of which are involved in the regulation of proper excitatory synapse development and maturation. This study is the first to investigate these factors within the context of FXS. Interestingly, we found altered levels of both hevin and SPARC in *Fmr1* KO mice compared to WT controls; however, protein expression patterns varied between the two brain regions examined. Interestingly, we found differences between WT and *Fmr1* KO groups that coincided with peak FMRP expression in the cortex (at P14; **Figures 1A,B**) and in the hippocampus (at P7; **Figures 1C,D**). These correlations may indicate time-periods during which *Fmr1* KO mice are particularly susceptible to deviations from appropriate astrocyte signaling, and thus, to the improper development of neuronal circuitry.

Normally, hevin is highly expressed in and largely restricted to astrocytes during development, and remains highly expressed in astrocytes during adulthood (Mendis et al., 1996; Cahoy et al., 2008; Eroglu, 2009). Microarray studies have shown an upregulation of *Hevin* transcripts present in the cerebellum of ASD patients (Purcell et al., 2001). Whole-genome sequencing has additionally identified possible ASD-associated mutations in *Hevin* (De Rubeis et al., 2014), which may alter the expression or function of hevin in these individuals. Here, we showed that alterations in the expression of hevin also occur in a mouse model of FXS. Perhaps this is not surprising given that hevin is a known target of FMRP (Darnell et al., 2011), but the differential expression across brain regions and developmental time points suggests that its role is not only spatially complex but also highly dependent upon temporal regulation.

In *Fmr1* KO mice, we observed a transient increase in hevin within whole cortical tissue and cortical astrocytes specifically, at age P14. Interestingly, Western blots from both the superior colliculus and whole cortical tissue homogenates have shown that hevin expression peaks at approximately P14–P25, a time-period roughly coinciding with peak synaptogenic activity (Kucukdereli et al., 2011; Risher et al., 2014). At this time intracortical and thalamocortical connections are actively being established and are not yet mature (Nakamura et al., 2005). In the cortex, excitatory synapses are primarily formed via thalamic and intracortical axonal projections that contact dendritic spines. Several lines of evidence indicate that hevin is required for the proper establishment and maintenance of thalamocortical connections. Risher et al. (2014) reported a profound reduction in thalamocortical synapses in Layer 1 of the primary visual cortex of *Hevin* KO mice at postnatal day 7, day 25 and week 12. Interestingly, this was accompanied by a transient increase of intracortical synapses at P25, a possible compensation for the reduced number of thalamocortical connections. These findings *in vivo* were supported by *in vitro* studies. When cultured cortical and thalamic neurons from *Hevin* KO mice were grown

together in the presence of hevin-containing growth media there was an increase in the number of thalamocortical synapses, compared to cultures grown in media that did not contain hevin. Moreover, a subsequent study found that hevin works to establish thalamocortical connections by bridging neurexin-1 alpha and neuroligin-1B (Singh et al., 2016), two trans-synaptic molecules abundantly expressed in the brain (Schreiner et al., 2015). The adhesion between presynaptic neurexin and postsynaptic neuroligin is crucial for the establishment and maturation of synapses (Baudouin and Scheiffele, 2010). Together these studies indicate that hevin directly influences the number of thalamocortical synapses, and in doing so, may also indirectly influence the formation of intracortical synapses.

Similar to the under expression of hevin, an excess of hevin during critical developmental windows could also contribute to alterations in thalamocortical and intracortical connectivity. This possibility is consistent with findings of altered cortical function and connectivity in FXS. In the barrel cortex of 2-week-old *Fmr1* KO mice, several defects in Layer III to IV synaptic connectivity have been reported, including reduced strength, diffuse axonal arbors and altered experience-dependent plasticity (Bureau et al., 2008). The critical period for thalamocortical plasticity in the barrel cortex of mice (somatosensory layer IV), which normally occurs during the first postnatal week, is also delayed in *Fmr1* KO mice and may reflect an increase in the number of silent synapses at earlier time points (Harlow et al., 2010). Wang et al. (2014) observed an increase in the number of thalamocortical synapses in layer IV of the somatosensory cortex of 4-month-old *Fmr1* KO mice, compared to their WT counterparts. Additionally, abnormal thalamocortical connectivity has been indicated in ASD (Mizuno et al., 2006; Cheon et al., 2011; Nair et al., 2013). The increase in cortical hevin levels in P14 *Fmr1* KO mice that we found, both in whole cortical tissue and in cortical astrocytes, may contribute to developmental delays in the maturation and stabilization of synapses in the cortex. Given the role of hevin in the establishment and maintenance of excitatory thalamocortical synapses (Risher et al., 2014; Singh et al., 2016), the increased density of thalamocortical synapses in cultures of *Fmr1* KO astrocytes vs. WT astrocytes found here supports the importance of hevin during this developmental window and the development of aberrant connections in the FXS cortex.

Although we also found group differences in hevin levels in the hippocampus, the pattern of hevin expression in this region was distinct from that of the cortex, suggesting an alternate mechanism by which astrocytes modulate the development of neuronal circuits in distinct brain regions. We found hevin expression in the hippocampus of P7 *Fmr1* KO mice was much lower than in WT controls, a time-point that directly coincided with maximal FMRP expression in the hippocampus (Lu et al., 2004; see also **Figures 1C,D**). While effects on spine and synapse phenotypes in the hippocampus of *Hevin* KO mice are unknown, pronounced deficits to excitatory synapses at P14 and P25 in the superior colliculus have been reported (Kucukdereli et al., 2011). Additionally, in Layer 1 of the primary visual cortex at P25, *Hevin* KO mice show an increase in the number of

filopodia-like immature dendritic spines, concomitant with a decrease in mature spines (Risher et al., 2014). Notably, these phenotypes are similar to neurobiological abnormalities found in the hippocampus of *Fmr1* KO mice, including a reduction in the number of spines that co-localize with synaptic markers (Antar et al., 2006) and delayed synapse maturation (Braun and Segal, 2000). Reduced expression of hevin in the hippocampus, such as we observed here, may contribute to the defects in dendritic spines and synapses found in the hippocampus of *Fmr1* KO mice.

Although very low at P7, protein expression of hevin in the hippocampus of *Fmr1* KO mice increased to WT levels by P14 and exceeded them by P21. This discrepancy may be indicative of a shift in the role of hevin at these time points. Early on, hevin promotes synapse formation during postnatal development and shifts to a more regulatory role in synaptic function and plasticity during adulthood. In agreement with this, hevin has been shown to exhibit anti-adhesive properties (Gongidi et al., 2004). The presence of hevin may enhance synaptic plasticity by reducing cell adhesion and promoting spine remodeling. Additionally, hevin contains a highly conserved calcium-binding domain (Hambrock et al., 2003) and may modulate synaptic function by regulating local calcium concentrations. Indeed, more studies are needed to further elucidate the role of hevin in the brain during development and adulthood, and particularly in regard to FXS.

In addition to hevin, we examined protein levels of SPARC. SPARC is highly expressed by astrocytes in the developing brain and is capable of inhibiting the synaptogenic function of hevin (Cahoy et al., 2008; Kucukdereli et al., 2011). Due to the antagonism between SPARC and hevin, we postulated that the expression of SPARC may also differ in *Fmr1* KO mice as part of a homeostatic mechanism to compensate for alterations in hevin. However, we found only modest decreases in SPARC in the cortex of *Fmr1* KO mice at P7 and P14; and SPARC expression did not differ between genotypes at P21 in the cortex or at any time-points examined for the hippocampus. Taken together, these findings indicate that SPARC does not compensate for alterations in hevin expression. In fact, the decrease in SPARC at P14 in the cortex coincides with a robust increase in hevin, thus providing a permissive environment for the synaptogenic activity of hevin. However, more research is required to more precisely discern the mechanism by which SPARC interacts with, and regulates, the function of hevin.

CONCLUSIONS

In this study, we found altered levels of hevin and SPARC in the *Fmr1* KO mouse that suggests aberrant astrocyte signaling in the absence of FMRP. Expression patterns of these factors differed between time-points and brain regions, implying both spatial and temporal differences in astrocyte regulatory mechanisms. These findings provide important groundwork for future studies focused on elucidating the roles of both hevin and SPARC throughout development and adulthood to help understand the

mechanisms of astrocyte-derived regulation of neural circuits. Moreover, these findings emphasize the temporal and regional specificity of FXS. Identifying the functional deficits associated with aberrant levels of astrocyte-based hevin and SPARC in the FXS brain would offer important insights into novel prospects for therapeutic intervention in FXS.

AUTHOR CONTRIBUTIONS

JW: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. ALS: data analysis and interpretation, manuscript writing, final approval of manuscript. KR: collection and/or assembly of data, data analysis and interpretation,

final approval of manuscript. LCD: conception and design, financial support, provision of study material, final approval of manuscript.

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Deficient Sleep in Mouse Models of Fragile X Syndrome

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In patients with fragile X syndrome (FXS), sleep problems are commonly observed but are not well characterized. In animal models of FXS (*dfmr1* and *Fmr1* knockout (KO)/*Fxr2* heterozygote) circadian rhythmicity is affected, but sleep *per se* has not been examined. We used a home-cage monitoring system to assess total sleep time in both light and dark phases in *Fmr1* KO mice at different developmental stages. *Fmr1* KOs at P21 do not differ from controls, but genotype $\times$ phase interactions in both adult (P70 and P180) groups are statistically significant indicating that sleep in *Fmr1* KOs is reduced selectively in the light phase compared to controls. Our results show the emergence of abnormal sleep in *Fmr1* KOs during the later stages of brain maturation. Treatment of adult *Fmr1* KO mice with a GABA_B agonist, R-baclofen, did not restore sleep duration in the light phase. In adult (P70) *Fmr1* KO/*Fxr2* heterozygote animals, total sleep time was further reduced, once again in the light phase. Our data highlight the importance of the fragile X genes (*Fmr1* and *Fxr2*) in sleep physiology and confirm the utility of these mouse models in enhancing our understanding of sleep disorders in FXS.

Keywords: sleep disruption, home-cage monitoring system, Fragile X, *Fmr1*, *Fxr2*

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INTRODUCTION

Fragile X syndrome (FXS) is an X-linked disorder caused by a CGG repeat expansion in the 5'UTR of *FMR1* resulting in gene silencing. FXS is a major inherited cause of intellectual disability and is also associated with autistic-like behaviors. Sleep abnormalities are a common symptom reported in patients with FXS (Picchioni et al., 2014) and are correlated with the severity of the behavioral phenotypes of the disorder (Kronk et al., 2010). The exact nature of sleep abnormalities is not well understood. The studies reporting sleep characteristics in FXS patients are confounded by the large age range of subjects studied, the variety of methods by which sleep is assessed, and medications used in the patients studied (Musumeci et al., 1995; Gould et al., 2000; Miano et al., 2008; Kronk et al., 2010).

Animal models of FXS provide a system in which many of the confounds of clinical studies can be avoided. Moreover, preclinical studies in animal models are critical to the investigation of efficacy of pharmacological interventions. To date, studies in animal models have focused more on circadian rhythm abnormalities than sleep deficiencies. In *Drosophila* models of FXS (*dfmr1*),

Abbreviations: CLAMS, comprehensive laboratory animal monitoring system; FXS, Fragile X syndrome; SEM, standard error of the mean.

an absence of circadian rhythm has been demonstrated (Dockendorff et al., 2002; Inoue et al., 2002; Sekine et al., 2008). In addition, two studies of the *dmfr1* model have shown that sleep is also dysregulated (Bushey et al., 2009; van Alphen et al., 2013). In mice, *Fmr1* deletion alone (*Fmr1* knockout, KO) results in a slightly shorter period length measured in free running mice housed in constant darkness (Zhang et al., 2008). In contrast to *Drosophila*, mammals also express *Fmr1* paralogs, *Fxr1* and *Fxr2*; both paralogs can interact with *Fmr1* (Zhang et al., 1995). Mice with mutations in both *Fmr1* and *Fxr2* have exaggerated behavioral phenotypes (Spencer et al., 2006) and a loss of circadian rhythm (Zhang et al., 2008). Sleep in these mice, however, has not been characterized.

Here, we report results of our studies of sleep in *Fmr1* KO mice. We studied mice at three ages, P21, P70 and P180, to determine the developmental course of sleep deficiencies. We also examined sleep in *Fmr1* KO/*Fxr2* Heterozygous (Het) animals at P70 to determine if the phenotype was made worse by the addition of an *Fxr2* mutation. We assessed sleep by means of a non-invasive home-cage monitoring-based system (Pack et al., 2007). Our results suggest that sleep disturbances increase over the lifecycle of *Fmr1* KO mice. At weaning (P21), total sleep time was not affected. At P70, *Fmr1* KO mice had reduced sleep in the light phase compared to controls. This phenotype persisted at P180 and was not rescued by treatment with a GABA_B agonist, R-baclofen. Additionally, P70 *Fmr1* KO/*Fxr2* Het animals had a further decrease in sleep in the light phase compared to *Fmr1* single mutants. These findings highlight the utility of *Fmr1* KO mice to understand sleep in FXS.

MATERIALS AND METHODS

Animals

All mice were group housed in a standard housing environment with up to five mice per cage (except during sleep analysis) in a climate-controlled central facility with a 12:12 h (6:00 AM–6:00 PM) light:dark environment. Food and water were available to mice *ad libitum*. All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

Fmr1 KO Breeding

These studies were conducted on male *Fmr1* hemizygous KO animals (*Fmr1* KO) and control littermates (on a C57BL/6J background), generated in house through Het female and WT male breeding pairs. Genotyping of mouse tail DNA by PCR amplification was previously described (Qin et al., 2002). In separate groups of animals, studies were initiated at 20–22 days of age (P21), 60–80 days of age (P70), or 170–190 days of age (P180).

Fmr1 KO/*Fxr2* Het Breeding

These studies were conducted on male *Fmr1* hemizygous KO animals, *Fxr2*^{+/+} (*Fmr1* KO/*Fxr2* WT) and *Fmr1* hemizygous KO animals, *Fxr2*^{+/-} (*Fmr1* KO/*Fxr2* Het) on a C57BL/6J background. Studies were conducted at 60–80 days of age.

These mice were generated from female *Fmr1*^{-/-}, *Fxr2*^{+/-} and male *Fmr1* hemizygous, *Fxr2*^{+/-} breeder pairs kindly provided by David Nelson (Baylor College of Medicine). The following primers were used to genotype *Fxr2*: (1) 5'-GTG ACA GTT TCC TGC TTT ACA GTC C; (2) 5'-TCT GCC TGC TTC CTG AGT GTT G; and (3) 5'-CGC CTT CTA TCG CCT TCT TGA C. Cycling conditions were as follows: 94°C for 5 min, (94°C for 60 s 54°C for 30 s and 72°C for 45 s) × 30 cycles, and 72°C for 7 min.

Home-Cage Assessment of Sleep

Sleep was assessed by home-cage activity monitoring. Mice were singly housed in a clean standard cage surrounded by a rectangular arena of oppositely positioned infrared emitters and sensors (Comprehensive Laboratory Animal Monitoring System (CLAMS); Columbus Instruments, Columbus, OH, USA). Photobeams were spaced 0.5 inches apart on the x and y planes to assess activity on a high-resolution grid. The CLAMS software discriminated between fine movements (multiple breaks of the same beam) and locomotor activity (breaking two adjacent beams). For the analysis, the sums of fine and locomotor activities were used. The CLAMS software detected beam breaks in 10 s epochs. A mouse was considered inactive if there was no xy movement over the 10 s epoch, and 40 s of such inactivity was recorded as sleep. Validation of these measures as indicators of sleep in C57BL/6J mice was reported previously (Pack et al., 2007). The total amount of time asleep was separated into light phase (time asleep between 6:00 AM and 6:00 PM) and dark phase (time asleep between 6:00 PM and 6:00 AM) and then recorded as a percentage of the 12 h total time per phase. Sleep was analyzed for each 24 h period. For *Fmr1* KO mice, sleep was analyzed for six consecutive days. For *Fmr1* KO/*Fxr2* Het mice, sleep was analyzed for three consecutive days.

R-Baclofen Treatment

In a separate group of animals, we used 18 *Fmr1* KO animals at 6 months of age to assess sleep duration prior to and during R-baclofen treatment. Mice were given saline injections, i.p., at 6:00 AM for 9 days. Sleep was assessed during the last 4 days of saline injections. R-baclofen was obtained from Seaside Therapeutics (Cambridge, MA, USA), dissolved in saline, and administered at 1.5 mg/kg i.p. at 6:00 AM for 2 days following the 9 days of saline injections (Days 10–11). The average sleep durations in the light and dark phases during saline injections were compared (Days 7–9) with sleep durations during R-baclofen injections (Days 10–11).

Statistical Analysis

Data were analyzed by means of a mixed model repeated measures (RM) ANOVA. The between subjects' variable was genotype. The within subjects' variables were day and phase (light, dark). A criterion of $p \leq 0.05$ was considered to be statistically significant. These results are indicated with an “*”. We compared sleep duration during saline injections with sleep duration during R-baclofen injections by means of a paired *t*-test.

RESULTS

Habituation to Home-Cage Monitoring

Recording commenced as soon as animals were housed in monitoring cages and continued for 6 days. We assessed the effect of day (habituation effect) on sleep in control and *Fmr1* KO mice at P21, P70 and P180. At P21, neither the day $\times$ phase nor the genotype $\times$ phase interaction was statistically significant. Furthermore, neither the main effect of day nor genotype was statistically significant. The main effect of phase was statistically significant. As expected in nocturnal animals, percent sleep time was longer in the light phase. Sleep was stable across the 6-day recording period and did not differ by genotype (**Figure 1A**). At P70, the day $\times$ phase interaction was statistically significant ($p < 0.001$). *Post hoc t*-tests revealed that sleep duration on Day 1 differed from Days 2–6 in the light phase only. The genotype $\times$ phase interaction was also statistically significant ($p = 0.002$; **Figure 1B**), indicating that *Fmr1* KO mice had a shorter sleep duration in the light phase. At P180, the day $\times$ phase interaction was statistically significant ($p < 0.001$). *Post hoc t*-tests revealed that Day 1 was different from all other days only in the light phase. The genotype $\times$ phase interaction was also statistically significant ($p = 0.004$; **Figure 1C**), indicating that the *Fmr1* KO mice had a shorter sleep duration in the light phase. These results show that habituation to the housing condition occurred during the first 24 h period, particularly in the adult animals, and that habituation was similar in both control and *Fmr1* KO mice.

Developmental Course of Sleep Deficiencies in *Fmr1* KO Mice

The genotype $\times$ phase interaction in P70 and P180 animals indicates that there are differences between the genotypes in sleep time that depend on phase. To eliminate the effect of habituation, we confirmed these effects by analyzing the genotype $\times$ phase effects on average sleep time over Days 2–6.

We assessed sleep in juvenile control ($n = 19$) and *Fmr1* KO ($n = 23$) mice at P21. At this age, neither the main effect of genotype nor the genotype $\times$ phase interaction were statistically significant (**Table 1**). Mean percent times asleep were similar for both genotypes in both phases (**Figure 2A**).

At P70 (young adult; **Figure 2B**), the phase $\times$ genotype interaction was statistically significant ($p < 0.001$; **Table 1**). *Post hoc t*-tests indicate that *Fmr1* KO mice ($n = 19$) had significantly ($p = 0.007$) reduced sleep only in the light phase compared to controls ($n = 19$). Mean differences were 6.39%.

At P180 (adult), the phase $\times$ genotype interaction was statistically significant ($p = 0.015$; **Table 1**; **Figure 2C**). *Post hoc t*-tests indicate that *Fmr1* KO mice ($n = 21$) slept less than controls ($n = 21$) in the light phase only ($p = 0.004$). Mean differences were 8.0%.

We asked if the sleep deficits in *Fmr1* KO mice at P180 could be reversed by treatment with R-baclofen,

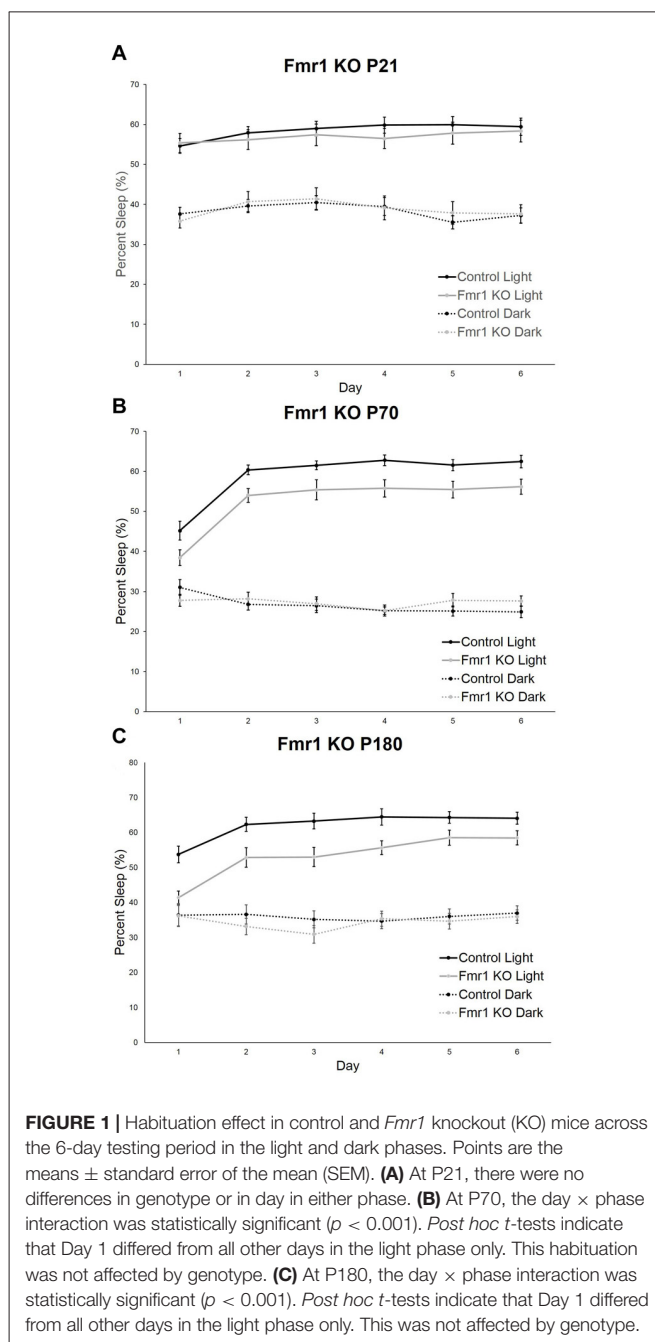


FIGURE 1 | Habituation effect in control and *Fmr1* knockout (KO) mice across the 6-day testing period in the light and dark phases. Points are the means $\pm$ standard error of the mean (SEM). **(A)** At P21, there were no differences in genotype or in day in either phase. **(B)** At P70, the day $\times$ phase interaction was statistically significant ($p < 0.001$). *Post hoc t*-tests indicate that Day 1 differed from all other days in the light phase only. This habituation was not affected by genotype. **(C)** At P180, the day $\times$ phase interaction was statistically significant ($p < 0.001$). *Post hoc t*-tests indicate that Day 1 differed from all other days in the light phase only. This was not affected by genotype.

a GABA_B agonist. R-baclofen treatment reverses other behavioral and physiological phenotypes in adult *Fmr1* KO mice (Henderson et al., 2012; Qin et al., 2015a). We administered R-baclofen by daily i.p. injections (1.5 mg/kg). We used a within subjects' design, i.e., sleep behavior was monitored in mice during daily i.p. injections of normal saline for 3 days followed by 2 days of daily R-baclofen i.p. injections. Prior to sleep monitoring, mice were acclimated to daily i.p. injections of saline for 6 days and 1 day of acclimation to the home-cage monitoring system. The sleep deficit in the light phase was not reversed by treatment with R-baclofen (57.7% sleep in the light phase during saline injections compared to 58.7%

TABLE 1 | Post hoc ANOVA results of average sleep times across Days 2–6 for the models presented.

Model/Age	Interaction	Main effect	$F_{(df,error)}$ value	P-value
<i>Fmr1</i> KO P21	Genotype × Phase		$F_{(1,40)} = 2.279$	0.139
		Phase	$F_{(1,40)} = 415.703$	<0.001*
		Genotype	$F_{(1,40)} = 0.037$	0.848
<i>Fmr1</i> KO P70	Genotype × Phase		$F_{(1,36)} = 11.687$	0.002*
		Phase	$F_{(1,36)} = 778.209$	<0.001*
		Genotype	$F_{(1,36)} = 2.204$	0.146
<i>Fmr1</i> KO P180	Genotype × Phase		$F_{(1,40)} = 6.498$	0.015*
		Phase	$F_{(1,40)} = 421.000$	<0.001*
		Genotype	$F_{(1,40)} = 4.090$	0.05*
<i>Fmr1/Fxr2</i> P70	Genotype × Phase		$F_{(1,61)} = 11.959$	0.001*
		Phase	$F_{(1,61)} = 350.108$	<0.001*
		Genotype	$F_{(1,61)} = 8.807$	0.004*

The genotype × phase interaction as well as main effects of phase and genotype are presented with the corresponding F values and p -values. Statistically significant results are indicated with a “*”.

sleep during R-baclofen injections; $p = 0.67$, paired t -test). Moreover, R-baclofen did not affect sleep duration in the dark phase.

Sleep Deficiencies in *Fmr1/Fxr2* Mice: Effects of Additional *Fxr* Deletion

We asked if the *Fxr2* paralog was involved in sleep regulation in *Fmr1* KO mice. The absence of *Fxr2* in *Fmr1* KO mice exacerbates circadian rhythm abnormalities (Zhang et al., 2008). To see if this role of *Fxr2* in circadian rhythm extends to sleep, we studied *Fmr1* KO mice with (*Fmr1* KO/*Fxr2* WT) or haploinsufficient (*Fmr1* KO/*Fxr2* Het) for *Fxr2* at P70. We found that the phase × genotype interaction was statistically significant ($p = 0.001$; **Table 1**). In the light phase, *Fmr1* KO/*Fxr2* Het animals slept significantly less than *Fmr1* KO/*Fxr2* WT animals ($p < 0.001$; mean difference of 10%), but in the dark phase percent times were similar for both genotypes (**Figure 3**).

DISCUSSION

The results of our study show that *Fmr1* expression plays a role in the regulation of sleep physiology, and that its influence becomes apparent in adulthood. Additionally, *Fxr2*, an *Fmr1* paralog, appears to have a further influence on sleep physiology in mice. Our data highlight sleep physiology as an important phenotype in FXS that needs further characterization in patients. Moreover, abundant data support the importance of sleep in behavior and brain function (Picchioni et al., 2014; Kreutzmann et al., 2015; Saré et al., 2016a). Accordingly, correction of sleep abnormalities in FXS patients offers a promising therapeutic strategy. The effects of such therapies on sleep and ultimately on behavioral outcomes can be tested in FXS mouse models.

There are several strengths to our current studies, as well as a few limitations. First, we conducted a cross-sectional study to investigate sleep across development. We had good statistical power for our analysis. We controlled for several of the variables problematic in human clinical studies. Our animals were well matched for age; we used the same measure

of sleep across all studies; all animals had not had any previous exposure to drugs. However, because of the nature of our study, we only have information about total sleep time, and we cannot measure sleep stages or sleep bout duration, which could inform us whether sleep fragmentation was occurring. For these questions, an electroencephalogram (EEG) study would be informative.

Hyperactivity is one of the common phenotypes detected in the *Fmr1* KO mouse model in both the active (Saré et al., 2016b) and inactive phases (Liu et al., 2011). Although hyperactivity and reduced sleep might be mediated by a similar mechanism, it is important to note that we are not detecting hyperactivity, *per se*. First, hyperactivity is traditionally assessed in a novel open-field environment larger than a typical mouse home-cage, whereas we are assessing sleep in the home-cage. Both tests use beam breaks to detect movement of the animal, but the criteria are different. A continuous measure of the number of beam breaks is used to measure activity in the open field. In the home-cage monitoring system, an animal is considered awake if it breaks a single beam in a 40 s epoch or if it breaks numerous beams in a 40 s epoch. Hyperactivity and decreased sleep duration may go hand in hand, but it is also possible that a hyperactive animal has the same number or even fewer awake epochs than a more sedentary animal.

Our finding of decreased sleep time in the light phase in *Fmr1* KO mice contrasts with results in the *dfmr1* model, in which sleep duration was increased (Inoue et al., 2002; Bushey et al., 2009). These phenotypic differences could reflect the absence of both *Fmr1* paralogs, *Fxr1* and *Fxr2*, in flies. In our study, both *Fmr1* KO and *Fmr1* KO/*Fxr2* Het mice had decreased sleep time in the light phase. The effects of loss of *Fxr1* in mice could not be tested due to the poor viability of *Fxr1* KO mice (Mientjes et al., 2004). Reduced sleep duration only in the light phase (the animal's inactive phase) suggests that the sleep effect is modulated by circadian rhythms, and that sleep deficiency and circadian rhythm disruption are linked in *Fmr1* KO animals. These results align with the circadian rhythm disruption previously reported in *Fmr1* KO animals (Zhang et al., 2008).

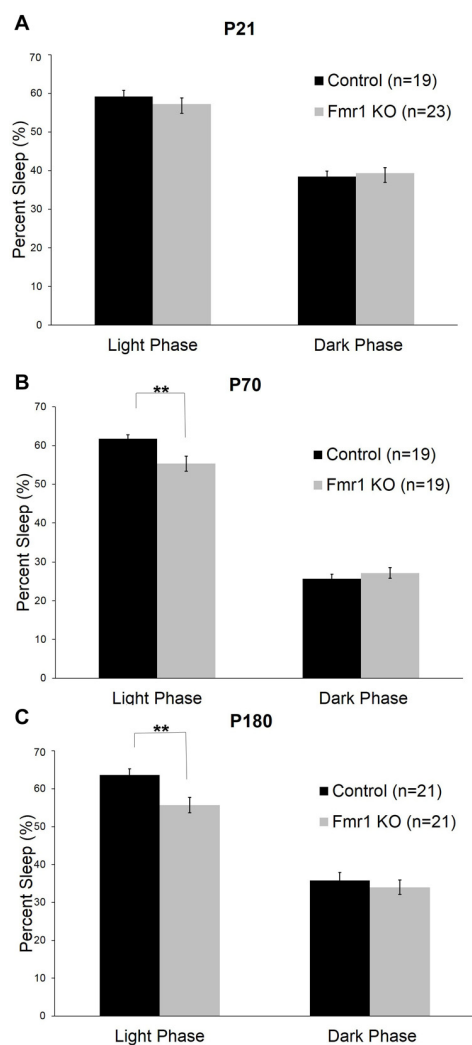


FIGURE 2 | Sleep duration in control and *Fmr1* KO mice in light and dark phases. Bars are the means $\pm$ SEM of sleep averaged across Days 2–6 of the number of animals indicated in parentheses. For each variable, full results of repeated measures (RM) ANOVA are reported in **Table 1**. **Denotes $p < 0.01$. (A) At P21, there were no differences between the genotypes in sleep duration in light and dark phases. (B) At P70, the genotype $\times$ phase interaction was statistically significant ($p < 0.001$). *Post hoc t*-tests indicate that *Fmr1* KO ($n = 19$) animals had less sleep than controls ($n = 19$) in the light phase ($p = 0.005$). (C) At P180, the genotype $\times$ phase interaction was statistically significant ($p = 0.015$). *Post hoc t*-tests indicate that *Fmr1* KO animals ($n = 21$) than controls ($n = 21$) in the light phase ($p = 0.004$).

Although we did not detect sleep deficiencies in *Fmr1* KO mice at P21 by activity monitoring, there is electrophysiological and calcium imaging evidence that cortical neuronal firing and synchrony during sleep are abnormally high in *Fmr1* KO mice at P14–P16 suggesting that Up/Down states are not normal (Gonçalves et al., 2013). How this may progress into circadian alterations and reduced sleep in the light phase is unknown. However, circadian rhythm in P21 animals is not as defined as in adult animals (Hagenauer et al., 2009), which may mask potential differences between genotypes. Additionally,

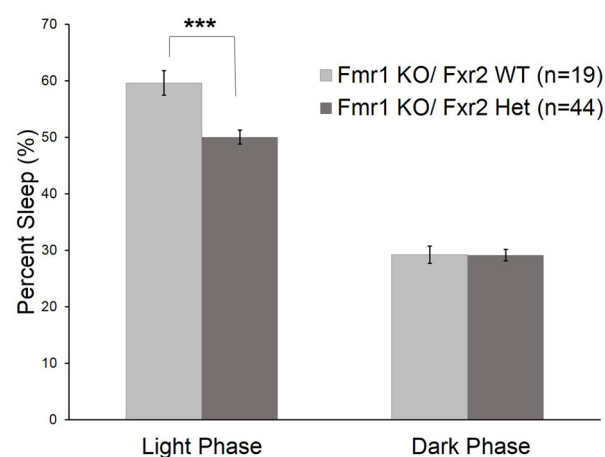


FIGURE 3 | Sleep duration in *Fmr1* KO/*Fxr2* WT ($n = 19$) and *Fmr1* KO/*Fxr2* Heterozygous (Het; $n = 44$) mice at P70. Bars are the means $\pm$ SEM of sleep averaged across Days 2–3 on the number of animals indicated in parentheses. Full results of RM ANOVA are reported in **Table 1**. The genotype $\times$ phase interaction was statistically significant ($p = 0.001$), indicating that total sleep time in *Fmr1* KO/*Fxr2* Het animals was reduced compared with *Fmr1* KO/*Fxr2* WT animals in the light phase ($p < 0.001$). *** $p < 0.001$.

at P21, the mouse brain is still developing, and is thought to be somewhat equivalent to a human of around 3 years old. By P70, the mouse brain is mature and equivalent to about a 20 year old human (Semple et al., 2013). It is during this period from P21 to P70 that *Fmr1* KO mice develop a statistically significant genotype $\times$ phase interaction, suggestive of a circadian rhythm disruption, implying that this abnormality unfolds during brain maturation. It is interesting to note that most behavioral abnormalities reported in *Fmr1* KO mice have been assessed at 2 months of age and later (Yan et al., 2004; Spencer et al., 2005; Zhao et al., 2005; Moon et al., 2006; Liu and Smith, 2009; Liu et al., 2011; Ding et al., 2014; Qin et al., 2015a,b), suggesting that sleep/circadian rhythm problems may develop before other behavioral impairments. The timing of the development of sleep abnormalities in the mouse may inform the timing of screening for sleep problems in FXS children. It also may help to determine the best window for treatment.

Although the differences in total sleep time between *Fmr1* KO and control mice are relatively small (6% in the light phase in P70 animals and 8% in the light phase in P180 animals), these differences may very well be biologically significant. Sleep has an important role in brain development and plasticity (Picchioni et al., 2014; Kreutzmann et al., 2015). Studies of chronic partial sleep loss have revealed that deficits are similar to those observed in acute total sleep deprivation. These deficits were in areas of cognition and neurobehavioral function (Van Dongen et al., 2003). Chronic sleep restriction in mice leads to long-lasting effects on behavior, even after restoration of normal sleep (Saré et al., 2016a). These behavioral changes could be mediated by changes in plasticity in the brain that are not recovered during subsequent sleep periods. Studies have shown that cellular processes implicated in plasticity

such as myelination, cellular stress and neurogenesis are affected by sleep restriction and may not recover even after regaining sleep (Tung et al., 2005; Picchioni et al., 2014; Kreutzmann et al., 2015). The consequences of chronically reduced sleep in the light phase in FXS may be an important contributor to the brain and behavioral manifestations of the disorder.

Given the potential impact of sleep deficits in the unfolding of fragile X phenotypes, it may be important to determine the mechanisms by which sleep is dysregulated in the *Fmr1* KO mice. There are two processes controlling the drive to sleep. One is by means of the circadian clock and the other is a homeostatic drive (Borbély and Achermann, 1999). In their study of circadian rhythm, Zhang et al. examined the expression of clock genes involved in circadian rhythm in both *Fmr1* KO and *Fmr1/Fxr2* double Het animals. They found that both models showed rhythmicity in the clock genes in the superchiasmatic nucleus (SCN). However, *Fmr1* KO/*Fxr2* Het animals did show increased expression of *Cry1* at the beginning of the active phase (Zhang et al., 2008). Given that both *Fmr1* KO and *Fmr1* KO/*Fxr2* Het animals show decreased sleep, this mechanism is unlikely to account for the change in sleep duration. Downstream of the SCN, in the liver which is noted as the peripheral clock, regulation of *Bmal1*, *mPer1*, *mPer2* and *Npas2* in the *Fmr1/Fxr2* Het animals was altered relative to controls (Zhang et al., 2008). Again, as these changes did not occur in *Fmr1* KO mice, they cannot fully explain the reduced sleep phenotype. Both *Fmr1* KO and *Fmr1/Fxr2* Het animals did have increased expression of *Cry1* in the liver at the beginning of the active phase (Zhang et al., 2008), so it is possible that *Cry1* regulation may contribute to the reduced sleep in both *Fmr1* KO and *Fmr1* KO/*Fxr2* Het animals.

The other process controlling sleep is the homeostatic drive. Understanding the molecular mechanisms that manage the homeostatic regulation of sleep in *Fmr1* KO mice is much more difficult because the process is less understood. One mechanism of sleep initiation, particularly nonREM sleep, is activation of GABA receptors (Lancel, 1999). It has been shown that *Fmr1* KO mice have downregulation of both GABA_A and GABA_B receptors (Pacey et al., 2011; Liu et al., 2013). In our study, R-baclofen, a GABA_B agonist, did not improve sleep in adult *Fmr1* KO mice. We controlled for effects of i.p. injections and acclimation to the monitoring system on sleep duration. We only tested mice at 6 months of age, and it is possible that the treatment might be effective in younger mice. Based on our

results, we think it unlikely that GABA_B receptors are involved in the sleep deficits observed in *Fmr1* KO and *Fmr1* KO/*Fxr2* Het animals. Future work will address the role of GABA_A receptors in mediating the sleep deficits in *Fmr1* KO and *Fmr1* KO/*Fxr2* Het animals.

Our findings highlight the importance of *Fmr1* and *Fxr2* in the regulation of sleep in adult mice. With loss of *Fmr1* (either alone or in combination with *Fxr2*), adult animals have reduced total sleep time in the light phase. Our data in conjunction with clinical reports (Musumeci et al., 1995; Gould et al., 2000) suggest that patients without *FMRI* expression are likely to have chronically reduced night-time sleep. Our findings in *Fmr1* KO mice suggest that sleep problems (such as reduced sleep) should be more thoroughly examined in FXS patients and considered as targets for therapeutic intervention. Additionally, these studies show that *Fmr1* KO mice and *Fmr1* KO/*Fxr2* Het mice may be useful for further examining the consequences and potential treatments for the sleep problems in FXS.

AUTHOR CONTRIBUTIONS

RMS designed the study, performed experiments, analyzed data, interpreted the data and drafted the manuscript; LH performed experiments; ML performed experiments and drafted the manuscript; AT performed experiments; CAS performed experiments; CBS designed the study, interpreted the data, and drafted the manuscript. All authors read and approved the final version of the manuscript.

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Fragile X-Associated Diminished Ovarian Reserve and Primary Ovarian Insufficiency from Molecular Mechanisms to Clinical Manifestations

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Fragile X syndrome (FXS), is caused by a loss-of-function mutation in the *FMR1* gene located on the X-chromosome, which leads to the most common cause of inherited intellectual disability in males and the leading single-gene defect associated with autism. A full mutation (FM) is represented by more than 200 CGG repeats within the *FMR1* gene, resulting in FXS. A FM is inherited from women carrying a FM or a premutation (PM; 55–200 CGG repeats) allele. PM is associated with phenotypes distinct from those associated with FM. Some manifestations of the PM are unique; fragile-X-associated tremor/ataxia syndrome (FXTAS), and fragile-X-associated primary ovarian insufficiency (FXPOI), while others tend to be non-specific such as intellectual disability. In addition, women carrying a PM may suffer from subfertility or infertility. There is a need to elucidate whether the impairment of ovarian function found in PM carriers arises during the primordial germ cell (PGC) development stage, or due to a rapidly diminishing oocyte pool throughout life or even both. Due to the possibility of expansion into a FM in the next generation, and other ramifications, carrying a PM can have an enormous impact on one's life; therefore, preconception counseling for couples carrying the PM is of paramount importance. In this review, we will elaborate on the clinical manifestations in female PM carriers and propose the definition of fragile-X-associated diminished ovarian reserve (FXDOR), then we will review recent scientific findings regarding possible mechanisms leading to FXDOR and FXPOI. Lastly, we will discuss counseling, preventative measures and interventions available for women carrying a PM regarding different aspects of their reproductive life, fertility treatment, pregnancy, prenatal testing, contraception and fertility preservation options.

Keywords: fragile X syndrome, fragile-X-associated primary ovarian insufficiency, diminished ovarian reserve, *FMR1* premutation carriers, fragile-X-associated diminished ovarian reserve

INTRODUCTION

Fragile X syndrome (FXS) represents the most common cause of inherited intellectual disability in males. It is also the leading single-gene defect associated with autism. The fragile X mental retardation (*FMR1*) gene is located near the end of the long arm of the X chromosome, locus Xq27.3. It includes a CGG (Cytosine-Guanine-Guanine) trinucleotide repeat within the 5' untranslated region. The name FXS arises from the characteristic chromosomal fragility in that locus observed during karyotyping (Sutherland and Ashforth, 1979).

A normal, unaffected gene contains less than 45 CGG repeats while having between 45 and 54 repeats is classified as intermediate, or gray zone, as this is when some level of CGG repeat instability in the gene transmission to the next generation has been reported (Nolin et al., 1996). The range of 55–200 CGG repeats is considered a premutation (PM) and more than 200 CGG repeats are categorized as a full mutation (FM), resulting in FXS (Kronquist et al., 2008). A recent meta-analysis reported the PM prevalence in the general population to be 1:150–300 females and 1:400–850 males (Hunter et al., 2014). Interestingly, the prevalence varies between different racial/ethnic groups; it is the highest in Colombia and Israel (1:100 females), and lowest in Japan (1:1674 females) (Seltzer et al., 2012; Hunter et al., 2014). The FXS prevalence is estimated to be 1 in 4000 males (Turner et al., 1996) and 1 in 8400 females (Pesso et al., 2000).

The PM is associated with increased level of *FMR1* gene transcription but decreased translation, resulting in low to normal levels of fragile X intellectual disability protein (FMRP) (Tassone et al., 2000b; Hagerman and Hagerman, 2002). The *FMR1* allele containing a FM is affected by DNA hypermethylation of the promoter and the CGG repeat region, causing its inactivation- transcriptional silencing. As a consequence, there is no FMRP production, which results in FXS in males (Fu et al., 1991; Heitz et al., 1991; Pieretti et al., 1991; Verkerk et al., 1991; Sutcliffe et al., 1992). In female FM carriers, however, due to random inactivation of one X chromosome, mRNA can be transcribed from the normal, but not the mutated-methylated allele, leading to lower but measurable FMRP levels.

In the past, individuals with fragile X mutations were divided either into affected (with more than 200 CGG repeats) or unaffected individuals (≤ 200 CGG repeats). Advances in diagnostic methods and increased awareness, however, have led to stratification of the previously “unaffected” group into normal, gray zone and PM, with associated clinical manifestations. This appears reasonable to consider the clinical spectrum of symptoms associated with fragile X as a continuum (McConkie-Rosell et al., 2005). The PM is associated with disorders distinct from FXS, including fragile-X-associated tremor/ataxia syndrome (FXTAS), an adult-onset neurological disorder (Hagerman et al., 2001) affecting primarily males, as well as fragile-X-associated primary ovarian insufficiency (FXPOI), and the fragile-X-associated diminished ovarian reserve (FXDOR) in female carriers: all of which we will expand upon in this review. In the recent years, more characteristic phenotypes associated

with a PM have been recognized: attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (Farzin et al., 2006), intellectual disability, childhood seizures (Bailey et al., 2008; Chonchaiya et al., 2012), adult-onset psychiatric conditions (Franke et al., 1996; Roberts et al., 2009), migraine headaches (Au et al., 2013), immune-mediated disorders, mainly thyroid disorders (Winarni et al., 2012), hypertension, fibromyalgia (Leehey et al., 2011) and chronic muscle pain (Rodriguez-Revenga et al., 2009).

The FXS phenotype, divergent from phenotypes associated with PM, varies by sex; males being more severely affected due to the X-linked inheritance and having only one X chromosome. Some of the distinctive facial features associated with FXS include an elongated face with a prominent forehead and large protruding ears, macrocephaly, strabismus, high arched palate with an occasional cleft palate. The facial characteristics often develop over time. Other symptoms include enlarged testicles (macroorchidism) and connective tissue disorders (hyperflexible joints; hyperextensible fingers, thumbs and wrists). The cognitive phenotype is characterized by a spectrum of features including developmental delay, intellectual and learning disabilities. The behavioral phenotype includes ADHD, speech and language delay, anxiety and autism spectrum disorders (McConkie-Rosell et al., 2005). Affected females may have a subtle phenotype, which makes it sometimes hard to establish the diagnosis based on clinical features alone. Up to 50% of females with a FM have some characteristic physical features associated with FXS. The intellectual impairment is usually less severe than observed in affected male (McConkie-Rosell et al., 2005).

Another sexual disparity when it comes to FM is the male's capability of reproducing. Hagerman et al. (2001) and Hagerman and Hagerman (2002) state that males with FXS have been documented to be fertile and capable of reproduction. On the other hand, Crawford states that most affected males do not reproduce, presumably due to the severity of intellectual disability (Crawford et al., 2001). This alleged disparity may be reconciled when we take the cognitive function into account. The majority of males with FXS are intellectually disabled, with severity ranging from profound (IQ < 20) to mild intellectual disability (IQ 50–70), with most being moderately disabled (IQ 40–54) (Hagerman and Hagerman, 2002). Male affected with the FM can reproduce, this is usually seen in about 15% of the FXS males, who have an IQ at 70 or higher. On the other hand, females are often less affected, most probably because of random X-inactivation, and are therefore at risk of transmitting a FM to their progeny. Interestingly, women with a PM have an increased risk of FXPOI compared to that of FM patients (Allingham-Hawkins et al., 1999; Uzielli et al., 1999). In this review article, we will elaborate on: (i) the clinical manifestations of POI, in specific the ovarian dysfunction found in female PM carriers; (ii) propose the definition of FXDOR (distinct from FXPOI); (iii) review recent scientific findings that might shed light on some potential mechanisms leading to FXDOR and FXPOI; and (iv) discuss counseling, preventative measures and interventions available for women carrying a PM regarding different aspects of their reproductive life, fertility treatment, pregnancy, prenatal testing, contraception and fertility preservation options.

CLINICAL MANIFESTATIONS OF POI IN GENERAL AND OVARIAN DYSFUNCTION IN PM CARRIERS IN PARTICULAR

POI- Primary Ovarian Insufficiency, and FXPOI

Normal ovarian function is a result of a continuous process that commences with primordial germ cells (PGC) formation, proliferation and migration, through the development of follicular units during fetal life (Baker, 1963). It then extends into neonatal and adult life, characterized by a steady follicular loss or atresia, and ends with a physiologic insufficiency of the ovary, or menopause (Faddy et al., 1992; De Felici et al., 2005). The ovary is susceptible to various intrinsic and extrinsic factors that might impair its normal formation and/or function i.e., genetic defects, smoking (Cooper et al., 1995), environment and medical issues including endometriosis, chemotherapy, radiotherapy, or ovarian surgery (De Vos et al., 2010; American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee, 2014; Rossetti et al., 2017; Vabre et al., 2017). The extreme form of ovarian dysfunction is manifested as POI.

POI is a diagnosis that accentuates the extreme spectrum of an impaired ovarian function. The term POI was coined by Albright et al. (1942), when he reported on a cluster of symptoms including amenorrhea, estrogen deficiency and menopausal follicle stimulating hormone (FSH) levels in 11 young women. He used this term to emphasize that the primary defect in this cohort was within the ovary. Today we still use his basic understanding for diagnostic purposes with some modifications. First, serum estrogen is not a mandatory criterion for the diagnosis. Many, but not all, women with POI develop symptoms of estrogen deficiency, including hot flashes, vaginal dryness and sleep disturbances. For example, lack of symptoms of estrogen deficiency might be due to intermittent ovarian function. On the other hand, some women experience hot flashes despite continued regular menses. Second, amenorrhea no longer represents the only criterion to characterize disturbance of menstrual cyclicity. With a better understanding of the phenotypic extent of POI, inclusion criteria have broadened and now include any cycle irregularities (oligomenorrhea, polymenorrhea, menometrorrhagia, dysfunctional uterine bleeding and amenorrhea- primary/secondary) which persist for more than four consecutive months. Nowadays, POI is characterized by the triad of cycle irregularities, as elaborated above, for at least 4 months, and two recordings of elevated levels of FSH >40 IU/L at least 1 month apart, in a woman younger than 40 (Coulam et al., 1986; Welt, 2008). POI can be primary, spontaneous, or secondary to external insults.

Although quite often used synonymously, POI should not be equated with menopause. The main difference lies in the fact that with POI, ovarian function can still be present albeit unpredictable and/or intermittent. Moreover, it is believed that roughly 50% of women with POI retain intermittent ovarian function for many years, may exhibit spontaneous follicular development, and commence menstruation

(Rebar and Connolly, 1990). This is strongly supported by the fact that 5%–10% of women with POI can conceive (van Kasteren and Schoemaker, 1999) and deliver a child (Rebar et al., 1982; Nelson et al., 2005) without any medical intervention, even years after diagnosis was established. Similarly, Hipp et al. (2016) reported that 12.6% of women diagnosed with FXPOI conceived spontaneously after diagnosis. The time to conception after diagnosis ranging up to 12 years (Hipp et al., 2016). Approximately 20% of women with *FMRI* PM will develop FXPOI. Among women with idiopathic sporadic or the rare form of familial POI, about 2%–6%, and 14%, respectively, carry the PM within the *FMRI* gene (Sherman, 2000; Sullivan et al., 2011). It's unknown whether the risk of FXPOI is higher in women carrying the PM who also have a family history of POI in comparison to those who don't.

POI is a multi-factorial disease, which affects about 1% of women under the age of 40 (Coulam et al., 1986). For the most part (90%), the cause cannot be determined (idiopathic), whereas approximately 10% have an etiology that can be identified. *FMRI* PM is one of the most common single-gene mutation causes of POI in women with a normal karyotype. POI associated with the *FMRI* gene PM is referred to as FXPOI. Other known single-gene mutations associated with POI are Bone morphogenetic protein 15 (BMP-15), Diaphanous homolog 2 (DIAPH2) and Inhibin alpha subunit (INHA).

Most probably, POI occurs through two main mechanisms: (i) inadequate formation of the follicular pool *in utero*; and (ii) abnormally extensive or fast depletion of the follicular pool via atresia during post-natal (neonatal, childhood and adult) life. Thus, it would be logical to conclude that POI would be preceded by some degree of DOR. The term "POI" can be described as a continuum of compromised ovarian function over time, rather than a dichotomous state (normal ovarian function followed by an early menopause). Ovarian function is to deteriorate over a period of months to years and progress from an occult stage, which may manifest only by reduced fecundity, through a phase of biochemical manifestation (also elevated FSH levels), reaching the final stage of overt ovarian insufficiency characterized by irregular or absent menses, along with reduced fecundity and elevated FSH levels (Welt, 2008).

An important consideration when diagnosing POI is that this diagnosis is usually devastating and life-changing for many women (Greil, 1997; Nelson, 2009). Indeed, impaired self-esteem, shyness, social anxiety and low level of social support are more common in women facing POI (Schmidt et al., 2006; Orshan et al., 2009). Taking into consideration that PM carriers have an increased risk of depression and anxiety, it was recommended by Nelson et al. (2005) that these women return for follow-up to screen for symptoms of depression and anxiety and in general be encouraged to find sources of emotional support.

Ovarian Dysfunction in PM Carriers

There is no apparent difference in age at menarche between PM carriers and healthy controls (Allen et al., 2007). Even so, the reproductive span is reduced in the former. First clinical hints for impaired ovarian function might be found during adolescence

when approximately 3% of the PM carriers will experience non-specific menstrual cycle irregularities (De Caro et al., 2008). Schwartz et al. (1994) noted for the first time that PM carriers reported irregular menses more often than non-carriers. The fact that PM carriers have shorter menstrual cycles in comparison to age-matched women additionally supports the idea that FXDOR precedes FXPOI (Table 1, Welt et al., 2004). Hormonal profile alterations might be evident as well.

FSH is a pituitary hormone, which stimulates the growth and recruitment of immature ovarian follicles. With ovarian aging and diminishing number of follicles, less Inhibin is being released from the ovary, which consequently weakens FSH negative feedback resulting in increased release of FSH. An elevated level of FSH on the 3rd day of menstrual cycle, therefore, indicates diminution in the ovarian pool and has been used as a marker of ovarian reserve (aging) for decades (Scott et al., 1989). PM carriers demonstrate significantly higher FSH levels in the follicular phase (cycle day 1–10) when compared to healthy, age-matched women (Murray et al., 1999). Considerably higher serum FSH levels were also found in the follicular and luteal phases in PM carriers. Furthermore, lower Inhibin A and Inhibin B levels have also been discovered in these patients, implying impaired follicular and luteal ovarian function (Table 1, Welt et al., 2004). Anti-Müllerian hormone (AMH), another marker of ovarian reserve, was measured by Rohr et al. (2008) and it appeared to be more sensitive than FSH in identifying an early decline in ovarian function among PM carriers. A subtle decrease in AMH levels in women carrying the PM was detected as early as the age of 18 years, in the absence of differences in FSH levels between controls and carriers, suggesting a low ovarian reserve for PM carriers even at this young age (Rohr et al., 2008). These findings support the notion of a continuous deterioration of ovarian function in these patients that may be detected only by sensitive biochemical markers.

There is an increased rate of infertility in women carriers compared to non-carriers (Allen et al., 2007). Despite the early DOR, no increase in the rate of miscarriages or chromosomal abnormalities due to maternal age-related chromosomal nondisjunction was demonstrated in offspring of women carrying a PM (Murray et al., 2000a; Allen et al., 2007). Thus, while there may be a relative drop in follicle number, oocyte competence continues to be related to chronological age. Lastly, these women enter menopause, on average, 5 years earlier than the women in general population (Partington et al., 1996; Murray et al., 2000a; Sullivan et al., 2005). Because of these substantial impairments associated with carrying a PM, the American College of Medical Genetics (ACMG) recommends testing for the *FMR1* PM in all women with POI (Wittenberger et al., 2007). We would make the case for testing all women presenting with any reproductive dysfunction (Zev Rosenwaks, personal communication).

Correlation between Number of Repeats and Ovarian Function

Some evidence suggests a correlation between CGG repeat length and severity of the phenotype. CGG repeat length had

previously been associated with FXTAS clinical features, such as the age of onset of tremor and executive dysfunction (Cornish et al., 2011). The repeat size is also considered a risk factor for developing FXTAS dementia (Seritan et al., 2016). Others reported a correlation between age and CGG repeat length, as they found that male carriers with over 100 CGG repeats are more susceptible to the effects of aging on measures of executive function (Cornish et al., 2011). Furthermore, similar correlations were found in other repeat expansion diseases. For instance, in Huntington's disease, longer CAG repeat length is associated with earlier onset of the disease (Ross and Tabrizi, 2011).

Interestingly, in female PM carriers, there appears to be a difference in the degree of ovarian function among different CGG repeat length subgroups. Although having below 45 repeats is considered normal, some studies have shown a direct correlation between the number of repeats and DOR (Bretherick et al., 2005; Bodega et al., 2006; Gleicher et al., 2009). However, other studies have refuted this finding (Schufreider et al., 2015; Pastore et al., 2017). Notably, the official statement of the ACMG is that a repeat length lower than 45 is not associated with an abnormal phenotype (Monaghan et al., 2013).

This is not the case when it comes to the PM CGG repeat range (55–200). Women carrying a PM exhibit impaired fertility compared to that observed in non-carriers (Allen et al., 2007). Women with a mid-sized PM (approximately 80–100 repeats) are at greater risk of developing FXPOI (Ennis et al., 2006; Allen et al., 2007; Wittenberger et al., 2007). It appears that the risk increases with increasing PM repeat size between 59 and 99, while it actually declines with >100 repeats (Sullivan et al., 2005). Additionally, when it comes to *in vitro* fertilization (IVF) treatment, it has been shown that fewer eggs are retrieved from PM carriers when compared those of age-matched controls carrying less than 55 CGG repeats (Elizur et al., 2014). In agreement with Sullivan's findings, Bibi et al. (2010) reported that PM carriers with less than 100 repeats demonstrate a lower response to controlled ovarian hyperstimulation (COH) and decreased fertilization rate, in comparison to those with more than 100 CGG repeats.

PROPOSING A NEW DEFINITION; FXDOR-FRAGILE X-ASSOCIATED DIMINISHED OVARIAN RESERVE

The concept of ovarian reserve defines the women's reproductive potential as a function of a number and quality of her remaining oocytes (Practice Committee of the American Society for Reproductive Medicine, 2015). DOR is a condition in which the ovary loses its normal reproductive potential, compromising fertility. The condition may result from disease or injury, but most commonly occurs as a result of normal aging. Overt POI might take several years to develop unless it's secondary to removal of the ovaries, chemotherapy or radiotherapy. On the other hand, DOR is not an overt phenotype and harder to diagnose because of its subtle nature, thus, as of today, there is no consensus on the definition of DOR

TABLE 1 | Menstrual cycle and hormonal milieu characteristics of premutation (PM) carriers compared to age-matched regularly cycling women.

	PM carries (n = 11)	Controls (n = 22)	P value
Total cycle length in days	26.1 ± 1.0	28.2 ± 0.4	<i>P</i> < 0.05
Follicular phase length in days	12.9 ± 0.8	14.5 ± 0.4	<i>P</i> < 0.05
Luteal phase length in days	13.2 ± 0.5	13.7 ± 0.3	NS
Mean follicular FSH levels IU/L	21.9 ± 3.5	11.2 ± 0.5	<i>P</i> < 0.001
Luteal FSH levels IU/L	14.6 ± 3.9	7.9 ± 0.5	<i>P</i> < 0.001
Follicular Inhibin B levels pg/ml	77 ± 11	104 ± 6	<i>P</i> < 0.05
Luteal Inhibin B levels pg/ml	35 ± 5	41 ± 3	<i>P</i> < 0.05
Follicular Inhibin A levels IU/ml	1.6 ± 0.2	2.1 ± 0.2	<i>P</i> < 0.05
Luteal Inhibin A levels IU/ml	3.4 ± 0.7	5.8 ± 0.5	<i>P</i> < 0.05

Adapted from Welt et al. (2004). Summarizes the cycle and follicular phase duration in days, in addition to the mean follicular and luteal levels of follicle stimulating hormone (FSH), Inhibin A and Inhibin B. Study group, comprising regularly cycling fragile X PM carriers, 24–41 year old (34.5 ± 5.7 year), was compared with age-matched, regularly cycling controls, 23–41 year old (34.6 ± 5.8 year), at each stage of the cycle. All values are presented as mean ± SEM.

(Ferraretti et al., 2011). However, compared with women of similar age, women with DOR commonly have regular menses but a reduced quantity of ovarian follicles. Therefore, patients with DOR may have a limited response to ovarian stimulation with fertility medications and reduced fecundity (Committee on Gynecologic Practice, 2015). Also, evidence of DOR does not necessarily equate with the inability to conceive (Practice Committee of the American Society for Reproductive Medicine, 2015).

We would like to propose a new term for PM carriers with reduced ovarian reserve: “FXDOR”. We believe this term is more clinically appropriate, as it best corresponds to this process of continuous deterioration along with its fluctuant nature. The diminished ovarian function/reserve, might or might not lead to overt FXPOI (depending on whether amenorrhea occurs at the age of 40, or later). FXDOR encompasses the phases of ovarian insufficiency previously termed by Welt as “biochemical” and “occult” (Welt, 2008). This diagnosis will be a diagnosis of exclusion, after excluding all other known reasons for infertility (for instance, male factor, endometriosis, mechanical factor, etc.), in a woman carrying a PM allele, with regular menstruations regardless to the levels of ovarian markers, younger than 40 years of age will be considered to suffer from FXDOR. There is no established gonadotrophin concentration cutoff to suggest the initiation of ovarian insufficiency (Panay and Kalu, 2009), most probably due to the fluctuant and reversible nature of the ovarian function. The difference between these two stages, of the occult and biochemical, is FSH levels, which might fluctuate. Therefore, it seems reasonable to utilize a single unifying term-FXDOR. Also, there is no difference in the clinical management of both stages either, so the division to these two categories becomes redundant and cumbersome.

As of now, the prevalence of FXDOR remains undetermined, as the clinical presentation is subtle and often associated with no symptoms besides possible subfertility or infertility, it might be completely asymptomatic (unlike FXPOI, characterized by alarming menstrual irregularities). If a PM carrier has already completed childbearing at a much younger age before developing significant FXDOR affecting fertility, or if she never attempted conceiving (lack of interest, delaying childbearing for socio-economic reasons), FXDOR might progress completely unnoticed, and the first symptom of her PM might present as

FXPOI. Any other phenotypic features associated with a PM are non-specific as well, and wouldn’t be alarming enough to justify genetic testing. Given that the definition of PM and having intermediate alleles are related to the potential for generational expansion and not of the possible ovarian function, the only way to reach a comprehensive understanding of the scope and determining especially the cutoff of the repeat size would be to screen the general population for FXDOR.

RECENT SCIENTIFIC FINDINGS PROPOSING POSSIBLE MECHANISMS FOR OVARIAN DYSFUNCTION IN PM CARRIERS

Successful development of primordial follicles during fetal life is critical for the establishment of the ovarian reserve, which in itself determines woman’s reproductive lifespan. In order to detect the link between the genetic impairment and the phenotype, we need to have a deeper understanding of the mechanisms involved. The etiology of ovarian dysfunction in carriers of the PM remains elusive. Elucidating mechanisms responsible for the development of FXDOR and FXPOI have proven to be challenging, largely due to the scarcity of suitable human samples and the lack of appropriate animal models available for research. Although FXDOR precedes FXPOI, it seems that only a portion of women having the phenotypic expression of FXDOR will eventually exhibit the extreme form of FXPOI. Expanded CGG repeats in the PM range are linked to the occurrence of both FXDOR and FXPOI; hence it is highly likely that the same mechanism accounts for both. Nevertheless, the alternative explanation that different mechanisms are involved in the development of FXDOR and FXPOI cannot be completely ruled out at this time.

Limitations and restrictions on the availability of human ovarian tissue and therefore existing studies on the mechanism leading to ovarian dysfunction in PM women force us to extrapolate findings from research in the field of FXTAS (Bourgeois et al., 2011; Seritan et al., 2013) onto FXDOR and FXPOI. One of the most commonly observed features in brain tissue of FXTAS patients are the ubiquitin-positive intranuclear inclusions (Galloway and Nelson, 2009). These

inclusions are composed of proteins and RNA. The presence of the FMR1 mRNA in the intranuclear inclusions (Tassone et al., 2004) together with the observed increase of FMR1 mRNA in PM carriers (Tassone et al., 2000a) led to the suggestion that a toxic RNA gain-of-function mechanism might be responsible for the development of FXTAS. It was proposed that the mutant FMR1 mRNA containing the expanded CGG repeats might sequester several RNA-binding proteins, preventing them from performing their normal intracellular functions (Galloway and Nelson, 2009). Willemsen et al. (2003) described an increase in both the number and the size of the inclusions during the course of life, which correlates with the progressive character of the cerebellar tremor/ataxia syndrome in humans. This suggests a correlation between the presence of intranuclear inclusions in distinct regions of the brain and the clinical features in symptomatic PM carriers (Willemsen et al., 2003).

A similar mechanism was suggested previously as a cause of two other repeat expansion disorders: myotonic dystrophy type 1 (DM1) and type 2 (DM2) (Mirkin, 2007). For DM1, it has been demonstrated that the RNA-binding protein MBLN co-localizes with the *DMPK* gene transcript containing the expanded repeats (Fardaei et al., 2001). This caused dysregulated splicing of MBLN targets and DM1 phenotype in transgenic mice (Kanadia et al., 2003). In addition, evidence of diminished ovarian reserve was reported in women with DM1 (Srebnik et al., 2014). It is possible that an accumulative process of RNA-protein complex over time represents the basis of this late-onset FXPOI as well. This observation suggests that a similar accumulation could perhaps occur in the ovary and may correlate with the onset and severity of the phenotype.

Besides the discovery of RNA aggregates in cells of FXTAS patients, ubiquitin-positive inclusions containing an FMR polyglycine protein (FMRpolyG) were found in brain cells of these patients (Todd et al., 2013). These intranuclear neuronal inclusions are generated by repeat-associated non-UTG (RAN)-initiated translation. RAN translation was also reported to occur in other repeat expansion diseases such as spinocerebellar ataxia type 8, ALS and frontotemporal patients (C9ALS/FTD) (DeJesus-Hernandez et al., 2011; Ash et al., 2013; Cleary and Ranum, 2014). Similar to the toxic RNA aggregates, FMRpolyG could sequester specific viable factors for proper cell function through protein-protein interaction. Furthermore, it was suggested that impairment in the protein quality control pathway, which is necessary for the cells in order to get rid of toxic and misfolded proteins, could contribute to the CGG repeat associated toxicity in human cells (Oh et al., 2015). In addition, reducing translation of FMRP was observed in PM carriers, probably due to decreased translation efficiency of the mutant FMR1 mRNA (Tassone et al., 2000a). FMRP is an RNA-binding protein which shuttles between the nuclear and cytoplasmic compartments (Jin and Warren, 2000). There is evidence that FMRP acts as a translational suppressor and functions in a dose-dependent manner as a regulator of gene expression at the post-transcriptional level (Laggerbauer et al., 2001; Li et al., 2001). Reduced FMRP

might cause some of the symptoms in PM women, such as a reduced germ cell population as seen in *Drosophila* model system.

The question remains whether a decrease in the number of primordial follicles arises from an insult during germ cell development or is it a result of an increased velocity of a diminishing oocyte pool by atresia or follicular destruction. Correspondingly, which mechanistic explanation is compatible with the phenotype of FXDOR and FXPOI? Is it a formation of abnormal potentially gonadotoxic RNA or protein aggregates during oocyte development, or later, during post-natal life? It is also possible that the explanation lies in a failure of the follicle to respond to gonadotropin stimulation. Herein we are about to propose some possible mechanisms that might explain the phenotype of FXDOR and FXPOI. The mechanisms postulate optional damage *in utero*, at the level of the establishment of the PGC, or later in postnatal life and the adult ovary (Figure 1).

Maintenance of PGC Require FMRP

During embryonic development at 6–8 weeks, germ cells begin to divide rapidly. By 16–20 weeks, fetal ovaries contain 6–7 million follicles, reaching its peak. In a mouse model, it was established that FMRP is expressed in PGC in the fetus (Hergersberg et al., 1995). It was also found that the PM allele does not affect the establishment of the primordial follicles pool (Sherman et al., 2014). This advocates that the expanded CGG repeats do not interfere with the assembly and the creation of the follicles. However, Yang et al. (2007) using *Drosophila* as a model, found that FMRP is required for preservation of germline stem cells (GSCs) in the ovaries. Ovaries of *Drosophila* female are composed of ovarioles. Each ovariole contains a functional unit called a germarium and differentiated egg chambers. GSCs are located at the tip of the germarium, and along with normal development, divide asymmetrically. GSCs generate some daughter cells for self-renewal, while other GSCs are displaced from the niche and become cystoblasts, which bud off the germarium as individual egg chambers and sustain oogenesis (Spradling et al., 1997). *Drosophila* ovaries of the *FMR1* null mutant contained fewer egg chambers and in some instances, the germaria were completely empty. These results indicate that in *Drosophila* FMRP is required for the maintenance of GSCs (Yang et al., 2007).

In concord with this observation, it has been shown in fetal ovarian samples that human FMRP is expressed in germ cells surrounded by FMRP-negative pregranulosa and interstitial cells (Rifé et al., 2004). FMRP expression in these germ cells coincides with the loss of expression of the pluripotency-associated protein (Rosario et al., 2016). Although the function of FMRP in fetal ovaries is unknown, a reduction of FMRP in the PM germ cells could affect the volume of the follicular pool. It has been shown that the translation of FMRP was strongly inhibited in cells containing the PM. This inverse correlation between decreased FMRP with increased repeat length is probably due to reduced translation efficiency of the mutant FMR1 mRNA (Primerano et al., 2002). The reduction in efficient translation thought to be caused by secondary mRNA (hairpin loops) structures disrupts

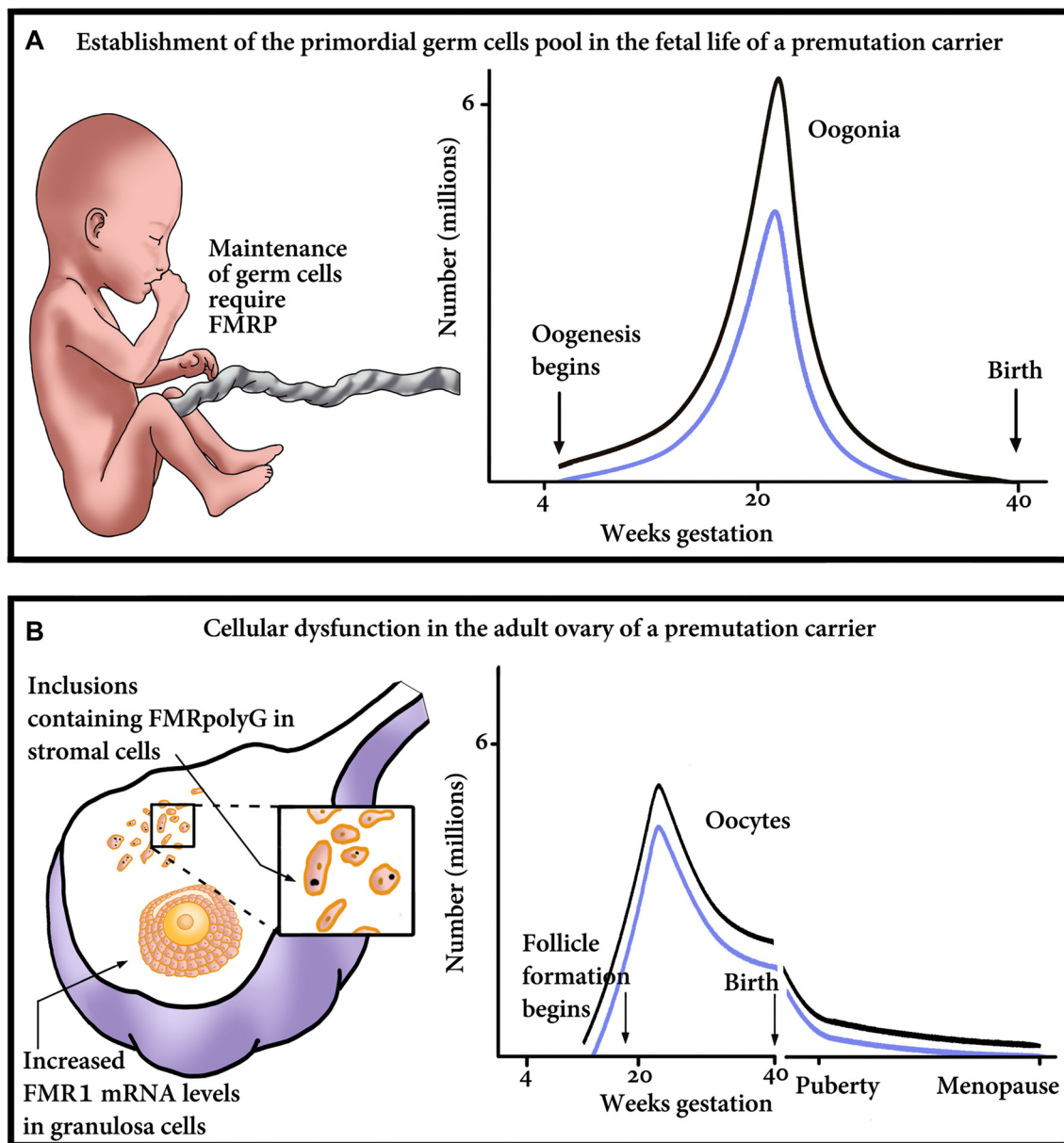


FIGURE 1 | Proposed model of major points of gonadal impairment consequent to *FMR1* premutation (PM) presence during fetal and adult life. **(A)** Establishment of the primordial germ cell (PGC) pool in a fetus with a PM. Due to reduced FMRP, the final endowment, as well as maintenance of the PGC, could be affected. The graph outlines the relationship between the number of oogonia and gestational age till birth. Damage to the PGCs and consequent reduction in the PGC pool size will result in reduced number of oogonia, thus shifting the graph downwards, from the non-carrier range (black line) to the PM carrier range (blue line). **(B)** Cellular dysfunction in the adult ovary of a PM carrier. The ovary suffers damage at the cellular level, which is clinically manifested as a diminished ovarian reserve. As seen on the left, the impairment occurs in the granulosa as well as the stroma cells of the ovary. Reduction in the number of oocytes/follicles could occur as a consequence of the mRNA-induced granulosa cell toxicity and subsequent dysfunction, detrimental effect of the inclusions containing FMRpolyG on the stroma cells, or both. On the right, a schematic representation of the number of oocytes, in non-carriers (black line) or PM patients (blue line). The graph emphasizes that the PM carriers' ovaries contain fewer oocytes than non-carriers', at any age.

proper ribosomal scanning, causing stalling at the expanded CGG repeat of *FMR1* (Fu et al., 1991; Tassone et al., 2000a). This reduction of FMRP expression could influence germ and stem cell maintenance, and lead to a reduced follicular pool in PM patients. Thus far, these models were not tested in humans.

Nonetheless, an international collaboration (Allingham-Hawkins et al., 1999) has established that in contrast to PM women, FXS patients have no elevated risk for FXPOI in comparison to that of the general population. However, it is uncertain whether FXS patients are at risk of developing FXDOR. FMRP, even though decreased, is still present in PGC of FM

patients since the *FMR1* locus is unmethylated in fetal oocytes (Malter et al., 1997). FMRP could play a role in the maintenance of the PGCs during fetal development and establishment of the follicle pool (**Figure 1A**). Reduction of FMRP in PM and FM individuals might conclude in less PGCs and a decreased follicle pool to begin with. Nevertheless, the rate of attrition could be normal, therefore, no FXPOI phenotype is apparent in FXS patients in contrast to PM carriers. PM, in addition, to a reduction in FMRP have an increase in *FMR1* mRNA, which could aggravate the ovarian dysfunction even further. Alternatively, there could be disparities between species and FMRP may not play a substantial role in the determination of the size of the follicular pool and consequently ovarian function in humans: therefore, FMRP could be less detrimental than the presence of toxic mRNA found in PM patients.

Increased mRNA Levels in Granulosa Cells of PM Women

During normal folliculogenesis, FMRP is predominantly expressed in granulosa cells (Hinds et al., 1993; Hergersberg et al., 1995; Schuettler et al., 2011). Although using mouse models, it was found that the PM allele does not hinder the establishment of the primordial pool, the number of more advanced subclasses of follicles was reduced (Sherman et al., 2014). This observation suggests that expanded CGG repeats do in fact interfere with the follicle development and assembly of the follicular unit. A toxic effect in human granulosa cells was exhibited, when granulosa cells were transduced with mRNA containing CGG repeats in the PM range (Hubayter et al., 2009). Recent findings also reported increased *FMR1* mRNA levels in mice ovary and human granulosa cells of PM carriers (Elizur et al., 2014; Sherman et al., 2014). Although the findings in PM women to date only show a correlation between the increased mRNA levels and low ovarian reserve, these results support a proposed toxic RNA-gain function mechanism similar to FXTAS in PM granulosa cells.

The mutant *FMR1* mRNA could sequester proteins by the formation of secondary RNA structures. RNAs containing CGG repeats are known to adopt secondary structures such as intramolecular hairpins (Zumwalt et al., 2007). Proteins could bind to these non-canonical RNA structures forming RNA-protein aggregates in the granulosa cells. Loss of function of these RNA-binding proteins in cells could compromise cell integrity and lead to early follicular decay (**Figure 1**). It has been shown in FXTAS-affected cells that CGG repeats bind to a large number of proteins, including hnRNP A2, Pur α , Lamina A/C and the miRNA biogenesis complex Drosha/DGCR (Jin et al., 2007; Sofola et al., 2007; Sellier et al., 2013). Another example is the RNA-binding protein Sam68, which is recruited to the RNA aggregates, generated by the *FMR1* mRNA containing the expanded CGG repeats. The sequestration of SAM68 causes an altered SAM68-regulated splicing in FXTAS patients (Sellier et al., 2010). Interestingly, SAM68 has been suggested to regulate the splicing of the mRNA of the FSH and the luteinizing hormone receptors (Bianchi et al., 2010). Indeed, altered splicing of these proteins could lead to ovarian resistance to FSH and LH at the receptor level.

In addition, an increased amount of *FMR1* mRNA in granulosa cells could also lead to a rise in R-loop formation, a secondary DNA-RNA hybrid structure formed by the repeats. R-loops could trigger genome instability and induce early decay of the follicles in PM women. These structures were observed by using the recently developed R-loop antibody in PM cells and also FXS cells, in which the *FMR1* gene transcription was reactivated by treatment with the DNA methylation inhibitor 5-aza-29-deoxycytidine (Groh et al., 2014; Loomis et al., 2014). Increased R-loops formation could lead to an increase in DNA damage in the cells. The formation of R-loops results in exposure to an unpaired single-stranded DNA due to the RNA-DNA hybridization (Santos-Pereira and Aguilera, 2015). Single-stranded DNA is more unstable and susceptible to lesions and transcription-associated mutagenesis or transcription-associated recombination (Aguilera, 2002). Another potential consequence of R-loop formation is the induction of genomic instability by interfering with DNA replication (Gan et al., 2011; Castellano-Pozo et al., 2012). Collisions of the transcription machinery with the replication fork have been shown to induce DNA breaks in budding yeast and mammals (Prado and Aguilera, 2005; Gottipati et al., 2008; Boubakri et al., 2010). Furthermore, replication fork stalling was detected at expanded CGG repeat sites in FXS stem cells and at expanded GAA repeats in Friedreich's ataxia stem cells, another repeat expansion disorder (Gerhardt et al., 2014, 2016). Prolong replication fork stalling could induce DNA breaks if unrepaired could result in cell apoptosis (Nowsheen and Yang, 2012). Yet, as of today, R-loops were not detected in human granulosa cells.

Inclusions Containing FMRpolyG in Stromal Cells

Intranuclear inclusions seem to be common in neurodegenerative conditions. Chang et al. (2011) demonstrated ubiquitin-positive inclusions within nuclei of the ovarian stromal cells. These inclusions appear to represent the ovarian counterparts of similar structures seen in the neurons of FXTAS patients. Interestingly, it was described by Sellier et al. (2017) that FMRpolyG interacts with the nuclear lamina protein LAP2b and disorganizes the nuclear lamina architecture in neurons differentiated from FXTAS iPSCs. Recently, FMRpolyG in ubiquitin-positive inclusions were found in ovarian stromal cells of a PM women (Buijsen et al., 2016; **Figure 1B**). Hypothetically, protein aggregates could be responsible for ovarian dysfunction leading to FXDOR and FXPOI. Perhaps an abnormal function of the stromal cells in the ovary will cause follicular atresia and an early decay of the ovarian pool.

Surprisingly, no inclusions containing FMRpolyG were found in the follicles *per se* (Buijsen et al., 2016). Since increased *FMR1* mRNA levels were observed in granulosa cells and FMRP seems to be expressed in all stages of the ovarian follicular development, we would expect a finding like this, even so, that wasn't described. One explanation could be that follicles containing inclusion are damaged, become atretic and are cleared away. However, FMRpolyG inclusions were only studied in the ovaries of one single PM woman so far, these results should be

confirmed in additional ovarian samples obtained from women carrying the PM.

Correlation between Repeat Size and the Severity of the Phenotype

Sherman et al. (2014) describe a non-linear association between the number of CGG repeats and symptoms in FXPOI patients. However, the mechanism leading to this phenomenon is not clear. An increase of *FMR1* mRNA level with the PM repeat length was observed in FXTAS-affected cells (Tassone et al., 2007) explaining the rise of symptoms with the repeat size until approximately 100 repeats. The only minimal decrease in the ovarian dysfunction in women with PM allele over 100 repeats could be explained by a different mechanism, skewing of the X-chromosome inactivation (García-Alegría et al., 2007). García-Alegría et al. (2007) found that the relationship between mRNA levels and repeat size is nonlinear; a significant positive correlation between CGG repeats and total mRNA levels has been found in the PM range <100 CGG, but this correlation diminishes from 100 onward. Nonetheless, when corrected for the X-inactivation ratio, García-Alegría et al. (2007) observed the mRNA levels increase as the number of CGG repeats increases, and this increase is highly significant over 100 CGG. They suggest that due to skewed X-inactivation, mRNA levels tend to normalize in females when the number of CGG repeats increases.

COUNSELING, PREVENTATIVE MEASURES AND INTERVENTIONS AVAILABLE FOR WOMEN CARRYING A PM

The Risk of Allele Expansion into a FM in the Next Generation and Clinical Implications

The PM can expand and be transmitted to the offspring in the form of a PM with a greater number of repeats or expand into a FM range. The pattern of inheritance suggests that the FM evolves during an intergenerational, multistep process characterized as anticipation (Pembrey et al., 1985). Both, the PM as well as the FM within the *FMR1* gene are inherited in an X-linked dominant fashion; female carriers transmit it to 50% of their offspring, while males transmit the PM to all of their daughters and none of their sons. The transmission to the offspring and its phenotype will depend on, the sex of the parent transmitting the gene, the sex of the child, the number of CGG repeats within the parental *FMR1* gene, and the stability of the affected allele, which depends on the presence of AGG trinucleotide interruptions within the allele.

A female PM carrier can transmit PM allele to both her male and female progeny. Due to its instability, the PM allele may expand into a PM with a higher number of repeats, or reach the range of a FM in the next generation (Nolin et al., 2011). The number of CGG repeats within the maternal premutated allele may undergo expansion during oogenesis (Malter et al., 1997) as well as during postzygotic mitoses in the embryo (Wöhrle et al., 1993). It has been long accepted that only maternally

inherited PM can expand into a FM in the next generation. On the other hand, PM as well as FM fathers can transmit only a PM to their daughters (Fisch et al., 1995). It is also believed that a paternally inherited PM does not expand to the same extent as the one inherited from the mother and can frequently contract. In fact, almost 40% of daughters of male PM carriers have PM with a lower number of CGG repeats than their fathers, in comparison to only 2% of daughters whose PM are shorter than their carrier mothers'. Moreover, when the transmission from the father expands it's by relatively fewer repeats compared to transmission from the mother (Fisch et al., 1995). A possible explanation lies in the fact that sequences with a high number of CGG repeats are highly unstable in the developing sperm and jeopardize their survival, as evidenced by only PM-size alleles found in spermatozoa of PM, as well as FM males (Reyniers et al., 1993). Nonetheless, this axiom has been challenged by at least one case report of a mentally disabled female child who inherited both a PM as well as a FM from her mosaic father (Zeesman et al., 2004). Although the father's peripheral blood cells demonstrated mosaicism, both premutated and fully mutated *FMR1* allele were present, his spermatozoa only contained the premutated allele, suggesting that the expansion to a FM found in the girl must have occurred post-zygotically.

The phenotype of a female child with a FM, as a result of either inheriting a maternal PM that expanded or inheriting an actual FM, is variable, ranging from severe intellectual impairment to apparently normal functioning. A male child, on the other hand, will almost invariably exhibit features of the FXS unless the *FMR1* allele is hypomethylated like in "high functioning males". When it comes to PM inheritance, female offspring are at risk of developing FXDOR or FXPOI regardless of the sex of the parent transmitting the PM (Murray et al., 2000b; Sullivan et al., 2005).

It has been previously demonstrated that the number of CGG repeats within the maternal PM allele is in direct correlation with the probability of expanding into a FM in the offspring (Fisch et al., 1995; Nolin et al., 2011). For instance, a maternal allele containing 55–59 CGG repeats carries a 3.7% risk of expanding into a FM in the next generation, as opposed to 98% if the allele contains ≥ 100 repeats (Nolin et al., 2011). The lowest number of CGG repeats reported to be associated with a single generation expansion into a FM was from a woman carrying a PM allele of 56 repeats (Fernandez-Carvajal et al., 2009). Women with an intermediate number of CGG repeats (45–54) do not transmit a FM, although expansion to a PM length in their offspring has been described (Nolin et al., 2011).

It has been shown that the number of AGG interruptions within the CGG repeat region is inversely correlated with the instability of a PM allele and the risk of its expansion to a FM (Eichler et al., 1994). Yrigollen et al. (2012) reported that the presence of AGG interruptions reduces the risk of transmission of a FM, specifically for maternal alleles containing <100 repeats. These findings were further validated by Nolin et al. (2015), strengthening the association between the number of AGG repeats with CGG repeat region stability and providing more accurate risk assessments of expansion to FM in the next generation for women with 45–90 CGG

repeats within the *FMRI* allele. Nolin et al. (2015) found that in each CGG repeat size category, those without any AGG interruptions had the greatest risk of instability and expansion into a FM. For instance, if a female carrier, whose allele contains 55–59 CGG repeats, has at least one AGG interruption within the allele, the risk of expansion into a FM in the next generation is reduced from 3.7% to less than 1%.

Preconception Counseling for Women Carrying the PM

Identifying PM in a timely fashion is of paramount importance. By doing so, two major problems associated with this disorder could potentially be avoided: (i) the development of FXDOR or FXPOI before childbearing, which could otherwise render conception difficult or even impossible; and (ii) the presence of a FM, and its clinical manifestations, in the offspring.

Screening and Patient Counseling

The American College of Obstetricians and Gynecologists (ACOG) recommends PM carrier screening for women with a family history of fragile X-related disorders or intellectual disability, who are considering pregnancy or are currently pregnant (Committee on Genetics, 2017). The College also stresses the importance of testing women who present with unexplained ovarian insufficiency and/or menopausal-range FSH levels before the age of 40. Southern blot and polymerase chain reaction (PCR) are the preferred methods of determining the number of CGG repeats within the *FMRI* gene for either screening or diagnostic purposes.

In the same committee opinion, the ACOG stated that “conditions included in an expanded carrier screening panel should meet the following criteria: have a carrier frequency of one in 100 or greater, have a well-defined phenotype, have a detrimental effect on quality of life, cause cognitive or physical impairment, require surgical or medical intervention, and have an onset early in life” (Committee on Genetics, 2017). Although the prevalence of the PM is not greater than 1:100, and the phenotype of the PM carriers is not well defined, FXS phenotype is defined. The FXS and the molecular biology of the *FMRI* gene are significantly more complex than the other single-gene screening targets. In particular, the carrier state being screened for, the PM allele, is also disease causing, unlike the heterozygous carrier mutations screened for autosomal recessive diseases such as Cystic fibrosis (Grody, 2011). The course of the disease, as well as transmission to the next generation, can be influenced by medical intervention. Population-based carrier screening has been already implemented in certain countries that experience a higher incidence of this disorder (Geva et al., 2000). Just recently, Haque et al. (2016) reported on 346,790 individuals undergoing expanded carrier screening and provided insights on carrier frequencies for many rare conditions in a large, diverse, albeit selected population. The findings indicated that an expanded testing panel identified more hypothetical fetuses at risk for severe or profound phenotypes than did testing based on current screening guidelines. Moreover, this study brings additional data to the debate on population screening for FXS (Grody, 2011;

Finucane et al., 2012). Interestingly, they reported that in every race/ethnicity category other than the Southeast Asian, FXS has been shown to be more common than spinal muscular atrophy, and more common than cystic fibrosis in all race categories. The authors suggest a reconsideration of FXS population screening (Haque et al., 2016). Given recent publications, and physician’s chance to intervene and improve the outcomes for these women on one side, and a relatively high incidence of this disorder in the general population on the other (Musci and Caughey, 2005; Berkenstadt et al., 2007), we support *FMRI* CGG repeat screening for all women of reproductive age.

Patients must have a clear understanding of what their results mean in order to be able to make informed decisions about their reproductive health or to prepare to care for an affected child. They should receive education and care tailored to their carrier screening results. Information regarding the likelihood of CGG repeat expansion, possibly to the level of a FM and its clinical consequences in subsequent generations should be discussed. In order to bypass the genetic inheritance risk, some couples may consider child-free living, no further children, adoption or foster care. Others may choose to use preventive measures; an egg/embryo or sperm donation from unaffected donors, or IVF with preimplantation genetic diagnosis (PGD) for the selection of unaffected embryos and a subsequent transfer. Of course, the couple can always decide to carry on with natural conception and perform fetal genetic testing or parent a child with FXS.

The Risk of Development of FXDOR and Progression into FXPOI

As previously outlined, a relatively high proportion (up to 20%) of females carrying the PM will develop FXPOI (Sherman, 2000). Symptoms including menstrual irregularities or difficulty conceiving will not necessarily precede the cessation of ovarian function and the first presenting symptom may be secondary (or less commonly primary) amenorrhea. Even though spontaneous conception is possible in all POI patients (Rebar et al., 1982; van Kasteren and Schoemaker, 1999; Nelson et al., 2005), including FXPOI (Hipp et al., 2016), the overall chances of pregnancy are low, this devastating diagnosis represents one of the greatest challenges patients and reproductive endocrinologists face. Even though the majority of PM carriers will fortunately not develop FXPOI, they are at risk of acquiring FXDOR (Nolin et al., 2003, 2011). Regardless of the etiology, the vast majority of patients with a DOR will exhibit regular menstrual cyclicity, and the diagnosis is usually established during an infertility evaluation (Friesen et al., 2006). Physiologic ovarian senescence, as well as the development of FXDOR and FXPOI, cannot be prevented or delayed. At this time, there is no known remedy that prevents continuous follicular atresia. In lieu of an overall increase of mean maternal age in the US as a result of delaying childbearing for socio-economic reasons (Mathews and Hamilton, 2016), identifying PM carriers early, stressing the importance of early childbearing, if possible is desired. Also, counseling them about possible consequences of delaying childbearing and fertility preservation options is of an essence. Women with a PM may feel pressured to pursue childbearing earlier than they planned due

to the significant ramifications of the carrier state. Identifying the PM earlier would give these women the opportunity to make an informed decision regarding their reproductive and family planning.

Genetic Counseling

All the individuals identified with either intermediate results or with CGG repeats in the PM or a FM range should be offered further genetic counseling (Committee on Genetics, 2017). During genetic consultation of a PM subject, the possible impact on other family members (female as well as male) should be emphasized. The counseling should explain the pathophysiology of the condition and educate a patient on possible clinical manifestations pertinent to her (such as cognitive impairment, FXDOR and/or FXPOI, FXTAS) as well as her future offspring (the possibility of expansion into a FM in the next generation and the risk of intellectual disability and autism). In the case of a PM, counseling should also encompass calculation of the risk of allele expansion into a FM range in the next generation, using the number of CGG repeats as well as the number of AGG interruptions within the maternal allele. Prenatal testing (PGD of embryos, or Chorionic villus sampling (CVS) or amniocentesis of a fetus) should be discussed and offered to any affected individual. Educating patients on the risk of FXDOR and FXPOI with associated infertility or subfertility is of paramount importance, as it might affect carriers' family planning. Patients should be advised to consider earlier childbearing if feasible, or otherwise offered fertility preservation via oocyte and/or embryo cryopreservation. Women carrying the PM should be advised to avoid risk factors that are known to decrease the age at menopause, such as smoking. It should also be recognized that use of hormonal contraception may mask POI symptoms.

Choosing the Right Diagnostic Test

Therapeutic and remedial options will depend on several factors: (i) age at diagnosis of a PM or FM of the affected individual; (ii) the risk of expansion to a FM in the following generation based on the number of CGG repeats and further refined by the number of AGG interruptions; (iii) ovarian reserve; and (iv) patient's preference. Ideally, diagnosis of PM or a FM in the affected female should be established prior to conception, nevertheless, that is not always the case. It is not uncommon that the diagnosis is made during early pregnancy.

Preimplantation Genetic Diagnosis

Establishing the diagnosis prior to conception, allows the performance of embryonic genetic testing and selection hence, avoiding the transfer of an embryo with abnormal CGG repeat, assuring that the offspring will have CGG in the normal range. Thus, virtually eliminating the possible need for termination of an otherwise affected pregnancy. PGD represents a technique by which embryos created via COH, oocyte retrieval and fertilized mainly by performing intracytoplasmic sperm injection (ICSI), are genetically tested and selected for embryo transfer based on the presence of the mutation of interest. The biopsy is performed on 1–2 blastomeres in the case of a 3-day embryo (Martin and Arici, 2008), or more (5–8) cells in the case of

an embryo at the blastocyst stage, followed by a chromosomal or genetic analysis. The aim is to achieve a pregnancy with an unaffected embryo. Given the limited amount of genetic material obtained via this technique (6 pg of genomic DNA/cell), determination of the actual number of CGG repeats within the embryonic *FMR1* allele using single cell PCR can be associated with amplification failure (Malcov et al., 2007; Reches et al., 2009) and inability to accurately distinguish between the PM and the FM. Instead, the approach called linkage analysis is more commonly utilized. Linkage analysis relies on the principle that certain DNA sequences that are close together on a chromosome are less likely to be separated during chromosomal crossover, and are therefore inherited together. It requires genetic testing of the couple's relatives (siblings, parents, or any living children) using either short tandem repeat (SRT) or less commonly single nucleotide polymorphisms (SNP) analysis, allowing an indirect identification of the affected maternal *FMR1* gene in the oocyte.

Even though it is costly, the major advantage of PGD is avoiding the need for termination of affected pregnancies. On the other side, one of the difficulties lies in the fact that a certain proportion of affected women exhibit FXDOR or FXPOI, which makes them less responsive to COH and can significantly diminish the availability of embryos for PGD. Fortunately, when PGD and embryo transfer were possible, the outcomes were comparable to those of other monogenic diseases (Tsafrir et al., 2010). Additionally, as the accuracy of PGD is 98%–99% (Liebaers et al., 2010), confirmation with prenatal testing (such as amniocentesis) later in pregnancy is recommended. Although determining the number of AGG interruptions within the affected allele might further stratify and lower the risk, it does not completely eliminate the risk of having a child with a FM. Additionally, PGD might reveal that all tested embryos are affected and are therefore not suitable for transfer. In this case, there are some other options available, which will be elaborated upon later in this manuscript.

Prenatal Diagnosis

If either the risk of allele expansion into a FM is reasonably low (<5%) based on the number of CGG repeats and AGG interruptions; the PM or a FM diagnosis is established post-conceptionally; or the patient chooses not to proceed with PGD for any reason (prohibitively low ovarian reserve, the cost of treatment or simply patient's preference), prenatal testing can be performed by fetal tissue sampling in the 1st or 2nd trimester. Its purpose is to identify a pregnancy with an affected fetus for termination or to prepare the parents for the birth of an affected child.

CVS is an invasive procedure by which placental cells are obtained for further genetic analysis. It is typically performed between 10–13 weeks of gestation, under ultrasound guidance. Depending on the location of the placenta, CVS can be performed either trans-abdominally or transvaginally. Its general risk of a miscarriage is <1% (Mujezinovic and Alfirevic, 2007). One of the advantages of CVS over amniocentesis is an earlier diagnosis, which allows for earlier termination of pregnancy when the procedure is less traumatic and generally associated

with fewer complications. Even though the placenta and the fetus have the same embryonic origin and should, therefore, be genetically identical, this rarely might not be the case and they could contain genetically different cell lines. This phenomenon is called placental mosaicism. Performing genetic testing on placental cells, therefore, might not be an accurate representation of the genetic material of the fetus, and this can be avoided by amniocentesis that yields actual fetal cells.

Amniocentesis is an invasive procedure by which a small amount of amniotic fluid containing fetal cells is obtained for further genetic testing. It is typically performed between 15–20 weeks of gestation, under ultrasound guidance. It overcomes the previously mentioned issue of placental mosaicism and is associated with even lower risk of a miscarriage (Mujezinovic and Alfrevic, 2007). One of the disadvantages of amniocentesis is that it establishes the diagnosis in a later stage of pregnancy when termination is procedurally more difficult, generally associated with more complications, and requires a skillful operator.

Further laboratory testing is performed in the same manner, regardless of the source of the cells, either CVS or amniocentesis. The first step in the genetic analysis is the determination of the number of CGG repeats within the allele by PCR and categorizing it as either a normal, intermediate, PM or a FM. Southern blot is then used to more accurately distinguish a large PM from a FM and to determine the allele's transcriptional activity by determining the extent of methylation. Alternatively, the CGG repeat length, AGG interruptions, and DNA methylation can be determined by AmpliX[®] PCR (Asuragen). Until approximately 10 weeks of gestation, FMRP is expressed normally in FM males, whereas at 12.5 weeks it's completely absent. FMRP expression in FM female >13 weeks is completely absent in a number of villi, whereas other villi show normal FMRP expression due to random X chromosome inactivation in females. X chromosome inactivation occurs very early in development before the villi start to proliferate, and it represents a clonal process. In addition, evidence indicates that X-inactivation occurs before the time of *FMR1* allele inactivation in the FM (Willemsen et al., 2002).

When analyzing cells obtained via CVS, the prenatal detection of the repeat number is accurate and reliable, but one should keep in mind that the methylation pattern observed in placental tissue retrieved at 10–12 weeks' gestation is incomplete and might not reflect that observed in the live born (Iida et al., 1994). Occasionally, a follow-up confirmation with amniocentesis is required, as the test is accurate and reliable regarding both the methylation status, as well as the number of the repeats (McConkie-Rosell et al., 2005). Nevertheless, since PCR assays are so accurate and able to identify all FM, the consultation is based mainly on the repeat number. In addition, if the CVS is not conclusive, for instance, due to placental mosaicism, the possibility of a follow-up amniocentesis to clarify the status of the fetus should be discussed. One of the challenges of prenatal testing is the difficulty in predicting intellectual, psychological and behavioral phenotype in a female FM carrier, even when the methylation status is known, due to mosaicism for X-chromosome inactivation.

Contraception

Women with FXDOR/FXPOI who do not wish to conceive should use contraceptives. As was published by Hipp et al. (2016), 12.6% of women diagnosed with FXPOI conceived spontaneously after diagnosis. Amazingly, the interval of time to conception after diagnosis was up to 12 years. It appears that there might be a temporary remission, which in rare cases may last for years. According to this data, we believe it is prudent to offer these patients appropriate contraception.

Fertility Preservation Options

Although today, we can't prevent or reverse the deterioration in the ovarian reserve, we do have a substantial experience in banking oocytes and embryos for the purposes of fertility preservation. That is the reason we believe that if a woman is diagnosed carrying a PM, she should be consulted regarding her risk of developing FXDOR and FXPOI, and be advised about her fertility preservation options. Two main options available for adult patients are oocyte or embryo banking.

CONCLUSION

This manuscript encompasses recent scientific findings which have led to a better comprehension of the effect the *FMR1* PM on fertility. Lack of a deeper understanding of the *FMR1* PM mechanisms involved is holding us back in terms of treating and curing PM women and helping them restore or prevent further damage to their ovarian reserve. By continuously gathering evidence derived from animal and human models, we are always on our way to solving this puzzle. Using evidence supporting the importance of FMRP during embryonic life for maintenance of PGCs, and subsequently the involvement of both RNA and protein in the pathologic processes, we created a hypothesis, which could explain the chain of events leading to the reduction in ovarian reserve. We hypothesize that the phenotype is derived from the combination of damage occurring at different stages of development and maintenance of follicular pool: (i) at the level of PGCs establishment and formation during embryonic life; and (ii) post-natal damage occurring at the level of the ovary in the granulosa and stroma cells (follicle unit) (**Figure 1**). Nonetheless, environmental exposure, genetic background and lifestyle decisions will contribute to the phenotype as well. We propose that the severity of the ovarian damage is a reflection of the accumulation of multiple hits along the development and maintenance of the ovary throughout the course of life, from the embryonic stage until menopause.

The mechanisms leading to both FXDOR and FXPOI are probably, at least in part, the same. Even so, our understanding is only partial. The evidence support that the PGCs need FMRP for their maintenance, hence reduction of FMRP expression could influence the germ cells and stem cell maintenance, and lead to a reduction in the follicular pool in PM patients. Moreover, an early decay of the follicles could be a result of increased *FMR1* mRNA or FMRpolyG protein toxicity through the sequestration of RNA- and non-RNA-binding proteins by the expanded CGG repeat length, thus leading to

a functional insufficiency of the sequestered proteins. Another possible insult might be an increase in R-loop formation at the *FMR1* gene locus that results in DNA damage and cell death. Interestingly, the *FMR1* gene containing a PM remains unmethylated and the gene is transcribed, while FXS women have lower levels of FMRP expression. Despite the lower levels, FXS women do not suffer from ovarian dysfunction. These findings accentuate the fact that the role of FMRP in folliculogenesis is uncertain and needs to be elucidated.

Women carrying the PM have variable expression and face many challenges in their life, including menstrual abnormalities, infertility, the risk for bearing a child with a PM or a FM, and earlier menopause. Strikingly, these women are at risk for other conditions including dementia, hypothyroidism, hypertension, seizures, fibromyalgia, autoimmune disease, neuropathies, migraines and psychiatric conditions including postpartum depression. Even so, the magnitude of long-term risks associated with the disorder (including cardiovascular disease and osteoporosis) and the optimal means of reducing these risks are uncertain.

Undoubtedly, more research is needed on strategies to improve fertility outcomes for women carrying a PM. In the

meantime, we see a benefit in determining the PM or the FM status earlier rather than later through a population-based screening program, as both of these conditions are actionable. Early detection will provide time for patient counseling and might affect individual's decision making in order to prevent ovarian failure before childbearing has occurred. It also allows for prevention of having a child with FXS. Given the high incidence of both a PM and a FM in the general population, we strongly believe that this is the time to take a step forward and offer to screen all reproductive age women. It will be beneficial for the carriers to be informed, to understand the condition and ramifications, and to plan reproduction and/or fertility preservation accordingly.

AUTHOR CONTRIBUTIONS

JG, LM, JL wrote the manuscript and JG, LM, JL and ZR edited the article.

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Combination Therapy in Fragile X Syndrome; Possibilities and Pitfalls Illustrated by Targeting the mGluR5 and GABA Pathway Simultaneously

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Fragile X syndrome (FXS) is the most common monogenetic cause of intellectual disability and autism. The disorder is characterized by altered synaptic plasticity in the brain. Synaptic plasticity is tightly regulated by a complex balance of different synaptic pathways. In FXS, various synaptic pathways are disrupted, including the excitatory metabotropic glutamate receptor 5 (mGluR5) and the inhibitory γ -aminobutyric acid (GABA) pathways. Targeting each of these pathways individually, has demonstrated beneficial effects in animal models, but not in patients with FXS. This lack of translation might be due to oversimplification of the disease mechanisms when targeting only one affected pathway, in spite of the complexity of the many pathways implicated in FXS. In this report we outline the hypothesis that targeting more than one pathway simultaneously, a combination therapy, might improve treatment effects in FXS. In addition, we present a glance of the first results of chronic combination therapy on social behavior in *Fmr1* KO mice. In contrast to what we expected, targeting both the mGluR5 and the GABAergic pathways simultaneously did not result in a synergistic effect, but in a slight worsening of the social behavior phenotype. This does implicate that both pathways are interconnected and important for social behavior. Our results underline the tremendous fine-tuning that is needed to reach the excitatory-inhibitory balance in the synapse in relation to social behavior. We believe that alternative strategies focused on combination therapy should be further explored, including targeting pathways in different cellular compartments or cell-types.

Keywords: Fragile X syndrome, *FMR1*, GABA, bumetanide, mGluR5, automated tube test, autism, *Fmr1* KO mouse

INTRODUCTION

Fragile X syndrome (FXS) is a common X-linked hereditary cause of intellectual disability and autism spectrum disorders (ASD), with a prevalence of about 1:7000 males and 1:11,000 females (Coffee et al., 2009; Hunter et al., 2014). FXS is mainly characterized by cognitive and behavioral symptoms (Garber et al., 2008; Hersh et al., 2011; Kidd et al., 2014; Lozano et al., 2014). The autistic behavior and social deficits lead to major disabilities and are important features of FXS to evaluate when testing efficacy of potential pre-clinical therapeutic interventions. FXS is currently treated symptomatically, using behavioral, educational and psychopharmaceutical strategies, often with unsatisfying results. A targeted treatment is lacking.

Since the discovery of the *FMR1* gene as the causative gene of the disease, and the generation of the *Fmr1* KO mouse model, research has focused on elucidating the molecular basis of the disorder. The discovery of several pathways involved has revealed possible targets for therapeutic intervention strategies, holding the promise for a disease modifying therapy. Targeting these pathways indeed could correct many FXS-related symptoms in animal models, however, these promising preclinical results could not be confirmed in clinical trials (reviewed in Braat and Kooy, 2014; Ligsay and Hagerman, 2016). Many reasons could explain this lack of translation from mice to human (Zeidler et al., 2015). One striking limitation in drug discovery research so far, is the oversimplification of the underlying molecular mechanisms of the disorder, by targeting only one pathway at a time. The vast amount of molecular targets of the *FMR1* gene product, FMRP, suggests that the use of a combination therapy, targeting multiple involved pathways simultaneously, is a promising new strategy in drug discovery for FXS. In this article we discuss the possible use of combination therapy in FXS. In addition, we present the first *in vivo* data on chronic combination therapy, targeting both the excitatory and inhibitory system in the synapse in *Fmr1* KO mice. Our data illustrate that the two synaptic pathways are interconnected, although tremendous fine-tuning is probably required to restore the synaptic excitatory/inhibitory balance.

MANY TARGETS, MANY DRUGS

The symptoms of FXS are caused by lack of FMRP, an RNA-binding protein that plays a critical role in the process which determines neuronal connectivity, called synaptic plasticity (Willemsen et al., 2011). In the *Fmr1* knock-out (*Fmr1* KO) mouse this synaptic plasticity is disrupted, leading to neuronal dysfunction. Several pathways are implicated in aberrant synaptic plasticity in FXS, revealing them as possible targets for therapy. The metabotropic glutamate receptor 5 (mGluR5) pathway and the γ -Aminobutyric acid (GABA) pathway are only two examples (Braat and Kooy, 2014). Many studies have shown that we can indeed target these pathways in the *Fmr1* KO mouse, in some cases leading to improvement of several disease characteristics (reviewed in Braat and Kooy, 2014, 2015; Gross et al., 2015; Scharf et al., 2015). Interestingly, FMRP is not only present in the postsynaptic compartment, but is also expressed in the presynaptic compartment and other cell-types in the brain, although little is known about its function there (Wang et al., 2004; Pacey and Doering, 2007; Christie et al., 2009; Akins et al., 2012, 2017; Giampetruzzi et al., 2013; Higashimori et al., 2013; Gholizadeh et al., 2014). This might implicate more options for targeted therapy.

The mGluR5-pathway was the first proposed and best studied pathway involved in the pathogenesis of FXS, leading in 2004 to the “mGluR5 theory” (Bear et al., 2004). Activation of mGluR5 leads to downstream local protein synthesis in the postsynaptic compartment, which is essential for synaptic plasticity. This local protein synthesis is controlled by FMRP and its absence results in exaggerated mGluR5-dependent protein

synthesis and consequently aberrant synaptic plasticity. Several studies have shown that either genetic or pharmacological reduction of mGluR5 restores FXS related phenotypes in *Fmr1* KO mice, including molecular, anatomical, electrophysiological and behavioral characteristics (Dölen et al., 2007; de Vrij et al., 2008; Osterweil et al., 2010; Thomas et al., 2011, 2012; Michalon et al., 2012; Gantois et al., 2013; Pop et al., 2014; Scharf et al., 2015; de Esch et al., 2015). Another important pathway implicated in FXS, is the GABAergic pathway, the major inhibitory pathway in the adult brain (D’Hulst et al., 2006, 2009; Gantois et al., 2006; Curia et al., 2009; Pacey et al., 2009; Adusei et al., 2010; Olmos-Serrano et al., 2010; Sabanov et al., 2017; Zhang et al., 2017). Drugs targeting the GABA_A or GABA_B receptor, have shown improvements of FXS features in *Fmr1* KO mice. The function of the ionotropic GABA_A receptor, a synaptic and perisynaptic chloride channel, can also be indirectly influenced with the Na⁺-K⁺-2Cl⁻-co-transporter 1 (NKCC1) blocker bumetanide (Tyzio et al., 2014). While the GABA_A receptor inhibits depolarization in adult neurons, its function in immature neurons during early development is excitatory, switching to inhibitory while the neurons mature. This important neurodevelopmental switch depends on the intracellular chloride levels, regulated by the chloride importer NKCC1 (Ben-Ari et al., 2012; Ben-Ari, 2015). It has been shown to be delayed or absent in *Fmr1* KO mice (He et al., 2014; Tyzio et al., 2014) and FXS derived human embryonic stem cells (Telias et al., 2016). Also in other disorders, a delayed GABAergic switch has been implicated, including autism (Ben-Ari, 2015), epilepsy (Holmes et al., 2015), Parkinson’s disease (Damier et al., 2016) and schizophrenia (Lemonnier et al., 2016). Reduction of chloride levels with bumetanide, forces the neuron to switch from immature to mature chloride concentrations and consequently also to mature GABA_Aergic function. This has been demonstrated by bumetanide treatment of pregnant mice, which restored electrophysiological and behavioral phenotypes in their *Fmr1* KO offspring (Tyzio et al., 2014). Several clinical trials in patients with autism, have demonstrated improvement after bumetanide treatment (Lemonnier and Ben-Ari, 2010; Lemonnier et al., 2012, 2017; Hadjikhani et al., 2015), rendering it a promising drug in FXS as well.

Translational Challenges

The promising preclinical results have motivated researchers to initiate clinical trials in FXS patients. Some randomized, placebo controlled clinical trials with the mGluR5 antagonists mavoglurant/AFQ056, fenobam (Berry-Kravis et al., 2009, 2016; Jacquemont et al., 2011) and basimglurant (Youssef et al., 2017) have been performed. However, despite the evidence for effectiveness of mGluR5 antagonists from animal model studies, these clinical trials did not result in improvement of symptoms in FXS patients. Also the larger clinical trials with the GABA_B agonist Arbaclofen, were terminated prematurely due to lack of efficacy (Berry-Kravis et al., 2017). In fact, none of the larger clinical trials have resulted in an effective treatment for FXS. This raises the question whether these observed preclinical treatment effects reflect a relevant and versatile treatment strategy. Major limitations that could account

for this lack of translation include the lack of reliable and robust outcome measures, aspects of study design and the validity of animal models in drug screening (Berry-Kravis et al., 2013; Zeidler et al., 2015). However, one important aspect is being consistently neglected: considering the vast amounts of targets of FMRP, probably multiple pathways will need to be targeted simultaneously in order to ameliorate the disease, a combination therapy. Current studies in mice as well as in humans have been consistently limited to targeting only one pathway at a time.

NEW STRATEGIES IN FXS DRUG DISCOVERY: COMBINATION THERAPY

Compelling evidence has demonstrated that aberrant synaptic plasticity in FXS is (partly) caused by an excitatory-inhibitory imbalance, due to malfunctioning of these pathways (reviewed in Braat and Kooy, 2014; Ligsay and Hagerman, 2016). Thus, we hypothesized that targeting both excitatory and inhibitory pathways simultaneously as combination therapy, might be more beneficial in treating FXS than targeting a single pathway.

Only two previous publications have reported data on combination therapy in *Fmr1* KO mice. Lim et al. (2014) observed a synergistic beneficial effect on synaptic plasticity and behavior when targeting serotonin and dopamine-pathways in *Fmr1* KO mice simultaneously. Pacey et al. (2011b) showed an additional synergistic effect of acute targeting of mGluR5 (MPEP) and GABA_B (R-baclofen) in *Fmr1* KO mice on seizures, while for both a lower dose was needed than when administered separately. However, these studies used acute treatment and did not address social behavior deficits. Especially when initiated later in life, treatment of FXS would probably require a life-long treatment. To our knowledge we are the first to investigate the effect of chronic combination therapy in *Fmr1* KO mice, and using social behavior as an outcome measure.

The results of our combination therapy experiments are depicted in **Figure 1**. We targeted the mGluR5 pathway by genetically reducing mGluR5 expression, and the GABAergic pathway using the commercially available diuretic bumetanide. These pathways were first targeted separately (**Figures 1A–F**) and then simultaneously (**Figures 1G–I**). We used *Fmr1* knock-out mice (Mientjes et al., 2006), their wild-type (WT) littermates and for the double transgenics, we crossed these with mice who were heterozygous for an mGluR5 deletion (*Grm5*^{+/-}; Lu et al., 1997). We measured the effect of the therapeutic interventions using a social behavior paradigm, the automated tube test (ATT). The protocols are extensively described in de Esch et al. (2015) and van den Berg et al. (2015). Mice received bumetanide (Centrapharm) dissolved in drinking water in a concentration of 0.01 mg/ml, based on Tyzio et al. (2014), and kept in light-tight bottles. Aspartame was added to reduce the bitter drug taste. Control mice of the experiments with bumetanide, received aspartame drinking water. Control drinking water containing aspartame has been shown to have no effect on the *Fmr1* KO

phenotype in the ATT (data not shown). Mice were chronically treated from weaning at postnatal week 4 until the end of the experiment, postnatal week 13–16. This study was carried out in accordance with the recommendations of Directive 2010/63/EU, European Commission. The protocol was approved by the Dutch Animal Ethical Committee (DEC).

Previously, we have published that *Fmr1* KO mice display a robust dominant ATT phenotype compared to WT littermates, resulting in significantly increased percentage of matches won by *Fmr1* KO mice (de Esch et al., 2015). **Figures 1A–C** display the results of previously published experiments, showing that genetic reduction of mGluR5 results in a partial correction of social behavior of *Fmr1* KO mice in the ATT (de Esch et al., 2015). A complete correction would lead to a 50%–50% distribution of the matches between WT and *Fmr1* KO mice. The correction is partial, since no change in the phenotype is observed in those matches, after genetic reduction of mGluR5 in the *Fmr1* KO animals (**Figure 1A**). However, compared to “untreated” *Fmr1* KO mice, they do lose their phenotype (**Figure 1B**), illustrating the treated mice do no longer behave as *Fmr1* KO mice. If there would have been no effect of treatment, a 50–50 distribution of wins over the two groups was expected. This partial correction indicates that targeting the mGluR5 pathway does significantly influence the social behavior phenotype, but is not sufficient to fully restore deficits in this type of social behavior. A quite similar effect was observed when targeting the GABAergic pathway, using chronic bumetanide treatment. **Figures 1D–F** depict the results of chronic bumetanide treatment, leading again to a partial correction of the FXS ATT phenotype. These results indicate that treatment with bumetanide by itself is insufficient as well. However, these results do underline that bumetanide might have a beneficial effect on social behavior in FXS patients. Since we administered bumetanide after the GABAergic developmental switch has occurred (He et al., 2014), the improvement we measure is encouraging in terms of treatment initiation later in life, although the exact underlying neurochemistry changes remain to be elucidated.

After the partial correction observed for both “treatment interventions” separately, we combined those. However, combination therapy leads to an opposite effect than expected. First, the *Fmr1* KO mice with combination therapy remain dominant in matches against WT animals (**Figure 1G**). Moreover, *Fmr1* KO mice with combination therapy show a mild but significant dominant phenotype against “untreated” *Fmr1* KO mice (**Figure 1H**), implicating worsening of the phenotype. Improvement of the phenotype would lead to dominant behavior of untreated mice, which is opposite to what we observed. To evaluate whether a subtle synergistic effect occurs with two treatments compared to one treatment alone, we performed the test comparing *Fmr1* KO mice with either mGluR5 reduction alone to *Fmr1* KO mice with a combination of mGluR5 reduction and bumetanide. A synergistic effect would have led to dominant behavior of *Fmr1* KO mice with one intervention, compared to those with a combination therapy. However, we did not observe a synergistic effect, but instead we observed a slight worsening of the ATT phenotype in *Fmr1* KO mice with a combination therapy, compared to

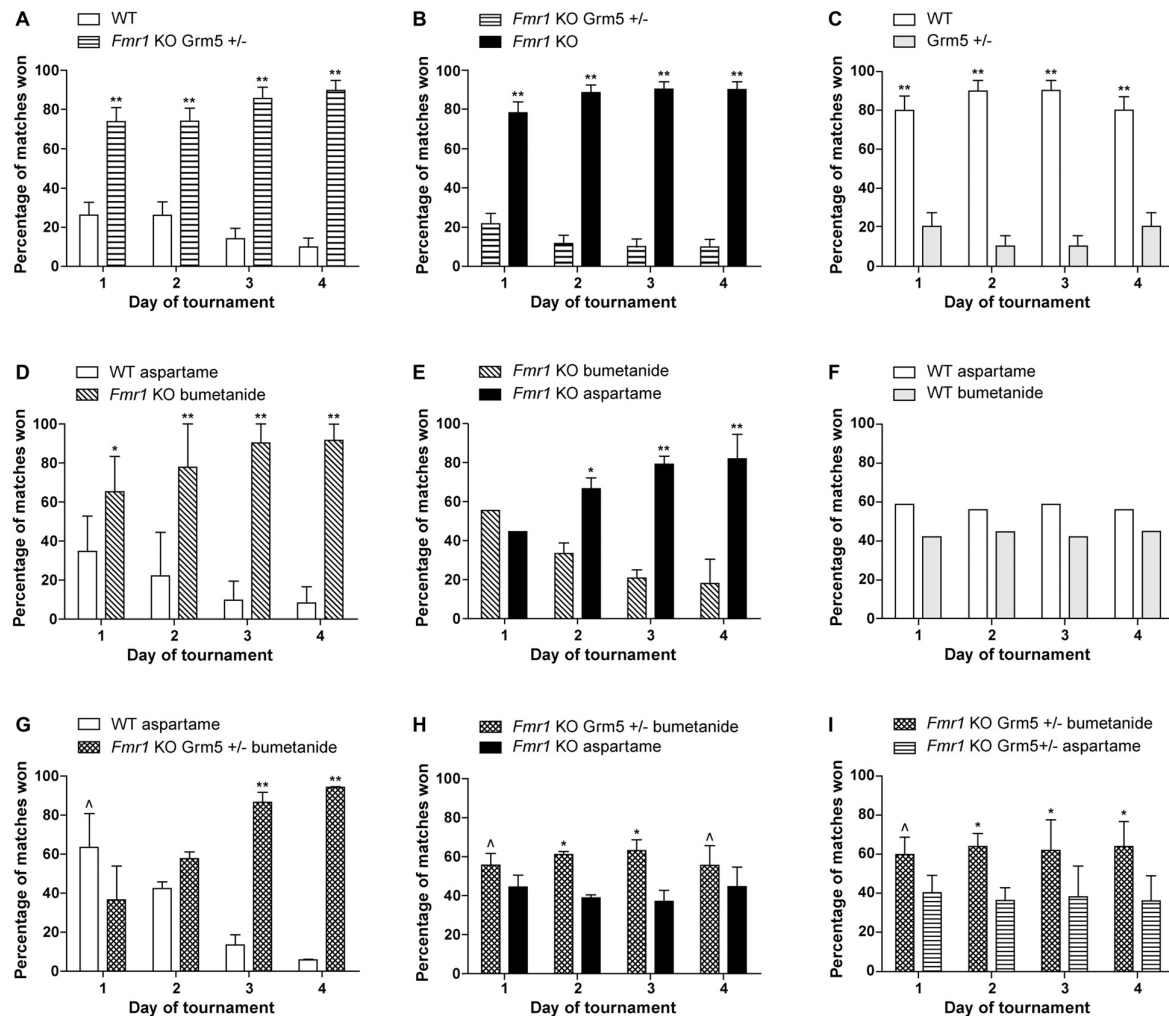


FIGURE 1 | Reduction of metabotropic glutamate receptor 5 (mGluR5) or enhancing γ -aminobutyric acid (GABA) separately, partially improves the Fragile X syndrome (FXS) phenotype, while a combination therapy slightly worsens this effect. Results are indicated as percentage of matches won by *Fmr1* KO mice and wild-type (WT) littermates. To explain what is meant by a partial correction in the tube test: a full correction would implicate a 50%–50% result of the matches between WT mice and treated *Fmr1* KO mice. In that case, both groups show a similar social behavior phenotype. When a partial correction is observed, there is a clear dominant phenotype of untreated *Fmr1* KO mice compared to treated *Fmr1* KO mice, while treated *Fmr1* KO mice do not show a correction in matches against WT mice. (A–C) Previously published results from de Esch et al. (2015) presenting that genetic reduction of mGluR5 partially corrects the automated tube test (ATT) phenotype in *Fmr1* KO mice. (A) *Fmr1* KO mice who are *Grm5*^{+/-} continue to show a strong phenotype compared to their WT littermates ($p < 0.001$, $n = 12$ per group). (B) Strong reduction of ATT phenotype with mGluR5 reduction: *Fmr1* KO win most matches against *Fmr1* KO mice who are *Grm5*^{+/-} ($p < 0.001$, $n = 10$ per group). (C) Genetic reduction of mGluR5 induces an inverse phenotype in the WT animals in the ATT ($p < 0.001$, $n = 6$ per group). (D–F) Chronic treatment with bumetanide partially corrects the ATT phenotype in *Fmr1* KO mice. (D) *Fmr1* KO mice treated with bumetanide continue to show a strong phenotype compared to their WT littermates receiving aspartame drinking water ($p < 0.01$ on day 1 and $p < 0.001$ on day 2–4, $n = 12$ per group). (E) Strong reduction of ATT phenotype after bumetanide treatment comparing treated and untreated *Fmr1* KO mice: *Fmr1* KO receiving aspartame water win most matches against *Fmr1* KO treated with bumetanide after day 1 ($p < 0.01$ on day 2, $p < 0.001$ on day 3 and 4, $n = 12$ per group). (F) WT mice receiving bumetanide in their drinking water and WT mice receiving aspartame drinking water win equal amounts of matches ($p > 0.1$ for all days, $n = 6$ per group). (G–I) Combination of genetic mGluR5 reduction and bumetanide treatment results in a slight worsening of the ATT phenotype compared to mGluR5 reduction or bumetanide treatment alone. (G) *Fmr1* KO who are *Grm5*^{+/-} and treated with bumetanide lose most matches against WT receiving aspartame drinking water on day 1 ($p = 0.02$) but win most matches on day 3 and 4 ($p < 0.001$, $n = 10$ mice per group). (H) *Fmr1* KO mice who are *Grm5*^{+/-} and treated with bumetanide win slightly more matches than *Fmr1* KO receiving aspartame drinking water ($p = 0.04$ on day 1 and 4 and $p < 0.01$ on day 2 and 3, $n = 18$ mice per group). (I) *Fmr1* KO who are *Grm5*^{+/-} and treated with bumetanide win slightly more matches than *Fmr1* KO *Grm5*^{+/-} receiving aspartame drinking water ($p = 0.01$ to $p = 0.002$, $n = 17$ mice per group). Data shown as mean percentage $\pm$ SEM. P -values were calculated using a binomial distribution test: in an experiment, both groups are similar if approximately 50% of matches are won per group. ** <0.001 , * <0.01 , ^ <0.05 .

one intervention alone (Figure 1I). This might be explained as an antagonistic effect. Although no synergy was observed, clearly targeting the two pathways simultaneously, does create

a combined effect, attenuating their therapeutic efficacy on FXS social behavior deficits, and confirming the pathways are interconnected.

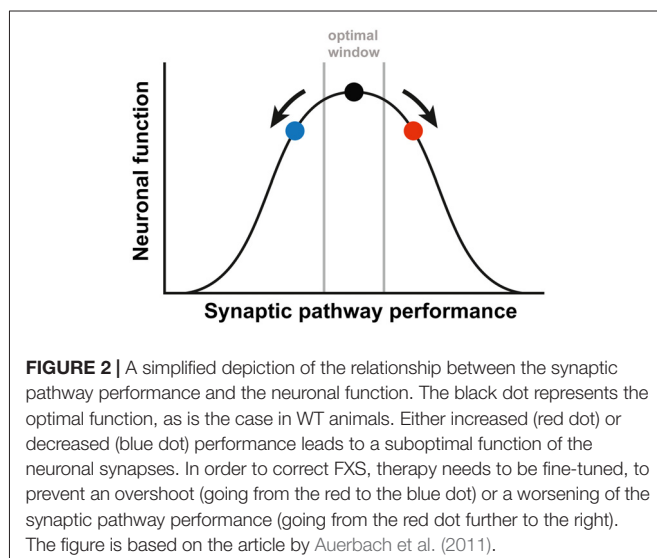
OPTIMAL WINDOW IN COMBINATION THERAPY

Interestingly, the partial rescue that we observed for both treatments separately, is reduced when they are combined, even leading to a slight worsening of the phenotype. These results implicate that the treatment effect might be managed by adding different interventions and titrating those to reach an optimal effect. It has been previously suggested by Auerbach et al. (2011) that synaptic plasticity is a tightly regulated process. The authors demonstrated an optimal window for protein synthesis levels. Deviations to either side of this optimum, resulted in decreased functioning of the synapse and aberrant synaptic plasticity. This idea of an optimal synaptic function due to a balanced interconnection of involved pathways, could be generalized to the excitatory/inhibitory balance of the synapse or to synaptic performance in general. Considering this optimum, our results might be explained by either an opposing effect of both treatments, or by an overshoot effect of both treatments when combined (**Figure 2**). In both cases, this means that restoring this tightly regulated balance will need tremendous fine-tuning. Unfortunately, it is poorly understood how the mGluR5 and GABAergic pathways are interconnected at the synapse and no biochemical read-out is available to test whether the right balance has been reached (Martin and Huntsman, 2012; Fatemi and Folsom, 2015). To complicate matters, the required balance might be significantly different in different brain regions or even differ at the synaptic level within one neuron, since FMRP is not localized in every spine (Feng et al., 1997; Antar et al., 2004).

Obviously, the results presented in **Figure 1** regard only one specific social behavior paradigm, which does not inform us on the effect on other FXS behavioral and cognitive phenotypes. In addition, only two pathways were targeted. Possibly, other pathways are more important for social behavior and targeting those might result in a beneficial effect. Even though we currently

cannot demonstrate a synergistic effect, it seems plausible that targeting only one pathway is not sufficient to ameliorate FXS completely. While considering the lack of translation from mice to human, combination therapy has received little attention, even though we are only starting to grasp the complex role of FMRP in synaptic plasticity. FMRP binds many post-synaptic mRNAs, that are involved in important neuronal synaptic pathways. Moreover, FMRP does not only have a postsynaptic function, but is also present in the presynaptic compartment and other cell types, including glia cells (Wang et al., 2004; Pacey and Doering, 2007; Pacey et al., 2011a; Giampetruzzi et al., 2013; Higashimori et al., 2013; Myrick et al., 2015). Thus, absence of FMRP potentially disrupts many cellular pathways, each with its own function. Recently, a missense mutation in *FMR1* has been identified in a patient, demonstrating a specific function of FMRP in the presynaptic compartment (Myrick et al., 2015). The patient only displayed ID and seizures, but did not display the behavioral problems associated with FXS, suggesting different pathways in different cellular compartments might be associated with specific FXS symptoms and phenotypes. Additionally, FMRP is present in other non-neuronal cell-types, where its function is even less understood (Wang et al., 2004; Pacey and Doering, 2007; Higashimori et al., 2013). For example, compelling evidence demonstrates the role of astrocytes, in neuronal maintenance, but also in active control of synaptic function, leading to the new concept of the tripartite synapse (Cheng et al., 2012). FMRP is present in the astrocytes, and its absence has been demonstrated to hamper normal astrocyte function, opening a new field of possible therapeutic strategies. An additional reason that advocates combination therapy, is the presence of compensational mechanisms that add to the individual differences. Targeting more than one unit of a pathway could be more effective and specific, with a lower dose needed, reducing the chance for side effects.

Other research fields have a longer history of combining targeted treatments to improve therapy. For example, studying the complex genetics of cancer has led to the identification of key-oncogenic cellular pathways, enabling the use of a combination of targeted pharmacological treatments to selectively block and kill tumor cells (Yap et al., 2013). However, these settings often have access to high throughput study models in cell culture and well-defined outcome measures, which are lacking in neurodevelopmental research. In recent years, combination therapy in neurodevelopmental syndromes have been proposed, for example in Rett syndrome (Sahin and Sur, 2015) and tuberous sclerosis complex (Lee et al., 2006). In FXS patients, one case report mentioned combination therapy with two drugs in combination with intensive educational treatment in two children, resulting in improvement of cognition and behavior (Winarni et al., 2012). In the near future, a clinical trial treating FXS patients with a combination of lovastatin and minocycline, will start (NCT02680379). New pre-clinical studies are needed to further evaluate the role of FMRP in other cell-types and to reveal new targets for therapy. Those targets should be used to investigate whether combination therapy is the key solution for FXS treatment, by targeting multiple pathways in different cellular compartments or cell-



types. Probably, all those interventions must be applied in combination with stimulating behavioral and cognitive therapy, to maximize therapeutic effects.

CONCLUSION

In conclusion, the complexity of the pathophysiology of FXS and the lack of translation from mouse to human, indicates that combination therapy is essential in the development of a targeted therapy for FXS syndrome. This approach needs to be further explored and might become successful, using other drugs, or targeting pathways in different cellular compartments, for example pre- and postsynaptic, or even other cell-types. However, combination therapy will need to be fine-tuned, in order to restore the tightly regulated synaptic pathway balance.

AUTHOR CONTRIBUTIONS

RW has been involved in design of experiments, supervising PhDs, correcting draft manuscript. SZ has been involved in performing experiments, design of experiments, interpretation

of results, statistics, writing draft manuscript. HB has been involved in performing experiments and interpretation of results, statistics, correcting draft manuscript. RKH has been involved in supervising PhDs, design of experiments, correcting draft manuscript.

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Negative Effects of Chronic Rapamycin Treatment on Behavior in a Mouse Model of Fragile X Syndrome

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Fragile X syndrome (FXS), the most common form of inherited intellectual disability, is also highly associated with autism spectrum disorders (ASD). It is caused by expansion of a CGG repeat sequence on the X chromosome resulting in silencing of the *FMR1* gene. This is modeled in the mouse by deletion of *Fmr1* (*Fmr1* KO). *Fmr1* KO mice recapitulate many of the behavioral features of the disorder including seizure susceptibility, hyperactivity, impaired social behavior, sleep problems, and learning and memory deficits. The mammalian target of rapamycin pathway (mTORC1) is upregulated in *Fmr1* KO mice and is thought to be important for the pathogenesis of this disorder. We treated *Fmr1* KO mice chronically with an mTORC1 inhibitor, rapamycin, to determine if rapamycin treatment could reverse behavioral phenotypes. We performed open field, zero maze, social behavior, sleep, passive avoidance, and audiogenic seizure testing. We found that pS6 was upregulated in *Fmr1* KO mice and normalized by rapamycin treatment, but, except for an anxiogenic effect, it did not reverse any of the behavioral phenotypes examined. In fact, rapamycin treatment had an adverse effect on sleep and social behavior in both control and *Fmr1* KO mice. These results suggest that targeting the mTOR pathway in FXS is not a good treatment strategy and that other pathways should be considered.

Keywords: Fragile X, rapamycin, mTORC1, hyperactivity, anxiety, social behavior, learning and memory, sleep

INTRODUCTION

Fragile X syndrome is the most common inherited form of intellectual disability. It is an X-linked disorder caused by an elongation of a CGG repeat (>200) in the 5'-untranslated region of the *FMR1* gene leading to silencing of the gene and a paucity of the protein product FMRP (Verkerk et al., 1991). FXS primarily affects males who experience a myriad of symptoms ranging from cognitive impairment, seizures, disordered sleep, and emotional instability

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ASD, autism spectrum disorders; FXS, Fragile X syndrome; KO, knockout; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide-3 kinase; PIKE, PI3K enhancer; SEM, standard error of the mean; TSC, tuberous sclerosis complex.

(Loesch et al., 2002; Jin and Warren, 2003; Penagarikano et al., 2007; Kronk et al., 2010). Up to 60% of FXS cases are diagnosed with ASD (Hagerman et al., 1986; Bailey et al., 1998). In a mouse model of FXS, *Fmr1* KO, behavioral characteristics paralleling those seen in FXS individuals have been demonstrated. These include hyperactivity, decreased preference for social novelty, sleep deficits, and learning and memory deficits (Mineur et al., 2002; Qin et al., 2002; Kooy, 2003; Spencer et al., 2005; Liu et al., 2011; Kazdoba et al., 2014; Saré et al., 2016, 2017).

It is thought that a dysregulation of protein synthesis, particularly at synapses, underlies these behavioral symptoms. Two signaling pathways that are critical nodes in the regulation of protein synthesis may be involved: mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and mTOR. mTOR in association with raptor forms mTOR complex 1 (mTORC1), a metabolic sensor and a key regulator of cell growth. Dysregulation of mTORC1 is associated with many neurological disorders including ASD, epilepsy, and neurodegenerative disorders (Lipton and Sahin, 2014). The MAPK/ERK cascade functions in cell proliferation, differentiation, and survival, and is activated through growth factors acting on receptor-activated tyrosine kinases. Both pathways have been shown to be elevated in studies of *Fmr1* KO mice (Osterweil et al., 2010; Sharma et al., 2010; Bhattacharya et al., 2012; Liu et al., 2012; Busquets-Garcia et al., 2013; Choi et al., 2016; Sawicka et al., 2016). However, there is some debate whether the mTORC1 pathway is actually elevated in *Fmr1* KO mice since some studies have not found increases in pathway components (Osterweil et al., 2010; Sawicka et al., 2016).

In the present study, we sought to determine the effects of rapamycin-induced mTORC1 inhibition on the behavioral phenotype of *Fmr1* KO mice. We chronically treated control and *Fmr1* KO mice by dietary administration of rapamycin. We found that p-S6 (a downstream target of both mTORC1 and ERK) in cortical lysates from vehicle-treated *Fmr1* KO mice was elevated compared to vehicle-treated controls. Levels of p-S6 were reduced in *Fmr1* KO mice following rapamycin treatment. We performed a battery of behavioral tests to examine sleep duration, activity, anxiety-like behavior, social behavior, learning and memory, and seizure susceptibility. Paradoxically, we found that rapamycin did not reverse most of the phenotypes examined. In fact, in both control and *Fmr1* KO mice, rapamycin decreased sleep duration and measures of social interaction. Our results suggest that the mTORC1 pathway is not causally involved in the behavioral phenotype of FXS and that alternate pathways should be considered for targeted treatments.

MATERIALS AND METHODS

Animals

Male *Fmr1* KO and control animals on a C57BL/6J background were generated from heterozygous and control breeder pairs in-house. Animals were weaned at 21 days of age. Genotyping was performed from tail biopsies as previously described (Qin et al., 2002). Animals were group housed in a climate controlled facility with access to food and water *ad libitum*. All procedures were performed in accordance with the National Institutes of Health

Guidelines on the Care and Use of Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

Rapamycin Treatment

At 21 days of age (P21), males in each litter were separated such that half received vehicle treatment and the other half received rapamycin treatment. Rapamycin was encapsulated with Eudragit (Rapamycin Holdings, San Antonio, TX, United States) and incorporated into mouse chow at 11 mg/kg food (Purina LabDiet, St. Louis, MO, United States). Based on how much food an average mouse consumes per day (4g; Tremblay et al., 2007), P21 animals received 4.4 mg/kg/day. Once the animals reached adult weight around 60 days of age, we estimate that the animals received 1.75 mg/kg/day. This dosage was based on a previous dosage used to reverse social behavior deficits in a mouse model of TSC (Reith et al., 2012).

Behavior Testing

Beginning at 60 days of age, animals were subjected to a battery of behavior tests with tests spaced 1 week apart. They were conducted from the least stressful to the most stressful in the following order: sleep testing, open field, zero maze, social behavior, and passive avoidance.

Sleep Monitoring

Sleep testing was conducted by means of a home-cage monitoring system (Columbus Instruments, Columbus, OH, United States) as previously described (Saré et al., 2017). Briefly, animals were individually housed and recorded for 72 consecutive hours. To eliminate the effect of habituation to the home-cage environment and single housing (Saré et al., 2017), we analyzed sleep duration from the last 48 h of recording. An animal was considered asleep if it had 40 s of consecutive inactivity. The percent time asleep was calculated separately for the light (inactive) and the dark (active) phases.

Open Field

Activity and anxiety were assayed by means of photobeam detection in a novel open field environment (Coulbourn Instruments, Whitehall, PA, United States) as previously described (Saré et al., 2015). Briefly, total horizontal distance moved was recorded over a 30-min session in 5 min epochs as a measure of total activity. The ratio of horizontal distance traveled in the center (more than 1.91 cm away from the arena walls) to total distance was calculated for each epoch as an inverse measure of anxiety.

Zero Maze

Anxiety was assayed by means of a zero maze (Med Associates, Fairfax, VT, United States). Animals were placed facing the open portion of the zero maze and allowed to explore the maze for 5 min. The times spent in the open and closed portions of the maze were determined. An animal was considered to be in a portion of the maze if both forepaws crossed into that portion. If an animal fell off the maze, it was eliminated from the analysis.

Social Behavior

Social behavior was assayed by means of a 3-chambered apparatus as previously described (Saré et al., 2016). The assay had three consecutive parts. First, the animal was allowed a 5 min habituation period to the empty chamber. Animals demonstrating a side preference of more than 3 min were eliminated from the test. Second, in the sociability phase, an age-matched stranger mouse was placed in a sociability enclosure (Noldus Information Technology, Inc., Leesburg, VA, United States) in one chamber. An empty sociability enclosure was placed in the opposite chamber. These locations were alternated between mice to avoid a side bias. The test animal was placed in the center chamber and allowed to freely explore all chambers for 5 min. Times spent sniffing the sociability enclosures were recorded by subsequent video analysis. Sniffing was analyzed by means of the TopScan software (Clever Systems, Reston, VA, United States). Parameters were set to define sniffing as the animal's nose directed toward the enclosure and within 20 mm of the enclosure. Third, in the social novelty phase, a novel stranger mouse was added to the previously empty sociability enclosure. The test animal was once again allowed to explore for 5 min, with measures recorded as before.

Passive Avoidance

Passive avoidance was conducted as previously reported (Saré et al., 2016). Briefly, habituation was conducted on Day 1. The animals were placed in the lighted chamber of the passive avoidance shuttle box with the door to the dark chamber closed. After 30 s, the door to the dark chamber opened. Once the animal entered, the test was ended. On Day 2, the animal was subjected to two training sessions. The animal was once again placed in the lighted chamber with the door to the dark chamber closed. After 30 s, the door was opened. Once the animal entered the dark chamber, it received a footshock (0.3 mA, 1 s). The animal was left in the dark chamber for 15 s and then moved to a holding cage for 120 s and was then placed in the lighted chamber and the process was repeated. On Day 3 and 24 h after the training session, animals were placed in the lighted chamber with the door to the dark chamber closed. The door was opened and the latency to enter the dark chamber was recorded with a cutoff of 570 s.

Audiogenic Seizure Susceptibility

In separate groups of animals, we conducted audiogenic seizure testing as previously reported (Miano et al., 2008). Testing began at P30. Animals were placed in a sound attenuating chamber with a viewing window (Med Associates, Fairfax, VT, United States). A siren (130 dB) sounded (Wal-Mart, Bentonville, AR, United States) for 5 min while seizure activity was observed. A seizure was defined as wild running, sometimes followed by myoclonic convulsions, sometimes followed by respiratory arrest. The frequency of each of these behaviors occurring in response to the tone was recorded.

Western Blotting

Mice used for Western blotting had been tested for behavior with the exception of passive avoidance and audiogenic seizure

susceptibility. A week after the last behavioral test, mice were decapitated and the frontal cortex was rapidly dissected for total protein extraction. The tissue was weighed and homogenized with 5% (weight/volume) solution of Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, United States) with 1% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) and 1% EDTA (Thermo Fisher Scientific). The homogenate was centrifuged at $15,000 \times g$ for 15 min at 4°C and the supernatant was collected. Protein extracts (10 µg) were treated with equal volume $2\times$ Laemmli buffer, incubated at 70°C for 10 min, and run on a 4–15% Mini-PROTEAN TGX Stain-Free gel (Bio-Rad Laboratories, Hercules, CA, United States). The gel was activated under ultraviolet light, proteins transferred to a nitrocellulose membrane, incubated with primary antibody overnight at 4°C, followed by secondary antibody [goat anti-rabbit horseradish peroxidase-linked at 1:10,000 (Bio-Rad Laboratories)] for 1 h at room temperature. We employed the Stain-Free Technology (Bio-Rad Laboratories) to normalize blots to total protein. The membrane was imaged under Stain-Free to determine total protein for loading control. The membrane was then incubated in Clarity substrate and imaged by means of a chemiluminescent signal on a ChemiDoc MP Imager (Bio-Rad Laboratories). Primary antibodies were used at a 1:1000 dilution and were as follows: FMRP (Abcam 27455), p-mTOR (Cell Signaling 5536), mTOR (Cell Signaling 2983), p-p70S6K (Cell Signaling 9234), p70S6K (Cell Signaling 2708), p-S6 235/236 (Cell Signaling 2211), p-S6 240/244 (Cell Signaling 2215), S6 (Cell Signaling 2217), p-ERK (Cell Signaling 4370), ERK (Cell Signaling 7124), p-AKT Ser473 (Cell Signaling 4060), and AKT (Cell Signaling 9272). Values presented are relative to the mean of vehicle-treated controls.

Statistical Analysis

Data are reported as means $\pm$ SEM. Data from the zero maze, passive avoidance tests, and Westerns blots were analyzed by means of two-way ANOVA with genotype (control, *Fmr1* KO) and condition (vehicle, rapamycin) as between subjects variables. Sleep and open field data were analyzed by means of a mixed-model repeated measures three-way ANOVA with genotype (control, *Fmr1* KO) and condition (vehicle, rapamycin) as between subjects variables and phase (sleep) or epoch (open field) as within subjects variables. In cases in which the interaction between or among variables was statistically significant, we compared groups and/or conditions by means of *post hoc t*-tests. Social behavior was analyzed by means of paired student's *t*-tests to compare stranger vs. object (sociability) or familiar vs. novel (social novelty). Audiogenic seizures were analyzed by means of a Fisher's exact test comparing the effect of treatment in *Fmr1* KO animals. Effects of $p \leq 0.05$ are considered statistically significant and are denoted with a “*.” Effects of $0.10 \geq p > 0.05$ are also reported here and are denoted with a “~.” A table reporting the *F*-values and corresponding *p*-values for interactions and main effects are listed for the Western blots (Table 1) and all behavior tests analyzed with ANOVA (Table 2).

TABLE 1 | ANOVA results for Western blots.

Protein	Interaction	Main effect	$F_{(df, error)}$ value	P-value
p-mTOR	Genotype $\times$ treatment		$F_{(1,10)} = 0.593$	0.459
		Genotype	$F_{(1,10)} = 0.002$	0.964
		Treatment	$F_{(1,10)} = 0.283$	0.606
mTOR	Genotype $\times$ treatment		$F_{(1,10)} = 0.433$	0.526
		Genotype	$F_{(1,10)} = 0.735$	0.411
		Treatment	$F_{(1,10)} = 0.060$	0.811
p-mTOR/mTOR	Genotype $\times$ treatment		$F_{(1,10)} = 0.003$	0.956
		Genotype	$F_{(1,10)} = 0.534$	0.482
		Treatment	$F_{(1,10)} = 0.084$	0.778
p-p70S6k	Genotype $\times$ treatment		$F_{(1,10)} = 0.059$	0.813
		Genotype	$F_{(1,10)} = 0.029$	0.868
		Treatment	$F_{(1,10)} = 0.090$	0.770
p70S6k	Genotype $\times$ treatment		$F_{(1,10)} = 0.148$	0.709
		Genotype	$F_{(1,10)} = 1.912$	0.197
		Treatment	$F_{(1,10)} = 0.785$	0.396
p-p70S6k/p70S6k	Genotype $\times$ treatment		$F_{(1,10)} = 0.096$	0.763
		Genotype	$F_{(1,10)} = 0.351$	0.567
		Treatment	$F_{(1,10)} = 0.007$	0.936
p-S6 235/236	Genotype $\times$ treatment		$F_{(1,10)} = 11.177$	0.007**
		Genotype	$F_{(1,10)} = 6.098$	0.033*
		Treatment	$F_{(1,10)} = 8.667$	0.015*
p-S6 240/244	Genotype $\times$ treatment		$F_{(1,10)} = 7.258$	0.023*
		Genotype	$F_{(1,10)} = 3.273$	0.101
		Treatment	$F_{(1,10)} = 6.800$	0.026*
S6	Genotype $\times$ treatment		$F_{(1,10)} = 0.446$	0.520
		Genotype	$F_{(1,10)} = 1.949$	0.193
		Treatment	$F_{(1,10)} = 2.55$	0.141
p-S6 (235/236)/S6	Genotype $\times$ treatment		$F_{(1,10)} = 4.843$	0.052~
		Genotype	$F_{(1,10)} = 9.880$	0.011*
		Treatment	$F_{(1,10)} = 0.004$	0.948
p-S6 (240/244)/S6	Genotype $\times$ treatment		$F_{(1,10)} = 4.138$	0.069~
		Genotype	$F_{(1,10)} = 4.226$	0.067
		Treatment	$F_{(1,10)} = 0.659$	0.436
p-AKT 473	Genotype $\times$ treatment		$F_{(1,10)} = 6.601$	0.028*
		Genotype	$F_{(1,10)} = 1.840$	0.205
		Treatment	$F_{(1,10)} = 3.844$	0.078~
AKT	Genotype $\times$ treatment		$F_{(1,10)} = 0.322$	0.583
		Genotype	$F_{(1,10)} = 0.215$	0.653
		Treatment	$F_{(1,10)} = 0.069$	0.797
p-AKT (473)/Akt	Genotype $\times$ treatment		$F_{(1,10)} = 1.705$	0.221
		Genotype	$F_{(1,10)} = 0.346$	0.570
		Treatment	$F_{(1,10)} = 3.008$	0.114
p-ERK	Genotype $\times$ treatment		$F_{(1,10)} = 1.489$	0.250
		Genotype	$F_{(1,10)} = 0.122$	0.734
		Treatment	$F_{(1,10)} = 6.925$	0.025*
ERK	Genotype $\times$ treatment		$F_{(1,10)} = 0.000$	0.986
		Genotype	$F_{(1,10)} = 0.477$	0.505
		Treatment	$F_{(1,10)} = 5.517$	0.041*
p-ERK/ERK	Genotype $\times$ treatment		$F_{(1,10)} = 1.001$	0.341
		Genotype	$F_{(1,10)} = 0.013$	0.912
		Treatment	$F_{(1,10)} = 1.151$	0.309

~0.05 $\leq p \leq 0.1$; *0.01 $\leq p \leq 0.05$; **0.001 $\leq p \leq 0.01$.

TABLE 2 | Repeated measures ANOVA results for behavior testing.

Behavior	Interaction	Main effect	$F(df, error)$ value	P-value
Open field				
Horizontal distance moved	Genotype $\times$ treatment $\times$ epoch		$F_{(4,366)} = 0.255$	0.919
	Treatment $\times$ epoch		$F_{(4,366)} = 2.292$	0.054~
	Genotype $\times$ epoch		$F_{(4,366)} = 0.559$	0.707
	Genotype $\times$ treatment		$F_{(1,84)} = 0.270$	0.605
		Genotype	$F_{(1,84)} = 8.154$	0.005*
		Treatment	$F_{(1,84)} = 1.536$	0.219
		Epoch	$F_{(4,366)} = 170.930$	<0.001*
Center/total distance	Genotype $\times$ treatment $\times$ epoch		$F_{(4,360)} = 0.899$	0.470
	Treatment $\times$ epoch		$F_{(4,360)} = 0.643$	0.643
	Genotype $\times$ epoch		$F_{(4,360)} = 0.823$	0.518
	Genotype $\times$ treatment		$F_{(1,85)} = 0.136$	0.713
		Genotype	$F_{(1,85)} = 3.081$	0.083~
		Treatment	$F_{(1,85)} = 3.115$	0.081~
		Epoch	$F_{(4,360)} = 7.724$	<0.001
Zero maze	Genotype $\times$ treatment		$F_{(1,87)} = 0.250$	0.619
		Genotype	$F_{(1,87)} = 13.059$	0.001*
		Treatment	$F_{(1,87)} = 5.111$	0.026*
Passive avoidance	Genotype $\times$ treatment		$F_{(1,74)} = 0.045$	0.832
		Genotype	$F_{(1,74)} = 5.004$	0.028*
		Treatment	$F_{(1,74)} = 0.027$	0.871
Sleep				
Total sleep time	Genotype $\times$ treatment $\times$ phase		$F_{(1,102)} = 0.020$	0.887
	Treatment $\times$ phase		$F_{(1,102)} = 0.236$	0.628
	Genotype $\times$ phase		$F_{(1,102)} = 3.558$	0.062~
	Genotype $\times$ treatment		$F_{(1,102)} = 0.374$	0.542
		Genotype	$F_{(1,102)} = 5.324$	0.023*
		Treatment	$F_{(1,102)} = 4.056$	0.047*
		Phase	$F_{(1,102)} = 1494.025$	<0.001*

~0.05 < $p \leq 0.1$; * $p \leq 0.05$.

RESULTS

Increased mTORC1 Activity in *Fmr1* KO Mice Normalized by Chronic Rapamycin Treatment

We analyzed lysates of frontal cortex from vehicle- and rapamycin-treated *Fmr1* KO and control mice. FMRP expression was absent in all *Fmr1* KO mice regardless of treatment (Figure 1A). In Figure 1, we present the results of the effects of genotype and rapamycin treatment on the phosphorylated forms (Figures 1B,E,H,K,N,Q), total (Figures 1C,F,I,L,O,R) and the ratio of phosphorylated to total (Figures 1D,G,J,M,P,S) for the signaling molecules measured. We did not find either an effect of genotype or of treatment on p-mTOR (Figure 1B and Table 1). We also did not find either an effect of genotype or treatment on p-p70S6k (Figure 1E and Table 1). We examined two phosphorylation sites on S6 (235/236 and 240/244), and in both cases, the genotype $\times$ treatment interactions were statistically significant (Table 1). *Post hoc t*-tests indicate that vehicle-treated *Fmr1* KO mice had significantly higher p-S6 than vehicle-treated controls ($p = 0.010$ for 235/236) ($p = 0.041$ for 240/244). For both sites, rapamycin reduced p-S6 levels in *Fmr1* KO mice ($p = 0.009$

for 235/236) ($p = 0.024$ for 240/244), but had no effect on controls (Figures 1A,H,K). To examine the mTORC2 pathway, we also found a statistically significant genotype $\times$ treatment interaction for p-AKT Ser473 (Table 1). *Post hoc t*-tests indicate that vehicle-treated *Fmr1* KO animals had increased p-AKT relative to vehicle-treated controls ($p = 0.02$). Rapamycin reduced p-AKT levels in *Fmr1* KO mice ($p = 0.013$), but had no effect on controls (Figures 1A,N). We also examined p-ERK. Although there was no genotype $\times$ treatment interaction, we did detect a statistically significant main effect of treatment for both p-ERK and total ERK. Regardless of genotype, rapamycin treatment decreased p-ERK and ERK levels (Figures 1A,Q,R and Table 1).

Given that p-S6 activity was higher in *Fmr1* KO mice and reduced by rapamycin treatment, we hypothesized that rapamycin treatment would ameliorate the behavioral phenotypes in *Fmr1* KO mice.

Rapamycin Did Not Alter Hyperactivity in *Fmr1* KO Mice

To examine hyperactivity, we conducted open field testing. We found a statistically significant main effect of genotype

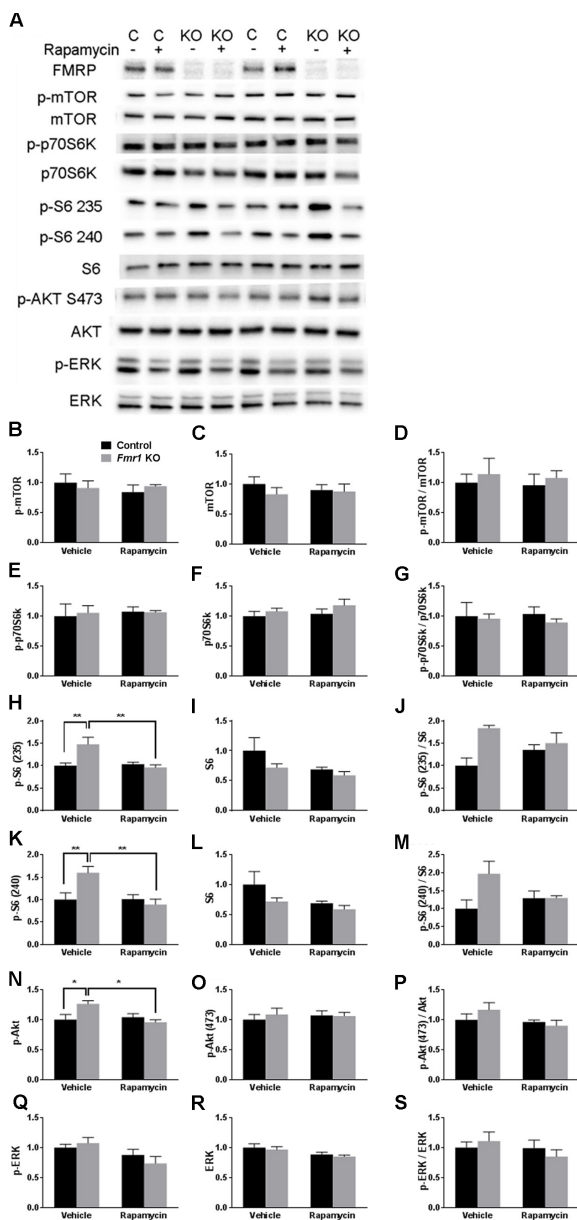


FIGURE 1 | Western blots of frontal cortex of *Fmr1* KO and control mice on vehicle and rapamycin treatment. **(A)** Representative Western blot images. **(B)** p-mTOR levels did not differ among the groups. **(C)** mTOR levels did not differ among the groups. **(D)** p-mTOR/Total mTOR did not differ among the groups. **(E)** p-p70S6K did not differ among the groups. **(F)** p70S6K did not differ among the groups. **(G)** p-p70S6K/Total p70S6K did not differ among the groups. **(H)** The genotype $\times$ treatment interaction for p-S6 235/236 was statistically significant. *Post hoc t*-tests revealed that vehicle-treated *Fmr1* KO animals had significantly higher p-S6 235/236 ($p = 0.002$) compared to vehicle-treated controls. This was significantly reduced by rapamycin treatment ($p = 0.002$). **(I)** S6 levels did not differ among groups. **(J)** The genotype $\times$ treatment interaction for p-S6 (235/236)/Total S6 approached statistical significance. We looked at individual differences by means of *post hoc t*-tests and found that the difference between vehicle-treated controls and vehicle-treated *Fmr1* KO mice was statistically significant ($p = 0.004$). **(K)** The genotype $\times$ treatment interaction for p-S6 240/244 was statistically significant.

(Continued)

FIGURE 1 | Continued

Post hoc t-tests revealed that vehicle-treated *Fmr1* KO animals had significantly higher p-S6 240/244 levels compared to vehicle-treated controls ($p = 0.010$). This was significantly reduced with rapamycin treatment ($p = 0.006$). **(L)** Total S6 levels did not differ among the groups. **(M)** The genotype $\times$ treatment interaction for p-S6 (240/244)/Total S6 approached statistical significance. We looked at individual differences by means of *post hoc t*-tests and found that the difference between vehicle-treated controls and vehicle-treated *Fmr1* KO mice was statistically significant ($p = 0.016$). **(N)** The genotype $\times$ treatment interaction for p-AKT Ser473 was statistically significant. *Post hoc t*-tests revealed that vehicle-treated *Fmr1* KO animals had higher p-AKT compared to vehicle-treated controls ($p = 0.020$). p-AKT Ser473 levels were reduced in *Fmr1* KO animals after rapamycin treatment ($p = 0.013$). **(O)** Total AKT levels did not differ among the groups. **(P)** p-Akt (473)/Akt did not differ among the groups. **(Q)** The main effect of treatment for p-ERK levels was statistically significant indicating that regardless of genotype, rapamycin reduced p-ERK. **(R)** The main effect of treatment for ERK levels was statistically significant indicating that regardless of genotype, rapamycin reduced ERK. **(S)** p-ERK/ERK did not differ among the groups. **(B–S)** Levels were normalized to total protein in the blot. Values presented are relative to the mean of vehicle-treated control values. Bars represent mean $\pm$ SEM. $\sim 0.05 < p \leq 0.1$, $*0.01 \leq p \leq 0.05$, $**0.001 \leq p \leq 0.01$ as determined by *post hoc t*-tests. $n = 4$ vehicle-treated control, $n = 4$ rapamycin-treated control, $n = 3$ vehicle-treated *Fmr1* KO, $n = 3$ rapamycin-treated *Fmr1* KO.

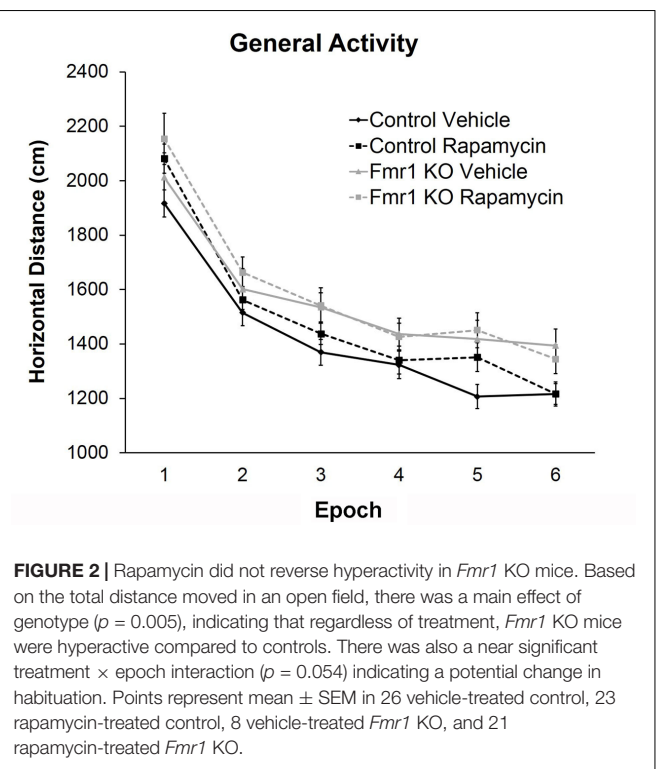
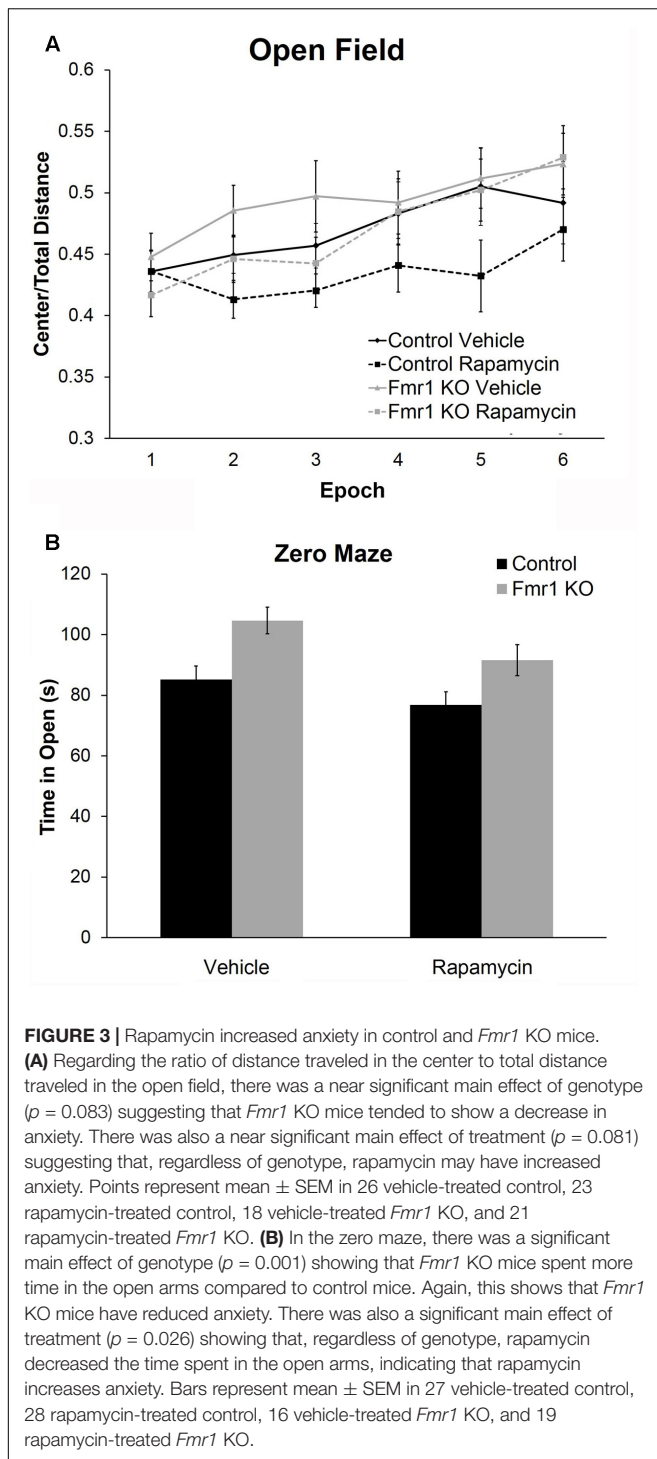


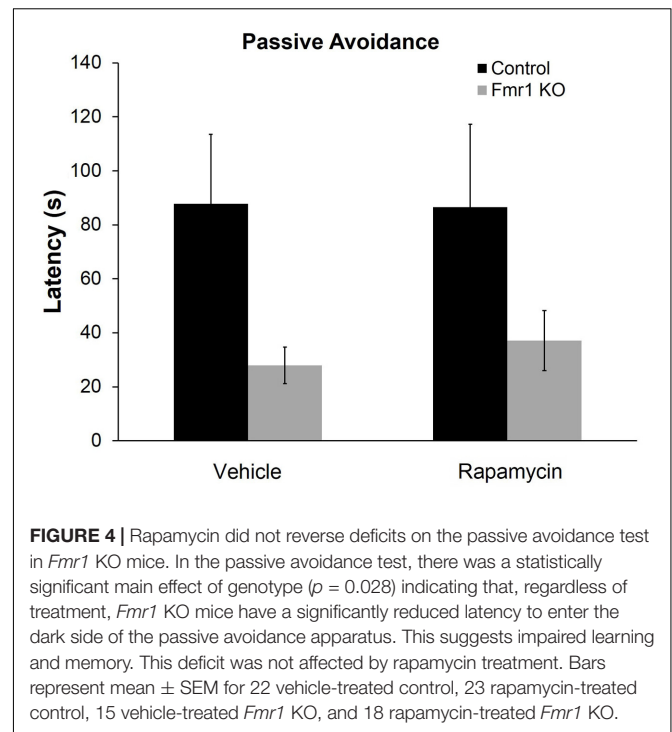
FIGURE 2 | Rapamycin did not reverse hyperactivity in *Fmr1* KO mice. Based on the total distance moved in an open field, there was a main effect of genotype ($p = 0.005$), indicating that regardless of treatment, *Fmr1* KO mice were hyperactive compared to controls. There was also a near significant treatment $\times$ epoch interaction ($p = 0.054$) indicating a potential change in habituation. Points represent mean $\pm$ SEM in 26 vehicle-treated control, 23 rapamycin-treated control, 8 vehicle-treated *Fmr1* KO, and 21 rapamycin-treated *Fmr1* KO.

regardless of treatment, indicating that *Fmr1* KO mice were hyperactive compared to controls, as previously reported (Liu et al., 2011; Figure 2 and Table 2). There was also a near statistically significant treatment $\times$ epoch interaction ($p = 0.054$) suggesting that rapamycin may enhance reactivity to the novel environment, regardless of genotype.



Rapamycin Increased Anxiety-Like Behavior in *Fmr1* KO and Control Mice

To examine anxiety, we analyzed the ratio of distance traveled in the center of the open field to total distance traveled. The main effect of epoch was statistically significant ($p < 0.001$) indicating that regardless of genotype or treatment, mice became less anxious as the test progressed (Figure 3A and Table 2).



Main effects of genotype and treatment approached statistical significance suggesting that anxiety may be lower in *Fmr1* KO mice regardless of treatment and that in both genotypes, rapamycin treatment tended to increase anxiety-like behavior.

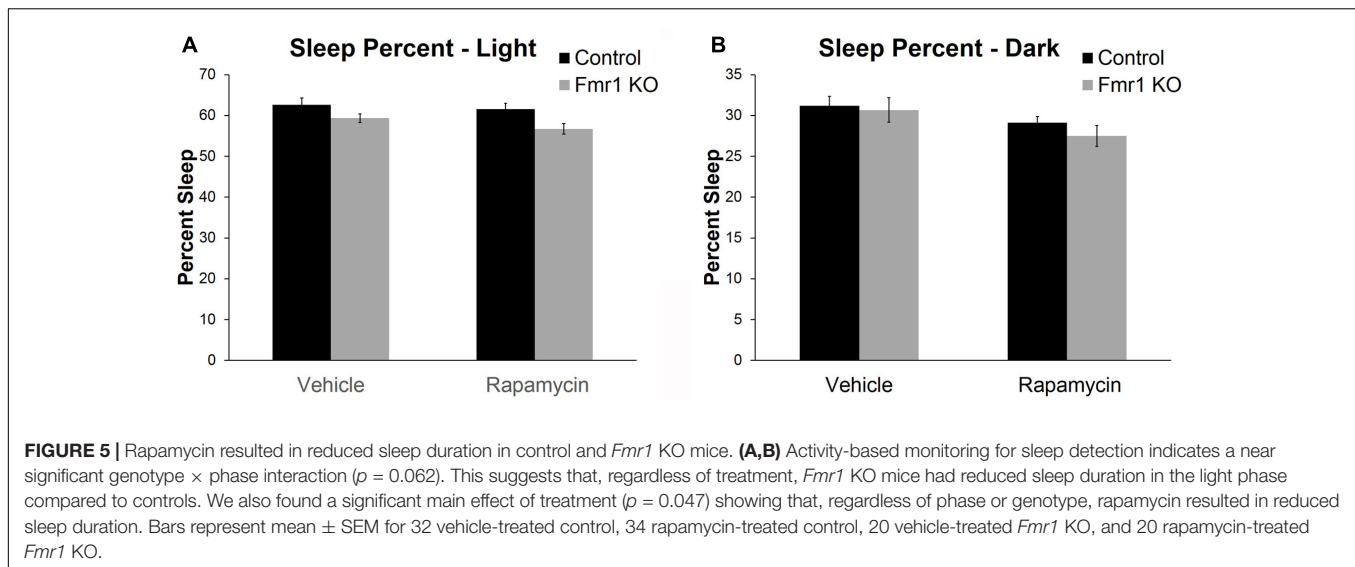
As another measure of anxiety-like behavior, we tested animals in the zero maze (Figure 3B). In this test, the main effects of both genotype and treatment were statistically significant (Table 2). Zero maze results are in accord with open field behavior. Our results show that regardless of treatment, *Fmr1* KO mice spent more time in the open arms indicating that *Fmr1* KO mice are less anxious compared to controls as previously reported (Liu et al., 2011). Moreover, regardless of genotype, rapamycin decreased the time spent in the open arms indicating that rapamycin increased anxiety-like behavior in both genotypes.

Rapamycin Did Not Improve Performance on the Passive Avoidance Test in *Fmr1* KO Mice

To examine a form of fear learning, we tested mice on passive avoidance (Figure 4). We found a statistically significant main effect of genotype ($p = 0.028$) (Table 2), indicating that regardless of treatment, *Fmr1* KO mice had a lower latency to enter the dark side. The main effect of rapamycin treatment was not statistically significant indicating that rapamycin did not enhance performance on this test.

Rapamycin Decreased Sleep Duration in *Fmr1* KO and Control Mice

Sleep in the active and inactive phases was assessed by home-cage monitoring. We found a nearly statistically significant



genotype $\times$ phase interaction ($p = 0.062$) (Table 2). This is consistent with previous work indicating that decreases in sleep duration occurred primarily in the inactive phase in *Fmr1* KO (Saré et al., 2017). We also found a statistically significant main effect of treatment ($p = 0.047$) (Table 2). These results indicate that rapamycin treatment reduced sleep duration across genotypes and phases (Figure 5).

Rapamycin Impaired Social Behavior in *Fmr1* KO and Control Mice

To examine social behavior, we used the three-chambered apparatus to assess sociability and preference for social novelty. In the test of sociability, vehicle ($p = 0.0003$)- and rapamycin ($p = 0.005$)-treated control mice and vehicle-treated *Fmr1* KO mice ($p = 0.02$) had a significant preference for the mouse compared to the object, but *Fmr1* KO mice on rapamycin treatment did not (Figure 6A). This indicates that rapamycin impaired sociability in *Fmr1* KO mice.

In the test of preference for social novelty, only the control mice on vehicle treatment had a significant preference for the novel mouse ($p = 0.003$) (Figure 6B). This was abolished with rapamycin treatment. Additionally and as previously reported (Liu et al., 2011), *Fmr1* KO animals did not show a preference for the novel mouse. This was not changed with rapamycin treatment (Figure 6B).

Rapamycin Did Not Reverse Audiogenic Seizure Susceptibility in *Fmr1* KO Mice

We did not observe audiogenic seizures in either vehicle- or rapamycin-treated control mice (Figures 7A,B). Vehicle-treated *Fmr1* KO mice had a 28% seizure incidence (11% wild running, 11% myoclonic convulsions, and 6% respiratory arrest) (Figure 7C). Rapamycin-treated *Fmr1* KO had a 37% seizure incidence (11% wild running, 16% myoclonic convulsions, and 11% respiratory arrest) (Figure 7D). There were no statistically

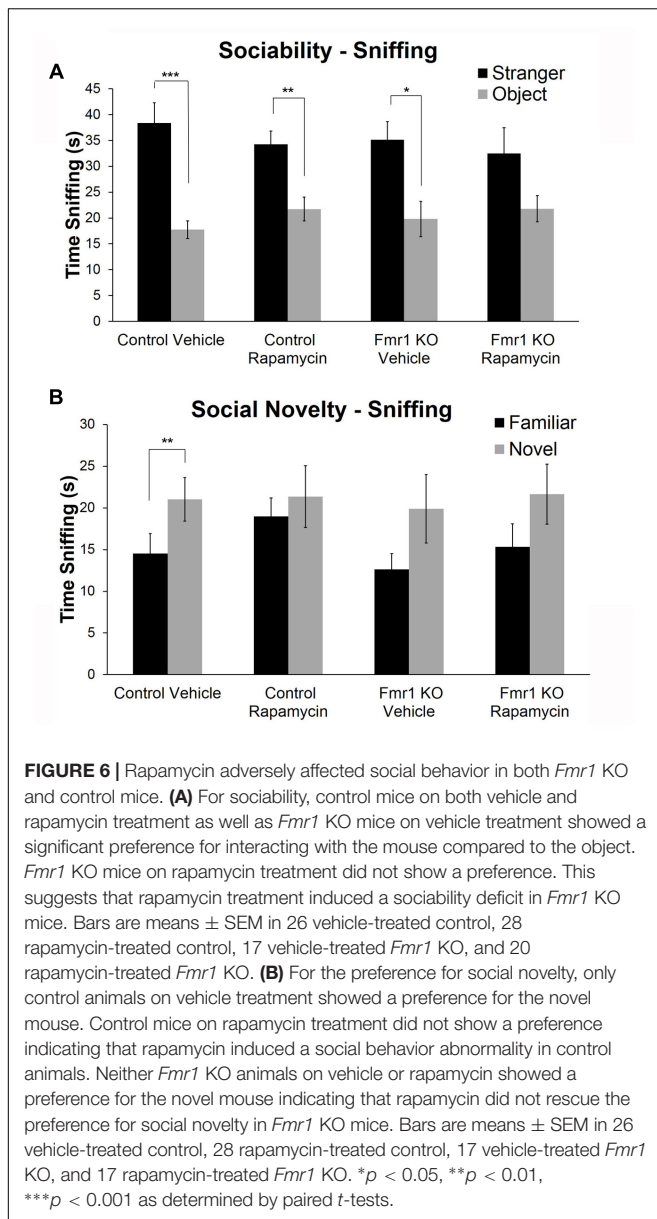
significant differences between vehicle and rapamycin-treated *Fmr1* KO mice.

DISCUSSION

The present study is, to our knowledge, the first study of the behavioral effects of chronic rapamycin treatment in *Fmr1* KO mice. We found that *Fmr1* KO mice have increased levels of p-S6 (a downstream target of mTORC1), and that rapamycin reverses this phenotype. However, rapamycin failed to reverse most of the behavioral phenotypes measured. Moreover, rapamycin exacerbated some of the abnormal behaviors resulting in further decreases in sleep duration and increased deficits in social interaction.

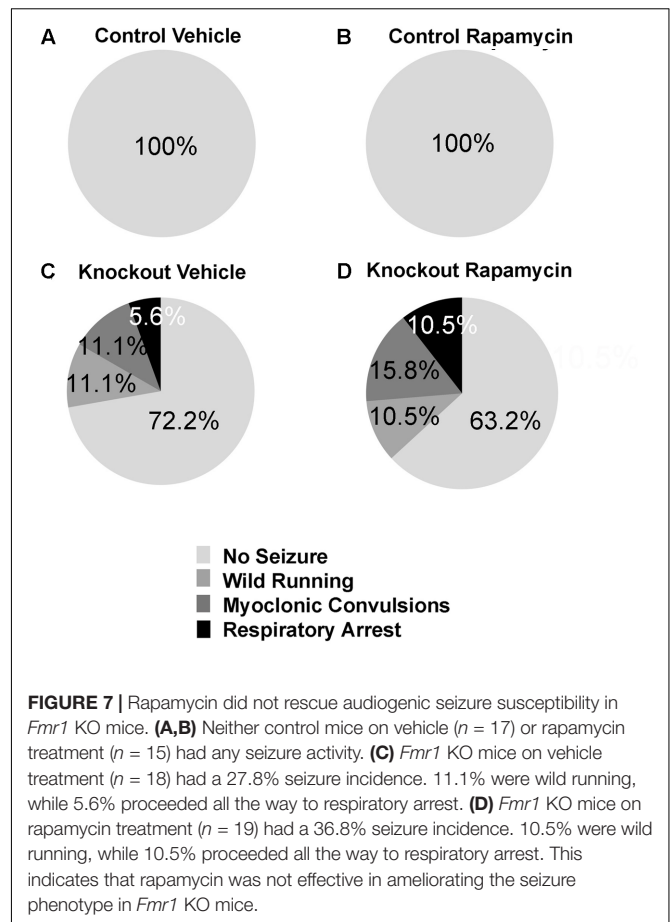
Increased activity of the mTORC1 pathway in the hippocampus of *Fmr1* KO mice has been reported previously (Sharma et al., 2010; Bhattacharya et al., 2012; Liu et al., 2012; Busquets-Garcia et al., 2013; Choi et al., 2016). Our present studies focused on the neocortex where we did not find an increase in p-mTOR or p-p70S6K, but we did find that p-S6, the downstream product of mTORC1, was increased at both phosphorylation sites (Ser235/236 and Ser240/244). Though rapamycin treatment reduced p-S6 levels, the lack of changes in p-mTOR and p-p70S6K suggest that rapamycin is reducing phosphorylation of S6 through a mechanism that is independent of mTORC1. This mechanism may be through mTORC2, which is supported by our results showing that rapamycin treatment reduced p-Akt at Ser473 in *Fmr1* KO mice. Whereas acute rapamycin is considered a selective mTORC1 inhibitor, chronic rapamycin treatment can also inhibit mTORC2 (Sarbasov et al., 2006). We posit that some of the effects of chronic rapamycin treatment on *Fmr1* KO mice may be due to mTORC2 inhibition.

In a recent paper that also examined signaling molecules in cortical lysates from young *Fmr1* KO mice (Sawicka et al., 2016), p-mTOR was not increased, but S6 phosphorylation



at Ser235/236 was increased via an ERK-dependent kinase (p90S6K); S6 phosphorylation at Ser240/244 (mTOR/p70S6K-dependent) was unchanged. In adult *Fmr1* KO mice, this study further showed elevated ERK/p90S6K/S6 signaling in neocortex suggesting ERK/p90S6K (but not mTORC1) signaling dysregulation in neocortex. We did find that rapamycin reduced ERK, but we did not find any baseline genotype differences in p-ERK. It is possible that some of the differences between our present study and the results from Sawicka et al. (2016) could be due to the use of isoflurane anesthesia in the Sawicka et al. (2016) study; isoflurane has been shown to effect changes in translational control signaling pathways (Antila et al., 2017).

The effects of rapamycin treatment on behavior were negative and, in some cases, detrimental. Other studies have shown negative effects of rapamycin on behavior in control animals.



In mice, acute rapamycin impaired performance on the Morris water maze (Ehninger et al., 2008), subacute rapamycin impaired performance on object place recognition (Zhou et al., 2013), and chronic rapamycin increased anxiety-like behavior (Russo et al., 2016) and resulted in deficits in social interaction (Reith et al., 2012). Similarly, studies in control rats have also demonstrated negative effects of rapamycin treatment. Acute rapamycin increased anxiety-like behavior (Hadamitzky et al., 2014), and chronic rapamycin treatment impaired performance on tests of learning and memory and increased anxiety-like behavior (Lu et al., 2015). In accord with these detrimental effects, it has been shown that long-term rapamycin treatment in young rats resulted in a decrease in progenitor cells in the dentate gyrus (Lu et al., 2015).

Whereas chronic rapamycin treatment has not been examined previously in *Fmr1* KO mice, there have been studies of the effects of acute treatment. In agreement with our results, acute rapamycin treatment in *Fmr1* KO mice also showed no effect on audiogenic seizure susceptibility (Osterweil et al., 2010). Moreover, in hippocampal slices, rapamycin treatment did not reverse the increased [35 S]methionine/[35 S]cysteine incorporation into protein (Osterweil et al., 2010). Another study (Price et al., 2007) addressed sensitization to pain. It was noted that acute rapamycin inhibited formalin- and DHPG-induced

nociception in control animals but had no effect in *Fmr1* KO mice (Price et al., 2007), suggesting that the role of mTORC1 in nociception is altered in the absence of FMRP.

Numerous studies in *Fmr1* KO mice have addressed targets upstream of mTORC1. For example, targeting elements of the phosphoinositide 3-kinase (PI3K) pathway was efficacious in FXS models (Gross et al., 2015; Gantois et al., 2017; Monyak et al., 2017). Reducing the expression of PIKE rescued phenotypes in *Fmr1* KO mice, but PIKE reduction is thought, at least in part, to exert some of its effects in an mTOR-independent manner (Gross et al., 2015). Metformin, an AMPK activator, rescued FXS behavioral symptoms in both *Drosophila* and *Fmr1* KO mice (Gantois et al., 2017; Monyak et al., 2017). AMPK can act as an mTORC1 inhibitor (Xu et al., 2012), but AMPK has many other targets in addition to mTORC1 (Canto and Auwerx, 2010). Moreover, there are AMPK-independent effects of metformin suggesting additional mechanisms of action that have not been identified (Rena et al., 2013). Downstream of the mTORC1 pathway, genetic reduction of p70S6K1 reversed many deficits seen in *Fmr1* KO mice including elevated hippocampal protein synthesis, dendritic spine abnormalities, and deficits in learning and memory and social novelty interaction (Bhattacharya et al., 2012). Whereas these results suggested that targeting the mTORC1 pathway would be beneficial in FXS, results of our study in which we used pharmacological inhibition of mTORC1 do not support this idea.

It is possible that mTORC1 mediates too many effects to be a good drug target. Although clearly this is not the case in mouse models of TSC. TSC mice exhibit many similar behavioral phenotypes to *Fmr1* KO mice including social behavior abnormalities and learning and memory deficits, and these were rescued by chronic rapamycin treatment (Ehninger et al., 2008; Reith et al., 2012; Sato et al., 2012; Tsai et al.,

2012). This suggests that although the mTORC1 pathway is implicated in both disorders with similar phenotypic outcomes, the molecular pathology underlying these disorders is quite different. Supporting this notion is the fact that a genetic cross between *Fmr1* KO and *Tsc2*^{+/-} mice normalized long-term depression as well as performance in a contextual fear conditioning task (Auerbach et al., 2011).

Our results collectively indicate that rapamycin treatment is not efficacious, and in some respects, is detrimental in *Fmr1* KO mice. Other pathways should be explored for mechanism-based treatment strategies in *Fmr1* KO mice.

AUTHOR CONTRIBUTIONS

RS designed the study, performed the experiments, analyzed the data, interpreted the data, and drafted the manuscript. AS, IL, AC, and IM performed the experiments. AL analyzed the data. CS performed the experiments. CBS designed the study, interpreted the data, and drafted the manuscript. All authors have read and approved the final version of the manuscript.

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Fragile X Mental Retardation Protein Regulates Activity-Dependent Membrane Trafficking and *Trans*-Synaptic Signaling Mediating Synaptic Remodeling

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Fragile X syndrome (FXS) is the leading monogenic cause of autism and intellectual disability. The disease arises through loss of fragile X mental retardation protein (FMRP), which normally exhibits peak expression levels in early-use critical periods, and is required for activity-dependent synaptic remodeling during this transient developmental window. FMRP canonically binds mRNA to repress protein translation, with targets that regulate cytoskeleton dynamics, membrane trafficking, and *trans*-synaptic signaling. We focus here on recent advances emerging in these three areas from the *Drosophila* disease model. In the well-characterized central brain mushroom body (MB) olfactory learning/memory circuit, FMRP is required for activity-dependent synaptic remodeling of projection neurons innervating the MB calyx, with function tightly restricted to an early-use critical period. FMRP loss is phenocopied by conditional removal of FMRP only during this critical period, and rescued by FMRP conditional expression only during this critical period. Consistent with FXS hyperexcitation, FMRP loss defects are phenocopied by heightened sensory experience and targeted optogenetic hyperexcitation during this critical period. FMRP binds mRNA encoding *Drosophila* ESCRTIII core component Shrub (human CHMP4 homolog) to restrict Shrub translation in an activity-dependent mechanism only during this same critical period. Shrub mediates endosomal membrane trafficking, and perturbing Shrub expression is known to interfere with neuronal process pruning. Consistently, FMRP loss and Shrub overexpression targeted to projection neurons similarly causes endosomal membrane trafficking defects within synaptic boutons, and genetic reduction of Shrub strikingly rescues *Drosophila* FXS model defects. In parallel work on the well-characterized giant fiber (GF) circuit, FMRP limits iontophoretic dye loading into central interneurons, demonstrating an FMRP role controlling core neuronal properties through the activity-dependent repression of translation. In the well-characterized *Drosophila* neuromuscular junction (NMJ) model, developmental synaptogenesis and activity-dependent synaptic remodeling both require extracellular matrix metalloproteinase (MMP) enzymes interacting with the heparan sulfate proteoglycan (HSPG) glypican dally-like protein (Dlp) to restrict *trans*-synaptic

Wnt signaling, with FXS synaptogenic defects alleviated by both MMP and HSPG reduction. This new mechanistic axis spanning from activity to FMRP to HSPG-dependent MMP regulation modulates activity-dependent synaptogenesis. We discuss future directions for these mechanisms, and intersecting research priorities for FMRP in glial and signaling interactions.

Keywords: fragile X syndrome, critical period, signaling, synapse, *Drosophila*

INTRODUCTION

Nascent neural circuitry, while functional, is nevertheless still developing and initially manifests activity-dependent refinement and optimization. During early-use critical periods, new neural circuits are highly sensitive to sensory experience, exhibiting a transient window of heightened synaptic remodeling capacity (Hensch, 2004). Sensory input driving downstream circuit activity can result in persistent, long-lasting structural and functional changes, which generally cannot be retrained once the critical period has past (Takesian and Hensch, 2013). During this activity-dependent refinement, excitatory and inhibitory synapses are balanced in circuits, generally by removing excess excitatory synapses and adding new inhibitory synapses, thereby establishing an optimized excitatory/inhibitory (E/I) balance (Doll and Broadie, 2014). Therefore, suitably primed activity-dependent mechanisms must be present to sculpt synaptic connectivity during these critical periods. The fragile X mental retardation protein (FMRP), which when lost through epigenetic silencing of the *FMR1* gene results in fragile X syndrome (FXS), is a prime candidate for mediating activity-dependent synaptic remodeling during critical periods. FMRP is directly regulated by activity (Weiler et al., 1997; Antar et al., 2004) and, in turn, regulates activity-dependent processes (Huber et al., 2002; Li et al., 2002). Importantly, considerable evidence supports the theory that FXS is caused by excessive excitatory neurotransmission (hyperexcitation theory), reduced inhibitory transmission (hypoinhibition theory), or some combination of both, resulting in an excitation/inhibition imbalance (E/I imbalance theory) (Gibson et al., 2008; Cea-Del Rio and Huntsman, 2014).

The *Drosophila* FXS disease model has established conserved requirements for *Drosophila FMR1 (dfmr1)* (Coffee et al., 2010, 2012). *Drosophila* FMRP has key roles in synaptic remodeling ranging from the larval neuromuscular junction (NMJ) and sensory circuits, to adult circadian clock neurons and the mushroom body (MB) olfactory learning/memory circuitry (Zhang et al., 2001; Pan et al., 2004; Gatto and Broadie, 2009; Gatto et al., 2014). Null *dfmr1* mutants display an elevated number of immature synaptic connections in these diverse circuits, as well as the loss of activity-dependent synaptic pruning (Gatto and Broadie, 2008; Tessier and Broadie, 2008). Importantly, *Drosophila* FMRP is developmentally regulated: FMRP levels are at their highest during very late pupal brain development and the first day of post-eclosion adulthood, with levels then decreasing dramatically at maturity (Tessier and Broadie, 2008). FMRP is required developmentally

for synaptogenesis, bouton elimination/pruning, activity-dependent refinement and calcium signaling (Gatto and Broadie, 2008, 2009; Tessier and Broadie, 2008, 2011; Doll and Broadie, 2015, 2016). For E/I balance, *Drosophila* FMRP drives use-dependent down-regulation of synaptic excitability via metabotropic glutamate receptors (mGluRs) (Pan and Broadie, 2007; Pan et al., 2008; Repicky and Broadie, 2008), and promotes GAD levels and GABAergic innervation (Gatto et al., 2014). Given E/I balance is established during the critical period, FMRP loss during this developmental window consistently causes differential activity regulation of excitatory vs. inhibitory neurons in the *Drosophila* FXS model, with defective activity-dependent synapse morphogenesis and Ca^{2+} signaling maturation (Doll and Broadie, 2015, 2016).

Fragile X mental retardation protein is an RNA-binding translation repressor (Laggerbauer et al., 2001; Li et al., 2001; Darnell et al., 2011; Ascano et al., 2012; Chen and Joseph, 2015), with translation enhancement also reported (Todd et al., 2003; Muddashetty et al., 2007; Kenny et al., 2014; Fernandez et al., 2015; Kenny and Ceman, 2016). Primary established targets of repression include cytoskeletal and signaling regulators (Zhang et al., 2001, 2005; Lee et al., 2003; Bongmba et al., 2011; Santoro et al., 2012; Friedman et al., 2013; Majumder et al., 2016). Genetic and pharmacological correction of protein levels or elevated signaling in FXS models can rescue synaptic defects. For example, FMRP binds the mRNA encoding microtubule-associated protein IB (MAP1B)/Futsch, *dfmr1* null animals overexpress Futsch, and genetic Futsch reduction corrects synaptic structure/function defects in the *Drosophila* FXS model (Zhang et al., 2001). Likewise, FMRP binds the mRNA encoding actin-binding Profilin/Chickadee to suppress Chickadee levels, with Chickadee overexpression phenocopying *dfmr1* null defects, and decreasing Chickadee levels correcting synaptic defects (Reeve et al., 2005). In signaling, *Drosophila* FMRP genetically interacts with a mGluR in a bidirectional mechanism controlling ionotropic glutamate receptor (iGluR) classes to regulate synaptic function (Pan and Broadie, 2007; Repicky and Broadie, 2008). *Drosophila* FMRP also limits two heparan sulfate proteoglycan (HSPG) co-receptors that modulate *trans*-synaptic signaling, and genetic reduction of these HSPGs suppresses synaptic structure/function defects in the *Drosophila* FXS model (Friedman et al., 2013). Thus, FMRP targets regulating cytoskeletal and signaling dynamics are causally related to synaptic defects characterizing the FXS disease state. The discovery/ordering of such targets is critical for understanding the FXS disease state.

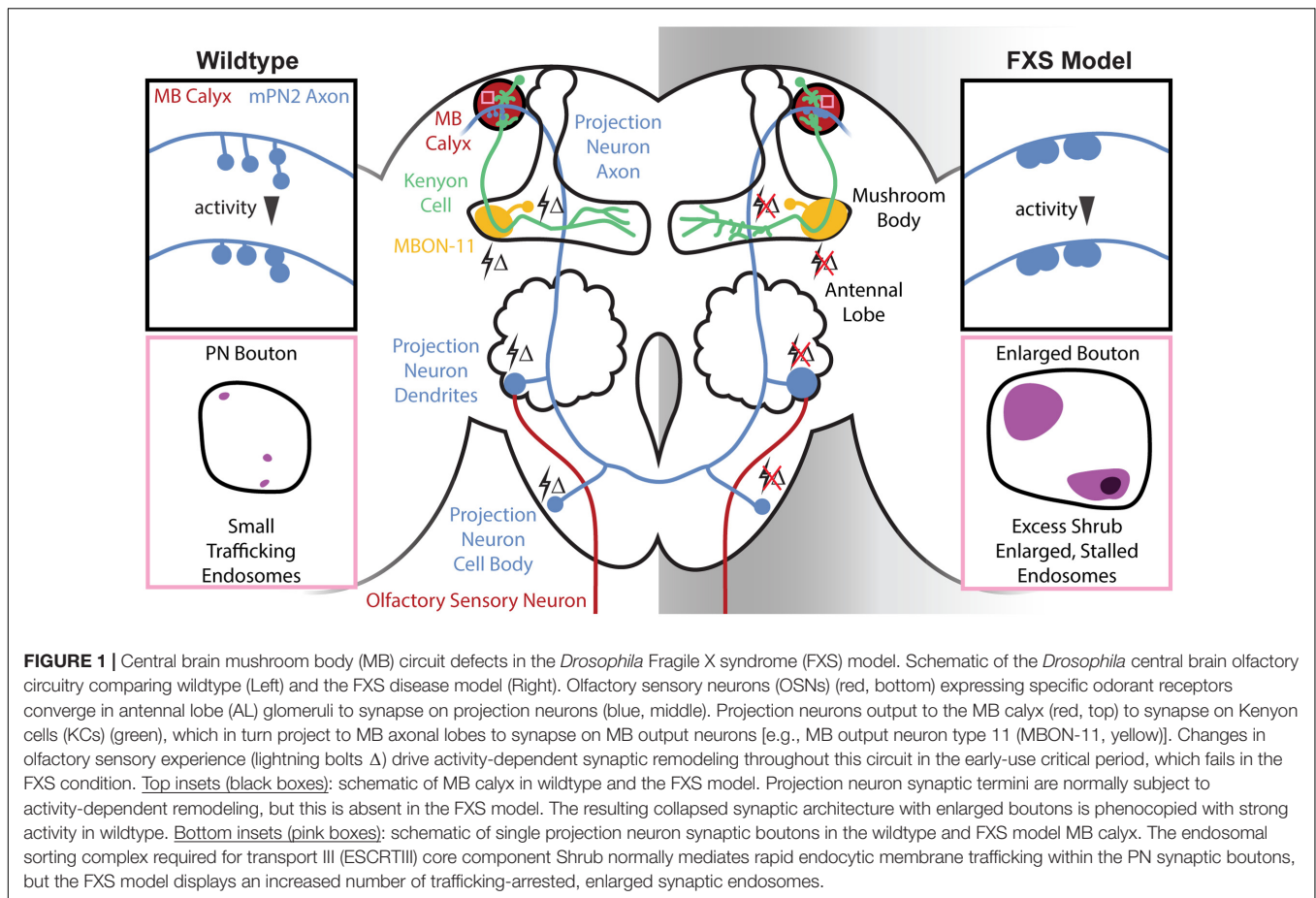
Mouse and *Drosophila* FXS models have been utilized to discover and test targets for therapeutic intervention. For example, inhibition of GSK3 β /Shaggy with lithium has mediated promising effects (Klein and Melton, 1996; Stambolic et al., 1996; McBride et al., 2005; Choi et al., 2010; Mines and Jope, 2011). Therapeutic targets regulating the cytoskeleton have long been a focus of FXS model tests. For example, FMRP translationally represses Rac1, and Rac1 activity is elevated in FXS models (Lee et al., 2003; Bongmba et al., 2011; Majumder et al., 2016). Importantly, many Rac1 inhibitors are known which may hold therapeutic potential for FXS treatments (Tejada-Simon, 2015). Downstream of Rac1, inhibition of p21-activated kinase (PAK) signaling can prevent phenotypes in the mouse FXS disease model (Dolan et al., 2013). Classic work showed FXS patient-derived cells have reduced cAMP levels and induction (Berry-Kravis and Huttenlocher, 1992; Berry-Kravis et al., 1995). Mouse and *Drosophila* FXS models similarly show reduced cAMP levels, with Forskolin stimulation of cAMP production significantly diminished, and genetic/pharmacological correction of cAMP levels preventing FXS phenotypes (Kelley et al., 2007; Kanellopoulos et al., 2012). As a final example, the MMP-9 inhibitor minocycline has been shown in mouse and *Drosophila* FXS models to correct FXS phenotypes (Bilousova et al., 2008; Siller and Broadie, 2011). These strategies highlight mechanisms causally involved in FXS, with the recurrent theme of efficacious inhibition of targets hyper-activated in the disease state. Further investigation of these core pathways in FXS patients and models will likely lead to future clinically relevant discoveries.

Fragile X mental retardation protein plays key roles in the regulation of intercellular interactions governing synaptic remodeling, including *trans*-synaptic signaling and glial pruning. Work over the last several years has established that FMRP regulates *trans*-synaptic signaling at the *Drosophila* NMJ model synapse, particularly in the control of the founding Wnt Wingless (Wg) signaling pathway (Siller and Broadie, 2011; Friedman et al., 2013). Wg *trans*-synaptic signaling regulates activity-dependent synaptic structure/function remodeling (Ataman et al., 2008), with the Wg secreted from synapse-associated glia selectively regulating post-synaptic assembly and transmission strength (Kerr et al., 2014). Activity-dependent Wg signaling occurs in a very rapid time frame; for example, the Wg-driven formation of nascent presynaptic boutons ("ghost boutons") occurs within minutes of stimulation (Ataman et al., 2008). Wg *trans*-synaptic signaling is modulated by extracellular HSPGs [e.g., dally-like protein (Dlp)] and matrix metalloprotease (MMP) enzymes that co-regulate each other in the synaptomatrix surrounding synaptic boutons (Dear et al., 2016). Importantly, HSPG/MMP levels and Wg signaling are altered in parallel in *dfmr1* null animals, and the genetic reduction of Dlp, or genetic/pharmacological reduction of secreted MMP1, both correct *Drosophila* FXS disease model phenotypes (Siller and Broadie, 2011; Friedman et al., 2013). In addition to the above glial involvement in *trans*-synaptic signaling, glia have also been implicated in neural phagocytosis pruning during remodeling (Tasdemir-Yilmaz and Freeman, 2014). Thus, glia may play central roles during FMRP-dependent synaptic refinement in response to activity states and intercellular signaling cues.

In this review, we focus on recent *Drosophila* FXS model studies of FMRP in activity-dependent synaptic remodeling. We highlight roles in a range of disparate neural circuits: (1) the adult central brain MB learning/memory circuit during an early-use critical period (Güven-Ozkan and Davis, 2014), (2) the adult giant fiber (GF) escape circuit connecting sensory input to motor output (Boerner and Godenschwege, 2010), and (3) the larval NMJ glutamatergic model synapse (Harris and Littleton, 2015). We concentrate on recent 2017 papers assaying different facets of FMRP biology in these circuits. In the MB circuit, FMRP functions in an activity sensor mechanism to mediate sensory experience refinement of olfactory projection neuron synapses during an early-use critical period, with loss of FMRP resulting in a hyper-excited state that is phenocopied in wildtype animals with intense stimulation (Doll et al., 2017). FMRP suppresses translation of ESCRTIII core component Shrub to enable endosomal membrane trafficking required for critical period activity-dependent synaptic refinement (Vita and Broadie, 2017). In the GF circuit, FMRP limits small molecule permeation in central interneurons, which is disrupted in the *Drosophila* FXS model (Kennedy and Broadie, 2017). At the NMJ, activity regulates extracellular HSPG/MMP co-localization in the synaptomatrix, within a FMRP-dependent mechanism driving synaptic remodeling (Dear et al., 2017). We end by discussing future directions stemming from this work, as well as emerging avenues on cAMP signal transduction, cytoskeleton regulation, glial-dependent refinement and activity-dependent *trans*-synaptic signaling impacting the FXS disease state.

FMRP REQUIREMENTS IN CRITICAL PERIOD ACTIVITY-DEPENDENT SYNAPTIC REMODELING

The *Drosophila* MB olfactory learning and memory circuit in the developing adult brain has numerous advantages for researching critical periods. With a particularly well-defined neural circuitry map, coupled to a host of genetic tools and transgenic markers, we can probe the mechanisms of activity-dependent remodeling in individually identified single neurons (**Figure 1**). Olfactory sensory experience can be manipulated in developmental time periods, or different neurons within the defined circuit targeted with bidirectional optogenetics or transgenic toxins, to dissect activity-dependent remodeling in this rapidly developing animal model. In this defined neural circuit, olfactory sensory neurons (OSNs) expressing the same odorant receptor converge on fully mapped antennal lobe (AL) synaptic glomeruli to innervate central brain projection neurons (PNs; **Figure 1**). PNs output information to the MB calyx by synapsing on Kenyon cells (KCs) involved in learning acquisition and memory consolidation (**Figure 1**). Using KC clonal analyses, we first discovered that FMRP is required for activity-dependent synaptic pruning downstream of olfactory sensory experience, and in response to targeted optogenetic depolarization (Tessier and Broadie, 2008). Sensory experience and activity both promote FMRP expression, with FMRP levels elevated during late pupariation and the first day post-eclosion (1 dpe), but much lower at



maturity (e.g., 7 dpe). During this transient window, FMRP represses overall protein levels as well as specific FMRP targets (e.g., Profilin/Chickadee; Tessier and Broadie, 2008). This work established an FMRP-defined critical period in the MB circuit for early-use, activity-dependent circuit refinement.

The recent emergence of new transgenic driver libraries allows for an unprecedented, circuit-level investigation of FMRP requirements during this critical period development (Jenett et al., 2012). These new generation, highly selective drivers allow neuron-specific visualization and optogenetic manipulation [e.g., excitatory olfactory PN type 2 (mPN2) and inhibitory MB output neuron type 11 (MBON-11); Aso et al., 2014; Ito et al., 2014]. Using these tools, the initial goal was to characterize activity-dependent synaptic remodeling during critical period development, and to test for FMRP requirements in this mechanism. In line with the excitatory vs. inhibitory neuron class (Figure 1), targeted optogenetic depolarization results in decreased dendritic size in mPN2 and opposite increase in dendritic arborization in MBON-11 neurons (Doll and Broadie, 2015). Consistently, prevention of depolarization through optogenetic hyperpolarization results in increased mPN2 dendritic arbors and a decrease in MBON-11 dendritic size. FMRP loss results in increased dendritic arborization in both neuron classes, and prevents activity-dependent remodeling due to either hyper- or hypo-polarization

(Doll and Broadie, 2015). Crucially, these activity-dependent changes normally only occur during the early-use critical period (0–1 dpe), and FMRP is necessary only during this window for synaptic remodeling (Doll and Broadie, 2015). Therefore, neurons without FMRP cannot respond to activity, eliminating their capacity to be refined during circuit optimization (Figure 1). The wider implication of this insensitivity is that FXS disease state neurons are no longer able to mature based on critical period experience in order to fine-tune behavioral responses.

Most critical period activity-dependent refinement studies in this FXS model have been restricted to structural analyses. The one exception is testing the maturation of calcium signaling dynamics with transgenic GCaMP reporters (Doll and Broadie, 2016). In the same excitatory input mPN2 and inhibitory output MBON-11 neuronal pair (Figure 1), *dfmr1* null mPN2 shows strongly elevated depolarization-induced Ca^{2+} transients, whereas MBON-11 manifests an opposite Ca^{2+} signaling depression during the critical period (Doll and Broadie, 2016). As above with architecture, these functional phenotypes are restricted to the 0–1 dpe critical period window, with activity-dependent Ca^{2+} transients largely normalized to wildtype levels in both neuron classes by maturity (e.g., 7 dpe). Excitatory mPN2s manifest a persistent functional defect, with depolarization-induced Ca^{2+} transients shifted from elevated in the critical period to slightly depressed at maturity (Doll and

Broadie, 2016). Importantly, cell-specific rescue of FMRP in the critical period restores Ca^{2+} signaling in both neuron classes, while conditional RNAi knockdown of FMRP phenocopies the *dfmr1* null defects, proving a cell-autonomous, critical period role for FMRP in Ca^{2+} signaling control (Doll and Broadie, 2016). In wildtype animals, targeted optogenetic depolarization during the critical period entrains increased Ca^{2+} transients in both neuron classes, but this activity-dependent plasticity is lost in the FXS model, with a slight timing delay in *dfmr1* null MBON-11 neurons (Doll and Broadie, 2016). These results suggest an E/I imbalance mechanism: excitatory neurons do not mature due to hyper-excitability, while inhibitory neurons do not mature due to hypo-excitability.

Most recently, mPN2 connectivity in the MB calyx learning/memory center was tested for FMRP requirements in activity-dependent remodeling (Doll et al., 2017). In these well-defined synapses, mPN2 axons project collateral branches with boutons into synaptic microglomeruli innervating KC dendrites (Figure 1). FMRP regulates mPN2-KC connectivity specifically during the critical period, with branch length decreased and synaptic bouton area increased in *dfmr1* nulls (Doll et al., 2017), causing a much more compact innervation pattern (Figure 1). In the mutants, mPN2 microglomeruli display a loss of the presynaptic active zone scaffold Bruchpilot during the critical period, suggesting delayed synaptogenesis (Doll et al., 2017). All defects are completely restricted to the critical period, with normal synaptic architecture and molecular differentiation restored by maturity (e.g., 7 dpe). GFP reconstitution across synaptic partners (GRASP) to test mPN2-KC connections (Feinberg et al., 2008; Pech et al., 2013) reveals that *dfmr1* null synaptic contacts are fewer in number, larger in size and more spatially restricted in the critical period, but not at maturity (Figure 1; Doll et al., 2017). EM ultrastructural analysis confirms that synaptic bouton size is increased in *dfmr1* null mutants during the critical period. Moreover, directly visualized T-bar synaptic active zones are drastically reduced in density in the FXS model, consistent with the loss of Bruchpilot labeling during the critical period (Doll et al., 2017). Given the activity-dependent remodeling during the normal critical period, and the activity insensitivity of *dfmr1* mutants, it was hypothesized that connectivity defects arise from activity-dependent refinement that occurs only in wildtype animals.

Odor response mapping studies demonstrate that IR75d OSNs respond to pyrrolidine upstream of mPN2 (Figure 1; Silbering et al., 2011; Münch and Galizia, 2016). Pyrrolidine exposure in the critical period, but not at maturity, phenocopies *dfmr1* synaptic defects and no changes occur in *dfmr1* mutants, demonstrating that FMRP is required for sensory experience synaptic remodeling (Doll et al., 2017). At maturity, pyrrolidine exposure causes no changes in wildtype animals, but does cause a reduction in *dfmr1* branch length, consistent with a shifted critical period. Optogenetic stimulation during the critical period also results in mPN2-KC connectivity changes in controls, but not *dfmr1* mutants (Doll et al., 2017). Conversely, targeted optogenetic hyperpolarization or tetanus toxin neurotransmission blockade both result in the opposite consequence of expanded MB calyx innervation in controls, but

not *dfmr1* mutants (Figure 1). All manipulations show FMRP is required for activity-dependent synaptic remodeling in the critical period. One exception is hyperpolarization causes partial rescue of *dfmr1* bouton area, which may indicate an inhibitory mechanism that can still promote some synaptic refinement despite FMRP loss and decreased GABAergic function in the FXS model (Gatto et al., 2014). Indeed, GABA agonists can rescue hyperexcitation in FXS models (Chang et al., 2008; Olmos-Serrano et al., 2010), and activating inhibitory neurons can rescue the experience-driven remodeling (Fagiolini and Hensch, 2000; Hensch, 2004). This may provide a parallel to FMRP critical period requirements, where a weakened inhibitory influence might suppress critical period hyperexcitation in the FXS disease state.

Taken together, these new studies show a transient requirement for FMRP during the early-use sensory experience critical period of synaptic remodeling (Doll et al., 2017). The *Drosophila* FXS disease model presents synaptic connectivity characteristics replicated by strong developmental activation of the brain circuitry. We conclude, therefore, that FXS is a hyper-activated state, or responsive as if hyper-activated, and that FMRP normally functions in an activity-dependent mechanism to enable circuit refinement during the critical period (Doll et al., 2017). Given the developmental and activity-dependent regulation of FMRP, coupled to its maintained requirement in learning and memory, it is tempting to speculate that loss of FMRP only during this transient window results in persistent network defects at multiple levels, including hyperactivity and improper connectivity (Pan et al., 2004; McBride et al., 2005; Bolduc et al., 2008; Tessier and Broadie, 2008; Doll and Broadie, 2015, 2016; Doll et al., 2017). Moreover, the appearance of a shifted critical period (Doll et al., 2017) is consistent with the argument that delays and developmental perturbations during neural circuit and E/I refinement may result in persistent behavioral abnormalities (Harlow et al., 2010; Takesian and Hensch, 2013). While our current metrics indicate rectification of structural and functional defects following the critical period (Bureau et al., 2008; Doll et al., 2017), there is also apparent overcorrection and blunted calcium signaling at maturity (Tessier and Broadie, 2008; Doll and Broadie, 2016). Future work needs to dissect both transient critical period and lasting mature consequences of FMRP loss in the FXS brain circuitry.

FMRP ROLE IN ENDOCYTIC MEMBRANE TRAFFICKING DURING SYNAPTIC REFINEMENT

Fragile X mental retardation protein acts primarily as an mRNA-binding translation suppressor, so this function was explored to test mechanisms of activity-dependent critical period synaptic remodeling (Vita and Broadie, 2017). A *Drosophila* brain developmental proteomics screen was done to identify candidate protein changes occurring during the critical period window (Tessier and Broadie, 2012). A secondary screen tested for activity-regulated proteins, consistent with a role in developmental plasticity. Finally, candidate hit overexpression

was assayed for predicted phenocopy of FXS defects, and protein level correction tested for predicted rescue of *dfmr1* null phenotypes during critical period development. A new FMRP target meeting all requirements is endosomal sorting complex required for transport III (ESCRTIII) core member Shrub (Vita and Broadie, 2017), *Drosophila* homolog of yeast Snf7/Vsp32 and human CHMP4 (Babst et al., 2002). Shrub exists as auto-inhibited monomers in the cytosol, which assemble in linear polymer arrays of spiral/helical filaments on membranes to drive inverse membrane budding (Teis et al., 2008). ESCRTIII mediates both plasma membrane and organelle trafficking (e.g., endosome-to-multivesicular body; MVB) in cooperation with other ESCRTs and the AAA-ATPase Vps4 (Henne et al., 2013). Canonically, ESCRTIII sorts ubiquitinated proteins to the lysosomal degradation pathway to remove targeted cell surface receptors (Sorkin, 1998; Babst et al., 2002). Importantly, ESCRTIII components are carefully regulated in endosome to MVB maturation, with loss or gain of ESCRTIII components resulting in similar trafficking aberrations, often in the form of greatly enlarged endosomal organelles (Teis et al., 2008).

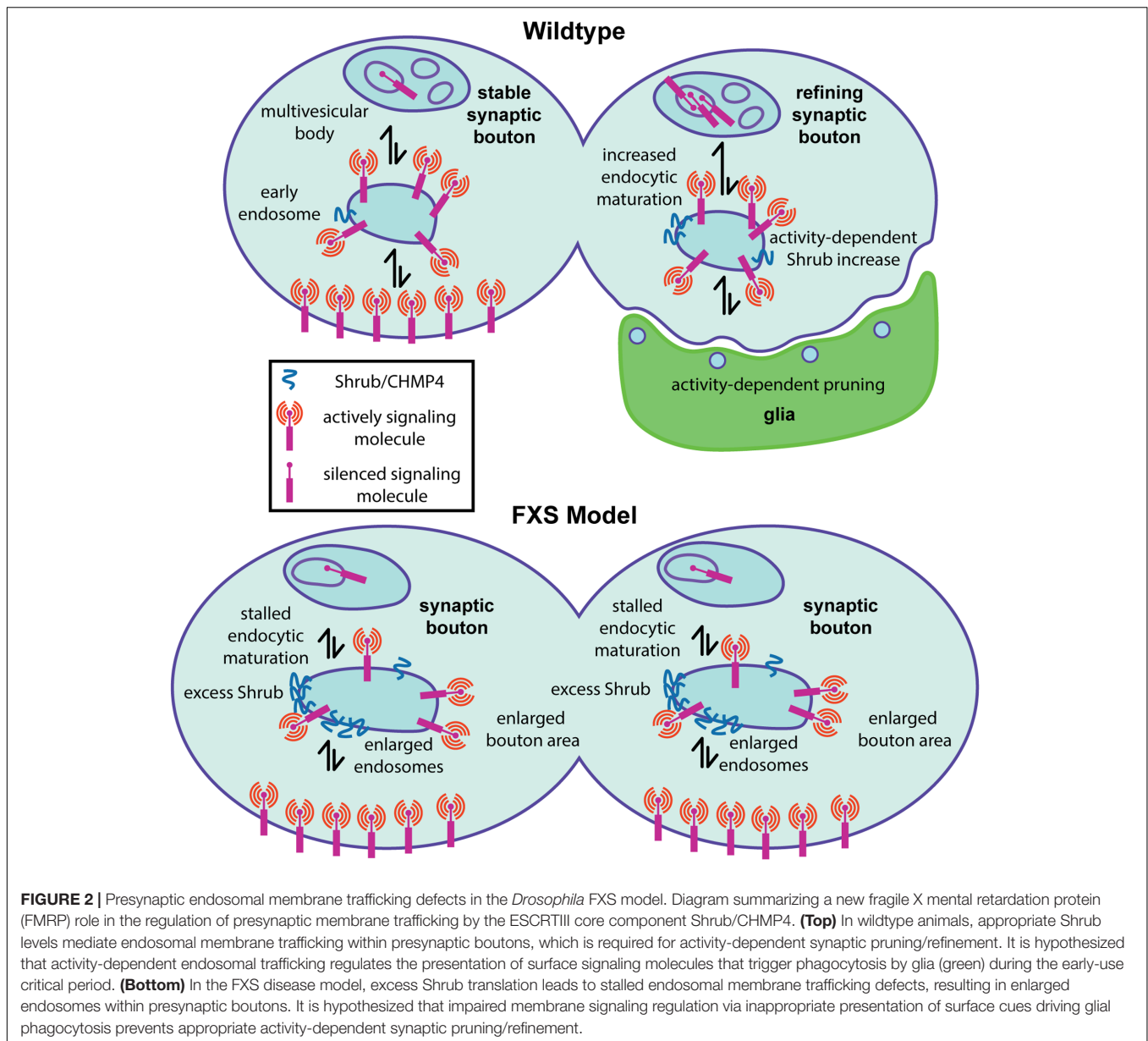
In *Drosophila*, Shrub is necessary for developmental axonal pruning, as well as for limiting dendritic arborization (Sweeney et al., 2006). These precedents support a role for Shrub downstream of FMRP translational suppression in synaptic remodeling. A key distinction is that Shrub levels are elevated in the FXS model (Vita and Broadie, 2017), predicting defects caused by excess Shrub. Consistent with selective involvement in the critical period, Shrub levels are elevated in *dfmr1* null brains during the 0–1 dpe window defined above, and FMRP expression rescues Shrub levels during this period (Vita and Broadie, 2017). Importantly, optogenetic stimulation drives increased Shrub levels in wildtype animals during the critical period, whereas *dfmr1* mutants display no Shrub protein level changes, indicating FMRP mediates activity-dependent regulation. Employing RNA immunoprecipitation, it was found that FMRP binds *shrub* mRNA (Vita and Broadie, 2017). Taken together, these results demonstrate FMRP limits Shrub levels during the critical period by repressing translation in an activity-dependent mechanism (Vita and Broadie, 2017). With a restricted PN driver (Nrv3-Gal4) for projection neurons innervating the MB calyx (Figure 1), it was shown that Shrub overexpression and FMRP loss similarly cause overelaborated synaptic contacts during the critical period (Vita and Broadie, 2017). Moreover, EM ultrastructural analyses revealed Shrub overexpression and FMRP loss both result in enlarged PN synaptic boutons within the MB calyx (Figures 1, 2). These results confirmed the importance of Shrub elevation in FXS phenotypes, and suggested that endocytic membrane trafficking is required for critical period synaptic refinement.

As a first step in assaying membrane trafficking, the endosome marker Rab5 was assayed in PN synaptic boutons innervating the MB calyx (Vita and Broadie, 2017). Both Shrub overexpression and FMRP loss result in an elevated number of enlarged Rab5-positive endosomes in PN synaptic boutons during the critical period (Figures 1, 2). Consistently, ultrastructural analyses reveal strikingly enlarged endosomal vacuoles within PN synaptic boutons in both the Shrub overexpressing and *dfmr1* null animals (Vita and Broadie, 2017). Interestingly,

both conditions also display an increased number of enlarged endosomal intraluminal vesicles, consistent with reports of Sfn7 overexpression and interpreted as a consequence of stalled MVB sorting (Teis et al., 2008). Taken together, these results suggest gain of Shrub or loss of FMRP similarly causes trafficking-arrested synaptic endosomes (Figures 1, 2). To definitively test the FMRP/Shrub interaction in the context of the FXS disease model, Shrub levels were corrected (*shrub/+* heterozygotes) in an otherwise *dfmr1* null mutant (Vita and Broadie, 2017). This correction rescues *dfmr1* phenotypes, with a significant restoration of PN innervation and synaptic bouton area, and complete rescue of endosome trafficking (Figure 2). This work establishes Shrub as an activity-dependent synaptic refinement protein, negatively regulated by FMRP during the critical period to mediate appropriate early-use neural circuit remodeling (Vita and Broadie, 2017). The mechanism likely involves Shrub-dependent endocytic trafficking, either of membrane being internalized during synaptic pruning, or in control of surface guidance molecules regulating activity-dependent synapse elimination (Figure 2).

It is tempting to speculate that stalled MVB maturation is a crucial determinant of the arrested critical period synaptic refinement characterizing the FXS disease state, operating via short-term plasma membrane and/or long-term signaling misregulation (Vita and Broadie, 2017). Evidence for the latter hypothesis comes from developmental pruning studies showing that reduction of cell adhesion molecule Neuroglian coincides with ESCRT-mediated pruning of sensory neuron dendrites during metamorphosis (Zhang et al., 2014). However, Neuroglian levels have not yet been demonstrated to be changed in the *Drosophila* FXS model, and Neuroglian is not known to be involved in MB synaptic pruning (Reeve et al., 2005; Zhang et al., 2014). Given Shrub is elevated with neuronal activity, we hypothesize it acts to sort activity-dependent reduction of as yet unidentified surface receptors regulating synaptic refinement (Vita and Broadie, 2017). One intriguing possibility is that Shrub-mediated membrane trafficking regulates cell surface signals for glial phagocytosis driving synaptic pruning during the early-use critical period (Figure 2). Consistently, *dfmr1* null mutants display delayed developmental MB gamma neuron pruning mediated by phagocytic glia and delayed glial engulfment of damaged axons, as well as clear deficiencies in immune cell-mediated engulfment (Tasdemir-Yilmaz and Freeman, 2014; O'Connor et al., 2017). Therefore, we hypothesize that activity-dependent defects in synaptic refinement in the FXS disease state could be due to improper intercellular interactions between neurons and glia (Figure 2), which depend on FMRP regulation of Shrub-mediated membrane trafficking.

Shrub misregulation is not the only aberrant translational repression in FXS, and there are broad consequences to neuron properties. Indeed, new evidence suggests this defect alters how molecules permeate *dfmr1* null neurons (Kennedy and Broadie, 2017). Iontophoresis of small polar dyes (e.g., neurobiotin, lucifer yellow) has long been used to assay gap junctions linking electrically coupled neurons (Lapper and Bolam, 1991; Hanani, 2012; Kudumala et al., 2013; Lee and Godenschwege, 2015), whereas large dyes (e.g., dextran-tetramethylrhodamine)



fill single neurons without transfer (Phelan et al., 1996). The electrically coupled *Drosophila* giant fiber interneuron (GFI) transmitting information from sensory neuron inputs to motor neuron outputs (Allen et al., 1998) has been used extensively for such dye injection studies (Boerner and Godenschwege, 2010). Null *dfmr1* mutants have strong defects in GFI-dependent behaviors (Martinez et al., 2007), and was therefore targeted for studies of electrical and chemical synaptic connectivity in our FXS model. However, a surprising discovery was made; mutant GFI axons, dendrites and cell bodies are much more easily dye-loaded (Kennedy and Broadie, 2017). The striking defect is specific to small polar dyes, but cannot be attributed to altered electrical synapse coupling. FMRP is absolutely required, since neuron-targeted FMRP fully rescues defects. Membrane properties do not account for the difference, which is due to

a highly elevated rate of cytosolic dye incorporation (Kennedy and Broadie, 2017). Our working hypothesis is that elevated protein levels caused by loss of FMRP translational suppression fundamentally alters the cytosolic milieu, to change molecular diffusion rates in FXS model neurons.

FMRP REQUIREMENT IN ACTIVITY-DEPENDENT PROTEOLYTIC SYNAPSE REMODELING

Up to this point, we have focused on cell-autonomous FMRP requirements, yet a crucial aspect of synaptogenesis and synaptic refinement is coordinated, *trans*-synaptic signaling between partners (Barros et al., 2011; Dani and Broadie,

2012). This highly dynamic intercellular communication influences innervation patterns, synaptic architecture and neurotransmission strength, although roles in activity-dependent mechanisms are less clear (Barros et al., 2011; Dani and Broadie, 2012). The extensive toolkit available for the *Drosophila* glutamatergic NMJ model synapse is ideally suited for testing *trans*-synaptic signaling within activity-dependent mechanisms (Broadie et al., 2011; Harris and Littleton, 2015). Enlarged presynaptic boutons at the NMJ are easily distinguishable from the post-synaptic subsynaptic reticulum (SSR), and numerous genetic tools, markers and assays separate pre- versus post-synaptic requirements (Harris and Littleton, 2015). Signaling ligands must necessarily traverse the extracellular synaptomatrix. Two key synaptomatrix regulatory factors are (1) HSPGs and (2) matrix metalloproteinases (MMPs; **Figure 3**). HSPGs contain a core protein and heparan sulfate (HS) glycosaminoglycan (GAG) chains, which bind MMPs and extracellular signaling ligands (Park et al., 2000; Tocchi and Parks, 2013). HSPGs also link MMPs to their targets, promoting proteolytic activation/specificity (Tocchi and Parks, 2013). At the *Drosophila* NMJ, HSPGs regulate synaptic architecture, presynaptic active zone size/number and post-synaptic function, and serve to localize Wnt signaling ligands (Johnson et al., 2006; Dani et al., 2012; Kamimura et al., 2013).

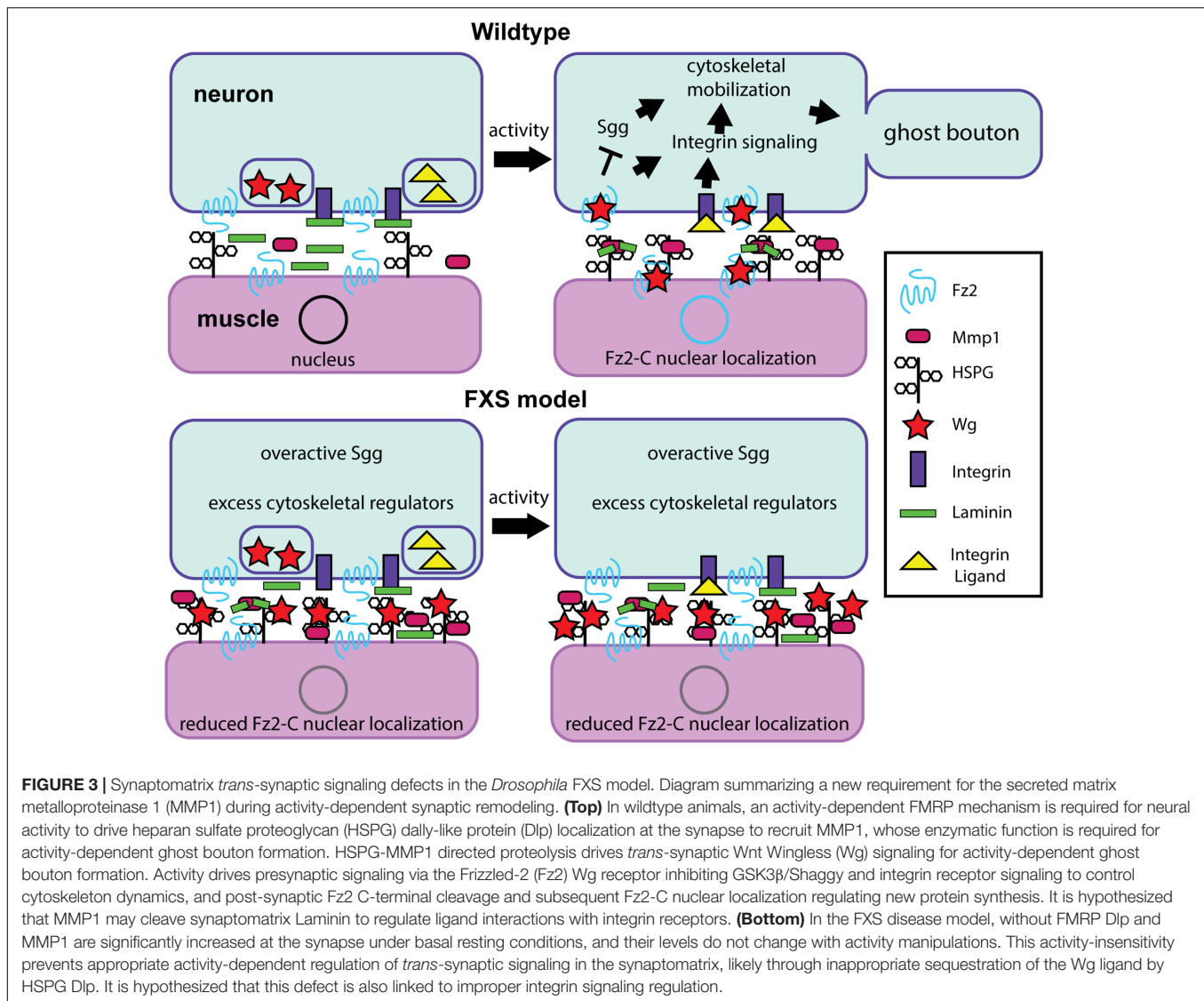
In both mammalian synapses and the *Drosophila* NMJ model, extracellular MMPs directly and indirectly regulate the *trans*-synaptic signaling ligands modulating synaptic structure and function (Włodarczyk et al., 2011; Dear et al., 2016). The mammalian genome encodes at least 24 MMPs with reportedly redundant/overlapping functions, many of which are localized to synapses. In contrast, *Drosophila* MMPs are represented by just two genes, *mmp1* and *mmp2*, which encode a single secreted and single GPI-anchored enzyme, respectively; although an anchored MMP1 has recently been described (Llano et al., 2000, 2002; LaFever et al., 2017). Compared to the MMP complexity in mammals, *Drosophila* enables reductionist testing of MMP roles in the FXS state. In *Drosophila*, both MMP1 and MMP2 regulate axonal and dendritic architecture (Kuo et al., 2005; Yasunaga et al., 2010; Depetris-Chauvin et al., 2014). At the *Drosophila* NMJ, both MMPs limit presynaptic growth, functional differentiation, and Wnt Wg *trans*-synaptic signaling (Dear et al., 2016). Interestingly, while MMP1 promotes MMP2 and HSPG Dlp localization, MMP2 limits MMP1 and Dlp localization at the synapse (Dear et al., 2016). These interactions suggest a complex level of interplay between MMPs and HSPGs within the synaptomatrix interface. Functionally, MMPs cleave not only extracellular matrix (ECM) targets during axon pathfinding (Miller et al., 2007, 2011), but also cell adhesion molecules (CAMs) in activity-dependent mechanisms sculpting synapse structure, turning off signaling and mobilizing membrane turnover in processes associated with neurological disorders including FXS (Nagappan-Chettiar et al., 2017).

In Wnt Wg signaling, presynaptic activity leads to Wg secretion, which binds to Frizzled-2 (Fz2) receptors on both pre- and post-synaptic cells (Koles and Budnik, 2012). Wg signaling drives both divergent and non-canonical Wnt cascades in pre- and post-synaptic cells (**Figure 3**), modulating both

synapse structure and function (Koles and Budnik, 2012). Importantly, activity-induced Wg secretion drives activity-dependent synaptic remodeling, which operates within a rapid time-frame to promote formation of “ghost boutons”; immature boutons with presynaptic but not post-synaptic specialization (Ataman et al., 2008). Critically, extracellular HSPGs are integrally involved in Wg *trans*-synaptic signaling (**Figure 3**), highlighting the importance of the synaptomatrix in Wg signaling regulation (Harris and Littleton, 2015). Specifically, the secreted HSPG Perlecan balances pre- and post-synaptic Wg signaling by promoting post-synaptic Wg localization (Kamimura et al., 2013). Moreover, the GPI-anchored HSPG Dlp regulates Wg signaling in a concentration-dependent manner (**Figure 3**): based on Dlp co-receptor levels relative to Fz2 receptor and Wg ligand, Dlp can either restrict or promote Wg signaling as a negative and positive signaling regulator (Yan et al., 2009). Importantly, FMRP restricts synaptic levels of two HSPGs (Dlp and Syndecan) to regulate Wnt Wg *trans*-synaptic signaling, which is strongly misregulated in the FXS disease model (Friedman et al., 2013). Given the complex interactions between neural activity states, MMP proteolytic function, HSPG coreceptors and signaling mechanisms, activity-dependent Dlp-MMP interactions badly needed to be compared in normal versus FXS model synapses (Dear et al., 2017).

To test activity-dependent mechanisms, temperature-sensitive dTRPA1 channels were used to acutely depolarize neurons over a 1-h period (Hamada et al., 2008; Pulver et al., 2009). These studies demonstrated that MMP1, but not MMP2, is required to form ghost boutons (Dear et al., 2017). Consistently, dTRPA1 activation, or high $[K^+]$ depolarization for just 10 min, rapidly increases MMP1 at the synapse (**Figure 3**). Conversely, MMP2 is reduced by stimulation, as predicted since MMP1 limits MMP2 (Dear et al., 2016). Moreover, stimulated synapses rapidly elevate Dlp, with increased Dlp and MMP1 co-localization (Dear et al., 2017), supporting previous findings of genetic interaction at the NMJ. Importantly, the Dlp-Mmp1 co-localization in synaptic subdomains is significantly increased following acutely elevated neuronal activity in just 10 min (**Figure 3**). Since HSPGs are known to anchor proteases in other contexts (Tocchi and Parks, 2013), the dependence of MMP1 localization on Dlp was next tested. Both genetic mutant and targeted RNAi reduction of Dlp reduce synaptic MMP1 levels dramatically, whereas Dlp overexpression causes an opposing MMP1 increase at the synapse (Dear et al., 2017). These results show that the GPI-anchored Dlp regulates secreted MMP1 localization (**Figure 3**). Moreover, overexpression of Dlp lacking HS-GAG chains causes no change in MMP1 localization, suggesting that the HS-GAG chains are necessary for MMP1 synaptic localization (Dear et al., 2017). These results are consistent with other studies that have established roles for HS-GAG chains in HSPG activity at the synapse (Baeg et al., 2001; Johnson et al., 2006; Yan et al., 2009).

Given activity recruits HSPG Dlp, which in turn localizes MMP1 at the synapse, there is expected to be an activity-dependent increase in proteolytic activity surrounding synaptic boutons. To test this prediction, a dye-quenched fluorogenic gelatin substrate was tested in *in situ* zymography studies of protease enzymatic function (Siller and Broadie, 2011; Shilts and



Broadie, 2017). Importantly, Dlp reduction results in reduced MMP-dependent proteolytic activity, while Dlp overexpression, with or without HS-GAG chains, elevates enzymatic function (Dear et al., 2017). These results are consistent with the hypothesis that synaptic Dlp levels tune synaptomatrix MMP1 proteolytic cleavage capacity, possibly via a Dlp core protein interaction resulting in protease activation (Figure 3). With the knowledge that basal MMP1 levels are tuned by membrane-anchored Dlp, it remained to be tested whether the acute neuronal activity-induced MMP1 increase also depends on Dlp. Indeed, Dlp loss suppresses activity-dependent MMP1 localization at the synapse, whereas Dlp overexpression elevates MMP1 levels and co-localization with Dlp (Dear et al., 2017). In line with above studies, overexpression of a Dlp isoform lacking HS-GAG chains results in a reduced activity-dependent enrichment of MMP1 at the synapse (Figure 3). Importantly, whereas synaptic MMP1 abundance tightly depends on Dlp, loss of MMP1 does not prevent the activity-dependent synaptic

enrichment of Dlp (Dear et al., 2017). Thus, synaptic MMP1 localization depends on Dlp, but not vice versa. Taken together, these results support the conclusion that Dlp is absolutely necessary for the rapid activity-dependent synaptic localization of MMP1.

In the *Drosophila* FXS model, Dlp is constitutively elevated at the NMJ synapse, and reduction of Dlp (or dependent MMP1) in otherwise *dfmr1* null mutants suppresses FXS synaptogenic phenotypes (Siller and Broadie, 2011; Friedman et al., 2013). Therefore, activity-dependent Dlp and MMP1 synaptic enrichment was tested in the FXS model (Dear et al., 2017). As expected, MMP1 synaptic localization is strikingly increased in *dfmr1* null mutants (Figure 3). One interpretation is that this enrichment reflects a FXS hyper-excited state, manifested in elevated Dlp localization at the synapse (Friedman et al., 2013). Consistent with this idea, genetic reduction of Dlp restores normal MMP1 levels in *dfmr1* null synapses (Dear et al., 2017). Importantly, acute stimulation in *dfmr1* mutants

causes no activity-dependent enrichment of MMP1 (Dear et al., 2017), demonstrating that MMP1 is insensitive to activity-dependent regulation in the FXS condition (**Figure 3**). Moreover, Dlp reduction restores activity-induced MMP1 enrichment in *dfmr1* null synapses (Dear et al., 2017). Just like stimulated controls, stimulated *dfmr1* nulls heterozygous for Dlp display striking synaptic enrichment of Mmp1 (**Figure 3**). Therefore, Dlp is the critical link determining activity-regulated synaptic MMP1 localization downstream of FMRP. These findings suggest MMP inhibition may ameliorate FXS phenotypes; for example, in the context of mGluR-induced MMP9 hyperactivity (Bilousova et al., 2008). These results also indicate that targeting the misregulated activity-dependent mechanism of Dlp mediating activity-dependent overabundance of synaptic MMP1 could potentially prevent inappropriate connections caused by hyperexcitability in the FXS condition.

This activity-FMRP-HSPG-MMP regulatory mechanism of synapse remodeling presents questions. A key question is the means by which activity-dependent MMP1 localization is restored by reducing Dlp in the FXS model. It is probable that an unidentified synaptomatrix player is involved. Since Dlp can activate and inhibit Wg signaling (Yan et al., 2009; Dani et al., 2012), reducing Dlp could restore proportionality between interacting synaptomatrix components (**Figure 3**). Altered Dlp sulfation may change protease activity (Tocchi and Parks, 2013), perhaps in concert with other effectors, such as HSPG-sulfating *hs6st* and *sulf1* genes that modulate Wg signaling (Dani et al., 2012). Alternatively, Wg *trans*-synaptic signaling is reduced in the FXS condition (Friedman et al., 2013), and Wg itself may feedback to restore activity-dependent MMP1 function (**Figure 3**). Another possibility is that a synaptomatrix regulator preventing excess Dlp from misregulating activity-dependent MMP1 could be lost in the FXS condition. Indeed, FMRP can promote protein levels (Feng et al., 1997; Derlig et al., 2013), and this could include synaptomatrix proteins. For example, other HSPGs (e.g., Perlecan) might consolidate Wg signaling, thus restoring a more normal activity-dependent dynamic (**Figure 3**). Relevant MMP1 catalytic targets are unclear, though it is tempting to speculate secreted MMP1 may cleave Laminin-A to enable activity-dependent integrin signaling (Tsai et al., 2012; Lee et al., 2017). Whatever further elements of the synaptomatrix mechanism have yet to be elucidated, the finding that activity-dependent regulation can be restored in the *Drosophila* FXS disease model opens exciting possibilities for new FXS therapeutic treatments, and may lead to the discovery of novel activity-regulated extracellular molecules critical for synaptic remodeling.

FUTURE DIRECTIONS

The very recent work discussed in this article highlights the utility of the *Drosophila* FXS disease model for the study of developmental activity-dependent mechanisms at synaptic connections, during use-dependent synaptic remodeling and in early-use critical periods. These new advances further

demonstrate FMRP requirements in activity-dependent regulation of protein translation and control of signaling mechanisms operating at the heart of synapse formation and refinement. The particularly well-characterized central brain MB olfactory learning and memory circuitry has become a powerful vehicle for determining molecular mechanisms disrupted by FMRP loss, cellular mechanisms of activity-dependent synaptic remodeling, and the means of establishing excitatory/inhibitory synapse balance during the critical period (Doll et al., 2017; Vita and Broadie, 2017). In parallel, the classic GF visual escape circuit linking sensory input, brain integration and motor output via particularly large and well-characterized interneurons has the promise of providing an exciting new avenue to dissect FMRP requirements (Kennedy and Broadie, 2017). Finally, the malleable NMJ provides a large and genetically tractable glutamatergic synapse model, which continues to be instrumental in the discovery and elucidation of FMRP synaptic requirements, including intracellular signaling, intercellular interactions, and *trans*-synaptic pathways that strongly contribute to the FXS disease state (Dear et al., 2017). These diverse circuits will continue to be the focus of future studies, as we seek to determine generalizable FMRP requirements throughout the entire nervous system, as well as selective FMRP roles in specific neural circuits and synapses.

Our current understanding of FMRP requirements during the critical period paves the way for future studies examining molecular mechanisms of activity-dependent refinement. Based on recent findings (Doll and Broadie, 2016), we hypothesize that developmental misregulation of activity-induced Ca^{2+} signaling is a core contributor to the FXS condition. Importantly, classic memory-linked pathways (e.g., cAMP pathway) connect directly and indirectly to Ca^{2+} signaling (Davis and Dauwalder, 1991; Skoulakis et al., 1993; Kanellopoulos et al., 2012), with pathway members enriched in *Drosophila* brain MB and AL (**Figure 1**; Crittenden et al., 1998). FXS patient-derived cells and models similarly show reduced cAMP levels, and genetic/pharmacological correction of cAMP levels prevents FXS model phenotypes (Berry-Kravis and Huttenlocher, 1992; Berry-Kravis et al., 1995; Kelley et al., 2007; Kanellopoulos et al., 2012). Downstream of cAMP, PKA phosphorylates a wide range of neuronal targets (Sassone-Corsi, 2012), and enhances excitability in both excitatory and inhibitory neurons to promote activity-dependent remodeling (Lee, 2015). A likely downstream target, the small GTPase Rac1, acts as a molecular switch in structural and functional synaptic plasticity, and is of interest in the context of FXS hyperexcitability (Lee et al., 2003; Schenck et al., 2003; Bongmba et al., 2011; Goto et al., 2013, 2014; Tejada-Simon, 2015). Interestingly, inhibition of PAK downstream of Rac1 prevents FXS model phenotypes (Dolan et al., 2013). We therefore hypothesize that FXS phenotypes associated with aberrant Ca^{2+} -cAMP-PKA-Rac1-PAK signaling likely occur in both the MB and AL during the early-use critical period (Doll et al., 2017; Vita and Broadie, 2017).

We are increasingly aware of possible intercellular interactions in the FXS state, such as neuron-glia roles in circuit refinement (Logan, 2017). Based on our recent work (Vita and Broadie, 2017), we hypothesize dysregulated neuronal surface signaling

cues impair glia-mediated phagocytosis driving synaptic pruning during activity-dependent refinement (**Figure 2**). Specifically, we propose disrupted membrane trafficking due to elevated ESCRTIII Shrub levels could alter a surface signal for glial phagocytosis (Vita and Broadie, 2017). Consistently, glia-mediated developmental phagocytosis pruning of MB gamma neuron collateral branches is reduced/delayed in the absence of FMRP (O'Connor et al., 2017). Studies to date have focused primarily on glial clearance via the Draper/Ced-1/MEGF-10 receptor pathway (Musashe et al., 2016; Lu et al., 2017). We propose that FMRP loss may cause improper refinement through signaling defects that prevent glial phagocytosis, delay signaling processes that promote phagocytosis, or manifest aberrations in glial inability to sufficiently regulate or respond to other cells during critical period refinement. Interestingly, glia modulate the excitation/inhibition balance via a GABA uptake mechanism regulating synaptogenesis (Muthukumar et al., 2014). We therefore hypothesize that neuron-glia interactions may also modulate synaptic excitation/inhibition balance in critical period refinement. Recent work in mice shows glial FMRP is necessary, but not sufficient, for FXS model dendritic spine dynamics (Hodges et al., 2017), and co-cultures with astrocytes lacking FMRP illustrate delayed development (Jacobs et al., 2016), indicating that FMRP can act directly in glia as well as in neurons.

In the NMJ model, misregulation of Wnt Wg *trans*-synaptic signaling is an established cause of FXS phenotypes (Friedman et al., 2013). FMRP is required for activity-dependent HSPG Dlp regulation of extracellular MMP1 synaptic localization and enzymatic function (Dear et al., 2017). MMP1, in turn, is absolutely required for rapid synaptic bouton formation in response to activity. Moreover, Tissue Inhibitor of MMP (TIMP) overexpression prevents synaptic defects in the *Drosophila* FXS model (Siller and Broadie, 2011), suggesting that synaptomatrix protease regulation is another avenue worth investigating in the FMRP-Dlp-MMP1 pathway (Dear et al., 2017). HSPG Syndecan is negatively regulated by FMRP (Friedman et al., 2013), and may therefore also be involved. Downstream of altered Wnt Wg *trans*-synaptic signaling, defective Fz2-C nuclear import is well described in the *Drosophila* FXS disease model (Friedman et al., 2013), but it remains to be tested whether autocrine Wg signaling is also impacted. Based on work showing that inhibition of the Wg divergent canonical target GSK3 β /Shaggy is a promising FXS therapeutic treatment (Klein and Melton, 1996; Stambolic et al., 1996; McBride et al., 2005; Choi et al., 2010; Mines and Jope, 2011), we hypothesize that overabundant synaptic Dlp sequesters Wg ligand, inhibiting Wg signaling, and therefore the activity-dependent suppression of GSK3 β /Shaggy. Future work will test whether Wg sequestration by excess Dlp explains the activity-insensitivity of *dfmr1* null synapses. Synapse-associated glia also regulate Wg *trans*-synaptic signaling (Kerr et al., 2014; Kopke

et al., 2017), indicating another plausible source of aberrant synaptomatrix regulation that needs to be explored in the FXS condition.

As we continue ongoing studies exploiting the *Drosophila* FXS disease model, we posit outstanding needs to dissect developmental activity-dependent synaptic remodeling and connectivity refinement mechanisms, both in the brain and at the NMJ. Within the brain AL-MB olfactory circuit (**Figure 1**), OSN, PN, and KC synaptic connections are well suited to pursue the mechanisms of *trans*-synaptic signaling (e.g., Notch, Wg), synaptomatrix regulation (e.g., HSPG, MMP), signal transduction (e.g., cAMP-PKA, actin cytoskeleton), and intercellular interactions (e.g., neuron-glia). This circuit is also ideal for testing mechanisms of excitation/inhibition balance (e.g., mPN2 vs. MBON-11; **Figure 1**) developing in response to sensory experience during early-use critical periods. Our work highlights a restricted, transient window of FMRP requirement coinciding with peak FMRP levels. In parallel, the NMJ glutamatergic model synapse will be instrumental for investigating the interplay of the multiple bidirectional *trans*-synaptic signaling pathways regulated by an increasingly defined synaptomatrix (**Figure 3**). This system is also ideal for testing activity-dependent synaptic remodeling mechanisms, including bouton addition and elimination, and glial involvement in the refinement of the pre- and post-synaptic sides of the synapse. In addition, NMJ findings will continue to inform and direct ongoing central brain studies. Our goal is to continue to discover cellular and molecular mechanisms of activity-dependent circuit remodeling that optimize behavioral performance, and to reveal the FMRP-dependent neurodevelopmental processes that go awry in FXS, so as to be able to devise effective new treatments for this devastating disease state.

AUTHOR CONTRIBUTIONS

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Reevaluation of *FMR1* Hypermethylation Timing in Fragile X Syndrome

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Fragile X syndrome (FXS) is one of the most common heritable forms of cognitive impairment. It results from a fragile X mental retardation protein (FMRP) protein deficiency caused by a CGG repeat expansion in the 5'-UTR of the X-linked *FMR1* gene. Whereas in most individuals the number of CGGs is steady and ranges between 5 and 44 units, in patients it becomes extensively unstable and expands to a length exceeding 200 repeats (full mutation). Interestingly, this disease is exclusively transmitted by mothers who carry a premutation allele (55–200 CGG repeats). When the CGGs reach the FM range, they trigger the spread of abnormal DNA methylation, which coincides with a switch from active to repressive histone modifications. This results in epigenetic gene silencing of *FMR1* presumably by a multi-stage, developmentally regulated process. The timing of *FMR1* hypermethylation and transcription silencing is still hotly debated. There is evidence that hypermethylation varies considerably between and within the tissues of patients as well as during fetal development, thus supporting the view that *FMR1* silencing is a post-zygotic event that is developmentally structured. On the other hand, it may be established in the female germ line and transmitted to the fetus as an integral part of the mutation. This short review summarizes the data collected to date concerning the timing of *FMR1* epigenetic gene silencing and reassess the evidence in favor of the theory that gene inactivation takes place by a developmentally regulated process around the 10th week of gestation.

Keywords: Fragile X syndrome, *FMR1*, DNA methylation, CGG expansion, epigenetic gene silencing

Fragile X syndrome (FXS; OMIM#300624) is one of the most common heritable forms of cognitive impairment (1 in 4000 male and 1 in 8000 female births), and is the leading known genetic cause of autism. It is inherited as an X-linked condition and results from a deficiency in the fragile X mental retardation protein (FMRP; McLennan et al., 2011). FMRP is an RNA-binding protein that is important for transport, stabilization and translation of mRNA into proteins that affect synaptic plasticity and connectivity in the central nervous system (Schaeffer et al., 2003; Santoro et al., 2011). In the absence of FMRP the dendritic spines are longer, thinner and less mature (Comery et al., 1997; Irwin et al., 2000).

Nearly all FXS patients lack FMRP due to an unusual loss-of-function mutation: a CGG tri-nucleotide repeat expansion in the 5'-UTR of the X-linked *FMR1* gene (Verkerk et al., 1991). As a function of the repeat tract size, four allele forms can be defined: normal (<45 CGG), intermediate (45–54 CGGs), premutation (55–200 CGG, PM) and full mutation (>200 CGG, FM)

alleles (Rajaratnam et al., 2017). In the normal range, the copy number of CGGs is steady and the gene is fully functional. In rare cases, the number of CGGs increases slightly (intermediate). This has no effect on gene function. However, it increases the risk of further increase to the PM range in future generations. Individuals with PM do not manifest FXS but are prone to premature fragile X-associated ovarian failure (FXPOI) in females and fragile X-associated tremor/ataxia syndrome (FXTAS), particularly in males (Streuli et al., 2009; Gleicher and Barad, 2010). FXPOI is a primary ovarian defect characterized by absent menarche (primary amenorrhea) or premature depletion of ovarian follicles before the age of 40 years while FXTAS is a neurodegenerative condition that is characterized by adult-onset progressive intention tremor and gait ataxia. Both, FXPOI and FXTAS, have been attributed to RNA/protein gain-of-function mechanisms (Sellier et al., 2017).

When the CGGs expand to a length exceeding 200 repeats (FM) they become extensively unstable and result in FXS pathology. This occurs when the PM allele is transmitted by mothers but not fathers (Pembrey et al., 1985; McLennan et al., 2011). Once the CGGs reach the FM range they lead to *FMR1* epigenetic gene silencing through the induction of DNA methylation (Oberlé et al., 1991). Hypermethylation takes place through a specific pattern of acquisition. It spreads out from the 5' flanking sequence (~650 bp upstream to the CGGs) to intron 1 of *FMR1*, spanning over the repeats and promoter region of the gene (Naumann et al., 2009). This occurs jointly with the loss of active histone modifications (H3K4me3) and the gain of repressive histone modifications (H3K9me2/3, H3K27me3), ultimately leading to a transcriptional block at the *FMR1* promoter (Coffee et al., 1999, 2002; Pietrobono et al., 2005; Tabolacci et al., 2005, 2008; Kumari and Usdin, 2010). Males with a FM are almost always severely affected by FXS, whereas females with a FM are generally less affected than males and manifest disease symptoms in only about 50% of the cases (Rajaratnam et al., 2017). This is because the *FMR1* gene is subject to X-inactivation in the somatic cells of females. A long-standing issue concerns the timing of *FMR1* epigenetic silencing in FXS. This is a fundamental question in the field of FXS research since the answer may provide new insights into the mechanism/s responsible for epigenetic gene silencing of *FMR1* in FXS.

Initially, when the gene for the disease had just been discovered, it was naturally assumed that hypermethylation was established and transmitted by the mother as an integral part of the FM. However, by monitoring for the repeat size and the methylation state of *FMR1* using methylation-sensitive Southern blot assays in fetal tissues and chorionic villus sampling (CVS) samples at 10–16 weeks of gestation, it became apparent that not all tissues are equally *FMR1*-hypermethylated during development. When the methylation status of the gene was examined, it was found to be heavily methylated in the majority of affected fetuses as early as 10 weeks of gestation, the time when embryo specification is already complete (Sutherland et al., 1991; Devys et al., 1992; Sutcliffe et al., 1992; Suzumori et al., 1993; Castellví-Bel et al., 1995; Willemsen et al., 2002). On the other hand, when the methylation status of *FMR1* was examined in the extra-embryonic tissues, the results were contradictory.

While some reports found hypermethylation in CVS as early as 10 weeks (Devys et al., 1992; Suzumori et al., 1993), others showed that the *FMR1* is often hypomethylated and remains active even after 13 weeks of age (Sutherland et al., 1991; Sutcliffe et al., 1992; Castellví-Bel et al., 1995). In fact, this is why prenatal genetic diagnosis for FXS by CVS often leads to ambiguous results and much confusion that demands a follow-up via amniocentesis.

In a different study by Willemsen et al. (2002), the timing of *FMR1* gene silencing was determined by monitoring FMRP expression. This was achieved by immuno-histochemical analysis that provided an opportunity to monitor protein expression at the single-cell level while preserving tissue structure. The authors showed that FMRP gradually disappears in chorionic villi of XY affected fetuses between 10 weeks and 12.5 weeks of gestation. In CVS from 13 week old female fetuses with a FM, each villus was either completely positive or entirely negative for FMRP expression, implying that the proliferation of villi is a clonal process. Given that *FMR1* is an X-linked gene that is liable to X-inactivation, these findings suggested that the timing of *FMR1* gene silencing follows X-inactivation since no single villus contained a mixture of FMRP-expressing and non-expressing cells. Altogether, this led to the general impression that *FMR1* inactivation is an ongoing, developmentally regulated process initiated after embryo implantation, completed in the fetus by the end of first trimester, and frequently on hold in extra-embryonic tissues (Devys et al., 1992; Sutcliffe et al., 1992; Suzumori et al., 1993; Iida et al., 1994; Willemsen et al., 2002). In addition, it led researchers to conclude that CGG expansion is necessary, but certainly not sufficient for gene inactivation, and that additional differentiation-dependent factors are required to achieve epigenetic silencing.

The notion that the timing of repeat expansion and hypermethylation are different and that hypermethylation is achieved by a post-zygotic event, led to the assumption that FMs would be hypomethylated in fetal gametes. In fact, by probing for expansion size and *FMR1* methylation, Malter et al. (1997) provided evidence for the presence of unmethylated FM exclusively in intact ovaries of female fetuses (16 and 17 weeks gestation) by Southern blot analysis. Ruling out the possibility of the existence of PM alleles, they argued that the vast majority of oocytes in the ovaries harbor a FM in its unmethylated form. Given these data, it was presumed that repeat expansion may have already occurred in the female germ line, or very early during embryogenesis, prior to *de novo* methylation.

Although this study was limited to only two fetuses, it was consistent with the under-methylated state of the mutation in the extra-embryonic tissues of affected fetuses. In addition, it corresponded to known human embryo developmental milestones. During early embryogenesis, the precursor cells for the extra-embryonic tissues (trophoblast, TE) and the primordial germ cells (PGCs) separate from the generally unmethylated epiblast just before genome wide *de novo* methylation takes place in the embryo proper at the time of implantation (Matsui and Mochizuki, 2014; Schroeder et al., 2015; Chatterjee et al., 2016). In mammals, DNA methylation patterns are initially established during gametogenesis. Almost

all of these patterns are erased at the time of pre-implantation development, and then re-established by a second wave of methylation during early gastrulation, when the embryo initiates implantation (Kafri et al., 1992). During this second wave of methylation, CpG islands (such as the one in the 5'-UTR of *FMR1*) remain protected from methylation, allowing housekeeping genes to remain expressed in all cells of the embryo (Kafri et al., 1992). Therefore, it would be extremely beneficial if isolated oocytes with a FM (instead of intact ovaries) could be analyzed for the methylation status of the locus prior to fertilization. Such oocytes can be obtained during IVF procedures for women who are carriers of the fragile X FM/PM.

It should be noted that unlike female germ line cells, mature sperm cells with a FM have never been observed in adult males with FXS (Malter et al., 1997). This is because during fetal development FM alleles undergo contraction by a selection for this subpopulation of cells, ultimately resulting in the exclusive production of mature sperm cells with alleles in the PM range (Reyniers et al., 1993). This strongly suggests that gene silencing drives selection against FM alleles during spermatogenesis. If correct, this would imply that there is a major difference in the induction of *FMR1* hypermethylation between male and female germ lines. Conversely, it has been shown in *FMR1* knockout mice that FMRP is dispensable for spermatogenesis (Bakker et al., 1994). In an FXS family with a large deletion that hampers *FMR1* transcription, the deletion did not hinder male fertility (Meijer et al., 1994), once again supporting the hypothesis that FMRP-deficiency by *FMR1* hypermethylation does not impede spermatogenesis.

The idea that *FMR1* is inactivated fairly late during embryogenesis is not well supported by the high rate of methylation mosaicism observed in FXS human embryonic stem cell lines (hESCs; Avitzour et al., 2014). hESCs are derived from the inner cell mass (ICM) of blastocyst stage embryos (7-days post fertilization). As such, they reflect the cells in the embryo at the time of implantation. In recent years, over a dozen FXS hESC lines have been established from mutant IVF embryos that were obtained from high risk couples undergoing preimplantation genetic diagnosis procedures for FXS (reviewed in Mor-Shaked and Eiges, 2016). Initially, when the first male hESC line was established, it was found to express *FMR1* at normal levels and to be completely unmethylated at *FMR1* (Eiges et al., 2007). However, as more FXS hESC lines became available, it turned out that *FMR1* hypermethylation is not restricted to somatic cells in patients, but can also be acquired in the undifferentiated state (Avitzour et al., 2014; Colak et al., 2014). In fact, of the FXS hESC lines examined so far, the majority present some levels of methylation (Avitzour et al., 2014), although no line was observed with completely methylated (100%) or entirely transcriptionally inactive *FMR1*. Nevertheless, the finding that most of these cell lines are, at least in part, already methylated raises doubts as to the actual timing of epigenetic gene silencing in FXS. Perhaps *FMR1* hypermethylation is established before/at the time of embryo implantation; i.e., the developmental stage when the stem cell lines are established. In addition, since methylation levels remain largely unchanged

over time in culture (more than 10 successive passages; Avitzour et al., 2014), unmethylated full expansions are most likely to arise from imperfect *de novo* methylation rather than from a failure to properly maintain aberrant methylation patterns. On the other hand, there is some evidence that when the size of the mutation drops below ~400 repeats but is still in the FM range (>200 repeats), methylation erodes (Zhou et al., 2016). This points to a threshold for the methylation of alleles bearing FM that may be higher than previously thought, and may change according to the type or differentiation state of the cell. To further corroborate these findings the threshold for methylation should be re-evaluated on a large sample of affected subjects. If correct, this will have major clinical implications for disease management and potential treatment. A higher threshold may also provide a plausible explanation for the lack of methylation acquisition in mice with more than 200 CGG repeats (Brouwer et al., 2007).

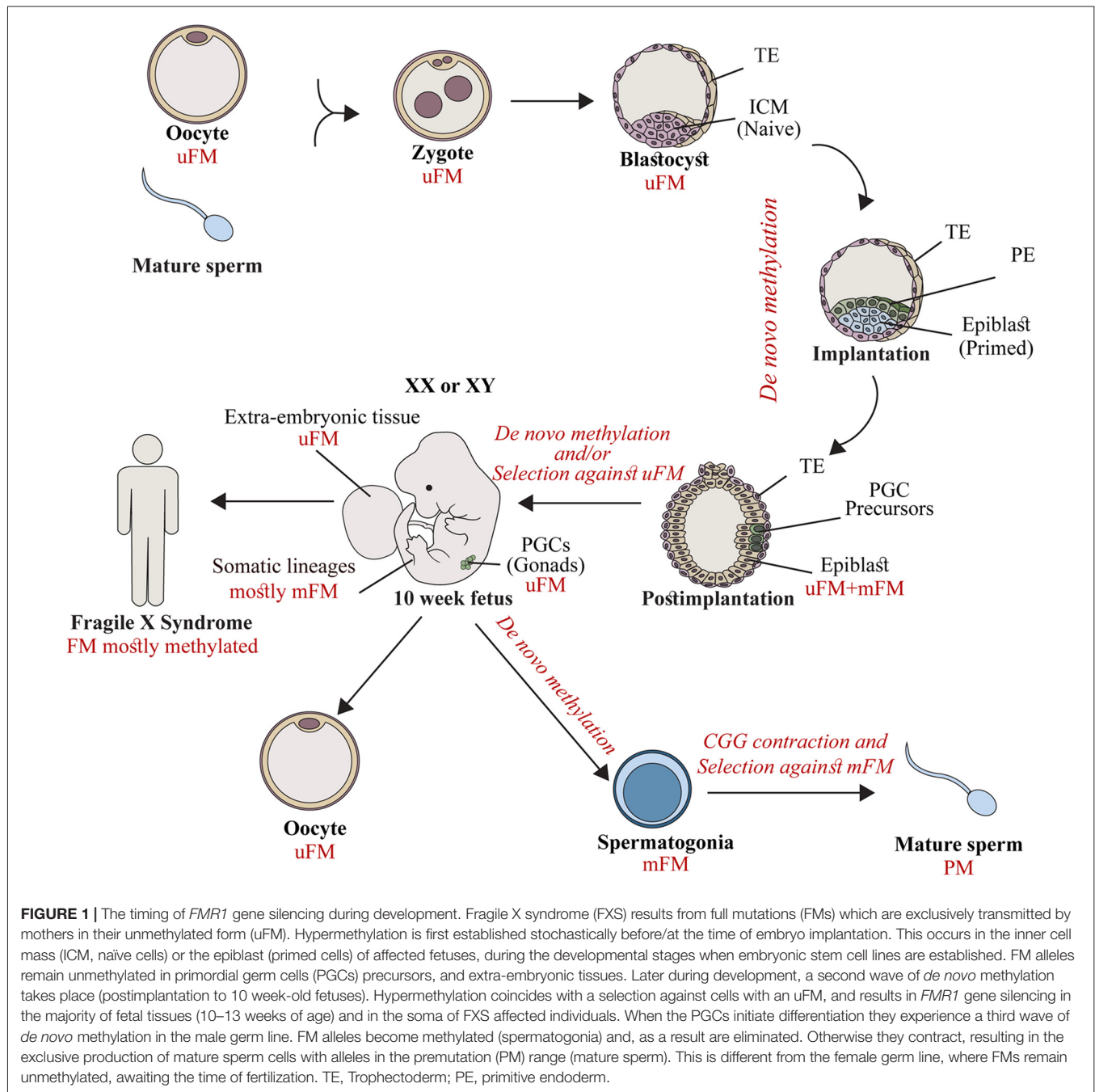
Interestingly, when examining female FXS hESC lines it was noted that like many other hESC lines, X-inactivation had already occurred in the majority of the cell lines, and was consistently skewed (Avitzour et al., 2014). Nevertheless, the nature of the skewing seemed to be unrelated to the activity of the gene. Whereas in some cell lines the maternal chromosome was inactivated, in others it was the paternal X. In addition, in certain cell lines where the normal X allele was inactivated, the repeat expansion was methylated whereas in others it was not. Therefore, and unlike in somatic cells of females with a FM (Salat et al., 2000), there appears to be no evidence for clonal selection of proliferating cells with FMRP expression in the undifferentiated state. This is in line with a previous report that selection for the unmethylated allele through skewed X-inactivation in FM carrier females is the most strongest between birth and puberty (Godler et al., 2013). The inverse correlation between age and the proportion of active X chromosomes harboring the FM in these females (Rousseau et al., 1991) further supports the undifferentiated state lack of clonal selection hypothesis.

Given that X-inactivation has already been induced in most female cell lines, hESCs may more closely resemble epiblast (primed ESCs) than the ICM cells (naïve ESCs) in the embryo. This is because X-inactivation does not initiate in the embryo until implantation, when the ICM converts into the epiblast. If so, these undifferentiated immortalized cell lines should reflect a less primitive ground state of pluripotency and exhibit higher methylation levels than originally assumed. In fact, in an earlier study researchers reported they had been able to re-activate the *FMR1* gene in FXS patients' cells with a hypermethylated full expansion by creating induced pluripotent stem cells (iPSCs) through transcription factor reprogramming (Gafni et al., 2013). By growing the cells in naïve supportive conditions they observed promoter CpG demethylation and upregulation of *FMR1* mRNA levels. However, under similar conditions other researchers failed to reverse/prevent *FMR1* hypermethylation in FXS iPSCs clones, respectively (de Esch et al., 2014). Careful examination of the parental cells in the earlier study indicated mosaicism for expansion size. Hence, it would be crucial to re-examine the size of the expansion following reprogramming before drawing any

conclusions. In addition, it would be equally important to explore the methylation status of the gene in FXS hESCs lines which were derived under naïve conditions from the very beginning. If *FMR1* is found to be consistently hypomethylated, the switch from naïve to primed hESCs, and vice versa, could provide a powerful tool to both trace and intervene in the process of *FMR1* epigenetic gene silencing by turning on and off the gene in a reversible fashion.

In any case, since the majority of FXS patients present heavily methylated expansions in their soma, it is expected that a second wave of *de novo* methylation will take place by cell differentiation.

However, reports on the effect of *in vitro* differentiation into neurons on the methylation status of the mutation are contradictory. One study reported epigenetic silencing achieved by a switch from active (H3K4me2) to repressive (H3K9me2) histone modifications by day 45 of neuronal differentiation (Colak et al., 2014), whereas in others neuronal differentiation of up to 90 days failed to trigger epigenetic silencing (Brykczynska et al., 2016; Zhou et al., 2016). In fact, it is becoming increasingly clear that the conversion of an active unmethylated FM allele into a silenced one appears to be conditioned on an increase in the length of the CGG tract rather than on the differentiation



status of the cell. This may imply that unmethylated FM are not subject to epigenetic silencing in a differentiation-dependent fashion as is commonly thought. Instead, there is some indication that *FMR1* hypermethylation may be gradually achieved through selection against unmethylated FM rather than by an active mechanism following cell specification (Zhou et al., 2016). In fact, in the earlier study on the induction of *FMR1* epigenetic silencing by neural differentiation (Colak et al., 2014), careful examination of the expansion by Southern blot analysis indicated certain levels of methylation on the FM alleles to begin with. Since unmethylated FM coincides with the formation of RNA foci in the nuclei of *in vitro* differentiated cells (Brykczynska et al., 2016), it would be useful to show that this subpopulation of cells is eventually eliminated in the embryo by an RNA gain-of-function mechanism. Indeed, there is increasing evidence that lengthy mRNAs are toxic to the cells and may be the leading cause of FXPOI (Elizur et al., 2014; Man et al., 2017) and FXTAS (Galloway and Nelson, 2009; Li and Jin, 2012) in PM carriers. It is expected that longer transcripts with greater number of repeats should have a more deleterious effect leading to cell death at a much earlier stage during development.

Importantly, contrary to original assumptions, hypermethylation of *FMR1* in FXS male patients is recurrently not complete. The degree of methylation can differ between or within different tissues in the same individual, giving rise to inter- or intra-tissue mosaicism (Dahl and Guldborg, 2007; Chen et al., 2011). These unmethylated alleles are transcriptionally active, and in many cases over-express *FMR1* (Tassone et al., 2001). Although partial methylation patterns in somatic cells of patients have been reported (Stöger et al., 1997; Dahl et al., 2007; Chen et al., 2011), the rate of such mosaicisms has not been extensively studied, and it is commonly believed to be infrequent. However, most of the methylation analyses performed on patients' cells, as well as the earlier studies with human fetal tissues and gametes were done using methylation-sensitive Southern blot assays. The limited sensitivity of Southern blot assays to accurately determine methylation levels near and at the CGG repeats is most likely the cause of the underestimated frequency of methylation mosaics in FXS patients and fetuses. Based on the analysis of 20 FXS patients using a PCR-based method (methylation-specific melting curve analysis), Dahl and Guldborg (2007) reported that 20% of the patients were actually methylation mosaics. Published and unpublished data suggest that this is actually a much more widespread event. The high rates of methylation mosaics are in line with the fact that the majority of the patients (60%) express significant levels of *FMR1* mRNA although none of them carry any PM alleles (Tassone et al., 2001). This is consistent with the heterogeneous levels of aberrant methylation observed in FXS hESC lines (Avitzour et al., 2014), and corresponds to the inability of FM to actively acquire hyper-methylation following cell differentiation *in vitro* (Brykczynska et al., 2016; Zhou et al., 2016).

To account for the data related to: (1) extensive *FMR1* methylation in fetal tissues by the end of the first trimester; (2) the high rates of methylation mosaics among male patients; and

(3) the wide variability in the epigenetic status of the expanded gene between and within the currently available FXS XY hESC lines, we proposed a temporal model of *FMR1* hypermethylation (Figure 1) which suggests that abnormal methylation is first acquired stochastically on full expansions during a restricted point in time before or during embryo implantation. Once established, it is clonally maintained. Expansions which escape abnormal methylation during this limited time frame remain unmethylated, and most likely are eliminated by a toxic RNA gain-of-function mechanism or are actively methylated as an outcome of cell differentiation. It would be extremely useful to explore whether FXS pre-implantation embryos are already *FMR1* methylated. If so, it would be just as important to determine whether methylation is uniformly induced in all cells of the embryo before the stage of stem cell line derivation. In addition, it would be key to extend the analysis to a greater number of oocytes by looking at cumulus-stripped mature eggs using more delicate methylation-sensitive assays. This would further strengthen the widely held assumption that methylation is beyond doubt a post-zygotic event. Alternatively, methylation may be established in the female germ line and transmitted to the fetus as an integral part of the mutation, but not properly maintained in the early embryo while the overall levels of the DNMT1 (maintenance methylase) are generally low.

There are a number of other avenues for further exploration in the area of epigenetic gene silencing of *FMR1*. It would be worthwhile exploring whether hypermethylation is induced by the conversion of mutation-bearing cells from the naïve to the primed pluripotent state. It would also be interesting to explore whether a second wave of *de novo* methylation occurs as a result of cell differentiation, or is a consequence of negative selection of cells bearing unmethylated FM over methylated FM alleles. Finally, it would be prudent to resolve the question of whether methylation plays a role in restricting repeat instability, and if so how the timing of *de novo* methylation impacts expansion size in the fetus as well as gamete precursors.

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HM-S and RE contributed to the conception, design and writing of this manuscript.

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Of Men and Mice: Modeling the Fragile X Syndrome

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The Fragile X Syndrome (FXS) is one of the most common forms of inherited intellectual disability in all human societies. Caused by the transcriptional silencing of a single gene, the fragile x mental retardation gene *FMR1*, FXS is characterized by a variety of symptoms, which range from mental disabilities to autism and epilepsy. More than 20 years ago, a first animal model was described, the *Fmr1* knock-out mouse. Several other models have been developed since then, including conditional knock-out mice, knock-out rats, a zebrafish and a drosophila model. Using these model systems, various targets for potential pharmaceutical treatments have been identified and many treatments have been shown to be efficient in preclinical studies. However, all attempts to turn these findings into a therapy for patients have failed thus far. In this review, I will discuss underlying difficulties and address potential alternatives for our future research.

Keywords: Fragile X Syndrome, mouse model, *FMR1*, microsatellite instability, E/I balance, behavior and cognition, primates, autism spectrum disorders

INTRODUCTION

The tremendous advance that has taken place in life sciences during the last decades has opened a variety of options and opportunities for research as well as for human societies, in particular in the field of genetics. One of these advances was the invention of the Crispr-Cas system (Crispr; reviewed in Donohoue et al., 2017; Huang et al., 2017; Petersen, 2017). The technique allows for a fast and relatively precise gene editing in a variety of different organisms ranging from plants and insects to vertebrates and primates including human cell lines and embryos. Being relatively efficient and easy to use, the system promises much progress not only for our understanding of complex biological systems, but also for the treatment of genetic disorders.

Of foremost interest in this context are monogenetic diseases with limited treatment options for patients, such as the Fragile X Syndrome (FXS), a nonetheless strikingly complex autism spectrum disorder (ASD). Combined with the advance of genetic screening methods for persons at risk (reviewed in Rajan-Babu and Chong, 2016) and assisted reproduction services, the Crispr technique opens not only new horizons, but also raises many ethical concerns, although there is no common agreement on ethical standards among mankind and not all people are sharing the concerns.

For our future research, it will therefore be important to critically evaluate what we are able to achieve, what we have achieved, and, on a society based level, what we do want to achieve. This article will review and discuss important results as well as ideas from the FXS field, and in particular address underlying difficulties arising from the current mouse models.

THE FRAGILE X SYNDROME — OF MEN

Phenotype

Affecting approximately 1 in 7000 males and 11,000 females (meta-analysis: Hunter et al., 2014), FXS represents one of the most frequent forms of monogenetically determined mental retardation in all human populations and ethnic groups (reviewed in Tzschach and Ropers, 2007). In the vast majority of cases, the disease is caused by the transcriptional silencing of a single gene on the X chromosome, the Fragile X Mental Retardation gene *FMR1*. In consequence, expression of the encoded protein FMRP is lost (reviewed in Saldarriaga et al., 2014; Usdin and Kumari, 2015).

FXS patients display a variety of intellectual deficits ranging from mild learning impairments to severe cognitive disabilities, but also autistic behaviors such as aggression, social anxiety and stereotypic acting characterize the disease (reviewed in Saldarriaga et al., 2014; Gross et al., 2015b; **Figure 1**). Men are in general more severely affected than women, achieving only average IQs¹ of 40–50, whereas women mainly present with mild to moderate cognitive impairments and an average IQ of about 80, though their abilities may range from severe deficits to superior performances (Freund et al., 1993; de Vries et al., 1996; Lewis et al., 2006; Chaste et al., 2012, reviewed in Huddleston et al., 2014). However, even men can be high functioning (Basuta et al., 2015).

Shyness, poor eye contact and attention difficulties are particularly characteristic to young women with FXS (*FMR1*^{-/-}), whereas increased aggression and the use of rote phrases are more typical to men (Murphy and Abbeduto, 2007; Hartley et al., 2011). In their adult life, women are mostly affected by deficits in interpersonal skills, while weak functional skills primarily concern men, though reduced social interaction skills are also prominent in men (Hartley et al., 2011). As a result, only 9% of the men affected by FXS achieve a high or very high level of independence in adult life, whereas 44% of the FXS women reach such a level. Contrary to most other X-linked diseases, approximately 35% of the women carrying a single mutated allele only (*FMR1*^{-/+}) also demonstrate cognitive disabilities (Hagerman et al., 1992).

The impairments observed in FXS patients are not uniform though, but rather specific to certain capabilities: several studies demonstrated that FXS patients perform particularly weak in tasks requiring abstract item reasoning, attention, the solution of new problems and goal-directed actions, as well as in tasks relying on short-term memory and visual-motor coordination. By contrast, FXS patients usually demonstrate normal skills in vocabulary knowledge, although they show a cluttered and less complex speech (Hanson et al., 1986; Dykens et al., 1987; Maes et al., 1994; Loesch et al., 2004; Lewis et al., 2006; Roberts et al., 2007; Van der Molen et al., 2010).

Recent research has linked some of the variability observed in the cognitive phenotype of FXS patients to residual FMRP expression and mosaic expression patterns (Kaufmann et al., 1999; Dyer-Friedman et al., 2002; Loesch et al., 2003, 2004; Pretto

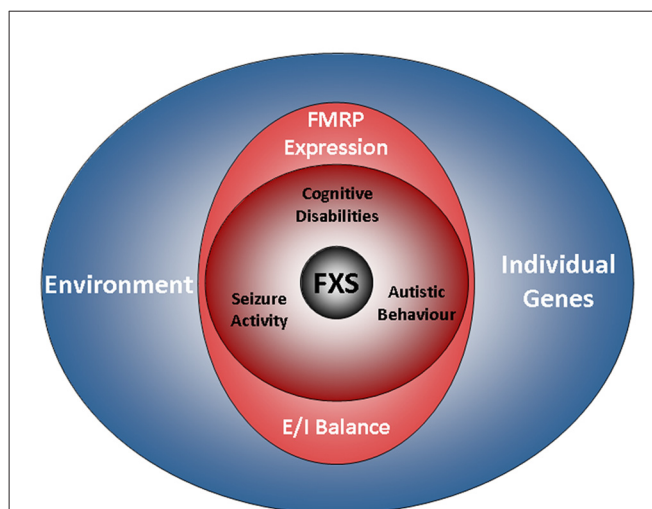


FIGURE 1 | The FXS world. The drawing illustrates factors influencing the disease. A characteristic of FXS is the presence of a broad range of deficits with a high degree of individual variation. The phenotype of the disorder includes cognitive disabilities as well as autistic behaviors and epilepsy. Aside from a loss of FMRP expression, the genetic background and environmental factors are emerging as determinants of the disease. However, while residual FMRP expression is known to correlate with the cognitive performance of FXS patients, the impact of individual genes and the relevance of the environment are less well understood (also see the section “Phenotype”). Recent findings indicate that autistic behaviors and epilepsy are influenced by the E/I balance, but not by residual FMRP expression. FXS, Fragile X Syndrome; FMRP, Fragile X Mental Retardation Protein; E/I balance, balance of excitation and inhibition in neuronal networks.

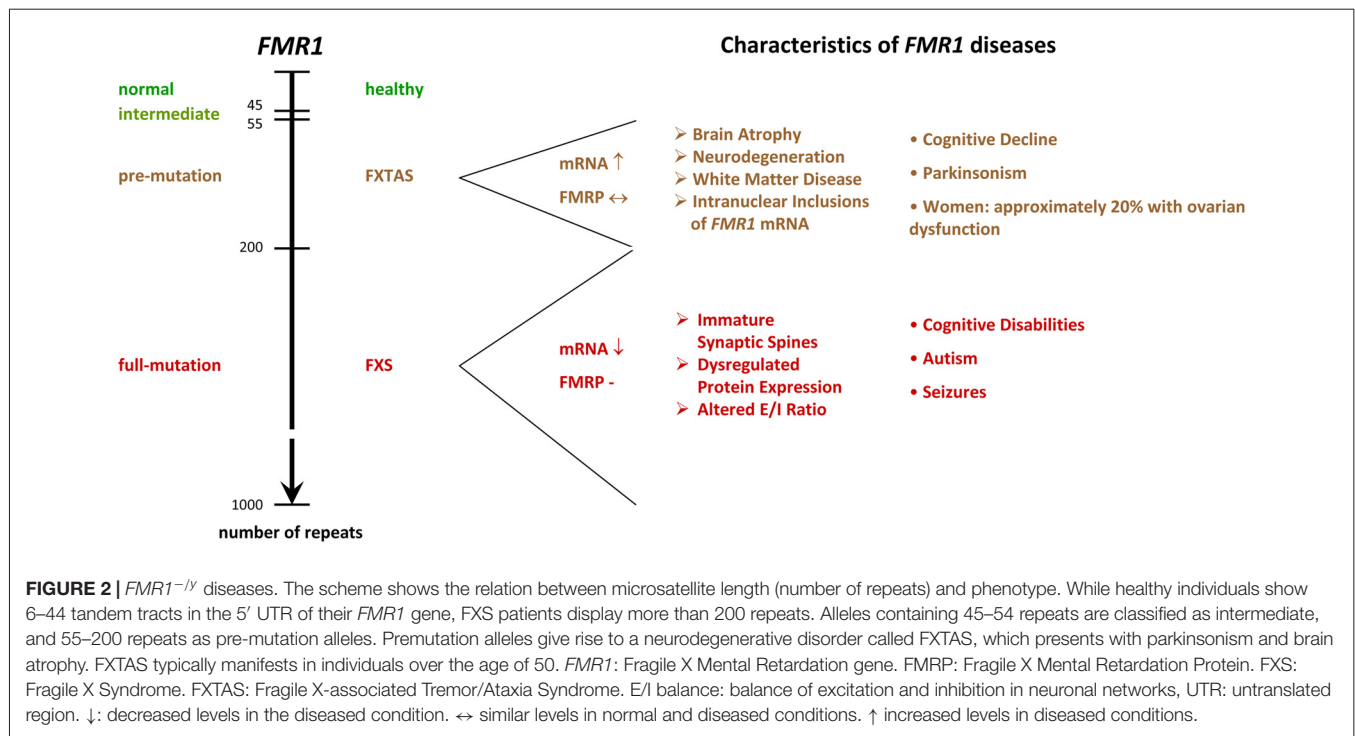
et al., 2014; Basuta et al., 2015). For instance, the cognitive abilities of FXS patients were shown to strongly correlate with FMRP expression levels, even when full-scale IQ scores are used. Although this holds true for most of the cases, exceptions exist: Govaerts and colleagues reported a case, in which residual FMRP expression could not explain the good cognitive performance observed (Govaerts et al., 2007), thus implying a role for individual genetic factors and/or environmental effects (cp.² **Figure 1**).

Further studies indeed support the significance of environmental factors in FXS (Dyer-Friedman et al., 2002; Kuo et al., 2002; Glaser et al., 2003): in particular maternal warmth and responsivity were demonstrated to ameliorate maladaptive as well as autistic behaviors, whereas maternal depression and criticism were indicated to increase FXS symptoms in children (Greenberg et al., 2012; Robinson et al., 2016; Smith et al., 2016). Contrary to cognitive deficits, autistic behaviors display no correlation with residual FMRP expression (Glaser et al., 2003; Pretto et al., 2014).

Particularly in children, seizures are frequent as well, affecting about 45% of the adolescent patients between 1 year and 14 years of age (Cowley et al., 2016; **Figure 1**). After the age of 20, seizure activity decreases, resulting in an overall prevalence of about 24% (Sabaratnam et al., 2001), although there is considerable variation among studies. Interestingly, some data suggest that the attention

¹intelligence quotient scores

²compare



deficits observed in FXS patients are related to seizure activity (Cowley et al., 2016).

Just like autistic behaviors, seizure activity was found not to correlate with residual FMRP expression (Pretto et al., 2014). Although an imbalance of excitatory and inhibitory neuronal activity has been associated with seizure activity and autistic behaviors in several ASDs as well as in corresponding animal models (reviewed in Frye et al., 2016; Uzunova et al., 2016; Lee et al., 2017), data on FXS patients are rare. Using EEG³ electrodes, increases in event related potentials were found in the auditory cortex of FXS children, suggesting enhanced excitability (Castrén et al., 2003). EEG-studies of oscillatory dynamics in males with FXS identified impaired theta oscillations indicative of an imbalance in excitatory and inhibitory neuronal circuit activity (Van der Molen and Van der Molen, 2013; Van der Molen et al., 2014) and implied a lack of coordination in information processing. Notably, two studies also found decreased activations, one in prefrontal regions and one in the fusiform gyrus (Dalton et al., 2008; Holsen et al., 2008). These findings suggest that FXS patients experience brain-region and most likely circuit-specific imbalances in neuronal excitation.

Genotype

Microsatellites — Sources of Complexity

The molecular mechanisms leading to the silencing of the *FMR1* gene during embryonic development are complex and result from expansions in the length of a microsatellite located in

the 5'UTR⁴ of *FMR1* (Fu et al., 1991; Pieretti et al., 1991; Verkerk et al., 1991; Eiges et al., 2007; Bar-Nur et al., 2012). In healthy individuals, the sequence consists of CGG/CCG tandem tracts and includes approximately 6–44 repeats, whereas FXS patients show more than 200 repeats. *FMR1* alleles containing 45–54 repeats are classified as intermediate, and 55–200 repeats as pre-mutation alleles (Figure 2). Contrary to regular tandem tracts, pre-mutation alleles are meiotically as well as mitotically unstable and may turn into full-mutation alleles within one generation, if transmitted by a female (Fu et al., 1991; Heitz et al., 1992; Yu et al., 1992).

Although microsatellites are often associated with diseases (reviewed in Nelson et al., 2013; Zhang and Ashizawa, 2017), they turned out to play crucial roles in many species, but in particular in humans. Due to their high variability, tandem tracts are thought to serve as a substrate for evolution (reviewed in Kashi and King, 2006; Hannan, 2012; Plohl et al., 2012). Most microsatellites are nonetheless only maintained by chance and total microsatellite numbers are rather species or clade specific than related to vertebrate evolution (Buschiazzi and Gemmell, 2010; Adams et al., 2016). Yet, tract length polymorphism turned out to be a major source for the emergence of variability and complexity in species: recent research revealed that tandem tracts located in regulatory regions contribute to the genesis of complexity (Liu H. et al., 2012; Namdar-Aligoodarzi et al., 2015). By triggering the formation of secondary structures in DNA as well as RNA molecules in a length dependent manner, these repeats redress the transcription and translation efficiency and, in doing so, control the expression level of

³Electroencephalography

⁴untranslated region

their proteins (reviewed in Kashi and King, 2006; Pezer et al., 2012; Sawaya et al., 2012; also see e.g., Zumwalt et al., 2007; Halder et al., 2009; Vines et al., 2009; Quilez et al., 2016). In consequence, the protein stoichiometry is altered, leading to modifications in a variety of protein interactions, while the functionality of the protein itself is preserved. It is therefore no surprise that tandem tracts are preferentially located in the proximity of transcription starts (Vines et al., 2009; Sawaya et al., 2013; Liang K. C. et al., 2015), that human promoter regions are particularly rich in microsatellites (Sawaya et al., 2013) and that the polymorphism and complexity of tandem tracts significantly increase in primates and humans (Zhang et al., 2004; Mohammadparast et al., 2014; Sabino et al., 2014; Bilgin Sonay et al., 2015; Namdar-Aligoodarzi et al., 2015; Ohadi et al., 2015; Rezazadeh et al., 2015).

Moreover, tandem tract polymorphism turned out to be astonishingly abundant in genes involved in the development of the nervous system (Riley and Krieger, 2009). Indeed, some studies even associated repeat variations with individual differences in behavioral traits—not only in humans (Gerra et al., 2005; Larsen et al., 2010; Simmons and Roney, 2011; Berry et al., 2013; Durdiaková et al., 2013; Valomon et al., 2014; Votinov et al., 2015), but also in birds (Stuber et al., 2016) and other mammals (Hammock and Young, 2005; Lucarelli et al., 2017). In addition, Bagshaw and colleagues showed that polymorphic microsatellites of genes involved in human personality traits and social behavior are able to integrate interactions with the environment, in this case maternal smoking, which caused anti-social acts in carriers of certain TBR1 alleles (Bagshaw et al., 2017). Their finding is in line with an earlier study showing that a CGG repeat variant of the glutathion peroxidase 1 gene is protective for autism (Ming et al., 2010). Indeed, it has been found that prenatal oxidative stress, such as caused by environmental toxicants, is involved in the establishment of autism, in particular when occurring in certain sensitive stages of embryogenesis (reviewed in Chauhan and Chauhan, 2006; Landrigan, 2010; Wells et al., 2016; Heyer and Meredith, 2017).

These findings illustrate that microsatellite polymorphism is an important component of individuality, complexity, neuronal development and gene-environment interactions. Since FMRP itself is developmentally regulated (Hinds et al., 1993) and was found to function in neuronal migration, differentiation and dendritic spine maturation (Hinton et al., 1991; Irwin et al., 2001; Saffary and Xie, 2011; Telias et al., 2013; La Fata et al., 2014; Khalfallah et al., 2017), it seems therefore possible that the microsatellite of *FMR1* might have currently unrecognized functions in the individual peculiarities characteristic to FXS.

To Silence or Not to Silence Expanded FMR1?

Transcript toxicity in humans

Mirroring the results on microsatellites and protein expression, the different *FMR1* alleles indeed give rise to different expression patterns: pre-mutation carriers are characterized by enhanced mRNA, but normal or slightly reduced protein levels, since the elongated transcripts are inefficiently translated,

but heavily transcribed (Tassone et al., 2000a,b; Kenneson et al., 2001; Primerano et al., 2002; Ludwig et al., 2014), whereas full-mutations cause FMRP deficiency due to DNA hypermethylation, Histone modification and subsequent heterochromatin formation (Pieretti et al., 1991; Sutcliffe et al., 1992; Hornstra et al., 1993; Coffee et al., 1999, 2002; Kumari and Usdin, 2010). Some residual mRNA is nonetheless still present in many men with FXS, but the mRNA is not translated (Tassone et al., 2001), probably due to secondary structure formation in the tandem tract.

It is noteworthy that pre-mutation carriers often develop a neurodegenerative disorder called the Fragile X-associated Tremor/Ataxia Syndrome (FXTAS, **Figure 2**), which presents with neurodegeneration, parkinsonism and brain atrophy, and which is associated with primary ovarian insufficiency in females (reviewed in Botta-Orfila et al., 2016; Hagerman and Hagerman, 2016). FXTAS is believed to arise from a toxicity of elongated mRNA transcripts and/or of a cryptic *FMR1* protein derived from CGG repeat triggered non-ATG translation (Handa et al., 2005; Hashem et al., 2009; Chen et al., 2010; Todd et al., 2013). Since the transcript levels are markedly reduced in FXS patients, these findings suggest that the silencing of full-mutation alleles in FXS serves to prevent from toxic effects, however, clear evidence for a toxicity of the full-mutation mRNA or the cryptic protein is missing. Indeed, the identification of several healthy and non-mosaic individuals carrying unmethylated, normally expressing full-mutation alleles (Smeets et al., 1995; Pietrobono et al., 2005; Tabolacci et al., 2008) argues against the idea of mutation-triggered toxicity in humans. Nonetheless, two cases were identified, where expression of a full-mutation gene caused severe FXTAS (Loesch et al., 2012; Santa Maria et al., 2014), thus supporting the idea that a fully mutated *FMR1* transcript can have toxic effects and that elongated transcripts are causative for FXTAS, but not for FXS, although some mRNA is present in many FXS patients (Tassone et al., 2001).

These apparently conflicting cases illustrate that individual genes and/or environmental effects may overcome the typical mechanisms and phenotypes observed in FXS. The relevance of the latter is further emphasized by the fact that alcohol abuse seemed to be involved in the case of severe FXTAS reported by Loesch and colleagues (Loesch et al., 2012). Recent studies revealed that alcohol is in fact exaggerating behavioral problems such as aggression and impulsivity in FXS patients (Salcedo-Arellano et al., 2016) and accelerating neurological deterioration in FXTAS (Muzar et al., 2014). It might be for these negative effects that, in contrast to cases of high-functioning autism, where patients used drinking to cope with social anxiety (Lalanne et al., 2015), alcoholism is at least in FXS patients rare (cp. Salcedo-Arellano et al., 2016).

For our future research, it will therefore be important to address questions such as: Why may fully mutated *FMR1* transcripts have toxic effects in one case, but not in the other? How does the environment influence the underlying mechanisms? Why may it be better to have a fully mutated gene silenced, and thus FXS, than unsilenced, and eventually FXTAS — from an evolutionary point of view? A deeper

understanding of the relevance of genetic individuality is required before attempting a reactivation of *FMRI* in patients (reviewed in Tabolacci et al., 2016b) — in particular, since full-mutation mRNA is not necessarily translated (Tassone et al., 2001; Dolskiy et al., 2017) and since transcribed alleles are able to cause FXTAS under circumstances currently unknown (Loesch et al., 2012; Santa Maria et al., 2014).

A question of secondary structures?

An alternative explanation for the silencing of full-mutation alleles is DNA stability. During germ cell generation as well as during early phases of embryogenesis and prior to the silencing of *FMRI*, CGG repeats may expand or contract through mechanisms under debate (reviewed in Mor-Shaked and Eiges, 2016; Gerhardt, 2017). Despite some uncertainty about the exact molecular events that cause repeat instability, it is believed that the formation of secondary DNA structures during recombination, DNA replication and DNA repair leads to the addition or deletion of repeats. Since no repeat instability has been observed in *FMRI* postnatally (Reyniers et al., 1993, 1999; Wöhrle et al., 1993), it is thought that the instability is related to events of the embryogenesis.

Little is known about the factors that could contribute to the instability of the CGG repeats in *FMRI*. It has been noticed that the number of repeats, the content of interspersed AGG and the haplotype are able to influence the stability of *FMRI* (Oberlé et al., 1991; Eichler et al., 1994; Gunter et al., 1998; Hirst and White, 1998; Taylor et al., 1999; Larsen et al., 2000; Dombrowski et al., 2002; Nolin et al., 2003, 2013, 2015; Yrigollen et al., 2012, 2013, 2014; Avitzour et al., 2014; Weiss et al., 2014). AGG interruptions, for instance, have been indicated to support the stability of *FMRI* by reducing secondary structure formation (Weisman-Shomer et al., 2000; Jarem et al., 2010) and promoting appropriate DNA conformations (Jarem et al., 2010) as well as adequate DNA packing (Mulvihill et al., 2005; Volle and Delaney, 2013). Furthermore, the number of repeats was shown to directly correlate with the instability of the tandem tracts (Oberlé et al., 1991; Eichler et al., 1994; Taylor et al., 1999; Avitzour et al., 2014); it is assumed that the size of G-rich tracts directly correlates with the formation of secondary structures and polymerase slippage during replication (Mornet et al., 1996; Freudenreich et al., 1997; Weitzmann et al., 1997; Hirst and White, 1998). In line with this idea, the total length of the CGG repeat allele turned out to be the best predictor for the risk of transmission (Yrigollen et al., 2014).

Aside from internal genetic properties, trans-acting factors have also been postulated to affect the stability of *FMRI* during mitosis and meiosis (Mornet et al., 1996; Nolin et al., 1996, 1999), but although it seems plausible that other genes might impact on the stability of *FMRI*, studies are rare. In an attempt to identify such trans-acting factors, Xu et al. (2013) analyzed two microarray sets containing data on the transcript expression in FXS patients and controls, and found a significant down-regulation of DNA damage/repair pathway transcripts, thus implying that impaired DNA repair pathways may support *FMRI* instability in FXS patients.

Remarkably, environmental factors have also been found to influence the instability of *FMRI*: maternal age was recently

related to increased instability (Yrigollen et al., 2014) and oxidative stress was demonstrated to interfere with the stability of *FMRI* (Adihe Lokanga et al., 2014). The latter finding is in line with other studies showing that different kinds of stress can induce instability in microsatellites (Chatterjee et al., 2015; Wu et al., 2017) and that oxidized DNA can trigger repeat expansion or contraction (Lai et al., 2013; Cilli et al., 2016). Given that chronic alcoholism causes oxidative stress (reviewed in Wu et al., 2006; Hernández et al., 2016), the data imply that alcohol abuse could contribute to the consolidation of *FMRI* diseases. Interestingly, a study conducted by Kogan and colleagues found alcoholism to be significantly more common in families of pre-mutation carriers than in control families (Kogan et al., 2008). Although this association does not tell whether the disease (FXTAS) is causing the alcoholism, for example by overcharging family members who care for their affected relatives, or whether the alcoholism is causing the disease (or both), the facts that maternal alcoholism seems to increase the risk for FXS (mentioned in Hagerman et al., 2010) and that fetuses are particularly vulnerable to alcohol (reviewed in Henderson et al., 1999; Dennerly, 2007) support a role for alcohol abuse in the emergence of FXS. Much more research is needed to establish the relation of toxins, such as derived from smoking or drinking, oxidative stress, microsatellite instability and the consolidation of *FMRI* diseases.

Repeat numbers exceeding 200 tandems trigger the epigenetic silencing of *FMRI* by initiating an abnormal 5'-C-phosphate-G-3' methylation and repressive Histone modifications in the promoter region (e.g., Coffee et al., 1999, 2002; Chandler et al., 2003; Kumari and Usdin, 2010; Brasa et al., 2016). As a consequence, CpG⁵ islands flanking the repeats as well as the repeats itself, which also function as a CpG island, become hypermethylated and render the gene inactive (Hansen et al., 1992). Studies in human FXS cell lines aiming to reactivate *FMRI* by either changing repressive Histone modifications (Kumari and Usdin, 2016; Dolskiy et al., 2017) or decreasing CpG methylation (Chiurazzi et al., 1998; Coffee et al., 2002; Tabolacci et al., 2005, 2016a), suggest that DNA methylation is the primary cause for gene inactivity, while repressive Histone methylations have a supportive function.

The molecular mechanisms by which CGG expansions trigger the epigenetic silencing of *FMRI* are currently not well understood though (for a review, please see Usdin and Kumari, 2015). It is thought that transient unpairing of the DNA during replication, transcription or repair provides an opportunity for the repeat region of *FMRI* to form secondary structures such as hairpins or G-quadruplexes (Fry and Loeb, 1994; Kettani et al., 1995; Mitas et al., 1995; Usdin and Woodford, 1995; Patel et al., 2000; Loomis et al., 2014). Similar structures occur in *FMRI* transcripts (Handa et al., 2003; Napierala et al., 2005; Zumwalt et al., 2007; Malgowska et al., 2014). Studies showed that these secondary structures hinder replication, transcription and translation (Fry and Loeb, 1994; Nadel et al., 1995; Usdin and Woodford, 1995; Subramanian et al., 1996) and that structure-

⁵5'-C-phosphate-G-3'

disrupting proteins are able to alleviate the situation (Fukuda et al., 2005; Khateb et al., 2007), thus suggesting that the formation of secondary structures is troubling the cells.

Not all structures are alike though: R-loops, a DNA-RNA hybrid formed during transcription, were recently indicated to prevent gene silencing by protecting DNA from *de novo* methylation (Ginno et al., 2012). Moreover, R-loops were shown to cause chromosome decondensation and transcription activation (Powell et al., 2013). Since *FMRI* has been observed to give rise to R-loops (Groh et al., 2014; Loomis et al., 2014), this data could nicely explain the enhanced expression of pre-mutation alleles observed in FXTAS, but experimental evidence is missing. In fact, Groh and colleagues found that the formation of R-loops on *FMRI* impedes gene expression (Groh et al., 2014), thus suggesting that R-loop formation is involved in the silencing of *FMRI*. Their data are in line with another study demonstrating that promoter-bound *FMRI* transcripts containing tandem tracts induce the silencing of *FMRI* (Colak et al., 2014).

The relevance of the number of repeats required to build R-loops and the role of the respective loop size are not yet clear though, since R-loops were found to form on normal, pre-mutation and full-mutation alleles (Colak et al., 2014; Groh et al., 2014; Loomis et al., 2014). It seems therefore possible that the role of R-loops in transcription regulation depends on the loop size: Loomis and colleagues provided evidence that the expansion of *FMRI* repeats causes an enhanced formation of aggrandized loops, which tend to form higher-order structures. These structures distinguish *FMRI* from other CpG islands containing promoters (Ginno et al., 2012, 2013) and could finally trigger repeat instability and hypermethylation (Loomis et al., 2014). Hence, R-loops formed on *FMRI* could enhance gene expression until they exceed a specific size and form secondary structures. Previous research on the methylation of different loci indeed indicated that it is the higher-order structures that trigger DNA methylation (Smrzka et al., 1995; Paoloni-Giacobino et al., 2007; Gentry and Meyer, 2013).

These findings imply that CpG methylation could serve to limit the formation of secondary structures. Nuclear magnetic resonance analyses revealed CpG methylations to decrease the dynamics of the DNA backbone (Geahigan et al., 2000), while molecular dynamic investigations illustrated DNA methylation to increase the rigidity of the DNA by steric hinderance and hydrophobicity (Derreumaux et al., 2001). Using density functional theory and nuclear magnetic resonance measurements, Taqi and colleagues further demonstrated that cytosine methylation impairs the conformational flexibility of short ssDNA⁶ molecules and their ability to form secondary structures (Taqi et al., 2012). Although these data support the idea that hypermethylation of *FMRI* could serve to prevent the formation of secondary structures, more evidence is required.

Further studies showed that the hypermethylation of *FMRI* indeed correlates with enhanced tract stability during mitosis (Gläser et al., 1999; Wöhrle et al., 2001; Nichol Edamura et al., 2005). Remarkably, Zhou and colleagues observed that cells

carrying fully mutated and hypermethylated alleles outcompete those carrying alleles with less repeats and no methylation when co-cultured, resulting in a loss of these cells (Zhou et al., 2016). Since no differences were seen in the viability of both cell lines, toxic effects are unlikely to account for the disappearing of cells with unsilenced alleles. The reasons for this effect remain to be investigated though.

The data imply that hypermethylation of *FMRI* should occur when cells start to divide a lot. Studies using fetal tissues showed that hypermethylation is established between the 10th and 12th week of gestation, but *FMRI* may remain partly active for some time (Devys et al., 1992; Sutcliffe et al., 1992; Suzumori et al., 1993; Iida et al., 1994; Willemsen et al., 2002; reviewed in Mor-Shaked and Eiges, 2018). Models of cell division in human embryos do not support high rates of mitosis during this time though, they rather show a decline in division rates (Luecke et al., 1999). Looking at the brain in specific, the situation is yet different: By the 9th week of gestation, neuronal tube formation is completed and shortly after, at the 12th week of gestation, neurogenesis as well as neuronal migration will reach their first peak (reviewed in Linderkamp et al., 2009). Nerve cells will then be proliferating at rates of about 15 million per hour (reviewed in Ackerman, 1992), thus implying that the silencing of *FMRI* could be related to cell line-specific changes serving in neurogenesis. Such a mechanism could be important to maintain the correct timing and pace during neuronal development, which is essential to establish the complex connections that characterize the brain.

Indeed, when Khalfallah and colleagues induced differentiation in a murine embryonic stem cell line lacking FMRP (sh*Fmr1* ES), they found an accelerated generation of both, progenitor and neuronal cells during the first steps of neurogenesis (Khalfallah et al., 2017; reviewed in Bardoni et al., 2017; Westmark, 2017). Their experiments further revealed that this phenotype is due to enhanced expression of a target of FMRP, APP⁷, which is able to accelerate neurogenesis following cleavage into the A-beta peptide. This mechanism might also provide an alternative or additional explanation for the observation that embryonic stem cells carrying a hypermethylated *FMRI* gene outcompete cells with the active gene as it was found by Zhou and colleagues (Zhou et al., 2016). It will be interesting to see how these changes affect neuronal maturation and signaling and how the findings of Khalfallah and colleagues relate to neuronal development in humans.

Notably, studies employing human embryonic stem cells (hESCs) showed that the epigenetic silencing of *FMRI* may occur prior to differentiation (Avitzour et al., 2014; Zhou et al., 2016) or upon differentiation (Eiges et al., 2007): of 11 hESC lines, 7 showed some levels of stable hypermethylation prior to differentiation. It is not yet clear though, when and how the hypermethylation is established. Current models suggest that fully expanded genes first acquire abnormal methylation patterns before or during embryo implantation and that *FMRI* silencing is achieved after the blastocyst stage. Microsatellites that escape the initial methylation changes are believed to remain unmethylated. Since standard reprogramming procedures serving to generate

⁶single-stranded DNA

⁷Amyloid-Precursor-Protein

induced pluripotent stem cells (iPSCs) from skin fibroblasts are unable to remove the exaggerated methylation marks inactivating *FMR1* (Urbach et al., 2010; Sheridan et al., 2011; Doers et al., 2014), hypermethylation is thought to be stable and irreversible once it is established.

This is not necessarily the case though: removing CGG repeats and the immediate 5'-flanking region from fully mutated microsatellites in the *FMR1* gene of a male iPSC line by the CRISPR/Cas9 system, Park and colleagues were able to restore *FMR1* gene expression as well as FMRP protein levels in a pair of clones (Park et al., 2015). The reactivation of *FMR1* led to a stable expression of the gene throughout differentiation into mature neurons. These findings are further supported by the previously mentioned study of Zhou and colleagues, who showed that hypermethylation present in embryonic stem cells is dynamic (Zhou et al., 2016): alleles containing over 400 repeats may contract to smaller repeat numbers, resulting in a permanent reactivation even when more than 200 tandem repeats are present. Moreover, de Esch and colleagues (de Esch et al., 2014) observed that the reprogramming of fibroblasts from an atypical individual carrying an active full mutation *FMR1* gene with 330 repeats into iPSCs recurrently resulted in a complete inactivation of the gene. Taken together, these studies not only illustrate the dynamics of the system, but also suggest that repeat length is not the only factor influencing the silencing of *FMR1* and that other factors, such as neurodevelopmental stage are involved. Indeed, the fact that the brother of the atypical individual also carried an unsilenced full-mutation implies that maternal-parental components, which were not present in fibroblasts or inactivated during the reprogramming procedure, and/or specific environmental factors caused their *FMR1* genes to remain active despite the high repeat length.

Aside from tandem repeat polymorphism, mutations in the coding region of *FMR1* have also been associated with the occurrence of FXS in patients (Quartier et al., 2017): a deletion of the last exon, which is giving rise to a truncated FMRP isoform, was recently identified in three brothers meeting FXS criteria (Hagerman's scores = 15). Moreover, two splice variants were detected in two unrelated patients showing the same outcome in the test, and several missense mutations have been identified in FXS patients (Siomi et al., 1994; Handt et al., 2014; Myrick et al., 2014). Although these cases represent rare exemptions, they nonetheless demonstrate that the correct functioning of FMRP is central to FXS.

THE FRAGILE X SYNDROME — OF MICE

The Genotype of Mice or the Charm of Simplicity

Mice are much different from humans, they are much smaller, live much shorter and have much less to learn. Nonetheless, mice and men are sharing almost 99% of their genes (Waterston et al., 2002) as well as most physiological functions and pathogenic mechanisms (see for example the reviews of Tecott, 2003; Eleftheriou and Yang, 2011; Van der Weyden and Adams, 2013; Jacobs et al., 2013; Eilam, 2014;

Hoehndorf et al., 2014; Vandamme, 2014; Lubojemska et al., 2016). Indeed, even their aging was recently shown to match human senescence surprisingly well (Dutta and Sengupta, 2016). Since mice are also easy to keep, they became the most widely used model organism in life sciences after their first documented employment almost 500 years ago (reviewed in Paigen, 2003; Goodman et al., 2015): of nearly 11.5 million animals used for scientific purposes in the European Union in 2011, 61% were mice (European Commission, 2010). Despite these massive research efforts, most attempts to translate the outcomes to humans have failed. In the FXS field for instance, more than 70 studies reporting rescues (excluding reviews) have been published on pubmed during the last 12 years, 63 clinical trials are registered on ClinicalTrials.gov, and not a single treatment is available for patients yet (2nd of October 2017; current state reviewed in Ligsay and Hagerman, 2016). Although several positive outcomes were observed during the trials, indicating at least some progress toward a better understanding of the disease and a treatment for patients, discrepancies between the data obtained in men and mice were common: benefits experienced by patients were often very subtle, limited to subgroups, outside the outcome measures or simply absent. How much wishful-thinking is involved in our mouse models?

Some facts on men and mice (for more details, please see **Table 1**):

- mice are 3000× smaller
- mice and humans diverged 75 million years ago (Waterston et al., 2002)
- laboratory mice are highly inbred
- their genome is approximately 14% smaller, probably due to deletions (Waterston et al., 2002)
- the DNA sequence identity is only 40% (Waterston et al., 2002)
- the average substitution rate in mice is twofold higher than in humans (Waterston et al., 2002)
- manipulations of their genome may induce mutations in their microsatellite sequences (Zuo et al., 2012; Du et al., 2013)
- mouse-specific promoter and enhancer regions are significantly enriched in repetitive sequences (Yue et al., 2014)
- only 12.6% of the murine DNA are associated with regulatory functions such as transcription factor binding, chromatin organization etc. (humans: 20%; Yue et al., 2014)
- approximately 50% of the regulatory sequences have no identifiable orthologs in human (Cheng et al., 2014; Yue et al., 2014)
- 38.5% of mouse-specific transcription enhancers do not show activity in human ES cells (Yue et al., 2014)
- mice have dozens of local gene family expansions related to reproduction, immunity and olfaction (Waterston et al., 2002)

Taken together, these studies show that although men and mice share many features, they differ in a variety of aspects when more detailed data are included (**Figure 3**). It is now widely accepted that the characteristics of mice and men mostly arise from alterations in the mechanisms

TABLE 1 | Differences between men and mice.

Category	Mice and men	Reference
Transposons	The mouse genome contains only 35.5% of transposon derived DNA (humans: >46%), but with 32.4% an higher amount of lineage-specific repeats (humans: 24.4%).	Waterston et al. (2002)
Breakpoint regions	Evolutionary breakpoint regions (intervals between segments of conserved gene order) of mice are mainly enriched for transposable elements of the SINE type (short interspersed nucleotide elements), whereas human breakpoint regions mainly contain the Alu type, a specific subtype of SINE elements.	Schibler et al. (2006)
Transcription	Only 22% of transcription factor footprints and 50% of transcription factor networks are conserved. Although the binding motifs of most sequence-specific transcription factors are conserved, the motifs for co-factors tend to be species specific.	Yue et al. (2014) Cheng et al. (2014)
Immune system	Differences in the immune system include in the balance of leukocyte subsets, in defensins, Toll receptors, inducible NO synthase, Ig subsets, the B cell and T cell signaling pathways, cytokines and cytokine receptors, Th1/Th2 differentiation, co-stimulatory molecule expression and function, antigen-presenting function of endothelial cells, and chemokine and chemokine receptor expression.	reviewed in Mestas and Hughes (2004)
Physiology	Several differences in the physiology and morphology of organs have been reported recently. Mice are indicated to have higher rates of reactive oxygen species production than humans, however, sufficient original evidence is missing. The fatty acid composition of the membrane is different in mice and men.	e.g.,: Gharib et al. (2010), Tabata et al. (2012), Barak et al. (2013), Dolensek et al. (2015), Schmidt et al. (2015) and Symonds et al. (2015) Ku et al. (1993), reviewed in Finkel and Holbrook (2000) and Demetrius (2006) Hulbert (2005)

The table summarizes genetic as well as physiological differences between mice and men, which may potentially affect the translation of research results between the two species. While genome studies in mice and humans showed that both mammals mainly differ in terms of gene regulation, differences in the physiology are not well characterized yet.

controlling gene expression, in particular from variations and polymorphisms of cis-elements (reviewed in Wittkopp and Kalay, 2011).

Genetics of the FXS Model Mouse

The fundamental difference between commonly used FXS model mice (*Fmr1*^{-/-} mice⁸) and patients is that mice never have any FMRP, beginning with their very first moment of existence, whereas in patients, the gene is active at least until the 10th week of gestation (Devys et al., 1992; Sutcliffe et al., 1992; Suzumori et al., 1993; Iida et al., 1994; Willemsen et al., 2002). Moreover, patients display mosaic expression patterns (Kaufmann et al., 1999; Dyer-Friedman et al., 2002; Loesch et al., 2003, 2004; Govaerts et al., 2007; Pretto et al., 2014; Basuta et al., 2015) as well as residual full-length mRNA (Tassone et al., 2001), but mice don't.

In order to overcome this dissatisfying situation, several CGG repeat knock-in mice were made to mimic the genotype found in humans (e.g., Bontekoe et al., 1997, 2001; Lavedan et al., 1997, 1998; Baskaran et al., 2002; Peier and Nelson, 2002; Fleming et al., 2003; Brouwer et al., 2007; Entezam et al., 2007; Alam et al., 2010). Similar to human pre-mutation carriers (Tassone et al., 2000a,b; Kenneson et al., 2001; Primerano et al., 2002; Ludwig et al., 2014), the knock-in mice display a direct correlation between the mRNA transcript level and the repeat length as well as an indirect correlation between the repeat length and the protein

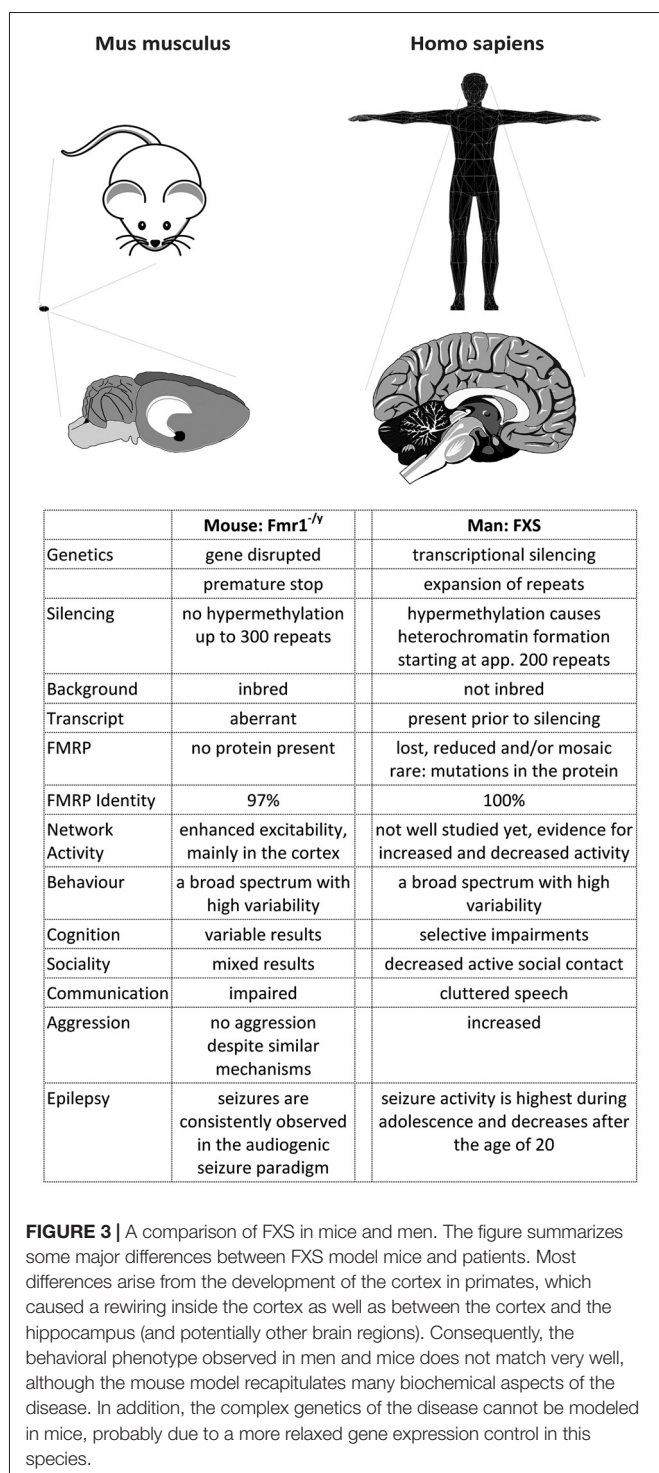
level, showing significant variation between individual animals (Ludwig et al., 2014).

In terms of pathological features, the knock-in mice reflect several biochemical, histological and behavioral symptoms of FXTAS patients (Willemsen et al., 2003; Van Dam et al., 2005; Entezam et al., 2007; Hunsaker et al., 2010, 2011; Wenzel et al., 2010; Cunningham et al., 2011) and pre-mutation transcripts were found toxic in mice (Handa et al., 2005; Hashem et al., 2009; Chen et al., 2010; Hukema et al., 2014). However, no explicit data on behavioral deficits or toxic effects were reported for the full-mutation situation (200–350 repeats; Entezam et al., 2007; Hunsaker et al., 2010, 2011; Ludwig et al., 2014) in specific, thus suggesting that contrary to humans, 350 repeats still represent a pre-mutation situation in mice.

Although some instability has been recognized in mice harboring pre-mutation alleles of about 100–260 repeats (Bontekoe et al., 2001; Peier and Nelson, 2002; Brouwer et al., 2007; Entezam et al., 2007), and based on these models, transacting mechanisms such as mismatch repair and transcript coupled repair were identified to support instability of the gene in mice (Zhao and Usdin, 2014; Zhao X. N. et al., 2015), instability is apparently not a major phenotype of the mouse (Bontekoe et al., 1997; Lavedan et al., 1997, 1998; Peier and Nelson, 2002; Fleming et al., 2003).

This situation first changed, when Baskaran and colleagues introduced the SV40 origin of replication along with their transcript to the gene (Baskaran et al., 2002) in order to exclude nucleosome formation. The transgene is driving the expression of *FMR1* Exon 1 by the SV40 early promoter encoded in the SV40 origin, which excludes chromatin formation at the transgenic locus. Exon 1 contains 26 copies of the CGG

⁸In 2006, an inducible *Fmr1*^{-/-} mouse model was generated to address aspects related to developmental or regional expression patterns, but the animals have only been used in a very few studies thus far, possibly due to limitations in the availability of appropriate Cre-lines. For more information, please refer to Table 2.



repeat together with the translational *FMR1* start codon. As a result, the transgene obtained an open chromatin structure compared to the normally nucleosome-flanked CGG repeats seen in other mouse models (Datta et al., 2011) and is therefore more prone to instability (cp. Oostra, 1998). Thereby, Baskaran and colleagues achieved expansions from 26 to 350 repeats within three generations only; however, the DNA remained unmethylated (Alam et al., 2010). Sadly, these results are in line

with two more recent studies which also achieved significant expansions (120–230 repeats; Brouwer et al., 2007; Entezam et al., 2007), but no methylation and no inactivation of *Fmr1*. Given that the expression of the full-mutation mRNA did apparently not cause unusual severe FXTAS or even FXS phenotypes, and given that the full-mutation alleles were neither hypermethylated nor inactivated or significantly unstable, these data support the idea that in mice, 200–350 repeats still represent a pre-mutation situation.

Taken together, these studies further suggest that the mechanisms leading to the inactivation of *FMR1* might be different in mice and humans (cp. the section “A Question of Secondary Structures?”). Indeed, Matsuo and colleagues found that CpG islands of men and mice have different properties: comparing 23 orthologous genes, they discovered that mice almost always have less pronounced islands, or even none at all. The authors speculate that the CpG islands in mice might have eroded during evolution as an adaption to the mouse’s small body mass and short life-span by allowing for a more relaxed control of gene activity (Matsuo et al., 1993). Their study is in line with an earlier report that also detected low numbers of CpG islands in mice (Aïssani and Bernardi, 1991), thus implying that mice may simply not have the capabilities to methylate genes the same way humans do, causing fully mutated *Fmr1* to remain active even if humanized transgenes are employed (e.g., Lavedan et al., 1998). This hypothesis is supported by comparative *in vivo* footprinting analyses across several human and mouse CpG islands that demonstrated striking differences in the protein-DNA interactions of both species (Cuadrado et al., 2001, reviewed in Antequera, 2003). Furthermore, Fu and colleagues found that the murine and the human DNA methyltransferase DNMT1, which is responsible for the maintenance of methylation patterns by preferentially adding methyl groups to hemi-methylated CpG sites, differ in their processivity (Fu et al., 2012). In particular, they demonstrate a high level of processivity for human DNMT1 at *FMR1*, which is possibly not achieved by the murine enzyme as it has much longer non-association tracts *in vivo*.

Phenotypes of the *Fmr1*^{-/-} Mouse

FMRP Functions to Regulate the mRNA Metabolism

Studying FMRP functions in humans is challenging. It is therefore no surprise, that most of the corresponding knowledge we acquired is derived from mouse models. However, recent advances in stem cell research contributed significantly to our understanding of the disease.

In healthy individuals, FMRP is widely expressed, yet most abundant in testes and brain, where it is present at particular high levels throughout the cerebral cortex, the hippocampus and the Purkinje cell layer as well as the granular layer of the cerebellum (Devys et al., 1993; Hinds et al., 1993; Bakker et al., 2000, also see <http://mouse.brain-map.org/gene/show/14042> and <http://www.gensat.org/GeneProgressTracker.jsp?gensatGeneID=339>). FMRP has been detected in glial and neuronal cells (Wang et al., 2004; Gholizadeh et al., 2015), but its special importance in synaptic signaling has drawn most attention

TABLE 2 | Fragile X Syndrome and Fragile X-associated Tremor/Ataxia Syndrome model mice.

Fmr1 mouse model	MGI	Aliases	Strains	First publication	Further publications and information
Fmr1 ^{tm1Cgr}	1857169	Fmr1 KO, Fmr1 ^{tm4Cgr} , FMRP KO, fmr-tm1Cgr, FraX, FMR1-	FVB.129P-Fmr1 ^{tm1Cgr} /J B6.129P2-Fmr1 ^{tm1Cgr} /J FVB.129P2(B6)-Fmr1 ^{tm1Cgr} /J	Bakker et al. (1994)	http://www.informatics.jax.org/reference/allele/MGI:1857169?typeFilter=Literature#myDataTable=results%3D100%26startIndex%3D0%26sort%3Dyear%26dir%3Ddesc%26typeFilter%3DLiterature
Fmr1 ^{tm1.1Cldz}	3808885	Fmr1 KO2	B6.129P2-Fmr1 ^{tm1.1Cldz} /J	Mientjes et al. (2006)	http://www.informatics.jax.org/reference/allele/MGI:3808885?typeFilter=Literature
Fmr1 ^{tm1Cldz}	3603442	Fmr1 CKO	Involves: 129S1/Sv* 129X1/SvJ	Koekkoek et al. (2005)	http://www.informatics.jax.org/reference/allele/MGI:3603442?typeFilter=Literature
Fmr1 ^{tm2Cgr}	2451086	CGG(98) Fmr1 CGG KI Fmr1 CGG KI (C57BL/6 congenic)	B6.129P2(Cg)-Fmr1 ^{tm2Cgr} /DlnJ	Bontekoe et al. (2001)	http://www.informatics.jax.org/reference/allele/MGI:2451086?typeFilter=Literature
Fmr1 ^{tm1Usdn}	3711215	CGG KI, Fmr1 ^{PM}	B6.129S6(Cg)-Fmr1 ^{tm1Usdn}	Entezam et al. (2007)	http://www.informatics.jax.org/reference/allele/MGI:3711215?typeFilter=Literature
Fmr1 ^{tm1Rbd}	3840615	Fmr1 ^{I304N} Fmr1 ^{tm1(I304N)Dmrl} Fmr1 ^{tm1(I304N)Rbd}	FVB.129-Fmr1 ^{tm1Rbd} /J B6.129-Fmr1 ^{tm1Rbd} /J	Zang et al. (2009)	http://www.informatics.jax.org/reference/allele/MGI:3840615?typeFilter=Literature
Tg(Fmr1-EGFP)HP76Gsat	4847053		B6;FVB-Tg(Fmr1-EGFP)HP76Gsat/Mmucd	-	- also see: http://www.informatics.jax.org/reference/allele/MGI:4847053?typeFilter=Literature

The table shows the mouse models most commonly used in FMR1 research. Embryonic stem cell lines are not included. The majority of studies (>260) has been performed with the first mouse model, the Fmr1 KO mouse (Bakker et al., 1994), which is characterized by a complete loss of FMRP and expression of abnormal mRNA (Yan et al., 2004; Mientjes et al., 2006). To investigate a potential effect of the aberrant mRNA, another model, the Fmr1 KO2 mouse was generated in 2006 (Mientjes et al., 2006), which does not express any mRNA, but no significant differences between the two animal models were found (Gaudissard et al., 2017). By now, the KO2 animals have been employed in more than 20 studies. Along with the Fmr1 KO2 mouse model, a conditional FXS mouse was developed as well (Fmr1 CKO), but thus far, the model has only been employed in approximately 10 studies. The knock-in animals have mostly achieved attention as models of FXTAS. In addition, one model of the I304N mutation (Zang et al., 2009), which affects mRNA binding by the KH domain, has been generated. Model mice are available to researchers with generous support from the FRAXA Research Foundation: <https://www.fraxa.org/fragile-x-mutant-mouse-facility/>.

on its function in neurons. Here, FMRP is primarily located in the cytosol and the nucleus (Feng et al., 1997), but it has also been found along dendrites, axons and at synaptic sites (Weiler et al., 1997; Greenough et al., 2001; Antar et al., 2006; Akins et al., 2017). Indeed, FMRP travels between these locations through microtubules (Feng et al., 1997; Antar et al., 2005).

The main function of FMRP is to regulate the mRNA metabolism. Interacting with five different RNA-motifs (U-Pentameres, Kissing complex, SosLip, G-quartets, G-rich regions), the protein may associate with a diversity of mRNAs, approximately 4% of all mRNAs in the mammalian brain (Brown et al., 2001; Miyashiro et al., 2003; Darnell et al., 2011). Detailed biochemical studies revealed that thereby, FMRP is able to regulate not only mRNA transport (Dicthenberg et al., 2008; Estes et al., 2008) and stability (Bagni and Greenough, 2005; De Rubeis and Bagni, 2010), but also mRNA translation (Ceman et al., 2003; Zalfa et al., 2003; Bechara et al., 2009). Furthermore, FMRP was found to influence the microRNA-pathway, thus gaining further control on the expression of its target proteins (reviewed in Kenny and Ceman, 2016).

Inside the nucleus, FMRP localizes to active transcription sites, where it binds to nascent mRNA (Eberhart et al., 1996; Kim et al., 2009) and may even take action in alternative splicing, since G-quartets present in the mRNA of FMRP itself were found to function as exonic splicing enhancers (Didiot et al., 2008). Studies of the transcription and splicing machinery actually revealed brain-region as well as cell type specific alterations in *Fmr1*^{-/-} mice (Derlig et al., 2013) and showed that loss of FMRP results in aberrant transcriptional regulation (Korb et al., 2017). Furthermore, FMRP was found to bind to DNA and to function in DNA damage response (Alpatov et al., 2014) as well as in Heterochromatin organization (Tan et al., 2016). These findings suggest that FMRP may control every step of protein expression from DNA organization to translation.

FXS and Group 1 Metabotropic Glutamate Receptors

On the other hand, FMRP itself turned out to be under control of mGluR1/5⁹ signaling cascades (Narayanan et al., 2008), hence allowing for an activity dependent regulation of the mRNA

⁹metabotropic glutamate receptors 1 and 5, = group 1 mGluRs

TABLE 3 | LTP protocols used in different studies.

Study	Stimulation	Recording	Study	Stimulation	Recording
Auerbach and Bear (2010)	<i>Hippocampus</i> : 1 s 100 Hz tetanus stimulation	Field recordings	Bostrom et al. (2015)	<i>Hippocampus</i> , CA1 and <i>dentate gyrus</i> : 4 trains of 50 pulses at 100 Hz, 30 s apart	Field recordings
Chen et al. (2014)	<i>Anterior cingulate cortex</i> : five trains of bursts with four pulses at 100 Hz and 200 ms interval; repeated five times at intervals of 10 s	MED64 probe (array)	Godfraind et al. (1996)	<i>Hippocampus</i> : data not available	Field recordings
Harlow et al. (2010)	<i>Somatosensory cortex</i> : pairing of 100 stimuli at 1 Hz with postsynaptic depolarization to 0 mV	Whole cell recordings	Hayashi et al. (2007)	<i>Cortex</i> : eight bursts (each four pulses at 100 Hz) every 200 ms	Field recordings
Hu et al. (2008)	<i>Hippocampus</i> : pairing of 200 pulses at 2 Hz at −5 mV within 5 min after formation of whole-cell configuration	Whole-cell recordings	Koga et al. (2015)	<i>Anterior cingulate cortex</i> : induction of pre-LTP with repetitive low-frequency stimulation at 2 Hz for 2 min	Whole-cell recordings (neurons), multielectrode array (slices)
Larson et al. (2005)	<i>Anterior piriform cortex</i> : 10 bursts at 100 Hz with four pulses repeated in 200 ms intervals, <i>hippocampus</i> : two pathways, one by five theta bursts, the other by 10 theta bursts	Field recordings	Lee et al. (2011)	<i>Hippocampus</i> : five theta burst stimuli	Field recording
Li et al. (2002)	<i>Hippocampus</i> : single tetanic train of 100 Hz, 1 s duration at maximal intensity, <i>cortex</i> : three tetanic trains of 200 Hz, 1 s with an interval of 10 min	Field recordings	Martin H. G. S. et al. (2016)	<i>Prefrontal Cortex</i> : five trains of burst with four pulses at 100 Hz and 200 ms interval, repeated four times at intervals of 10 s	Whole-cell recordings
Padmashri et al. (2013)	<i>Motor cortex</i> : chemical LTP via bath application of bicuculline (6.3 μ m) for 3 min, followed by forskolin (50 μ m) and the phosphodiesterase inhibitor rolipram (0.1 μ m) in Mg^{2+} -free ACSF for 15 min	Field recordings	Paradee et al. (1999)	<i>Hippocampus</i> : stimulation 1x every 30 s for 20 min for baseline response, followed by induction of L-LTP by three trains (10 bursts at 5 Hz, each burst consisting of a 40 ms burst at 100 Hz) of theta bursts, 1 min apart	Field recordings
Shang et al. (2009)	<i>Hippocampus</i> : stimulation intensity adjusted so that a half-maximal fEPSP was elicited	Field recording, whole-cell recording	Wilson and Cox (2007)	<i>Neocortex</i> : three trains of 100 Hz, 1-s duration at 5 min intervals	Field recordings
Xu et al. (2012a)	<i>Prefrontal cortex</i> : 80 pulses at 2 Hz, and then paired with postsynaptic depolarization at +30 mV	Whole-cell recordings	Xu et al. (2012b)	<i>Anterior cingulate cortex</i> : 80 pulses at 2 Hz paired with postsynaptic depolarization at +30 mV	Whole-cell recordings
Yang et al. (2014)	<i>Auditory cortex</i> : three repetitions of 100-Hz stimulation of 1-s duration.	Field recordings	Zhang et al. (2009)	<i>Hippocampus</i> : L-LTP induced by four 1 s trains of 100 Hz with a 5 min interval	Field recordings
Zhao et al. (2005)	<i>Anterior cingulate cortex</i> : 80 pulses at 2 Hz paired with postsynaptic depolarization at +30 mV	Whole-cell recordings			

Literature providing only general information is not included. The data illustrates the variety of induction protocols used to study LTP.

metabolism in various aspects by FMRP. Detailed studies on the function of mGluRs in FXS have lead to the advance of the mGluR-Theory (Huber et al., 2002; Bear et al., 2004; Dolen et al., 2007; Nakamoto et al., 2007): the theory states that FMRP normally acts as a repressor of mRNA translation downstream of group 1 mGluRs, which is released after mGluR activation and thereby induces the translation of proteins required for the expression of LTD. Hence, in the absence of FMRP, persistent and mGluR stimulation independent synthesis of LTD-proteins causes ongoing AMPAR¹⁰ internalization. The

theory further posits that exaggerated mGluR-signaling, perhaps as a consequence of malfunctioning feedback inhibition, is causing many of the symptoms observed in FXS.

There is indeed good evidence for this theory: the pharmacological down-regulation of mGluR5 signaling has been shown to improve a variety of typical symptoms in *Fmr1*^{−/y} mice, including aberrant neuronal morphology, hyperactivity, social behavior, seizure susceptibility and learning and memory (Yan et al., 2005; de Vrij et al., 2008; Levenga et al., 2011; Su et al., 2011; Michalon et al., 2012; Vinueza Veloz et al., 2012; Gantois et al., 2013; de Esch et al., 2015) and so has a genetic reduction of group 1 mGluRs (Dolen

¹⁰ α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

et al., 2007; de Esch et al., 2015). One group reported some contradictory findings (Thomas et al., 2011), but the promise of the results prompted clinical trials with mGluR antagonists such as AFQ056 (Novartis) and RO4917523 (Roche) to down-regulate the exaggerated mGluR signaling. Although patients treated with these substances were initially reported to experience some behavioral improvements (Jacquemont et al., 2011), all trials were discontinued during phases IIb/III since the studies did not show any significant improvements in abnormal behaviors compared to placebo (reviewed in Scharf et al., 2015). It has been speculated that cross-reactions with other drugs used to treat FXS patients, the long time of perpetuation in adult patients, irreversible changes during early brain development and difficulties in the outcome measures might have caused the failure, but the underlying reasons have remained unclear.

Remarkably, several targets of FMRP belong to the two major mGluR-signaling cascades controlling the expression of related proteins, the ERK¹¹ - and the mTOR¹² - pathway: ERK, PI3K¹³, PIKE¹⁴, GSK3¹⁵ and mTOR are target mRNAs of FMRP (Darnell et al., 2011; Ascano et al., 2012). Both signaling pathways turned out to be exaggerated not only in the hippocampus of *Fmr1*^{-/-} mice (ERK pathway: Hou et al., 2006; Michalon et al., 2012; mTOR pathway: Sharma et al., 2010; Liu Z. H. et al., 2012; Bhattacharya et al., 2016; Choi et al., 2016), but also in humans (Weng et al., 2008; Hoeffler et al., 2012; Wang X. et al., 2012; Kumari et al., 2014; Pellerin et al., 2016).

There has been some dispute about the status of ERK activation in FXS though (mice: Hu et al., 2008; Gross et al., 2010; Osterweil et al., 2010; patients: Yrigollen et al., 2016), but pathway-specific inhibitors of ERK-signaling constantly rescued characteristic deficits in FXS models (Chuang et al., 2005; Osterweil et al., 2010; Wang X. et al., 2012), thus supporting the idea that enhanced mGluR1/5 cascades are causative for some symptoms of the disease. Further studies found that inhibition of ERK-signaling with Metformin (Gantois et al., 2017) or Lovastatin (Osterweil et al., 2013) indeed ameliorates many deficits in *Fmr1*^{-/-} mice. Strikingly though, mTOR inhibition with Rapamycin turned out to induce adverse effects on sleep and social behavior in both, control and *Fmr1*^{-/-} mice (Sar   et al., 2018), although positive effects of Rapamycin were reported for the BTBR *T⁺Itpr3^{tf}/J* mouse model of autism (Burket et al., 2014). In fact, some data even suggest that mTOR inhibition with Rapamycin might cause neurodegeneration (Lin et al., 2013). The hopes are therefore now on clinical trials with Lovastatin (Caku et al., 2014; Pellerin et al., 2016) and Metformin (Dy et al., 2018).

Brain Region Specific Mechanisms in FXS

All the above mentioned strategies to amend FXS suffer from the same difficulty though; contrary to drugs, the outlined mechanisms only apply to the hippocampus. There is evidence

that the signaling mechanisms of the cortical and hippocampal networks are differentially affected by the loss of FMRP. In cortical synaptosomes of *Fmr1*^{-/-} mice for example, mTOR activity was found normal (Sawicka et al., 2016), whereas in the hippocampus, mTOR signaling is exaggerated (Sharma et al., 2010; Liu Z. H. et al., 2012; Bhattacharya et al., 2016; Choi et al., 2016). Cortical ERK was demonstrated to be erroneously deactivated following mGluR stimulation in *Fmr1*^{-/-} mice (Kim et al., 2008), whereas it was illustrated to be normal (Osterweil et al., 2010) or even over-activated in hippocampal tissue (Hou et al., 2006; Michalon et al., 2012). Remarkably, the study of Sawicka and colleagues showed that ERK signaling in the neocortex of *Fmr1*^{-/-} mice is impinging on ribosomal protein S6, which usually receives input from mTOR, though in this study, cortical ERK activity was found exaggerated.

The data on patients are mostly derived from fibroblasts (Kumari et al., 2014; Yrigollen et al., 2016) or thrombocytes (Weng et al., 2008; Pellerin et al., 2016), making conclusions on the characteristics of specific brain regions difficult. Using post-mortem tissue, one study detected no differences in ERK activation in the frontal lobe between FXS patients and controls (Hoeffler et al., 2012), while another one found increased levels of phosphorylated ERK in the frontal cortex of patients (Wang X. et al., 2012). Despite the unclear status of ERK activation in the cortex, these studies suggest that mGluR1/5 signaling pathways are functioning differently in the cortex and the hippocampus. In line with this notion, a recent study of mTOR activity and exercise demonstrated that the activation status of mTOR is depending on the brain region, the cell type (neuron or glia) and the type of exercise (sedentary, voluntary or forced; Lloyd et al., 2017).

Long term potentiation

Further support for the relevance of brain region specific mechanisms in FXS comes from a variety of studies on synaptic plasticity. Synaptic plasticity is mostly investigated by the induction of LTP¹⁶ or LTD¹⁷, two paradigms which are considered cellular models of learning and memory (reviewed in Kandel, 2001; Bliss et al., 2003; Neves et al., 2008). Both, LTP and LTD, ultimately depend on the modulation of synaptic signaling and have been studied intensively in *Fmr1*^{-/-} mice using manifold induction protocols (please see Table 3 for more details).

While some protocols have led to the discovery of disturbances in certain forms and aspects of hippocampal LTP (Hu et al., 2008; Shang et al., 2009; Lee et al., 2011), most examinations found the expression of hippocampal LTP in *Fmr1*^{-/-} mice to be normal (Godfraind et al., 1996; Paradee et al., 1999; Li et al., 2002; Larson et al., 2005; Zhang et al., 2009; Auerbach and Bear, 2010). Two of these studies also investigated cortical LTP: while they observed no abnormalities in hippocampal LTP, both detected significantly impaired LTP in the cortex of *Fmr1*^{-/-} mice (Li et al., 2002; Larson et al., 2005). The result is line with several studies demonstrating defective

¹¹extracellular signal-regulated kinase

¹²mammalian target of rapamycin

¹³Phosphoinositid-3-Kinase

¹⁴PI3K Enhancer

¹⁵Glycogen synthase kinase 3

¹⁶long term potentiation

¹⁷long term depression

LTP in different regions of the cortex (Zhao et al., 2005; Hayashi et al., 2007; Wilson and Cox, 2007; Harlow et al., 2010; Xu et al., 2012b; Padmashri et al., 2013; Yang et al., 2014; Koga et al., 2015).

LTP includes two distinct phases, an early phase (E-LTP), which does not require protein synthesis, and a late phase (L-LTP), which depends on protein synthesis and gene expression (Frey et al., 1993; Abel et al., 1997, reviewed in Kandel, 2001, 2009). While L-LTP is unaffected in the hippocampus of *Fmr1*^{-/-} mice (Paradee et al., 1999; Zhang et al., 2009), it is blocked in the cingulate cortex (Chen et al., 2014). Since pharmacological inhibition of mGluR5 or GSK3 rescued L-LTP in the cingulate cortex of *Fmr1*^{-/-} mice, these results show that exaggerated mGluR signaling is involved. Indeed, two other studies also reported rescues of cortical LTP based on mGluR1/5 antagonists (Xu et al., 2012a; Martin H. G. S. et al., 2016), but the effects of mGluR5 or GSK3 inhibition on (L-)LTP in the hippocampus were never investigated although hippocampal L-LTP is known to depend on mGluR1/5 activation (Riedel and Reymann, 1996; Francesconi et al., 2004; Neyman and Manahan-Vaughan, 2008; Fan, 2013).

The differences between the hippocampus and the cortex are further emphasized by the fact that age-related deficits in LTP were discovered specifically in the cortex (Larson et al., 2005; Martin H. G. S. et al., 2016). While *Fmr1*^{-/-} mice older than 6 months displayed significant defects in the expression of cortical LTP, the mice never displayed any impairment in hippocampal LTP. In fact, Bostrom et al. (2015) even demonstrated differences within the hippocampus itself: while the loss of FMRP caused impairments in NMDAR¹⁸-dependent LTP in the dentate gyrus, NMDAR-LTP was found normal in the CA1¹⁹ region of the hippocampus.

Long term depression

The most prominent and best studied plasticity model in FXS is mGluR-LTD, a type of LTD that depends on the activation of mGluR1/5, protein synthesis and the internalization of AMPA receptors. However, while numerous studies demonstrated enhanced mGluR-LTD in the hippocampus of *Fmr1*^{-/-} mice (Huber et al., 2002; Hou et al., 2006; Nosyreva and Huber, 2006; Volk et al., 2007; Park et al., 2008; Ronesi and Huber, 2008; Zhang et al., 2009; Auerbach and Bear, 2010; Choi et al., 2011; Bhattacharya et al., 2012; Michalon et al., 2012; Niere et al., 2012; Costa et al., 2015; Toft et al., 2016; Thomson et al., 2017), nobody studied mGluR-LTD in the cortex yet. There is evidence though that cortical mGluR-LTD is existing (reviewed in Kang and Kaang, 2016). Using a protocol to induce spike-time-dependent plasticity (STD-LTP or STD-LTD) in the neocortex of *Fmr1*^{-/-} mice, Desai and colleagues found no impairment in LTD, but a significant reduction in LTP (Desai et al., 2006). Experiments with MPEP²⁰, a mGluR5 antagonist, revealed that cortical STD-LTP is not depending on mGluR5 activation, whereas

cortical STD-LTD is. Remarkably, the application of anisomycin, an inhibitor of protein synthesis, revealed that cortical STD-LTD does not require protein synthesis, thus suggesting that FMRP is not necessary for cortical STD-LTD despite the dependence on mGluR5. Sadly though, STD-LTD has not been investigated in the hippocampus yet.

It is worth noting that the biological relevance of mGluR-LTD has recently been questioned, arguing that mGluR-LTD in the absence of previous LTP is artificial (discussed in Jones, 2017). Nonetheless, since more than 15 years of mGluR-LTD research in *Fmr1*^{-/-} mice did not provide any evidence for abnormal mGluR-LTD in the cortex, it seems likely that the mechanisms underlying this form of plasticity differ among brain regions.

Keeping the balance

FXS is associated with a vast misregulation of protein expression, not only in the context of mGluR signaling, but also with respect to proteins regulating other aspects of neuronal excitability such as Calmodulin or Neuroligin for instance (Liao et al., 2008; Matic et al., 2014; Kalinowska et al., 2015; Tang et al., 2015). Although it is not clear yet whether abnormal mGluR-LTD directly impacts on the balance of excitatory and inhibitory activity in neuronal networks (E/I balance), mGluR signaling is able to alter the excitability of neurons by increasing the intrinsic conductance (Bianchi et al., 2009; Tang and Alger, 2015). Furthermore, FMRP itself may bind to ion channels such as Calcium, Slack²¹ and BK²² channels, thereby providing an additional level of FMRP mediated control (Brown et al., 2010; Deng et al., 2013; Ferron et al., 2014).

Deviations in the balance of excitation and inhibition have been associated with seizure activity, hypersensitivity and cognitive deficits in several ASDs and animal models (reviewed in Frye et al., 2016; Uzunova et al., 2016; Lee et al., 2017; e.g., Orekhova et al., 2008; Tebartz van Elst et al., 2014; Robertson et al., 2016), including *Fmr1*^{-/-} mice (e.g., D'Hulst et al., 2006; Zhong et al., 2009; Dahlhaus and El-Husseini, 2010; Aguilar-Valles et al., 2015; Deng and Klyachko, 2016). Using these animals, in particular neocortical circuits have been shown to experience enhanced excitation (Gibson et al., 2008; Goncalves et al., 2013; Patel et al., 2013; Zhang Y. et al., 2014; Westmark et al., 2016), partially due to inhibitory deficits (Selby et al., 2007) and potentially supported by inhibitory dysfunctions in the cortico-hippocampal pathway and inhibitory defects in feed-forward circuits (Wahlstrom-Helgren and Klyachko, 2015).

Indeed, not only glutamatergic signaling has been demonstrated to be altered in *Fmr1*^{-/-} mice, but also gabaergic and dopaminergic mechanisms have been found malfunctioning. Studies showed that *Fmr1*^{-/-} mice experience diminished GABAa/b²³ receptor expression, reduced GABA release, decreased dopamine receptor expression and malfunctioning interneurons (D'Hulst et al., 2006; Selby et al., 2007; Pacey et al.,

¹⁸N-methyl-D-aspartate receptor

¹⁹Cornu Ammonis region 1

²⁰2-Methyl-6-(phenylethynyl) pyridine

²¹sodium-activated potassium channel Slack

²²large-conductance calcium-activated potassium channels

²³gamma-aminobutyric acid

2011b; Paluszkiwicz et al., 2011; Henderson et al., 2012; Heulens et al., 2012; Patel et al., 2013; Paul et al., 2013; Berzhanskaya et al., 2016a; Kang et al., 2017). Deficiencies in inhibitory conductance are therefore characteristic to many circuits of the murine FXS brain including circuits of the striatum, amygdala, hippocampus, subiculum and the somatosensory as well as the prefrontal cortex (Centonze et al., 2008; Curia et al., 2009; Olmos-Serrano et al., 2010; Paluszkiwicz et al., 2011; Vislay et al., 2013; Martin et al., 2014; Sabanov et al., 2016). In addition, the loss of interactions between FMRP and BK channels causes uncontrolled Glutamate release, altered action potential waveforms and exaggerated excitability (Zhang Y. et al., 2014; Myrick et al., 2015; Deng and Klyachko, 2016).

Recent research indicates that the alterations in the E/I balance may include brain region and circuit specific mechanisms. For instance, while dendrites of hippocampal neurons from *Fmr1*^{-/-} mice display increased HCN1-channel²⁴ expression and reduced input resistance (Brager et al., 2012), dendrites of cortical layer 5 neurons show the opposite (Zhang Y. et al., 2014). The intrinsic membrane excitability of cortical layer 4 excitatory neurons is exaggerated (Gibson et al., 2008), while that of excitatory hippocampal neurons is normal (Deng et al., 2013; Luque et al., 2017). Hippocampal neurons demonstrate significantly longer action potential durations and higher firing frequencies in the absence of FMRP than under normal conditions (Luque et al., 2017), whereas layer 2/3 neurons in the prefrontal cortex present significantly narrower and taller action potentials in *Fmr1*^{-/-} mice than in their wildtype litter mates (Routh et al., 2017). Although both alterations indicate enhanced excitability, the sharpened action potentials observed in the cortex of *Fmr1*^{-/-} mice are in contrast with the broadened action potentials seen in the hippocampus. Since two studies even demonstrated increased inhibition early in development (Berzhanskaya et al., 2016b; Truszkowski et al., 2016), the data show that the alterations in neuronal activity depend on the specific circuit, aspect and age.

Despite the high complexity of the system, several studies were able to rescue the aberrant neuronal activity based on restorations of mGluR1/5 signaling (Meredith et al., 2011; Ronesi et al., 2012; Westmark et al., 2016; Aloisi et al., 2017), GABA signaling (Olmos-Serrano et al., 2010, 2011; Martin B. S. et al., 2016; Kang et al., 2017) or ion channel function (Zhang Y. et al., 2014; Deng and Klyachko, 2016; Aloisi et al., 2017). In doing so, phenotypes such as epileptiform activity (Ronesi et al., 2012; Zhang Y. et al., 2014; Deng and Klyachko, 2016; Westmark et al., 2016), hyperactivity (Olmos-Serrano et al., 2011; Ronesi et al., 2012) and hypersensitivity (Zhang Y. et al., 2014) were normalized. A few studies have also investigated network oscillations in FXS model animals (Gibson et al., 2008; Goncalves et al., 2013; Rotschafer and Razak, 2013; Radwan et al., 2016; Westmark et al., 2016; Berzhanskaya et al., 2017). In these experiments, cortical neurons displayed increased synchrony in their activity as well as a threefold higher firing rate during Up states (Gibson et al., 2008; Goncalves et al., 2013; Westmark

et al., 2016), increased high-frequency as well as reduced low-frequency power during rest (Berzhanskaya et al., 2017) and elevated responses to auditory stimuli (Rotschafer and Razak, 2013).

The findings support the theory that neuronal hyperexcitability is a leading cause for many symptoms of FXS and fit well with the Intense World Theory of Autism (reviewed in Markram and Markram, 2010), which posits that hyperactive micro-networks cause many of the cognitive deficits characteristic to ASDs, in particular hypersensitivity, hyperattention, hyperemotionality and seizure susceptibility. Although the studies of oscillatory dynamics in FXS patients currently available mostly confirm the idea of imbalanced circuit activity (Castrén et al., 2003; Dalton et al., 2008; Holsen et al., 2008; Van der Molen and Van der Molen, 2013; Van der Molen et al., 2014; Wang et al., 2017), not all of them are in favor of the theory: two studies detected decreased activity, one in prefrontal regions and one in the fusiform gyrus (Dalton et al., 2008; Holsen et al., 2008), while a third study found intracortical inhibition in FXS patients to be normal (Oberman et al., 2010).

FROM MICE TO MEN

Literature Summary

Most rescues reported from *Fmr1*^{-/-} mice are based on investigations of the hippocampus (Table 4). Some studies included data on further brain regions or transferred approaches to cortical regions, analyzing different aspects of mGluR signaling for example; however, only 4 studies were found that focussed on the cortex in terms of their strategy and their experiments. No studies could be identified that are reporting behavioral rescues based on mechanisms characteristic to the amygdala, cerebellum, striatum or any other brain region in specific, although the available data argue for different deficits in different brain regions (see for instance Chen et al., 2014; Bostrom et al., 2015; Sawicka et al., 2016; Lloyd et al., 2017) and despite the fact that rescues of neuronal activity have been reported for the amygdala (Olmos-Serrano et al., 2010, 2011; Suvrathan et al., 2010; Martin B. S. et al., 2016). These findings show that there is a significant imbalance between the current hippocampus centered investigations and the multifarious mechanisms found in the brain of wildtype as well as FXS model animals.

The Cortex, the Hippocampus and FXS

Problems in translating findings from mice to men are a common phenomenon in brain research. A major difficulty arises from the development of the cerebral cortex in primates and in particular in humans. Recent research revealed that the cognitive performance of vertebrates is best reflected by a combination of the number of neurons, neuron density and axonal conduction velocity in the cortex (reviewed in Dicke and Roth, 2016). In this regard, mice are not well set, since their cerebral cortex exhibits only a relatively low density of neurons: if mice would have a brain the size of a human, their cerebral cortex would contain

²⁴hyperpolarization-activated cyclic nucleotide-gated cation channel

TABLE 4 | Brain-region bias in FXS research.

	Rescue of behavior	Rescue of neuronal function only
Hippocampus based strategies and/or studies	Yan et al. (2005), Dolen et al. (2007), de Vrij et al. (2008), Gross et al. (2010, 2015a), Levenga et al. (2011), Westmark et al. (2011), Bhattacharya et al. (2012), Goebel-Goody et al. (2012), Guo et al. (2012), Liu Z. H. et al. (2012), Michalon et al. (2012), Ronesi et al. (2012), Vinuesa Veloz et al. (2012), Chen et al. (2013), Gantois et al. (2013), Osterweil et al. (2013), Udagawa et al. (2013), Boda et al. (2014), Franklin et al. (2014), Hébert et al. (2014), Sidhu et al. (2014), Sun et al. (2014, 2016), Tian et al. (2015), de Esch et al. (2015), Aloisi et al. (2017), Martinez and Tejada-Simon (2017), Pardo et al. (2017) and Thomson et al. (2017) total: 30	Lauterborn et al. (2007), Nakamoto et al. (2007), Zeier et al. (2009), Choi et al. (2011), Gross et al. (2011), Meredith et al. (2011), Costa et al. (2012, 2015), Deng et al. (2013), Bostrom et al. (2015), Choi C. H. et al. (2015), Ghilan et al. (2015), Tang and Alger (2015), Zhao W. et al. (2015), Deng and Klyachko (2016), Toft et al. (2016), Westmark et al. (2016) and Yau et al. (2016) total: 18
Studies including data on several brain regions	Yuskaitis et al. (2010), Liu et al. (2011), Pacey et al. (2011a), Ronesi et al. (2012), Xu et al. (2012a), Gkogkas et al. (2014), Lim et al. (2014), Braat et al. (2015), Bhattacharya et al. (2016) and Li et al. (2016) total: 10	
Cortex based strategies and/or studies	Hayashi et al. (2007), Dolan et al. (2013), Gross et al. (2015c) and Yang et al. (2015) total: 4	Henderson et al. (2012), Kim et al. (2013), Chen et al. (2014) and Lovelace et al. (2016), Westmark et al. (2016) total: 5
Amygdala based strategies and/or studies	none	Olmos-Serrano et al. (2010, 2011), Suvrathan et al. (2010) and Martin B. S. et al. (2016) total: 4
Brain regions not specified/studied	Veeraragavan et al. (2011), Heulens et al. (2012), Gholizadeh et al. (2014) and Pietropaolo et al. (2014) total number: 4	

The table lists all publications that were identified on pubmed or PMC in October 2017, and found to report at least functional rescues of symptoms related to FXS using murine model systems. Studies reporting only morphological rescues were not included. The data show that there is a strong bias toward investigations of hippocampal functions in the literature.

2 billion neurons, whereas the human cerebral cortex does in fact have 16 billion (cp. Herculano-Houzel, 2009). However, neuron density is not the only disadvantage mice are confronted with:

- the neocortex constitutes 80% of the human brain (Azevedo et al., 2009), but only 40% of a mouse brain (reviewed in Herculano-Houzel, 2009)
- while mice have about 20 different cortical areas, humans have more than 200 (Kaas, 1989, 2011; Changizi and Shimojo, 2005)
- most cortical areas in mice are related to sensory and motor functions, whereas the majority of the human areas function in association (reviewed in Buckner and Krienen, 2013)
- some parts of the human neocortex are specifically enlarged compared to the rest of the neocortex:
 - the prefrontal cortex and area 10 (Semendeferi et al., 2002, 2011), which is important to higher cognitive functions,
 - the insula, which usually functions in sensory information processing, but has new areas in humans that are functioning in empathy and social awareness (reviewed in Keysers et al., 2010) and
 - the posterior parietal cortex (reviewed in Orban et al., 2006), which is important to planning, imitation and the highly skilled use of tools.
- by contrast, the primary sensory and motor areas have maintained their relative sizes during the evolution from mice to men (Hill et al., 2010; Preuss, 2011)

- several cell types found in the human brain do not exist in mice (reviewed in Buckner and Krienen, 2013).

In line with these findings, rodents perform relatively poor in behavioral tasks when compared to monkeys possessing similar sized brains (e.g., capybara (Macdonald, 1981) and capuchin monkey (Anderson et al., 2013; De Moraes et al., 2014; Takahashi M. et al., 2015)), thus suggesting that primate brains have properties, which are significantly different from those of rodents.

This notion is emphasized by an elegant study of Han and colleagues (Han et al., 2013), who showed that human glia cells are much more competent than murine glia cells when it comes to supporting brain functions: replacing the glia of mice with human cells, they observed that the engrafted mice were able to propagate calcium signals three times faster than allografted mice, exhibited sharply enhanced LTPs and performed excellent in cognition tests. Indeed, astrocytes are able to coordinate and modulate neural signal transmission (reviewed in Allen, 2014; Allen and Eroglu, 2017), but human astrocytes differ from their murine counterparts in that human astrocytes are larger, more complex and more diverse, have much more synaptic contacts and are more efficient in calcium signaling (Andriezen, 1893; Colombo, 1996; Colombo et al., 1997, 2000; Reisin and Colombo, 2002; Oberheim et al., 2009).

Remarkably, this is particularly true for the cerebral cortex: there are at least four morphologically distinct astrocyte classes within the primate cortex, as compared to two in rodents.

The two novel classes are interlaminar astrocytes and varicose projection astrocytes (Andriezen, 1893, reviewed in Vasile et al., 2017). Varicose projection astrocytes have hitherto been observed only in cortical layers 5–6 of humans and chimpanzees, but the human cells are more complex. By contrast, interlaminar astrocytes reside in upper cortical layers and extend long processes to cortical layers 3 and 4 (Colombo, 1996; Colombo et al., 1997; Reisin and Colombo, 2002; Korzhhevskii et al., 2005; Oberheim et al., 2009). Although interlaminar astrocytes are present in both, monkeys and men, humans have higher numbers of interlaminar astrocytes. Despite the fact that more than 120 years have passed since their initial description, the role of these primate-specific astrocytes is still elusive.

Nonetheless, astroglia seem to have a role in FXS: though FMRP is predominantly expressed in neurons at all ages, it is also seen in oligodendrocyte precursor cells and astrocytes. While neurons display a gradual decrease in FMRP expression during development (Davidovic et al., 2011; Bonaccorso et al., 2015), astrocytes and oligodendrocyte precursor cells mainly express FMRP during early and mid-postnatal stages of brain maturation (Wang et al., 2004; Gholizadeh et al., 2015), thus suggesting a role for glia and FMRP in development. Indeed, it was shown that FMRP deficient astrocytes cause developmental delays in dendrite maturation and synaptic protein expression of hippocampal wildtype neurons (Jacobs et al., 2010, 2016), whereas normal astrocytes prevent abnormal dendritic development in hippocampal FXS neurons (Jacobs and Doering, 2010).

Investigations on the underlying mechanisms are also still in their infancy. In line with the role of FMRP in regulating protein expression, it was recently shown that the astrocyte-secreted factors Hevin²⁵ and SPARC²⁶, which function to control excitatory synapse development, display abnormal expression patterns in hippocampal and cortical tissues from *Fmr1*^{-/-} mice (Wallingford et al., 2017): while hippocampal Hevin expression is gradually increasing from a reduced expression at P7²⁷ to elevated levels at P21, Hevin expression in the cortex displays only a transient increase at P14. SPARC, on the other hand, shows a modest decrease at P7 and P14 in the cortex, but no abnormalities in the hippocampus. Emphasizing the relevance of this data, Cheng and colleagues were able to demonstrate that astrocyte-conditioned medium is sufficient to prevent morphological deficits in hippocampal neurons cultured from FXS model mice (Cheng et al., 2016). Furthermore, Higashimori and colleagues showed that a selective knock-out of astroglial FMRP in mice modestly increases spine density and size in cortical neurons, whereas a selective re-expression is able to attenuate the abnormal spine morphology characteristic for FXS (Higashimori et al., 2016). The group also described a normalization of the FXS-typical gain in body weight, but no data on cognitive performances were reported. Studies with human astroglia would be desirable, but are currently missing.

The expansion of neocortical areas in primates caused a profound rewiring of the cortex. As a consequence, the human association cortex lacks the strict hierarchical organization of circuits seen in rodents, although certain projections follow the canonical form (e.g., parieto-prefrontal projections), but is instead characterized by multiple, large-scale distributed and highly interwoven networks termed non-canonical circuits. These circuits are highly active during cognitive performances (reviewed in Goldman-Rakic, 1988; Buckner and Krienen, 2013; Margulies, 2017).

Notably, not only the internal pathways of the cortex changed during the evolution from mice to men, also the wiring between the cortex and the hippocampus was modified: in men, the hippocampus preferentially connects to cortical association networks, whereas in mice, it preferentially associates with sensory networks (Bergmann et al., 2016). Although there are still two conserved parallel pathways between the cortex and the hippocampus in mice and men, which transfer object and context related information (reviewed in Ranganath and Ritchey, 2012), the finding of Bergmann and colleagues is of quiet some significance since cortical-hippocampal pathways are required for important brain functions including spatial working memory (studies in rodents and humans; reviewed in Sigurdsson and Duvarci, 2016), long-term memory (studies in rodents, primates and humans; reviewed in Sigurdsson and Duvarci, 2016), motivation and emotion (studies in rodents; reviewed in Sigurdsson and Duvarci, 2016), and social recognition (studies in rodents and humans; reviewed in Bicks et al., 2015). Indeed, weaknesses in working memory performance, in particular when requiring abstract item reasoning, are characteristic to FXS patients (Munir et al., 2000; Cornish et al., 2001; Kwon et al., 2001; Ornstein et al., 2008; Baker et al., 2011; Wang et al., 2013), but not to model mice (Leach et al., 2016). While *Fmr1*^{-/-} mice perform as well as their wildtype littermates even when their working memory is significantly challenged (Leach et al., 2016), individuals with FXS are unable to modulate activation of the prefrontal and parietal cortex in response to an increasing working memory load (Kwon et al., 2001), implying a lack of circuit control. Indeed, a fMRI²⁸ study showed that decreased levels of FMRP correlate with decreases in parahippocampal activation and reduced connectivity between the hippocampus and the prefrontal cortex in patients (Wang J. M. et al., 2012).

Emotional and social difficulties are frequently observed in individuals with FXS as well (Cohen et al., 1988; Mazzocco et al., 1994; Hall et al., 2009; Cordeiro et al., 2011; Kim et al., 2014), but the data on mice are inconsistent (reviewed in Kazdoba et al., 2014). Similar to the impairments observed in working memory performance, social deficits were associated with impairments in the activation of prefrontal regions in individuals with FXS (Holsen et al., 2008). Taken together, these data demonstrate that the cortical and cortical-hippocampal circuits, which characterize the human brain, are critical to FXS and cannot be appropriately modeled in mice. Even though mice may perform the same tasks as men, it is

²⁵high endothelial venule protein, also known as SPARC like protein

²⁶secreted protein, acidic and rich in cysteine

²⁷postnatal day 7

²⁸functional magnetic resonance imaging

obligatory to investigate whether they also do it in the same way.

In the hippocampus, the situation is slightly different. Current research does not support major differences in the structure and function of hippocampi from mice and men (reviewed in Clark and Squire, 2013). A study comparing human and murine spatial navigation showed that both species use the same strategies (Eilam, 2014) to solve this basic and evolutionary old task. Spatial navigation in mammals is assumed to rely on hippocampal place cells, entorhinal grid cells (reviewed in Moser et al., 2008) and hippocampal theta oscillations, albeit neuronal firing in distant brain regions such as the somatosensory or prefrontal cortex is phase-locked to hippocampal theta oscillations (reviewed in Jacobs, 2013). Interestingly, the frequencies of these theta oscillations differ among species: while rats typically display oscillations at 4–8 Hz, human theta oscillations have a frequency of approximately 1–4 Hz (reviewed in Jacobs, 2013).

In line with this data, both, *Fmr1*^{−/y} mice as well as FXS patients were found to show elevated error rates in the Hebb-Williams maze (MacLeod et al., 2010), a test that analyses spatial memory performance in humans and rodents under comparable conditions. In a follow-up study, the group was able to rescue the deficit in *Fmr1*^{−/y} mice using MPEP, an antagonist of mGluR5 (Gandhi et al., 2014), however, contrary to humans, mice did not show increased latencies, implying differences between FXS individuals and model mice. The authors suggest that the discrepancy might arise from differences in the presentation of the maze, that is real vs. virtual, but since the human control group faced the same difficulty, this would rather argue for a different navigation strategy in FXS individuals, a strategy, that relies more on real information, such as obtained from walking, touching or smelling for instance. Indeed, difficulties in abstract thinking are characteristic for FXS patients, but not for model mice, which do as well as their wildtype litter mates in tests employing touch screens (cp. the review of Huddleston et al., 2014 and Leach et al., 2016). Since the Hebb-Williams maze is rarely used for rodents and was not employed to measure outcomes in clinical trials, conclusions are difficult to draw, in particular in the light of the role of cortical connections in human cognition and of the mixed results obtained from mice (reviewed in Kazdoba et al., 2014). However, even under conditions clearly favoring a translation between mice and men, such as in spatial navigation paradigms, the rodent model apparently reflects the situation in humans only partially.

Alternatives

Poor translatability represents indeed a major issue in most preclinical brain research. In order to overcome this obstacle, touch screen paradigms have recently been developed for rodents, which center on the Cambridge Neuropsychological Test Automated Battery for humans (reviewed in Horner et al., 2013; Hvorslef-Eide et al., 2016; Kangas and Bergman, 2017). Although these assays analyze specific cognitive abilities known to be affected in patients and achieve a high degree of standardization as well as high throughputs, *Fmr1*^{−/y} mice failed to recapitulate the working memory impairments characteristic

to individuals with FXS in corresponding tests even when their cognitive performance was challenged in a non-match to position task with increasing delays (Leach et al., 2016). The reasons are elusive. It is possible that the excessive pre-training as well as the correction trials required for mice affect the interpretation of results. Also, since working memory relies on the prefrontal cortex, differences in the cognitive processing can be expected, but studies are missing. More research is required to validate the neurocognitive mechanisms underlying each behavioral paradigm in our species of interest and to identify routes as well as boundaries of translation across species.

The outlined data illustrate that the current FXS mouse models fail to mirror important genetic as well as behavioral aspects of FXS, probably due to the evolutionary distance of mice and men. To address this issue, two rat models were recently developed, *Fmr1* KO²⁹ rats (Engineer et al., 2014; Till et al., 2015; Berzhanskaya et al., 2016b; Kenkel et al., 2016) and *Fmr1* exon4 KO rats (Tian et al., 2017). The investigations revealed that the new model animals indeed recapitulate many features of the murine model, including enhanced basal protein synthesis, exaggerated hippocampal mGluR-LTD, elevated dendritic spine densities and cortical hyper-excitability (Till et al., 2015; Berzhanskaya et al., 2016b; Tian et al., 2017). However and much to a surprise, the rats neither reflect the behavioral phenotype of mice very well (Till et al., 2015), nor reproduce the symptoms of FXS patients any better than the murine model: although the animals demonstrated deficits in hippocampus-dependent learning (Till et al., 2015; Tian et al., 2017), they failed to display defects in spatial reference memory and reversal learning (Till et al., 2015), thereby contrasting not only the majority of data obtained from *Fmr1*^{−/y} mice (D'Hooge et al., 1997; Paradee et al., 1999; Van Dam et al., 2000; Baker et al., 2010, reviewed in Kazdoba et al., 2014), but also the weak performance of FXS patients in tasks requiring the solution of new problems (Dyken et al., 1987; Maes et al., 1994; Loesch et al., 2004; Lewis et al., 2006; Van der Molen et al., 2010). Instead, these results rather imply that there is a clade-dependent component in the behavioral phenotype observed in FXS, causing the same molecular and cellular changes to give rise to different behavioral phenotypes. Given the high number of proteins affected by the loss of FMRP, numerous options exist, through which the genetic background may impact on the symptoms of the disorder. It is indeed long known that the specific phenotype of FXS model mice varies significantly among strains (reviewed in Bernardet and Crusio, 2006; Kazdoba et al., 2014). Further studies would be needed to characterize the performance of FXS model rats in cognition as well as sociality in more detail and to explain certain discrepancies between the two rat models (see Till et al., 2015; Tian et al., 2017), but it might not be worth the effort.

Considering the evolution of cognition in primates, the logical model organism for human brain function would be a closely related primate. Although virus-mediated transgenesis

²⁹knock-out

(Sasaki et al., 2009; Liu et al., 2016; Park et al., 2016) or ZFN³⁰/TALEN³¹-driven genome editing (Sato et al., 2016) have been used to generate primate models, the invention of Crispr-mediated genome editing and its successful employment in primates (Niu et al., 2014; Chen et al., 2015; Tu et al., 2017; Zhao et al., 2017; Zuo et al., 2017) and even in (nonviable) human embryos (Liang P. et al., 2015; Tang et al., 2017) have brought this option within much closer reach. Compared to rodents, primates have several advantages when it comes to modeling cognition (for a comprehensive review, please also see “Why primate models matter”; Phillips et al., 2014), in particular with respect to ASDs:

- the cell types and circuits seen in primates are more similar to those found in humans, which is relevant to many cognitive tasks, but particularly important to spatial working memory and social recognition (see Hopkins, 2013; Frey et al., 2014; Neubert et al., 2014; Morecraft et al., 2015; Wilson et al., 2015a; and the section: “The Cortex, the Hippocampus and FXS”)
- the prolonged prenatal development of the cortex, which is characteristic to humans and primates, is not present in rodents, hampering neuro-developmental studies in rodents
- group living, co-operative behavior and cultural intelligence are much more sophisticated in primates (cp. the section: “The Cortex, the Hippocampus and FXS” and Decasien et al., 2017; Street et al., 2017)
- the basic communication features characteristic to human language are already present in primates, including the ability to utilize symbolization, basic semantic representation, categorical representation and rudimentary grammar (Moore et al., 2003, 2005, 2006; Joly et al., 2012; Morrill et al., 2012; Ghazanfar et al., 2013; Wilson et al., 2013, 2015b)
- many tests developed to analyze cognition in humans can easily be adapted for primates, e.g., eye-tracking to study abnormal gaze (Machado and Nelson, 2011; Rosati et al., 2016), a typical symptom of autism spectrum disorders, or computerized cognition tests (Spinelli et al., 2004; Harris et al., 2007; Barner et al., 2008; Diester and Nieder, 2010; Jones et al., 2010; Takemoto et al., 2011; Verrico et al., 2011; Beran et al., 2012, 2015; Brosnan et al., 2012; Evans and Beran, 2012; Basile and Hampton, 2013; Klein et al., 2013; Bramlett-Parker and Washburn, 2016; Oikonomidis et al., 2017), thereby facilitating the translation of results

Taken together, these studies show that many features characterizing human behavior and typically affected in ASDs, such as communication capabilities and social skills or abstract thinking for instance, are represented in primates. A primate model would hence facilitate investigations addressing core symptoms of the autism phenotype, which are absent or only rudimentary developed in rodents.

There is another important feature of the disease, which cannot be modeled in mice: hypermethylation. Contrary to

mice, in which most CpG-islands have apparently eroded during evolution (Aïssani and Bernardi, 1991; Matsuo et al., 1993), primates show methylation patterns highly similar to those of humans. Approximately 90% of the methylation patterns are in fact conserved between men and chimpanzees (Martin et al., 2011; Molaro et al., 2011; Pai et al., 2011), in particular at CpG-islands and promoter regions (Illingworth et al., 2010; Hernando-Herraez et al., 2013, 2015; Long et al., 2013). However, when comparing those genes that are differentially methylated, human genes turned out to exhibit lower levels of promoter methylation than genes from chimpanzees (Gama-Sosa et al., 1983; Zeng et al., 2012), suggesting that gene expression control is less strict in humans. Since a disproportional high number of differentially methylated genes is associated with human diseases (Zeng et al., 2012; Fukuda et al., 2013; Hernando-Herraez et al., 2013), it seems possible that the reduced control of certain genes, which finally made us human, also makes us more susceptible to certain pathologies such as senescence and Alzheimer's disease, disorders, which are not present in our closest relatives, the great apes (reviewed in Finch and Austad, 2015; Lowenstine et al., 2016). Notably, though some of the genes shown to be differentially methylated in humans are known for their association with ASDs, such as GABAA and GABAB receptors for instance (Zeng et al., 2012; Hernando-Herraez et al., 2013), *FMR1* was not associated with human-specific methylation patterns yet.

Despite these promising premises, primate models also have some disadvantages when compared to rodent models: Working with primates, in particular with great apes, is expensive by all means, it requires a lot of training and experience, bears the risk of transmitting diseases and the longevity of great apes hampers investigations of senescence. Significant n-numbers are often hard to achieve. Hence, primate species which are small, relatively short lived and easy to keep could help to overcome these obstacles.

The evolutionary next closest alternatives to great apes are old world monkeys. Consisting of about 160 species, old world monkeys represent the largest group of primates. Among them are the macaque monkeys, which include two species, that have been used in science for a long time, the crab-eating (alias: cynomolgus; *M. fascicularis*) and the rhesus macaque (*M. mulatta*). These two species are the widest spread primates next to humans and are categorized as species with Least Concerns by the Red List. Facilitating housing and breeding, macaques live in large multi-male, multi-female groups, mature at the age of app. 3.5 years (great apes: 6–10 years) and have a gestation period of 160–180 days (great apes: 225–270 days), giving birth to one infant per parturition (reviewed in Lindburg, 1991).

Notably, crab-eating macaques have recently been used to model ASDs (Liu et al., 2016; Zhao et al., 2017). Liu and colleagues expressed human MeCP2³² in the macaque brain to mimic an ASD resulting from duplications of the *MECP2*-containing genome segment, whereas Zhao and colleagues

³⁰zinc-finger nucleases

³¹transcription activator-like effector nucleases

³²methyl CpG binding protein 2

introduced deletions to *SHANK3*³³, which are known to cause ASD. Remarkably, both studies found striking differences compared to the corresponding mouse models: while the *MECP2*-overexpression model demonstrated clear autism-like behaviors in primates, but not in mice, the *SHANK3*-deletion model revealed significant differences in the expression pattern of Shank3, and found an impaired neurogenesis in the prefrontal cortex of macaques.

Despite these promising results, macaque models also have some disadvantages: The monkeys are carriers of Herpes B viruses and may transmit this potentially fatal disease to humans. Their long adolescence (3.5 years) hampers breeding, and their longevity (approximately 30 years in captivity) is clearly not supporting studies of senescence. Although highly developed, their social system is not in favor of ASD research as well, since macaques live in large multi-male/female groups, whereas humans live in (extended) family groups. As a result, proactive prosociality, which is believed to be based on shared infant care, is well developed in humans, but not in chimpanzees or macaques (Burkart and van Schaik, 2013).

For these reasons, marmosets have recently garnered interest as model animals for studying brain function (reviewed in Saito, 2015; Tokuno et al., 2015), for a general review on the species, please see (Schiel and Souto, 2017). Also known as zaris, these new world monkeys split from the primate lineage approximately 40 million years ago, 35 million years before humans and chimpanzees separated. Compared to great apes and macaques, marmosets have the advantage of being small, easy to handle, easy to breed and less expensive: with a life span of about 12 years in captivity, marmosets mature more quickly than great apes or macaques, starting reproduction at 18 months of age and giving birth to twins after a gestation period of 140–150 days. Contrary to macaques, marmosets do not represent a natural reservoir of Herpes B viruses.

Though lissencephalic (smooth), their brain shares many features of the human brain, and their cerebral cortex shows the neuronal architecture of all primates (Bendor and Wang, 2005; Burman et al., 2006; Elston et al., 2006; Burman and Rosa, 2009; de la Mothe et al., 2012; Chaplin et al., 2013), from which only humans differ in that neurons of their prefrontal cortex are more spiny and more complex than those of their primate relatives (Elston et al., 2006). Consequently, marmosets have the high cognitive abilities characteristic to all primates: they are not only able to perform true imitation (Bugnyar and Huber, 1997; Voelkl and Huber, 2007), transposition and generalization (Yamazaki et al., 2014) but to also solve string problems (Halsey et al., 2006; Gagne et al., 2012) and to understand physical causality (Yamazaki et al., 2011). Considering the weaknesses of FXS individuals in abstract item reasoning and in addressing new problems, the cognitive abilities of marmosets might help to develop tasks which directly translate results between the two species.

Of particular interest for the design of ASD models is the sociality of marmosets. In a convergent evolution to humans, marmosets developed a high level of group living (reviewed

in Graham, 2016). At a state of sophistication not known from other mammals including most primates, marmosets demonstrate altruistic behaviors such as proactive prosociality and third-party reciprocity (Burkart et al., 2007; Burkart and van Schaik, 2013), social learning during infancy (Dell'Mour et al., 2009) as well as adulthood (Caldwell and Whiten, 2004), and behavioral adaptations to social environments (Koski and Burkart, 2015). Similar to humans, marmosets live in extended family groups, pair-bond and care for their offspring in a cooperative manner (Digby and Barreto, 1993; Sousa et al., 2005; Birnie et al., 2013; Yamamoto et al., 2014). Since parental care and in particular maternal warmth are known to influence FXS symptoms in children (Dyer-Friedman et al., 2002; Kuo et al., 2002; Glaser et al., 2003; Greenberg et al., 2012; Robinson et al., 2016; Smith et al., 2016), the latter could be useful to further investigate the role of the social environment for FXS.

Like other primates, marmosets mostly rely on vocalizations (Miller and Wang, 2006; Chen et al., 2009; Miller et al., 2010; Watson and Caldwell, 2010; Bakker et al., 2014; Kato et al., 2014; Agamaite et al., 2015) and visual cues (de Boer et al., 2013; Massen et al., 2016) for their communication, although social grooming (Lazaro-Perea et al., 2004) and scent marking (Epplé, 1970; Massen et al., 2016) are present as well. Since ASDs are characterized by gaze avoidance, it is particularly interesting that marmosets are able to use a variety of facial expressions for communication (Kemp and Kaplan, 2013) and to gain information by geometrical gaze following, even from human experimenters (Burkart and Heschl, 2006). Analyzing the face scanning patterns performed by marmosets, Kotani et al. (2017) showed that these primates primarily view the eye region during contact and that this behavior can be employed to evaluate the influence of drugs.

Due to the dense vegetation of their natural habitat, vocalizations are unusual rich in marmosets, even for primates (Epplé, 1968; Morrill et al., 2013; Agamaite et al., 2015). Marmoset conversations involve a cooperative vocal control and require the infants to learn when to talk and when to listen (Takahashi et al., 2013, 2016; Choi J. Y. et al., 2015; Chow et al., 2015), thereby paralleling human development. Indeed, recent research revealed that marmoset infants need to transform their babbling and crying into mature vocalizations in order to properly communicate with other group members (Margaret Elowson et al., 1998; Pistorio et al., 2006; Takahashi D. Y. et al., 2015; Ghazanfar and Zhang, 2016) and that this transformation requires social reinforcement from caregivers (Margaret Elowson et al., 1998; Takahashi et al., 2017). Given the ability of marmosets to even learn grammar (Wilson et al., 2013) and the cluttered speech observed in individuals with FXS (Hanson et al., 1986; Belser and Sudhalter, 2001; Roberts et al., 2007; Klusek et al., 2014), the high level of vocal communication in marmosets could be valuable to the development of treatments for FXS and ASD patients.

Though their sociality and their cognitive abilities make marmosets a promising model organism for FXS and ASD research, it is just this very same point, which also bears the disadvantages. The high level of communication and

³³SH3 and multiple ankyrin repeat domains 3, alias: proline-rich protein 2

understanding of social interactions increases the risk that relations between researchers and experimental animals arise, which may impact on experiments (cp. Herzog, 2002), for example, if individual animals dislike or prefer being handled or cared for by a certain person and subsequent stress levels influence the outcome of an experiment. Since the n-numbers used in primate research are at the minimum, such effects might easily turn out “significant”.

Another important concern is morality. Although all mammals are able to feel pain, stress and fear in a similar way, no matter whether they are rodents, primates or of any other clade, the high cognitive capabilities and social skills of primates have raised particular ethical concerns regarding their use in biomedical research (cp. the reviews of Coors et al., 2010; Phillips et al., 2014; Zhou, 2014; Bailey and Taylor, 2016; Arnason, 2017). As a consequence, many countries have implemented policies and regulations to ensure the physiological as well as psychological well-being of all research animals (Pereira et al., 2004; Luy, 2007; Hansen et al., 2017), and the standards are particularly high for primates (see Tardif et al., 2013; Weiss and Hampshire, 2015). However, the ethical viewpoints often differ, not only within a society (cp. Buckley et al., 2011; Phillips et al., 2014; Arnason, 2017 vs. Bailey and Taylor, 2016), but also among societies (cp. Cyranoski, 2004; Zhang X. L. et al., 2014), causing the hurdles for the approval of a project to vary significantly between countries and sometimes even within countries. The same project might thus be approved within a few weeks in one country, within a few months in another, and not in years in the next.

These circumstances raise several questions:

Is it ethically justified to approve therapies developed in countries with lower animal welfare standards in countries with

higher standards, considering that such approvals would torpedo all animal welfare efforts? Is it ethically justified to deny patients a therapy developed in a country, which had lower animal care standards? Is it ethically justified to have wealthy people traveling to countries offering corresponding therapies, while others cannot afford to do so?

Moreover: can cognitive abilities indeed be a measure to define welfare standards and requirements? Where is the red line? These two questions are intimately connected, since the use of cognitive capabilities as an argument for increased standards in turn causes diminished abilities to result in the opposite. Since everything goes the way of least constrains, this is the point where the seemingly high moral standards cause ethics and science to start losing ground: is it ethically and scientifically justified to use thousands of mice in preclinical research, to enrol hundreds of patients in clinical studies, to put patients at risk, albeit the risk might be marginal, and to cause hopes and disappointments in their families, just to learn how hard it is to find out how to keep the Concorde flying from studying a biplane?

AUTHOR CONTRIBUTIONS

RD wrote the manuscript.

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Utility of the Hebb–Williams Maze Paradigm for Translational Research in Fragile X Syndrome: A Direct Comparison of Mice and Humans

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To generate meaningful information, translational research must employ paradigms that allow extrapolation from animal models to humans. However, few studies have evaluated translational paradigms on the basis of defined validation criteria. We outline three criteria for validating translational paradigms. We then evaluate the Hebb–Williams maze paradigm (Hebb and Williams, 1946; Rabinovitch and Rosvold, 1951) on the basis of these criteria using Fragile X syndrome (FXS) as model disease. We focused on this paradigm because it allows direct comparison of humans and animals on tasks that are behaviorally equivalent (criterion #1) and because it measures spatial information processing, a cognitive domain for which FXS individuals and mice show impairments as compared to controls (criterion #2). We directly compared the performance of affected humans and mice across different experimental conditions and measures of behavior to identify which conditions produce comparable patterns of results in both species. Species differences were negligible for Mazes 2, 4, and 5 irrespective of the presence of visual cues, suggesting that these mazes could be used to measure spatial learning in both species. With regards to performance on the first trial, which reflects visuo-spatial problem solving, Mazes 5 and 9 without visual cues produced the most consistent results. We conclude that the Hebb–Williams mazes paradigm has the potential to be utilized in translational research to measure comparable cognitive functions in FXS humans and animals (criterion #3).

Keywords: Fragile X syndrome, human, mouse, spatial learning, Hebb–Williams maze, translational research

INTRODUCTION

“By carefully selecting tasks for animals with high construct validity to human tasks, reliability and accuracy of translational efforts will not be lost and meaningful progress can be made in ameliorating the cognitive deficits that affect the lives of those suffering from mental illness.”

(Gilmour et al., 2013, p. 2126).

The quotation above articulates the potential benefits of translational research for society and stresses the need to carefully select tasks that allow extrapolation from animal models to humans in order to reap these benefits. Surprisingly, there is little consensus on what criteria should be used for validating translational paradigms, a fact that significantly hinders selection of appropriate tasks

(see Willner, 1986 for validation criteria for animal models) and therefore limits the likelihood that laboratory research will translate to human treatments. We address this limitation by outlining three criteria for validating translational paradigms. We then use these criteria to evaluate the Hebb-Williams maze paradigm using Fragile X syndrome (FXS) as a model disease.

Fragile X syndrome is the most common identifiable genetic cause of intellectual disability and a leading genetic cause of autism spectrum disorder (ASD) (e.g., Hagerman, 1987; Farzin et al., 2006). The disorder arises from mutation of a single gene, the FMR1 (Fragile X Mental Retardation 1) gene, which codes for a protein that plays a key role in experience-dependent synaptic plasticity (e.g., Huber et al., 2002). The mutation is caused by intergenerational expansion of a trinucleotide region upstream of the coding sequence results in methylation and silencing of the FMR1 gene [Online Mendelian Inheritance in Man® (OMIM) 309550¹; Verkerk et al., 1991]. The silencing of the FMR1 gene results in several significant behavioral and cognitive impairments including deficits in attention (Backes et al., 2002; Baumgardner et al., 1995), visual-spatial cognition (Crowe and Hay, 1990; Cornish et al., 1998, 1999), working memory (Schapiro et al., 1995; Jakala et al., 1997), and visual-perceptual processing (Kogan et al., 2004a). Because it is the outcome of a single gene defect, FXS offers a remarkable opportunity to investigate the validity and feasibility of translational paradigms by comparing the animal model with affected individuals. The animal model for the disease, the *fmr1* knock-out (KO) mouse, has significantly contributed to our understanding of the neurobiology and synaptic mechanisms of the disorder and to the identification of potential therapeutic agents (e.g., Berry-Kravis et al., 2011). Despite these advances, translation of drug discovery research from the mouse model to humans has proven difficult (e.g., Arbaclofen trial: Berry-Kravis et al., 2017), highlighting the need for the identification of valid translational tasks.

Drawing from the literature (Willner, 1986; Gilmour et al., 2013; McGonigle and Ruggeri, 2014; Gabel et al., 2016), we propose that to allow for appropriate extrapolation from animal to human studies, translational paradigms must: (1) allow direct comparison of humans and animals on tasks that are behaviorally equivalent, (2) measure constructs that are fundamental to the disorder, and (3) engage comparable underlying neural mechanisms and cognitive functions in both species². To our knowledge, only three studies have directly compared the performance of FXS humans and KO mouse on behaviorally equivalent paradigms (Frankland et al., 2004; Koekkoek et al., 2005; MacLeod et al., 2010; see, e.g., Gilmour et al., 2013; Gabel et al., 2016 for other conditions). Two studies (Frankland et al., 2004; Koekkoek et al., 2005) employed paradigms that measure prepulse inhibition, a function that is thought to be impaired in

FXS as indicated by heightened sensitivity to sensory stimulation (mouse: Chen and Toth, 2001; humans: Miller et al., 1999). The other study employed a paradigm that measures spatial processing (MacLeod et al., 2010), a central feature of cognitive impairment in affected individuals (e.g., Crowe and Hay, 1990; Cornish et al., 1998, 1999; Kogan et al., 2004a) and the murine model (e.g., Baker et al., 2010; Gandhi et al., 2014b, but see Fisch et al., 1999). We review their findings below.

Frankland et al. (2004) measured prepulse inhibition to acoustic startle in *fmr1* KO mice, affected individuals and their respective comparison groups. Whereas prepulse inhibition was enhanced in KO mice as compared to controls, it was significantly reduced in humans. The authors concluded that the *FMR1/fmr1* gene impacts sensory processing in both species. However, because the results were opposite in direction for mice as compared to humans, it is unclear whether different mechanisms might be measured by this paradigm across the two species. Koekkoek et al. (2005) used an eye blink reflex paradigm to measure prepulse inhibition. They report a comparable reduction in prepulse inhibition in FXS humans and KO mice as compared to their respective comparison groups. This eye blink reflex paradigm has also been used in translational research to successfully demonstrate efficacy of a metabotropic glutamate receptor antagonist (i.e., Fenobam) for reversing prepulse inhibition in affected humans (Berry-Kravis et al., 2009). While these results point to the utility of the eye blink paradigm for translational research, it measures a discrete sensorimotor gating function that provides limited insight in to the central cognitive impairments in FXS.

MacLeod et al. (2010) used the Hebb-Williams mazes paradigm to measure visuo-spatial abilities in *fmr1* KO mice, affected individuals, and their respective comparison groups. Hebb-Williams mazes require successful navigation through different mazes whose configurations can be varied to provide problems of varying difficulty (Rabinovitch and Rosvold, 1951; Meunier et al., 1986). MacLeod et al. (2010) employed this paradigm because both traditional animal-based and computer versions of the task exist, allowing researchers to test mice and humans under equivalent conditions (Shore et al., 2001). Moreover, performance on the mazes appears to be dependent on the integrity of brain areas with known impairment in FXS humans and KO mice. Lesion studies in mice have demonstrated that successful performance on paradigms such as the radial arm maze, T-maze, and water maze rely on intact hippocampal processing (Mitchell et al., 1982; Morris et al., 1982; Hock and Bunsey, 1998; Okada and Okaichi, 2009). Equivalent results are found with similar visuo-spatial tasks in humans (Rogers and Kesner, 2006; Hunsaker et al., 2008). Structural abnormalities of the hippocampus are seen in FXS patients (see Bostrom et al., 2016 for a review). Although similar gross morphological differences have not been observed in the *Fmr1* KO mice, significant neuronal pathology has been described in the form of longer dendritic spines in pyramidal cells in subfield CA1 (Grossman et al., 2006), smaller intra-infra pyramidal mossy fiber terminal fields (Mineur et al., 2002), as well as shorter dendrites, fewer dendritic spines and functional synaptic connections (Braun and Segal, 2000). Studies of human

¹<http://www.ncbi.nlm.nih.gov/omim>

²Note that criterion #3 is derived from Willner (1986) and Sjöberg (2017). While Willner's discussion of validity focuses on the cognitive processes that underlie psychopathology (e.g., memory, perception, motivation, etc.), Sjöberg extends the discussion to include underlying biological mechanisms. While both are relevant and important in FXS, the present study, which was conducted from an experimental psychology perspective, focuses on the cognitive functions measured by the Hebb-Williams paradigm, namely visuo-spatial abilities.

hippocampal neuronal dysmorphology reveal a higher density of dendritic spines suggestive of abnormal synaptic pruning in FXS (Irwin et al., 2000). Another area involved in maze performance is the posterior parietal cortex. This area is recruited during spatial navigational tasks in humans (Hyvarinen and Poranen, 1974; Andersen and Buneo, 2002; Spiers and Maguire, 2007) and mice (Harvey et al., 2012). Kogan et al. (2004b) demonstrated that visual-spatial processing associated with the posterior parietal cortex is selectively impaired in humans with FXS. Finally, the paradigm assesses cognitive functions that are related to the clinical features of the disorder and that are the principle targets for interventions. MacLeod et al. (2010) found comparable impairments on the mazes for both KO mice and affected humans which manifested as a lack of improvement in performance across trials, indicative of a spatial learning deficit. At the molecular level, Gandhi et al. (2014a) showed that a metabotropic glutamate receptor antagonist (i.e., MPEP) reversed these deficits in KO mice.

These findings suggest that the Hebb–Williams paradigm meets two of the criteria for validating translational paradigms: it allows for direct comparison of humans and animals on a paradigm that is behaviorally equivalent (criterion #1), and it measures cognitive functions and underlying neural mechanisms that are fundamental to the disorder (criterion #2). In addition, unlike other cognitive tasks, the Hebb–Williams maze paradigm is not too difficult for individuals with FXS to complete nor does it produce unacceptable levels of variability (Berry-Kravis et al., 2006). In the present study, we evaluated whether the paradigm meets criterion #3, with an emphasis on whether the paradigm engage comparable underlying cognitive functions in both species. We directly compared the performance of FXS humans and KO mice across different experimental conditions and measures of behavior. Our goal was to identify which conditions, if any, yield similar performance and hence which conditions might be used to measure comparable underlying cognitive functions in both species. This *direct comparison* approach complements the *homology of impairments* approach (Sjoberg, 2017) adopted by the aforementioned studies where mutant mice were compared to wild-type controls and FXS individuals were compared to typically developing participants or those matched for intellectual ability. While the homology of impairments approach provides valuable information for the identification of key features of the disorder, it is less appropriate for identifying tasks that engage comparable underlying mechanisms across species. Indeed, this approach assumes that reduced performance of affected participants compared to controls on equivalent tasks reflects impairments in the same underlying mechanism(s) in humans and animals (Sjoberg, 2017). This assumption might not always be valid for the following reasons. First, there are difficulties inherent to matching comparison groups to participants affected by FXS. Human studies typically match on the basis of chronological age or intellectual ability (e.g., Frankland et al., 2004; Kogan et al., 2004a, 2009; Lightbody et al., 2006; MacLeod et al., 2010; Klusek et al., 2015; McDuffie et al., 2015). Such techniques limit test implementation and interpretation of group effects. For example, choosing chronological age matched comparisons might yield

ceiling effects for the unaffected group or floor effects in the FXS group. Moreover, impairments in the FXS group might be better explained by differences in understanding instructions rather than an impairment in the cognitive function of interest. On the other hand, similarities and/or differences between FXS individuals and mental-age matched comparisons, who are typically younger, might be better explained by differences in their stage of development rather than serve as evidence for preservation or impairment in a specific cognitive domain. Similarly, in mouse studies, there is no universal methodology for matching the comparison group to the KO group on the basis of intellectual abilities. Instead, behavior of KO mice is typically compared to that of age-matched wild type littermates, making it impossible to determine whether group differences arise from general cognitive impairments or from impairment of a distinct cognitive function. Second, the neuroconstructivist view of development (reviewed by Karmiloff-Smith, 1998, 2009) suggests that when there are no differences in performance in special populations vs. typically developing individuals, one cannot necessarily conclude that cognitive function is normal in the special population because the two groups may rely on different underlying mechanisms to perform the task. Differences in underlying mechanisms may arise because alterations at the level of gene expression (e.g., see Kooy, 2003; Erriegers and Kooy, 2004) could compensate for perturbations in the course of development such that affected individuals demonstrate normal or near normal performance at the behavioral level. It is also possible that affected individuals compensate by employing different neuronal structures to achieve the same outcome as typically developing individuals. Such considerations limit inferences that can be drawn on the basis of the homology of impairments approach, especially with respect to the measure of equivalent underlying mechanisms in humans and animals.

In the present study, we adopted a direct comparison approach to address these limitations. Indeed, to be informative and ultimately predictive of therapeutic response, translational paradigms should measure the same underlying construct across species (Willner, 1986; Gilmour et al., 2013). While there is no perfect solution, introducing a procedural modification or new variable to a paradigm with the goal of examining whether both species react similarly to this modification provides compelling evidence that equivalent underlying mechanisms are being measured (Willner, 1986; Shore et al., 2001; van der Staay et al., 2010; Sjoberg, 2017). This approach circumvents some of the limitations associated with the homology of impairments approach. It is important to note that we conceptualize these two approaches as complementary in yielding evidence in favor of the validity of a specific translational paradigm. We do not question the valuable contribution made by studies adopting the homology of impairments approach but instead highlight limitations of this approach to advancing the field of translational research. An optimal outcome is one whereby a behaviorally equivalent paradigm is first shown to measure key features of the disease by yielding homologous performance differences between comparison and affected participants across species, followed by a direct comparison of humans and animals on variations of the paradigm to identify conditions yielding comparable patterns of

performance, which would suggest that the paradigm taps into comparable underlying mechanisms.

Having already established the potential for the Hebb–Williams mazes to serve as a translational paradigm using a homology of impairments approach (MacLeod et al., 2010), we sought complementary evidence in the present study using a direct comparison approach. We compared the profile of performance of FXS participants with that of KO mice on two variants of the Hebb–Williams paradigm and on multiple measures of performance. We compared data obtained on the standard version of the paradigm (MacLeod et al., 2010) as well as new data obtained on a variation with visual cues. We also performed multiple new analyses to directly compare humans and mice on various indicators of cognitive function and performance. Performing multiple cross-species comparisons across experimental conditions and measures (Vorhees and Williams, 2014) allowed us to identify which conditions, if any, produce comparable patterns of performance across species. As such, the present study seeks to identify which conditions of the paradigm meet *all* of the validation criteria outlined herein by accruing new data with respect to criterion three. This study therefore seeks to validate the Hebb–Williams maze paradigm for use in translational and drug discovery research and to provide practical information regarding which conditions and measures offer the best potential for extrapolating from mice to humans.

Notwithstanding the variable nomenclature and experimental manipulations that have been used to describe and investigate the impact of visual cues on spatial learning, evidence from both animals and humans suggest that the presence of visual cues in maze environments improves learning in typically developing participants (Heft, 1979; Jansen-Osmann and Fuchs, 2006; Waller and Lippa, 2007; reviewed by Chan et al., 2012). However, in certain populations visual cues may be ignored rather than being used to assist navigation (Wilkniss et al., 1997; Moffat and Resnick, 2002; Hanlon et al., 2006; Robaey et al., 2016). Particularly relevant to FXS, Robaey et al. (2016) showed that children who exhibit symptoms of Attention Deficit Hyperactivity Disorder (ADHD) do not rely on visual cues while navigating in an eight-arm radial maze. Because a majority of males with Fragile X exhibit symptoms of ADHD (e.g., Hagerman, 1987; Hatton et al., 2002; Farzin et al., 2006; Sullivan et al., 2006), introducing visual cues to the Hebb–Williams paradigm provides a critical experimental variation upon which to compare humans and mice. We also conducted new analyses to determine whether the two species display comparable patterns of results for the two main processes involved in solving mazes, namely basic visual functioning necessary for solving a novel spatial task and learning/memory. Indeed, while performance on the first trial is thought to reflect visuo-spatial processing and problem solving (Hebb and Williams, 1946), performance on subsequent trials reflects memory for the configuration of the maze and goal location as indicated by rate of learning across trials. Finally, we examined the relative difficulty of the mazes across the two species (Meunier et al., 1986). Level of difficulty is an index of problem-solving complexity and comparable patterns would suggest similar approaches to maze problem-solving in both species.

MATERIALS AND METHODS

Mice

Two groups of male FVB.129P2-*Fmr1*^{tm1Cgr}/J mice (JAX Stock #004624) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, United States). The first group of mice provided data which has not been previously published on the paradigm with visual cues. The second group corresponds to mice tested in MacLeod et al. (2010) on the standard paradigm. Each strain had been backcrossed for 11 generations. Mice are pigmented (gray), do not carry the *rd1* mutation and possess the wild type *Pde6b* allele indicating that they do not suffer from blindness due to retinal degeneration. For the standard group, 11 animals were shipped at 4 weeks of age and were tested when they were approximately 3 months old. For the visual cue group, which was tested a year after the standard group, 10 animals were tested when they were approximately 5 months old. Eight days prior to behavioral testing, all subjects were individually housed in a climate-controlled vivarium (20–22°C) that was maintained on a 12 h light-dark cycle with lights on from 0700 to 1900. All testing was conducted during the light phase of the cycle. Mice were fed Harlan Global Rodent Chow and tap water. They were maintained at 85–90% of their *ad lib* body weight. Mice were weighed daily and fed their individually weighed ration of food 30 min after completion of the session. The mice were treated in accordance with the guidelines and principles set by the Canadian Council on Animal Care and tested under a protocol approved by the University of Ottawa Animal Care Committee.

Mouse Apparatus

Mice were tested using the Hebb–Williams maze apparatus as described by Meunier et al. (1986). The apparatus was constructed using black opaque Plexiglas and was covered with a clear Plexiglas top (Plastics of Ottawa Ltd., Ottawa, ON, Canada). It consisted of a square open field (60 cm × 60 cm × 10 cm) with start and goal box compartments (20 cm × 10 cm × 10 cm) located at diagonally opposite corners. These compartments were fitted with clear Plexiglas lids that were attached with hinges and could be blocked with removable clear Plexiglas barriers. The goal box was fitted with a ledge (8 cm × 2.5 cm) with a recessed food cup in the center (2.5 cm diameter). The floor of the maze was divided into 36-equal squares that were clearly outlined in white. The squares were used as markers for placing the barriers in different maze configurations and to define error zones. The same maze configurations as in MacLeod et al. (2010) were employed, namely mazes 2, 4, 5, 8, 9, 11, and 12 (Rabinovitch and Rosvold, 1951). Testing was in the following order: Maze 12, 2, 8, 4, 5, 9, and 11. Pilot data with humans from our laboratory suggested that the other mazes were too easy and might yield ceiling effects. The order was determined on the basis of maze difficulty (less to more difficult) as assessed using pilot data. Removable barriers (10 cm high) were created using black opaque Plexiglas and each was supported by two permanent bases (2.5 cm × 2.5 cm). Extra-maze cues were minimized by conducting the study in an all-black enclosure and by having a dim light as the only source of illumination.

For the visual cue group, six images of simple geometric shapes (e.g., circle, square, and triangle) surrounded by a white background were added to each of the mazes (10 cm × 10 cm). An identical set of six shapes was used in each maze configuration. Each set of shapes was the same color as that of the test maze with the white background clearly distinguishing the shape from the maze wall. The visual cues were distributed within a test maze such that at least one image was visible from any position within the maze. Visual cues were laminated and adhered to the interior of the maze with double-sided tape.

Mouse Procedure

The protocol consisted of three consecutive phases: habituation, acquisition, and testing. Initially, mice were habituated to the maze environment for 20 min per day on 4 consecutive days with barriers and doors to the start and goal box removed. During the last two sessions the goal box was baited (Rodent Chow, 100 mg) and mice had *ad lib* access to the food for the duration of the session. Subsequently, mice from both groups were trained on six acquisition mazes without visual cues as described by Rabinovitch and Rosvold (1951) (Figure 1a). On any given day, mice were tested such that they completed five trials for each of two of the six acquisition mazes. Mice completed all six acquisition mazes in sequence as many times as necessary for them to attain the criterion performance, which was defined as two consecutive sessions completed successfully in less than 30 s each. The acquisition phase required an average of 7 days to complete. On each acquisition trial, mice received a small reinforcer (Rodent Chow, 20 mg). Immediately following acquisition, mice were given a selection of the standard test

mazes over 4 days (Rabinovitch and Rosvold, 1951) according to the same training protocol used during acquisition sessions. None of the acquisition or testing sessions exceeded 180 s. Mice completed the Rabinovitch and Rosvold (1951) maze configurations. Latency and number of errors were recorded. Latency was recorded from the moment the barrier at the start box was removed until the animal took its first bite of food. An error was scored each time the animal's two front paws crossed into an error zone (Figure 1b). Experimenters were blind to the animal genotypes and were never visible to the mice during the runs. The maze was thoroughly cleaned between trials and all trials were recorded using a closed-circuit camera mounted on the ceiling directly above the maze.

Human Participants

Twenty-six participants were recruited from patient contact lists at Rush University Medical Center, Chicago, IL, United States. Participants either completed the mazes with visual cues ($n = 10$, mean chronological age = 22 years, $SD = 3.84$, mean verbal mental age = 6.26 years, $SD = 3.02$) or without visual cues ($n = 15$, mean chronological age = 24 years, $SD = 4.9$, mean verbal mental age = 7.57 years, $SD = 1.29$). The first group of participants provided data, which has not been previously published, on the paradigm with visual cues. The second group corresponds to participants tested in MacLeod et al. (2010) on the standard paradigm. All had a DNA-confirmed diagnosis of FXS. Informed consent was obtained from caregivers and assent was obtained from the individuals with FXS. All participants were paid \$25 per hour for their participation in the study and were treated in accordance to the ethical principles established

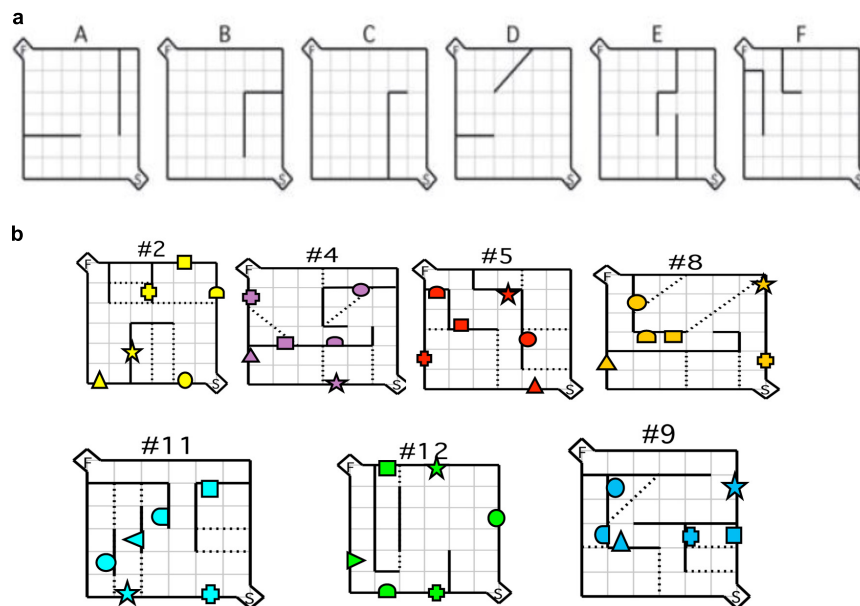


FIGURE 1 | Maze configurations. (a) Testing was conducted using the six practice mazes (A–F) and (b) the seven test mazes depicted. For each maze configuration, the (S) depicted in the bottom right hand corner represents the start box, and the (F) in the top left corner represents the goal box. Error zones are depicted by the dotted lines. Location of visual cues is depicted by geometric shapes. Reprinted in part from MacLeod et al. (2010). A comparative study of the performance of individuals with Fragile X syndrome and *Fmr1* knockout mice on Hebb–Williams mazes. *Genes, Brain and Behavior*, 9(1), 53–64.

by the Research Ethics Board at the University of Ottawa. Both the ethics committees of the School of Psychology, University of Ottawa, and of the Rush University Medical Center approved the study. Written informed consent was obtained from carers of the participants. Participants also provided their written assent to participate in the study.

Human Measures

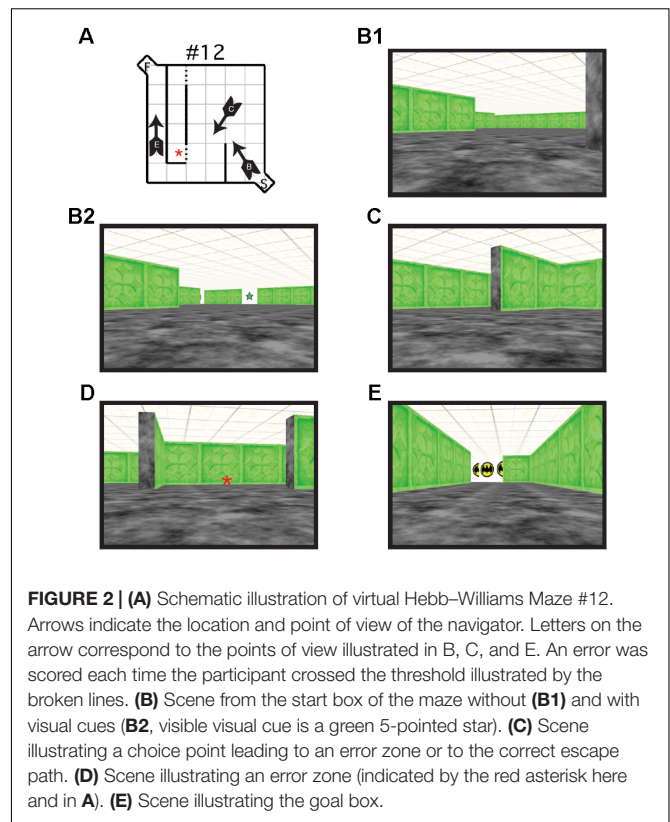
A brief Medical History Questionnaire was administered to all caregivers of participants to screen for any problems that would exclude them from the study. Exclusion criteria were any significant health or vision difficulties (e.g., color blindness, amblyopia, astigmatism, etc.) that would impact controlling a joystick or viewing the maze stimuli. Four FXS participants were excluded from the study because they did not complete at least half of the mazes. These participants reported and exhibited symptoms of anxiety and appeared distracted throughout the administration.

Human Apparatus

All participants were tested using a version of the virtual Hebb–Williams maze designed by Shore et al. (2001). Five mazes were eliminated from the original Hebb–Williams set for the purpose of this project because our pilot studies indicated that participants found these mazes too easy. In order to reduce administration time, a factor that is particularly important when testing participants affected by intellectual disability, only the most challenging mazes were used. All participants were tested on the remaining subset of mazes.

Experiments were performed on an Asus PC with a 19-inch Acer LCD monitor. Mazes were displayed at a resolution of 640×480 in full-screen mode. Participants navigated through the virtual environment at a constant velocity of 12 km/hr (forward, backward) and a turn rate of 50 degrees per second (left, right) using a Logitech Attack 3 joystick. Assuming a viewing height of 5 ft 6 in., the projection of the whole maze appeared to participants as 20 m^2 , and the diagonal straight line from start to finish was perceived as being located at a distance of 28.3 m.

Each maze was made up of a 6×6 room, with a 1×1 alcove at the entrance (start area) and exit (goal area) of the maze. Walls were created using textured rectangles that differed in color depending on the maze configuration. A different color was used for each maze configuration to indicate to participants that a new maze was being presented. The start alcove and the floors were textured with black and gray marble effect. Each wall of the goal alcove was white and contained the image of a comic book character to provide motivation and reward for the participants. The roof was textured using beige and brown mottled square tiles (Figure 2). For the visual cue group, the Hebb–Williams virtual maze was identical to that used in the standard condition, with the exception of the addition of visual cues. For each test maze, six images of simple geometric shapes (e.g., circle, square, and triangle) surrounded by a white background were added to the maze environment ($10 \text{ cm} \times 10 \text{ cm}$). Geometric shapes were used because of they are easy to recognize and discriminate across age ranges. An identical set of six shapes was placed in each maze configuration. Each set was the same color as that of the test maze



with the white background clearly distinguishing the shape from the maze wall (Figure 1b). Visual cue placement was the same as was used for the mouse apparatus.

Human Procedure

All participants were individually tested by a research assistant, in a quiet room without their caregivers present. The tasks were administered during a 1–1.5 h session and presented in a standardized order as described above. Participants were trained on two types of practice mazes. An alley maze was presented first and enabled participants to establish how to adaptively maneuver through the virtual environment, while maintaining direct visual contact with the goal area. After meeting this criterion, a T-maze was presented in which participants had to choose a virtual navigational pathway in order to practice searching for the goal area of the maze. Visual cues were not provided during practice. Criterion was achieved in both acquisition mazes when participants could complete three consecutive maze trials in less than 30 s each. At any time if a participant exceeded 120 s during a trial, the trial was considered finished and the participant proceeded to the next maze. For both the acquisition and testing mazes, participants received a sticker as reward after each trial, and after completing all three trials of a maze they received a small piece of candy to be saved and consumed after the experiment was terminated.

After the acquisition sessions, human participants completed three trials of each test maze (Figure 1b). In between testing for each maze, participants were provided with a 2-min break,

at which time a children's DVD was played. After completing the fourth maze (#8) all participants were given a 10-min break. Latency for solving the maze (time taken from the maze entrance to exit) and number of errors (number of times a participant crossed a predefined error line – see **Figure 1b**) were calculated.

Statistical Analyses

Because of floor effects and large variability across participants for both error and latency, data from Maze 11, the last maze tested, is excluded from this study. Outliers were removed from the data such that any score that was more than 2.5 SD away from the group mean was replaced by the next lowest or highest score that is within 2.5 SD of the mean. Because error and latency measures did not appear to meet the assumption of normality upon visual inspection, both measures were square root transformed.

Inverse Efficiency

Inverse efficiency allows for direct cross-species comparisons within the same statistical analysis (Shore et al., 2001). Moreover, inverse efficiency is the most appropriate measure for cross-species comparisons because humans and mice can adopt different strategies with respect to speed vs. accuracy to solve a maze (Shore et al., 2001). It is calculated by standardizing raw latency and raw error scores into Z-scores using the overall grand means and standard deviations from all subjects of the same species. Performance inverse efficiency scores were also calculated as follows: $Z(\text{Latency}) + Z(\text{Error})/2$. Larger inverse efficiency scores indicate relatively poorer maze performance. This composite measure weights increases in latency and error equally and therefore accounts for differences between species in the relative contribution of errors and latency to overall performance (Shore et al., 2001).

Rate of Learning

To compare learning and memory across Species and Condition, we calculated a rate of learning measure using individual difference scores on inverse efficiency as follows: $[(T1 - T2) + (T2 - T3)]/2$. A positive value indicates that efficiency increased across trials.

Difficulty

Two indices of difficulty were computed: one for learning and one for performance on the first trial. For learning, difficulty was computed using the method described in Meunier et al. (1986). We examined the relative difficulty of each Maze for humans and mice and the Standard and With Visual Cue conditions separately. A difficulty index (D) was calculated as follows: mean number of errors across trials/number of error zones. The number of error zones were determined according to Rabinovitch and Rosvold (1951). We also computed an index of difficulty for performance on the first trial by modifying the computation proposed by Meunier et al. (1986). Difficulty was calculated as follows: mean number of errors for the first trial/number of error zones.

Analysis of Variance (ANOVAs)

All statistical analyses were conducted using SPSS. To examine the influence of adding a Visual Cue, a $2 \times 2 \times 6 \times 3$ mixed-design ANOVA was conducted on latency, error, and inverse efficiency with Species (Humans, Mice) and Condition (Standard, Visual Cue) as independent-groups variables and Maze (2, 4, 5, 8, 12, and 9) and Trial (1, 2, and 3) as repeated-measures variables. Note that whereas mice were tested on five trials, human participants were tested on three trials to cater to the limited attention span of affected individuals. To allow direct comparison of the two species within the same analysis, only trials one, two, and three were used from the mouse data. We did not analyze trials 4 and 5 because these additional trials may have engendered some additional fatigue/practice that might affect learning and that was not experienced by the human participants. Results are shown in **Tables 1–3**. The rate of learning variable was submitted to a $2 \times 2 \times 6$ mixed-design ANOVA with Species (Humans, Mice) and Condition (Standard, Visual Cue) as independent-groups variables and Maze (2, 4, 5, 8, 9, and 12) as repeated-measures variable. Finally, we examined performance on Trial 1 only to compare the two species on visuo-spatial processing performance. A 2×6 mixed-design ANOVA with Species (Humans, Mice) as independent groups variable and Maze (2, 4, 5, 8, 12, and 9) as repeated-measures variable was conducted on the inverse efficiency measure for the Standard and Visual Cue conditions separately (**Table 4**). Because Mauchly's test of sphericity was significant for most conditions, we applied the Greenhouse-Geisser correction to all effects involving repeated-measures factors.

TABLE 1 | Analysis of variance (ANOVA) of latency between factors Species (humans and mice), Condition (standard and visual cues), Maze (2, 4, 5, 8, 9, and 12), and Trials (1, 2, and 3).

Test of between-subjects effects	df	F	η_p^2	p
Species	1	189.22	0.82	0.00
Visual Cue	1	1.12	0.03	0.30
Species * Visual Cue	1	4.89	0.10	0.03
Error	43			
Trial	1.98	10.95	0.20	0.00
Trial * Species	1.98	1.08	0.02	0.34
Trial * Visual Cue	1.98	0.96	0.02	0.39
Trial * Species * Visual Cue	1.98	0.11	0.00	0.89
Error (Trial)	85.23			
Maze	3.76	24.49	0.36	0.00
Maze * Species	3.76	10.06	0.19	0.00
Maze * Visual Cue	3.76	3.44	0.07	0.01
Maze * Species * Visual Cue	3.76	3.37	0.07	0.01
Error (Maze)	161.52			
Trial * Maze	7.54	1.19	0.03	0.31
Trial * Maze * Species	7.54	2.64	0.06	0.01
Trial * Maze * Visual Cue	7.54	0.39	0.01	0.92
Trial * Maze * Species * Visual Cue	7.54	1.01	0.02	0.43
Error (Trial * Maze)	324.16			

df, degrees of freedom; F, F statistic; η_p^2 , partial eta squared; p, probability of a Type I error.

TABLE 2 | Analysis of variance (ANOVA) of errors between factors Species (humans and mice), Condition (standard and visual cues), Maze (2, 4, 5, 8, 9, and 12), and Trials (1, 2, and 3).

Test of between-subjects effects	df	F	η_p^2	p
Species	1	1.39	0.03	0.25
Visual Cue	1	1.01	0.02	0.32
Species * Visual Cue	1	0.00	0.00	0.97
Error	41			
Maze	3.84	36.10	0.47	0.00
Maze * Species	3.84	15.77	0.28	0.00
Maze * Visual Cue	3.84	2.10	0.05	0.09
Maze * Species * Visual Cue	3.84	0.77	0.02	0.54
Error (Maze)	157.37			
Trial	1.97	13.25	0.24	0.00
Trial * Species	1.97	0.43	0.01	0.65
Trial * Visual Cue	1.97	0.07	0.00	0.93
Trial * Species * Visual Cue	1.97	1.35	0.03	0.27
Error (Trial)	80.64			
Maze * Trial	7.32	1.07	0.03	0.39
Maze * Trial * Species	7.32	2.87	0.07	0.01
Maze * Trial * Visual Cue	7.32	0.50	0.01	0.84
Maze * Trial * Species * Visual Cue	7.32	1.05	0.03	0.40
Error (Maze*Trial)	300.07			

df, degrees of freedom; F, F statistic; η_p^2 , partial eta squared; p, probability of a Type I error.

TABLE 3 | Analysis of variance (ANOVA) of inverse efficiency between factors Species (humans and mice), Condition (standard and visual cues), Maze (2, 4, 5, 8, 9, and 12), and Trials (1, 2, and 3).

Test of between-subjects effects	df	F	η_p^2	p
Species	1	0.12	0.00	0.73
Visual Cue	1	4.05	0.09	0.05
Species * Visual Cue	1	5.09	0.11	0.03
Error	41			
Maze	3.11	36.30	0.47	0.00
Maze * Species	3.11	13.43	0.25	0.00
Maze * Visual Cue	3.11	4.83	0.11	0.00
Maze * Species * Visual Cue	3.11	3.73	0.08	0.01
Error (Maze)	127.59			
Trial	1.90	15.69	0.28	0.00
Trial * Species	1.90	1.91	0.04	0.16
Trial * Visual Cue	1.90	1.36	0.03	0.26
Trial * Species * Visual Cue	1.90	0.89	0.02	0.41
Error (Trial)	78.02			
Maze * Trial	5.90	1.77	0.04	0.11
Maze * Trial * Species	5.90	4.34	0.10	0.00
Maze * Trial * Visual Cue	5.90	0.47	0.01	0.83
Maze * Trial * Species * Visual Cue	5.90	1.17	0.03	0.32
Error (Maze*Trial)	241.78			

df, degrees of freedom; F, F statistic; η_p^2 , partial eta squared; p, probability of a Type I error.

Alpha adjustment was not performed because it was deemed too conservative on the grounds that a valid paradigm is likely to yield non-significant differences between species and because

TABLE 4 | Analysis of variance (ANOVA) of rate of learning between factors Species (humans and mice), Condition (standard and visual cues), Maze (2, 4, 5, 8, 9, and 12).

	df	F	η_p^2	p
Species	1	3.10	0.07	0.09
Visual Cue	1	1.73	0.04	0.20
Species * Visual Cue	1	0.30	0.01	0.59
Error	41			
Maze	4.08	2.35	0.05	0.06
Maze * Species	4.08	2.32	0.05	0.06
Maze * Visual Cue	4.08	0.70	0.02	0.59
Maze * Species * Visual Cue	4.08	0.84	0.02	0.50
Error (Maze)	167.18			

df, degrees of freedom; F, F statistic; η_p^2 , partial eta squared; p, probability of a Type I error.

of the potentially large number of mean comparisons following significant interactions. Instead, we focused on effect sizes for comparing relevant means operationalized as follows: no effect: Cohen's *d* of 0.0–0.2; small effect: Cohen's *d* of 0.2–0.5; medium effect: Cohen's *d* of 0.5–0.8; large effect: Cohen's *d* of 0.8 and more.

RESULTS

Latency

As expected, the main effect of Trial was significant with latency decreasing from Trial 1 ($M = 5.13$; $SE = 0.12$) to Trial 2 ($M = 4.75$; $SE = 0.13$) to Trial 3 ($M = 4.53$; $SE = 0.11$), indicating that learning occurred whereby participants took progressively less time to complete a maze from Trial 1 to Trial 3. We focus on effects involving a Species $\times$ Condition interaction since significant findings involving this interaction suggest that the two species react differently to the presence of visual cues. The Species $\times$ Condition $\times$ Maze interaction was significant. For humans, comparing the Standard Condition to the Visual Cue Condition for each maze, participants took less time to find the goal with the Visual Cue for mazes 4 ($d = -0.4$), 5 ($d = -0.6$), and 9 ($d = -0.7$). Participants took more time to find the goal with the Visual Cue for mazes 8 ($d = 0.2$), 12 ($d = 0.3$). There was no effect of Visual Cue for maze 2 ($d = 0.0$). Mice took more time finding the goal with the Visual Cue for mazes 4 ($d = 0.3$), 5 ($d = 0.4$), 8 ($d = 0.4$), and 9 ($d = 0.7$). There was no effect of Visual Cue for mazes 2 ($d = 0.0$) and 12 ($d = 0.1$). Hence, there was consistency between the two species only for Maze 2 where the Visual Cue did not improve the speed at which the maze was solved for both humans and mice.

Errors

As expected, the main effect of Trial was significant with number of errors decreasing from Trial 1 ($M = 1.65$; $SE = 0.06$) to Trial 2 ($M = 1.43$; $SE = 0.05$) to Trial 3 ($M = 1.31$; $SE = 0.05$), indicating that learning occurred whereby participants made progressively fewer errors from Trial 1 to Trial 3. The Species $\times$ Condition Interaction was not significant, nor were any of the interactions involving the Species $\times$ Condition effect. The main effect of

Condition was not significant. These results suggest that both species reacted similarly to the Visual Cue whereby adding a Visual Cue did not influence errors committed while solving the maze for both humans and mice.

Inverse Efficiency

Results are illustrated in **Figure 3**. As expected the main effect of Trial was significant with efficiency increasing from Trial 1 ($M = 0.20$; $SE = 0.05$), to Trial 2 ($M = -0.02$; $SE = 0.05$), to Trial 3 ($M = -0.15$; $SE = 0.05$), indicating again that learning occurred whereby participants became progressively more efficient at solving the mazes from Trial 1 to Trial 3. This improvement in performance is best captured by the Rate of Learning analyses presented below. The Species $\times$ Condition Interaction was significant. The Species $\times$ Condition $\times$ Maze interaction was also significant. For humans, comparing the Standard Condition to the Visual Cue Condition for each maze, performance was more efficient with the Visual Cue for Maze 2 ($d = -0.2$), 4 ($d = -0.7$), 5 ($d = -0.5$), and 9 ($d = -1.0$). Performance was less efficient with the Visual Cue for Mazes 12 ($d = 0.2$), and there was no effect of Condition for maze 8 ($d = 0.1$). For mice, performance was more efficient with the Visual Cue for Mazes 12 ($d = -0.8$), 2 ($d = -0.6$), 4 ($d = -0.4$), 5 ($d = -0.2$). Performance was less efficient with Visual Cue for

Maze 8 ($d = 0.8$) and 9 ($d = 0.8$). Hence, for both species, the addition of a visual cue improved efficiency for mazes 2, 4, 5 but not for the other mazes.

Considering that comparing the Standard with the Visual Cue conditions yielded inconsistent results, we also compared humans and mice on each maze for the Standard and Visual Cue conditions separately. For the Standard condition, Maze 4 ($d = 0.4$), Maze 5 ($d = 0.0$), Maze 9 ($d = 0.0$), and Maze 12 ($d = -0.4$) yielded no or small species differences. Maze 2 ($d = -1.4$) and Maze 8 ($d = -1$) yielded large species differences. For the Visual Cue condition, only Maze 12 ($d = 0.2$) yielded a small species difference. The differences between humans and mice were large for Maze 2 ($d = -0.7$), Maze 4 ($d = 1.3$), Maze 5 ($d = 0.7$), Maze 8 ($d = -2.2$), and Maze 9 ($d = 0.7$). Hence, performance was generally consistent across species for the Standard condition but not for the Visual Cue condition.

For performance on the first trial only, we report findings with respect to the Species variable only. For the Standard Condition, the main effect of Species was significant [$F(1,24) = 4.28$, $p = 0.05$, $\eta_p^2 = 0.15$]. The Maze $\times$ Species interaction was also significant [$F(3.65,87.33) = 2.77$, $p = 0.04$, $\eta_p^2 = 0.10$]. For the Visual Cue Condition, the main effect of Species was not significant [$F(1,24) < 1$]. The Maze $\times$ Species interaction was significant [$F(2.88,48.96) = 8.17$, $p < 0.01$, $\eta_p^2 = 0.33$]. To explore

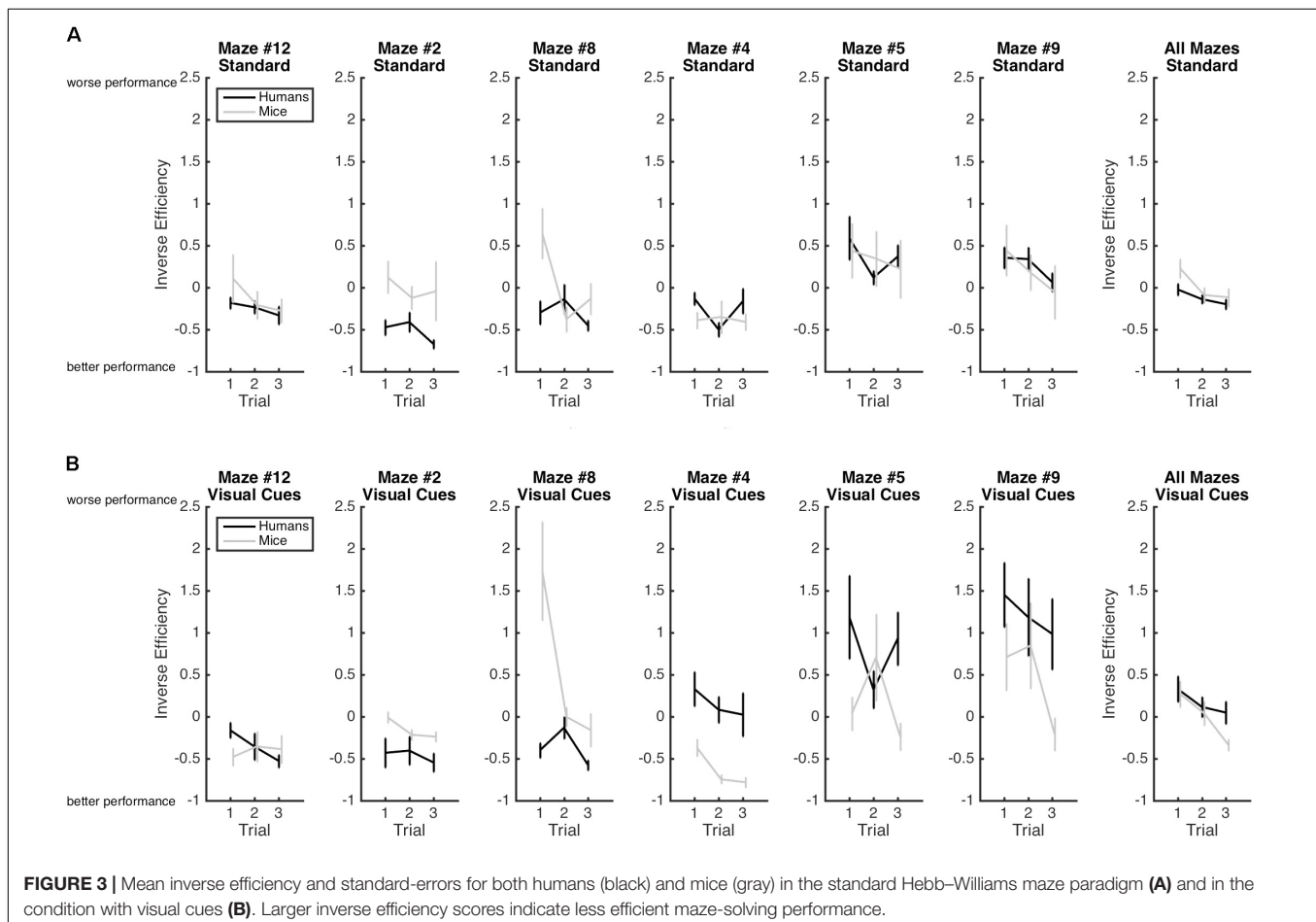


FIGURE 3 | Mean inverse efficiency and standard-errors for both humans (black) and mice (gray) in the standard Hebb-Williams maze paradigm (A) and in the condition with visual cues (B). Larger inverse efficiency scores indicate less efficient maze-solving performance.

these significant interactions, we compared humans and mice on each maze for the Standard and Visual Cue conditions separately. For the Standard condition, Maze 5 ($d = 0.4$) and Maze 9 ($d = -0.2$) yielded small species differences. Maze 4 ($d = 0.7$) and Maze 12 ($d = -0.7$) yielded medium species differences. Maze 2 ($d = -1.7$) and Maze 8 ($d = -2.7$) yielded large species differences. For the Visual Cue condition, Maze 9 ($d = 0.6$) yielded medium species differences. All other mazes yielded large species differences (Maze 2: $d = -0.8$; Maze 4: $d = 1.1$; Maze 5: $d = 0.9$; Maze 8: $d = -4.4$; Maze 12: $d = 0.8$). These results suggest that performance obtained on Mazes 5 and 9 of the standard paradigm provide the best measure of visuo-spatial processing and problem solving across species.

Rate of Learning

Rate of Learning indicates the amount by which efficiency increased across trials. Results are illustrated in **Figure 4**. The Species $\times$ Condition interaction was not significant. The Species $\times$ Condition $\times$ Maze interaction was significant. For humans, comparing the Standard Condition to the Visual Cue Condition for each maze, rate of learning was superior with the Visual Cue for Maze 4 ($d = -0.4$), Maze 5 ($d = -0.3$), Maze 8 ($d = -0.3$), Maze 9 ($d = -0.2$), and Maze 12 ($d = -0.6$). Rate of learning was inferior with the Visual Cue for Maze 2 ($d = 0.2$). For mice, rate of learning was superior with the Visual Cue for Maze 12 ($d = -0.2$). Rate of learning was inferior with the Visual Cue for Mazes 8 ($d = 0.2$), 4 ($d = 1$), and 9 ($d = 0.2$). There was no effect of Condition for Mazes 2 ($d = 0.1$) and 5 ($d = 0.1$). Except for Maze 12 for the humans and Maze 4 for the mice, effect sizes were generally small in both species, suggesting that adding

a Visual Cue had very little impact on Rate of Learning for both humans and mice.

We also compared humans and mice on each maze for the Standard and Visual Cue conditions separately. For the Standard condition Maze 2 ($d = 0.04$), Maze 4 ($d = 0.02$) and Maze 5 ($d = 0.00$), Maze 9 ($d = -0.17$) and Maze 12 ($d = -0.28$), yielded no or small species differences. Differences between humans and mice were medium for Maze 8 ($d = -0.77$). For the Visual Cue condition, Maze 2 ($d = -0.42$), Maze 4 ($d = -0.17$), Maze 5 ($d = -0.01$), and Maze 9 ($d = -0.28$) yielded no or small species differences. Differences between humans and mice were large for Maze 8 ($d = -1.02$) and Maze 12 ($d = 1.15$). Hence, performance was generally consistent across species for both the Standard and the Visual Cue condition for three out of the six mazes tested (2, 4, and 5).

Difficulty

Difficulty was computed for each maze for humans and mice and for the standard and the visual cue conditions separately for performance across trials and for performance on the first trial. Mazes were then ordered in ascending levels of difficulty to examine similarities/differences in the pattern of performance across the two species. For performance across trials (**Table 5**), while patterns of difficulty were not identical across species some consistencies were observed. Mazes 2 and 4 were among the three easiest mazes and Mazes 5 and 12 were among the three hardest mazes for both humans and mice. Moreover, for each species, mazes 5, 9, and 12 were most difficult and mazes 2, 4, and 8, were easiest irrespective the presence of visual cues. For performance on the first trial (**Table 6**), Mazes 2 and 4 were among the three

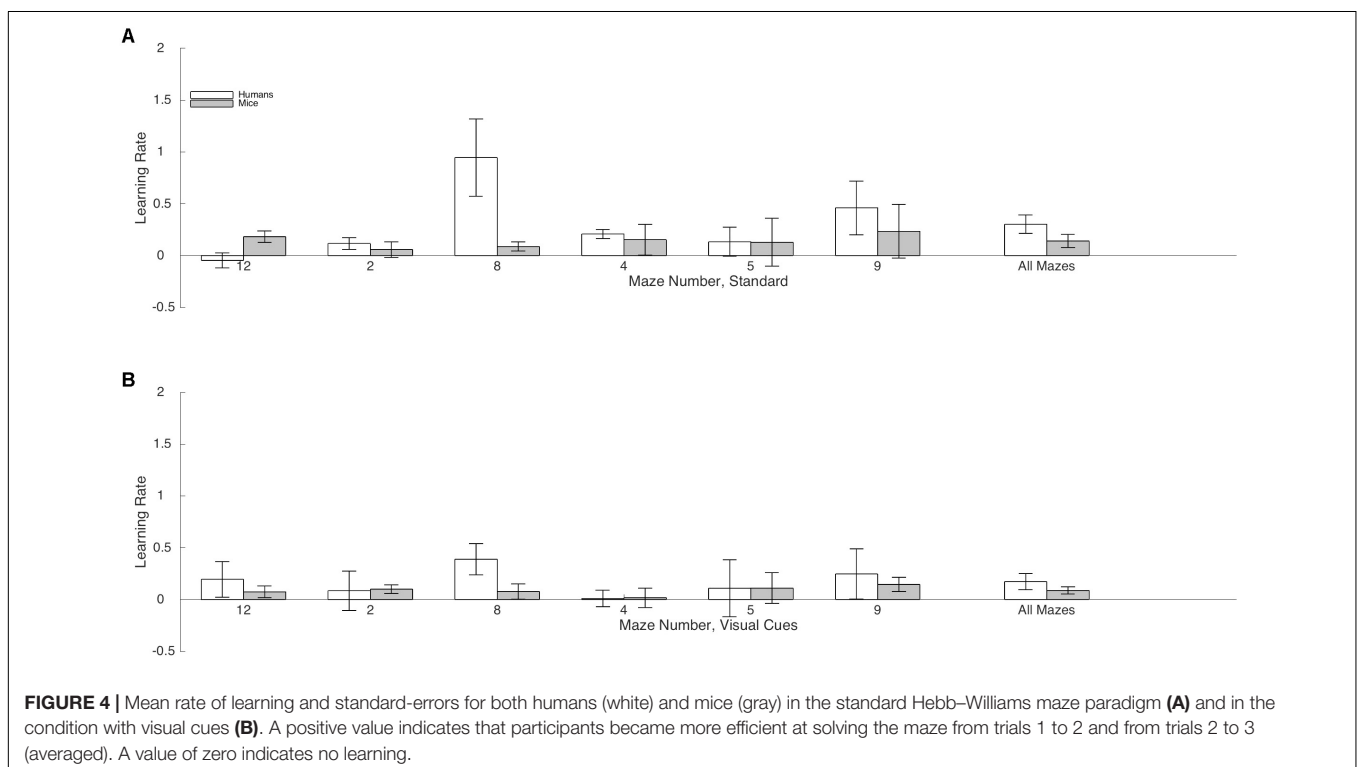


TABLE 5 | Relative difficulty (D) of the different mazes across trials for the Standard and Visual Cue conditions for Humans and Mice separately.

Humans				Mice			
Standard		Cue		Standard		Cue	
Maze	D	Maze	D	Maze	D	Maze	D
2	0.21	2	0.27	4	0.26	4	0.34
8	0.41	8	0.48	9	0.52	9	0.38
4	0.44	4	0.66	2	0.58	2	0.57
9	0.61	12	0.73	5	0.9	8	0.81
5	1.08	9	0.91	12	1.1	5	0.92
12	1.12	5	1.34	8	1.23	12	1.67

Results are presented in ascending order with the higher the D index, the more difficult the maze. Details are provided in the text.

easiest and Maze 5 was among the three most difficult for both species irrespective of visual cues.

Activity Level in Mice

Previous studies suggest that FXS KO mice may exhibit increased activity levels as compared to wild type mice (e.g., Mineur et al., 2002). Thus, we assessed activity levels by obtaining a count of the number of line crosses per unit of time for trial 1 of maze 12. The latter maze was chosen because it has the least number of partitions thus allowing for the clearest observation of locomotion. We restricted our analysis to trial 1 because performance on this trial is independent of learning and memory and reflects exploratory behavior. An independent samples *t*-test revealed no significant differences in activity levels between the group tested in the standard paradigm and the group tested with visual cues.

DISCUSSION

Using FXS as a model disease, we examined the potential utility of the Hebb–Williams maze paradigm (Hebb and Williams, 1946; Rabinovitch and Rosvold, 1951) for translational and drug discovery research on the basis of three validation criteria. The paradigm allows direct comparison of humans and animals on tasks that are behaviorally equivalent (criterion #1) (Shore et al., 2001; see also Gabel et al., 2016) and measures visuo-spatial abilities, a cognitive domain for which FXS individuals and KO mice show impairments as compared to their respective control groups (condition #2) (e.g., Crowe and Hay, 1990; Cornish et al., 1998, 1999; Kogan et al., 2004a). We compared the performance of affected humans and mice across multiple conditions and measures to evaluate whether the paradigm engages comparable cognitive mechanisms in both species (criterion #3). These cross-species comparisons allowed us to identify which conditions, if any, produce comparable patterns of performance across species and therefore offer the best potential for extrapolating results from mice to humans (Willner, 1986; Shore et al., 2001; van der Staay

TABLE 6 | Relative difficulty (D) of the different mazes for performance on the first trial for the Standard and Visual Cue conditions for Humans and Mice separately.

Humans				Mice			
Standard		Cue		Standard		Cue	
Maze	D	Maze	D	Maze	D	Maze	D
4	0.00	2	0.28	4	0.35	4	0.41
2	0.29	8	0.47	2	0.62	2	0.61
8	0.38	4	0.86	9	0.87	12	0.75
9	0.94	12	1.44	5	0.91	5	0.75
5	1.27	5	1.67	8	1.29	9	1.00
12	1.47	9	1.68	12	2.00	8	2.15

Results are presented in ascending order with the higher the D index, the more difficult the maze. Details are provided in the text.

et al., 2010; Sjöberg, 2017). The discussion of our results focuses on measures of performance that allow direct cross-species comparisons, namely efficiency, rate of learning, and difficulty.

We compared performance of FXS humans and KO mice on the standard paradigm as well as on a variation of the paradigm where visual cues were added to the mazes. Our goal was to examine whether this variable has a comparable impact on humans and mice, which would provide support for the notion that the paradigm taps into comparable underlying cognitive mechanisms in both species (Willner, 1986; Shore et al., 2001; van der Staay et al., 2010; Sjöberg, 2017). Our results provide mixed evidence regarding the influence of this manipulation. Specifically, the addition of a visual cue improved performance efficiency for both species for three out of six mazes (i.e., 2, 4, and 5). In contrast, rate of learning was enhanced by the presence of visual cues for both species for only one maze (i.e., 12). Finally, looking at the human and mice data separately, we find that relative maze difficulty was comparable with and without the presence of visual cues, either for difficulty in learning the maze across trials, or for performance on the first trial. Taken together, these results suggest that visual cues do not consistently improve spatial information processing in FXS with the exception of specific mazes where results were consistent across species (information pertaining to specific mazes is presented below). While visual cues have been shown to improve spatial learning performance in typically developing human adults and mice (reviewed by Chan et al., 2012), this is not the case for other populations. Consistent with our findings, there is evidence that visual cues do not improve spatial learning in individuals with ADHD (Robaey et al., 2016), a diagnosis that is commonly comorbid with FXS (Sullivan et al., 2006). FXS is also highly co-morbid with ASD (Hatton et al., 2006) and in this population, the literature on the influence of visual cues on spatial learning is inconclusive, in part because comparisons are often made between conditions that differ with respect to many variables (reviewed by Smith, 2015). Looking at the general effect of landmarks on navigation performance, and seemingly in agreement with our findings, it appears

that impairments in navigation are not diminished by the presence of visual cues in participants with ASD (Lind et al., 2013).

Because there is considerable variation in nomenclature and interpretation of results involving visual cues, it is difficult to provide a simple cohesive interpretation of our findings. The presence of visual cues is thought to shift the strategies implicated in solving mazes from spatial learning toward response-based learning (e.g., Packard and McGaugh, 1996). Response-based strategies rely on reinforcement of stimulus-response contingencies, allowing participants to solve mazes by learning that they can reach the goal by making a specific body turn at a choice point in the maze. Because humans affected by FXS have been shown to display perseverative behaviors (Van Dam et al., 2000; Kogan et al., 2009), and because we used the same cues across mazes, it is possible that learning of stimulus-response contingencies in the earlier mazes interfered with learning new stimulus-response contingencies in the later mazes, which would have rendered the visual cues ineffective with time. To explore this possibility, we conducted separate analyses to examine Species $\times$ Condition interactions for the first maze tested and for the last maze tested on inverse efficiency. In partial agreement with this interpretation, the interaction was not significant for the first maze but approached significance for the last maze (first maze: $F = 0.827$, $p = 0.368$, ns; last maze: $F = 3.702$, $p = 0.061$). However, inspection of **Figure 4** suggests that rate of learning was not linked to testing order for either condition. Another possible explanation for our results is that participants may have had difficulty discriminating between cues that provided information about relative position and those that could be used for a turning response. Indeed, while the visual cues were informative because they were fixed and therefore could indicate to the participant their location in the maze, they were not all located at decision points in the mazes. Additional studies are needed to resolve these discrepancies and to confirm under which conditions visual cues should be used in this paradigm for cross-species extrapolation. Future research should include probe trials where visual cues are removed after a maze has been solved to determine whether they contributed to improvements across trials (e.g., Moffat and Resnick, 2002; Vorhees and Williams, 2014). Moreover, distinct visual cues should be used across different mazes to avoid the possibility of perseveration of responding to similar cues from one maze to the next. Finally, because there is lower reliance on the visual modality for navigation in mice (Brown and Wong, 2007), it would be interesting to examine whether using cues that are optimal for each species (e.g., olfactory in mice, visual in humans) at decision points in a maze generates more comparable findings across species³.

³Using different navigational cues across species might have implications for criterion #1. However, one must consider all criteria when making methodological decisions in translational research. If presenting each species with tasks that are more ecologically relevant leads to the measurement of more comparable underlying functions (criterion 3), then we would consider this more important than using tasks that are identical across species (see Willner, 1986, for a discussion of face vs. construct validity in animal models).

It should be noted that humans and mice displayed more consistent results with respect to the influence of visual cues for measures that incorporate errors (error, inverse efficiency, rate of learning) than for the measure of latency. Pollard and Lysons (1969) have suggested that measures based on errors are more indicative of learning and memory, whereas those based on time are more indicative of exploratory and motivational factors. Hence, it is possible that humans and mice reacted similarly to visual cues with respect to learning as indexed by measures of error, but not with respect to some of the other behaviors triggered by the maze environment (i.e., those indexed by latency, including efficiency). Gandhi et al. (2014a) also found that it was the measure of errors that was sensitive to the effects of the mGluR antagonist MPEP, which concomitantly reversed the deficits in PSD-95 reactivity to Hebb–Williams maze learning. These data suggest that future studies focusing on molecular pathways mediated by FMRP (Fragile $\times$ Mental Retardation protein) and involved in synaptic plasticity should include errors as a dependent variable to evaluate the effect of pharmacological, genetic, or other manipulations.

Cross-species comparisons revealed comparable patterns of performance for FXS humans and mice for some mazes but not others. Focusing on the measure of efficiency, which reflects overall performance on the mazes by combining error rates and latency, four mazes (4, 5, 9, and 12) produced consistent results in humans and mice in the standard condition and one maze (12) in the visual cue condition. For rate of learning, which reflects improvements in performance across trials, three out of the six mazes tested (i.e., 2, 4, and 5) produced consistent results for humans and mice for both conditions. We also conducted cross-species comparisons by measuring the level of difficulty of each maze for each species and each condition separately. The relative difficulty of four mazes (2, 4, 5, and 12) was consistent in humans and mice, irrespective of the presence of visual cues. Finally, we compared efficiency and difficulty across species and for each condition separately for performance on the first trial only. This allowed us to distinguish the effects of learning/memory across trials from the ability to utilize visual information to solve a novel spatial task on the first trial (Hebb and Williams, 1946). Only two mazes (i.e., 5 and 9) tested in the standard condition yielded comparable results for humans and mice. These two mazes also produced consistent results in humans and mice in terms of difficulty for the visual cue condition. At the time of this writing, we retrieved only two studies that have directly compared humans and mice on the Hebb–Williams maze paradigm (Shore et al., 2001; Gabel et al., 2016). These studies also report heterogeneous cross-species consistencies across mazes tested in typically developing participants. Shore et al. (2001) report consistent efficiency and rate of learning for three mazes (6, 8, and 12), however, statistical results for these group comparisons are not provided. Gabel et al. (2016) report consistent efficiency and rate of learning for all mazes tested (5, 6, 11, and 12). The discrepancy between our results and those obtained with typically developing participants underline the relevance of directly comparing affected humans and mice to evaluate the validity of translational paradigms in FXS.

To provide practical advice regarding which conditions and mazes should be used for extrapolating results from mice to humans in translational research, we examined the overall pattern of consistencies across the different measures and identified those that provided at least two equivalent cross-species comparisons. Irrespective of visual cues, Mazes 2, 4, and 5 provided no or small species differences for at least two measures of performance, suggesting that these conditions have good potential to measure spatial learning/memory across species. With regard to performance on the first trial, which reflects visuo-spatial problem solving, Mazes 5 and 9 without visual cues produced consistent results.

Notwithstanding the aforementioned cross-species consistencies, many conditions failed to produce comparable results in humans and mice, which highlights the need for additional research focusing on the nature of the cognitive processes implicated in this paradigm. This desideratum is not merely theoretical but has very practical implications to clinical researchers submitting their rationale and predictions about variables that will change in drug trials to the drug regulatory bodies (e.g., FDA in the United States). Specific outcomes have not been scaled from mice to humans in recent trials such as the trial of Arbaclofen in FXS, which was intended to improve social avoidance (Berry-Kravis et al., 2017). One important obstacle to demonstrating efficacy may have been the lack of a translational measure validated on the basis of criteria such as those specified in the present article. Within this context, it would be particularly important to further investigate the purported dissociation between the cognitive mechanisms underlying performance on the first trial vs. improvements in performance across trials. Indeed, there were no differences between FXS participants and controls for performance on the first trial in MacLeod et al. (2010). In contrast, deficits in spatial learning have been reported using both the Hebb–Williams maze paradigm (MacLeod et al., 2010) as well as other maze paradigms (e.g., radial maze: Mineur et al., 2002; cross shaped maze: Dobkin et al., 2000). If our findings of comparable and intact performance on the first trial vs. comparable and impaired learning across trials were corroborated, then it would support the utility of these two measures to target distinct cognitive functions in drug efficacy trials.

Finally, differences between mice and humans may have arisen from the use of a virtual environment with humans. Indeed, virtual navigation only assesses visually-based learning without input from the other modalities recruited when participants are actually moving through a real space (e.g., proprioception, motor). Despite these differences, studies in the elderly generally indicate that more often than not, results with virtual mazes generalize to real maze paradigms (Moffat, 2009). Whether this is also the case in individuals affected by FXS has yet to be determined.

As whole, our results support the potential utility and validity of the Hebb–Williams maze paradigm for measuring visual-spatial abilities in translational research in FXS. First, it is the only paradigm that has shown comparable patterns of results in humans and mice using both the homology of impairments approach (MacLeod et al., 2010) and the direct comparison

approach described here. Second, the paradigm measures visuo-spatial problem solving as well as spatial learning and memory, two processes that have been shown to be impacted by the lack of FMRP and important targets for treatment (Cornish et al., 1999). Third, while more work is needed in this area, there is evidence that performance on the Hebb–Williams maze paradigm can be examined and related at multiple levels of analysis including cognitive and behavioral functioning (e.g., MacLeod et al., 2010), anatomical pathways (e.g., Hunsaker et al., 2008), and molecular pathways (e.g., Gandhi et al.). In light of these promising results, we feel that increased emphasis needs to be directed toward specifying the practical parameters for the Hebb–Williams paradigm as well as other paradigms that allow direct comparison of humans and animals (e.g., object-discrimination learning and reversal, radial mazes, see Boutet et al., 2007; Kogan et al., 2009; Gilmour et al., 2013; McGonigle and Ruggeri, 2014; Gabel et al., 2016; see also Watase and Zoghbi, 2003). These efforts are critically important to extrapolating results of drug discovery as well as basic cellular and molecular research from animal studies to humans and therefore in ultimately improving the lives of those affected by FXS.

AUTHOR CONTRIBUTIONS

IB drafted the manuscript and contributed to the conceptualization and data analyses. CC contributed to the conceptualization and methodological design for the human Hebb–Williams Mazes, as well as aiding in analysis. He was available for consultation and reviewed the manuscript. LM contributed to the conceptualization and design of the experiments described, as well as tested participants as part of her dissertation research. CM contributed to the conceptualization, methodological design, and data collection for the animal testing. He also provided the necessary laboratory space to conduct testing. MH provided guidance on the conceptualization of the paradigm with visual cues and reviewed the manuscript. EB-K assisted with the conceptualization of the project and was responsible for the recruitment of participants at RUSH University Medical Center. She also reviewed the manuscript and provided feedback. RG contributed to the behavioral testing of mice. CK is lab director. He conceptualized the research project, obtained funding, and provided guidance and assistance in all phases of the project and reviewed and revised the manuscript.

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Mental Illnesses-Associated Fxr1 and Its Negative Regulator Gsk3 β Are Modulators of Anxiety and Glutamatergic Neurotransmission

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Genetic variants of the fragile X mental retardation syndrome-related protein 1 (*FXR1*) have been associated to mood regulation, schizophrenia, and bipolar disorders. Nonetheless, genetic association does not indicate a functional link of a given gene to neuronal activity and associated behaviors. In addition, interaction between multiple genes is often needed to sculpt complex traits such as behavior. Thus, modulation of neuronal functions by a given gene product, such as Fxr1, has to be thoroughly studied in the context of its interactions with other gene products. Glycogen synthase kinase-3 beta (GSK3 β) is a shared target of several psychoactive drugs. In addition, interaction between functional polymorphisms of *GSK3b* and *FXR1* has been implicated in mood regulation in healthy subjects and bipolar patients. However, the mechanistic underpinnings of this interaction remain unknown. We used somatic CRISPR/Cas9 mediated knockout and overexpression to investigate the impact of Fxr1 and its regulator Gsk3 β on neuronal functions directly in the adult mouse brain. Suppression of Gsk3 β or increase of Fxr1 expression in medial prefrontal cortex neurons leads to anxiolytic-like responses associated with a decrease in AMPA mediated excitatory postsynaptic currents. Furthermore, Fxr1 and Gsk3 β modulate glutamatergic neurotransmission via regulation of AMPA receptor subunits GluA1 and GluA2 as well as vesicular glutamate transporter VGlut1. These results underscore a potential mechanism underlying the action of Fxr1 on neuronal activity and behaviors. Association between the Gsk3 β -Fxr1 pathway and glutamatergic signaling also suggests how it may contribute to emotional regulation in response to mood stabilizers, or in illnesses like mood disorders and schizophrenia.

Keywords: fragile X proteins, GSK-3, mood disorders, CRISPR/Cas9, frontal cortex, AMPAR

INTRODUCTION

The fragile X mental retardation syndrome-related protein 1 (Fxr1) is a member of a small family of RNA binding proteins that also comprises the fragile X mental retardation protein 1 (Fmr1) and Fxr2 (Siomi et al., 1995). FXR family proteins are enriched in the brain with Fxr1 being expressed in neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells of mouse cortex (Tamanini et al., 1997; Bakker et al., 2000; Cook et al., 2011; Thomsen et al., 2013;

Zhang et al., 2014). The neuronal functions of this family have mostly been studied in the context of fragile X syndrome and autism spectrum disorders (Bardoni et al., 2001; Pfeiffer and Huber, 2009). However, genome-wide association studies (GWAS) have linked *FXR1* to schizophrenia and bipolar disorders (Consortium, 2014; Hauberg et al., 2016; Liu et al., 2016; Takata et al., 2017), therefore indicating its possible wider roles in mental illnesses. Nonetheless, genetic association does not always indicate a direct mechanistic link to neuronal activity and associated behavior (Boyle et al., 2017). Moreover, complex traits are often influenced by interactions between multiple genes.

We identified genetic polymorphisms in *FXR1* and *GSK3B* that are linked to differential expression of their respective mRNAs in the human dorsolateral prefrontal cortex (DLPFC) (Del'Guidice et al., 2015). Interaction between these polymorphisms contributes to mood regulation in healthy subjects in whom higher *FXR1* expression is associated to greater emotional stability, except in the context of higher *GSK3B* expression (Del'Guidice et al., 2015). Furthermore, an interaction between these genetic variants has also been linked to symptom severity in bipolar patients (Bureau et al., 2017). The *GSK3B* gene encodes glycogen synthase kinase-3 beta (Gsk3 β), a serine-threonine kinase. Inhibition of Gsk3 β is a consequence of treatment with several psychoactive drugs including antipsychotics, antidepressants, ketamine and mood stabilizers (Beaulieu et al., 2009; Beurel et al., 2011). Fxr1 is directly phosphorylated by Gsk3 β and negatively regulated by this kinase (Del'Guidice et al., 2015). Conversely, chronic treatment with the mood stabilizers—lithium, lamotrigine or valproate—or other manipulations leading to an inhibition of Gsk3 β , elevate Fxr1 levels (Del'Guidice et al., 2015).

Mental illnesses are believed to be associated to a misregulation of the neuronal excitation/inhibition balance (Nelson and Valakh, 2015; Foss-Feig et al., 2017; Lener et al., 2017). Ionotropic glutamate receptors, α -Amino-3-hydroxy-5-methyl-4-isoxazole Propionic-Acid (AMPA) and *N*-methyl-D-aspartate (NMDA) are the major mediators of excitatory transmission in the brain. Changes in AMPA or NMDA receptors could be one of the causes of imbalance of neuronal activity. Moreover, alterations in glutamatergic neurotransmission have been widely implicated in mental illnesses (Javitt, 2004; Lener et al., 2017). Thus, mechanistic contribution of genetic risk factors for schizophrenia and mood disorders to the regulation of glutamatergic neurotransmission in the nervous system is of a particular interest.

We used CRISPR/Cas9 mediated somatic gene knockout (sKO) in combination with adeno-associated viral vector (AAV) driven gene overexpression to investigate the consequences of altered Fxr1 and Gsk3 β expression in the adult medial prefrontal cortex (mPFC) on neuronal activity and associated behaviors. Augmentation of Fxr1 and reduction of Gsk3 β expression resulted in anxiolytic-like behaviors and decrease in AMPA mediated spontaneous excitatory currents. Further investigation of underlying mechanism revealed that increase in Fxr1 and decrease in Gsk3 β expression leads to AMPA receptor composition change most likely due to alteration of trafficking of both synaptic GluA1 and GluA2 subunits. Changes

in AMPA receptor subunits were accompanied with a decrease in vesicular glutamate transporter 1 (Vglut1) indicating pre- and post-synaptic changes of glutamatergic neurotransmission. Overall, our results uncovered an implication of Fxr1 and its regulator Gsk3 β in the control of synaptic components of glutamatergic neurotransmission. These results underscore a mechanism by which Fxr1 contributes to the regulation of neuronal activity and suggest how it could be implicated in emotional regulation.

MATERIALS AND METHODS

Experimental Animals

All experiments conducted in this study were approved by either the Université Laval or University of Toronto Institutional Animal Care Committee in line with guidelines from the Canadian Council on Animal Care. For all the experiments C57BL/6J male (Jackson Laboratory, Bar Harbor, ME, United States) mice were used. Littermates were housed 3–4 per cage in a humidity-controlled room at 23°C on a 12 h light dark cycle with *ad libitum* access to standard mouse chow and water. At the time of experiment, mice were 3–4 months old and weighed approximately 25–30 g. Animals were all drug naïve and were used only for single experiments.

DNA Constructs

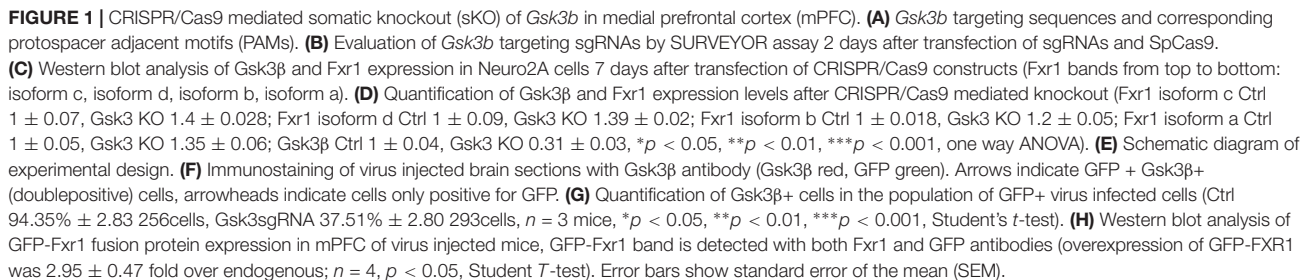
To knockout (KO) *Gsk3b* gene 20-nt target sequences in exons of the gene were selected using online CRISPR design tool¹ to minimize off-target activity. For *in vitro* evaluation of *Gsk3b* KO by SURVEYOR assay (Figure 1B), guide oligonucleotides were cloned into pX330 [pX330-U6-Chimeric_BB-CBH-hSpCas9 was a gift from Feng Zhang (Addgene # 42230)] (Cong et al., 2013) all in one vector by single step cloning using BbsI restriction sites (Ran et al., 2013). For *in vitro* evaluation of *Gsk3b* KO by Western blot (Figures 1C,D), the most active guide (gRNA3) was cloned into pX459 vector [pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988)] (Ran et al., 2013). Sequences of all constructs were verified.

To generate sgRNA expressing AAV viral vector (pAAV Gsk3sgRNA/GFP) preparation the most active guide (gRNA3) was cloned into pX552 [pX552 was a gift from Feng Zhang (Addgene plasmid # 60958)] (Swiech et al., 2015) vector by single step cloning using SapI restriction sites. AAV SpCas9 (pX551) was a gift from Feng Zhang (Addgene plasmid # 60957) (Swiech et al., 2015). AAV GFP-Fxr1 (Fxr1 over) neuron-specific AAV vector was described previously (Del'Guidice et al., 2015).

Cell Line Culture and Transfection

Neuro-2A (N2A) cells were grown in high glucose DMEM containing 10% FBS, penicillin/streptomycin and L-glutamine (HyClone-GE Healthcare, Logan, UT, United States). Cells were maintained at 37°C in 5% CO₂ atmosphere and transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, United States) according to manufacturer's protocols.

¹<http://crispr.mit.edu/>



vectors with guides targeting *Gsk3b*). Cells transfected with pX330 only served as negative control. Cells were lysed 48 h after transfection by lysis buffer (Tris pH = 8.0 0.1M, NaCl 0.2M, EDTA 5mM, SDS 0.4% and proteinase K 0.2 mg/ml), and DNA was precipitated using isopropanol followed by centrifugation (13000 g 15 min). DNA was resuspended in TE Buffer (10 mM Tris pH 8.0, 0.1 mM EDTA) and used for downstream analysis. Functional testing of individual sgRNAs was performed by SURVEYOR nuclease assay (Transgenomics, Omaha, NE, United States) using PCR primers listed in **Table 1**. Band intensity quantification was performed as described (Ran et al., 2013).

For all viral vector preparations, AAV serotype 5 viral particles were produced by the University of North Carolina (UNC) Vector core facility. AAV GFP (AAV5 hSYN EGFP)

TABLE 1 | PCR primers used in the SURVEYOR assay.

Gene exon	Forward primer sequence	Reverse primer sequence
Gsk3b exon 1	TCTTCCAGGAAAGGG AGGTGA	AGGCACTGGAGCACTT GAAA
Gsk3b exon 3	GGTTCCTCTTGCCCC TATTA	TTCTCATTGGCATTTC ACGC
Gsk3b exon 6	GCTAACACCTGACACT CACTT	CTGTGAGCACGTCTTT TTGC
Gsk3b exon 10	TAGCAAGCAGTTTGC CCCAC	AGTCCATGATAGTGGAG GGGA

and AAV KORD (AAV9-CaMKII-HA-KORD-IRES-mCitrine) (Vardy et al., 2015) were purchased from UNC Vector core facility (Chapel Hill, NC, United States).

Stereotaxic Injections

Three weeks before the behavioral tests and electrophysiology recordings, bilateral injection of the virus was made in the mPFC. Mice were anesthetized with a preparation of ketamine 10 mg/ml and xylazine 1 mg/ml (0.1ml/10 g, i.p.). The animal was placed in a stereotaxic frame, and the skull surface was exposed. Two holes were drilled at injection sites and 1 μ l of virus (AAV GFP-Fxr1 4.4×10^{12} vg/ml or AAV GFP or AAV KORD 3×10^{12} vg/ml or 1:1 AAV mixture: AAV SpCas9 2.6×10^{12} vg/ml and AAV Gsk3sgRNA/GFP 5.4×10^{12} vg/ml or AAV SpCas9 and AAV GFP 4.5×10^{12} vg/ml) was injected using nanoliter injector with microsyringe pump controller (WPI) at the speed of 4 nl per second. Following coordinates were used: anterior-posterior (AP), +2.4 mm anterior to bregma; mediolateral (ML), $\pm$ 0.5 mm; dorsoventral (DV), 1.7 mm below the surface of the brain. All measures were taken before, during, and after surgery to minimize animal pain and discomfort. These measures included: using local analgesics on the site of incision, using heating pad during surgery and recovery period to keep an optimal body temperature for mice, making minimal incisions on the head skin and minimal size of the hole drilled in the skull, making a proper suture of the skin, so it's not itchy for mice, using analgesics 24–48 h post surgery.

Acute Slice Preparation

Mice were anesthetized with isoflurane followed by rapid cervical dislocation. Cortical slices (300 μ m) were prepared from mice (3 weeks after injection of viruses) using a vibrating blade microtome (Leica Biosystem, Wetzlar, Germany). Slices were prepared using ice-cold artificial cerebrospinal fluid (ACSF) containing: NaCl 87 mM, NaHCO₃ 25 mM, KCl 2.5 mM, NaH₂PO₄ 1.25 mM, MgCl₂ 7 mM, CaCl₂ 0.5 mM, glucose 25 mM and sucrose 75 mM. Right after sectioning, slices were placed in oxygenated ACSF at 32°C for 30 min, transferred to extracellular ACSF and maintained at room temperature prior to experiments. All recordings were performed with extracellular ACSF containing: NaCl 124 mM, NaHCO₃ 25 mM, KCl 2.5 mM, MgCl₂ 1.5 mM, CaCl₂ 2.5 mM and glucose 10 mM, equilibrated with 95% O₂/5% CO₂,

pH7.4, maintained at 31–33°C and perfused at a rate of 2–3 mL/min.

Electrophysiology

Whole-cell current-clamp and voltage-clamp recordings were made with glass electrodes (4–6.5 M Ω) filled with a solution containing: K-gluconate 120 mM, KCl 20 mM, MgCl₂ 2 mM, EGTA 0.6 mM, MgATP 2mM, NaGTP 0.3 mM, Hepes 10 mM, phosphocreatine 7 mM or Cs-gluconate 100 mM, NaCl 8 mM, MgCl₂ 5 mM, EGTA 0.6 mM, MgATP 2 mM, NaGTP 0.3 mM, Hepes 10 mM, phosphocreatine 7 mM, QX-314 1, spermine 0.1 mM (Cs-methanesulfonate-based solution was used to investigate I-V relationships of evoked EPSCs, **Figure 6**).

Biocytin (0.2%) was routinely added to the patch solution for further cell reconstruction. Pyramidal neurons expressing GFP (green) were visually identified in acute slices (mPFC layer III-V) using fluorescence microscope. Electrophysiological recordings were made using a Multi Clamp 700A amplifier (Axon Instruments, Union City, CA, United States), operating under current-clamp and voltage-clamp mode. Data were filtered at 4 kHz and acquired using pClamp 10 software (Molecular devices, Sunnyvale, CA, United States). Local cortical inputs were electrically stimulated via a patch micropipette placed in the mPFC layer II. All recordings were done at a holding potential -70 mV. For the I-V curve experiments holding potential was varied from -100 mV to 60 mV. Paired-pulse stimulation was delivered with 50 ms interval. Action potentials (APs) were triggered using 500 ms depolarizing pulses of various amplitudes. The uncompensated series resistance was monitored by the delivery of -10 mV steps throughout the experiment, only recordings with less than 15% change were analyzed.

Drugs

10 μ M CNQX, 50 μ M AP5 and 10 μ M bicuculline methiodide (Sigma-Aldrich, Oakville, ON, Canada) were dissolved in extracellular ACSF and applied through the perfusion system (at least 5 min before recordings).

Immunofluorescent Staining

Mice were euthanized 3 weeks after viral delivery by a lethal dose of ketamine/xylazine and perfused with phosphate buffer saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were incubated in 4% PFA 24 h at 4°C. Fixed tissue was sectioned using vibratome (Leica, VT1000S). Next, 40 μ m sections were boiled for 2 min in sodium citrate buffer (10 mM tri-sodium citrate dehydrate, 0.05% Tween-20, pH 6.0) and cooled down at room temperature (RT) for 20 min. Sections were blocked and permeabilized with a permeabilization solution containing 10% normal goat serum (NGS) and 0.5% Triton X-100 (Sigma) in PBS for 2 h. Sections were incubated with primary antibodies diluted in permeabilization solution overnight at 4°C. After three washes in PBS, samples were incubated with secondary antibodies for 2 h at room temperature. After washing with PBS three times, sections were mounted using DAKO mounting medium (DAKO, Mississauga, ON, Canada) and visualized with a

confocal microscope (Zeiss LSM 700, Zen 2011 Software, Oberkochen, Germany). Quantification was performed using ImageJ (National Institute of Health (NIH), Bethesda, MD, United States).

For immunofluorescent staining of biocytin-filled neurons, acute brain slices were fixed in 4% PFA overnight at 4°C. Slices were washed 3 times in PBS and incubated in permeabilization solution containing 10% normal goat serum (NGS) and 0.5% Triton X-100 (Sigma-Aldrich, Oakville, ON, Canada) in PBS for 2 h at RT. After sections were incubated with streptavidin-Alexa 546 conjugated antibodies diluted in permeabilization solution overnight at 4°C. After washing with PBS three times, sections were mounted using the mounting medium (DAKO, Mississauga, ON, Canada). Pictures were taken using a confocal microscope (Zeiss LSM 700) with a voxel size of $0.1 \times 0.1 \times 0.55 \mu\text{m}$. Spines counting and dendrite length measurements were performed blindly using NeuronStudio (Icahn School of Medicine at Mount Sinai (ISMMS), New York, NY, United States).

Following primary antibodies were used: mouse anti-Gsk3 β (1:500, Abcam 93926, Cambridge, United Kingdom, for **Figure 1**), rabbit anti-Gsk3 β (1:500, Cell Signal Technology 9315, Danvers, MA, United States, for Supplementary Figure S1) and Streptavidin-Alexa546 (1:200, Life Technologies/Thermo Fisher Scientific, S11225, Waltham, MA, United States) Secondary antibodies: Alexa Fluor 568 (Life Technologies/Thermo Fisher Scientific, Waltham, MA, United States, 1:1000).

Tissue Dissection

Mice were killed by rapid cervical dislocation. Heads of animals were immediately cooled by immersion in liquid nitrogen for 6 s. mPFC tissues were dissected rapidly (within 30 s) on an ice-cold surface and frozen in liquid nitrogen. For synaptosome extraction experiments (**Figure 7**), first, 500 μm thick serial coronal sections were prepared using ice-cold adult mouse brain slicer and matrix (Zivic instruments), second, mPFC was dissected on ice cold surface using a microsurgical knife (KF Technology).

Synaptosome Isolation and Western Blot

Synaptosomes were isolated using Syn-PER reagent according to manufacturer's recommendations (Thermo Fisher Scientific). Briefly, dissected and frozen brain tissue was lysed in Syn-PER solution supplied with protease inhibitor cocktail, 10 mM NaFluoride, 25 mM β glycerophosphate, 10 mM Na Orthovanadate (Sigma-Aldrich, Oakville, ON, Canada). Samples were centrifuged for 10 min at 1200 g. After discarding the pellet, samples were centrifuged for another 20 min at 15000 g to obtain synaptosomes in the pellet. Neuro2A cells and dissected brain tissue were lysed in lysis buffer containing: 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, Protease inhibitor cocktail, 1% SDS, 0.5% Na-deoxycholate, 1% NP-40, 10 mM NaFluoride, 25 mM β glycerophosphate, 10 mM Na Orthovanadate (Sigma-Aldrich, Oakville, ON, Canada). Lysates were centrifuged 10000 g for 30 min and supernatants were collected. Protein concentration was measured by using a DC-protein assay (Bio-Rad, Hercules, CA, United States). Protein extracts were separated on precast

10% Tris-glycine gels (Thermo Fisher Scientific, Waltham, MA, United States) and transferred to nitrocellulose membranes. Blots were immunostained overnight at 4°C with primary antibodies. Immune complexes were revealed using appropriate IR dye-labeled secondary antibodies from Li-Cor Biotechnology (Lincoln, NE, United States). Quantitative analyses of fluorescent IR dye signal were carried out using Odyssey Imager and Image Studio Lite 5.2 software (Licor Biotechnology, Lincoln, NE, United States). For quantification, GAPDH (Actin in case of Neuro2A cells) was used as a loading control for the evaluation of total protein levels. For measurement of synaptic receptor levels, the ratio of p845GluA1/GluA1 and p880GluA2/GluA2 were calculated. Results were further normalized to respective control conditions to allow for comparison between separate experiments. Following primary antibodies were used in the experiments: mouse anti-Actin (1:10000, Millipore, MAB1501), mouse anti-GAPDH (1:5000, Santa Cruz sc-322333) rabbit anti-Gsk3 β (1:500, Cell Signal Technology 9315, Danvers, MA, United States), rabbit anti-Fxr1 (1:1000, Abcam 129089), mouse anti-GFP (1:1000, Rockland/VWR 600-301-215), mouse anti-GluA1 (1:1000, Millipore MAB2263), rabbit anti-p845 (GluA1) (1:1000, Millipore 06-773), mouse anti-GluA2 (1:1000, Millipore MAB397), rabbit anti-p880 (GluA2) (1:1000, Abcam ab52180), mouse anti-NR1 N308/48 (1:1000, Antibodies incorporated 75-272), mouse anti-Vglut1 N28/9 (1:5, Antibodies incorporated 75-066), mouse anti-GABA α R alpha 1 N95/35 (1:1000, Antibodies incorporated 75-136), mouse anti-Neurologin 1 (1:1000, Synaptic systems 129111), rabbit anti-Neurologin 2 (1:1000, Synaptic systems 129202) and mouse anti-PSD95 (1:250, BD transduction 610495) Secondary antibodies: goat anti-mouse IR Dye 680 (1:10000, Mandel 926-68020), goat anti-rabbit IR Dye 800 (1:10000, Mandel 926-32211).

Chemogenetic Inhibition

To activate KORD receptors and silence neurons, Salvinorin B (10 mg/kg) (SalB) (Cayman chemical, Ann Arbor, MI, United States) [or dimethyl sulfoxide (DMSO) as a vehicle] was administered to mice 4 weeks after stereotaxic injection of AAV9 KORD virus. SalB was dissolved in DMSO and injected subcutaneously (s.c.) at a volume of 1 $\mu\text{L/g}$ body weight 10 min prior to behavioral test as described (Vardy et al., 2015).

Behavioral Tests

Open field Test (OFT)

It was performed for 30 min in an automated Omnitech Digiscan apparatus (AccuScan Instrument, Columbus, OH, United States). Each mouse was placed in a corner of the large plexiglass box and the exploratory activity was recorded. Time spent in the center, number of entries and horizontal activity were recorded separately for the central (25% of the total surface) and peripheral areas.

Dark-Light Emergence Test (DLET)

It was performed for 5 min with mice placed initially at the center of the dark chamber. Tests were conducted using

an automated open field activity apparatus with light/dark insert (Med-Associates, St Albans, VE, United States) with the light compartment illuminated at 800 lux. The total time spent in the dark and light compartments, the total distance traveled, and the delay to cross from the dark to the light chamber were used as parameters for analysis.

Elevated Plus Maze (EPM)

It was performed for 10 min with mice initially placed at the far end of the close arm. The time spent in the open arm was measured manually (by the observer being unaware of the treatment) and used for the analysis.

Behavioral Z Scoring

To obtain integrated measures in each group, emotionality- and locomotion-related data were normalized using a Z-score methodology (Guilloux et al., 2011). Z-scores for individual animals were calculated using the formula: $Z = (X - \mu) / \sigma$, which indicates how many standard deviations (σ) an observation (X) is above or below the mean of a control group (μ). Z-scores for behavioral measures were first averaged within the test, and then across all three tests (OFT, DLET, EPM). OFT (time spent in the center), DLET (time spent in the light chamber), EPM (time in open arms) were used to obtain emotionality Z-scores. Locomotion Z-scores were obtained from DLET (total distance traveled) and OFT (total distance traveled) data.

Quantification and Statistical Analysis

Synaptic events were analyzed using pClamp 10 software within at least 3 min of recordings, individual events were detected using automatic template search. Templates were created using the average of at least 10 events aligned by the rising of their slopes. The peak amplitude of evoked EPSCs (eEPSCs) was measured for an averaged response (5 trials). Paired-pulse ratio was calculated as average for 15–20 trials. Rectification index (RI) was calculated, as a ratio of $I-V$ slopes, $RI = s_2/s_1$ (Adesnik and Nicoll, 2007; Lallane et al., 2016). First we calculated slope 1 (s_1) using linear regression to AMPA currents recorded at holding potential ≤ 0 mV, as well as an AMPAR reversal potential, E_{rev} . Next, we estimated slope 2 (s_2) using linear fit of $I-V$ data recorded at positive holding potentials and constrained to intersect the x -axis at E_{rev} . This method allows taking into account variations of AMPA reversal potential between recordings. Threshold current necessary to evoke single AP, as well as maximal firing rate, AP amplitude, half width and time to peak (TTP) were calculated to investigate excitability.

The data are presented as means $\pm$ SEM. For comparison between two groups, two-tailed t -test is used. For comparison between multiple groups one-way ANOVA is used followed by Bonferroni-corrected pair-wise comparisons using GraphPadPrism 5 software (La Jolla, CA, United States) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

CRISPR/Cas9 Mediates Efficient Somatic Knockout of Gsk3 β

Gsk3 β activity or expression can be manipulated systemically by using various drugs or systemic genetic manipulations (Hoeflich et al., 2000; McManus et al., 2005; Beaulieu et al., 2009). Cell type or brain region specific inactivation of Gsk3 β has also been achieved using the Cre-Lox system in transgenic mouse models (Latapy et al., 2012; Del'Guidice et al., 2015; Ochs et al., 2015). To avoid developmental compensation and preserve cell type and brain region specificity we took advantage of a non-conventional CRISPR/Cas9 method to induce sKO of *Gsk3b* in neurons of the adult mouse medial prefrontal cortex (mPFC, the mouse homolog of human DLPFC). First, we designed guide RNAs (gRNAs) targeting several exons of the *Gsk3b* gene using online CRISPR design tool to minimize off-target activity (Figure 1A). Efficacy of single-guide RNAs (sgRNAs) to target genomic DNA was tested *in vitro* by SURVEYOR assay following transfection into mouse cells (Figure 1B). Efficacy of the most active gRNA for *Gsk3b* (gRNA3) was further established, as compared to a scrambled gRNA, using a puromycin selection system (Ran et al., 2013) in mouse neuroblastoma cells. Expression of CRISPR/Cas9 against *Gsk3b* in transfected Neuro2A cells resulted in a massive decrease in expression levels of the Gsk3 β protein. Moreover, KO of *Gsk3b* resulted in an increase of Fxr1 levels further validating the negative regulation of Fxr1 by Gsk3 β (Figures 1C,D) (Del'Guidice et al., 2015).

A dual AAV viral delivery of CRISPR/Cas9 (Swiech et al., 2015) was used for *in vivo* applications. This system comprises one AAV vector encoding the Cas9 nuclease expressed under the neuron-specific short mecp2 promoter (AAV spCas9). The second AAV vector encodes *Gsk3b* targeting sgRNA3 expressed under a U6 promoter and a GFP-KASH fusion protein under the neuron-specific human synapsin (hSYN) promoter (AAV Gsk3 sgRNA/GFP) (Swiech et al., 2015). AAV SpCas9 and AAV Gsk3 sgRNA/GFP viral particles were mixed in 1:1 ratio and injected into the mouse medial prefrontal cortex (Gsk3 sKO condition). A mixture of AAV spCas9 and AAV GFP viral particles were used as a control (CRISPR-Ctrl condition) (Figure 1E). Mice were sacrificed 3 weeks after infection and Gsk3 β expression was evaluated by immunofluorescent staining of brain slices using two different antibodies (Figure 1F and Supplementary Figure S1). Intense signal was detected throughout all brain slices since Gsk3 β is ubiquitously expressed in neurons, astrocytes, and microglia (Perez-Costas et al., 2010). All GFP expressing control neurons (infected with AAV spCas9 + AAV GFP) showed expression of Gsk3 β in their cell bodies. In contrast, 63% of GFP expressing Gsk3 sKO neurons (infected with AAV spCas9 + AAV Gsk3 sgRNA/GFP) had undetectable levels of Gsk3 β (Figures 1E,G). Moreover, on the same brain slice absence of Gsk3 β expression was only noted in virus infected neurons (infected with AAV spCas9 + AAV Gsk3sgRNA/GFP), while neurons outside of the infection area expressed Gsk3 β (Supplementary Figure S1). Hence, *in vivo* delivery of CRISPR/Cas9 resulted in efficient, brain region targeted and neuron-specific sKO of *Gsk3b* gene.

To overexpress Fxr1 (Fxr1 over condition), we delivered a GFP-Fxr1 fusion construct to the mPFC using AAV (Del'Guidice et al., 2015). AAV GFP was used as a control (Ctrl condition) (**Figure 1E**). Mouse mPFCs were dissected and expression of Fxr1 was evaluated by western blot (**Figure 1H**). Expression of GFP-Fxr1 protein was detected by both anti-GFP and anti-Fxr1 antibodies in mice from the Fxr1 over condition, as opposed to Ctrl mice where only expression of brain-specific Fxr1 isoforms and GFP protein were detected (**Figure 1H**).

Medial Prefrontal Cortex Specific Overexpression of Fxr1 or Gsk3 β Somatic Knockout Result in Reduced Anxiety-Related Behaviors

The interaction between functional polymorphisms of *GSK3B* and *FXR1* has been associated to the regulation of mood and emotionality in healthy subjects (Del'Guidice et al., 2015). Thus, we evaluated anxiety-related behavioral outcomes after augmentation of Fxr1 and reduction of Gsk3 β levels. Mice were injected into mPFC with either: AAV GFP-Fxr1 (Fxr1 over condition), AAV GFP (Ctrl condition), AAV spCas9 + AAV Gsk3sgRNA/GFP (Gsk3 sKO condition) or AAVspCas9 + AAV GFP (CRISPR-Ctrl condition). Mice were subjected to behavioral tests 3 weeks after viral infection. Mice from the CRISPR-Ctrl and Ctrl condition did not show difference in behavioral tests, indicating that expression of Cas9 does not affect behavioral responses by itself in these tests (Supplementary Figures S2A–K). From this point on, Ctrl group consisted of an equal number of mice from CRISPR-Ctrl and Ctrl conditions. Fxr1 overexpression and sKO of *Gsk3b* in mPFC resulted in anxiolytic-like behaviors compared to controls in three separate behavioral paradigms: the open field exploration tests (**Figures 2A–D**), the dark light emergence tests (**Figures 2E–H**) and the elevated plus maze (**Figure 2I**). To obtain integrated measures for each group of mice and summarize results across all the tests, we performed behavioral Z-scoring (Guilloux et al., 2011). Mice from Fxr1 over and Gsk3 sKO groups showed a decrease in emotionality Z score compared to Control mice, while locomotion Z score was unaffected (**Figures 2J,K**). This indicates that either selective increase in the expression of Fxr1 or knockout of *Gsk3b* in mPFC neurons is sufficient to reduce anxiety.

Prefrontal Overexpression of Fxr1 or Gsk3 β sKO Reduce Excitatory Synaptic Currents

To evaluate the impact of elevated Fxr1 and reduced Gsk3 β levels on neuronal activity acute brain slices were obtained from mice and whole cell patch clamp recordings were performed on mPFC layer III–V pyramidal neurons. Fxr1 overexpression and *Gsk3b* sKO resulted in decreased spontaneous excitatory postsynaptic current (sEPSC) amplitude and frequency as compared to control (**Figures 3A–D**). In contrast, no changes of spontaneous inhibitory postsynaptic currents (sIPSCs) were detected (**Figures 3E–G**). Neuronal excitability (**Figures 3H–J**) and action potentials properties (**Figures 3K–N**) were unaffected

by overexpression of Fxr1 and sKO of *Gsk3b*. Overall, this data indicates that augmentation of Fxr1 and reduction of Gsk3 β levels has a major impact on spontaneous excitatory activity.

KORD Mediated Silencing of mPFC Pyramidal Neurons Reduce Anxiety-Related Behaviors

To verify if reduced excitatory neuronal activity in pyramidal neurons of the mPFC can be associated to a reduction in anxiety-related behaviors, we used κ -opioid derived DREADD (KORD) (Vardy et al., 2015) mediated silencing of mPFC neurons. KORD is an engineered G α i protein-coupled kappa opioid receptor that can be specifically activated by the biologically inert drug salvinorin B (SalB) leading to neuronal silencing (Vardy et al., 2015). One limitation of the DREADD technology is that CNO, the activator of muscarinic receptor derived DREADDs, is metabolized to Clozapine *in vivo* thus leading to potential side effects other than activation of DREADDs (Gomez et al., 2017; Mahler and Aston-Jones, 2018). The use of KORD allows to circumvent this limitation since SalB has no biological activity *in vivo* (Vardy et al., 2015). Since inhibitory neurotransmission is not affected by manipulations of Gsk3 β and Fxr1 expression (**Figures 3E–G**), we sought to silence only excitatory neurons. To achieve this, an AAV vector with a CamKII α promoter was used to express KORD only in pyramidal neurons of the mPFC (Wang et al., 2013). Four weeks after AAV KORD injection, mice were subjected to behavioral testing. One group of mice received vehicle (veh) and a second SalB (**Figure 4A**). Silencing of mPFC pyramidal neurons in response to the activation of KORD by SalB resulted in anxiolytic-like behaviors similar to those observed in mice from the Fxr1 over and Gsk3 sKO conditions (**Figures 4B–E**) thus establishing a functional association between reduced excitatory neuronal activity and behavioral outcomes.

Prefrontal Overexpression of Fxr1 and Gsk3 β sKO Does Not Affect Spine Density

Excitatory synapses are mostly localized in dendritic spines of pyramidal neurons (Peters, 2007). Furthermore, congenital reductions in expression of Fragile X family proteins have been shown to result in alterations of synaptic spine density (Comery et al., 1997; Cook et al., 2014; Guo et al., 2015). We have performed morphological analysis to address whether observed reduction in the frequency of EPSCs was a result of decreased spine density. Since spine density on apical dendrite of pyramidal neurons may vary, we subdivided apical dendrite into distal and proximal parts to minimize variability (**Figures 5A,D** dotted squares). No differences were found on distal (**Figures 5A–C**) or proximal (**Figures 5D–F**) apical dendrite spine density between Fxr1 over, Gsk3 sKO and Ctrl conditions. This indicates that changes in Fxr1 or Gsk3 β expression levels do not induce major morphological alterations in synaptic spines of pyramidal neurons.

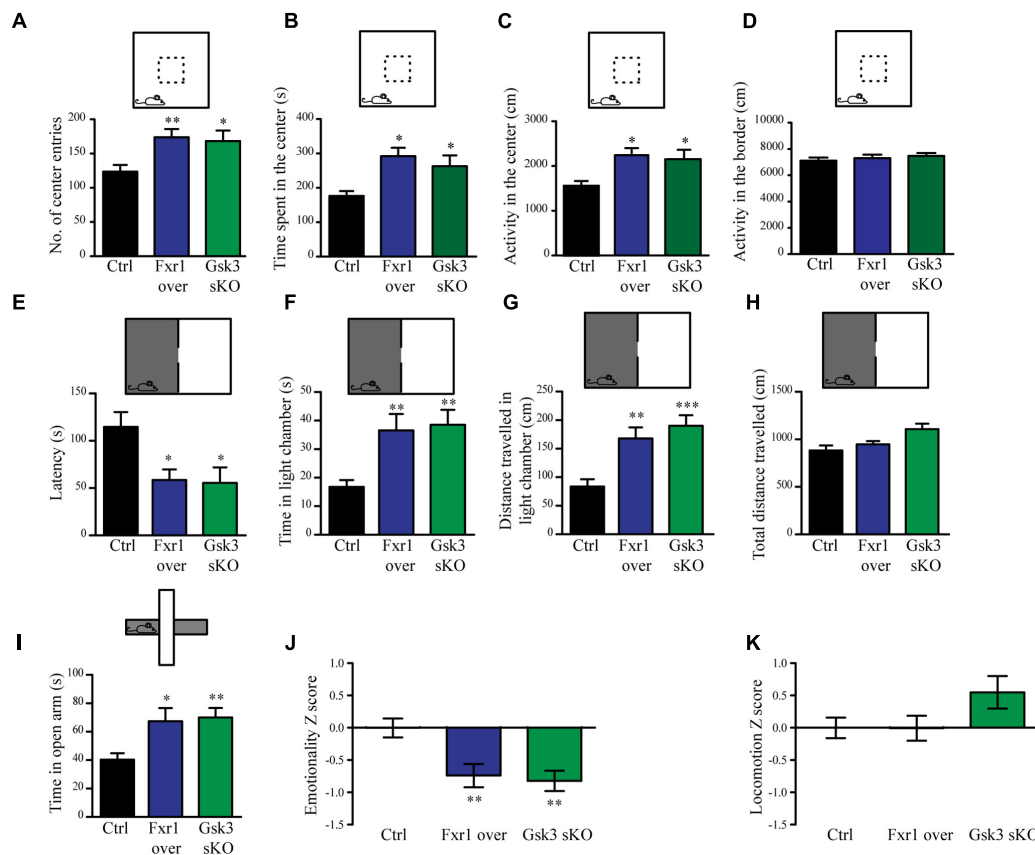


FIGURE 2 | Prefrontal CRISPR/Cas9 mediated *Gsk3b* sKO or *Fxr1* overexpression modulate mood related behaviors. **(A–D)** Open field test for control ($n = 18$), *Fxr1* overexpressing ($n = 16$) and *Gsk3* sKO ($n = 17$) mice. Graphs represent **(A)** number of center entries (Ctrl 123.6 ± 9.8 , *Fxr1* over 173.8 ± 11.9 , and *Gsk3* sKO 168.2 ± 15.3), **(B)** time spent in the center (Ctrl 176.4 ± 14.2 , *Fxr1* over 292.3 ± 24.3 , *Gsk3* sKO 262.8 ± 31.5), **(C)** horizontal activity in the center (Ctrl $1553 \text{ cm} \pm 114$, *Fxr1* over $2244 \text{ cm} \pm 156$, *Gsk3* sKO $2154 \text{ cm} \pm 108$) and **(D)** horizontal activity in the border (Ctrl $7106 \pm 242 \text{ cm}$, *Fxr1* over $7298 \pm 267 \text{ cm}$ and *Gsk3* sKO 7468 ± 218). **(E–H)** Dark/light emergence test for control ($n = 17$), *Fxr1* overexpressing ($n = 11$) and *Gsk3* sKO ($n = 10$) mice. Graph represents **(E)** latency to cross from the dark to the light compartment (Ctrl 114.5 ± 15.8 , *Fxr1* over 58.5 ± 11.1 , and *Gsk3* sKO 55.4 ± 11.4), **(F)** time spent in the light chamber (Ctrl 16.7 ± 2.3 , *Fxr1* over 36.5 ± 5.7 , *Gsk3* sKO 38.5 ± 5.2), **(G)** distance traveled in the light chamber (Ctrl $83.5 \text{ cm} \pm 12.6$, *Fxr1* over $168.1 \text{ cm} \pm 19$, *Gsk3* sKO $190.2 \text{ cm} \pm 18.4$) and **(H)** total distance traveled during all 5 min of the test (Ctrl $882.8 \text{ cm} \pm 52.3$, *Fxr1* over $947.1 \text{ cm} \pm 35.1$, *Gsk3* sKO $1107 \text{ cm} \pm 57.5$). **(I)** Elevated plus maze test for control (40.3 ± 4.5 , $n = 20$), *Fxr1* overexpressing (67.3 ± 9.2 , $n = 16$) and *Gsk3* sKO (70 ± 6.6 , $n = 20$) mice. Graph represents time spent in open arms during all 10 min of the test. **(J)** Emotionality Z-score for control (-0.0001719 ± 0.1489 , $n = 21$), *Fxr1* overexpressing (-0.7383 ± 0.1835 , $n = 18$) and *Gsk3* sKO (-0.7108 ± 0.1589 , $n = 20$) mice. **(K)** Locomotion Z-score for control ($3.6 \times 10^{-6} \pm 0.157$, $n = 21$), *Fxr1* overexpressing (-0.004789 ± 0.196 , $n = 18$) and *Gsk3* sKO (0.545 ± 0.255 , $n = 20$) mice. Error bars show standard error of the mean (SEM). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one way ANOVA).

Prefrontal Overexpression of *Fxr1* or *Gsk3b* sKO Alters AMPA Receptor Mediated Currents

We have observed a reduction of sEPSC amplitude in *Fxr1* over and *Gsk3* sKO conditions. Ionotropic AMPA and NMDA receptors are major players in mediating excitatory neurotransmission. Thus, we performed recordings in the presence of channel blockers to identify the main source of reduced excitatory currents. Bath application of AMPA receptor blocker CNQX drastically reduced amplitude and almost completely abolished frequency of recorded sEPSCs from all conditions (**Figure 6A**), indicating that recorded sEPSCs were mainly mediated by AMPA receptors. AMPA receptors are heterotetramers composed of a combination of four subunits (GluA1–4) (Hollmann and Heinemann, 1994). GluA1/GluA2

heterotetramers are the predominant AMPA receptors in the adult forebrain (Craig et al., 1993). GluA1 homotetramer AMPA receptors are Ca^{+2} permeable and inwardly rectifying (Jonas and Burnashev, 1995), thus changes in rectification index may indicate changes of AMPA receptor subunit composition. In order to calculate rectification index, we conducted current-voltage relationship (I–V curve) experiments using electric stimulation to evoke EPSCs. Overexpression of *Fxr1* had no effect on I–V curve of evoked mixed AMPA + NMDA currents (**Figure 6B**) and isolated NMDA currents, recorded in the presence of CNQX (**Figure 6C**). In contrast, recordings of AMPA mediated EPSCs revealed a decrease of the rectification index in *Fxr1* over and *Gsk3* sKO conditions (**Figure 6D**), thus suggesting a change in the AMPA receptor composition corresponding to the prevalence of GluA1 homotetramer mediated currents.

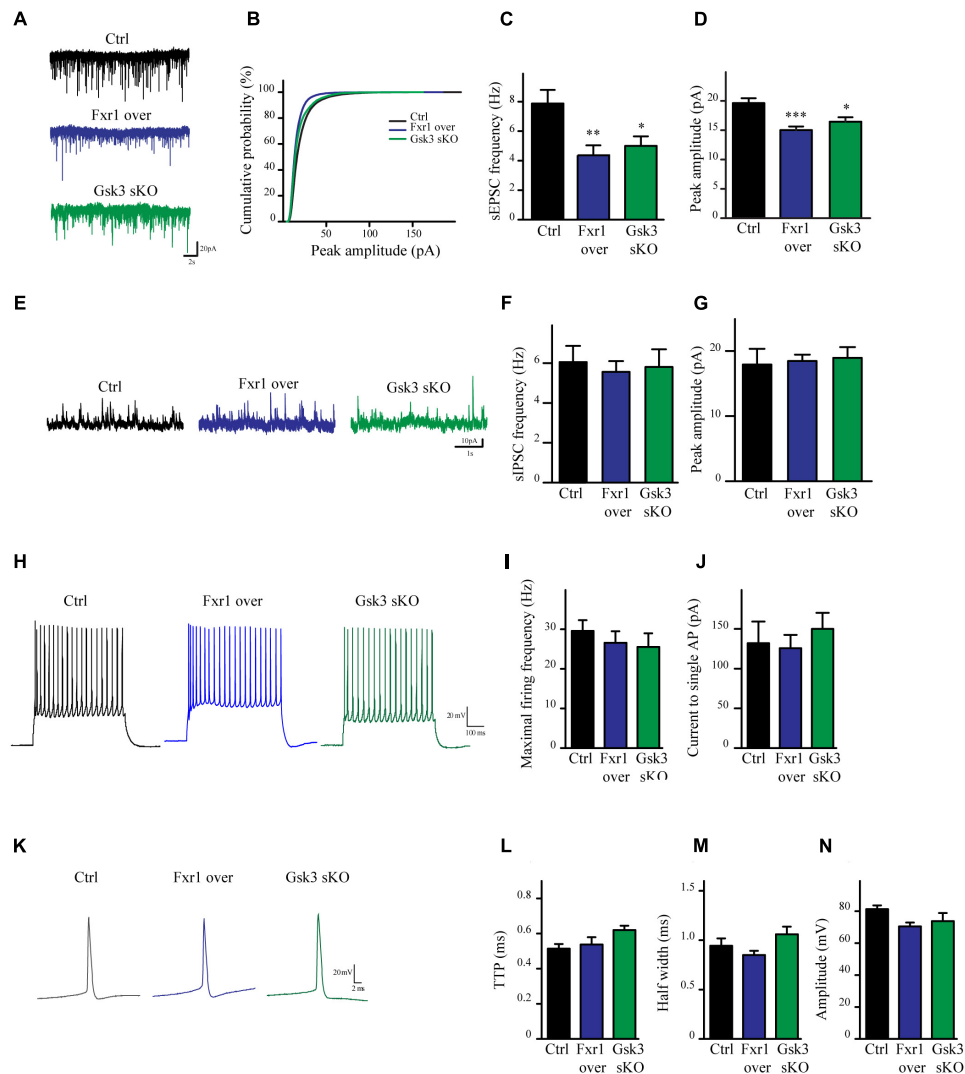
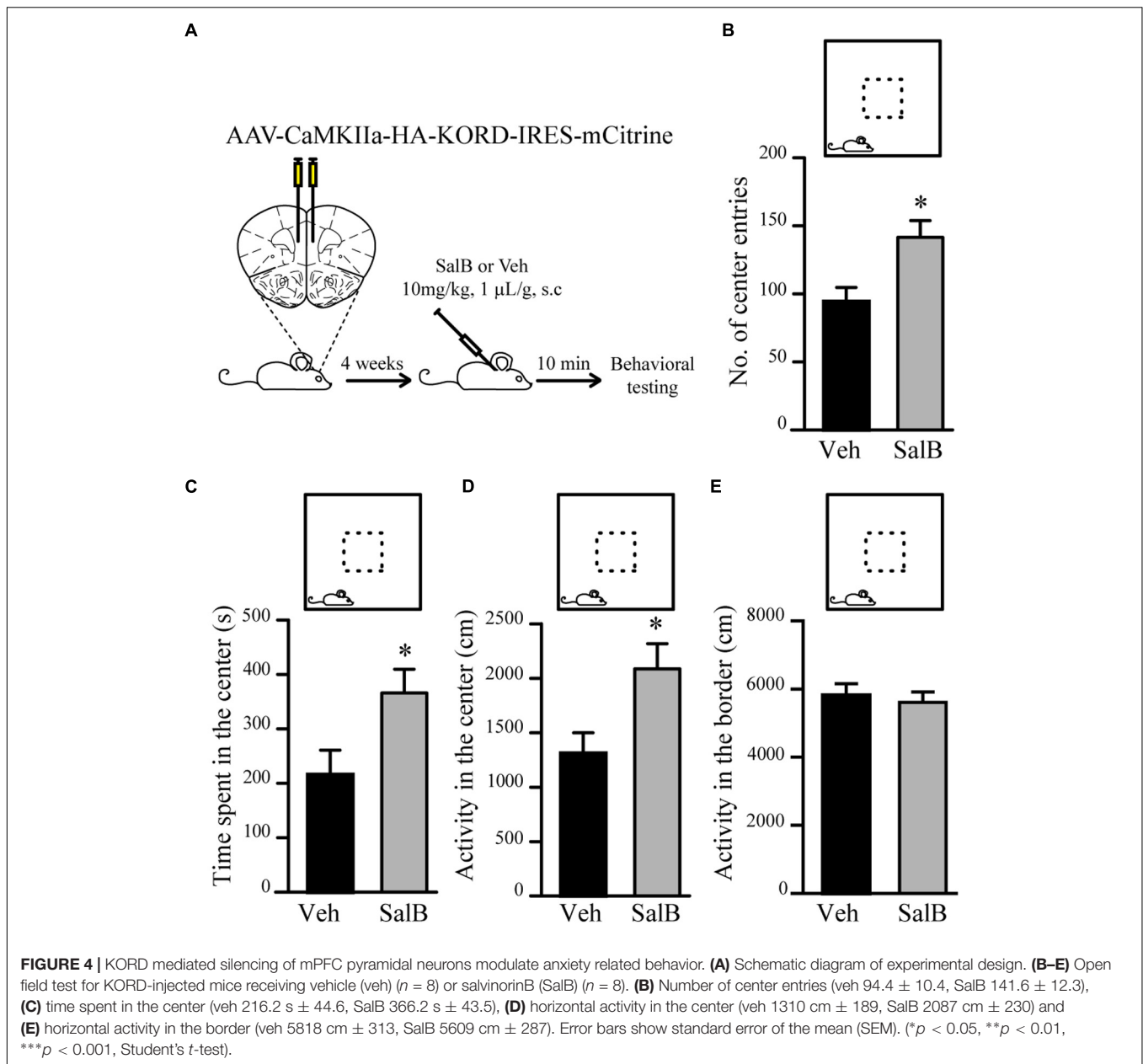


FIGURE 3 | Prefrontal CRISPR/Cas9 mediated *Gsk3b* sKO or *Fxr1* overexpression modulate the spontaneous neuronal activity. **(A)** Individual traces showing spontaneous EPSCs recorded from cortical slices of control, *Fxr1* overexpressing and *Gsk3* sKO mice. **(B)** Cumulative probability plot of sEPSCs amplitude (500 events per cell, control $n = 26$, *Fxr1* overexpression $n = 20$, or *Gsk3* sKO $n = 19$). sEPSCs **(C)** frequency and **(D)** peak amplitude in control (Frequency $7.87\text{ Hz} \pm 0.92$, amplitude $19.61\text{ pA} \pm 0.82$), *Fxr1* overexpressing (Frequency $4.36\text{ Hz} \pm 0.67$, amplitude $15.01\text{ pA} \pm 0.62$) and *Gsk3* sKO (Frequency $4.99\text{ Hz} \pm 0.65$, amplitude $16.45\text{ pA} \pm 0.75$) neurons. **(E)** Individual traces showing spontaneous sEPSCs recorded from cortical slices of control, *Fxr1* overexpressing and *Gsk3* sKO mice. Summary bar graphs showing **(F)** frequency and **(G)** peak amplitude of control (frequency $6.05\text{ Hz} \pm 0.81$, amplitude $17.9\text{ pA} \pm 2.41$), *Fxr1* overexpressing (frequency $5.56\text{ Hz} \pm 0.54$, amplitude $18.46\text{ pA} \pm 0.96$) and *Gsk3* sKO neurons (frequency $5.80\text{ Hz} \pm 0.88$, amplitude $18.93\text{ pA} \pm 1.65$). **(H)** Individual traces showing trains of action potentials recorded from cortical slices of control, *Fxr1* overexpressing and *Gsk3* sKO mice. **(I, J)** Graphs showing excitability of control, *Fxr1* overexpressing and *Gsk3* sKO cells, **(I)** maximal firing rate (Ctrl 29.6 ± 3.5 , *Fxr1* over 26.5 ± 3.4 , *Gsk3* sKO 25.5 ± 3.5), **(J)** current necessary to evoke single action potential (Ctrl 132 ± 27 , *Fxr1* over 125 ± 16 , *Gsk3* sKO 150 ± 20). **(K)** Individual traces showing single action potential recorded from cortical slices of control, *Fxr1* overexpressing and *Gsk3* sKO mice. **(L–N)** Graphs showing action potential properties of control, *Fxr1* overexpressing and *Gsk3* sKO cells, **(L)** time to peak (TTP) (Ctrl 0.51 ± 0.02 , *Fxr1* over 0.53 ± 0.04 , *Gsk3* sKO 0.62 ± 0.02), **(M)** half-width (Ctrl 0.94 ± 0.07 , *Fxr1* over 0.85 ± 0.04 , *Gsk3* sKO 1.06 ± 0.07) and **(N)** amplitude (Ctrl 81.2 ± 2.3 , *Fxr1* over 70.5 ± 2.3 , *Gsk3* sKO 73.8 ± 4.25). Error bars show standard error of the mean (SEM). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one way ANOVA).

Prefrontal Overexpression of *Fxr1* or *Gsk3b* sKO Affect Components of the Glutamatergic Synapse

Fxr1 overexpression and *Gsk3b* sKO resulted in augmentation of GluA1 homomer mediated currents, while overall AMPA receptor-mediated sEPSCs were decreased. These changes can

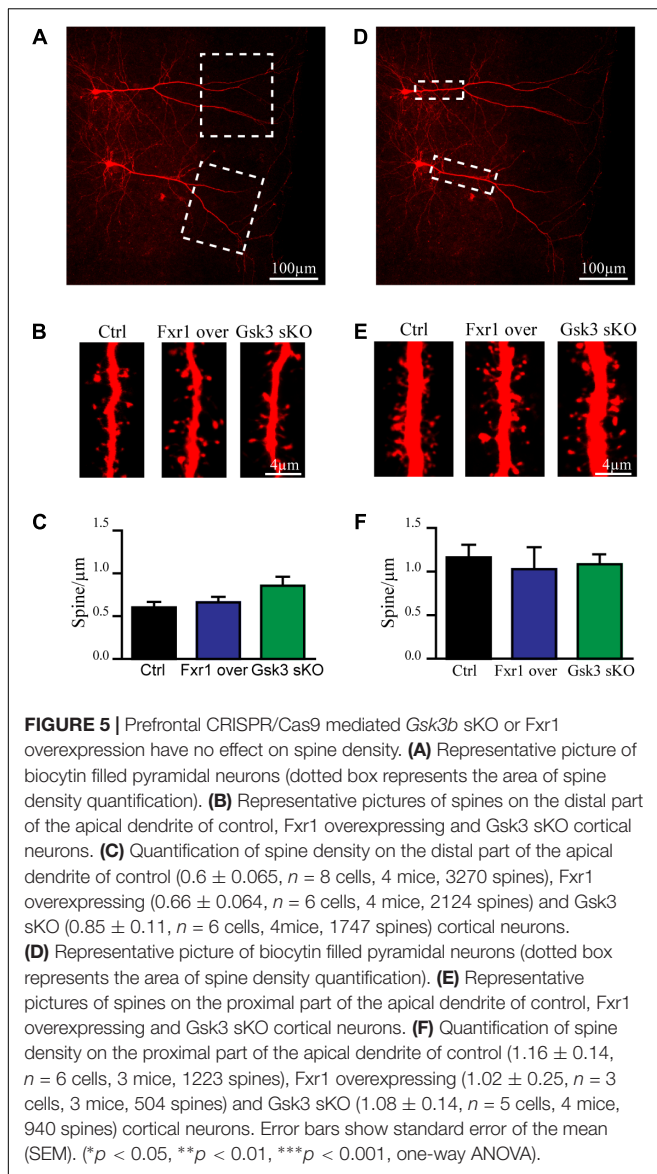
originate either from altered local translation in spines or from changes in trafficking and subsequent insertion of GluA1 and GluA2 subunits into the glutamatergic synapse. To address those questions we investigated the impact of *Fxr1* overexpression and *Gsk3b* sKO directly on the level of GluA1 and GluA2 subunits. To be more selective for local protein expression in spines and enrich samples for synaptic proteins, we performed crude



synaptosome isolation from dissected brain tissue (**Figure 7A**). As a validation, we found enrichment of synaptic proteins in our synaptosomal preparation (**Figure 7B**). Overexpression of Fxr1 and sKO of *Gsk3b* did not result in changes in the levels of GluA1 and GluA2 subunits in the synaptosomal preparation (**Figures 7C,D**). This indicates that local expression of AMPA receptor subunits may not be altered in these conditions. It has been shown that phosphorylation of GluA1 and GluA2 may be involved in regulation of their trafficking and anchoring to postsynaptic density, hence in their surface expression (Banke et al., 2000; Chung et al., 2000; Ehlers, 2000; Esteban et al., 2003; Seidenman et al., 2003; Steinberg et al., 2006; Man et al., 2007; Diering et al., 2014). Levels of both p845 GluA1 and p880 GluA2 were reduced in synaptosomes following *Gsk3b* sKO or Fxr1

overexpression (**Figures 7C,D**). This shows that synaptic AMPA receptor composition changes in these conditions are likely not due to changes in local synthesis, but rather altered trafficking of GluA1 and GluA2 subunits.

In addition to changes in synaptic AMPA receptor subunit trafficking, we identified a decrease in vesicular glutamate transporter Vglut1 indicating possible presynaptic alterations under Fxr1 over and *Gsk3* sKO conditions (**Figure 7E**). No changes in expression level of synaptosomal PSD95 (**Figure 7F**), NMDA receptor subunit 1 (**Figure 7G**), GABA A receptor subunit alpha 1 (**Figure 7H**), Neuroligin1 and Neuroligin 2 (**Figure 7I**) were observed between Fxr1 over, *Gsk3* sKO and Control conditions. Overall, augmentation of Fxr1 and reduction of *Gsk3 β* in mPFC decrease both p845 GluA1 and p880



GluA2 subunits, as well as synaptosomal Vglut1. This indicates a broad impact of *Fxr1*-Gsk3 β signaling on glutamatergic neurotransmission potentially affecting both pre- and postsynaptic compartments.

DISCUSSION

FXR1 recently has been identified as a risk factor for schizophrenia and bipolar disorder (Consortium, 2014; Hauberg et al., 2016; Liu et al., 2016; Takata et al., 2017). Interaction between polymorphisms affecting cortical expression of the *FXR1* and *GSK3B* genes have been shown to regulate mood-related behavioral dimensions in healthy humans and patients with bipolar disorder (Del'Guidice et al., 2015; Bureau et al., 2017). This genetic interaction may be explained by the negative regulation of *Fxr1* following its phosphorylation by Gsk3 β

(Del'Guidice et al., 2015; Qie et al., 2017). Results presented here demonstrate how neuronal activity and related behavior are impacted by Gsk3 β and *Fxr1*. Mice with reduced Gsk3 β or elevated *Fxr1* expression in mPFC showed decreased anxiety-related behaviors and reduced AMPA mediated excitatory postsynaptic currents. Our results indicate that these effects originate from the capacity for the Gsk3 β and *Fxr1* to alter AMPA mediated glutamatergic neurotransmission by affecting synaptic GluA1 and GluA2 subunits as well as vesicular glutamate transporter Vglut1.

Systemic inhibition of Gsk3 β activity has been shown to result in anti-depressant and anxiolytic-like behavioral effects in mice (Kaidanovich-Beilin et al., 2004; Beaulieu et al., 2008a,b, 2009). Similar behavioral signatures were reported using a conventional Cre-lox system to suppress Gsk3 β expression in all CamKII expressing forebrain pyramidal neurons (Latapy et al., 2012) or in all the cells of the prefrontal cortex (Del'Guidice et al., 2015). Overexpression of *Fxr1* in mPFC has been shown to have anxiolytic-like effect in DLET (Del'Guidice et al., 2015). Here we used CRISPR/Cas9 mediated sKO to achieve brain region targeted and neuron-specific modulation of *Gsk3b* gene expression in adult mice. Moreover, we expended the characterization of anxiety-related behaviors and obtained an integrated index from all the tests. Reduction of Gsk3 β or elevation of *Fxr1* levels in mPFC neurons resulted in similar anxiolytic-like behaviors, further validating *in vivo* relationship between these two proteins. The modulation of mood-related behaviors by *Fxr1* and Gsk3 β is in line with observations in human subjects carrying functional polymorphisms for *FXR1* and *GSK3B* genes (Del'Guidice et al., 2015). Interestingly, anxiety symptoms are highly comorbid in schizophrenia patients and *FXR1* being a risk factor for schizophrenia can represent a potential molecular target to study mood related problems in these patients (Braga et al., 2013; Temmingh and Stein, 2015). Overall, our results illustrate that alteration of Gsk3 β and *Fxr1* expression levels in mPFC neurons of adult mice is sufficient to modulate mood-related behaviors.

A reduction in the frequency and amplitude of sEPSC has been reported following the Cre/Lox mediated suppression of Gsk3 β expression in CA1 pyramidal neurons in adult mice (Ochs et al., 2015). These effects have been suggested to result from increased beta-catenin levels. Here we show that modulation not only of Gsk3 β but also its substrate *Fxr1* in mPFC can result in similar electrophysiological outcomes with behavioral consequences. This shows the need to expand studies of Gsk3 β targets and their involvement in the various functions of this kinase. This may lead to the identification of converging or diverging functional pathways involving different Gsk3 β targets.

The modulation of neuronal activity by Gsk3 β and *Fxr1* in the mPFC is most probably linked to their effects on anxiety-related behaviors. Indeed, chemo-genetic KORD mediated silencing of mPFC pyramidal neurons caused anxiolytic-like responses, therefore supporting a link between neuronal activity and behavior. Interestingly, inhibition of the direct excitatory input from ventral hippocampus (vHPC) to mPFC has been shown to decrease anxiety in the elevated plus maze and open field test (Padilla-Coreano et al., 2016). The decrease of mPFC

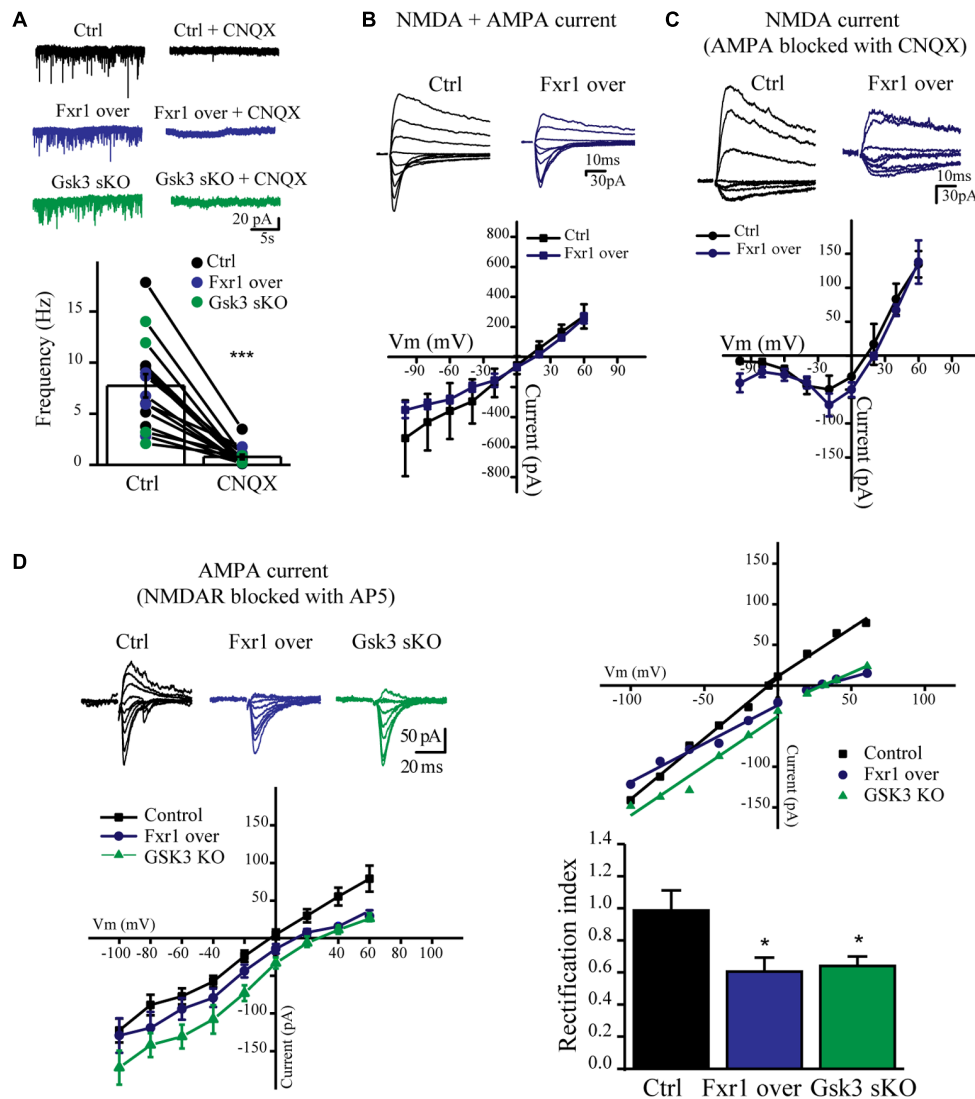


FIGURE 6 | Prefrontal CRISPR/Cas9 mediated *Gsk3b* sKO or *Fxr1* overexpression affect AMPA current. **(A)** Frequency of sEPSCs recorded from cortical slices in control ($7.74\text{Hz} \pm 1.18$, $n = 15$) and after application of CNQX ($0.78\text{Hz} \pm 0.24$, $n = 15$) ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, paired Student's *t*-test). **(B–C)** I-V curves of evoked EPSC amplitude recorded in control (black, $n = 3$) and *Fxr1* overexpressing (blue, $n = 3$) brain slices **(B)** before and **(C)** after application of CNQX in the presence of bicuculline. Representative examples of recordings are shown as inserts on top. **(D)** Top Left: Representative traces of evoked EPSC amplitude recorded in control (black), *Fxr1* overexpressing (blue) and *Gsk3* sKO (green) slices in the presence of bicuculline and AP-5. Average graph (Bottom Left) and representative examples (Top Right) of I-V curves of evoked EPSC amplitude recorded in control (black), *Fxr1* overexpressing (blue) and *Gsk3* sKO (green) neurons from brain slices (Top Right: lines show linear fit to the data and calculated reversal potential is also included among data points). Bottom Right: summary bar graphs showing rectification index of control (black, 0.99 ± 0.13 , $n = 8$), *Fxr1* overexpressing (blue, 0.51 ± 0.08 , $n = 8$) and *Gsk3* sKO (green, 0.64 ± 0.06 , $n = 9$) neurons. ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, one-way ANOVA). Error bars show standard error of the mean (SEM).

neuronal activity has also been associated with resilience in the learned helplessness model of depression (Wang et al., 2014). In contrast, increase of mPFC activity using chemogenetics has been reported to trigger helplessness in resilient mice in this same model (Wang et al., 2014). Along with our observations, this supports a role for the decreased neuronal activity of mPFC neurons in maintaining low emotionality and greater mood stability.

Recordings from brain slices showed that reduction of Gsk3 β or augmentation of *Fxr1* expression affects excitatory

postsynaptic activity through modulation of AMPA receptors, which includes not only decrease in the sEPSC amplitude, but also change in the rectification index. Further investigation revealed that augmentation of *Fxr1* and reduction of Gsk3 β levels resulted in a decrease of both synaptic GluA1 and GluA2 subunits. These results indicate an overall decrease in synaptic AMPA receptors along with a possible switch from predominantly heteromeric GluA1/GluA2 containing to homomeric GluA1 AMPA receptors with higher rectification properties.

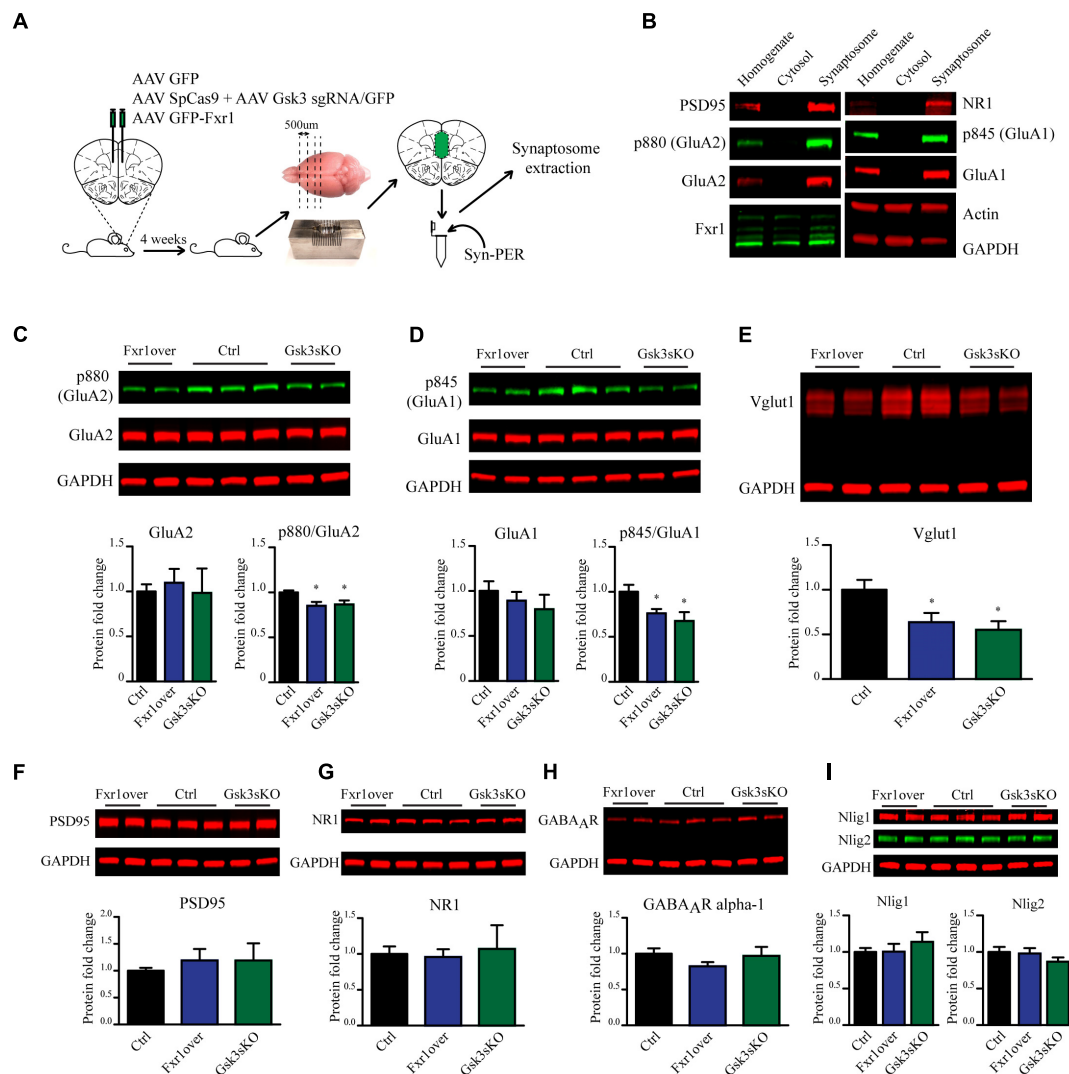


FIGURE 7 | Prefrontal CRISPR/Cas9 mediated *Gsk3b* sKO or *Fxr1* overexpression affect pre- and postsynaptic components of the glutamatergic synapse. **(A)** Schematic diagram of experimental design. **(B)** Visualization of candidate proteins by Western blot in Homogenate, Cytosol and Synaptosomal fractions. **(C)** Expression of AMPA receptor subunit GluA2 and its p880 phosphorylated form in synaptosomes from Ctrl (GluA2/GAPDH 1 ± 0.079 , p880/GluA2 1 ± 0.021 , $n = 6$ mice), *Fxr1* overexpressing (GluA2/GAPDH 1.09 ± 0.15 , p880/GluA2 0.85 ± 0.04 , $n = 7$ mice) and *Gsk3* sKO (GluA2/GAPDH 0.98 ± 0.27 , p880/GluA2 0.86 ± 0.04 , $n = 5$ mice) mice. A representative picture is shown on the top panel. **(D)** Expression of AMPA receptor subunit GluA1 and its p845 phosphorylated form in synaptosomes from Ctrl (GluA1/GAPDH 1 ± 0.05 , p845/GluA1 1 ± 0.07 , $n = 6$ mice), *Fxr1* overexpressing (GluA1/GAPDH 1.17 ± 0.09 , p845/GluA1 0.76 ± 0.04 , $n = 7$ mice) and *Gsk3* sKO (GluA1/GAPDH 1.16 ± 0.09 , p845/GluA1 0.67 ± 0.09 , $n = 5$ mice) mice. A representative picture is shown on the top panel. **(E)** Expression of Vglut1 in synaptosomes from Ctrl (Vglut1/GAPDH 1 ± 0.11 , $n = 6$ mice), *Fxr1* overexpressing (Vglut1/GAPDH 0.63 ± 0.1 , $n = 7$ mice) and *Gsk3* sKO (Vglut1/GAPDH 0.55 ± 0.09 , $n = 4$ mice) mice. A representative picture is shown on the top panel. **(F)** Expression of PSD95 in synaptosomes from Ctrl (PSD95/GAPDH 1 ± 0.05 , $n = 6$ mice), *Fxr1* overexpressing (PSD95/GAPDH 1.19 ± 0.2 , $n = 7$ mice) and *Gsk3* sKO (PSD95/GAPDH 1.18 ± 0.03 , $n = 5$ mice) mice. A representative picture is shown on the top panel. **(G)** Expression of NMDA receptor subunit NR1 in synaptosomes from Ctrl (NR1/GAPDH 1 ± 0.1 , $n = 6$ mice), *Fxr1* overexpressing (NR1/GAPDH 0.95 ± 0.1 , $n = 7$ mice) and *Gsk3* sKO (NR1/GAPDH 1 ± 0.3 , $n = 5$ mice) mice. A representative picture is shown on the top panel. **(H)** Expression of GABA A receptor subunit alpha 1 in synaptosomes from Ctrl (GABA $_A$ R/GAPDH 1 ± 0.07 , $n = 6$ mice), *Fxr1* overexpressing (GABA $_A$ R/GAPDH 0.8 ± 0.05 , $n = 7$ mice) and *Gsk3* sKO (GABA $_A$ R/GAPDH 0.96 ± 0.12 , $n = 5$ mice) mice. A representative picture is shown on the top panel. **(I)** Expression of Neuroligin 1 and Neuroligin 2 in synaptosomes from Ctrl (Nlig1/GAPDH 1 ± 0.04 , Nlig2/GAPDH 2 ± 0.05 , $n = 6$ mice), *Fxr1* overexpressing (Nlig1/GAPDH 1 ± 0.1 , Nlig2/GAPDH 0.97 ± 0.07 , $n = 7$ mice) and *Gsk3* sKO (Nlig1/GAPDH 1.13 ± 0.12 , Nlig2/GAPDH 0.86 ± 0.05 , $n = 5$ mice) mice. A representative picture is shown on the top panel. Error bars show standard error of the mean (SEM). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t -test).

Apart from the autosomal *Fxr1*, the fragile X gene family comprises two other members *Fmr1*, which is encoded on the X chromosome and *Fxr2*, which is also autosomal. These proteins show strong structural homology but do not have fully

overlapping functions (Siomi et al., 1995; Kirkpatrick et al., 2001; Bontekoe et al., 2002; Spencer et al., 2006; Zhang et al., 2009; Xu et al., 2011; Guo et al., 2015). In line with this, it has been shown that *Fmr1*, and *Fxr2*, are involved in the regulation of

AMPA receptor subunits via distinct mechanisms (Guo et al., 2015). Fxr2 directly binds to the coding sequence of GluA1 and regulates its expression by stabilizing its mRNA, while Fmr1 only regulates surface levels of this AMPA receptor subunit with no effect on its expression levels (Guo et al., 2015). Regulation of GluA2 by Fxr2 has also been reported albeit with variable results (Cook et al., 2014; Guo et al., 2015). In hippocampal slices, Fxr1 has been reported to negatively regulate the *de novo* synthesis of the GluA2 subunit of AMPA receptors by directly binding to the 5'UTR of its mRNA, during chemically induced long-term potentiation. However, possible alterations of GluA1 subunit were not thoroughly investigated (Cook et al., 2014). Our results suggest a regulation of both AMPA receptor subunits by Fxr1. Interestingly Fxr1 and Gsk3 β altered only p845 GluA1 and p880 GluA2 with no apparent changes in total synaptosomal expression levels of these AMPA receptor subunits. This could be indicative of a regulation on the level of receptor trafficking, however, the exact mechanism by which Fxr1 may regulate GluA1 and GluA2 subunits in this system remains to be investigated.

Changes in Fxr1 and Gsk3 β levels did not result in alterations of synaptosomal PSD95, NMDA receptor subunit 1, GABA A receptor alpha 1, Neuroligin 1 and 2. This is in line with the absence of alterations in spine density as well as NMDA receptor and GABA receptor-mediated currents observed under the Fxr1 over and Gsk3 sKO conditions. However, those conditions resulted in a decrease of Vglut1 indicating possible alterations in presynaptic glutamate release. The decrease in Vglut1 has been shown to affect the quantal size and result in a reduction of frequency and amplitude of EPSCs (Wojcik et al., 2004). Thus, reduction in Vglut1, along with a reduction in GluA1 and GluA2, may contribute to decrease in amplitude and explain the decrease in the frequency of spontaneous EPSCs found in Fxr1 over and Gsk3 sKO conditions. Overall, this indicates that alteration in Fxr1 and Gsk3 β expression may have both pre- and post-synaptic impact on spontaneous glutamatergic neurotransmission.

CONCLUSION

Our results showcase that a disease-associated factor Fxr1 and its regulator Gsk3 β modulate components of neuronal signaling and impact behavioral manifestations in the same manner.

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Inhibition of GSK3 activity has been suspected for a long time to contribute to the behavioral actions of psychoactive drugs such as lithium, antidepressants, and antipsychotics (Beaulieu et al., 2009; Beurel et al., 2011). The correlation between the effects of Gsk3 β inactivation and Fxr1 overexpression suggests that this RNA binding protein may be one of the major substrate through which Gsk3 β exerts these effects by modulating glutamatergic synapses. Further manipulation of Gsk3 β -Fxr1 signaling in different brain regions and cell types may allow uncovering the molecular and circuit level underpinnings of various phenotypes impacted by this signaling. This, in turn, could shed light on the pathophysiology of mental disorders and lead to the rational development of novel therapeutics.

AUTHOR CONTRIBUTIONS

J-MB and JK conceived the study and designed the experiments. J-MB, JK, and AE wrote the manuscript. JK performed the design and testing of CRISPR/Cas9 *in vitro* and *in vivo*, stereotaxic injections, brain dissections and synaptosome preparations, protein expression analysis *in vitro* and *in vivo*, mouse behaviors, spine counting, and data analysis. AE and SC performed whole cell patch clamp recordings and data analysis. AM performed CRISPR/Cas9 KO experiments with puromycin selection followed by detection of Gsk3 β and Fxr1 expression. AB performed behaviors of KORD-injected mice. KT provided technical, financial, and intellectual support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2018.00119/full#supplementary-material>

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Modeling Fragile X Syndrome in *Drosophila*

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Intellectual disability (ID) and autism are hallmarks of Fragile X Syndrome (FXS), a hereditary neurodevelopmental disorder. The gene responsible for FXS is Fragile X Mental Retardation gene 1 (*FMR1*) encoding the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein involved in RNA metabolism and modulating the expression level of many targets. Most cases of FXS are caused by silencing of *FMR1* due to CGG expansions in the 5'-UTR of the gene. Humans also carry the FXR1 and FXR2 paralogs of *FMR1* while flies have only one *FMR1* gene, here called *dFMR1*, sharing the same level of sequence homology with all three human genes, but functionally most similar to *FMR1*. This enables a much easier approach for *FMR1* genetic studies. *Drosophila* has been widely used to investigate *FMR1* functions at genetic, cellular, and molecular levels since *dFMR1* mutants have many phenotypes in common with the wide spectrum of *FMR1* functions that underlay the disease. In this review, we present very recent *Drosophila* studies investigating FMRP functions at genetic, cellular, molecular, and electrophysiological levels in addition to research on pharmacological treatments in the fly model. These studies have the potential to aid the discovery of pharmacological therapies for FXS.

Keywords: Fragile X Syndrome, *FMR1*, Fragile X Mental Retardation Protein, *Drosophila*, *dFMR1*, neuromuscular junction, mushroom bodies, behavior

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INTRODUCTION

The Fragile X Syndrome (FXS), previously known as Martin-Bell syndrome or marker X syndrome or FRAXA, is the first X-linked intellectual disability (ID) syndrome described involving a DNA alteration and the most frequent heritable monogenic form of ID (reviewed in Penagarikano et al., 2007; Santoro et al., 2012; Hayward et al., 2017). Human FXS patients present severe ID often accompanied by an increase in Autism Spectrum Disorder (ASD) traits and other phenotypes like delayed development, hyperactivity, attention deficit, hypersensitivity to sensorial stimuli, anxiety, aggression, sleep, cardiac disorders, and epileptic seizures (reviewed in Hagerman, 2002; Garber et al., 2008; Utari et al., 2010; Santoro et al., 2012; Hagerman et al., 2014; Kidd et al., 2014; Maurin et al., 2014; Schaefer et al., 2015; Dahlhaus, 2018). These abnormalities can be explained by defects in neuronal development and maturation. Some patients also present characteristic morphological facial traits, macrocephaly, flat feet, and male macroorchidism. The first morphological phenotype observed in FXS patients was the presence of abnormalities in the spines (Comery et al., 1997; Irwin et al., 2001). More recently, ElectroEncephaloGraphy (EEG) and Magnetic Resonance Imaging (MRI) have evidenced volume and Event Related Potential (ERP) defects in FXS patients (Devitt et al., 2015).

FXS was initially associated with an X-chromosome fragile site (an isochromatid gap in metaphase chromosomes) in position Xq27.3 (Harrison et al., 1983). In 1991, this site was mapped to a CGG trinucleotide expansion in the 5' non-coding region of a gene named *Fragile X Mental Retardation 1* (*FMR1*), the first gene associated with an X-linked ID (Verkerk et al., 1991). *FMR1* is 38 kb long and transcribed in a 4.4 kb full length mRNA that encodes a 632 aa protein called Fragile X Mental Retardation Protein (FMRP). Through alternative splicing, at least 12 different isoforms of 67–80 kD are produced. The CGG repeats are polymorphic in the population ranging from 5 to 54 repeats in normal individuals to more than 200 (full mutation) in severely affected patients (reviewed in Hayward et al., 2017). The repeat expansion results in hypermethylation of the CGG repeat, of a 5' CpG island, and of flanking promoter sequences causing the reduction or absence of *FMR1* expression through an epigenetic mechanism involving *FMR1* mRNA (Colak et al., 2014). Several deletions and point mutations leading to the production of non-functional proteins have also been described (Okray et al., 2015 and references therein). Individuals with 55–200 CGG repetitions (premutation) do not present FXS symptoms, but may develop two other disorders: Fragile-X Primary Ovarian Insufficiency (FXPOI) (reviewed in Sherman et al., 2014) or Fragile X Associated Tremor/Ataxia Syndrome (FXTAS) (reviewed in Hall et al., 2016; Dahlhaus, 2018). FXTAS has been modeled in *Drosophila* by overexpressing 90 rCGG repeats alone fused to GFP, which causes a neuron-specific degeneration and the formation of inclusions (Jin et al., 2003; Qurashi et al., 2012).

In mammals, FMRP is nearly ubiquitous, present mainly in neurons (particularly in the cortex, hippocampus, and Purkinje cells) and in testes and absent from muscles and the heart (Devys et al., 1993). FMRP has two paralogs: Fragile X Related 1 (*FXR1*) and Fragile X Related 2 (*FXR2*). While *FXR2* has a distribution comparable to that of FMRP, some isoforms of *FXR1* display a specific expression in brain while other isoforms are only present in muscle and heart (Khandjian et al., 1998; Bechara et al., 2007). These three proteins are members of the same family, namely the Related Fragile X Protein family, and are RNA-binding proteins mainly localized in the cytoplasm, although they carry a Nuclear Localization Signal (NLS) and a Nuclear Exportation Signal (NES) (Bardoni et al., 2000). Indeed, some isoforms of FMRP have also been localized in the nucleus (Eberhart et al., 1996; Bardoni et al., 1997). Collectively, these results have suggested that the three FXR proteins are able to shuttle between nucleus and cytoplasm to export their target mRNAs.

Three RNA-binding sequence motifs are the hallmarks of FMRP that may explain its function, i.e., two K homology (KH) domains and one Arginine-Glycine-Glycine (RGG) box. The main function of FMRP is to regulate translation and indeed it has been found associated with polyribosomes in different cell lines and, importantly, in the brain (Khandjian et al., 2004). Although FMRP mainly acts as a repressor, an activator function has been observed (reviewed in Maurin et al., 2014). Many methods have been used to identify FMRP targets (reviewed in Maurin et al., 2014; Davis and Broadie, 2017; Hayward et al., 2017): binding to biotinylated RNAs (Ashley

et al., 1993), Cross-Link Immuno Precipitation (CLIP) (Darnell et al., 2011; Ascano et al., 2012; Anderson et al., 2016), Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Chen et al., 2003), yeast two-hybrid system (Ma et al., 2016), yeast three-hybrid system (Dolzanskaya et al., 2003), and Antibody-Positioned RNA Amplification (APRA) (Miyashiro et al., 2003). Many of the identified targets have been involved in autism, other neuronal pathologies or gonadal development and many of them encode synaptic proteins (reviewed in Maurin et al., 2014). Finally, FMRP has been linked to the microRNA (miRNA) and Piwi-interacting RNA (piRNA) pathways in Mammals, *Drosophila*, and zebrafish (reviewed in Kelley et al., 2012; Specchia et al., 2017).

FUNCTIONAL INSIGHTS ON FXS FROM *DROSOPHILA* STUDIES

The first model of FXS was the mammalian mouse model (The Dutch-Belgian Fragile X Consortium, 1994; Mientjes et al., 2006), which recapitulates some major patients' phenotypes (Dahlhaus, 2018 and references therein). However, ever since then, also research on *Drosophila melanogaster* has brought important knowledge on the basic mechanisms underlying FMRP function. The *Drosophila* homolog of *FMR1* was first identified in 2000 (Wan et al., 2000) and named *dfmr1*. Over the years, it has been called by many different names that are listed in the *Drosophila* database FlyBase (<http://flybase.org/reports/FBfr0174476.html>). It is now named *Fmr1* with a capital F, meaning that it has been identified through the human homolog *FMR1*. Here, we will call it *dFMR1* to distinguish it from the mouse gene (*Fmr1*). FlyBase names the protein *Fmr1*, but here we will call it *dFMRP* with the "d" indicating *Drosophila*. The *dFMR1* gene exhibits high sequence homology with all three human genes (FMRP, *FXR1*, and *FXR2*; Zhang et al., 2001; Coffee et al., 2010), but is most functionally related to *FMR1* (Coffee et al., 2010; see below). *dFMR1* is 8.7 kb long and transcribed in many different mRNAs of 2–4 kb encoding many different proteins of different sizes (<http://flybase.org/reports/FBgn0028734.html>). All functional domains are highly conserved with the two KH domains being 75% identical and 85% similar between *dFMR1* and *hFMR1* (Wan et al., 2000).

The gene expression of *dFMR1* in embryos was explored soon after its cloning and observed in the Central Nervous System (CNS), in the somatic musculature, in pole cells, in the gut and in the gonads (Wan et al., 2000; Zhang et al., 2001; Schenck et al., 2002). In **Figure 1**, we show the expression of *dFMR1* at stage 14 by *in situ* hybridization with a full-length probe using the Tyramide Signal Amplification (TSA) (Tevy et al., 2014). High levels of expression are found in the brain (**Figure 1A**, arrowhead), in the CNS (**Figure 1A**, arrow) and in muscle precursors (**Figure 1B**), confirming the previously described pattern of *dFMR1* expression at this stage through a sensitive method.

In embryos, *dFMRP* has been localized in the brain, ventral nerve cord, and mesoderm (Zhang et al., 2001; Schenck et al., 2002; Dolzanskaya et al., 2003; Ma et al., 2016), muscle

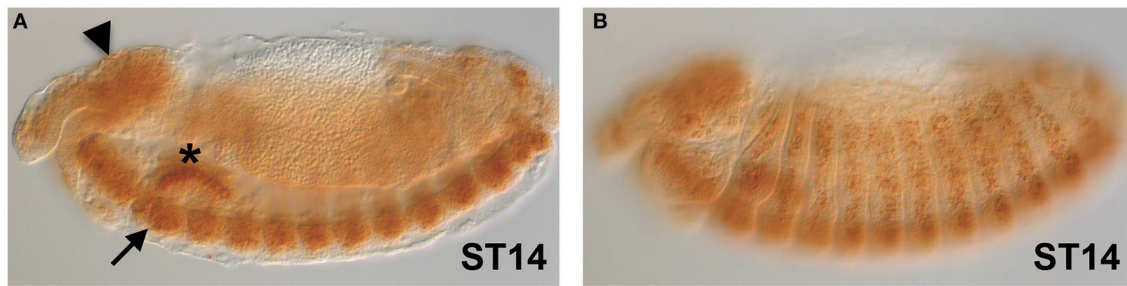


FIGURE 1 | *dFMR1* expression in stage 14 *Drosophila* embryos. **(A)** Lateral view of a stage 14 embryo (middle focus) showing expression in the brain (arrowhead) and in the CNS (arrow). The salivary gland (asterisk) is non-specific background. **(B)** Lateral view of the same stage 14 embryo (surface focus) showing expression in several muscle precursors. The *dFMR1* anti-sense probe was synthesized from the full length EST-clone LD09557 (Drosophila Genomics Resource Center, Bloomington, IN, USA) linearized with EcoRI and transcribed with the T7 RNA polymerase using the Riboprobe Combination System kit (Promega, Madison, WI) and the DIG RNA Labeling Mix (Roche, Indianapolis, IN). *In situ* hybridization was performed as in Tevy et al. (2014) except that SA-HRP and TSA were diluted at 1:250. Images were acquired at the SPIBOC imaging platform of the Institut Sophia Agrobiotech (Sophia Antipolis, France) on an Axioplan II microscope using the ZEN software (Carl Zeiss, Germany).

attachment sites and dendritic tips of chordotonal organs, dendrites of the trachea-innervating neurons (Schenck et al., 2002), developing egg chambers (Zarnescu et al., 2005; Pepper et al., 2009), and in punctate cytoplasmic structures in cleavage-stage embryos in association with cytoplasmic RNP bodies (Monzo et al., 2006; Papoulas et al., 2010). In larvae, dFMRP has been detected in the CNS, the PNS, the eye disk, the testis, at low levels in muscles, in the Mushroom Bodies (MBs, the *Drosophila* learning and memory center; Schenck et al., 2002) and in dendritic arborization (DA) neurons (Lee et al., 2003). dFMRP is found exclusively in the cytoplasm of the soma of neurons (Zhang et al., 2001). Finally, in adults dFMRP has been found in the central brain and eyes (Zhang et al., 2001), in pupal and adult brain neurons (Morales et al., 2002), in specific cells of the adult brain (Morales et al., 2002), in antennal lobe projection neurons (Sudhakaran et al., 2014), and in the cell bodies of specific neurons of the MBs (Pan et al., 2004). Although the main tissue in which dFMRP is present is the CNS, these studies suggest that this protein also has non-neuronal functions, most of which still have to be dissected. Indeed, *dFMR1* has been involved by FlyBase (<http://flybase.org/reports/FBgn0028734.html>) in 58 biological processes, which are summarized in **Table 1**.

It is interesting to note that the phenotypes of *dFMR1* mutants largely recall the pathological symptoms of FXS patients. For instance, some FXS patients have delayed motor development, which can be compared to uncoordinated behavior of flies measured by flight or climbing assays. Moreover, olfactory learning and courtship conditioning of *Drosophila* can serve to test learning and memory behaviors that are often impaired in FXS patients. Also the changes in neuron structure observed in FXS patients are mimicked in the fly model (reviewed in van Alphen and van Swinderen, 2013). The first *dFMR1* mutants were generated soon after its cloning (Zhang et al., 2001) and resulted viable and fertile as in humans. Nevertheless, mutant viability is highly sensitive to the genetic background, such that *dFMR1* mutants can become fully lethal in some backgrounds (see Morales et al., 2002) in a generation-dependent manner, a

phenomenon that requires further study. These original alleles were loss-of-function excisions of hypomorphic EP insertions (producing over-expression of the gene in which they are inserted) presenting phenotypes upon over-expression in the eye (Zhang et al., 2001). Other null mutants were obtained by excision of other EP elements (Dockendorff et al., 2002; Inoue et al., 2002) or by EMS mutagenesis (Lee et al., 2003). Altogether, *dFMR1* mutants show defects in many different biological functions listed in **Table 1**. Over-expression studies in flies have also provided evidence for understating the mis-functions of mutant human FMRP as in the case of the assessment of a neomorphic function for a frameshift FMRP mutant (Okray et al., 2015). In summary, *dFMR1* mainly plays a crucial role in synaptic plasticity and this affects many neuronal processes that are important for fly behavior.

RNA-mediated interference (RNAi) enables the knockdown of a gene of interest at the post-transcriptional stage (Fire et al., 1998). Combining the RNAi with the UAS/GAL4 system (Brand and Perrimon, 1993) in flies makes it possible to down-regulate gene expression in certain tissues and/or at a desired stage of development. Using this combined mechanism, the promoter-GAL4 fusion drives the expression of the RNAi hairpin fragment under the control of UAS sequences (Piccin et al., 2001). Tissue- or stage-specific expression is achieved by the use of specific GAL4 drivers. For instance, *elav-GAL4* is usually used to trigger pan-neuronal expression, *mef2-GAL4* for pan-muscular expression and *Act5c-GAL4* for ubiquitous expression. Since 2007, the Vienna Drosophila Resource Center (VDRC; <http://stockcenter.vdrc.at/control/main>; Dietzl et al., 2007) has established many RNAi lines of *D. melanogaster*. These comprise around 12,671 (91%) of *Drosophila* protein-coding genes, making it the largest collection of RNAi lines for all model systems. Currently, there are three different types of UAS-RNAi stocks available: GD and KK with long hairpins and shRNA with short hairpin micro RNAs. The existence of such a broad array of RNAi lines provides many experimental benefits for biological studies, including those focusing on FMRP.

TABLE 1 | Main phenotypes of loss of function *dFMR1* mutants.

Phenotype	References
BEHAVIOR	
Adult locomotion/Climbing	Zhang et al., 2001; Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Martinez et al., 2007; Banerjee et al., 2010; Adewoye et al., 2015; Novak et al., 2015; Monyak et al., 2016
Circadian rhythm	Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Banerjee et al., 2007, 2010; Gatto and Broadie, 2009; Siller and Broadie, 2011; Xu et al., 2012; Adewoye et al., 2015; Monyak et al., 2016
Courtship	Dockendorff et al., 2002; McBride et al., 2005; Banerjee et al., 2007, 2010; Chang et al., 2008; Choi et al., 2010; Tauber et al., 2011; Gross et al., 2015
Grooming	Tauber et al., 2011
Larval crawling	Xu et al., 2004; Coyne et al., 2015; Günther et al., 2016; Kashima et al., 2017
Olfactory learning and memory	Morales et al., 2002; Bolduc et al., 2008; Kanellopoulos et al., 2012; Andlauer et al., 2014; Sudhakaran et al., 2014; Choi et al., 2015; Monyak et al., 2016
Social behavior	Bolduc et al., 2010
Sleep	Bushey et al., 2009; van Alphen et al., 2013
Touch perception	Cvetkovska et al., 2013
NEURAL PHYSIOLOGY/STRUCTURE	
Bouton area	Pan et al., 2004; Gatto and Broadie, 2008; Reeve et al., 2008; Coffee et al., 2010; Friedman et al., 2013; Cavolo et al., 2016; Doll et al., 2017
Bouton/synapse number	Zhang et al., 2001; Zarnescu et al., 2005; Banerjee et al., 2007, 2010; Gatto and Broadie, 2008; Xu et al., 2008; Pepper et al., 2009; Coffee et al., 2010; Beerman and Jongens, 2011; Bhogal et al., 2011; Siller and Broadie, 2011; Friedman et al., 2013; Kashima et al., 2016, 2017; Mansilla et al., 2017
Calcium signaling	Tessier and Broadie, 2011; Gatto et al., 2014; Sudhakaran et al., 2014; Doll and Broadie, 2016
M B β -lobe crossing	Michel et al., 2004; McBride et al., 2005; Banerjee et al., 2007; Bolduc et al., 2008; Chang et al., 2008; Beerman and Jongens, 2011; Gross et al., 2015
Neural branching	Morales et al., 2002; Lee et al., 2003; Pan et al., 2004; Reeve et al., 2005, 2008; Gatto and Broadie, 2008; Tessier and Broadie, 2008; Xu et al., 2008; Cziko et al., 2009; Pepper et al., 2009; Coffee et al., 2010; Bhogal et al., 2011; Siller and Broadie, 2011; Friedman et al., 2013; Kim et al., 2013; Doll and Broadie, 2015; Myrick et al., 2015; Kashima et al., 2016
Neurite extension	Morales et al., 2002; Michel et al., 2004; Pan et al., 2004; Reeve et al., 2005; Gatto et al., 2014
Neural fasciculation	Schenck et al., 2003; Reeve et al., 2005
Neurotransmission	Zhang et al., 2001; Martinez et al., 2007; Gatto and Broadie, 2008; Friedman et al., 2013; Doll et al., 2017; Franco et al., 2017
Synaptic growth	Zhang et al., 2001; Morales et al., 2002; Schenck et al., 2003; Abekhoukh et al., 2017; Doll et al., 2017
Branch/Neurite/NMJ/Synaptic length	Lee et al., 2003; Schenck et al., 2003; Reeve et al., 2008; Tessier and Broadie, 2008; Bhogal et al., 2011; Siller and Broadie, 2011; Cvetkovska et al., 2013; Kim et al., 2013; Gatto et al., 2014; Bozzetti et al., 2015; Myrick et al., 2015; Sterne et al., 2015; Abekhoukh et al., 2017; Doll et al., 2017; Kennedy and Broadie, 2017
Synapse structure	Zhang et al., 2001; Morales et al., 2002; Lee et al., 2003; Schenck et al., 2003; Michel et al., 2004; Pan et al., 2004; McBride et al., 2005; Reeve et al., 2005, 2008; Zarnescu et al., 2005; Banerjee et al., 2007, 2010; Bolduc et al., 2008; Chang et al., 2008; Gatto and Broadie, 2008, 2009; Tessier and Broadie, 2008; Xu et al., 2008; Cziko et al., 2009; Pepper et al., 2009; Coffee et al., 2010; Beerman and Jongens, 2011; Bhogal et al., 2011; Siller and Broadie, 2011; Cvetkovska et al., 2013; Friedman et al., 2013; Kim et al., 2013; Gatto et al., 2014; Bozzetti et al., 2015; Doll and Broadie, 2015; Gross et al., 2015; Myrick et al., 2015; Sterne et al., 2015; Cavolo et al., 2016; Kashima et al., 2016, 2017; Abekhoukh et al., 2017; Doll et al., 2017; Kennedy and Broadie, 2017; Mansilla et al., 2017
Synapse volume	Mansilla et al., 2017
OTHERS	
Adult eclosion	Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002
Aging	Martinez et al., 2007; Bushey et al., 2009
Apoptosis	Gatto and Broadie, 2011
Blastoderm cellularization	Deshpande et al., 2006; Monzo et al., 2006; Papoulas et al., 2010
Cell cycle	Deshpande et al., 2006; Monzo et al., 2006; Epstein et al., 2009; Callan et al., 2010, 2012; Papoulas et al., 2010
Germline development	Zhang et al., 2004, 2014; Costa et al., 2005; Deshpande et al., 2006; Megosh et al., 2006; Epstein et al., 2009; Yang et al., 2009; Bozzetti et al., 2015; Jiang et al., 2016
Heart rate	Novak et al., 2015
Oviposition	Kacsoh et al., 2015a,b; Jiang et al., 2016
Phagocytosis	O'Connor et al., 2017

For example, tissue-specific RNAi studies uncovered the role of dFMRP in early developmental stages of fly larvae. It was shown that dFMRP regulates glial-dependent proper timing of

neuroblast reactivation during brain development (Callan et al., 2012). In another study, RNAi knockdown of *Torsin* revealed its involvement in locomotion. *Torsin* probably works together with

dFMR1 to regulate synaptic plasticity and *dFMR1* expression is altered in *Torsin* mutant flies (Nguyen et al., 2016). By creating the cardiac-specific *dFMR1* RNAi knockdown, *dFmr1* was also shown to be involved in regulating heart rate during development using the climbing assay. In this simple assay, adults are placed in a vial and tapped down. The time by which they reach the height of 5 cm is then measured (Novak et al., 2015). Some FMR1 patients present cardiac defects (Kidd et al., 2014) and changes in FMRP levels have been associated with structural and functional defects in zebrafish and mice (Mientjes et al., 2004; Van't Padje et al., 2009). On the contrary, in *Drosophila* no structural defects have been found, suggesting that *dFMR1* and *FMR1* regulate distinct targets. In a different study, hemocyte-specific *dFMR1* knockdown by RNAi causes a defect in immune cell phagocytosis of bacteria and increases *Drosophila* sensitivity to bacterial infections, suggesting that *dFMR1* is involved in the regulation of phagocytosis (O'Connor et al., 2017; reviewed in Logan, 2017). This study can provide further insights into the engagement of the immune system in FXS pathogenesis, especially considering the fact that some FXS patients exhibit defects in this system (O'Connor et al., 2017 and references therein). Aberrations in calcium homeostasis are connected with changes in neuron structure that probably cause the learning and memory deficits seen in FXS patients (Tessier and Broadie, 2011 and references therein). This was supported by RNAi knockdown of *dFMR1* during a critical period of development, which proved the importance of the dFMRP role in regulating calcium signaling in the learning and memory circuitry (Doll and Broadie, 2016). These studies highlight the value of the *Drosophila* system in detailed phenotypic analyses of FMRP function.

NeuroMuscular Junctions (NMJs) of *Drosophila* are simple synapses that resemble those present in the Vertebrate CNS. Thus, they are a good model for the study of synapses (reviewed in Menon et al., 2013). The neuromuscular system of *Drosophila* contains 32 motor neurons in each abdominal hemisegment. NMJ synapses show developmental and functional plasticity. They are large and individually specified, enabling their visualization. NMJs are composed of branches and three types of boutons (I, II, and III) that are oval structures hosting synapses differing in size, shape, physiology, and in the amount of sub-synaptic reticulum surrounding them. Type I boutons are glutamatergic and have been the focus of FXS studies. Immunohistological staining can be used to visualize these structures and observe different NMJ phenotypes. *dFMR1* is expressed pre-synaptically in motor neurons (Zhang et al., 2001), but post-synaptically in muscles (Zhang et al., 2001; Schenck et al., 2002). In *dFMR1* mutants, NMJs display increased synapse arborization and branching, increased synaptic bouton numbers, and elevated neurotransmission, whereas larvae over-expressing *dFMR1* show the opposite phenotypes (Zhang et al., 2001). These phenotypes recall the dendritic spine over-growth observed in mammalian mutants and in FXS patients (Irwin et al., 2001).

In addition to defects in NMJ synaptic architecture in neurons, *dFMR1* mutants also exhibit fecundity and testes dysfunctions, which can be used to evaluate non-neuronal requirements (Zhang et al., 2004). Coffee et al. examined the evolutionary conservation of *FMR1* and its paralogs in the *Drosophila* FXS

model at the neuronal and non-neuronal levels (Coffee et al., 2010). In this study, out of the three human genes, only *FMR1* turned out to be able to restore the normal number of boutons in *dFMR1* null mutants. On the other hand, all three homologs rescued the sterility and testicular phenotypes. These results indicate that in neurons *FMR1* has a unique evolutionarily conserved role. In contrast, in non-neuronal tissues *FMR1*, *hFXR1*, and *hFXR2* are able to substitute each other (Coffee et al., 2010).

The larval NMJ has proved a powerful system to study genetic interactions occurring in the actin remodeling pathway that is altered in mammalian FMRP-null neurons (Castets et al., 2005; Pyronneau et al., 2017). FMRP has been shown to interact with Cytoplasmic FMRP Interacting Protein 1 (CYFIP1) *in vitro* (Schenck et al., 2001, 2003) and also in both mammals (Schenck et al., 2001) and *Drosophila* (Schenck et al., 2003; Abekhouk et al., 2017). CYFIP1 is part of the WAVE regulatory complex (WRC) along with five other proteins involved in actin polymerization (reviewed in Cory and Ridley, 2002; Takenawa and Suetsugu, 2007). Human CYFIP1 has been linked to neurodevelopmental disorders such as ID, autism, schizophrenia, epilepsy, and Burnside-Butler (15q11.2 BP1-BP2 micro-deletion) syndrome (Madrigal et al., 2012; Waltes et al., 2014; Huang, 2015; Wang et al., 2015). Abekhouk et al. (2017) utilized the fine genetic tools of *Drosophila* to investigate the genetic interactions between dFMRP and dCYFIP1. Through loss- and gain-of-functions studies, the authors showed that *dFMR1* and dCYFIP1 have opposing functions on larval NMJ length: dFMRP represses while dCYFIP1 promotes synaptic growth at the NMJ in gene dosage studies using the presynaptic *elav-Gal4* driver. A rescue of the reciprocal NMJ length phenotypes is observed in double homozygous mutant animals. It should be noted that double homozygous mice are lethal, thus preventing a similar epistatic genetic analysis in the mouse model. Here, the advantage of *Drosophila* for synaptic plasticity and actin studies is that specific parameters can easily be monitored. Since *dFMR1* and *CYFIP1* are candidates for ID and autism, these studies on the fly pave the way to deeper and more refined studies in mice and in humans.

The first *Drosophila* target of dFMRP was the gene *futsch*, identified by RNA immunoprecipitation (Zhang et al., 2001). This gene encodes a microtubule-associated protein with homology to mammalian MAP1B. *futsch* and *dFMR1* have opposite phenotypes (undergrowth and overgrowth of synaptic boutons, respectively) and dFMRP binds *futsch* mRNA, negatively regulating its translation (Zhang et al., 2001). Binding of dFMRP was also found for the mRNA of the actin monomer binding protein *profilin* (encoded by *chickadee*; Reeve et al., 2005) and of the small GTPase Rac1 (Lee et al., 2003), whose loss- and gain-of-functions also have opposite phenotypes to those of *dFMRP* that are rescued by over-expression of both genes. dFMRP also binds the mRNAs of BMPR2 (Kashima et al., 2016), DSCAM (Cvetkovska et al., 2013), and the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) mRNA together with Ataxin-2 (Sudhakaran et al., 2014), confirming the involvement of *dFMR1* in the Ca²⁺ pathway. Nevertheless, there is still a need for high throughput studies to identify novel dFMRP targets.

MBs consist of bilateral clusters of ~2,500 neurons in the fly brain. In MBs, there are three types of intrinsic neurons: $\alpha\beta$, $\alpha'\beta'$, and γ (Crittenden et al., 1998). $\alpha'\beta'$ are a prerequisite for gaining olfactory memory, whereas $\alpha\beta$ are required to retrieve memory (Krashes et al., 2007). MBs play a major role in olfactory learning and memory in *Drosophila*. Odor and courtship-based tests are frequently used to assess memory dysfunctions in this system (reviewed in Weisz et al., 2015). MBs are also involved in visual context generalization, information processing, locomotion, sleep, courtship conditioning, and choice behavior. The MB axons in *dFMR1* null mutants show architectural defects such as an increase in volume and branching, as well as abnormalities in synapse formation (Pan et al., 2004; Chang et al., 2008). *dFMR1* null mutants show morphological MB defects in the lobes, the most frequent of which is the failure of β -lobe arrest at the brain midline (Michel et al., 2004 and see **Table 1**), whereas over-expression of *dFMR1* causes the opposite phenotype (Reeve et al., 2005). These MB malformations can be restored through pharmacological treatment, but they are not indispensable for restoring memory (McBride et al., 2005). It has been shown that *dFMR1* expression requirements differ between lobe types in MBs. Simultaneous expression of *dFMR1* in α , β , and γ lobes is essential for learning skills. The lack of *dFMR1* expression in α and β lobes is sufficient to impair associative olfactory learning and memory, whereas the knock-down of *dFMR1* only in γ lobes does not exhibit detrimental effects on learning (Kanellopoulos et al., 2012). The MBs have also been the focus of calcium-signaling studies using a transgenic GCaMP calcium sensor (Doll and Broadie, 2016 and references therein). The MB neurons have been shown to be involved in activity-dependent processes during critical period development thanks to their ability to respond when illuminated by a blue light (see below; reviewed in Doll and Broadie, 2014). All the *dFMR1* phenotypes in the MBs correlate with learning and memory dysfunctions and make the FXS *Drosophila* model very appealing because of its easy *in vivo* analysis and the wide range of tools that have been developed (Ugur et al., 2016; Chow and Reiter, 2017).

Electrophysiological techniques have been used in *Drosophila* to study the effect of *dFMR1* loss-of-function and over-expression on synaptic transmission. Two-Electrode Voltage Clamp (TEVC) studies at NMJs in *Drosophila* showed that FMRP has clear pre-synaptic functions (Zhang et al., 2001), although photoreceptor synaptic transmission is normal in the mutants (Morales et al., 2002). Through TEVC recordings, Gatto and Broadie (2008) showed that presynaptic *dFMR1* expression in *dFMR1* mutants rescues the defects in NMJ structure, but not in neurotransmission, suggesting that dFMRP also has post-synaptic functions. On the other hand, in mice, FMRP was first shown to act post-synaptically (Huber et al., 2002) and only recently has also been found to play a pre-synaptic role in signal transmission (Koga et al., 2015; Myrick et al., 2015; Zhang et al., 2015) as in the fly. The possibility to target gene expression to specific cells through the UAS/GAL4 system (Brand and Perrimon, 1993), the wide availability of genetic mutants and the precise spatial and temporal resolutions make electrophysiology a very informative technique for FXS modeling in flies, and a useful complement to the advanced imaging studies described below.

Optogenetics is an *in vivo* technique that uses light to measure neuronal activity in living tissues and has also been exploited in the FXS *Drosophila* model. For example, dFMRP has been shown to play a cell-specific role in the regulation of activity-dependent calcium transients that is restricted to the early critical period (Tessier and Broadie, 2011; Doll and Broadie, 2016). Using optogenetic stimulation, it was recently established that *dFMR1* mutants show increased circuit excitability (probably due to reduced GABAergic lateral inhibition; Franco et al., 2017) and that dFMRP is required for the activity-dependent regulation of synaptic connectivity (Doll et al., 2017). These data may explain the deficits in olfactory behaviors and the hyper-excitation found in FXS patients. *Drosophila* has been used to study even sleep patterns in *dFMR1* mutants by electrophysiology and optogenetics. *dFMR1* mutants show deeper night-like sleep during the day (van Alphen et al., 2013), which is likely because of the FMRP function in synaptic remodeling. It is clear that, through *Drosophila*, the cellular and physiological processes involved in FXS pathology can be studied at a deeper level than in any other model system. In addition, flies have been used for pharmacological studies on FXS through their complex behaviors like for example olfactory learning and memory, courtship, circadian rhythm, crawling and sleep (see **Table 1** and below).

FXS IN OTHER MODEL SYSTEMS

Drosophila is not the only animal model used to study FXS. Here, we report some examples of other animal models that can help in FXS studies. Mammalian mouse and rat models have been predominantly used for FXS studies. Two different mouse (The Dutch-Belgian Fragile X Consortium, 1994; Mientjes et al., 2006) and rat (Hamilton et al., 2014; Tian et al., 2017) KO models have been generated. It should be noted that, in mice, the full CGG expansion does not cause methylation and *Fmr1* silencing (Brouwer et al., 2007) as it does in humans. Thus, all FXS studies in mice have been carried out using the KO animal (The Dutch-Belgian Fragile X Consortium, 1994; Mientjes et al., 2006). In general, the *Fmr1*-KO mouse is considered to be a good model for this disorder since it recapitulates most FXS phenotypes (deficits in learning and memory, hyperactivity, altered volumes of some brain regions, altered morphology of dendritic spines, and increased size of testis) and because it allows genetic experimentation. Also KO mutant rats present behavioral abnormalities related to ASD-like altered patterns of social interaction (Tian et al., 2017) and social play behavior (Hamilton et al., 2014), defects in visual attention (Berzhanskaya et al., 2016), and speech and auditory dysfunctions (Engineer et al., 2014). Rats have provided an excellent model for neuroscience and pharmacology as they have bigger brains than mice, are easier to train, can learn sophisticated behaviors and have an elaborated social repertoire; however, they are more expensive and much less genetically amenable than mice and flies. These recent studies on *Fmr1* KO mice and rats show that both of these models are useful to study the complex phenotypes of FXS patients. Nevertheless, although Vertebrate model organisms provide more precise insights into the human disease pathogenesis,

they are much more difficult than flies to maintain, more time-consuming and incur considerable expenses. In addition, performing experiments on Vertebrate models is much more restrictive in the context of animal laws and often triggers ethical issues.

Other animal models have been generated to understand the physiopathology of FXS, such as the zebrafish, a small fresh water fish endogenous to South-East Asia, the frog *Xenopus laevis* and the marine mollusk *Aplysia californica*. Although zebrafish *fmr1* mutants generated by morpholino knock-down showed gross morphological defects in neurons (Tucker et al., 2006), true genetic null alleles later obtained by random mutagenesis resulted viable, fertile, and of normal morphology (den Broeder et al., 2009) likely due to off-targeting effects of the morpholino technology. Importantly, mutant zebrafish exhibit hyperactivity, learning deficits, impaired anxiety, and increased social behaviors like shoaling (Ng et al., 2013; Kim et al., 2014; Wu et al., 2017) maybe because of hyperactivity as in mice (Sorensen et al., 2015). The frog *fmr1* mutant was also obtained by morpholino KO and showed behavioral (Truszkowski et al., 2016) and FMRP level-sensitive neuronal defects (Faulkner et al., 2015). Finally, in *Aplysia*, basic neurobiological studies have evidenced the pre- and post-synaptic control of plasticity regulating long-term memory and a functional interaction with the Na⁺-activated K⁺ channel (K_{Na}) Slack (reviewed in Abrams, 2012). Overall, these simpler systems have also facilitated further insights into the mechanisms of FXS pathology.

In comparison to vertebrate models, the more complex human behaviors do not always correspond to those of flies and many neurological diseases can only be modeled in certain aspects. This is mainly due to the fact that *Drosophila* and humans differ in anatomy and despite of having many orthologs in common their pathways exhibit many differences. In addition, although drug administration is much simpler in *Drosophila*, the potential toxicity is much tougher to predict in humans because of significant metabolic differences and complexities (Pandey and Nichols, 2011).

SUCCESSFUL PHARMACOLOGICAL TREATMENTS OF FXS PHENOTYPES IN *DROSOPHILA*

Since the *Drosophila dFMR1* phenotypes recapitulate the patients' symptoms, this model has been used to develop pharmacological treatments for the disease. One reason is that drugs can easily be administered in the standard fly food in which larvae feed and grow. This food is composed of cornmeal, agar and yeast and requires boiling. Recently, the Formula 4–24 (Carolina Biological Supply Company) medium has been exploited because it does not require heating and can simply be dissolved in room temperature water so that even heat-sensitive drugs can be tested (Kashima et al., 2017). Simple feeding has been used in the articles reported below, but many other methods to feed embryos, larvae or adults have been developed (reviewed in Pandey and Nichols, 2011). Another reason is that *Drosophila* does not carry vessels, but all of its organs bathe in hemolymph, which circulates thanks to a

tubular heart in an open circulatory system. The fly blood/brain barrier is only made of a thin layer of glial cells presenting septate and gap junctions contrary to the Vertebrate one that is composed of glial cells and of endothelial cells forming tight junctions. Thus, the fly blood/brain barrier is simpler and allows for a better pharmacokinetic penetration (Pinsonneault et al., 2011; Limmer et al., 2014). Here, we discuss some examples of drugs successfully tested on flies to cure *dFMR1* phenotypes that affect the different pathways listed below.

Glutamate is the main excitatory neurotransmitter in the CNS. Metabotropic glutamate receptors (mGluRs) at synapses control Long Term Depression (LTD), which mediates synaptic plasticity, thus weakening the synaptic response to stimuli. mGluR-dependent LTD was initially found to be enhanced in *FMR1* mutant mice (Huber et al., 2002) and later in *Drosophila* (McBride et al., 2005). After the establishment of the “mGluR theory” of FXS by Bear et al. (2004), it was in *Drosophila* that inhibition of mGluR signaling was first shown to alleviate the behavioral fly phenotypes mimicking human FXS symptoms (McBride et al., 2005). In addition, a rescue of the defects in the fibers crossing the β -lobe of the MBs was observed in the treated *dFMR1* mutants. Interestingly, adult therapy is sufficient to restore normal courtship behavior and short-term memory, but not β -lobe crossing, suggesting that other morphological defects are responsible for the memory defects (McBride et al., 2005). Since then, much research has been carried out to pharmacologically correct this defect both in mice (reviewed in Hagerman et al., 2014) and in flies (Choi et al., 2010; Kanellopoulos et al., 2012). *dFMR1* mutants have less vigorous courtship behavior, learn normally, but forget. Treatments of larvae and adults either with the non-competitive mGluR antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP), three competitive mGluR antagonists or LiCl rescue naive courtship behavior, immediate recall memory and short-term memory of *dFMR1* mutants (Choi et al., 2010). Subsequently, Choi et al. (2010) compared naive courtship, locomotion, olfactory capabilities, learning, and memory between 20 day-old flies and 5 day-old flies. They found that the inhibitors of the Glutamate receptor pathway and Lithium rescue these phenotypes of *dFMR1* mutants to different degrees. The best rescue is obtained when the treatment is applied at both larval and adult stages. Learning is defective only in old flies, without the involvement of cell death. Treatment with four different mGluR inhibitors or LiCl exclusively during development rescues the learning, but not the courtship defects. Despite these successful treatments in pre-clinical trials, all clinical trials carried out so far have failed, although others are currently underway (<https://clinicaltrials.gov>). One reason could be the timing of the treatment or the need to combine different drugs since many other molecular mechanisms have been evoked as causative aspects of this disease. Importantly, in addition to FXS, many other neurodevelopmental disorders are correlated with defects in mGluR signaling.

As opposed to glutamate, GABA is the main inhibitory neurotransmitter in the brain. It functions through Gamma-Aminobutyric Acid (GABA)_A and GABA_B receptors (R) that are fast-acting inotropic and metabotropic receptors, respectively.

A decrease in GABA_A R expression and defects in GABAergic signaling were found in the *FMRI* Knock Out (KO) mouse (Idrissi et al., 2005; D'Hulst et al., 2006; Gantois et al., 2006; Sabanov et al., 2017). GABA_B R has been linked to mGluR signaling and its reduction associated with autism (reviewed in Hagerman et al., 2014). Many clinical treatments targeted to the GABAergic pathway have been attempted or are underway (reviewed in Braat and Kooy, 2015). Altered GABAergic circuitry, like depressed glutamic acid decarboxylase (GAD) levels, was also found in the *Drosophila* FXS model, particularly in neurons expressing *dFMRI* (D'Hulst et al., 2006; Gatto et al., 2014). In this system, through a fine mapping method of Mosaic Analysis with a Repressible Cell Marker (MARCM), Gatto et al. (2014) showed that *dFMRI* is not required for GABAergic neuron survival, but it regulates the architecture of GABAergic neurons innervating the MBs. In addition, *dFMRI* GABAergic neurons display elevated calcium signaling (Gatto et al., 2014). To further underline the critical role of the GABA pathway in defining the FXR phenotype in the fly, it is worth mentioning that the down regulation of the GABA receptors in projection neurons of the antennal lobe observed in *dFMRI* mutants is sufficient to produce olfactory behavioral defects (Franco et al., 2017).

Thanks to the toxicity of glutamate for the *dFMRI* mutant, Chang et al. (2008) were able to carry out an unbiased screen for small molecules that can rescue the lethality of glutamate-treated *dFMRI* mutants, using the mGluR5 non-competitive antagonist MPEP as a positive control. The active compounds belong to biochemical pathways not targeting mGluR signaling, namely the GABAergic, the muscarinic, the serotonin, and hormone-related pathways. Notably, GABA and MPEP treatments were also able to rescue the β -lobe crossing in the MBs and the courtship behavior phenotypes. Indeed, a decrease in GABA_A R expression and defects in GABAergic signaling were found in the *FMRI* KO mouse (Sabanov et al., 2017 and references therein) and in the *Drosophila* FXS model, particularly in neurons expressing *dFMRI* (Gatto et al., 2014). This is the first chemical screen for FXS in flies and was made possible by the easily scorable phenotype of lethality, which revealed novel pathways implicated in FXS (Chang et al., 2008). GABA_B R has been linked to mGluR signaling and its reduction associated with autism (reviewed in Hagerman et al., 2014). Many clinical treatments targeting the GABAergic pathway have been attempted or are underway (reviewed in Braat and Kooy, 2015).

Another pathway that has been shown to be involved in FXS alterations is the Bone Morphogenetic Protein (BMP) signal transduction pathway (Kashima et al., 2016), controlling a number of developmental processes including nervous system development (Liu and Niswander, 2005). This pathway has been linked to anxiety and object exploration in mice (McBrayer et al., 2015). It was previously established that the LIM domain kinase 1 (LIMK1) co-localizes with the BMP type II receptor (BMPR2) in the neuronal terminals (Lee-Hoeflich et al., 2004). These two proteins bind to each other leading to activation of the LIMK1 catalytic activity. Once activated by BMPR2, LIMK1 promotes neural growth and dendritogenesis through phosphorylation and inhibition of cofilin (Meng et al., 2002; Lee-Hoeflich et al., 2004). The C-terminal domain (CTD) of

BMPR2 plays a role as a repressor of BMPR2 translation and as an activator of LIMK1 function. Kashima et al. (2016) found that FMRP binds to the CTD of the BMPR2 and inhibits the translation of full-length BMPR2 in humans and mice. These data have been genetically confirmed in the *Drosophila* model. In the NMJs of *dFMRI* mutants, synaptic boutons are overgrown, whereas they are under-grown in mutants for the BMPR2 homolog *wishful thinking (wit)* (see also Eaton and Davis, 2005). Double *wit*, *dFMRI* mutants show a number of boutons as in single *wit* mutants, indicating that *wit* is negatively regulated by *dFMRI*. These results obtained in the fly encouraged the authors to carry out pharmacological studies in mice that revealed the *Fmr1* rescue potential of a LIMK1 inhibitor (Kashima et al., 2016). These cellular phenotypes correlate with the crawling behavior of *Drosophila* larvae, which has been used as a simple genetic and pharmacological screening tool for FXS treatment (Kashima et al., 2017). *dFMRI* mutant larvae crawl faster than wild-type larvae: heterozygotes for three different *dFMRI* alleles show increased larval locomotor activity correlated with an increased bouton number at the NMJ of muscle 6/7 in A3. This phenotype is rescued by a loss-of-function allele of *wit* in heterozygosis suggesting that it is due to an up-regulation of the BMPR2 homolog. The oral treatment with a pharmacological inhibitor of LIMK1 (downstream target of BMPR2, see above) restores the number of boutons in *dFMRI* mutants. Using a newly developed larval crawling assay and a sophisticated algorithm (LarvaTrack) for drug screening, the locomotion defects (distance and velocity) of *dFMRI* larvae were used as a readout of the larval bouton number phenotype (Kashima et al., 2017). In this case, LIMK1 inhibitors and puromycin rescue the *dFMRI* locomotion phenotypes in correlation with the bouton number. Hyperactivity and anxiety of *Fmr1*-KO mice are also ameliorated by treatment with a LIMK inhibitor, showing that this specific and simple assay developed in flies has considerable potential in the assessment of new drug therapies. Indeed, all of these results can be applied to the human condition because an increase in BMPR2 is observed in the prefrontal cortexes of FXS patients (Kashima et al., 2016), paving the way to drug trials in humans.

Neuronal Calcium Sensor 1 (NCS-1) and its *Drosophila* homolog *Frequenin (Frq) 2* are Ca²⁺-binding proteins that play a role in the control of the synapse number: loss-of-function mutations increase the synapse number and over-expression decreases it. Human NCS-1 is involved in schizophrenia (Koh et al., 2003; Torres et al., 2009) and autism (Piton et al., 2008). Frq2 physically interacts with the guanine nucleotide-exchange factor *Ric8a* and the structure of their interaction has been resolved (Romero-Pozuelo et al., 2014). The *Ric8a* protein is localized at larval motor neuron terminals and is particularly abundant in boutons. *Ric8a* knockdown causes a reduction in the number of synapses, thus producing a phenotype opposite to that of Frq2 with Frq2 acting as a negative regulator of Ric8a in the control of the synapse number (Romero-Pozuelo et al., 2014). On the other hand, as in humans and mice (Irwin et al., 2001; Hutsler and Zhang, 2010), loss of *dFMRI* causes an increase in the synapse number and neuron volume (see **Table 1**). Mansilla et al. (2017) identified the aminophenothiazine derivative FD44

as a potent inhibitor of the *Drosophila* NCS-1/Ric8a interaction and showed that treatment of *dFMR1* mutant adults and larvae with this drug reduces their synapse number and volume at the glutamatergic NMJ of larval muscle fibers 6/7 of A3. In addition, FD44 restores normal associative learning. This indicates that, in the *dFMR1* mutant, the interaction between NCS-1 and Ric8a is unbalanced, probably because dFMRP controls *Frq2* transcription (Tessier and Broadie, 2011). Consistently, over-expression of both NCS-1 and *Ric8a* restores a normal number of synapses (Romero-Pozuelo et al., 2014). FD44 is an interesting drug because it is small and able to cross the blood/brain barrier and because the structure of the interaction with its target proteins has been clearly illustrated (Mansilla et al., 2017).

The Down Syndrome Cell Adhesion Molecule (DSCAM) is a conserved protein the levels of which have been found to be elevated in many brain disorders including FXS. This has been confirmed in the mouse and *Drosophila* models where it has been shown that FMRP binds the *Dscam* mRNA and down-regulates its translation (Darnell et al., 2001; Cvetkovska et al., 2013; Kim et al., 2013). *Dscam* encodes a transmembrane protein that is a member of the immunoglobulin superfamily of cell adhesion molecules involved in self-avoidance, synaptic target selection, and axon guidance. In pre-synaptic terminals, *Dscam* has been shown to interact with *Abelson tyrosine kinase* (*Abl*), which mediates the exaggerated presynaptic terminal growth followed by *Dscam* over-expression probably because it is activated by DSCAM (Sterne et al., 2015). As multiple FDA-approved *Abl* inhibitors were available, Sterne et al. (2015) tested whether *ABL* inhibition could restore normal presynaptic terminal growth in larvae over-expressing *Dscam* or mutant for *dFMR1*. The nilotinib inhibitor was found to have this property, which suggests that *ABL* is as a promising therapeutic target for FXS.

Insulin and insulin-like growth factors (IGF) are evolutionarily highly conserved proteins that play an important role in growth and metabolism, but also in neurogenesis through their role in neuronal stem-cell homeostasis (reviewed in Lee et al., 2016; Ali et al., 2018). In *Drosophila*, expression of *dFMR1* specifically in the insulin-producing cells (IPs) or in the whole nervous system is able to rescue the defective free-running locomotor rhythmicity of *dFMR1* mutants, as well as short-term and long-term memory defects in olfactory learning tests (Monyak et al., 2016). Protein levels of the major insulin-like peptide DILP2 are elevated in the IPs of *dFMR1* mutants, suggesting a post-transcriptional control, and markers of the insulin pathway are up-regulated. Through fine genetic manipulations aimed at reducing insulin signaling (IS), the authors were also able to obtain a rescue of the circadian rhythmicity of *dFMR1* flies. These data are consistent with the fact that *FMR1* is expressed in the IPs of the mammalian pancreas and that insulin and IGFs have been involved in many neurological events, including synaptogenesis and progenitor cell growth (reviewed in Fernandez and Torres-Alemán, 2012). Metformin is a drug used to cure type 2 diabetes that acts as a sensitizer of IS signaling by increasing Phosphatase and TENsin homolog protein (PTEN) expression, AMP-activated protein Kinase (AMPK) activation and decreasing Target Of

Rapamycin (TOR) signaling. Treatment of *dFMR1* mutants with metformin restores normal short-term courtship memory and normal olfactory long-term memory (Monyak et al., 2016). Using GAL80, the temperature-sensitive repressor of GAL4, Monyak et al. (2016) also tested the precise stage at which reduction of IS is necessary to rescue the different behavioral phenotypes of *dFMR1* mutants. While lowering IS during adulthood is sufficient to rescue learning and memory in the olfactory-based paradigm, reduction during the pupal stages is indispensable to rescue circadian behavior. This may mean that dFMRP is required at different developmental steps to temporally modulate IS. The weight of KO *Fmr1* mice is significantly increased compared to wild-type (Dölen et al., 2007) and some FXS patients are obese (Nowicki et al., 2007) and show elevated IS through the mTor pathway in blood and brain (Hoeffer et al., 2012), implying that mis-regulation of the insulin pathway is likely to be one cause of the disease and a promising target for therapy (reviewed in Castagnola et al., 2017).

CONCLUSIONS

Individuals affected by FXS show a broad spectrum of clinical presentations, with a great variability of signs, symptoms and severity levels (Hagerman, 2002). Indeed, due to the high number of *FMR1* targets, FXS can be considered as a multifactorial disorder. The possibility to study null *dFMR1* flies and mice brought significant advantages for understanding the functional roles of FMRP. These systems made it possible to decrypt several pathways responsible for phenotypes recalling human symptoms, such as cognitive and learning deficits. In comparison to the other animal models, *Drosophila* is less expensive and easier to maintain, lays many eggs that enable to perform genetic screens, has a shorter lifespan and generation time, a smaller entirely sequenced genome (Adams et al., 2000) and sophisticated genetic tools to study human diseases like the easiness to make transgenics or to carry out *in vivo* functional studies (reviewed in Wangler et al., 2015; Ugur et al., 2016; Chow and Reiter, 2017). The fly genome has many orthologs displaying similar roles to human disease genes (Fortini et al., 2000; Doronkin and Reiter, 2008) that can be potential targets for functional and therapeutic studies. *dFMR1* mutants exhibit defects that resemble those observed in human FXS patients, and flies and humans share many pathways that are altered in the disease and likely responsible for a specific set of phenotypes. Studies on FXS in *Drosophila* have set a paradigm to validate drug targets and gain a deeper insight into their molecular mechanisms for future research on FXS and other neurodevelopmental diseases. This is also because the dissection of the correlation between pathways and genotypes can be more easily realized in the fly than in any other model system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Human DNA Helicase B as a Candidate for Unwinding Secondary CGG Repeat Structures at the *Fragile X Mental Retardation* Gene

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The fragile X syndrome (FXS) is caused by a CGG repeat expansion at the *fragile X mental retardation (FMR1)* gene. *FMR1* alleles with more than 200 CGG repeats bear chromosomal fragility when cells experience folate deficiency. CGG repeats were reported to be able to form secondary structures, such as hairpins, *in vitro*. When such secondary structures are formed, repeats can lead to replication fork stalling even in the absence of any additional perturbation. Indeed, it was recently shown that the replication forks stall at the endogenous *FMR1* locus in unaffected and FXS cells, suggesting the formation of secondary repeat structures at the *FMR1* gene *in vivo*. If not dealt with properly replication fork stalling can lead to polymerase slippage and repeat expansion as well as fragile site expression. Despite the presence of repeat structures at the *FMR1* locus, chromosomal fragility is only expressed under replicative stress suggesting the existence of potential molecular mechanisms that help the replication fork progress through these repeat regions. DNA helicases are known to aid replication forks progress through repetitive DNA sequences. Yet, the identity of the DNA helicase(s) responsible for unwinding the CGG repeats at *FMR1* locus is not known. We found that the human DNA helicase B (HDHB) may provide an answer for this question. We used chromatin-immunoprecipitation assay to study the *FMR1* region and common fragile sites (CFS), and asked whether HDHB localizes at replication forks stalled at repetitive regions even in unperturbed cells. HDHB was strongly enriched in S-phase at the repetitive DNA at CFS and *FMR1* gene but not in the flanking regions. Taken together, these results suggest that HDHB functions in preventing or repairing stalled replication forks that arise in repeat-rich regions even in unperturbed cells. Furthermore, we discuss the importance and potential role of HDHB and other helicases in the resolution of secondary CGG repeat structures.

Keywords: replication, helicases, fragile X syndrome, fragile sites, secondary structure, repeats

INTRODUCTION

The fragile X syndrome (FXS) is the most common inherited form of intellectual disability. FXS is caused by a CGG repeat expansion on the X chromosome in the 5'UTR of the *fragile X mental retardation (FMR1)* gene (Nelson et al., 2013). CGG repeat expansion leads to *FMR1* gene silencing. FXS is inherited from women carrying a premutation, 55–200 CGG repeats. More than 200 CGG repeats are categorized as a full mutation (FM), resulting in FXS (Kronquist et al., 2008). Furthermore, expansion of CGG repeats in premutation range within *FMR1* gene is also associated with other disorders; fragile X-associated primary ovarian insufficiency (FXPOI; Sherman, 2000), fragile X-associated diminished ovarian reserve (DOR; Man et al., 2017) and fragile X-associated tremor ataxia syndrome (FXTAS; Hagerman et al., 2001). In contrast to FXS patients, the *FMR1* gene is transcribed in premutation carriers. A toxic RNA gain-of-function and the expression of an abnormal FMRpolyglycin protein is suggested to cause the symptoms in premutation carriers (Hagerman and Hagerman, 2013; Todd et al., 2013).

The expanded repeats at the *FMR1* gene locus in FXS cells are characterized as rare fragile sites (RFS). While RFS are only found in some individuals, common fragile sites (CFS; Durkin and Glover, 2007) and early replicating fragile sites (ERFS; Barlow et al., 2013) are found in every individual. In contrast to RFS, CFS and ERFS contain non-expanding repetitive DNA sequences, and are therefore stable under normal conditions. However, RFS as well as CFS and ERFS tend to break upon replicative stress, which could result in chromosomal deletions, translocations and sister chromatid exchanges (Glover and Stein, 1987, 1988; Wang et al., 1997). It was suggested that these chromosomal alterations are consequences of prolonged replication fork stalling at repetitive DNA sequences located at these fragile sites.

It was reported that DNA and RNA containing CGG repeats are able to form secondary structures, such as hairpins (Gacy et al., 1995; Gacy and McMurray, 1998), G-quadruplexes (Fry and Loeb, 1994; Khateb et al., 2004) and R-loops (Groh et al., 2014; Loomis et al., 2014). DNA templates with such secondary structures stall replication forks *in vitro* and *in vivo* (Samadashwily et al., 1997; Pelletier et al., 2003; Voineagu et al., 2009; Gerhardt et al., 2014). Consistent with prolonged fork stalling at CGG repeats, replication of the *FMR1* locus is delayed in FXS cells compared to unaffected cells (Hansen et al., 1993; Subramanian et al., 1996). Prolonged replication fork stalling and uncompleted DNA replication at this fragile site can lead to genomic instability such as DNA break-induced chromosomal alterations or repeat expansions due to DNA polymerase slippage (Madireddy and Gerhardt, 2017). On the other hand, non-canonical secondary structures may turn CGG repeat containing *FMR1* mRNA into toxic RNA, which may be pathogenic through sequestering RNA-binding proteins. Thus, molecular mechanisms that disrupt these secondary structures are crucial for genome stability and cellular function.

Here we discuss mechanisms that may prevent chromosomal fragility and repeat expansion at the *FMR1* locus upon replication fork stalling at CGG repeats, and the possible involvement of

Human DNA helicase B (HDHB) at unwinding CGG repeat structures to aid replication fork progression. We present data that show HDHB localization to fragile sites specifically during S-phase even in unperturbed cells, suggesting that HDHB may help prevent or repair replication fork stalling. Furthermore, we discuss other possible DNA and RNA helicases that are capable of unwinding secondary CGG repeat structures.

DNA HELICASES INVOLVED IN RESOLUTION OF SECONDARY REPEAT STRUCTURES DURING REPLICATION FORK PROGRESSION

Despite the threats to genome stability posed by the secondary structures adopted by repeats, replication most often proceeds through repeat regions without the expression of chromosomal fragility. This suggests the presence of molecular mechanisms that help replication fork progression through repeat regions. At the heart of these mechanisms are DNA helicase(s) responsible for unwinding the secondary structures adopted by repeats. Helicases separate strands of a DNA double helix or a self-annealed RNA molecule using the energy from ATP hydrolysis, a process characterized by the breaking of hydrogen bonds between annealed nucleotide bases. DNA helicases translocate on double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) to unwind dsDNA into ssDNA or remove proteins bound to DNA. There is a plethora of helicases encoded by the genome, each performing a specialized function dictated by their enzymatic properties as well as their interaction partners.

Conserved among vertebrates, DNA helicase B (DHB) contains seven helicase motifs of superfamily 1 with sequence similarity to homologous recombination proteins prokaryotic RecD and bacteriophage T4 dda. It works with 5' to 3' polarity. The C-terminus of HDHB contains a phosphorylation-dependent subcellular localization domain (PSLD; **Figure 1A**). PSLD is responsible for nuclear localization in G1-phase and phosphorylation-dependent nuclear export of HDHB at the G1/S transition (Gu et al., 2004). HDHB was shown to associate with pre-replication complex components Cdc45 and TopBP1 (Taneja et al., 2002; Gerhardt et al., 2015). HDHB also interacts with Cyclin E and A (Gu et al., 2004) and ssDNA-binding protein RPA (Guler et al., 2012). It was reported that its helicase activity is necessary for replication initiation (Taneja et al., 2002). At G1/S, majority of HDHB is exported from the nucleus. However, a low level of HDHB is retained on bulk chromatin during S-phase and this fraction is increased in cells exposed to agents that stall fork progression. Furthermore, DNA damage leads to HDHB accumulation on chromatin particularly during S-phase (Guler et al., 2012). Single stranded DNA-bound RPA at stalled replication forks recruit HDHB (**Figure 1A**) by a direct protein interaction between HDHB and RPA.

In addition to HDHB, there may be other helicases to help manage replication stress induced by repeat structures. Particularly, helicases capable of resolving G-quadruplexes adopted by guanine-rich stretches of DNA such as CGG

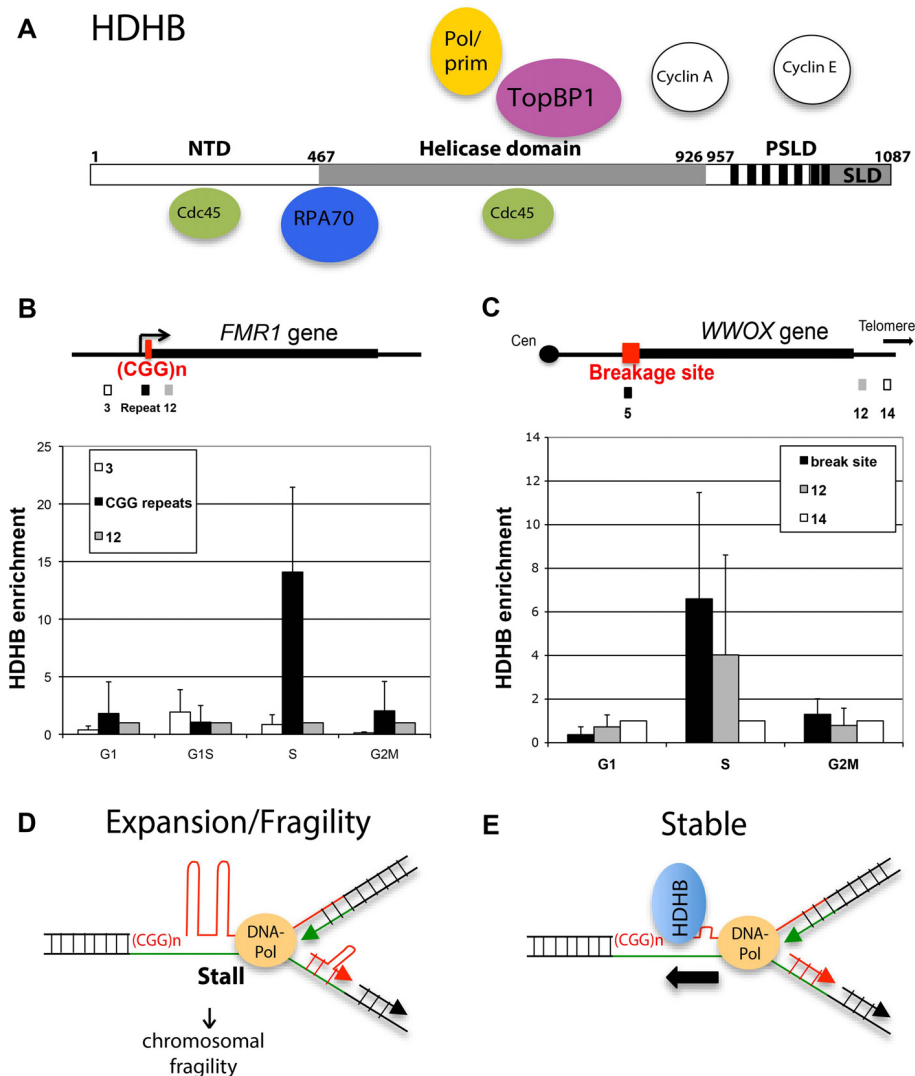


FIGURE 1 | Human DNA helicase B (HDHB) resides at fragile sites in particular at the CGG repeats at human *fragile X mental retardation (FMR1)* gene locus. **(A)** Functional regions of HDHB. An N-terminal domain (NTD) is uncharacterized. The central domain (gray; residues 467–926) contains the seven superfamily I helicase motifs. The C-terminal domain contains consensus CDK phosphorylation sites (vertical black bars) and a subcellular localization domain (SLD), which together constitute a phosphorylation-dependent SLD (PSLD). Replication proteins, which are binding to HDHB are indicated. **(B)** Maps showing *FMR1* gene locus and three primer sets and **(C)** the common fragile sites (CFS) FRA16D and three primer sets. ChIP experiments were performed using U2OS cells synchronized in G1, S and G2/M cells and affinity-purified polyclonal HDHB antibody or rabbit IgG as control as described before (Gerhardt et al., 2006, 2015). HDHB enrichment at each site in each of three independent experiments is plotted on the Y-axis. Horizontal bars show the average HDHB enrichment at each time point and error bars are indicated. **(D,E)** Model for repeat expansion and fragility after fork stalling at secondary CGG repeat structures. HDHB could prevent genome instability by resolving the non-canonical repeat structures.

repeats are of interest. G-quadruplexes may require specialized machinery to unwind them so that replication fork can progress through. One of the helicases capable of resolving G-quadruplexes is another superfamily 1 member with sequence similarity to RecD, called Pif1 helicase. Closely related to *S. cerevisiae* Rrm3 helicase, Pif1 was shown to promote replication fork progression through genomic regions that contain G-quadruplex sites (Paeschke et al., 2011). Furthermore, Pif1 functions to prevent G-quadruplex-associated DNA damage (Paeschke et al., 2013). Another helicase implicated at resolving

G-quadruplexes is Dna2 nuclease-helicase, which also has roles in telomere maintenance in addition to Okazaki fragments-processing while traveling with the fork (Lin et al., 2013). Moreover, RecQ helicase members Bloom's syndrome helicase (BLM), Werner's syndrome helicase (WRN), and Fanconi anemia group J (FANCI) helicase are all capable of resolving G-quadruplexes and accumulate at stalled replication forks (London et al., 2008). Of particular note, WRN was previously reported to unwind CGG repeats *in vitro* (Fry and Loeb, 1999). Even though none of these helicases were yet shown to associate

with the *FMRI* gene, it remains to be investigated whether these helicases can help replication forks progress through the CGG repeats at the *FMRI* locus.

HDHB IS RECRUITED TO THE REPEATS AT THE *FMRI* GENE AND AT COMMON FRAGILE SITES

Replication forks may stall naturally without any additional perturbation at hard to replicate regions such as repetitive regions. Replication fork stalling initially results in long stretches of ssDNA that get coated with RPA. To facilitate replication fork recovery, ssDNA-bound RPA recruits DNA damage response proteins, including helicases like HDHB (Guler et al., 2012).

To investigate whether HDHB associates with replication forks stalled at repetitive DNA sequences, in particular to the CGG repeats at *FMRI* locus and AT-rich repeats at FRA16D, we performed chromatin immunoprecipitation (ChIP) experiments. U2OS cells were blocked in G2/M-phase and released for 5 h (G1), or synchronized in G1/S-phase and released for 6 (S) or 9 h (G2/M), followed by formaldehyde treatment to crosslink chromatin. Chromatin was isolated, sheared and immunoprecipitated using purified polyclonal HDHB antibody or non-immune control antibody (Gerhardt et al., 2015). HDHB enrichment in immunoprecipitated chromatin was measured by quantitative real-time PCR using one primer pair amplifying DNA segment containing the repeat region/break site (**Figures 1B,C**) and two primer pairs in a distal region (*FMRI* gene: primer pair 3 and 12 (Gray et al., 2007); FRA16D/*WWOX* gene primer pair 12 and 14). In S-phase cells, HDHB was significantly enriched on chromatin in the repeat region of fragile sites relative to the distal regions. HDHB enrichment in both repeat regions was reduced in G1- and G2/M-phase. We found similar results at a second CFS, FRA3B (data not shown). These results show that HDHB is enriched in S-phase at the repetitive DNA sequences and suggest that HDHB is recruited to stalled replication forks at repetitive regions such as CGG repeats at the *FMRI* gene.

Upon recruitment to the fork, HDHB can unwind the secondary repeat structures formed by CGG repeats ahead of DNA-polymerase (**Figures 1D,E**). Repeat expansion following DNA polymerase slippage at stalled replication forks can be so prevented (**Figure 1E**) as well as chromosomal fragility, which could result from DNA breaks induced by replication fork stalling. It would be interesting to determine whether FXS and premutation patients have a decreased HDHB protein level or HDHB helicase activity, and whether such differences can affect replication fork stalling, chromosomal fragility and CGG repeat expansion.

RNA HELICASES INVOLVED IN PREVENTION OF SECONDARY CGG REPEAT RNA STRUCTURES

The presence of *FMRI* mRNA in intranuclear inclusions (Tassone et al., 2004) in premutation patients, as observed in

brain tissues from FXTAS patients (Galloway and Nelson, 2009), and increased *FMRI* mRNA level in PM carriers (Tassone et al., 2000) led to the suggestion that a toxic RNA gain-of-function mechanism might be responsible for FXTAS development. An increased *FMRI* mRNA level was also noticed in ovarian granulosa cells of female carriers with a PM (Elizur et al., 2014). Since RNAs containing CGG repeats can adopt secondary structures *in vitro* (Napierala et al., 2005), it is likely that *FMRI* mRNA assumes non-canonical RNA structures *in vivo* as well.

It was previously shown that the expansion to FM is reduced by AGG interruptions within the premutated allele (Yrigollen et al., 2012; Nolin et al., 2014). AGG interruptions are located at the 5' end of the CGG repeat sequence in *FMRI* gene (Kunst and Warren, 1994; Kunst et al., 1996, 1997). These interruptions were proposed to stabilize the repeats (Nelson et al., 2013), potentially by preventing the formation of secondary repeat DNA structures within the cell. AGG interruptions could also prevent formation of secondary structures in *FMRI* mRNA. Indeed, we recently found that an increase in number of AGG interruptions, from none or one to two, is associated with lower risk of FXDOR in patients carrying a PM (Lekovich et al., 2017). We propose a model where AGG interruptions lowers the probability of secondary repeat structure formation in *FMRI* mRNA, and hence pathogenesis, in the ovaries of women carrying a PM.

Other than cis-acting elements like AGG interruptions described above, RNA helicases could also prevent repeat-containing RNA to form non-canonical secondary structures. One candidate for unwinding rCGG repeat structures is Rm62, *Drosophila* ortholog of p68/DDX5 RNA helicase. p68 RNA helicase is a prototypical DEAD-box RNA helicase that has been implicated in transcriptional regulation, pre-mRNA splicing, and nucleocytoplasmic shuttling. Rm62 overexpression rescued neurodegeneration in flies expressing 90 CGG repeats (Qurashi et al., 2011). Additionally, RNA helicases p68/DDX5 and DDX6 were reported to unwind expanded CUG repeats in myotonic dystrophy (Laurent et al., 2012; Pettersson et al., 2014), making these helicases of therapeutic interest. Another helicase able to unwind G-quadruplexes and R-loop structures is RNA helicase A (RHA, also called DHX9, and nuclear DNA helicase II). RHA unwinds DNA-DNA, RNA-RNA and DNA-RNA duplexes with 3' to 5' direction. It acts preferentially on RNA substrates.

Additional proteins, involved in preventing the formation of secondary RNA structures, are heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1). hnRNP A2/B1 have a role in packaging nascent mRNA, alternative splicing and cytoplasmic RNA trafficking, translation and stabilization. Found in intranuclear inclusions of FXTAS patients (Iwahashi et al., 2006), hnRNP A2/B1 were described to act as an RNA chaperone destabilizing RNA structures formed by CGG repeats (Khateb et al., 2004; Ofer et al., 2009). hnRNP A2/B1 overexpression rescues neurodegeneration in *drosophila* expressing 90 CGG repeats (Jin et al., 2007; Sofola et al., 2007). It remains to be determined if these helicases or proteins unwinding secondary structures are deregulated in patients and whether helicase deficiency promotes the pathogenicity observed in patients with a premutation.

CONCLUSION

Repeats present a challenge for the replication machinery and other cellular processes involving repetitive DNA and RNA. Therefore, mechanisms to resolve secondary structures that repeats might adopt are needed. Specialized helicases are able to unwind secondary DNA and RNA structures, which can otherwise lead to replication fork stalling or toxic RNA, respectively. Replication fork stalling at endogenous repeats is reported in cells derived from FXS and FRDA patients (Gerhardt et al., 2014, 2016). This can lead to genomic instability by DNA polymerase slippage-induced repeat expansion or chromosomal fragility when occurred at RFS and CFS (Madireddy et al., 2016). Toxic RNAs, on the other hand, may disrupt cellular processes particularly by sequestering proteins important for RNA function. Therefore, resolution of secondary structures, which DNA or RNA repeats adopt, by DNA/RNA helicases is

a crucial mechanism that could help prevent repeat-induced diseases. Consequently, helicases that resolve such secondary structures adopted by DNA or RNA repeats may constitute a crucial toolbox cells employ to help prevent repeat-induced diseases such as fragile X syndrome.

AUTHOR CONTRIBUTIONS

GG and JG performed the experiments. GG, JG and ZR wrote and edited the manuscript.

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Stress Odorant Sensory Response Dysfunction in *Drosophila* Fragile X Syndrome Mutants

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Sensory processing dysfunction (SPD) is present in most patients with intellectual disability (ID) and autism spectrum disorder (ASD). Silencing expression of the Fragile X mental retardation 1 (*FMR1*) gene leads to Fragile X syndrome (FXS), the most common single gene cause of ID and ASD. *Drosophila* have a highly conserved *FMR1* ortholog, *dfmr1*. *dfmr1* mutants display cognitive and social defects reminiscent of symptoms seen in individuals with FXS. We utilized a robust behavioral assay for sensory processing of the *Drosophila* stress odorant (dSO) to gain a better understanding of the molecular basis of SPD in FXS. Here, we show that *dfmr1* mutant flies present significant defects in dSO response. We found that *dfmr1* expression in mushroom bodies is required for dSO processing. We also show that cyclic adenosine monophosphate (cAMP) signaling via PKA is activated after exposure to dSO and that several drugs regulating both cAMP and cyclic guanosine monophosphate (cGMP) levels significantly improved defects in dSO processing in *dfmr1* mutant flies.

Keywords: Fragile X syndrome, sensory response dysfunction, *Drosophila*, cAMP, cGMP, avoidance response, IBMX

INTRODUCTION

Sensory processing dysfunction (SPD) is a key symptom seen in 90% of individuals with intellectual disability (ID) and autism spectrum disorder (ASD) (Marco et al., 2011; Chang et al., 2014) where the response to a given stimulus is different from the typically developing population. The most common clinical features of SPD are under-responsiveness, sensory seeking, auditory filtering, and tactile sensitivity (Tomchek and Dunn, 2007). This reflects that multiple senses are affected, including audition, touch, vision, and oral sensation (Kern et al., 2006). For instance, some individuals with ASD will perceive sound much louder than typically developing individuals and this will affect their behavior. Indeed, they will either block their ears or become increasingly anxious. SPD affects patients with mild to severe ID equally (Engel-Yeger et al., 2011). Sensory processing deficits have also been linked to stereotypical movements and anxiety (Joosten and Bundy, 2010). SPD predicts communication competence and maladaptive behaviors (Lane et al., 2010), which are the drivers of socio-economic impact (Bailey et al., 2012). Importantly, SPD is present in both children and adults (Crane et al., 2009). While brain magnetic resonance imaging (MRI) studies have provided some insights (Owen et al., 2013), the molecular basis and treatment of SPD remain poorly understood.

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Fragile X syndrome (FXS) is the most common single gene cause of ID and ASD (Androschuk et al., 2015). FXS is caused by a trinucleotide CGG repeat expansion that leads to the methylation and transcriptional silencing of the Fragile X mental retardation 1 (*FMR1*) gene. This results in the loss of Fragile X mental retardation protein (FMRP), an mRNA-binding protein that functions in neuronal mRNA metabolism, namely in the translation of neuronal mRNAs involved in synaptic structure and function. Individuals with FXS frequently present with SPD (Goldson, 2001), which has a major impact on their ability to function (Baranek et al., 2002). We reasoned that response to sensory stimulation may serve as endophenotype of the processing of information.

Drosophila have a conserved *FMR1* ortholog, *dfmr1*. *dfmr1* mutants present with the circadian, cognitive, and social defects also observed in individuals with FXS. Little is known about the response to sensory signal in *dfmr1* mutant flies. Normal shock and olfactory stimuli used for olfactory memory training have not provided a model to study sensory processing. Suh et al. (2004) discovered that *Drosophila* avoided systematically an environment in which other flies had previously been submitted to mechanical stress. Indeed, the *Drosophila* stress odorant (dSO) is a signal emitted when flies are subjected to electrical or mechanical stressors, and elicits an innate and robust avoidance behavioral response in wild-type (WT) *Drosophila* (Suh et al., 2004). Here, we show that *Drosophila dfmr1* mutant flies present significant defect in responding to dSO.

MATERIALS AND METHODS

Drosophila melanogaster Stocks and Crosses

Fly stocks were maintained at 22°C on standard cornmeal-yeast media from Cold Spring Harbor Laboratory. WT stocks were backcrossed to *w¹¹¹⁸isoCJ1* for 6 generations. *dfmr1^{B55}* flies were obtained from Dr. Kendal Broadie (Vanderbilt University). *dfmr1³* flies and *dfmr1³* flies containing a WT rescue transgene (*dfmr1³WTR*) were obtained from Dr. Tom Jongens (University of Pennsylvania). *Elav-Gal4*, *OK107-Gal4*, *C747-Gal4*, *MB247-Gal4*, and *Feb170-Gal4* flies were obtained from Dr. Tim Tully. To determine the spatial requirement of FMRP in mediating dSO avoidance, we used RNA interference (RNAi) against FMRP in order to knockdown/reduce expression of FMRP. Using the Gal4-UAS system (Brand and Perrimon, 1993), we generated crosses by mating *Elav(Embryonic lethal vision)-Gal4*, *OK107-Gal4*, *Feb170-Gal4*, *MB247-Gal4*, and *747-Gal4* virgin females to *UAS-dfmr1RNAi¹⁻⁷* males generated previously in our laboratory (Bolduc et al., 2008). To assess the spatio-temporal requirement of *dfmr1*, we used *Gal80^{ts}*; *Elav-Gal4* (from Dr. Tom Jongens) to drive the expression *UAS-dfmr1RNAi¹⁻⁷*. WT and transgenic flies were tested in parallel. WT and transgenic flies were raised at 18°C (restricting the expression of ELAV-Gal4) and then transferred for 3 days at 30°C allowing its expression, or kept at 18°C, to restrict the expression of ELAV-Gal4, as before for memory experiments in our laboratory (Bolduc et al., 2008).

Behavioral Paradigm

The T maze avoidance assay was conducted, as previously described by Suh et al. (2004), with modifications (Androschuk, 2016). All testing was performed in an environment controlled room which was maintained at 25°C and 70% humidity. To produce dSO a group of 50 flies (mixed sex, termed ‘emitter’ flies) were vortexed (Fisher Vortex Mixer) for 1 min (alternating between 3 s of vortexing followed by 5 s of rest for the entire duration) in a 10 mL Falcon tube sealed with Parafilm (Fisher Scientific 149598) at maximum speed. Emitter flies were then removed from the Falcon tube and the dSO-containing Falcon tube was placed into a T maze. A new dSO-free Falcon tube was placed opposite the dSO-containing tube. Subsequently, 50 naïve flies (termed ‘responder’) were transferred into a new Falcon tube and loaded into the elevator of the T maze. Responder flies were then given 1 min to choose between the dSO-containing and the dSO-free Falcon tubes. Following the 1-min testing period, flies were sequestered and avoidance response was scored. Avoidance was scored as a Performance Index (PI), calculated as follows:

$$PI = \frac{(\text{No. of responder flies in dSO-free tube}) - (\text{No. of responder flies in dSO tube})}{\text{Total no. of responder flies}}$$

Statistical Analysis

For unplanned comparisons between more than 2 groups, we used one-way ANOVA followed by Tukey’s test. For all analysis between 2 groups, we used a two-tailed Student’s *t*-test. Analysis was performed using GraphPad (PRISM7).

CO₂ Avoidance

Gaseous CO₂ was used in place of emitter flies in CO₂ avoidance testing. A flow-meter set at 0.2 mL/min or 0.5 mL/min was used to administer CO₂ into Falcon tubes for 30 s, which were then momentarily sealed using Parafilm prior to being loaded into the T maze. Responder flies were given 1 min to choose between the CO₂-containing and the CO₂-free Falcon tubes. Flies were then sequestered and avoidance response was scored as a PI, as above.

Drug Administration

Using previously published feeding protocols for Lithium in the classical olfactory conditioning assay (Choi et al., 2015), we performed dose response curves for the avoidance assay. For drugs not previously tested in our laboratory (IBMX, dipyrindamole, 8-CPT), we assessed response at 1 day as well as longer treatment if there was no response after 1 day. The treatments were provided only in post-natal set up to reflect the potential clinical application at this time. For all experiments, only responder flies were treated with vehicle or treatment.

IBMX

The 3-isobutyl-1-methylxanthine (IBMX; Sigma I7018) was added to standard food media for drug administration. The 1-day-old flies were placed in food bottles containing 0.05 mg/mL IBMX or the food alone for 4 days and transferred to food vials containing 0.05 mg/mL IBMX or the food alone the day prior to testing (Androschuk, 2016).

8-CPT

The 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (8-CPT; Sigma C3912) was administered to flies on 2.1-cm Whatman filter paper (Fisher WHT1540321). The 1-day-old flies were placed in vials containing 225 μ L of 8-CPT with 5% sucrose or 5% sucrose only and treated for 5 days prior to testing. Flies were transferred daily to new vials containing fresh 8-CPT with sucrose or sucrose alone (Androschuk, 2016).

LiCl

Lithium chloride (LiCl; Sigma L9650) was added directly to the standard food media for drug administration. The 1-day-old flies were set up in food bottles containing 10 mM LiCl or the food alone for 4 days and transferred to food vials containing 10 mM LiCl or the food alone the day prior to testing (Androschuk, 2016).

Dipyridamole

The 0.8 mM dipyridamole (Sigma D9766) was added directly to standard food media for drug administration with 0.8% DMSO. The 1-day-old flies were placed for 24 h in food bottles containing either dipyridamole or vehicle.

Immunohistochemistry

After 1 min exposure to dSO, naïve responder flies avoiding the dSO were placed on ice for 2 min and heads of female responder flies were removed and placed in cold PBS for dissection. Unexposed flies were placed in a dSO-free 10 mL Falcon tube sealed with Parafilm for 1 min. Then flies were processed blind in parallel. Flies were then placed on ice for 2 min and heads of female flies were removed and placed in cold PBS for dissection. Fly heads were dissected as before (Bolduc et al., 2008). Protein kinase A (PKA) was identified with 1:1000 α -PKA catalytic subunit (phospho T198) (Abcam ab118531).

Following overnight incubation with the secondary antibody (1:200 Cy3 α -Rabbit Jackson ImmunoResearch 111-165-003) and 1% PBS triton (PBST) with 0.25% NGS, brains were washed three times with 1% PBST and mounted using FocusClear (Cedarlane FC-101). Imaging was completed using a Zeiss LSM 700 Confocal Microscope and images were quantified using ImageJ (Androschuk, 2016). Gain was set the same for both groups.

Pathway Analysis

In silico pathway analyses were performed with Ingenuity Pathway Analysis (IPA, Qiagen) to identify interactions with cAMP and cGMP by genes associated with ASD from the SFARI Gene database (<https://www.sfari.org/>) and genes implicated in ID from published literature (Gilissen et al., 2014).

RESULTS

dfmr1 Is Required for dSO Response

In order to determine the role of FMRP in the processing and modulation of dSO avoidance behavior in *Drosophila*,

we utilized the two null alleles, *dfmr1*³ and *dfmr1*^{B55}, known to have olfactory and courtship memory defects, as well as social interaction defect (McBride et al., 2005; Bolduc et al., 2008; Bolduc et al., 2010). We found that *dfmr1*³ and *dfmr1*^{B55} flies exhibited a significant decrease in dSO avoidance compared to flies with the appropriate genetic control (*dfmr1*³ with a genomic rescue fragment, *FMR1*³ WTR, and WT flies) (Figure 1A). Similarly, transheterozygous *FMR*^{B55}/*FMR1*³ mutants exhibited a significant decrease in dSO avoidance behavior compared to WT flies (Figure 1B). Next, we tested if FMRP was involved in dSO emission or dSO response. We conducted avoidance trials in which WT flies were utilized as the emitter or responder and tested with the *dfmr1* mutant flies. WT flies exhibited normal avoidance in response to dSO emitted by *FMR*^{B55} and *FMR1*³ (Figure 1C). *FMR*^{B55} and *FMR1*³ flies exhibited decreased avoidance as compared to their genetic controls when WT flies were utilized as emitter flies (Figure 1D). Considering the normal avoidance of WT flies when using *dfmr1* flies as emitters, we considered that FMRP is involved in sensory processing and not emission of dSO.

dfmr1 Is Required in Mushroom Bodies (MB) for dSO Processing

We first used the pan-neuronal driver *ELAV-Gal4* and *UAS-FMR* responder with RNAi to knockdown FMRP in neurons. Pan-neuronal knockdown of FMRP resulted in a significant decrease in dSO avoidance response, which we confirmed causes a dSO processing defect and not emission deficiency from knockdown of FMRP (Figures 2A,B). Next, we asked whether loss of FMRP in two higher-order processing centers, the mushroom bodies (MB) and the central complex, are involved in dSO avoidance. We showed previously that FMRP was required in MB for olfactory memory (Bolduc et al., 2008). Bräcker et al. (2013) showed that MB were required for CO₂ avoidance response in the context of food deprivation or food-related odors. Knockdown of FMRP using the MB-specific driver *OK107* resulted in a significantly decreased avoidance response compared to WT flies (Figures 2C,D). To confirm the requirement of FMRP in the MB in mediating dSO avoidance behavior, we utilized the MB-specific driver *MB247* to knockdown FMRP, which resulted in a significant defect in dSO avoidance (Figures 2E,F). Unlike the significant decrease in dSO avoidance that resulted from using the *OK107-Gal4* and *MB247-Gal4* driver lines to knockdown FMRP in the MB, use of the *C747-Gal4* driver line did not result in a significant decrease in dSO avoidance (results not shown). These differences are likely due to regional specificity and strength of expression of each individual driver within the MB. The *OK107-Gal4* and *MB247-Gal4* driver lines strongly target expression in α , β , and γ Kenyon cells, while *C747-Gal4* expression is weaker in γ Kenyon cells (Aso et al., 2009). Knockdown of FMRP in the central complex using *FEB170-Gal4* did not result in any significant changes in dSO avoidance (Supplementary Figures 1A,B). In addition, we did not observe significant defects in the avoidance after

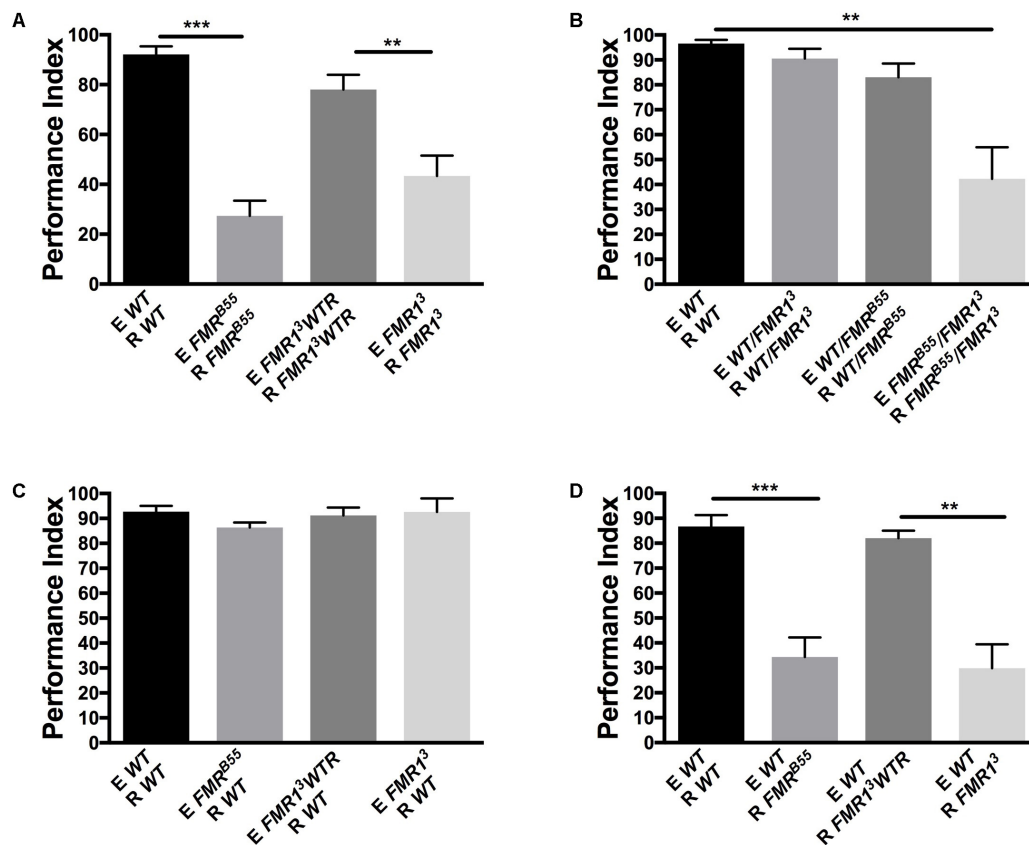


FIGURE 1 | Fragile X mental retardation protein (FMRP) is required for avoidance of *Drosophila melanogaster* stress odorant (dSO). For all figures, the flies emitting the dSO (E) are submitted to the vortexing protocol. The flies tested for their response to tubes exposed to dSO or not are considered the responders (R) of dSO signaling. **(A)** *FMR^{B55}* mutants exhibit a significantly lower avoidance in response to dSO compared to WT flies (Student's *t*-test $P < 0.0001$; $N = 8$); avoidance is quantified as Performance Index (PI). *FMR¹³* exhibit decreased avoidance compared to *FMR¹³WTR* flies, the avoidance of which is rescued genetically through the addition of a genomic *dfmr¹³* fragment (Student's *t*-test $P = 0.0049$; $N = 8$). dSO avoidance behavior is scored as PI. **(B)** *FMR^{B55}/FMR¹³* flies exhibit decreased avoidance compared to WT flies (Tukey's test $P = 0.0001$; $N = 7$). Avoidance behavior is genetically rescued in *FMR^{B55}/WT* (Tukey $P = 0.9348$; $N = 7$) and *FMR¹³/WT* (ANOVA $P = 0.5638$; $N = 7$) flies. *FMR^{B55}/FMR¹³* flies exhibit decrease avoidance behavior compared to *FMR¹³/WT* (Tukey's test $P = 0.0004$; $N = 7$) and *FMR^{B55}/WT* (Tukey's test $P = 0.0028$; $N = 7$) flies. **(C)** WT flies did not exhibit decreased avoidance behavior to dSO emitted by *FMR^{B55}*, (Student's *t*-test $P = 0.0988$; $N = 5$), *FMR¹³* (Student's *t*-test $P = 0.9897$; $N = 5$), and *FMR¹³WTR* flies (Student's *t*-test $P = 0.7153$; $N = 5$). **(D)** *FMR^{B55}* flies exhibit decreased avoidance behavior to WT dSO (Student's *t*-test $P < 0.0001$; $N = 12$). *FMR¹³* also flies exhibit diminished avoidance behavior to WT dSO as compared to *FMR¹³WTR* flies (Student's *t*-test $P = 0.0018$; $N = 12$). ** $P < 0.01$, *** $P < 0.001$.

post-natal variation in FMRP levels [using Gal80^{ts}; ELAV-gal4 with *UAS-dfmr1RNAi¹⁻⁷* to lower FMRP level as before (Bolduc et al., 2008)], which is different to what was observed in long-term olfactory memory defects in *dfmr1* mutants previously and more similar to short-term memory (Figure 2G; Bolduc et al., 2008).

Targeting cAMP/cGMP Signaling Pharmacologically in Adult Flies Rescues dSO Response in *dfmr1* Mutants

Next, we explored if pharmacological intervention could improve *dfmr1* mutant avoidance response and help decipher the molecular mechanism related to the dSO defects in *dfmr1* mutant flies. We first considered the seminal report from Suh et al. (2004) who showed that CO₂ was a key component of the dSO. Lin et al. (2013) further showed that CO₂ olfactory information was

conveyed by 2 types of projection neurons depending on the concentration of CO₂ present in the environment (Lin et al., 2013). We therefore tested response to CO₂ for *dfmr1* mutants and found that *dfmr1¹³* and *dfmr1^{B55}* had significant response deficits to CO₂ at 0.2 mL/min and 0.5 mL/min (Supplementary Figures 1C,D). As cAMP signaling is required for CO₂ sensing (Klengel et al., 2005) and cAMP signaling dysregulation is linked to FXS early on in human (Berry-Kravis et al., 1984) and in *Drosophila* (Kanellopoulos et al., 2012), we investigated if cAMP regulation could be involved in the defective dSO response in *dfmr1* mutants. Activity dependent reactivity of cAMP is abnormal in FXS (Berry-Kravis et al., 1995). Moreover, FMRP binds to adenylyl cyclase (AC) and phosphodiesterase (PDE) mRNAs (Darnell et al., 2011). Importantly, PDE4 inhibitors Rolipram and Lithium, which lead to increased cAMP levels, have been found to rescue memory and long-term depression (LTD) defects in FXS mice and flies (Choi et al., 2015, 2016).

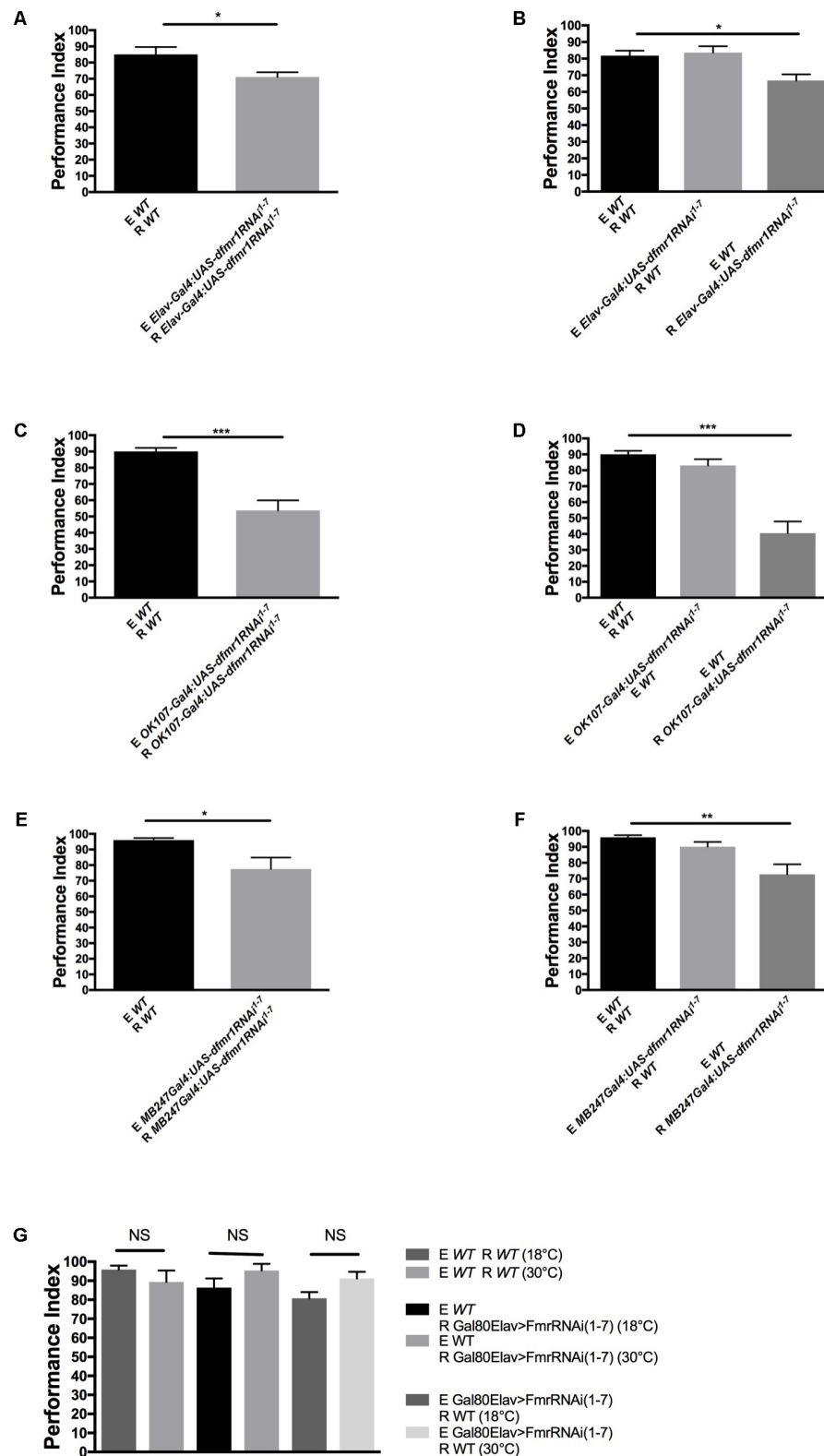


FIGURE 2 | FMRP expression in mushroom bodies and glia is required for dSO avoidance. **(A)** Pan-neuronal knockdown of FMRP by *Elav-Gal4:UAS-dfmr1RNAi¹⁻⁷*, results in decreased avoidance to dSO compared to WT flies (Student's *t*-test $P = 0.0409$; $N = 20$). **(B)** WT flies did not exhibit any significant decrease in avoidance (Continued)

FIGURE 2 | Continued

behavior to dSO emitted by *Elav-Gal4;UAS-dfmr1RNAi¹⁻⁷* flies (Student's *t*-test $P = 0.7653$; $N = 10$). *Elav-Gal4;UAS-dfmr1RNAi¹⁻⁷* flies exhibit decreased avoidance behavior to WT dSO as compared to WT flies (Student's *t*-test $P = 0.00285$; $N = 12$). **(C)** *OK107-Gal4;UAS-dfmr1RNAi¹⁻⁷* flies exhibit significantly decreased avoidance to dSO compared to WT flies (Student's *t*-test $P < 0.0001$; $N = 12$). **(D)** *OK107-Gal4;UAS-dfmr1RNAi¹⁻⁷* flies exhibit a significantly decreased avoidance response when tested against dSO emitted by WT flies (Student's *t*-test $P < 0.0001$; $N = 8$). WT flies exhibited normal avoidance behavior when tested against dSO emitted by *OK107 > FmrRNAi(1-7)* flies (Student's *t*-test $P = 0.1240$; $N = 8$). **(E)** *MB247Gal4;UAS-dfmr1RNAi¹⁻⁷* flies exhibited diminished avoidance behavior as compared to WT flies (Student's *t*-test $P = 0.0239$; $N = 10$) when tested with same genotype pairs. **(F)** *MB247Gal4;UAS-dfmr1RNAi¹⁻⁷* flies exhibit a significantly decreased avoidance response when tested against dSO emitted by WT flies (Student's *t*-test $P = 0.0016$; $N = 8$). WT flies exhibited normal avoidance behavior when tested against dSO emitted by *MB247Gal4;UAS-dfmr1RNAi¹⁻⁷* flies (Student's *t*-test $P = 0.0707$; $N = 8$). **(G)** WT (Student's *t*-test $P = 0.27$; $N = 5$) and *Gal80ts;ELAV-Gal4 > UAS-dfmr1RNAi¹⁻⁷* flies present no significant defect in avoidance performance comparing their performance at restrictive (18°C) versus permissive (30°C) either as responder to dSO (R) (Student's *t*-test $P = 0.1689$; $N = 5$ PI per group) or as emitter of dSO (E) (Student's *t*-test $P = 0.059$; $N = 5$ PI per group). NS, not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

We assessed if dSO exposure was associated with activation of the cAMP pathway using brain immunohistochemistry first. Activation of cAMP leads to concomitant activation of cAMP-dependent protein kinase A (PKA). Using confocal imaging, we examined the relative levels of the phosphorylated catalytic subunit of PKA in WT fly brains in response to dSO exposure by utilizing a free catalytic subunit-specific PKA (phospho T198) antibody. PKA catalytic subunit mRNA and protein have been shown to be expressed throughout the brain with increased signal in the MB Kenyon cells especially in the dorsal aspect (Skoulakis et al., 1993). PKA is activated when cAMP binds to regulatory subunits, resulting in the disassociation of catalytic subunits. The catalytic-PKA phosphorylation levels were significantly elevated overall in WT brains following dSO exposure compared to naïve, unexposed WT flies, suggesting that cAMP signaling participates in modulating dSO avoidance behavior (Figures 3A,B). Interestingly, high expression was noted in cells located dorsally in the brain in the region corresponding to the Kenyon cells of the MB, similar to the previous report (Skoulakis et al., 1993). Nonetheless, further confirmation with a functional PKA activity assay and measurement of constituents of the cAMP pathway or downstream targets (CREB for instance) will be important to conduct in the future to measure treatment efficacy and could be used as biomarkers.

We wanted to determine whether dSO avoidance behavior could be rescued through pharmacological intervention targeting the cAMP and/or cGMP signaling pathway restricted to the post-natal period as this is closer to potential clinical interventions in individuals with FXS. We first asked whether IBMX, a non-specific cAMP and cGMP PDE inhibitor, could rescue avoidance behavior in FXS flies. IBMX administration for 5 days resulted in a significant increase in avoidance behavior in *FMR^{B55}* and *FMR1³* flies (Figures 3C,D). Interestingly, Rolipram, a selective PDE4 (cAMP specific) inhibitor shown to improve olfactory and courtship memory (Choi et al., 2015, 2016), did not lead to significant improvement in avoidance (data not shown). This may suggest that both cAMP and cGMP need to be modulated for rescue of avoidance behavior. We reasoned that other PDEs may be required for dSO rescue in *dfmr1* mutants. Therefore, we used 8-(4 Chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium (8-CPT) which is an activator of cAMP-dependent PKA and inhibitor of cGMP dependent PDE. Administration for 5 days of 8-CPT resulted in

a significant rescue of dSO avoidance in *FMR^{B55}* and *FMR1³* flies (Figures 4A,B). Then, we tested dipyrindamole, a United States Food and Drug Administration (FDA) approved drug which increases cAMP levels via both inhibition of PDE-dependent cGMP degradation and adenosine-dependent cAMP synthesis. Dipyrindamole is an inhibitor of PDE 6 which inhibits cGMP and PDE 11 which inhibits both cAMP and cGMP degradation. We observed a significant improvement of *FMR^{B55}* (Figure 4C) mutants' avoidance response after 1 day of treatment. We also tested another drug already FDA approved, Lithium, with effect on cAMP signaling and shown to improve learning and memory in *FMR^{B55}*, *FMR1³* flies and *FMR1* KO mice (Choi et al., 2015, 2016). We observed significant rescue of avoidance response in *FMR^{B55}* (Figure 4D) mutants with Lithium administration after 5 days of treatment (no effect was seen after 24 h treatment – not shown). Together, our pharmacological results strengthen the previous molecular work in *FMR1* KO mice showing that FXS may involve both production and degradation of cAMP considering that *FMRP* binds to mRNAs for PDE regulating cAMP (PDE4B, PDE4DIP, PDE8B), but also cAMP and cGMP (PDE2A) (Darnell et al., 2011).

cAMP and cGMP Are Linked to Several ID and ASD Genes

Based on the recent report of interaction between *FMR1* and several novel ASD candidate genes, we asked if other ID and ASD genes were linked to cAMP/cGMP signaling (Iossifov et al., 2014; Ronemus et al., 2014). This is important as treatment identified for FXS may then be tried in priority with other ID/ASD genes related molecularly. Using an *in silico* gene pathway analysis approach, we identified both ID and ASD genes interacting with cAMP (Figures 5A,C) and to a lesser extent cGMP (Figures 5B,D).

DISCUSSION

Our work provides a novel application of dSO avoidance response assay as an endophenotype model to study sensory response behavior in *Drosophila* models of FXS and possibly other ID and autism causes. We show that sensory response required developmental *dfmr1* expression while emission of the sensory cue (dSO) did not. Our results illustrate the importance of *dfmr1* expression in the MB for typical dSO response. This

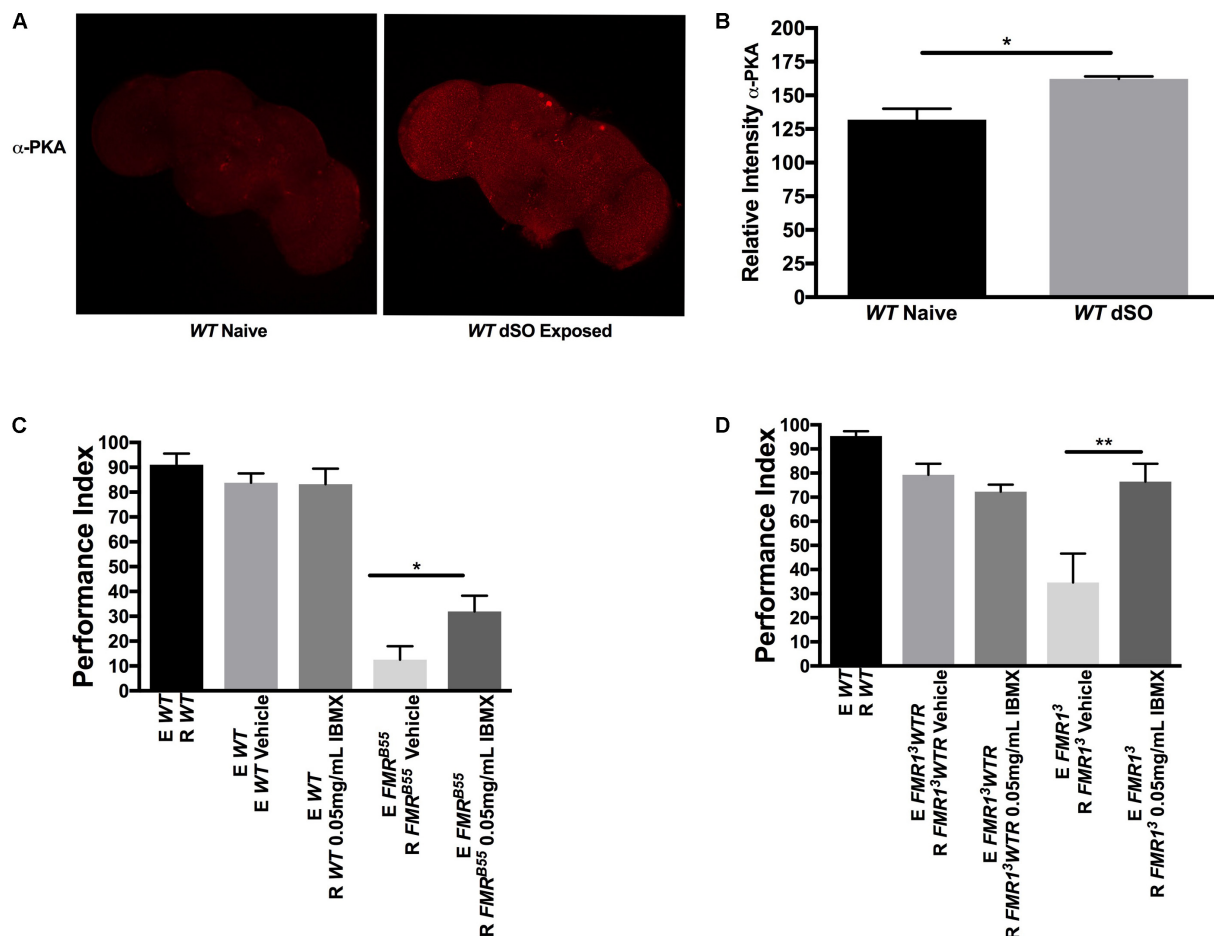


FIGURE 3 | Pharmacological intervention targeting cyclic adenosine monophosphate (cAMP) rescues dSO avoidance in Fragile X syndrome flies. **(A)** Confocal imaging of WT flies catalytic subunit PKA (phospho T198) levels in dSO exposed and unexposed WT fly brains processed in parallel and imaged with same gain. **(B)** dSO exposure results in an overall significant increase in PKA catalytic subunit (phospho T198) levels in WT brains compared to unexposed control (Student's *t*-test $P = 0.0226$; $N = 3$). All graphs depict mean $\pm$ SEM. **(C)** 5-day treatment of *FMR^{B55}* flies with 0.05 mg/mL IBMX results in significantly increased avoidance compared to *FMR^{B55}* on vehicle (Student's *t*-test $P = 0.0282$; $N = 14$). No significant difference in avoidance behavior observed in WT flies following 5-day treatment with 0.05 mg/mL IBMX as compared to vehicle (Student's *t*-test $P = 0.9379$; $N = 14$). **(D)** 5-day treatment of *FMR1³* flies with 0.05 mg/mL IBMX resulted in a significantly increase in avoidance compared to *FMR1³* fed vehicle (Student's *t*-test $P = 0.0068$; $N = 13$). No significant difference in avoidance behavior observed in *FMR1³WTR* flies following 5-day treatment with 0.05 mg/mL IBMX as compared to vehicle (Student's *t*-test $P = 0.02077$; $N = 13$). * $P < 0.05$, ** $P < 0.01$.

parallels our previous finding showing that *dfmr1* expression in MB was required for learning and memory (Bolduc et al., 2008) although the developmental but also acute expression of FMRP was linked to memory formation.

To our knowledge, this is the first time that dSO defects are rescued pharmacologically in a post-natal setting in *dfmr1* mutants. This is a promising avenue for individuals with FXS suffering of SPD as both Lithium and dipyridamole are FDA approved drugs. As there is pre-clinical evidence showing a conserved deficit of cAMP across species in FXS (Kelley et al., 2007) and recent evidence of improvement of cognitive symptoms in fly and mouse models of FXS (Choi et al., 2015, 2016) with PDE4 inhibitors, our results underline the importance of a symptom specific approach in ID and ASD pharmacological intervention testing. Moreover, PDE-specific inhibitors are currently undergoing clinical trial

for behavioral defects in FXS and it may be interesting to assess improvement in SPD. PDEs are well-conserved in flies and include highly conserved critical domains compared to human PDEs (Day et al., 2005). In *Drosophila*, there are seven genes encoding PDEs. The most studied is *dunce* which encodes a PDE4 ortholog and is required for olfactory learning and memory (Kauvar, 1982). More recently, orthologs for PDE1, PDE5, PDE6, PDE8, PDE9, and PDE11 were identified. Our results with IBMX show a strong effect and indicate that multiple signaling cascades may be impacted in FXS. Pharmacologically, IBMX is a complex drug. It inhibits PDE1, PDE2, PDE3, PDE4, PDE5, PDE7, and PDE 11, while PDE8 and PDE9 are insensitive to IBMX. In addition though, apart from its inhibitory effects on PDEs, IBMX has been shown in rat adipocytes to block the inhibitory regulatory protein, G_i , thereby stimulating AC and increasing intracellular

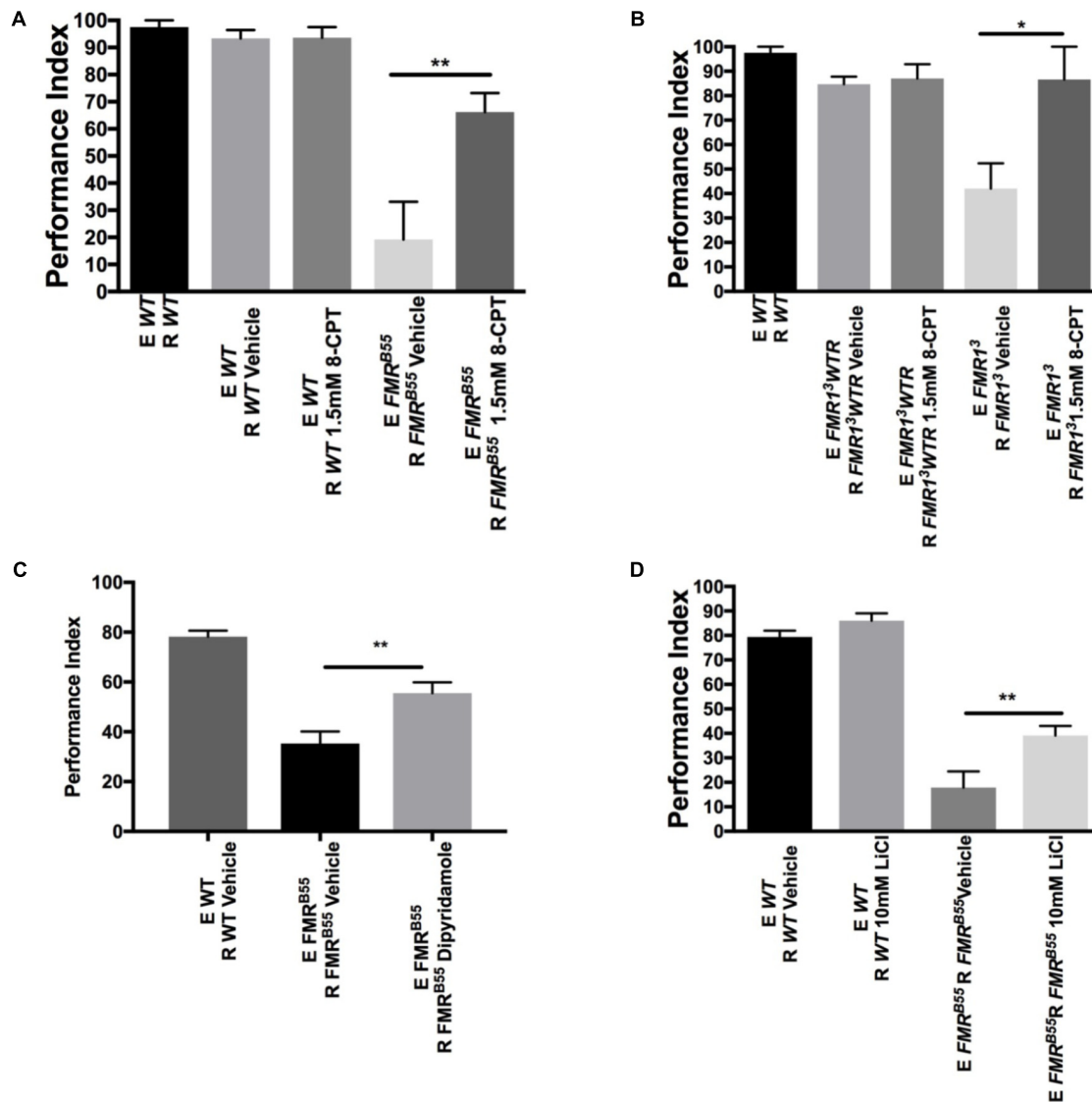
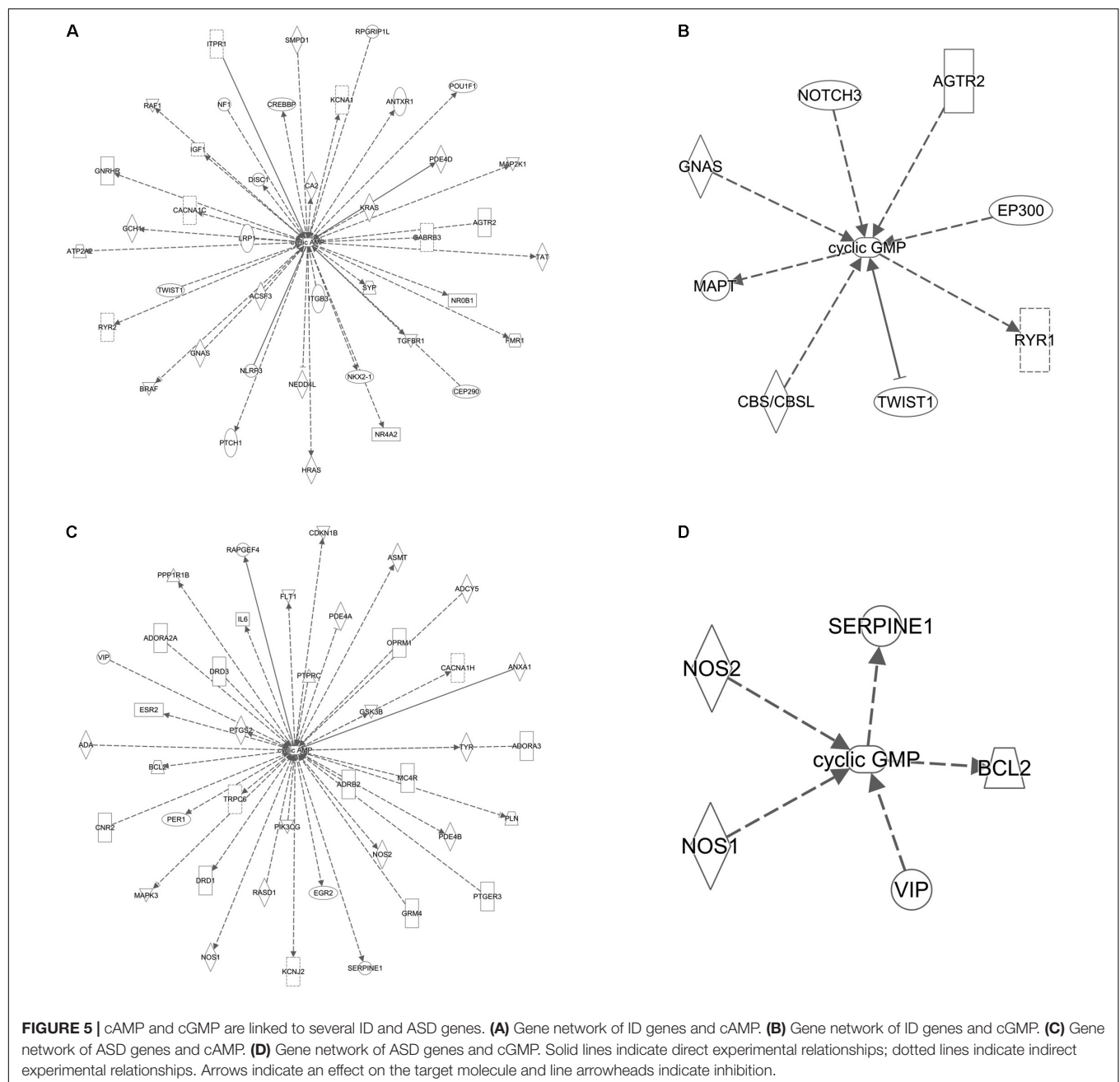


FIGURE 4 | Pharmacological rescue of dSO avoidance with PDE antagonists in *dfmr1* mutant flies. **(A)** *FMR^{B55}* flies treated for 5 days with 1.5 mM 8-CPT exhibited significantly increased avoidance behavior as compared to vehicle (Student's *t*-test $P = 0.0073$; $N = 5$). 5-day treatment of WT flies with 1.5 mM 8-CPT did not result in any significant difference in avoidance behavior as compared to vehicle (Student's *t*-test $P = 0.9688$; $N = 5$). **(B)** *FMR1^{3WTR}* flies treated for 5 days with 1.5 mM 8-CPT exhibited significantly increased avoidance behavior as compared to vehicle (Student's *t*-test $P = 0.0252$; $N = 6$). 5-day treatment of *FMR1^{3WTR}* flies with 1.5 mM 8-CPT did not result in any significant difference in avoidance behavior as compared to vehicle (Student's *t*-test $P = 0.7334$; $N = 6$). **(C)** *FMR^{B55}* flies treated for 1 day with 0.8 mM Dipyridamole exhibited significantly increased avoidance as compared to vehicle (Student's *t*-test $P = 0.0064$; $N = 8$). **(D)** *FMR^{B55}* flies treated for 5 days with 10 mM LiCl exhibited significantly increased avoidance behavior as compared to vehicle (Student's *t*-test $P = 0.0094$; $N = 15$). 5-day treatment of WT flies with 10 mM LiCl did not result in any significant difference in avoidance behavior as compared to vehicle (Student's *t*-test $P = 0.99$; $N = 15$). All graphs depict mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

cAMP levels (Parsons et al., 1988). IBMX and other xanthine-derived PDE inhibitors are also well-known adenosine receptor antagonists, consequently increasing cAMP production, which could also be a mode of action as it is “hypoactive” in FXS (Daly et al., 1981; Morgan et al., 1993). Indeed, our results and previous molecular evidence showing that FMRP binds to mRNA of PDEs regulating cGMP suggest that both cAMP and cGMP need to be considered in FXS. As cGMP has been shown to modulate cholinergic and dopaminergic

signaling, it is possible that sensory processing requires a tight balance of both cAMP and cGMP (Moody et al., 1981). Maurin et al. (2018) recently showed the importance of PDE2a in FMR1KO mice which has been shown to regulate both cAMP and cGMP. Thus, further molecular dissection studies, for instance using neurons derived from induced pluripotent cells from FXS patients, with more specific PDE inhibitors and AC activators will be required prior to clinical trials.



In addition, our genetic manipulation of FMRP suggests that the defect in avoidance is routed in developmental defects. Importantly though, despite the absence of a clear effect in modulation of FMRP level in adult fly brain on avoidance response, pharmacological treatment of adult *dfmr1* mutants can still improve avoidance performance defects. This implies a potential developmental origin of cognitive dysfunction, but also illustrates that pharmacological treatment should be considered even in absence of acute effect of the target gene on behavior. This raises the possibility that downstream consequences of the absence of *dfmr1* during development, such as dysregulation in epigenetic marks (Korb et al., 2017) and/or structural defects

(spine or neuronal network) (Comery et al., 1997; Mansilla et al., 2017) affecting cAMP equilibrium, established during development may be key to the avoidance defects and not the level of FMRP itself. This may be an important consideration when assessing the potential benefit of post-natal treatment in animal models of neurodevelopmental disability.

Finally, treatment targeting cAMP and cGMP may be of benefit to other individuals with neurodevelopmental disorders considering how ID and ASD genes are linked to cAMP-cGMP signaling *in silico*. This raises the need for high-throughput, but clinically relevant systems, to test not only multiple candidate drugs, but several genes.

AUTHOR CONTRIBUTIONS

AA co-designed the experiments, performed most of the experiments, analyzed the results, and co-wrote the manuscript. RH performed the *in silico* pathway analyses and assisted in manuscript preparation. SW performed some of the behavioral experiments. CR assisted with the drug preparation and advice on the experimental design. FB co-designed the experiments, analyzed the results, and co-wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2018.00242/full#supplementary-material>

FIGURE 1 | Spatial requirement and CO₂ response in *dfmr1* mutants. **(A)** *Feb170-Gal4:UAS-dfmr1RNAi^{1–7}* flies did not exhibit any defect in avoidance response compared to WT flies (Student's *t*-test $P = 0.8973$; $N = 10$). **(B)** *Feb170-Gal4:UAS-dfmr1RNAi^{1–7}* flies did not exhibit any defect in avoidance when tested against WT dSO (Student's *t*-test $P = 0.2119$; $N = 10$). **(C)** *FMR^{B55}* (Student's *t*-test $P < 0.0001$; $N = 6$) and *FMR1³* (Student's *t*-test $P = 0.0013$; $N = 6$) flies exhibited significantly decreased avoidance to CO_{2(g)} at a concentration of 0.2 mL/min compared to WT flies. **(D)** *FMR^{B55}* (Student's *t*-test $P < 0.0001$; $N = 10$) and *FMR1³* (Student's *t*-test $P = 0.0009$; $N = 13$) flies exhibited significantly decreased avoidance to CO_{2(g)} at a concentration of 0.5 mL/min compared to WT flies. All graphs depict mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Targeted Reactivation of *FMR1* Transcription in Fragile X Syndrome Embryonic Stem Cells

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Fragile X Syndrome (FXS) is the most common inherited cause of intellectual disability and autism. It results from expansion of a CGG nucleotide repeat in the 5' untranslated region (UTR) of *FMR1*. Large expansions elicit repeat and promoter hyper-methylation, heterochromatin formation, *FMR1* transcriptional silencing and loss of the Fragile X protein, FMRP. Efforts aimed at correcting the sequelae resultant from FMRP loss have thus far proven insufficient, perhaps because of FMRP's pleiotropic functions. As the repeats do not disrupt the FMRP coding sequence, reactivation of endogenous *FMR1* gene expression could correct the proximal event in FXS pathogenesis. Here we utilize the Clustered Regularly Interspaced Palindromic Repeats/deficient CRISPR associated protein 9 (CRISPR/dCas9) system to selectively re-activate transcription from the silenced *FMR1* locus. Fusion of the transcriptional activator VP192 to dCas9 robustly enhances *FMR1* transcription and increases FMRP levels when targeted directly to the CGG repeat in human cells. Using a previously uncharacterized FXS human embryonic stem cell (hESC) line which acquires transcriptional silencing with serial passaging, we achieved locus-specific transcriptional re-activation of *FMR1* messenger RNA (mRNA) expression despite promoter and repeat methylation. However, these changes at the transcript level were not coupled with a significant elevation in FMRP protein expression in FXS cells. These studies demonstrate that directing a transcriptional activator to CGG repeats is sufficient to selectively reactivate *FMR1* mRNA expression in Fragile X patient stem cells.

Keywords: Fragile X Syndrome, human embryonic stem cells, CRISPR-dCas9, transcriptional activation, VP-192, nucleotide repeat expansion

INTRODUCTION

Fragile X Syndrome (FXS) is an X-linked disorder affecting approximately 1 in 4,000 males and 1 in 8,000 females worldwide (Tassone et al., 2012; Hunter et al., 2014). It is the leading inherited cause of intellectual disability and autism. Many FXS patients also experience attention deficit hyperactivity disorder (ADHD), increased seizure susceptibility, anxiety and language difficulties. FXS results from expansion of a CGG trinucleotide repeat within the 5' untranslated region (UTR) of the fragile X gene, *FMR1*. Normally, *FMR1* has between 25 and 40 CGG repeats.

Instability of the CGG repeat over multiple generations leads to large (>200) expansions that markedly alter the epigenetic profile of the *FMR1* locus (reviewed in Usdin and Kumari, 2015). In most FXS patients, both the CGG repeat and the *FMR1* promoter are hypermethylated at cytosine residues (Oberlé et al., 1991; Pieretti et al., 1991). This hypermethylation is associated with epigenetic marks consistent with heterochromatin formation over the locus and a partial or complete loss of *FMR1* messenger RNA (mRNA) transcription (Coffee et al., 2002). Although the exact mechanism and order of events leading to transcriptional silencing remains incompletely understood, the net result of these epigenetic alterations is the absence of the Fragile X Mental Retardation Protein, FMRP.

There is strong evidence that loss of FMRP causes FXS symptoms, as rare patients with mutations or deletions elsewhere in *FMR1* also present with FXS (Gedeon et al., 1992; De Boulle et al., 1993; Bhakar et al., 2012; Santoro et al., 2012). Moreover, *Fmr1* knockout (KO) mouse models recapitulate many key features of the human disease, including learning deficits, abnormal socialization and anxiety behaviors, enhanced seizure susceptibility and dendritic spine morphologic abnormalities (Bhakar et al., 2012; Santoro et al., 2012). FMRP is an RNA-binding protein that binds ~4% of brain mRNAs, including an enriched fraction of synaptic transcripts from genes associated with autism (Brown et al., 2001; Darnell et al., 2011; Ascano et al., 2012). FMRP regulates activity-dependent protein translation at synapses (Bhakar et al., 2012), where it suppresses translation of bound transcripts, either through direct interactions or via association with translating ribosomes (Feng et al., 1997; Darnell et al., 2011; Chen et al., 2014). Upon activation of Group 1 metabotropic glutamate receptors (mGluRs), FMRP is dephosphorylated and rapidly degraded, allowing for local translation of FMRP-associated mRNAs (Ceman et al., 2003; Hou et al., 2006; Nalavadi et al., 2012).

Dysregulation of mGluR signaling is thought to play a central role in disease pathogenesis, and both genetic and pharmacologic targeting of these receptors suppresses phenotypes in mice (Bear et al., 2004; Dölen et al., 2007; Michalon et al., 2012). However, studies of mGluR inhibitors in humans were unsuccessful (Berry-Kravis et al., 2016; Berry-Kravis E. M. et al., 2017). Other preclinical studies in *Fmr1* KO mice and *Drosophila* models demonstrated dysfunction in GABAergic signaling (Chang et al., 2008; Braat et al., 2015). This too led to a series of clinical trials that failed to meet their primary endpoint (Berry-Kravis E. et al., 2017; Berry-Kravis E. M. et al., 2017; Ligsay et al., 2017). More recently, FMRP was found to have additional functions in targeting of ion channel proteins in neurons through direct protein-protein interactions, and these functions underlie some of the phenotypic and electrophysiological abnormalities in *Fmr1* KO mice (Brown et al., 2010; Lee et al., 2011; Deng et al., 2013). FMRP also functions as part of the RNA Induced Silencing Complex (RISC) complex in microRNA translational silencing and has poorly understood nuclear functions which may be relevant to disease phenotypes (Cheever and Ceman, 2009; Kim et al., 2009; Alpatov et al., 2014; Korb et al., 2017). Thus, one potential explanation for the lack of success in human clinical trials to date is that

the pleiotropic functions played by FMRP in neurons and other cell types may be difficult to correct with any treatment targeting only one dysregulated pathway (Berry-Kravis E. M. et al., 2017).

An alternative approach to therapeutic development in FXS involves directly targeting the proximal event in disease pathogenesis—the transcriptional silencing of the *FMR1* gene (Tabolacci et al., 2016). While most FXS patients exhibit CGG repeat methylation, in a fraction of cases this methylation is incomplete or absent, allowing for continued *FMR1* transcription (Nolin et al., 1994; Jacquemont et al., 2011). However, large transcribed repeats still exhibit marked translational inefficiency, presumably due to the repeat element precluding ribosomal scanning through the start codon utilized to generate FMRP (Feng et al., 1995). Despite this, in cases where some *FMR1* transcription occurs, expression correlates with both symptom severity and response to therapeutics (Tassone et al., 1999; Jacquemont et al., 2011). These findings suggest that even small changes in *FMR1* mRNA expression might lead to phenotypic improvements in patients.

Previous work utilizing pharmacological approaches to reactivation of the *FMR1* locus met with some success. Application of non-specific demethylating agents such as 5-azadeoxycytidine (5-azadC) to Fragile X patient derived cells is sufficient to at least transiently enhance *FMR1* transcription and in some cases recover FMRP expression (Chiurazzi et al., 1998). Similarly, treatment with agents that alter the epigenetic landscape, such as the SIRT1 histone deacetylase inhibitor splitomycin, can also re-activate *FMR1* transcription in patient-derived lymphoblastoid cell lines, suggesting that other epigenetic manipulations may also be effective (Biacsi et al., 2008). Approaches coupling these two techniques hold promise at extending the potential effects in patient cells (Kumari and Usdin, 2016). However, many of these agents are toxic in humans and have the potential for significant off-target activity elsewhere in the genome, potentially confounding their use clinically in FXS patients.

An important step in developing methods for reactivation of *FMR1* transcription is identifying a model that demonstrates the developmental epigenetic silencing that occurs in FXS patients. Human embryonic stem cells (hESCs) are important disease models for studying developmental processes for which no other suitable models exist. Previous studies in FXS hESC show that some full mutation hESC remain unmethylated following derivation and exhibit gradual loss of *FMR1* mRNA during directed neuronal differentiation (Eiges et al., 2007; Telias et al., 2013; Colak et al., 2014), similar to the silencing observed in human FXS fetuses (Malter et al., 1997). In other lines, however, gene silencing occurs absent differentiation and appears to be repeat-length dependent, with expansions beyond 400 repeats demonstrating greater silencing (Avitzour et al., 2014; Brykczynska et al., 2016; Zhou et al., 2016). However, many hESC lines derived and characterized to date are not currently available in the United States for federally funded research.

More recently, researchers have taken a more targeted approach to *FMR1* gene reactivation using the Clustered

Regularly Interspaced Palindromic Repeats-CRISPR associated protein 9 (CRISPR-Cas9) system (Doudna and Charpentier, 2014). This technique utilizes either one or a set of single guide RNAs (sgRNAs) to target the CRISPR-Cas9 complex to specific genomic loci. The Cas9 endonuclease then nicks the DNA, allowing for either introduction of a deletion or for homology-directed repair. Two separate groups have now utilized CRISPR-Cas9 to delete expanded CGG repeats in Fragile X patient derived cells (Park et al., 2016; Xie et al., 2016). In both cases, removal of the repeat led to reactivation of the *FMR1* gene and production of FMRP.

In addition to endonuclease-mediated gene editing, the CRISPR-Cas9 system can also be modified to allow for targeted gene expression modulation in multiple systems (Hsu et al., 2014). Of particular interest is the use of CRISPR-Cas9 to activate gene expression by using an endonuclease-deficient Cas9 (dCas9) fused to a transcriptional activator (Perez-Pinera et al., 2013). Here, we show evidence for the targeted activation of the *FMR1* gene using a dCas9 fused to multiple domains of the VP16 transcriptional activator. Our initial studies in cell lines show differential activity for the various dCas9-VP16 fusion constructs with the most robust activity seen with the dCas9-VP192 construct. This system was used in a newly characterized hESC line derived from a Preimplantation Genetic Diagnosis (PGD) FXS embryo. The FXS hESCs show a passage-dependent silencing of the *FMR1* transcript. The dCas9-VP192 construct coupled with guide RNAs targeting the CGG repeat elicited significant activation of *FMR1* transcription in both the early and late passage FXS hESCs and in patient derived Neural Progenitor Cells (NPCs). Overall, these data provide proof-of-principle evidence that CRISPR-dCas9 based transcriptional activation approaches can reactivate *FMR1* transcription even in the setting of large methylated repeats. Targeting the repeat itself may enhance such efforts by providing multiple sequential binding sites for sgRNAs, effectively leveraging the disease mutation to greater efficacy.

MATERIALS AND METHODS

CRISPR Guide RNA Design and Plasmids

Promoter-targeted gRNA sequences were identified within 500 nucleotides upstream of the main transcriptional start site based on the prediction of on-target to off-target effect in the human genome and arrangement within the region using the CRISPR design web portal (Hsu et al., 2013). These sequences and the CGG repeat sequence were cloned into the pSPgRNA plasmid by replacing the sequence between the BbsI sites using the Q5 site-directed mutagenesis kit (NEB) and the primers listed in **Supplementary Table S1**. All dCas9 expression plasmids were obtained from Addgene. pcDNA-dCas9-VP64, pSPgRNA and pLV hUbc-dCas9 VP64-T2A-GFP were gifts from Charles Gersbach (Addgene plasmid # 47107, 47108, 53192, respectively; Perez-Pinera et al., 2013). SP-dCas9-VPR was a gift from George Church (Addgene plasmid # 63798; Chavez et al., 2015). pAC93-pmax-dCas9VP160 was a gift from Rudolf Jaenisch (Addgene plasmid # 48225; Cheng

et al., 2013). pCXLE-dCas9VP192-T2A-EGFP was a gift from Timo Otonkoski (Addgene plasmid # 69536; Balboa et al., 2015). pcDNA3.1(+) and pEGFP-N1 served as control plasmids.

Cell Culture and Transfection of HEK293T Cells

HEK293T cells (ATCC) were maintained at 37°C, 5% CO₂ in Dulbecco's modified Eagle's Medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin following standard procedures. Transfections were performed using Lipofectamine 3000 transfection reagent (Invitrogen) according to manufacturer's instructions. Transfection efficiencies were typically 80%, as determined by fluorescence microscopy after delivery of a control eGFP expression plasmid, and only samples with transfection efficiencies in this range were utilized for further experiments. dCas9 expression plasmids were transfected at a mass ratio of 3:1 to either the CGG gRNA expression plasmid or the identical amount of gRNA expression plasmid consisting of a mixture of equal amounts of the four promoter-targeted gRNAs. Cells were harvested 48 h after transfection.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated using the Quick RNA Miniprep kit (Zymo research) with on-column DNase I treatment followed by cDNA synthesis using the iScript Reverse Transcriptase kit (Biorad) according to the manufacturer's protocol. Quantitative RT-PCR was performed on the Bio-Rad iCycler real-time detection system using the iQ SYBR Green Supermix (Biorad) and the primers (IDT) listed in **Supplementary Table S1**. Primer specificity was confirmed by gel electrophoresis and melting curve analysis. Relative fold expression for genes of interest was calculated using the comparative CT method (Schmittgen and Livak) with HPRT as the internal control. Technical triplicates were averaged and recorded for each sample. To identify potential off-target genes, a blast search of the human transcriptome was performed with a sequence of 10 CGG repeats (30 nucleotides). The hits were sorted based on their total score. Primers for qPCR were designed for all genes with a score greater than or equal to *FMR1*. All of these genes contained repeats in the 5'UTR similar to *FMR1*.

Western Blot

Cells were washed with cold PBS and lysed on ice in RIPA buffer (50 mM Tris-Cl pH-8.0, 150 mM NaCl, 0.1% SDS, 0.1% Sodium deoxycholate, 1% NP-40) with complete protease inhibitor cocktail (Roche). Samples were centrifuged at 14,000 RPM for 5 min at 4°C and supernatant was transferred to a clean tube. For western blot, protein lysates were boiled in Laemmli buffer and separated on SDS-PAGE gels. Gels were transferred to PVDF membranes, blocked with 5% nonfat dry milk and probed with mouse anti-FMRP (6B8; Biolegend 834601), rabbit anti-Cas9 (Clontech 632607) and rat anti-tubulin (Abcam ab-6160) primary antibodies. Secondary antibodies were goat anti-mouse HRP (Jackson Immunoresearch), IRDye

800 goat anti-rabbit IgG (LI-COR) and IRDye 800 goat anti-rat IgG (LI-COR), respectively. Antibodies were detected using an Odyssey imager or using Western Lightning Plus-ECL substrate (Perkin-Elmer) and developed on film. Quantification of western blot signal were performed as previously described (Renoux et al., 2014). ImageJ was used for quantification. Band intensity was confirmed to be in the linear range by densitometry measurements on control samples with 0.5× or 2× the amount of protein on left edge of blot. Experiments were performed in technical triplicate, and FMRP/tubulin ratio was determined for each sample. These ratios were averaged, normalized to the mean control value for each experiment, and expressed as % control. As multiple groups were compared simultaneously, a Kruskal-Wallis one-way ANOVA was used with Dunn's multiple comparison test applied to account for repeated measures.

Immunocytochemistry

Cells were cultured in chamber slides or on coverslips. The media was removed and cells were washed with 1× PBS and fixed in 4% PFA/4% Sucrose solution for 15 min at room temperature (RT). The cells were permeabilized with 0.1% Triton X-100 for 5 min at RT and were blocked in a 5% Normal Goat Serum solution for 1 h at RT. The cells were stained with primary antibodies overnight at 4°C followed by three washes in 1× PBS for 5 min each. The antibodies used were: mouse anti-FMRP (6B8) at 1:250 dilution (Biolegend 834601), rabbit anti-Cas9 (Clontech 632607) at 1:150 dilution, anti-MAP2 (Millipore Ab5622) at 1:1000 dilution, SOX2 and PAX6. The cells were then stained with species specific secondary antibodies conjugated to Alexa 488, 568, or 635 fluorophores and mounted using Prolong Gold with DAPI. Images were captured on an inverted Olympus FV1000 laser-scanning confocal microscope.

ES Cell Line Derivation and Characterization

Human embryos were donated, under two conditions, to MStem Cell Laboratory's Institutional Review Board (IRB) approved study (HUM00028742) entitled "*Derivation of human Embryonic Stem Cells*." Written informed consent was obtained for all embryo donations. First, embryos made for reproductive purposes, not genetically tested, frozen and no longer required for reproduction were donated (e.g., UM4-6). Second, partners with a known history of familial Fragile-X elected to perform *in vitro* fertilization and PGD, irrespective of embryo donation, to reduce the risk of having a child with Fragile X-spectrum disorder. The female partner was an *FMR1* pre-mutation carrier with a mutant allele determined to have 108 and 115 CGG repeats on two separate evaluations. The female partner had three paternal uncles with Fragile X-spectrum disorder. *In vitro* produced embryos were biopsied as blastocysts on day 5 of development, and trophectoderm cells were genetically assessed by an off-site genetic analysis company. Blastocysts were vitrified and cryo-stored until PGD results were obtained. Embryos with PGD results showing the mutant maternal haplotype and

no paternal X chromosome (affected male) where consented for donation and shipped to MStem Cell Laboratory (e.g., UM139-2).

Following hESC production and characterization, documents demonstrating adherence to NIH-established guidelines for embryo donation and hESC production of UM4-6 and UM139-2 were submitted to NIH for placement on the NIH hESC Registry and approvals were granted on 02/02/2012 (Registration # -0147) and 09/29/2014 (Registration # -0292), respectively. Derivation of hESCs and their derivatives prior to acceptance on the NIH registry were performed with non-federal funds. Additionally, studies after placement on the NIH registry were also supported by non-federal funds. Briefly, blastocyst morphology was assessed 4 h after embryos were warmed and dictated the mode of hESC derivation. Laser-dissected inner cell masses (ICMs) were plated on human foreskin fibroblast (HFF)—feeders to obtain early hESC colonies that were manually split after 5–7 days and expanded on HFF for establishment and characterization of hESC lines. hESC lines were tested for pluripotency marker expression (Oct4, Nanog, Sox2) by Q-PCR and protein expression by immunofluorescence (Oct4, Nanog, Sox2, SSEA4 and TRA-1-60). hESCs were differentiated for 21 days in culture as embryoid bodies and tested for expression of lineage markers by Q-PCR of endoderm (α -fetoprotein (AFP) and GATA4), mesoderm (brachyury and VE-Cadherin (VE-Cad)) and ectoderm (TUI-1 and keratin-18 (Krt-18)). Finally, G-band karyotyping of UM4-6 and UM139-2 demonstrated euploid hESC lines.

Culture and Transfection of hESCs

Undifferentiated hESCs were cultured in mTeSR1 media (Stem Cell Technology) on Matrigel-coated plates with daily media changes and were passaged at 1:5–1:10 using L7 passaging media (Lonza) or 1 mM EDTA. For transfections, undifferentiated hESCs or NPCs derived by directed differentiation of hESCs were plated on Matrigel-coated plates in mTeSR1 media containing 10 μ M Rock Inhibitor and grown overnight. Media was replaced with mTeSR1 the next day. Cells were allowed to recover for at least 4 h and media was replaced again just prior to transfections. Transfections were performed using plasmids as described above and TransIT LT1 transfection reagent (Mirus Bio) according to manufacturer's instructions. For NPC studies, transfections were done after a 14-day differentiation. Typical transfection efficiencies in hESCs and in NPCs were 40%–50% as measured by GFP fluorescence, and only samples with sufficient transfection rates were used in subsequent studies. Cells were cultured with daily media changes and harvested 48 h after transfection for RNA isolation and 72 h after transfection for western blots.

Southern Blot

Southern blotting was performed as in Gold et al. (2000) with modifications. Briefly, genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue kit. 10 μ g of genomic DNA from each cell line was digested with HindIII and EagI overnight. The digoxigenine (DIG)-labeled probe was amplified from the pE5.1 plasmid, using forward primer:

CGCCAAGAGGGCTTCAGGTCTCCT and reverse primer: GAGACTGTTAAGAACCTAAACGCGGG. The digested genomic DNA was resolved on a 0.7% agarose gel prior to Southern blotting. The nylon membrane was processed using the commercially available DIG Easy Hyb solution and DIG Wash and Block Buffer Set (Roche). DIG was antibody labeled with Anti-DIG-AP, Fab fragments (Sigma), processed using CDP-Star substrate (ThermoFisher) and detected on film. A wild-type band (~20 repeats) in Control lines appears at ~2.8 kb, whereas the expanded and methylated repeat in the Fragile X line appears at ~7.6 kb (800 + repeats) which is ~2.4 kb above where a non-expanded, methylated DNA fragment would appear (~5.2 kb).

Methylation qPCR

FMR1 methylation determination was made as previously described with minor modifications (de Esch et al., 2014). Briefly, genomic DNA was isolated from cell lines using the Qiagen DNeasy Blood and Tissue Kit. 2 µg of DNA from each was bisulfite converted using the EpiTect Bisulfite kit (Qiagen). qPCR was performed on 100 ng of bisulfite converted DNA using the iQ SYBR Green supermix (BioRad). The primers used were designed against the sense strand of *FMR1* and are previously described (de Esch et al., 2014). *FMR1* methylation-specific primers, forward: GGTCGAAAGATA GACGCGC and reverse: AAACAATGCGACCTATCACCG; *FMR1* unmethylated-specific primers, forward: TGTTGG TTTGTTGTTTGTGTTAGA and reverse: AACATAATTTCATATTTACACCC; and primers for the housekeeping gene *CLK2*, which is constitutively active in stem cells and should therefore not undergo CpG methylation in the region of amplification, forward: CGGTTGATTTTGGGTGAAGT and reverse: TCCCGACTAAAATCCCACAA. Methylation-specific and unmethylated-specific qPCR analyzed neighboring but non-overlapping regions with 11 and 15 methylation sites, respectively. The efficiency of the primers was between 95% and 100%. Amplification of both methylated and unmethylated *FMR1* was normalized to *CLK2*, then a ratio was created using the two values. In control fibroblasts where no amplification was detected with the methylation-specific primers, methylation was set at 0%.

Directed Differentiation of hESC to NPCs and Neurons

Neural induction was performed using a dual-SMAD inhibition (Shi et al., 2012) protocol with modifications. In brief, undifferentiated hESCs in two wells of a 6-well plate were grown to approximately 80% confluence, dissociated with EDTA, and plated into a single well of a Matrigel-coated 6-well plate with TeSR-E8 containing 10 µM Rock Inhibitor (Y-27632). The cells were confluent the next day and neural differentiation was induced using neural maintenance media (referred to here as 3N) containing 1 µM dorsomorphin and 10 µM SB431542. The cells were cultured for 12–14 days with daily media changes. Neuroepithelial sheets were then combed into large clumps, passaged and maintained on Matrigel-coated plates in rosette media (3N containing 20 ng/ml FGF2) with

daily media changes until neural rosettes appeared. Rosettes were manually picked and dissociated into single cells using Accutase. NPCs were plated onto Matrigel-coated plates, grown in neural expansion media (3N containing 20 ng/ml FGF and 20 ng/ml EGF) with media changes every other day, and passaged as needed using Accutase. For differentiation into neurons, NPCs were plated at a density of approximately 1.5×10^5 cells/mL in neural expansion media on PLO-laminin coated plates or coverslips, allowed to grow for 24 h, and switched to neural maintenance media. Neurons were maintained for up to 6 weeks with half media changes every other day and a full media change supplemented with 1 µg/ml laminin every 10 days.

RNA Sequencing and GO Analysis

Sequencing was performed by the UM DNA Sequencing Core, using the Illumina Hi-Seq 4000 platform, single end, 50 cycles, mRNA prep. At the UM Bioinformatics Core, files from the Sequencing Core's storage were concatenated into a single fastq file for each sample. The quality of the raw reads data for each sample was checked using FastQC¹ (version 0.11.3) to identify features of the data that may indicate quality problems (e.g., low-quality scores, over-represented sequences, inappropriate GC content). The Tuxedo Suite software package was used for alignment, differential expression analysis and post-analysis diagnostics (Langmead et al., 2009; Trapnell et al., 2009, 2013). Briefly, the reads were aligned to the reference mRNA transcriptome (hg19²) using TopHat (version 2.0.13) and Bowtie2 (version 2.2.1). Default parameter settings for alignment were used, with the exception of: “—b2-very-sensitive” telling the software to spend extra time searching for valid alignments. FastQC was used for a second round of quality control (post-alignment), to ensure that only high-quality data would be input to expression quantitation and differential expression analysis. Cufflinks/CuffDiff (version 2.2.1) was used for expression quantitation, normalization and differential expression analysis, using hg19.fa as the reference genome sequence. For this analysis, the parameter settings were: “—multi-read-correct” to adjust expression calculations for reads that map in more than one locus, as well as “—compatible-hits-norm” and “—upper-quartile-norm” for normalization of expression values. Diagnostic plots were generated using the CummeRbund R package. Locally developed scripts were used to format and annotate the differential expression data output from CuffDiff. Briefly, genes and transcripts were identified as being differentially (DE) expressed based on three criteria: test status = “OK”, FDR ≤ 0.05, and fold change ≥ ±1.5. Genes and isoforms were annotated with NCBI Entrez GeneIDs and text descriptions. iPathwayGuide (Advaita Corporation³) was used to model the biological relevance of DE genes for each algorithm, as well as a meta-analysis comparing the two approaches.

¹<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

²<http://genome.ucsc.edu/>

³<http://www.advaitabio.com>

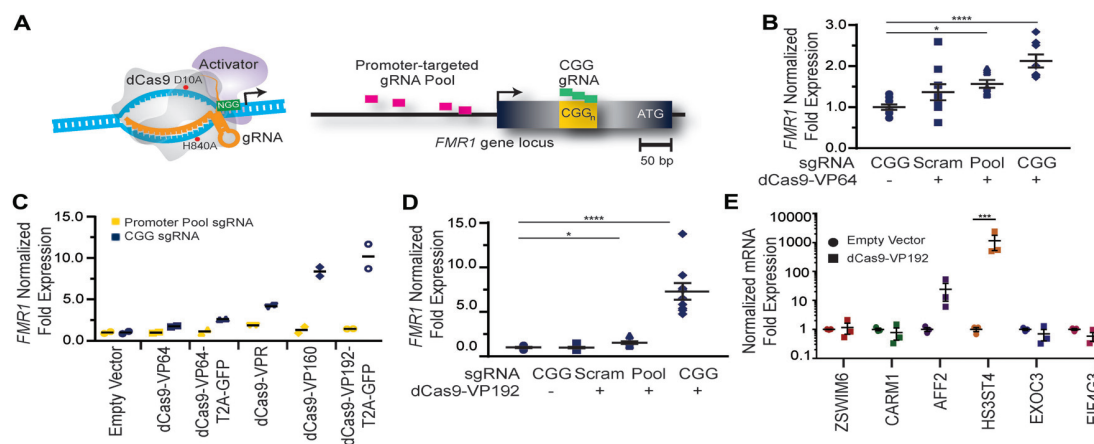


FIGURE 1 | *FMR1* messenger RNA (mRNA) increases with Clustered Regularly Interspaced Palindromic Repeat (CRISPR)-mediated targeting of transcriptional activators to either the *FMR1* promoter or the CGG repeat. **(A)** Illustration of nuclease-inactive Cas9 (deficient Cas9, dCas9) fused to a transcriptional activator (left) and guide RNA (gRNAs) targeting regions within the *FMR1* promoter or the CGG repeat (right). The promoter-targeted gRNA pool (pink) consisted of four gRNAs with unique targeting sequences, while the CGG gRNA (green) represents a single targeting sequence capable of tiling across the CGG repeat. **(B)** Relative *FMR1* mRNA expression from three independent experiments in HEK293T cells transfected with empty vector or dCas9 fused to VP64 (dCas9-VP64) and non-targeting guide RNA (Scram), a pool of four guide RNAs within the *FMR1* promoter (Pool), or a single CGG repeat guide (CGG). **(C)** Relative *FMR1* mRNA expression from a single experiment in HEK293T cells transfected with an empty vector or dCas9 “second generation” activators and the *FMR1* promoter gRNAs or the CGG gRNA. **(D)** Relative *FMR1* mRNA expression from three independent experiments in HEK293T cells transfected with a control plasmid or dCas9 fused to VP192 (dCas9-VP192) and the indicated gRNA (for panels B–D, * $p < 0.05$, **** $p < 0.0001$, Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparison test). **(E)** Relative expression of select CGG repeat-containing genes after transfection of HEK293T cells with CGG gRNA and dCas9-VP192 constructs (*** $p < 0.001$, two-way ANOVA with Sidak’s multiple comparisons test). For all scatter plots shown, each data point represents an individual well and error bars on all graphs represent SEM.

RESULTS

Transcriptional Activation of the *FMR1* Gene by CRISPR-dCas9 Fused to VP16 Activation Domains

To determine whether use of CRISPR targeted transcriptional activators could augment *FMR1* expression, we first tested them in HEK 293T cells that have a normal sized (23) CGG repeat in the 5′UTR of the *FMR1* gene. We designed multiple guide RNAs (gRNAs) to the promoter region or to the CGG repeat of the *FMR1* gene. These gRNAs were used along with the catalytically-inactive dCas9 fused to different versions of the VP16 transcriptional activation domain (Figure 1A). At 48 h post transfection, we observed a significant increase in *FMR1* transcript levels using the dCas9-VP64 construct with both the promoter pool and CGG gRNAs (Figure 1B) compared to a scrambled control gRNA or to cells transfected with only GFP. We next compared activation efficiencies for both sets of gRNAs with different versions of dCas9 fused with either the chimeric activation domain VPR (composed of the activation domains of VP64, p65 and Rta linked in tandem), or multiple domains of VP16 (Figure 1C). The strongest transcriptional activation in heterologous cells was achieved with a CGG repeat-targeted dCas9-VP192, which yielded approximately an 8-fold increase in *FMR1* mRNA levels (Figures 1C,D). The CGG repeat targeted guide robustly increased *FMR1* transcript levels compared to the promoter-pool gRNAs, suggesting that its repetitive binding sites augment the targeting strategy (Figure 1D).

Because CGG tandem microsatellites are not unique within the genome, we also queried six candidate genes with CGG repeats in their 5′UTR for off target effects in HEK293T cells. We observed an increase in transcript levels for the *AFF2* gene (also called *FXR2*) and *HS3ST4* gene suggesting potential off-target effects in this cell type with a repeat- targeting strategy (Figure 1E). However, the effects on *AFF2* are potentially interesting clinically. Expansion of this CCG repeat triggers hypermethylation and transcriptional silencing of the *AFF2* gene, in a fashion that is quite similar to FXS (also known as FRAXA). This results in FRAXE, a rare genetic form of autism and intellectual disability (Knight et al., 1993; Gecz et al., 1996). Together, these data demonstrate that the dCas9-VP192 system can effectively activate transcription of the *FMR1* gene. Additionally, CGG gRNA provide more robust activation compared to promoter pool gRNAs but with a greater potential for off-target effects.

dCas9-VP192 Increases FMRP Levels in HEK293T Cells

We next determined if the observed transcriptional changes correlated with enhanced production of FMRP. FMRP levels were measured in HEK293T cells transfected with either the promoter pool or CGG-repeat targeted gRNAs and the dCas9-VP192 construct. By immunocytochemistry, cells transfected with CRISPR constructs show an increase in FMRP signal with either CGG or promoter targeted gRNAs, but not with scramble guide RNA (Figure 2A). Western blot analysis of transfected cells demonstrated a significant increase in FMRP protein in

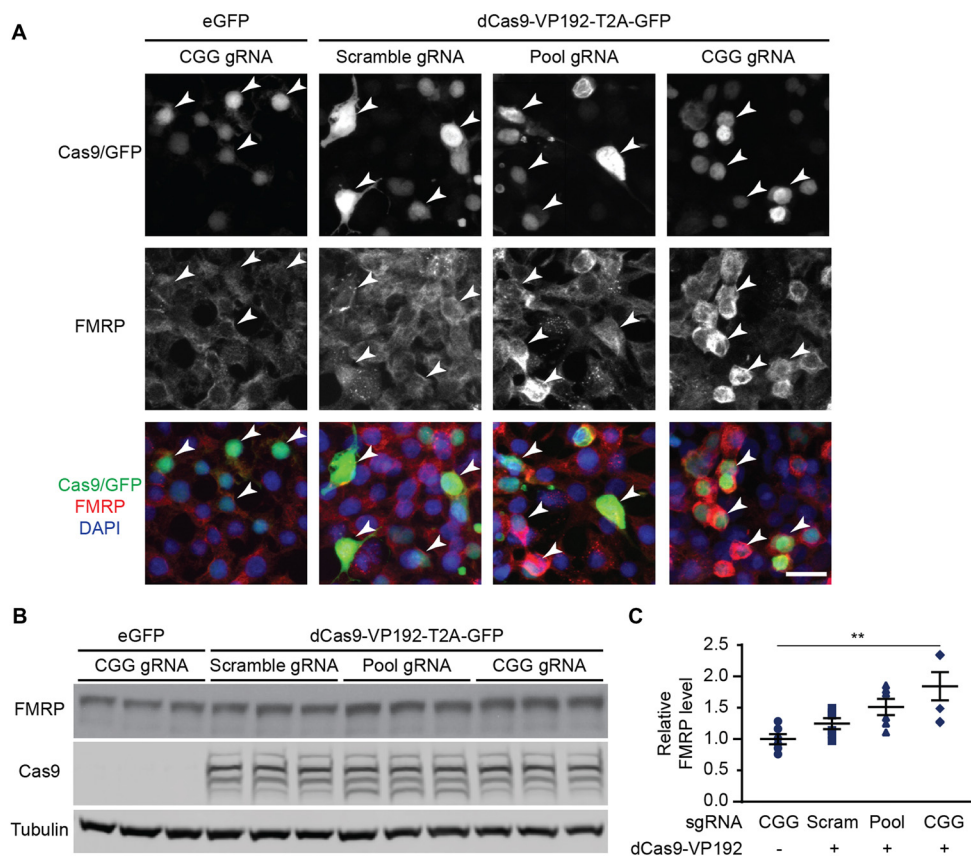


FIGURE 2 | CRISPR-mediated transcriptional activation increases FMRP protein abundance at normal CGG repeat sizes. **(A)** Immunocytochemistry of HEK293T cells transfected with eGFP or dCas9-VP192 and gRNAs, as indicated. Single channel and merged images are shown with Cas9/GFP (green), FMRP (red), and DAPI (blue). White arrowheads indicate transfected cells. Scale bar represents 20 μ m. **(B)** Western blots showing triplicate samples of HEK293T cells transfected with control plasmid (eGFP) or with dCas9-VP192 and indicated gRNAs and immunoblotted for FMRP, Cas9 and Tubulin. **(C)** Quantification of western blots from HEK293T cells transfected as indicated. Data are shown as FMRP normalized to tubulin and relative to the control plasmid ($n = 6$ /group evaluated over at least two independent experiments. $**p < 0.01$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). Error bars represent SEM.

CGG repeat targeted gRNAs compared to control transfections (**Figures 2B,C**). Thus, targeted activation of the *FMR1* gene using a dCas9-VP192 system increases both *FMR1* mRNA and FMRP levels in human cells at normal repeat sizes.

FXS hESCs Exhibit Passage-Dependent Silencing of *FMR1* Prior to Neuronal Differentiation

An important first step in developing a method for reactivation of *FMR1* transcription is identifying a model that recapitulates the developmental epigenetic silencing that occurs in FXS patients. Until recently, the UM139-2 PGD hESC line was the only Fragile X hESC line on the NIH registry of approved lines for federally-funded research in the United States⁴. However, different Fragile X hESC lines exhibit variability in terms of their methylation and *FMR1* transcription (Avitzour et al., 2014). We therefore characterized this new FXS hESC line.

⁴<https://stemcells.nih.gov/research/registry.htm>

The embryo from which this hESC line (UM 139-2) was derived was determined to be affected with FXS through PGD. This blastocyst was cryopreserved after testing and sent to MStem Cell laboratories, where derivation of hESCs took place (**Figures 3A–C**). Pluripotency and fidelity of this line was confirmed by RT-PCR and immunohistochemistry for pluripotency markers (**Figures 3D,E**). The line was capable of embryoid body formation containing all three germ layers, consistent with pluripotency (**Figure 3F**). DNA fingerprinting and karyotyping demonstrated a 46XY euploid genetic background (**Figure 3G**).

We next characterized the line in terms of its Fragile X mutation. Southern blot analysis indicated that this hESC line contains a Fragile X full mutation with approximately 800 CGG repeats (**Figure 4A**). The first characterized FXS hESC line, HE-FX, exhibited no methylation in the hESC state, but instead demonstrated methylation and transcriptional silencing with cellular differentiation (Eiges et al., 2007). However, more recent studies suggest that this property is not universal, with some Fragile X hESCs exhibiting early

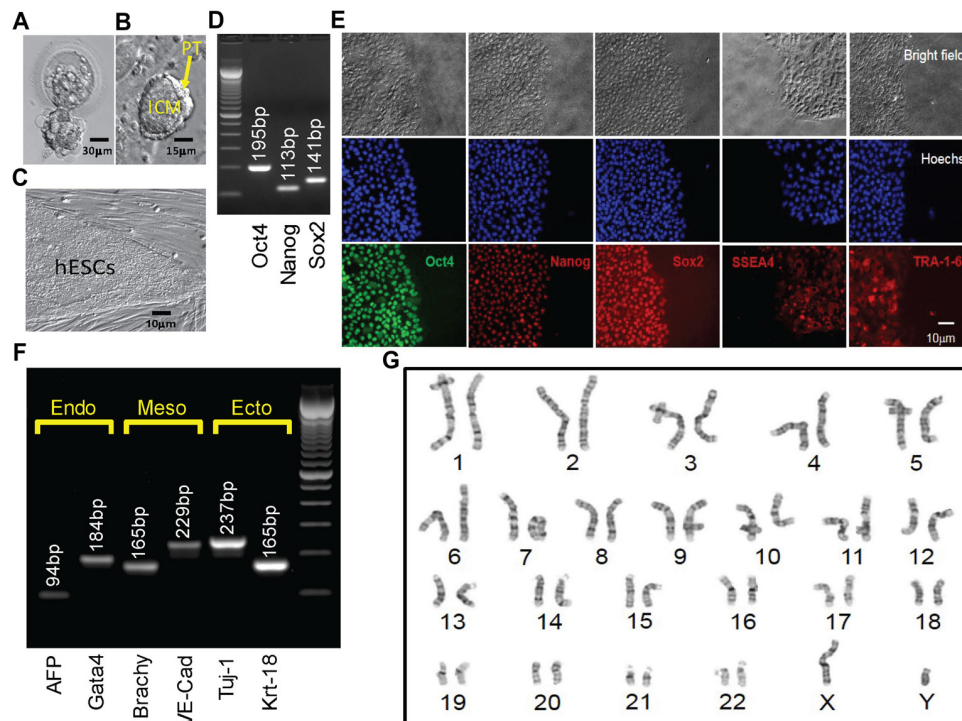


FIGURE 3 | Derivation, expansion and characterization of Fragile X-disease specific human embryonic stem cell (hESC; vFrag-X-ds-hESC; UM139-2 PGD) line. **(A)** Human blastocyst with *FMR1* expansion that was cryopreserved, donated, shipped and warmed prior to attempting hESC derivation. Scale bar represents 30 μ m. **(B)** The inner cell mass (ICM) with surrounding polar trophectoderm (PT) was laser-dissected from the blastocyst and plated/attached on human foreskin fibroblast (HFF). This micrograph represents the early Frag-X-ds-hESC colony before the first passage, 5 days after laser-dissection and plating of the ICM/PT (POD5). Scale bar represents 15 μ m. **(C)** Expanding undifferentiated Frag-X-ds-hESCs with tight colony borders on a HFF feeder layer (P3D3). Scale bar represents 10 μ m. **(D)** Undifferentiated Frag-X-ds-hESCs expressed pluripotency markers (Oct4, Nanog, Sox2) as assessed by qPCR. Electrophoresis demonstrated anticipated amplicons for each pluripotency marker PCR primer sets. **(E)** Expanded undifferentiated Frag-X-ds-hESCs with tight colony borders on Martigel (brightfield micrographs) expressed pluripotency marker proteins in the nucleus (same location as Hoechst staining; Oct4, Nanog, Sox2) or cytoplasmic/cell membrane associated (SSEA4 and TRA-1-60). **(F)** Frag-X-ds-hESCs were differentiated into embryoid bodies for 21 days in culture. Differentiated Frag-X-ds-embryoid bodies expressed lineage marker RNA of endoderm [α -fetoprotein (AFP) and GATA4], mesoderm [brachyury (Brachy) and VE-Cadherin (VE-Cad)] and ectoderm [neuron-specific class III β -tubulin (Tuj-1) and keratin-18 (Krt-18)] with anticipated amplicon size by electrophoresis for each lineage marker PCR primer set. Scale bar represents 10 μ m. **(G)** Passage 6 undifferentiated Frag-X-ds-hESCs were sent to Cell Line Genetics (Madison, WI, USA) for G-B and karyotyping and reported to be a 46XY, euploid hESC line.

methylation and silencing. To evaluate whether the repeat was methylated in UM 139-2 hESC line, we performed methylation-specific quantitative-PCR on early passage (<20 passages) and late passage (>30 passages) FXS hESCs. This demonstrated a passage-dependent methylation of the *FMR1* promoter region with earlier passages displaying incomplete methylation and later passages displaying complete methylation as compared to control hESCs and FXS fibroblasts, respectively (Figure 4B).

To determine the impact of this methylation on *FMR1* transcriptional activity, we measured *FMR1* mRNA expression by qRT-PCR. This demonstrated a passage-dependent shift in expression in FXS hESCs. At early passages, *FMR1* mRNA levels were only modestly decreased (30%) compared to controls. However, after continued passages (typically >30 passages, with some variability), *FMR1* mRNA levels became nearly undetectable (0.4% of control levels, Figure 4C). We observed a similar passage-dependent change in FMRP protein level,

although there was a significant deficit in FMRP expression even at early passage numbers, perhaps due to translational blockade (Figure 4D; Feng et al., 1995; Chen et al., 2003; Iliff et al., 2013).

To confirm that the absence of FMRP does not preclude differentiation into neurons from FXS hESCs, we performed directed neuronal differentiation using a dual SMAD inhibitor-based differentiation protocol (Shi et al., 2012). This method successfully produced hESC-derived PAX-6 and MAP2 positive neural rosettes and FXS neurons (Figures 4E–G). As reported previously for other FXS lines, we also observed a slight delay in neural rosette formation as well as a lower density of neurons from the UM 139-2 FXS line (Telias et al., 2013). Combined, these data suggest that UM 139-2 FXS hESCs are a good model for investigating methods of reactivating *FMR1* transcription, and the feature of time-dependent transcriptional silencing allows for targeting of reactivation at expanded repeats in different epigenetic contexts.

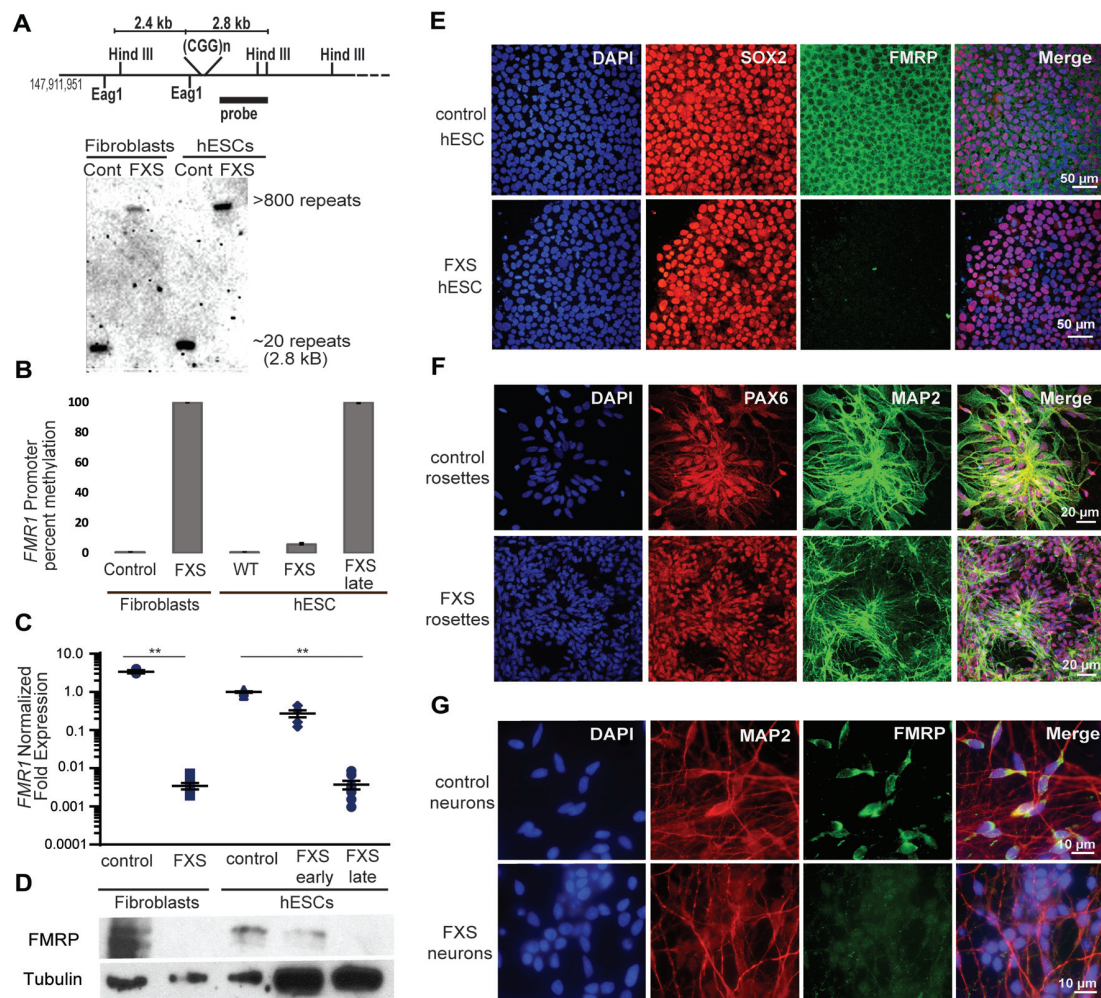


FIGURE 4 | Fragile X hESC line (UM139-2 PGD) carries a large CGG repeat and undergoes passage dependent transcriptional silencing. **(A)** Top panel: schematic representing the *EagI* and *HindIII* restriction sites on the *FMR1* genomic locus, the digoxigenine (DIG)-labeled probe used for Southern blotting in this study and the expected fragment sizes. The figure is not to scale. Bottom panel- Southern blot shows CGG repeat length and methylation status for genomic DNA from control and Fragile X Syndrome (FXS) patient-derived fibroblasts and hESCs. Repeat size is estimated as <800. **(B)** Bisulfite-qPCR using methylation-specific primers reveals a passage dependent methylation at the *FMR1* promoter for genomic DNA from indicated cells. Data shown for two independent experiments. **(C)** Relative *FMR1* transcript levels in control and FXS patient-derived fibroblasts and hESCs. FXS hESCs were assessed at early passages (P13–20) and late passages (P30+). ****** $p < 0.01$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test, data from five independent experiments, error bar represents SEM. **(D)** Western blots showing FMRP and tubulin in control and FXS patient-derived fibroblasts as well as control hESC and early and late passage FXS hESCs. One-tenth of the lysate was loaded for control fibroblasts and control hESCs. **(E)** Undifferentiated control and FXS hESC colonies immunostained for FMRP (green), pluripotency marker SOX2 (red) and DAPI (blue). Scale bars represent 50 μ m. **(F)** Neural rosettes derived from control and FXS hESCs with neuronal lineage marker MAP2 (green), neuroectoderm maker PAX6 (red), and DAPI (blue) immunostaining. Scale bars represent 20 μ m. **(G)** Neurons derived from control and FXS hESCs shown with FMRP (green), MAP2 (red) and DAPI (blue) immunostaining. Scale bars represent 10 μ m.

dCas9-VP192 Activates *FMR1* Transcription in FXS hESCs and NPCs

Based on our success using dCas9-VP192 to activate transcription of the *FMR1* gene in HEK293T cells, we first tested the same constructs and gRNAs in control hESCs. Control hESCs showed a significant increase in *FMR1* transcript levels using the promoter targeted gRNAs with dCas9-VP192 only (**Figure 5A**), although the effects were more variable and less robust than those observed in HEK293T cells. We next evaluated whether this increase in mRNA was associated with

changes in protein expression. By immunohistochemistry, there was a clear relationship between cells expressing the dCas9 construct and an increase in FMRP expression for the promoter pool gRNAs but not the CGG repeat gRNAs (**Figure 5B**). By western blot as well, only the promoter pool targeted gRNAs demonstrated a significant change in protein expression (**Figures 5C,D**). This discrepancy may reflect differences in efficiency of translation and expression from these vectors between HEK293T cells and hESCs.

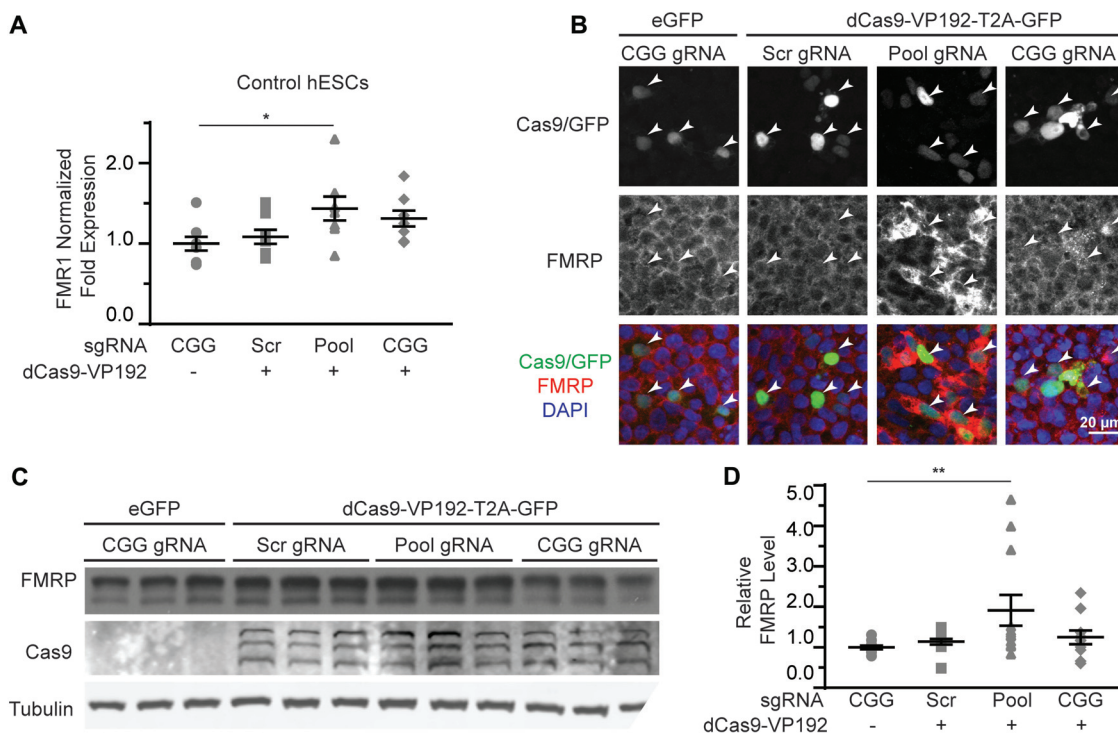


FIGURE 5 | CRISPR-mediated activation enhances *FMR1* transcription and FMRP protein abundance in control hESCs. **(A)** Relative *FMR1* mRNA expression in control (UM4-6) hESCs transfected with a control plasmid or dCas9-VP192 and the indicated gRNAs (* $p < 0.05$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). **(B)** Images of control hESCs transfected with eGFP or dCas9-VP192 and gRNAs, as indicated. Single channel and merged images are shown with Cas9/GFP (green), FMRP (red) and DAPI (blue). Arrowheads indicate transfected cells. Scale bar represent 20 μ m. **(C)** Western blots showing triplicate samples of control hESCs transfected and immunoblotted as indicated. **(D)** Quantification of western blots from control hESCs transfected as indicated. Data are shown as FMRP levels normalized to tubulin and relative to the control plasmid (**indicates $p = 0.0061$ by Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). For all scatter plots, each data point represents an individual well. Data were obtained from three **(A)** or four **(D)** independent experiments. The mean with error bars (SEM) is shown for each condition.

We next tested whether the dCas9-VP192 system could re-activate or enhance transcription from the *FMR1* locus in UM139-2 FXS hESCs. Because of their baseline differences in *FMR1* transcription, we evaluated both early and late passage hESCs. In early passage FXS hESCs, both the promoter and CGG gRNAs elicited a 1.3-fold and a 1.8-fold increase, respectively in *FMR1* transcript levels compared to the scrambled guide RNA in the same line (Figure 6A). However, this increase was significantly greater with the CGG guide RNAs compared to the promoter targeting gRNAs (Figure 6A).

In late passage FXS hESCs *FMR1* mRNA levels were very low basally (Figure 4C). Treatment with scrambled gRNA or promoter targeted gRNAs in the setting of dCas9-VP192 had no impact on *FMR1* RNA expression. However, CGG targeted gRNA coupled with dCas9-VP192 led to a marked increase in *FMR1* mRNA expression-upwards of 20-fold in some samples (Figure 6B). Thus, at both a partially and completely transcriptionally silenced CGG full mutation locus, we observed that targeting a transcriptional activator directly to the repeats elicited the greatest enhancement of *FMR1* mRNA expression. Next, we differentiated the control and late FXS hESCs to NPCs and tested for *FMR1* transcript levels after treating them with

the dCas9-VP192 and gRNAs. The NPCs differentiated from the late FXS hESCs were selected since they did not have any baseline *FMR1* transcription, which reflected the disease state more closely. Similar to our observations in undifferentiated hESCs, control NPCs showed a statistically significant increase in *FMR1* levels using the promoter pool targeted gRNAs (Figure 6C) while the FXS NPCs showed the same increase in transcript level with the CGG gRNA only (Figure 6D). Thus, the effects of specific gRNAs with dCas9-VP192 on *FMR1* mRNA expression are different in the setting of large CGG repeat expansions, but consistent across cell differentiation states.

CGG Repeat Targeted gRNA Shows Minimal Off Target Effects in FXS hESCs

We next evaluated whether there were off-target effects elicited by the CGG repeat targeted gRNAs. We first queried the six candidate genes identified in HEK293T cells (Figure 1E). Unlike the case in HEK293T cells, we saw no increase in their mRNA levels in FXS hESCs expressing CGG gRNA and dCas9-VP192 (Figure 7A). To evaluate genome-wide off-target effects elicited by expression of CGG gRNA and dCas9-VP192, we performed

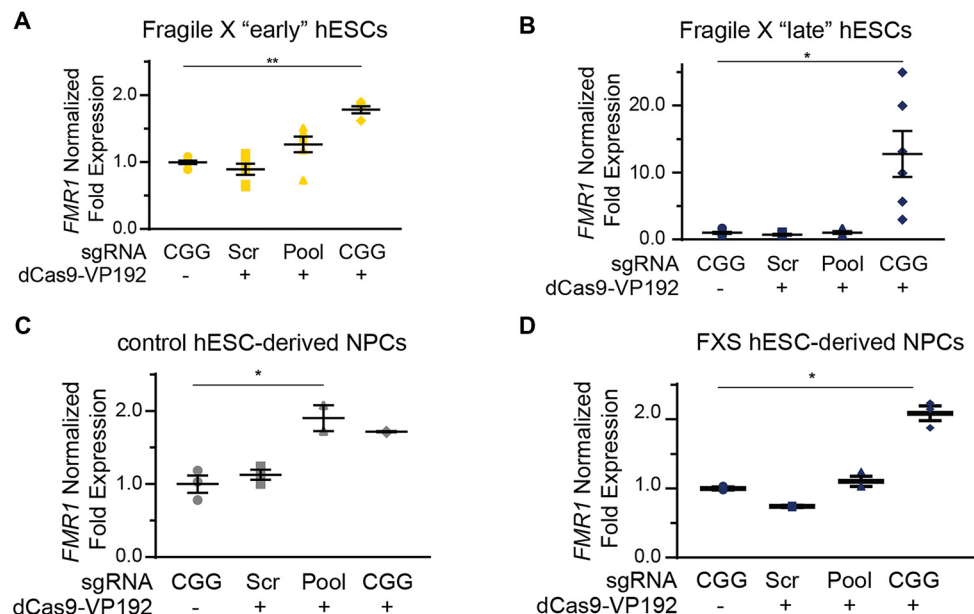


FIGURE 6 | Targeting CRISPR-dCas9-VP192 to the CGG repeat overcomes transcriptional silencing and selectively enhances transcription of *FMR1* in FXS hESCs and neural progenitor cells (NPCs). **(A)** Relative *FMR1* mRNA expression in early (P23–25) passage FXS hESCs transfected with control plasmid or dCas9-VP192 and the indicated gRNA (** $p < 0.01$, * $p < 0.05$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). **(B)** Relative *FMR1* mRNA expression in late (P53–57) passage FXS hESCs transfected with control plasmid or dCas9-VP192 and the indicated gRNA (** $p < 0.01$, * $p < 0.05$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). **(C)** Relative *FMR1* mRNA expression in control hESC-derived NPCs at 48 h after transfection with control plasmid or dCas9-VP192 after differentiation with the indicated gRNAs (* $p < 0.05$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). **(D)** Relative *FMR1* mRNA expression in late FXS hESC-derived NPCs transfected with control plasmid or dCas9-VP192 with the indicated gRNAs (* $p < 0.05$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test). For all scatter plots, each data point represents an individual well. Data were obtained from two independent experiments. The mean with error bars (SEM) is shown for each condition.

RNA-seq analysis of FXS hESCs treated with scramble gRNA vs. CGG gRNA and dCas9-VP192 (**Figures 7B,C**). A comparison between FXS hESCs treated with scrambled or CGG gRNA and dCas9-VP192 showed only 35 genes out of 23,394 that were differentially expressed (DE) between these two conditions (**Figure 7C**).

In parallel, we also performed RNA-seq to identify if there were any significant transcriptional differences between our FXS hESC line and our control hESC line (**Figure 7B**). A total of 1,797 genes were found to be DE between untreated WT and FXS hESCs. As expected, *FMR1* expression was much lower in the FXS hESCs. Gene Ontology analysis comparing the WT and FXS hESCs datasets identified nervous system development and neurogenesis as particularly different between these two hESC lines (**Supplementary Figure S1A**). Additionally, DE genes between these two hESC lines significantly map to cancer pathways (**Supplementary Figure S1B**). This data is consistent with studies suggesting that FMRP regulates mRNAs involved in cancer progression and metastasis (Lucá et al., 2013; Zalfa et al., 2017). However, one must be cautious in interpreting these differences in expression as resulting from the *FMR1* repeat expansion or loss of FMRP as these two hESC lines are not isogenic. Of note, treatment with CGG gRNA and dCas9-VP192 did not significantly revert FXS hESCs back towards the WT hESC transcriptomic profile (data not shown).

dCas9-VP192 Activation Does Not Increase FMRP Levels in FXS hESC

In order to test whether the increase in *FMR1* transcript levels would cause a subsequent increase in FMRP, we tested the early and late FXS hESC lines with the promoter pool and CGG targeted gRNAs along with dCasVP-192. Despite a significant increase in mRNA, we did not observe a statistically significant increase in FMRP levels in either early or late passage FXS hESCs (**Figures 8A–D**). Similar results were obtained with ICC measurements in these cells (data not shown). Thus, there is a dissociation at least in these cells between transcriptional reactivation and recovery of FMRP expression.

DISCUSSION

FXS results primarily from transcriptional silencing of the *FMR1* locus. Here, we report reactivation of *FMR1* transcription utilizing a CRISPR- dCas9 coupled transcriptional activator selectively targeted to the expanded CGG repeat. Enhanced transcription occurs at very large CGG repeat expansions in hESCs in the setting of incomplete and complete transcriptional silencing and despite DNA methylation of the locus. This transcriptional reactivation is also greatest when we use a guide RNA that directly targets the CGG repeat, and this effect is enhanced in the setting of a large CGG repeat expansion.

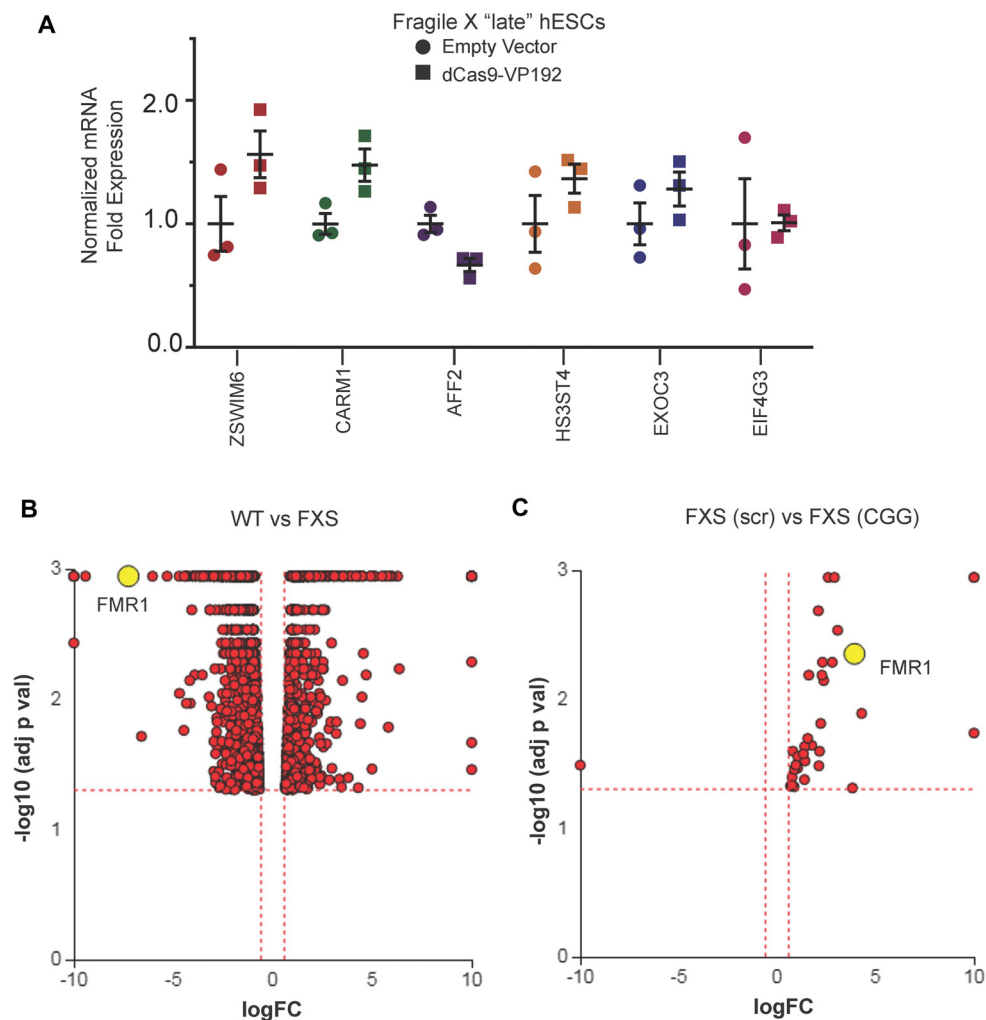


FIGURE 7 | Targeted reactivation of *FMR1* by CGG guide RNAs has minimal off target effects compared to scramble guide RNA. **(A)** Relative fold expression of select CGG repeat-containing genes after transfection of late passage FXS hESCs with CGG gRNA and eGFP (empty vector) or dCas9-VP192 constructs. Each data point represents a technical replicate and error bars represent SEM. **(B)** Volcano plot showing RNA-seq analysis of WT and FXS hESCs. All 1784 significant differentially expressed (DE) genes are represented in terms of their measured fold change (x-axis) and the significance represented as the negative log (base 10) of the *p*-value (y-axis). The yellow dot shows the position of *FMR1* mRNA. **(C)** Volcano plot showing RNA-seq analysis of FXS (scramble gRNA treated) and FXS (CGG gRNA treated) late passage hESCs expressing dCas9VP192. All 35 DE genes are represented as in terms of fold change. The axes are as described in **(B)**. Yellow dot represents position of *FMR1* mRNA. Images **(B,C)** were obtained from iPathwayGuide (<http://www.advaitabio.com/ipathwayguide>).

Unfortunately, while transcriptional activation correlated with an increase in FMRP expression in human cell lines with normal repeat sizes, we did not observe a significant increase in FMRP expression in FXS hESC cells. These findings provide proof-of-concept for a CRISPR based approach to gene reactivation in FXS patient cells with the potential for translation to *in vivo* systems, but with the caveat that large transcribed CGG repeats may introduce an additional blockade on FMRP translation.

Our approach uses a nuclease deficient Cas9 to target the CGG repeats for the reactivation of the *FMR1* gene. The use of a nuclease-deficient Cas9 fused to transcriptional activators or suppressors is a powerful tool for studying genome-scale events as well as specific processes (Wang et al., 2016). Similar transcriptional activator systems have been used previously

to successfully reverse disease symptoms in mouse models of Duchenne Muscular Dystrophy, highlighting the potential applicability of this system for *in vivo* treatment of disease (Long et al., 2016; Nelson et al., 2016). In our hands, dCas9-VP16 fusion constructs show the highest activation with a VP192 fusion construct along with the CGG repeated targeted gRNA. The robust activation observed using the CGG gRNA vs. a promoter-targeted construct is particularly intriguing, as it suggests that the repetitive nature of the CGG guide may serve to augment its targeting strategy by providing a promoter-proximate tiling site for dCas9-VP192 complexes. This is consistent with findings obtained from other groups targeting repeats as a method of transcriptional silencing of the locus (Pinto et al., 2017). It thus appears that repeat expansions

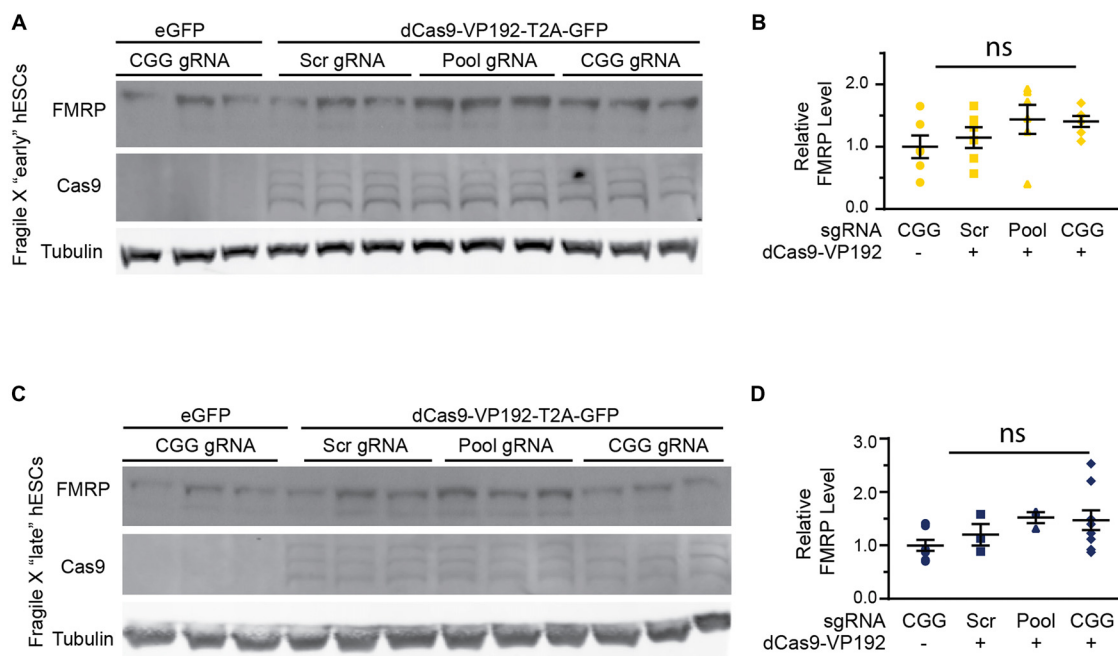


FIGURE 8 | Targeted reactivation of *FMR1* does not significantly enhance FMRP expression in FXS hESCs. **(A)** Western blots showing triplicate samples of early (P25–28) passage FXS hESCs transfected with control plasmid (eGFP) or with dCas9-VP192 and gRNAs immunoblotted for FMRP (by Femto ECL), Cas9 and tubulin as a loading control. **(B)** Quantification of western blots from early passage FXS hESCs transfected as indicated. **(C)** Western blots showing triplicate examples of late (P47–64) passage FXS hESCs transfected as in **(A)**. **(D)** Quantification of western blots from late passage FXS hESCs transfected as indicated. For all scatter plots, each data point represents an individual well. Data were obtained from two **(B)** or three **(D)** independent experiments. The mean with error bars (SEM) is shown for each condition. ns, not significant.

can recruit multiple dCas9-VP192 complexes simultaneously, with greater recruitment and potentially greater effect at larger repeat sizes. Additionally, evaluation of potential off-targets for this gRNA suggests that the presence of a large repeat element in FXS hESCs may suppress effects at other CGG repeat sites throughout the genome. Alternatively, there may be differences between hESCs and HEK293T cells in their basal transcriptomes that make them differentially sensitive to CGG repeat targeted gRNAs. This approach of directly targeting the repeats leverages the very nature of the repeat to achieve greater efficacy and specificity and has recently been used in other repeat expansion disorders to great effect (Batra et al., 2017; Pinto et al., 2017).

This work characterizes a new FXS hESC line, which was recently added to the NIH registry allowing for its use in United States federally-funded research. Despite a lack of FMRP, the FXS hESCs were effectively differentiated into neural rosettes and finally neurons (Figure 4; Eiges et al., 2007; Telias et al., 2013). This line exhibits a passage dependent silencing, including a passage-dependent methylation of the *FMR1* promoter that occurs in the absence of any neuronal differentiation (Figure 4). This is consistent with some published findings suggesting selection against expression of large expanded CGG repeat containing RNAs and (potentially) RAN translation products (Brykczynska et al., 2016; Zhou et al., 2016). However, it disagrees with work in the first characterized and widely used hESC FXS

line that exhibits a neuronal differentiation-dependent silencing that appears dependent on an RNA induced transcriptional silencing mechanism (Eiges et al., 2007; Colak et al., 2014). Our work does not delineate between these two possibilities, but future studies over longer time courses using stable transfection systems will be needed to determine both the sustainability of the enhanced transcription observed and the impact of enhanced production of large CGG repeat RNAs on cell viability and differentiation.

This study is complementary to a series of recent articles utilizing the CRISPR-Cas9 system to reactivate transcription from the *FMR1* locus in FXS. Two studies took a more direct approach of cutting out the repeat with the Cas9 nuclease and both achieved correction of the transcriptional silencing and a reactivation of FMRP expression (Park et al., 2016; Xie et al., 2016). More recently, a third study used a strategy more akin to our approach, targeting gRNAs to the CGG repeat and coupling that with a dCas9 fused to the active domain of the TET DNA demethylase (Liu et al., 2016, 2018). Using this approach in an iPSC line with ~500 CGG repeats, they were able to achieve both transcriptional reactivation of *FMR1* as well as at least partial recovery of FMRP expression (Liu et al., 2018). As with our work, it is intriguing that reactivation of *FMR1* transcription can occur even at a fully methylated and transcriptionally silenced locus observed in the late passage FXS hESCs (Figure 4B). This suggests that methylation and heterochromatinization of the locus

do not preclude access of the gRNAs and dCas9 complex to the repeat sequence. Our study adds the additional element that even targeting a transcription factor to the repeat, which does not directly target the epigenetic alterations present at the locus in FXS, is sufficient to reactivate the gene. Taken together, these findings imply that the silenced CGG repeat expanded *FMR1* locus may be more dynamic than previously thought—at least in the setting of hESCs where such boundaries may be more permissive to epigenetic change. Moreover, these results imply that *FMR1* transcriptional reactivation can be achieved through multiple potentially complementary approaches.

While the dCas9-VP192 activation system in control HEK293T cells elicited relatively equivalent effects on both *FMR1* transcription and FMRP production, in hESCs with pathologic repeat expansions the impact of transcriptional upregulation on FMRP expression was significantly blunted (Figure 7). There are a number of potential explanations for this finding. First, the method of dCas9-VP192 complex delivery utilized in these studies (transient transfection) was different from those used in studies with dCas9-Tet1 (Viral delivery with extension of measures of FMRP synthesis for weeks after transduction). Transfection rates empirically determined in our studies in hESCs were ~50%, meaning that any effects were diluted by the contribution of un-transfected cells. Viral delivery, especially in NPCs, is more efficient and expression is prolonged, which may explain their greater impact on both *FMR1* transcription and FMRP production. These delivery issues may also limit our ability to accurately exclude off-target effects if an insufficient number of cells were effectively transfected. Second, delivery of a transcriptional activator absent DNA demethylation may be less efficient at reactivating *FMR1* mRNA expression compared with a targeted demethylation. Direct head-to-head experiments with identical delivery mechanisms and multiple cell lines as well as evaluation for potential synergistic impacts on gene reactivation will need to be evaluated in the future.

An alternative explanation for the observed discrepancy between *FMR1* mRNA transcriptional reactivation and FMRP production could be the larger repeat size of the hESC line studied in these experiments (>800 CGG repeats). Transcribed and expanded CGG repeats elicit a significant impedance to ribosomal scanning and downstream initiation of FMRP translation (Feng et al., 1995; Chen et al., 2003; Khateb et al., 2007; Iliff et al., 2013). How large of a factor such a translational blockade might play in any transcriptional re-activation strategy is unclear. Very large un-methylated repeats that are efficiently transcribed can still produce a FXS phenotype, although some cases of methylation mosaicism and repeat length mosaicism have only modest or no clear clinical symptoms (Burman et al., 1999; Tassone et al., 2000). It may be that the underlying repeat size is the critical determinant. Most cases of unmethylated full mutation patients described to date have repeat sizes that are less than 400 CGGs and these typically have very mild if any clinical phenotypes (Pietrobono et al., 2005; Tabolacci and Chiurazzi, 2013). In cases of methylation mosaicism, somatic instability complicates data interpretation, meaning that effects on FMRP production may be cell specific (Jiraanont et al., 2017).

If transcribed repeats preclude recovery of FMRP expression in FXS patients with very large expansions, then concomitant approaches specifically targeting this translational blockade will be needed to achieve reactivation in these cases. However, given that less CGG DNA methylation, more *FMR1* mRNA transcription and more FMRP production in even a subset of cells in FXS patients all correlate with better clinical outcomes and differential responses to pharmacological agents (Nolin et al., 1994; Tassone et al., 1999; Jacquemont et al., 2011), even modest successes targeting these proximal events in pathogenesis may elicit meaningful effects on clinical phenotypes. Thus, this proof-of-principle study provides additional hope that such approaches will eventually lead to effective therapeutics in patients with FXS while also raising concerns related to the generalizability of the approach to all cases.

DATA AVAILABILITY

The datasets generated for this study can be found in the GEO database GEO identifier GSE112031 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112031>).

AUTHOR CONTRIBUTIONS

PT and JH conceived the project. JH conducted all of the experiments in HEK293T cells and all initial experiments in hESC cells. GS validated hESC experiments, did NPC and neuronal work and performed the RNA-seq experiments. GS wrote the manuscript with significant input from PT and JH. GSmith and AMR derived the UM139-2 hESC line. CR initially characterized the UM 139-2 hESC line. JP provided assistance with stem cell work. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2018.00282/full#supplementary-material>

FIGURE S1 | Gene ontology analysis and pathways for WT vs. late FXS hESCs. **(A)** Graph depicting top Gene Ontology (GO) terms for the RNA-seq data comparing WT and FXS hESCs. X-axis shows the GO terms and Y-axis shows the negative log (base 10) of the *p*-values for each category. **(B)** All pathways from the WT and FXS hESC RNA-seq analysis are plotted in terms of the two

types of evidence computed by iPathwayGuide using Impact Analysis (Tarca et al., 2009): over-representation on the x-axis (pORA: Over Representation Analysis) and the total pathway accumulation on the y-axis (pAcc: Accumulated perturbation of the pathway). Each pathway is represented by a single dot, with significant pathways shown in red, non-significant in black. Both *p*-values are shown in terms of their negative log (base 10) values. Yellow dot represents cancer pathways. The adjacent red dots show pathways for melanoma and breast cancer. Figure obtained from iPathwayGuide (<http://www.advaitabio.com/ipathwayguide>).

TABLE S1 | List of all primers used to construct sgRNA plasmids and for qPCR analysis in this study.

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New Insights Into the Role of Ca_v2 Protein Family in Calcium Flux Deregulation in *Fmr1*-KO Neurons

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Fragile X syndrome (FXS), the most common form of inherited intellectual disability (ID) and a leading cause of autism, results from the loss of expression of the *Fmr1* gene which encodes the RNA-binding protein Fragile X Mental Retardation Protein (FMRP). Among the thousands mRNA targets of FMRP, numerous encode regulators of ion homeostasis. It has also been described that FMRP directly interacts with Ca²⁺ channels modulating their activity. Collectively these findings suggest that FMRP plays critical roles in Ca²⁺ homeostasis during nervous system development. We carried out a functional analysis of Ca²⁺ regulation using a calcium imaging approach in *Fmr1*-KO cultured neurons and we show that these cells display impaired steady state Ca²⁺ concentration and an altered entry of Ca²⁺ after KCl-triggered depolarization. Consistent with these data, we show that the protein product of the *Cacna1a* gene, the pore-forming subunit of the Ca_v2.1 channel, is less expressed at the plasma membrane of *Fmr1*-KO neurons compared to wild-type (WT). Thus, our findings point out the critical role that Ca_v2.1 plays in the altered Ca²⁺ flux in *Fmr1*-KO neurons, impacting Ca²⁺ homeostasis of these cells. Remarkably, we highlight a new phenotype of cultured *Fmr1*-KO neurons that can be considered a novel cellular biomarker and is amenable to small molecule screening and identification of new drugs to treat FXS.

Keywords: Fragile X syndrome, Ca_v2.1, calcium homeostasis, ratiometric calcium imaging, *Cacna1a*

INTRODUCTION

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID) and the leading identified monogenic cause of autism (Maurin et al., 2014; Castagnola et al., 2017). FXS is caused by the silencing of the *Fmr1* gene encoding the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein modulating the expression of thousands of mRNAs primarily at the translational level in particular, it has been shown to regulate translation at the synaptic level. Furthermore, FMRP has been reported to be involved in different steps of RNA metabolism, indeed it is a component of various ribonucleoproteic complexes (mRNPs), including the RNA granules, the mRNP involved in transport along neurites (Maurin et al., 2014, 2018a).

Several reports have shown that FMRP binds multiple RNAs encoding regulators of ion homeostasis and more particularly involved in the calcium ion pathway (Brown et al., 2001; Miyashiro et al., 2003; Darnell et al., 2011; Ascano et al., 2012; Maurin et al., 2018a).

Furthermore, the search for FMRP-interacting proteins has resulted into the identification of dozens of partners, including ion channels (Bardoni et al., 2006; Ferron, 2016; and this study). Consistently with these findings, ion homeostasis defects in FXS neurons have been described (Chen et al., 2003; Meredith et al., 2007; Brown et al., 2010; Deng et al., 2013; Ferron et al., 2014; Hebert et al., 2014; Zhang et al., 2014; Contractor et al., 2015; Myrick et al., 2015; Wahlstrom-Helgren and Klyachko, 2015; Achuta et al., 2018). In particular, FMRP has been reported to directly interact with two members of the Voltage Gated Calcium Channels (VGCC) family, namely $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ (Ferron et al., 2014).

Cytosolic calcium concentration is set by the balance between calcium influx and efflux as well as by the exchange of calcium ion with internal stores. Calcium homeostasis is tightly controlled and involves multiple protein complexes such as ATPase pumps, transporters and ion channels in various cellular compartments (Clapham, 2007).

VGCCs respond to plasma membrane depolarization by allowing extracellular calcium ions to flow into cells according to their concentration gradient. Calcium can then act as a second messenger of cell depolarization activating various key intracellular signaling pathways, inducing contraction in muscle cells, protein phosphorylation, secretion and synaptic transmission. VGCCs are heteromers composed by the assembly of a pore-forming subunit (encoded by the corresponding $\alpha1$ gene) and auxiliary β and $\alpha2\delta$ proteins (Dolphin, 2016). VGCCs can be distinguished as L-, N-, R- and P/Q-type channels depending on the identity of the pore-forming subunit. L- and T-type VGCCs are found in a great variety of cells, while N-, P/Q- and R-type are mostly expressed in neurons (Catterall, 2011).

The *Cacna1a* gene encodes the P/Q-type VGCC $\text{Ca}_v2.1$, which is critical for the depolarization-evoked release of neurotransmitters at the presynaptic terminals (Simms and Zamponi, 2014). $\text{Ca}_v2.1$ is mostly expressed in the cerebellum, consequently mutations in the *Cacna1a* gene are associated with several neurological disorders such as episodic ataxia and spino-cerebellar ataxia (Zhuchenko et al., 1997). More recently, new mutations in this gene have been identified in four unrelated families with ID, attention deficit, hyperactivity and autism spectrum disorder (Damaj et al., 2015). This suggests that $\text{Ca}_v2.1$ may play a previously under-appreciated role in brain regions other than the cerebellum and could have been implicated roles in cognition, memory and social interaction regulation. Indeed, regulation of $\text{Ca}_v2.1$ channels by calcium sensor proteins is required for normal short-term synaptic plasticity, LTP, and spatial learning and memory in mice (Nanou et al., 2016).

We thus investigated calcium homeostasis using ratiometric calcium imaging in *Fmr1*-KO neurons. Our results show that neurons lacking FMRP are not only more sensitive to $\text{Ca}_v2.2$ inhibition but also less sensitive to $\text{Ca}_v2.1$ inhibition compared to wild-type (WT) neurons and this is a consequence of an impaired membrane expression of this channel in the absence of FMRP. We propose here a model in which FMRP is involved in the regulation of the relative membrane expression of P/Q- and N-type VGCCs.

MATERIALS AND METHODS

Primary Neuronal Cultures

Cultures were prepared from the cortex of embryonic stage E14.5 WT and *Fmr1*-KO embryos as previously described (Abekhoukh et al., 2017; Maurin et al., 2018a). Neurons (250,000 cells) were plated on ornithine-coated glass coverslips (35 mm diameter) and cultivated in complete medium: Neurobasal (Invitrogen) supplemented with B-27 (Invitrogen) and glutamax (Invitrogen). Neurons were fed weekly by removing 10% of the culture medium and replacing it with fresh complete medium.

Ratiometric Calcium Imaging

Primary cortical neurons Day-*In-Vitro* 19–23 (DIV 19–23; 13 independent cultures) grown on coverslips were incubated in neurobasal containing 20 μM Fura2-AM (Invitrogen) for 30 min at 37°C. After two washes with HEPES-buffered Tyrode's calcium solution (in mM: 139 NaCl, 15 glucose, 1.25 Na_2HPO_4 dibasic heptahydrate, 1.8 MgSO_4 heptahydrate, 1.6 CaCl_2 dihydrate, 3 KCl, 10 HEPES), coverslips were placed in a metal chamber on an inverted microscope (AxioObserver, Carl Zeiss) equipped with a 300W Xenon lamp (Sutter Instruments) and a Fluor 40 $\times$ NA 1.4 oil immersion objective. Cells were perfused at 22°C throughout the recording with Tyrode's calcium solution. The pharmacological stimulations were performed by supplementing the calcium recording solution with either DiHydroxyPhenylGlycine (DHPG, 100 μM) or KCl (50 mM) or VGCC antagonist (ω -agatoxin-Iva (100 nM); ω -conotoxin GV1a (1 μM); Nitrendipine (1 μM)) or VGCC antagonist (same concentrations) + KCl. A calibration step was performed at the end of every recording by applying successively 0 Ca^{2+} (in mM: 129 NaCl, 15 glucose, 1.25 Na_2HPO_4 dibasic heptahydrate, 1.8 MgSO_4 heptahydrate, 0.5 EGTA, 3 KCl, 10 HEPES), then 0 Ca^{2+} + ionomycin (5 μM) and finally 10 Ca^{2+} + ionomycin (5 μM ; in mM: 129 NaCl, 15 glucose, 1.25 Na_2HPO_4 dibasic heptahydrate, 1.8 MgSO_4 heptahydrate, 10 CaCl_2 dihydrate, 3 KCl, 10 HEPES) solutions. This calibration step allows to quantify the lowest and the highest probe fluorescence $F_{340/380}$ ratio for every Region of Interest (ROI); the maximal value was used subsequently to normalize the fluorescence $F_{340/380}$ measurements. Every recording experiment followed the same protocol:

Tyrode's—40 s; Tyrode's + DHPG—40 s; Tyrode's—60 s; Tyrode's + KCl—20 s; Tyrode's—60 s; Tyrode's + KCl—20 s; Tyrode's—60 s; Tyrode's + VGCC antagonist—60 s; Tyrode's + KCl + VGCC antagonist—20 s; Tyrode's + VGCC antagonist—60 s; Tyrode's + KCl + VGCC antagonist—20 s; Tyrode's + VGCC antagonist—60 s; Tyrode's + KCl + VGCC antagonist—20 s; Tyrode's + VGCC antagonist—60 s; Tyrode's (0 Calcium)—80 s; calibration (see above).

Fura2 was sequentially excited at 340 nm and 380 nm, and the emission monitored at 510 nm. Images were acquired with a cascade 512 EMCCD camera every 2 s using the Metafluor software (Roper Scientific). For each recorded cell, the intracellular calcium concentration $[\text{Ca}^{2+}]_i$ was estimated by measuring the $F_{340/380}$ nm ratio of fluorescence normalized to the

maximal probe fluorescence measured when cells were perfused with the 10 Calcium + ionomycin solution. ω -agatoxin-IVA and ω -conotoxin GVIA were purchased from Smartox, Nitrendipine from Sigma-Aldrich. Resting calcium levels (“baseline”) were measured as the average fluorescence from the first 40 s of each recording. For KCl stimulation, for each cell analyzed we report the results of the mean of two maximal $F_{340/380}$ in two consecutive stimulations. The Drug Response (DR) represents the mean of the two max $F_{340/380}$ in two consecutive stimulations over the mean of the three max $F_{340/380}$ in three consecutive stimulations in the presence of antagonist. The results of the pharmacological stimulations (DHPG, KCl) are reported as fold change over baseline levels. Only cells for which the DHPG stimulation elicited a fold change greater than 1.1 times the baseline levels in $F_{340/380}$ ratio were considered responsive cells.

Immunoprecipitation

Cerebella from WT and *Fmr1*-KO mice were grinded in liquid nitrogen into fine powder and resuspended in 5 v/w with PBS containing 1% Igepal. Samples were cleared with 15 μ l of naked Dynabeads A (ThermoFisher) for 30 min at 4°C on a rotating wheel. During this time, 30 μ l of Dynabeads A were incubated with anti-FMRP primary antibody for 1 h at room temperature on a rotating wheel, with 100 μ g of tRNA, ssDNA and BSA. The “pre-clear” beads were then removed and samples were centrifuged for 10 min at 14,000 rpm at 4°C. Supernatants were incubated with antibody-coated beads overnight at 4°C on a rotating wheel. Beads were washed three times with PBS containing 0.1% Igepal and incubated for 15 min at 55°C with 100 mM dithiothreitol and 2 $\times$ Laemmli sample buffer. Eluted proteins were then resolved on 4%–12% gradient SDS-PAGE using MOPS buffer (Invitrogen).

Biotinylation

Primary neurons plated at the density of 200,000 cells per well were used for biotinylation experiments at DIV 15. Neurons were washed twice with PBS and incubated with EZLink Sulfo-NHS-LC-Biotine (0.3 mg/ml in PBS, Thermo Scientific) for 10 min at 4°C. After a quick wash with PBS, unbound biotin molecules were quenched with 50 mM NH₄Cl for 5 min. After two washes with ice-cold PBS, proteins were extracted using lysis buffer containing 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% mammalian protease inhibitor cocktail (Sigma-Aldrich). Two-hundred microgram of proteins from each condition were incubated overnight at 4°C with streptavidin-conjugated beads (Sigma-Aldrich). Beads were then washed three times with lysis buffer and resuspended in Laemmli buffer. Proteins were separated in 7% acrylamide-bis-acrylamide gel. Primary antibodies anti β -Actin (Sigma, #A5441; 1/1,000), anti-Ca_v2.1 (Alomone Labs, #ACC-001; 1/1,000) and anti β 3-tubulin (Synaptic Systems, #302302; 1/1,000) were used.

RNA extraction and RT-qPCR were performed as previously described (Maurin et al., 2018a). The sequences of the primers used in this study are provided in Table 1.

TABLE 1 | Sequences of the primers used in this study.

	Forward	Reverse
<i>Cacna1a</i>	GAGTATGACCCTGCTGCCTG	TGCAAGCAACCCTATGAGGA
<i>Cacna1b</i>	TGCGTTCTCGAGCTTCATGG	CGCTTGATGGTCTTGAGGGG
<i>Cacna1c</i>	GAACCATATCCTAGGCAATGCAG	AAGAGCCCTTGTGCAGGAAA
<i>Cacna1e</i>	TGAGTTTGTCCGTGTCTGGG	GAGGGACATCTCTTGCCGAG
<i>c-Kit</i>	GGAGTGTAAAGGCCTCCAACG	TGGGCCTGGATTTGCTCTTT
<i>Klf4</i>	CAGGATTCCATCCCCATCCG	TGGCATGAGCTCTTGATAATGGA
<i>Gfap</i>	CAGATCCGAGGGGGCAAA	TGAGCCTGTATTGGGACAACT
<i>Dlg4</i>	GGCGGAGAGGAACCTGTCC	AGAATTGGCCTTGAGGGAGGA
<i>Tbp</i>	AGGCCAGACCCCAACTC	GGGTGGTGCCTGGCAA

Sequences are presented from 5' to 3' end.

Polyribosome Fractionation

Samples from polyribosome fractionation were described previously (Maurin et al., 2018a). Polyribosome fractionation was performed as described previously (Bechara et al., 2009) on 20%–50% (w/w) continuous sucrose gradients. Fractions were separated on a BR-188 Density Gradient Fractionation System (Brandel). Fold changes in *Cacna1a* mRNA levels between WT and *Fmr1*-KO were assessed by RT-qPCR and were calculated for individual fractions 6–14 according to the formula 2^{-ddCp} where ddCp is $(Cp \text{ Pde2a KO fraction}_x - Cp \text{ Gapdh KO fraction}_x) - (Cp \text{ Pde2a WT fraction}_x - Cp \text{ Gapdh WT fraction}_x)$. Results from fractions 6 to 8 (light), 9 to 11 (medium) and 12 to 14 (heavy) were pooled and analyzed together.

Protein Extraction and Western Blot Analysis

Cells and tissues extracts were processed as described previously (Maurin et al., 2018a). Primary antibodies anti β -Actin (Sigma, clone AC-74; 1/1,000) and anti-Ca_v2.1 (Alomone Labs, #ACC-001; 1/1,000) were incubated overnight at 4°C in PBS 0.05%.

Immunocytochemistry on Primary Neurons

Primary neurons grown on glass coverslips were washed three times with PBS at room-temperature and then fixed using 4% Paraformaldehyde (PFA) in PBS for 10 min at room temperature. After rinsing briefly with PBS, free aldehydes were blocked with 50 mM NH₄Cl in PBS for 5 min. Then, a saturation step was performed with PBS containing 10% Fetal Bovine Serum and 0.1% Triton X-100 for at least 20 min. Neurons were incubated with antibodies diluted in PBS containing 10% Fetal Bovine Serum and 0.1% Triton X-100 in a humidified chamber overnight at 4°C. After three PBS washes, neurons were incubated with secondary antibodies for 1 h at RT. After three PBS washes cells were incubated for 3 min in a PBS solution containing DAPI (10 μ g/ml). The glass coverslips were finally washed once with ddH₂O and mounted (Dako Fluorescent Mounting Medium) on glass slides and stored in the dark at 4°C. The polyclonal anti-Ca_v2.1 (Alomone Labs, #ACC-001) antibody was used at a dilution of 1/50. The 1C3 antibody against FMRP was used at a dilution of 1/200 (Castets et al., 2005). Colocalization quantifications of FMRP and Ca_v2.1 in one confocal plan (average of three scans) were carried out using the JACoP plugin for ImageJ (Bolte and Cordelières, 2006). Cells were examined on a TCS SP5 confocal microscope (Leica).

Cell Shape Analysis

We designed an ImageJ (Schneider et al., 2012) dedicated macro to analyze simultaneously the cell shape and the Fura2 fluorescence ratio variations (in time) obtained by sequential excitation at 340 and 380 nm. First, kinetics images of 340 and 380 nm excitation were stacked together and any lateral drift was corrected using the StackReg plugin (Thévenaz et al., 1998). A mask and a list of ROIs for each cell was obtained on the last 340 nm image after a filtering (recursive TopHat followed by an unsharp mask) and a Huang intensity thresholding. Then the 340 and 380 nm images were separated in two stacks and their $F_{340/380}$ ratio calculated after a background measurement and subtraction in each image of the stack. The ROIs were then used on the 340/380 stack to get individual cell measurements of shape parameters (Aspect Ratio, Roundness, Area, Solidity) and $F_{340/380}$ fluorescence ratios during time.

Multivariate Analysis of the Cell Morphology Parameters

Baseline and KCl data were extracted and normalized to the maximal calcium value obtained for each cell with the 10 mM Calcium + ionomycin solution and combined to cell morphology parameters extracted from the images. Both cell morphology, normalized baseline and KCl data were then used for unsupervised analysis. Data were first log10 transformed, then mean-centered and scaled. Then, dimension reduction was performed using Barnes-Hut implementation of t-Distributed Stochastic Neighbor Embedding (tSNE), with perplexity parameter set to 40. K-means clustering was performed on the two-dimension tSNE projection and the optimal number of clusters was determined using the Gap statistic. Significance of the differences between continuous variable distributions was assessed using either Mann-Whitney or Kruskal-Wallis rank sum tests as appropriate. All analyses and graphical representations were performed using the R statistical package or Prism Software 6-2 version (GraphPad Software, Inc., San Diego, CA, USA).

Statistics

The Kolmogorov-Smirnov test was used to assess the normality of the distribution of the datasets. To compare non-normally distributed data, two non-parametric tests were used: the Mann-Whitney test was applied to data of two unpaired samples, while the Kruskal-Wallis test was used to examine the significance of four unpaired groups. Data are expressed as mean $\pm$ SEM, and the *P* values (or adjusted *P* values) < 0.05 were considered statistically significant. RT-qPCR analysis of mRNA expression were analyzed using ANOVA TWO WAY with Sidak's multiple comparisons *post hoc* test. The statistical analysis was performed using Prism Software 6-2 version (GraphPad Software, Inc.).

Animal Experiments

The experiments were performed following the ARRIVE (Animals in Research: reporting *in vivo* Experiments) guidelines (Kilkenny et al., 2010). Animal care was conducted in accordance with the European Community Directive

2010/63/EU. The experiments were approved by the local ethics committee (Comité d'Ethique en Expérimentation Animale CIEPAL-AZUR N. 00788.01; APAFIS#4985-2016032314169426 v4APAFIS#8100-2016112217148206 v3).

RESULTS

Calcium Homeostasis Is Impaired in *Fmr1*-KO Cells

We investigated calcium homeostasis using Fura2 ratiometric imaging in primary neuron cultures derived from the cortex of E14.5 WT and *Fmr1*-KO embryos. According to our immunocytochemistry results, these cultures are enriched in neurons and have limited mature astrocyte content (less than 10% of cells) that are mostly present in cell aggregates (Supplementary Figures S1A,B). Therefore, these regions were avoided in subsequent calcium recordings. RT-qPCR analysis of the expression of GFAP and PSD95 markers showed that the absence of FMRP does not affect the relative amounts of astrocytes and neurons in *Fmr1*-KO cultures compared to WT (Supplementary Figure S1C). We systematically applied a series of consecutive drug treatments followed by a calibration step that allowed us to quantify the minimum and maximum fluorescence of Fura2 in each analyzed cell. We used the normalized fluorescence ratio ($[F_{340/380}]/\max[F_{340/380}]$) as an indirect quantification of the actual intracellular calcium concentration. By this imaging approach we investigated the functionality of several key parameters of calcium homeostasis in neurons in the presence or in the absence of FMRP.

Cellular Analysis

Our imaging data clearly show the heterogeneity of the neuronal types present in primary neuron cultures (Figures 1A–C). Cells differ in size, shape, resting intracellular calcium levels and maximum calcium entry upon KCl stimulation. We wondered whether the absence of FMRP could have different impacts on calcium homeostasis in different cell types. The Fura2 fluorescence ratio and the shape analysis of the ROIs were simultaneously quantified by an ImageJ lab-made macro giving the shape descriptors for each ROI (area, roundness, solidity, circularity). Roundness reflects how circular a ROI is, while solidity and circularity indicate how soft (high scores) or rough (low scores) are the contours of the region. We then performed an unsupervised multivariate analysis (Supplementary Figures S2A,B) to group cells according to their size, shape and calcium homeostasis parameters (baseline levels, maximum calcium levels upon KCl stimulation) identifying four distinct and homogeneous groups of cells (Supplementary Figures S2C–H). Representative images of ROIs detected in each cluster are shown in Supplementary Figure S3. Cells in group 1 and 3 differ in size and in the complexity of their contour, have a higher resting calcium concentration and high calcium entry upon KCl stimulation. Group 2 cells are small with rough contours and display a limited calcium entry following KCl stimulation, characteristics that suggest an astrocytic lineage. Group 4 ROI are small elongated objects that mostly correspond

to neurites (**Supplementary Figure S3**). We considered the repartition of WT and *Fmr1*-KO cells in these clusters and our results indicate a homogeneous distribution of cells from the two genotypes in all clusters (**Supplementary Figures S2I–L**). The number of DHPG-responding cells was also similar in both genotypes (**Supplementary Figure S4**). We focused our analysis on cells belonging to group 1 and 3 which according to this analysis, have neuron characteristics. These cells were subsequently analyzed together. The steady state intracellular Ca^{2+} concentration, measured prior to any pharmacological treatment during the first 40 s of the recording, is elevated in the absence of FMRP (Mann-Whitney test, $P < 0.0001$; **Figure 1D**).

The metabotropic Glutamate receptor pathway has been described to be deregulated in FXS (Huber et al., 2002; Bear et al., 2004). The activation of this pathway with pharmacological agonists like DHPG triggers calcium release from internal stores through IP3 receptors as a consequence of the activation of the Phospholipase C and IP3 second messenger pathway. The calcium ion release from intracellular stores in response to DHPG is variable and not significantly different in the absence of FMRP compared to WT cells at the population level (Mann-Whitney test, $P = 0.9963$, not significant; **Figure 1E**).

We next induced cell depolarization by applying a 50 mM KCl solution onto the cultures, as in these conditions VGCCs are the main determinants of calcium entry in neurons (Mao et al., 2001). VGCCs respond to cell depolarization, upon which they open and allow calcium ion entry through their pore-forming subunit. We thus analyzed for each cell the fold change in $F_{340/380}$ induced by KCl over baseline levels. Our results show that calcium entry through voltage-dependent plasma membrane channels upon KCl-induced neuron depolarization is slightly decreased in *Fmr1*-KO neurons (Mann-Whitney test, $P < 0.0001$; **Figure 1F**). Last, we observed that after the KCl stimulations *Fmr1*-KO neurons had significantly higher mean $F_{340/380}$ ratio over the 40 s that followed the KCl stimulation compared to WT, suggesting a deregulated return to baseline levels in the absence of FMRP (Mann-Whitney test, $P < 0.005$; **Figure 1G**).

Highly specific pharmacological blockers have been identified for all these VGCC subfamilies (Zamponi et al., 2015). For instance, we used specific pharmacological blockers of VGCCs: dihydropyridines, such as nitrendipine, block L-type VGCCs (Peterson et al., 1996) by binding to transmembrane domains of the $\alpha1$ subunit hence affecting the gating mechanism of the L-type VGCCs. ω -Conotoxin-GV1a (Conotoxin) blocks N-type VGCCs (Ichida et al., 2005) by interacting with the channel pore. ω -Agatoxin IVa (Agatoxin) inhibits P/Q-type VGCCs (Adams et al., 1993) by binding to two extracellular loops of the $\alpha1$ subunit that are close to the sensor domain of the P/Q-channel. Thus, we used some of these blockers in order to further investigate the molecular determinants of such calcium homeostasis deregulations. Within each neuron expressing or not FMRP, we measured the DR as the ratio of the mean of the maximal depolarization-induced Ca^{2+} entry in the presence of a VGCC-specific antagonist on the mean calcium entry in the absence of a

VGCC-specific antagonist. All the antagonists tested significantly reduced calcium ion entry upon KCl stimulation. Indeed, each antagonist treatment produced a DR that was statistically different from 1, the DR value expected for a drug having no effect (one sample *t*-test, $P < 0.0001$; **Figures 2A–C**). Nevertheless, Nitrendipine (1 μM) reduced KCl-triggered calcium ion entry similarly in WT and *Fmr1*-KO cells (Mann Whitney test, n.s.: $P = 0.2968$; **Figure 2A**). The ω -Conotoxin-GV1a (Conotoxin; 1 μM) was more efficient in *Fmr1*-KO cells (Mann Whitney test, $P < 0.0001$; **Figure 2B**). On the contrary, the ω -Agatoxin IVa (Agatoxin; 100 nM) had a fainter effect in *Fmr1*-KO than in WT cells (Mann Whitney test, $P < 0.0001$; **Figure 2C**). These findings strongly suggest that N- and P/Q-type channels are deregulated in *Fmr1*-KO neurons. These results are recapitulated in **Table 2**.

***Cacna1a* Expression Is Altered in *Fmr1*-KO Primary Neurons**

The pore forming unit of P/Q-type VGCC is encoded by the *Cacna1a* gene, whose mRNA is a target of FMRP (Darnell et al., 2011), in particular also during early brain development (at Post-Natal Day 13, PND 13; Maurin et al., 2018a). We therefore investigated how FMRP regulates *Cacna1a* expression in *Fmr1*-KO primary cultured neurons and in cortical extracts of *Fmr1*-KO mouse. We precisely characterized the time course of various $\alpha1$ gene expression in WT and *Fmr1*-KO primary neurons by RT-qPCR. *Cacna1a* is the most upregulated $\alpha1$ gene of the Ca_v2 family between DIV 14 and 21, and its expression is reduced in *Fmr1*-KO neurons (**Figures 3A–D**) at DIV 21 compared to WT cells. We therefore investigated whether FMRP modulates *Cacna1a* mRNA half-life by measuring *Cacna1a* stability together with control RNAs in primary neurons treated with the polymerase II inhibitor Actinomycin D. We observed that, consistent with a previous report (Sharova et al., 2009), Actinomycin D treatment triggers a strong decrease in *Klf4* transcript expression (**Figure 3E**) which is not due to cell toxicity, as we could show that in the same conditions *c-Kit* expression is stable over time (**Figure 3F**). In these conditions, *Cacna1a* expression is affected to a similar extent in WT and *Fmr1*-KO neurons (**Figure 3G**), excluding a role of FMRP in regulating *Cacna1a* mRNA stability. We concluded that the decreased expression levels of *Cacna1a* mRNA in *Fmr1*-KO cells do not depend on the half-life of this mRNA in the absence of FMRP but it is likely due to a decreased transcription level. Thus, we analyzed *Cacna1a* translation in the cortex of WT and *Fmr1*-KO mice by quantifying *Cacna1a* mRNA levels in different fractions of polyribosome preparations obtained from WT and *Fmr1*-KO PND 13 mouse cortex. Our results show that *Cacna1a* mRNA polyribosome association is increased in the light and medium polyribosome fractions, which argues in favor of an increased translation of this mRNA in the absence of FMRP (**Figure 3H**).

Western blot analysis of total $\text{Ca}_v2.1$ protein levels in DIV 17–21 primary neurons showed no statistically significant difference between WT and *Fmr1*-KO cells (Mann-Whitney test, $P = 0.7$, not significant; **Figures 4A,B**). We also analyzed $\text{Ca}_v2.1$ expression at the plasma membrane of *Fmr1*-KO and

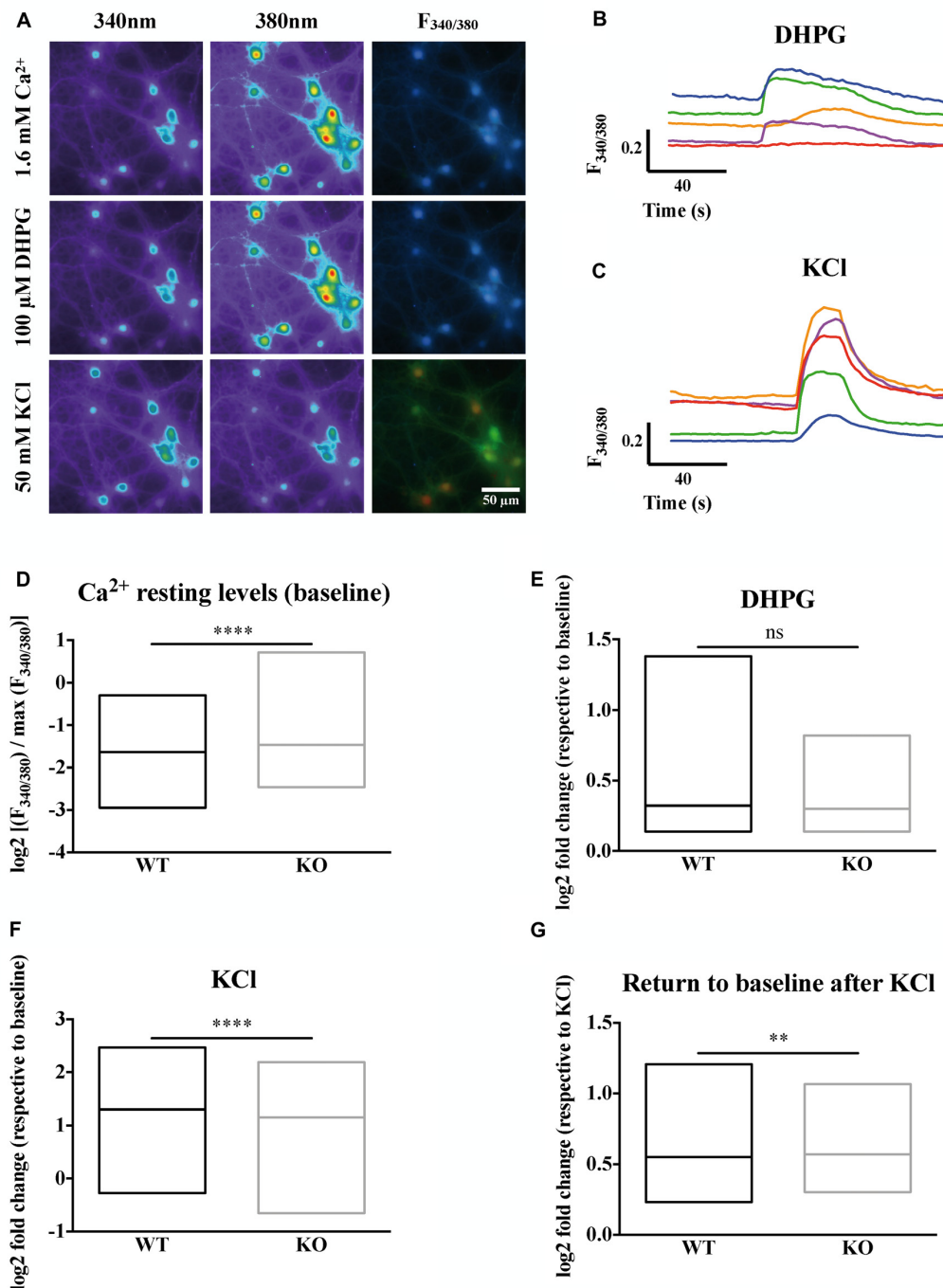
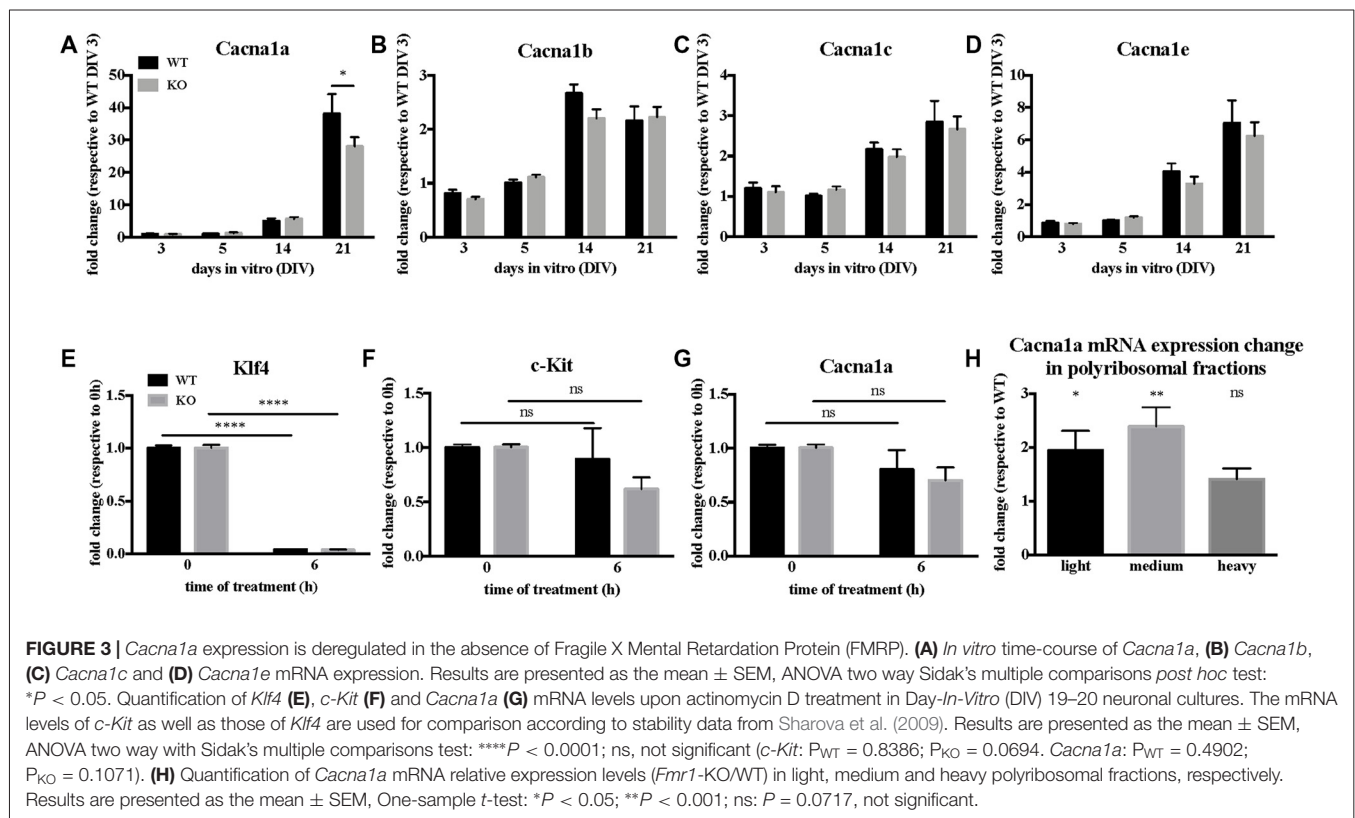
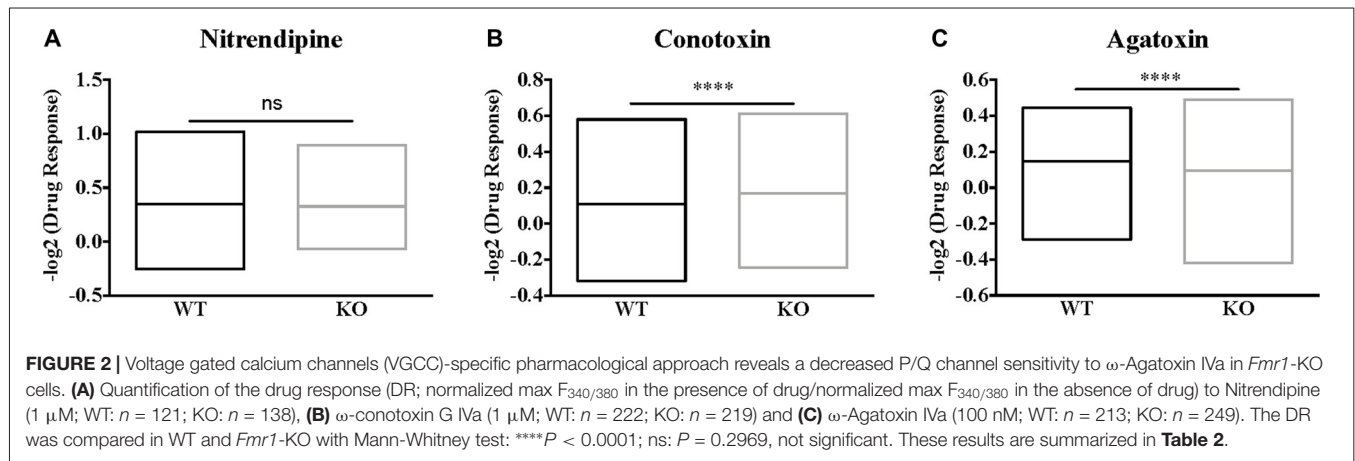


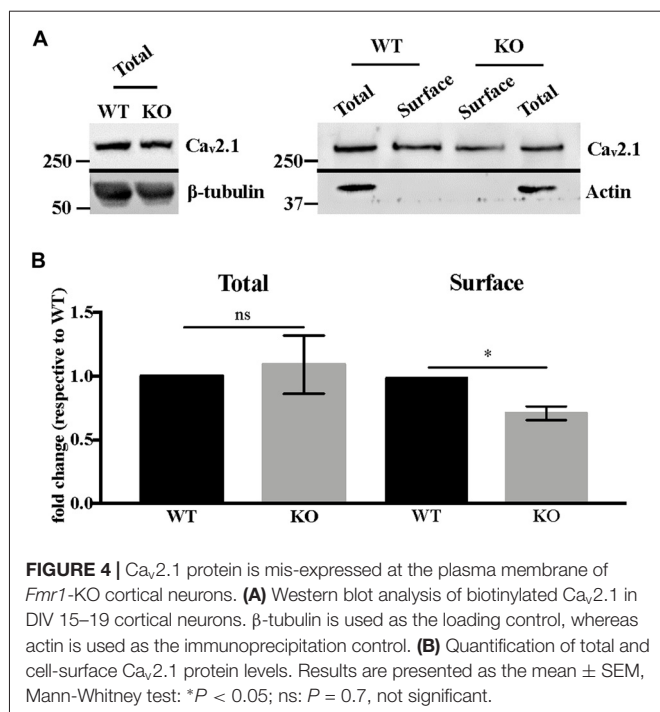
FIGURE 1 | Calcium homeostasis is deregulated in Fragile X mental retardation 1-knockout (*Fmr1*)-KO neurons. **(A)** Profiles of the ratiometric calcium imaging response. Left panels show the emission of Fura2 at 340 nm. Middle panels show the emission of Fura2 at 380 nm. Right panels show the 340 nm/380 nm ratio of fluorescence ($F_{340/380}$). Upper panels show the emission of Fura2 upon 1.6 mM Ca^{2+} perfusion. Middle panels show the emission of Fura2 upon 100 μM DiHydroxyPhenylGlycine (DHPG) perfusion. Lower panels show the emission of Fura upon 50 mM KCl perfusion. The scale bar of each panel is 50 μm . **(B)** Sample traces of Fura2 recording upon metabotropic glutamate receptor stimulation with DHPG (100 μM) or **(C)** depolarization with KCl (50 mM) in wild-type (WT) cells. For each cell recorded, the Fura2 fluorescence at each time was normalized to the maximum Fura2 fluorescence ratio observed in the presence of a solution containing 10 mM CaCl_2 and ionomycin (5 μM). The mean stabilized $F_{340/380}$ ratio of Fura2 fluorescence during the first 40 s of recording in the absence of any stimulation is represented in **(D)**. The $\log_2$ fold change in normalized $F_{340/380}$ after 100 μM DHPG stimulation over baseline normalized ratio is presented in **(E)**. The $\log_2$ fold change in normalized $F_{340/380}$ after 50 mM KCl stimulation over baseline normalized ratio is presented in **(F)**. The return to baseline following a KCl stimulation is shown for WT and *Fmr1*-KO neurons **(G)**. Mann-Whitney test: **** $P < 0.0001$; ** $P < 0.005$; ns: $P = 0.9963$, not significant. WTn = 697; KO n = 744. These results are summarized in **Table 2**.

TABLE 2 | Results summary.

	Mean $\pm$ SEM WT (n)	Mean $\pm$ SEM KO (n)	P value	P value significance
Figure 1D resting	-1.636 ± 0.016 (697)	-1.459 ± 0.0141 (744)	<0.0001	****
Figure 1E DHPG	0.3221 ± 0.0150 (222)	0.2989 ± 0.0105 (211)	0.9963	ns
Figure 1F KCl	1.297 ± 0.0142 (697)	1.154 ± 0.0147 (744)	<0.0001	****
Figure 1G After KCl	0.5509 ± 0.0054 (697)	0.5709 ± 0.0051 (744)	0.0038	**
Figure 2A Nitrendipine response	0.3497 ± 0.0215 (121)	0.3273 ± 0.0196 (138)	0.2968	ns
Figure 2B Conotoxin response	0.1088 ± 0.0087 (222)	0.1681 ± 0.0090 (219)	<0.0001	****
Figure 2C Agatoxin response	0.1478 ± 0.0085 (213)	0.0961 ± 0.0087 (249)	<0.0001	****

Mann-Whitney test was used to assess statistical significance.



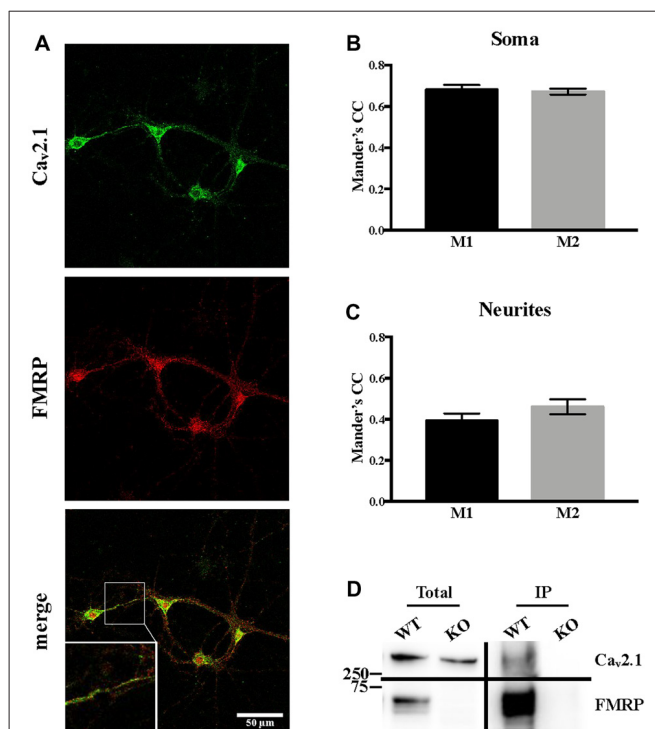


WT primary neurons by performing biotinylation assay. Our results show that $\text{Ca}_v2.1$ protein is less expressed at the cell surface of *Fmr1*-KO neurons (Mann-Whitney test, $P < 0.05$; Figures 4A,B).

Since it was reported that, when overexpressed, FMRP directly interacts with both $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ (Ferron et al., 2014), we assessed whether $\text{Ca}_v2.1$ and FMRP are colocalized in cortical neurons. Using double immunofluorescent staining and confocal microscopy, we observed and quantified their colocalization using Mander's coefficients both in soma and in neurites (Figures 5A–C). These findings were also confirmed by biochemistry experiments performed on cerebellar extracts from PND 13 mice in which we showed that endogenous $\text{Ca}_v2.1$ co-immunoprecipitates with FMRP (Figure 5D).

DISCUSSION

We and others have shown that among the FMRP mRNA targets many encode ion channels, sensors of intracellular ion concentration and other regulators of ion homeostasis (Brown et al., 2001; Darnell et al., 2011; Maurin et al., 2018a). Nonetheless, the direct interaction of FMRP with ion channels has been reported previously (Brown et al., 2010; Ferron et al., 2014; Myrick et al., 2015; Ferron, 2016). Also, it is not surprising that deregulations of expression levels as well as activities of ion channels have been shown in *Fmr1*-KO neurons (Chen et al., 2003; Meredith et al., 2007; Brown et al., 2010; Deng et al., 2013; Ferron et al., 2014; Zhang et al., 2014; Deng and Klyachko, 2016), some directly implicating VGCC deregulation in FXS (Chen et al., 2003; Meredith et al., 2007; Deng et al., 2013; Ferron et al., 2014; Zhang et al., 2014). Even if some of the conclusions of various studies were not completely convergent (Meredith et al.,



2007; Ferron et al., 2014; Zhang et al., 2014), collectively these works suggest that the Ca^{2+} signaling-associated pathways may be involved in the physiopathology of FXS. For this reason, we decided to study calcium homeostasis in live, cultured neurons in the presence and in the absence of FMRP, using calcium imaging.

FMRP Regulates VGCC Expression and Function

VGCCs play key roles in neurons, notably by regulating membrane excitability, neurotransmitter release and gene expression modulation (Simms and Zamponi, 2014). Alterations in the plasma membrane expression of these channels lead to pathological phenotypes, ranging from ataxia, ID, ASD and epilepsy (Yue et al., 1997; Damaj et al., 2015). Thus, to gain further insight in the Ca^{2+} pathway-associated molecular pathology in FXS, we carried out a pharmacological approach using VGCC-specific antagonists in our cellular model. We showed that both N- and P/Q-type VGCC inhibition differently affected KCl-mediated entry in WT and *Fmr1*-KO neurons. Indeed, blocking N-type VGCCs was more efficient in *Fmr1*-KO than in WT neurons and conversely, P/Q-type inhibition had less effect in *Fmr1*-KO neurons, suggesting that both $\text{Ca}_v2.2$

and Ca_v2.1 activities are deregulated in the absence of FMRP. Interestingly, *Cacna1a* mRNA is a target of FMRP in various brain regions (Maurin et al., 2018a) and here we show that:

1. The membrane levels of Ca_v2.1 channels are reduced in *Fmr1*-KO neurons, consistent with the reduced sensitivity to P/Q-type VGCC inhibition with Agatoxin. Since the intracellular levels of Ca_v2.1 do not appear to be altered (Figure 4), we conclude that Ca_v2.1 direct interaction with FMRP could play a role in its function/localization in the absence of the partner. Also, the altered actin cytoskeleton organization described in different FXS cell lines (Castets et al., 2005; Nolze et al., 2013; Abekhoukh and Bardoni, 2014; Abekhoukh et al., 2017) may explain the reduced membrane expression of Ca_v2.1, since cytoskeleton is the route for the correct subcellular localization of mRNAs (Bramham and Wells, 2007). It is worth reminding that altered sublocalization of membrane proteins (encoded by mRNA targets of FMRP) have been already described, such as diacylglycerol lipase- α (DGL- α ; Jung et al., 2012), Homer 1 (Giuffrida et al., 2005; Aloisi et al., 2017) and Kv4.2, (Gross et al., 2011). Similarly, Ca_v2.1 could be one of the deregulated elements. Interestingly, FMRP binds the mRNAs of other of its interacting proteins such as FMRP, CYFIP2, FXR1, Ca_v2.2 (Darnell et al., 2011; Maurin et al., 2018a), suggesting a tight regulation of a FMRP-containing complex in a FMRP-dependent manner. Furthermore, the multiple mRNA targets of FMRP likely generate a network of interactions among FMRP-dependent pathways whose functional consequences are not easily predictable only considering the main role of FMRP as a translational repressor.
2. Even if the level of the mRNA encoding *Cacna1a* is slightly decreased in *Fmr1*-KO neurons at DIV21 (Figure 3A), the translational upregulation of this mRNA (as predicted by the increased polyribosome association of Ca_v2.1 mRNA in *Fmr1*-KO brain compared with WT; Figure 3H) counterbalances the reduced mRNA level of *Cacna1a* in mature neurons. As in a yin-yang effect, this leads to unaltered total Ca_v2.1 levels. We did not find any FMRP-dependent effect on RNA stability of *Cacna1a*, leading to the conclusion that the reduced level of *Cacna1a* mRNA in *Fmr1*-KO neurons is rather due to an indirect transcriptional deregulation.

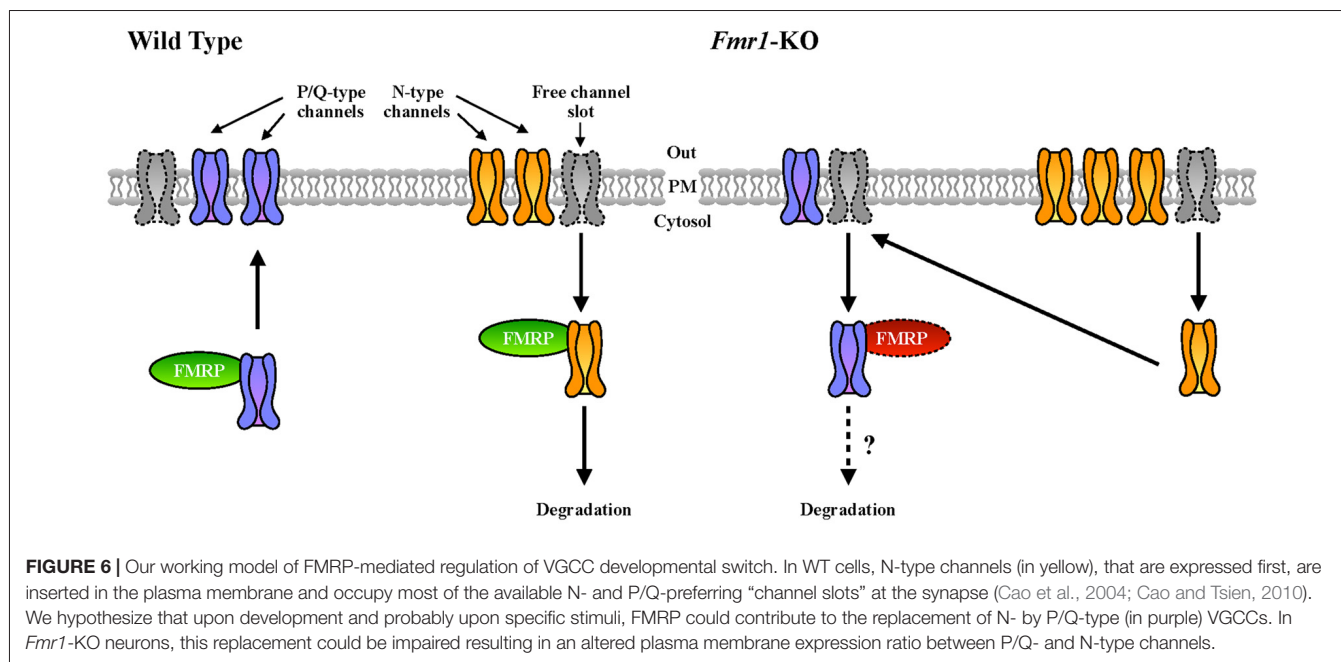
Pre-synaptic Calcium Channels in FXS and ASD

Ca_v2.2 was previously described to be more expressed and present at the plasma membrane of cells in the absence of FMRP (Ferron et al., 2014). This is consistent with the increased sensitivity to conotoxin that we observed in *Fmr1*-KO neurons compared to WT. At the molecular level, this abnormality was explained on the basis of the interaction (by overexpression) between FMRP and both Ca_v2.2 and Ca_v2.1 channels (Ferron et al., 2014). Interestingly, we confirmed here this latter finding by showing that the interaction between the endogenous proteins also occurs in brain (Figure 5D). Remarkably, we showed here that in *Fmr1*-KO cells Ca_v2.1

expression deregulation is opposite to the one of Ca_v2.2 (Ferron et al., 2014). As we already stated, FMRP also binds Ca_v2.1 mRNA transcripts, indeed strongly suggesting a central role of FMRP in the regulation of P/Q- and N-type channels relative expression. Interestingly, it was shown that in cultured hippocampal synapses, P/Q- and N-type channels have preferred plasma membrane slots (Cao et al., 2004; Cao and Tsien, 2010) and according to this model, there are exclusive N-type channel slots and P/Q- preferring slots that can be used by N-type channels. For instance, in neurons expressing mutated P/Q-channels that lead to familial hemiplegic migraine type disease, N-type channel currents are increased, either by an increased release probability or rather by an increased N-type expression at the plasma membrane (Cao and Tsien, 2010). Collectively, these findings suggest that some P/Q-type channel slots can actually be occupied by N-type channels upon P/Q-type deficiency. Since FMRP has been shown previously to regulate N-type expression by targeting this channel to the proteasome (Ferron et al., 2014), it is tempting to speculate that FMRP is a molecular adaptor regulating the relative plasma membrane expression of N- and P/Q-type channels. In addition, by regulating the subcellular mRNA localization and/or translation of these channel types, it may also directly modulate their presence at the plasma membrane (Figure 6). Future studies will clarify the precise molecular mechanisms underpinning this deregulation in FXS, but it is interesting to underline here that an imbalance between the levels and the activities of N- and P/Q-type channels, could have some impacts on the physiopathology of FXS. Indeed, the differences in N- and P/Q-type inactivation kinetics, their various effects on short term plasticity (Inchauspe et al., 2004) and their different sensitivity to G-protein-coupled receptor-mediated inhibition of neurotransmitter release may have strong impacts on the functioning of synapses (Bourinet et al., 1996). Noteworthy, P/Q-type channel activity, but not N-type, mediates GABA release in fast spiking interneurons in rat pre-frontal cortex (Zaitsev et al., 2007). This suggests that abnormal GABA secretion at the temporoammonic branch of the perforant path in the *Fmr1*-KO mouse model (Wahlstrom-Helgren and Klyachko, 2015) could be related to Ca_v2.1 expression defects. Furthermore, it was reported that the maximal inhibition by the GABAB receptor agonist baclofen was greater for EPSCs mediated by N-type channels than for those mediated by P/Q-type channels (Ishikawa et al., 2005). Consequently, in *Fmr1*-KO mice it is likely that the compensation of P/Q- by N-type channels have strong consequences on GABAB inhibition by weakening its effect on presynaptic release, likely leading to network hyper-excitability.

Impairment of Calcium Homeostasis as a New Phenotype of *Fmr1*-KO Neurons. Is It a Novel Biomarker?

Implications of our findings are twofold, biological and clinical. Indeed, the FXS research field actively seeks new treatments and biomarkers to evaluate their efficiency (Castagnola et al., 2017; Maurin et al., 2018b) and, to date, the main cellular



biomarker of cultured *Fmr1*-KO neurons is represented by their abnormal dendritic spine morphology, whose analysis requires exquisite expertise (Khayachi et al., 2018). Conversely, using spectroscopy, calcium concentration measurements can be routinely performed in most laboratory settings, making it an easy and robust marker to monitor drug efficacy. Here, we applied this technique to primary cultured neurons but it will also be possible to perform it in iPS-derived neurons thus obtaining, for the first time, a molecular marker that can be functionally quantified. This can be useful for diagnostic purposes and particularly as a follow-up for specific therapies. Indeed, the search for specific and easily measurable biomarkers for FXS as well as for ASD is urgent. For instance, since 2009 one of the conclusions of the Outcome Measures Working Groups for Fragile X was “...research on biomarkers for detecting treatment response in FXS was in its infancy, but this was an area of utmost importance” (Berry-Kravis et al., 2013). More recently, the accurate analysis of 22 double-blind controlled clinical trials in FXS finalized between 2008 and 2015 led to the conclusion that the readouts employed to evaluate the outcome of treatments were in general of moderate/poor quality (Budimirovic et al., 2017). Last but not least, this cellular biomarker could be used as the readout for screenings of small-molecule (singular) libraries (Bardoni et al., 2017) to define new treatments opportunities for FXS.

Study Limitations

There are several limitations of this study that one may consider:

1. Our ImageJ macro analysis resulted in the identification of four types of cells, which is clearly underestimating the complexity of the cell population. We nevertheless trust that this approach will be useful to identify a cell type of

interest in the future, associating morphological parameters with molecular/physiological determinants; interestingly, Ota et al. (2018) very recently published a study highlighting the benefits of identifying cells according to their shape;

2. In agreement with the expression levels of $Ca_v2.1$, we focused our study on mature neuron cultures. This VGCC deregulation may not be observed in different culture settings;
3. The polyribosome fractionation experiments were performed on cortex extracts from PND 13 mice, preventing the identification of actively translating ribosomes through pharmacological inhibition. Therefore, we can only speculate that the increased presence of *Cacna1a* mRNA in light and medium fractions reflects an increased translation of this mRNA in *Fmr1*-KO mice;
4. The working model describing the putative role of FMRP in the regulation of N- and P/Q-type VGCCs at the plasma membrane (Figure 6) awaits a molecular mechanism and therefore is speculative. It nevertheless may be considered as a starting point for future analyses.

AUTHOR CONTRIBUTIONS

TM, FD and AF designed the experiments. SC, SD, AF, MB, MJ, MG and TM performed the experiments. FB designed and wrote the macro in ImageJ. AP performed the unsupervised multivariate analysis. TM, SC, AF, AP, MM, SM and BB analyzed the data. TM, SC and BB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2018.00342/full#supplementary-material>

FIGURE S1 | Cortical primary neuronal cultures show a negligible level of astrocytic growth. **(A)** Fluorescent analysis showing the level of GFAP (in green) in Day-In-Vitro (DIV) 12 WT primary neuronal cultures compared to the total number of cells (DAPI staining in blue for nuclei). **(B)** Percentage of GFAP-positive cells (20 imaged regions; $n = 261$ DAPI-positive cells; $n = 23$ GFAP-positive cells).

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(C) Quantification of *Gfap* and *Psd95* (*Dlg4* transcript) mRNA levels in DIV 20 cortical neurons ($n = 3$ independent cultures). Results are presented as the mean $\pm$ SEM, Mann-Whitney test: ns, not significant (*Gfap*: $P = 0.3701$; *Psd95*: $P = 0.6200$).

FIGURE S2 | Unsupervised analysis of the shape and calcium homeostasis parameters of primary neuron cultures leads to the identification of four different groups of Regions-of-Interest (ROIs). Shape and calcium homeostasis parameters were first visualized in 2-dimension space using t-Distributed Stochastic Neighbor Embedding (t-SNE), then K-means clustering was performed on the 2-dimension t-SNE projection, and the optimal number of clusters, was determined using the Gap statistic. **(A)** t-SNE representation of the data, with cells colored by genotype. The distribution of WT (black dots) and *Fmr1*-KO (red dots) is homogeneous and vastly overlapping in all clusters. **(B)** t-SNE representation of the data, with cells colored by cluster. The distribution of the parameters of interest by clusters are presented using boxplots. The boxplots are defined as 25th percentile–75th percentile, the horizontal line corresponds to the median value, and whiskers extend to the min-max values. **(C)** Area covered by the cells, **(D)** cell circularity, **(E)** cell solidity, **(F)** cell roundness, **(G)** cell resting intracellular calcium concentration and **(H)** maximal KCl-triggered intracellular calcium concentration. **(I–L)** Number of cells from each genotype in the various experiments: ω -agatoxin-IVa (Aga), ω -conotoxin GVIA (Cono), Nitrendipine (Nitren) or in the absence of VGCC antagonist (NoDrug) show the homogeneous WT and *Fmr1*-KO cell distribution in all identified clusters.

FIGURE S3 | Representative images of ROIs identified using the ImageJ macro. Left panels are pseudo-colored images of stabilized unstimulated cells **(a, d, g, j)**. Middle panels represent the same cells during KCl stimulation **(b, e, h, k)**. Right panels show the macro output result **(c, f, i, l)**. The ROIs are encircled by a yellow line and the numbers indicate to which cluster ROI were attributed.

FIGURE S4 | The percentage of DHPG-responding cells is similar in WT and *Fmr1*-KO neurons. Cells in which the pharmacological stimulation elicited at least a 1.1 fold change in the $F_{340/380}$ ratio compared to baseline $F_{340/380}$ were considered DHPG-responsive and were counted in each cell cluster.

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Activation of Serotonin 5-HT₇ Receptors Modulates Hippocampal Synaptic Plasticity by Stimulation of Adenylate Cyclases and Rescues Learning and Behavior in a Mouse Model of Fragile X Syndrome

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We have previously demonstrated that activation of serotonin 5-HT₇ receptors (5-HT₇R) reverses metabotropic glutamate receptor-mediated long term depression (mGluR-LTD) in the hippocampus of wild-type (WT) and *Fmr1* Knockout (KO) mice, a model of Fragile X Syndrome (FXS) in which mGluR-LTD is abnormally enhanced. Here, we have investigated intracellular mechanisms underlying the effect of 5-HT₇R activation using patch clamp on hippocampal slices. Furthermore, we have tested whether *in vivo* administration of LP-211, a selective 5-HT₇R agonist, can rescue learning and behavior in *Fmr1* KO mice. In the presence of an adenylate cyclase blocker, mGluR-LTD was slightly enhanced in WT and therefore the difference between mGluR-LTD in WT and *Fmr1* KO slices was no longer present. Conversely, activation of adenylate cyclase by either forskolin or Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) completely reversed mGluR-LTD in WT and *Fmr1* KO. 5-HT₇R activation reversed mGluR-LTD in WT and corrected exaggerated mGluR-LTD in *Fmr1* KO; this effect was abolished by blockade of either adenylate cyclase or protein kinase A (PKA). Exposure of hippocampal slices to LP-211 caused an increased phosphorylation of extracellular signal regulated kinase (ERK), an intracellular effector involved in mGluR-LTD, in WT mice. Conversely, this effect was barely detectable in *Fmr1* KO mice, suggesting that 5-HT₇R-mediated reversal of mGluR-LTD does not require ERK stimulation. Finally, an acute *in vivo* administration of LP-211 improved novel object recognition (NOR) performance in WT and *Fmr1* KO mice and reduced stereotyped behavior in *Fmr1* KO mice. Our results indicate that mGluR-LTD in WT and *Fmr1* KO slices is bidirectionally modulated in conditions of either reduced or enhanced cAMP formation. Activation of 5-HT₇ receptors reverses mGluR-LTD by activation of the cAMP/PKA intracellular pathway.

Importantly, a systemic administration of a 5-HT₇R agonist to *Fmr1* KO mice corrected learning deficits and repetitive behavior. We suggest that selective 5-HT₇R agonists might become novel pharmacological tools for FXS therapy.

Keywords: serotonin, 5-HT₇ receptor, fragile X syndrome, cyclic AMP, mGluR-LTD, learning, PACAP

INTRODUCTION

Fragile X Syndrome (FXS), the most common single-gene cause of intellectual disability, autism and epilepsy (Garber et al., 2008), is caused by transcriptional silencing of the FMR1 gene coding for Fragile X Mental Retardation Protein (FMRP), an mRNA-binding protein that regulates translation of several synaptic proteins (Pfeiffer and Huber, 2009). An abnormal morphology and density of dendritic spines was observed in the brain cortex of FXS patients (Irwin et al., 2001) and in the cortex and hippocampus of *Fmr1* knockout (KO) mice (Comery et al., 1997; Nimchinsky et al., 2001; Grossman et al., 2010), a model of FXS, suggesting a dysfunction of excitatory synaptic transmission in several brain regions. Accordingly, studies on the mouse model of FXS revealed altered synaptic plasticity mediated by metabotropic glutamate receptors (mGluRs; Pfeiffer and Huber, 2009). Metabotropic glutamate receptor-mediated long term depression (mGluR-LTD), a form of plasticity playing a crucial role in cognition and in behavioral flexibility (Luscher and Huber, 2010; Sanderson et al., 2016), is pathologically enhanced in the hippocampus of *Fmr1* KO mice (Huber et al., 2002) and is regarded as the electrophysiological readout of synaptic malfunction in the mouse model of FXS (Bear et al., 2004; Waung and Huber, 2009).

In the last few years, a decrease of cyclic AMP (cAMP) has been proposed to be involved in FXS pathogenesis (Kelley et al., 2008). Different data support a “cAMP theory” of FXS: early observations indicated a reduction of basal cAMP levels in blood platelets from FXS patients and a decrease in cAMP production following adenylate cyclase stimulation (Berry-Kravis and Huttenlocher, 1992; Berry-Kravis and Sklena, 1993). Reduced cAMP production was later detected in the brain of *dfmr1* null drosophila, in brain and blood platelets of *Fmr1* KO mice and in neural precursor cells from human FXS fetal tissues (Kelley et al., 2007). Besides, pharmacological manipulation with agents that potentially increase cAMP, i.e., inhibitors of group II mGluRs and phosphodiesterase IV inhibitors (PDE4-Is), reversed the mGluR-LTD alteration in FXS mouse models (Choi et al., 2011, 2015, 2016), leading to the hypothesis that increasing cAMP formation might become a potential therapeutic strategy to rescue FXS phenotype.

We have previously demonstrated that exaggerated mGluR-LTD in *Fmr1* KO mice was reversed by activation of 5-HT₇ receptors (5-HT₇Rs) for serotonin (Costa et al., 2012a, 2015). 5-HT₇Rs are positively coupled to adenylate cyclase and are highly expressed in the hippocampus, where they are believed to regulate learning and memory (Matthys et al., 2011; Ciranna and Catania, 2014).

In the present work, we have tested the hypothesis that a dysregulation of cAMP pathway might play a role in abnormal mGluR-LTD in *Fmr1* KO hippocampal neurons. In this perspective, we evaluated whether the activation of 5-HT₇Rs, coupled to Gs, rescues mGluR-LTD by increasing cAMP levels. We also tested whether *in vivo* administration of a 5-HT₇R agonist could rescue learning ability and the behavioral phenotype in a mouse model of FXS.

MATERIALS AND METHODS

Animals

All experiments were performed in mice obtained from a breeding colony kept at the University of Catania. We used *Fmr1* KO mice and wild-type (WT) littermates from C57BL/6J strain for electrophysiology and behavioral experiments; both FVB and C57BL/6J strains were used for Western blotting. We crossed homozygous *Fmr1* KO females with hemizygous *Fmr1* KO males and both male and female pups were used in our experiments. Mice were maintained with a controlled temperature (21°C ± 1°C) and humidity (50%) on a 12 h light/dark cycle, with *ad libitum* food and water. All animal experimentation was conducted in accordance with the European Community Council guidelines (2010/63/EU) and was approved by the University Institutional Animal Care and Use Committee (Projects #181; 250—approval number: 352/2016-PR, Project #286—approval number: 174/2017-PR).

Electrophysiology

Acute hippocampal slices were prepared as previously described (Costa et al., 2012b) from WT and *Fmr1* KO mice on a C57BL/6J background [postnatal (PN) age 14–23 days]. The brains were removed, placed in oxygenated ice-cold artificial cerebrospinal fluid (ACSF; in mM NaCl 124; KCl 3.0; NaH₂PO₄ 1.2; MgSO₄ 1.2; CaCl₂ 2.0; NaHCO₃ 26; D-glucose 10, pH 7.3) and cut into 300 μm slices with a vibratome (Leica VT 1200S). Slices were continually perfused with oxygenated ACSF and viewed with infrared microscopy (Leica DMLFS). Schaffer collaterals were stimulated with negative current pulses (duration 0.3 ms, delivered every 15 s by A310 Accupulser, WPI, USA). Evoked excitatory post synaptic currents (EPSCs) were recorded under whole-cell from CA1 pyramidal neurons (holding potential −70 mV; EPC7-plus amplifier HEKA, Germany). Stimulation intensity was set to induce half-maximal EPSC amplitude. Series resistance (Rs) was continuously monitored by 10 mV hyperpolarizing pulses; recordings were discarded from analysis if Rs changed by more than 20%. EPSC traces were filtered at 3 kHz and digitized at 10 kHz. Data were acquired

and analyzed using Signal software (CED, England). The recording micropipette (resistance 1.5–3 M Ω) was filled with intracellular solution (in mM: K-gluconate 140; HEPES 10; NaCl 10; MgCl₂ 2; EGTA 0.2; Mg-ATP 3.5; Na-GTP 1; pH 7.3).

To isolate AMPA receptor-mediated EPSCs, bath solution (ACSF; flow rate of 1.5 ml/min) routinely contained (-)-bicuculline methiodide (5 μ M, Hello Bio) and D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 μ M, Hello Bio).

(S)-3,5-dihydroxyphenylglycine (DHPG; 100 μ M; Hello Bio), forskolin (20 μ M; Tocris), PACAP-38 (PACAP, 10 nM, Tocris) and LP-211 (10 nM) were dissolved in ACSF and applied by bath perfusion. LP-211 was synthesized and provided by the research group of Prof. Leopoldo (University of Bari, Italy). SQ-22536 (10 μ M, Tocris), protein kinase A (PKA) inhibitor fragment 6–22 (PKI; 20 μ M, Tocris) and PD-98059 (40 μ M, Tocris) were included in the intracellular solution.

LTD Data Analysis

Peak amplitude values of EPSCs were averaged over 1 min and expressed as % of baseline EPSC amplitude (calculated from EPSCs recorded during at least 15 min before DHPG application). % EPSC values from groups of neurons were pooled (mean $\pm$ standard error of mean, SEM) and graphically represented as a function of time. For each neuron studied, the amount of mGluR-LTD was calculated by averaging EPSC values recorded during 5 min between 40 min and 45 min after LTD induction and was expressed as percentage of baseline (% EPSC amplitude). Cumulative bar graphs indicate % EPSC amplitude (mean $\pm$ SEM from groups of neurons) after application of DHPG alone (control LTD) or DHPG with the 5-HT₇ receptor agonist under different experimental conditions. EPSC amplitude values from two groups of neurons were compared using unpaired Student's *t*-test, with *n* indicating the number of neurons tested in each condition; several groups of data were compared by one-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Prism 6, La Jolla, CA, USA).

Western Blotting

We used brains of WT (FVB and C57BL/6J strains) and *Fmr1* KO (C57BL/6J strains) mice for stimulation assay. Hippocampi from mice at PN age 14–23 days were dissected, quickly cut into 350 μ m slices using a McIlwain tissue chopper and transferred to oxygenated ice-cold artificial cerebrospinal fluid (ACSF; in mM NaCl 124, KCl 3, CaCl₂ 2, NaHCO₃ 25, NaH₂PO₄ 1.1, MgSO₄ 2, D-glucose 10, pH 7.4) containing protease inhibitor cocktails EDTA free (Roche) and (+)-MK 801 maleate (1 μ M; Tocris). Thereafter, slices were pre-incubated with ACSF containing protease inhibitor cocktails EDTA free (Roche) and (+)-MK 801 maleate (1 μ M; Tocris) for 35 min at 32°C. Then, slices were exposed to LP-211 (10 nM) for 5 min; when present, H-89 dihydrochloride (1 μ M; Tocris) was added 5 min before stimulation with LP-211 and maintained during LP-211 stimulation. Then, slices were washed three times in ice-cold ACSF and immediately frozen at –80°C until use.

Frozen slices were homogenized in ice-cold extraction buffer [Tris 50 mM pH 8; NaCl 150 mM; MgCl₂ 1.5 mM; NP40 1%; protease inhibitor cocktail EDTA free and phosphatase inhibitor cocktail (Roche)], and centrifuged for 30 min at 18,000 $\times$ g at 4°C. The supernatant was collected and one aliquot was used for protein determination using the BCA methods (Pierce BCA protein assay kit). 80–100 μ g of proteins were eluted with SDS sample buffer and separated onto 9% SDS-PAGE, as previously described (Bonaccorso et al., 2015). The following primary antibodies were used for Western blotting: polyclonal rabbit p44/42 MAP-kinase antibody (1:1,000, Cell Signaling), polyclonal rabbit phospho-p44/42 MAP-kinase antibody (1:1,000, Cell Signaling), while polyclonal rabbit GAPDH antibody (1:1,000, Cell Signaling) was used as a loading control.

Blots were developed by using the specific Western Breeze Chemiluminescent Immunodetection Kit (Invitrogen). Band densities were measured using ImageJ 1.49 software (Figure 5; Supplementary Figure S2) or the VersaDoc 4000 Imaging System (Bio-Rad, Figure 5; Supplementary Figure S3). The expression levels of phospho-extracellular signal regulated kinase (ERK)1/2 and ERK1/2 were normalized first against the levels of respective GAPDH and then calculated as ratio of total ERK signal.

Behavior

Novel object recognition (NOR) test was performed as previously described (Puzzo et al., 2013) on sex-balanced WT and *Fmr1* KO mice (C57BL/6J background; age 3–4 months). After 3 days of habituation (10 min/day), mice underwent the training session (T1). They were placed in the arena for 10 min, a time sufficient to learn the task, and allowed to explore two identical objects, i.e., two glass beakers upside-down placed in the central part of the box, equally distant from the perimeter. Thirty minutes before T1 they received a i.p. injection of LP-211 (3 mg/Kg) or vehicle. Twenty-four hours after T1 mice underwent the second trial (T2) where a “familiar” (i.e., the one used for T1) and a “novel” object (ceramic cup) were presented to test memory retention. The novel object was placed on the left or the right side of the box in a randomly but balanced manner, to minimize potential biases because of a preference for particular locations or objects. To avoid olfactory cues, the objects and the apparatus were cleaned with 70% ethanol after each trial. Animal exploration, defined as the mouse pointing its nose toward the object from a distance not >2 cm (as marked by a reference circle), was evaluated in T2. We analyzed: (i) % exploration of the novel and % exploration of the familiar object; (ii) discrimination (D) index calculated as “exploration of novel object minus exploration of familiar object/total exploration time”; (iii) latency to first approach to novel object; and (iv) total exploration time.

Marble burying test was performed as previously described (Thomas et al., 2009), under standard room lighting and noise conditions. Twenty green glass marbles (15 mm in diameter) were arranged in a clean standard cage filled with a sani-chips bedding in a 4 $\times$ 5-cm pattern. Each mouse was gently placed into the cage and allowed to explore for 20 min. The

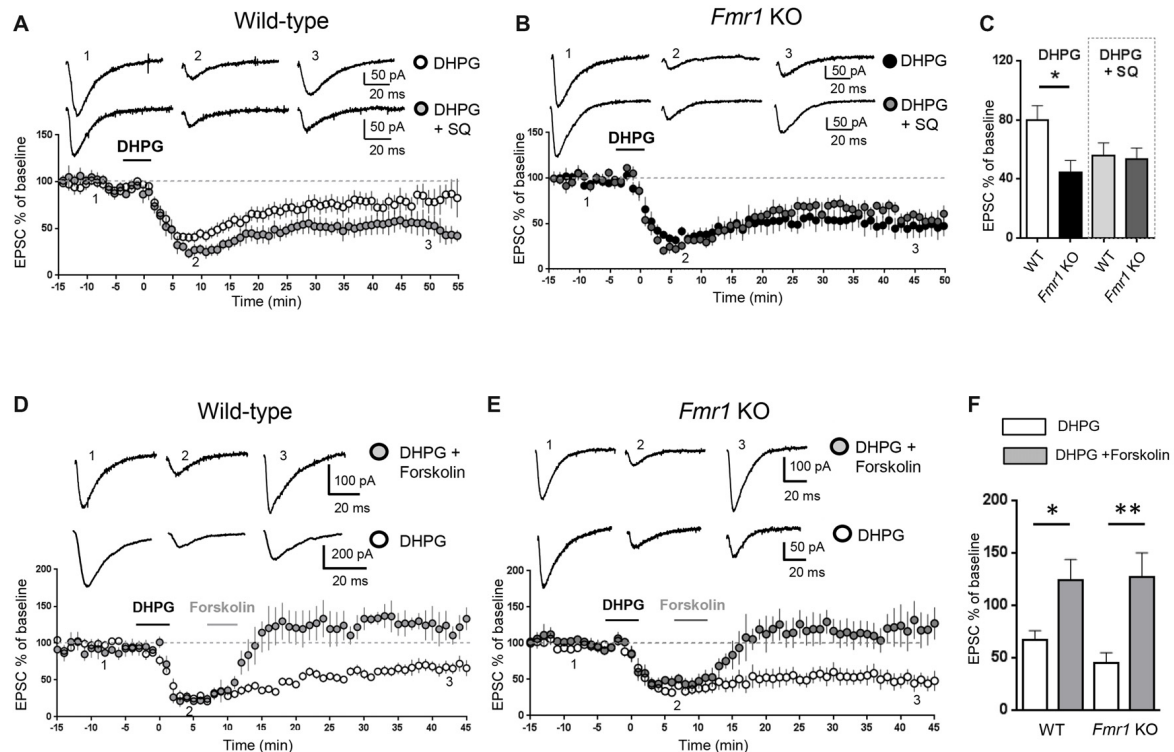


FIGURE 1 | Modulation of adenylyl cyclase activity modified metabotropic glutamate receptor-mediated long term depression (mGluR-LTD) in wild-type (WT) and *Fmr1* Knockout (KO) slices. AMPAR-mediated excitatory post-synaptic currents (EPSCs) were recorded in the presence of D-AP5 (50 μ M) and bicuculline (5 μ M) under whole-cell patch clamp in the CA3–CA1 synapse in hippocampal slices from WT and *Fmr1* KO mice. **(A)** In WT slices, bath application of the group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG; 100 μ M, 5 min) induced a long-term depression (mGluR-LTD) of EPSC amplitude (white dots, $n = 11$). When the adenylyl cyclase blocker SQ 22536 (SQ, 10 μ M) was added to intracellular medium, DHPG-induced mGluR-LTD was slightly enhanced (gray dots, $n = 8$) with respect to control. Individual representative EPSC traces are shown on top of the graph (1: baseline; 2: acute EPSC reduction; 3: mGluR-LTD). **(B)** In *Fmr1* KO slices, DHPG-induced mGluR-LTD (black dots, $n = 9$) was enhanced with respect to WT **(A)**, white dots) and was not further enhanced in the presence of intracellular SQ 22536 (SQ, 10 μ M; dark gray dots, $n = 6$). **(C)** The bar graph shows the amount of mGluR-LTD (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude). The amount of mGluR-LTD was compared by one-way ANOVA followed by Tukey's multiple comparisons test in the four experimental conditions illustrated in **(A,B)** ($*P = 0.03$). In control conditions (DHPG), mGluR-LTD in *Fmr1* KO was significantly enhanced with respect to WT ($*P = 0.025$, by unpaired t -test). In the presence of intracellular SQ 22536 (DHPG+SQ), the amount of mGluR-LTD in WT was not significantly different from *Fmr1* KO ($P = 0.83$, by unpaired t -test) and was also comparable to that observed in *Fmr1* KO in control conditions ($P = 0.35$ by unpaired t -test). **(D)** When DHPG application was followed by bath application of forskolin, a direct activator of adenylyl cyclase (20 μ M, 5 min), mGluR-LTD was reduced (gray dots, $n = 4$) with respect to control conditions (white dots; $n = 5$). **(E)** The same result was observed in *Fmr1* KO slices: DHPG-induced mGluR-LTD (white dots; $n = 9$) was completely reversed by application of forskolin (20 μ M, 5 min; gray dots, $n = 5$). **(F)** Reversal of mGluR-LTD by forskolin was statistically significant both in WT ($*P = 0.025$; by unpaired t -test) and in *Fmr1* KO ($**P = 0.0026$).

number of marbles buried (covered by >50% bedding) was recorded.

Open field (OF) was performed as previously described (Palmeri et al., 2016). Each mouse was gently put in the arena (a white plastic box divided into sectors by black lines) and was allowed to freely explore the environment for 5 min. The test was performed in a quiet, darkened room and one light bulb provided a bright illumination. We scored the following parameters: (i) % time spent into the center; (ii) number of entries into the center; (iii) “horizontal activity”, time spent moving into the arena; (iv) rearing or “vertical activity”, time spent erected on its hind legs; (v) grooming (time spent scratching itself with the forepaws); (vi) freezing (time of immobility); and (vii) defecation (number of fecal boli produced).

RESULTS

Modulation of Adenylyl Cyclase Activity Modified the Amount of mGluR-LTD in WT and *Fmr1* KO Hippocampus

Excitatory post-synaptic currents (EPSCs) mediated by AMPA receptors were recorded in the CA3–CA1 synapse on hippocampal slices from WT and *Fmr1* KO mice. mGluR-LTD of EPSCs was chemically induced by bath application of the mGluR agonist DHPG (100 μ M, 5 min). We have previously confirmed (Costa et al., 2012a) that mGluR-LTD in *Fmr1* KO slices is enhanced compared to WT, consistent with previous findings (Huber et al., 2002). To test if changes in intracellular cAMP levels might affect mGluR-LTD, we measured the amount

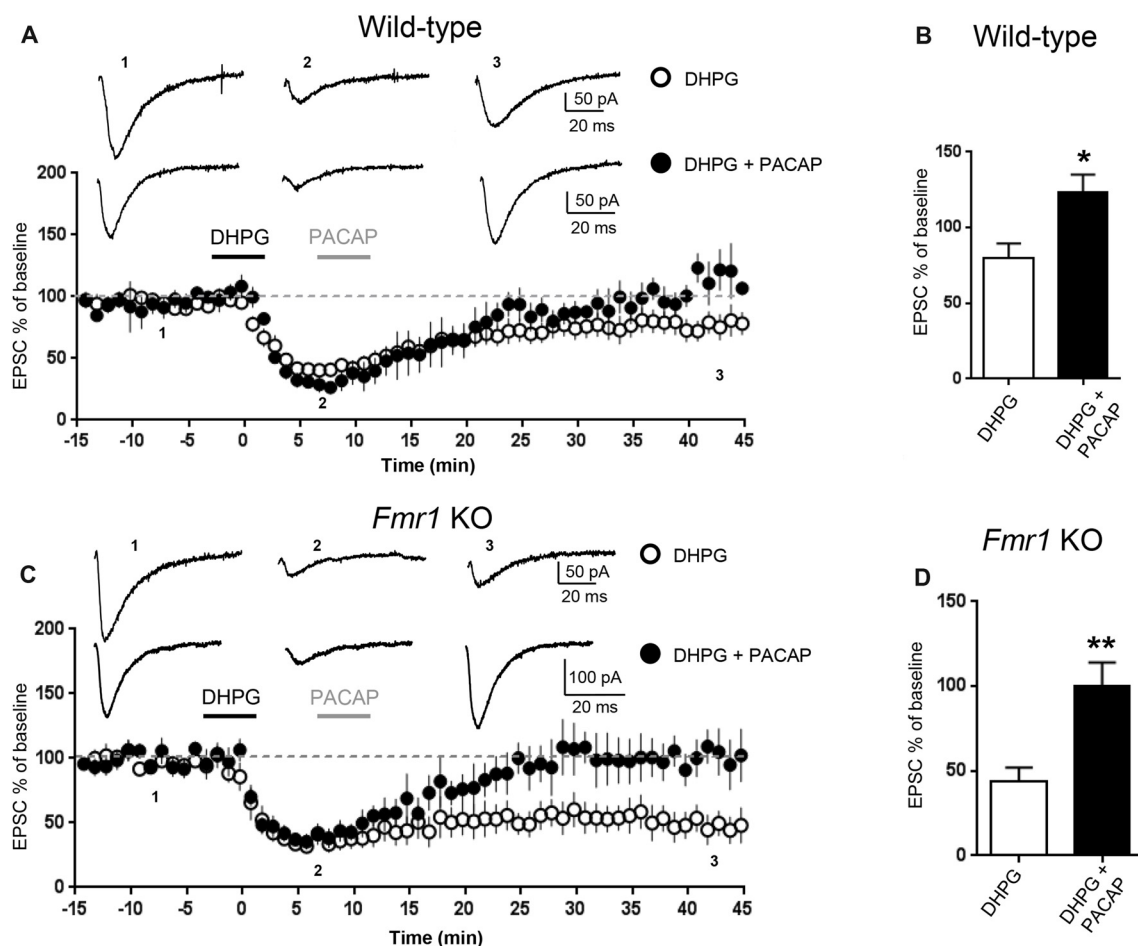


FIGURE 2 | Pituitary adenylate cyclase activating peptide (PACAP) reversed mGluR-LTD in WT and in *Fmr1* KO hippocampus. mGluR-LTD was induced by application of DHPG (100 μ M, 5 min). Application of PACAP (10 nM, 5 min) fully reversed DHPG-induced mGluR-LTD in WT (**A**) and in *Fmr1* KO slices (**C**). (**B,D**) Reversal of mGluR-LTD by PACAP was statistically significant both in WT (* P = 0.04, by unpaired t -test) and in *Fmr1* KO slices (** P = 0.0025, unpaired t -test).

of mGluR-LTD in hippocampal slices from WT and *Fmr1* KO mice under experimental conditions reducing or enhancing cAMP levels in the recorded neuron. When the adenylylase inhibitor SQ 22536 (10 μ M) was included in the intracellular pipette solution (**Figure 1A**), the amount of mGluR-LTD in WT slices showed a trend towards an enhancement compared to control conditions, although not statistically significant (EPSC % amplitude measured 45 min after LTD induction: 79.5 ± 10 , vs. 56 ± 9 comparing control vs. SQ 22536, $n = 11/7$; unpaired t -test: $t_{(16)} = 1.56$; $P = 0.07$).

In *Fmr1* KO slices, the amount of mGluR-LTD in control conditions was significantly higher than in WT (EPSC %: 44 ± 8 vs. 79.5 ± 10 , comparing *Fmr1* KO vs. WT, $n = 9/11$; $t_{(18)} = 2.44$; $P = 0.025$; **Figure 1C**) and was not further enhanced by the adenylylase blocker (EPSC %: 44 ± 8 vs. 53 ± 7 , comparing *Fmr1* KO control vs. *Fmr1* KO + SQ 22536, $n = 9/6$; $t_{(13)} = 0.76$; $P = 0.45$; **Figures 1B,C**).

When comparing the amount of mGluR-LTD in all the different experimental conditions (**Figure 1C**), a significant difference was found only between WT and *Fmr1* KO in

control conditions ($P = 0.03$ by one-way ANOVA followed by Tukey's multiple comparisons). In the presence of SQ 22536, the amount of mGluR-LTD was comparable in WT and *Fmr1* KO slices (EPSC %: 55 ± 10 vs. 53 ± 7 comparing WT + SQ 22536 vs. *Fmr1* KO + SQ 22536, $n = 7/6$; $t_{(11)} = 0.21$; $P = 0.83$) and was not significantly different from that measured in *Fmr1* KO slices without SQ 22536 ($t_{(14)} = 0.96$; $P = 0.35$).

Conversely, a direct stimulation of adenylylase by bath application of forskolin (20 μ M, 5 min) completely abolished DHPG-induced mGluR-LTD in both WT (EPSC %: 67 ± 8 vs. 124 ± 20 , $n = 5/4$; $t_{(7)} = 2.8$; $P = 0.025$, comparing DHPG vs. DHPG + forskolin, **Figures 1D,F**) and *Fmr1* KO neurons (EPSC %: 45 ± 10 vs. 127 ± 23 , $n = 9/5$; $t_{(12)} = 3.7$; $P = 0.0026$, comparing DHPG vs. DHPG + forskolin, **Figures 1E,F**), showing that mGluR-LTD was reversed by increasing cAMP levels.

To further study the effect of increasing cAMP levels on mGluR-LTD, we tested the effects of Pituitary Adenylylase Activating Polypeptide (PACAP), a potent endogenous stimulator

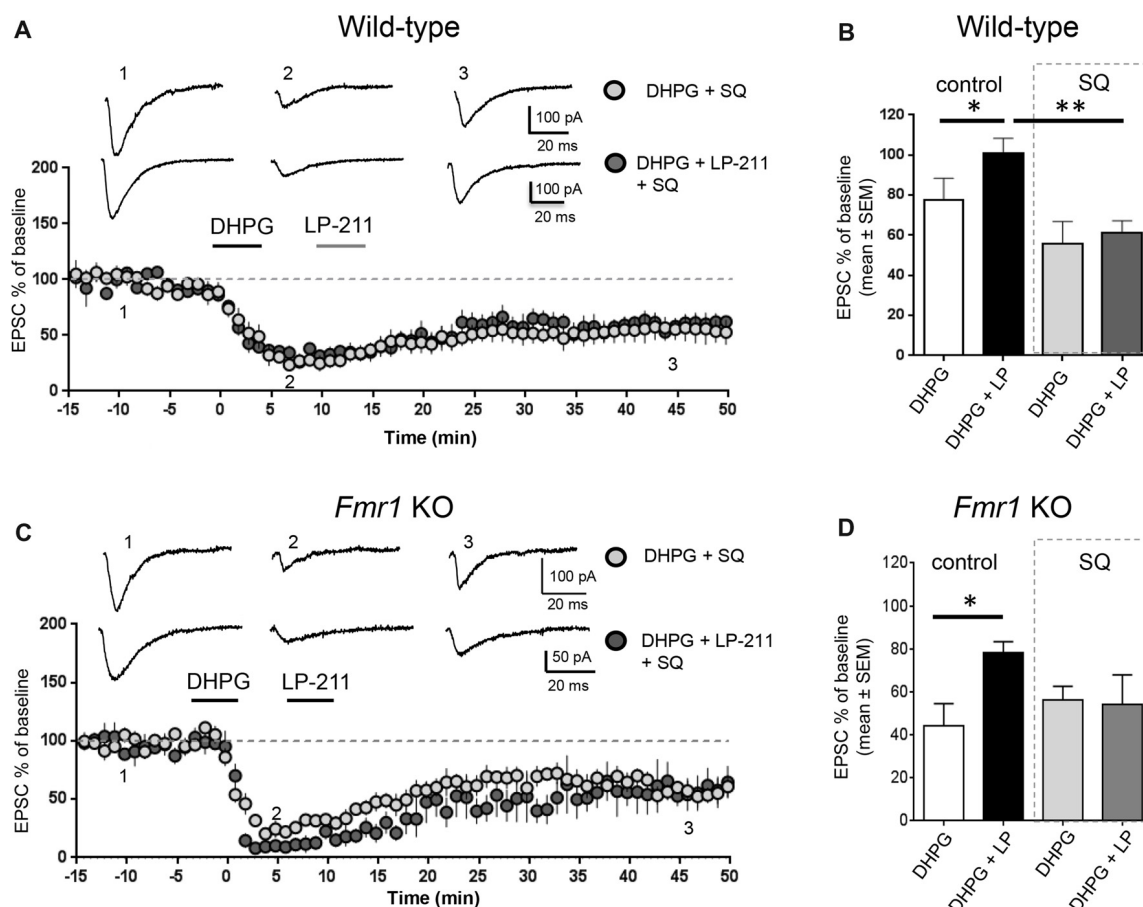


FIGURE 3 | Activation of 5-HT₇ receptors reversed mGluR-LTD by stimulation of adenylate cyclase. **(A)** In hippocampal WT slices, application of LP-211 did not modify mGluR-LTD in the presence of the adenylate cyclase blocker SQ 22536 (10 μ M, included in intracellular pipette solution; dark gray dots, $n = 6$). **(B)** The bar graph shows the amount of mGluR-LTD measured 45 min after LTD induction (mean EPSC amplitude in all tested neurons, expressed as % of baseline EPSC amplitude). In WT slices, bath applications of DHPG-induced mGluR-LTD (white column; $n = 11$) that was completely reversed when DHPG application was followed by application of the 5-HT₇R agonist LP-211 (10 nM, 5 min; black column, $n = 7$; $*P = 0.03$ by unpaired t -test). In the presence of intracellular SQ 22536 (10 μ M; gray column, $n = 6$), the amount of mGluR-LTD was slightly increased with respect to control and was not reversed by application of LP-211 (10 nM, 5 min; dark gray column, $n = 6$; significantly different from the effect of LP-211 in control conditions, $**P = 0.0026$ by unpaired t -test). **(C)** Similarly, in *Fmr1* KO slices application of LP-211 (10 nM, 5 min) had no effect on mGluR-LTD in the presence of intracellular SQ 22536 (10 μ M; dark gray column, $n = 6$). **(D)** In *Fmr1* KO slices, application of LP-211 reversed DHPG-induced mGluR-LTD only in control conditions (black column, $n = 6$; $*P = 0.02$ by unpaired t -test) but not in the presence of SQ 22536 (SQ, 10 μ M; gray column, $n = 6$), showing that 5-HT₇R-mediated effect required stimulation of adenylate cyclase also in *Fmr1* KO slices.

of adenylate cyclase activity (Harmar et al., 2012). Application of PACAP (10 nM, 5 min) reversed DHPG-induced mGluR-LTD both in WT (EPSC% 79.5 ± 10 vs. 123 ± 11 , DHPG vs. DHPG + PACAP, $n = 11/6$, $t_{(15)} = 2.72$, $P = 0.015$, **Figures 2A,B**) and in *Fmr1* KO hippocampus (EPSC% 44 ± 8 vs. 100 ± 14 , DHPG vs. DHPG + PACAP, $n = 9/7$, $t_{(13)} = 3.66$, $P = 0.0025$, **Figures 2C,D**).

Activation of 5-HT₇ Receptors Reversed mGluR-LTD by Stimulation of Adenylate Cyclase and Protein Kinase A

We tested the effect of LP-211, a 5-HT₇R agonist, on mGluR-LTD in the presence of pharmacological modulators of the cAMP/PKA pathway. LP-211 was applied at 10 nM

dose in order to activate selectively 5-HT₇ receptors without activating the 5-HT_{1A} subtype, based on its reported binding affinity for 5-HT₇ and 5-HT_{1A} receptors (K_i 0.58 and 188 nM respectively, see compound 25 in Leopoldo et al. (2008)). In the presence of intracellular SQ 22536 (10 μ M), application of LP-211 did not modify the amount of mGluR-LTD (EPSC %: 55 ± 10 vs. 61 ± 6 , $n = 7/6$; $t_{(11)} = 0.39$, $P = 0.69$ comparing DHPG + SQ 22536 vs. DHPG + SQ 22536 + LP-211; **Figures 3A,B**). Therefore, in the presence of SQ 22536 we did not observe the 5-HT₇R-mediated reversal of mGluR-LTD that we described in control conditions (Costa et al., 2012a, 2015).

Similar to WT, in *Fmr1* KO 5-HT₇R-mediated reversal of mGluR-LTD was abolished by SQ-22536 (EPSC %: 53 ± 7 vs. 54 ± 13 , $n = 6/5$; $t_{(9)} = 0.04$; $P = 0.96$, comparing DHPG

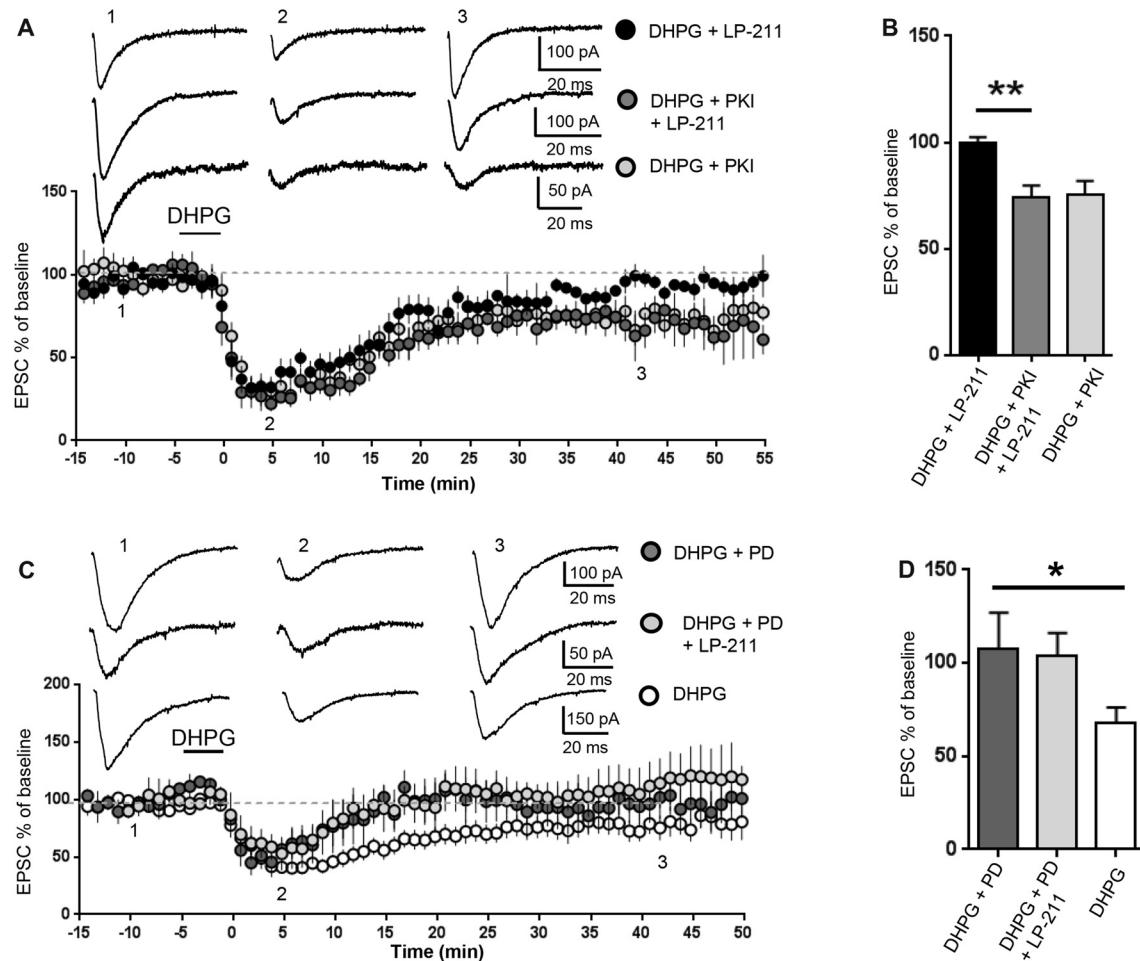


FIGURE 4 | 5-HT₇ receptor-mediated reversal of mGluR-LTD required protein kinase A (PKA) and was occluded by inhibition of extracellular signal regulated kinase (ERK). mGluR-LTD was induced by bath application of DHPG (100 μ M, 5 min) in WT slices in control conditions and in the presence of peptide fragment 6–22 (PKI, added to intracellular solution), an inhibitor of PKA. **(A)** Application of LP-211 (10 nM, 5 min) reversed mGluR-LTD in control conditions (black dots, $n = 7$) but had no effect in the presence of PKI (dark gray dots, $n = 5$). **(B)** In the presence of PKI, the effect of LP-211 was significantly reduced (** $P = 0.0013$, by unpaired t -test), indicating that PKA activation was necessary for 5-HT₇R-mediated reversal of mGluR-LTD. **(C)** mGluR-LTD was induced by bath application of DHPG (100 μ M, 5 min) in WT slices in control conditions (white dots, $n = 11$) and in the presence of the mitogen-activated protein kinase (MAPK)/ERK blocker PD-98059 (PD, 40 μ M, added to intracellular solution). Intracellular PD-98059 completely reversed mGluR-LTD (dark gray dots, $n = 5$) and occluded the effect of LP-211 (10 nM, 5 min; gray dots, $n = 5$). **(D)** mGluR-LTD was significantly reversed by PD-98059 (* $P = 0.03$) and was not further modified by LP-211 ($P = 0.52$).

+ SQ22536 vs. DHPG + SQ22536 + LP-211; **Figures 3C,D**), showing that reversal of mGluR-LTD by 5-HT₇R activation was mediated by cAMP.

We then tested a possible involvement of PKA, one of the main cAMP effector targets (Taylor et al., 1990). The PKA inhibitor peptide fragment 6–22 (PKI, 20 μ M) was added to the intracellular solution (**Figures 4A,B**): in this condition, the amount of DHPG-induced mGluR-LTD was not significantly different from control (EPSC % amplitude: 75 ± 6 vs. 79.5 ± 10 , $n = 10/11$; $t_{(19)} = 0.32$; $P = 0.75$, comparing PKI vs. control) but the effect of LP-211 was significantly reduced (EPSC %: 74 ± 5 vs. 99.5 ± 3 , $n = 6/7$; $t_{(11)} = 4.26$; $P = 0.0013$, comparing LP-211+PKI vs. LP-211; **Figures 4A,B**), indicating an involvement of PKA in 5-HT₇R-mediated reversal of mGluR-LTD.

5-HT₇R-Activation Stimulated Extracellular Signal-Regulated Kinase (ERK)

The cAMP pathway can also interact with the RAS/MEK/ERK signaling cascade (Dumaz and Marais, 2005), which is required for mGluR-LTD and dysregulated in the mouse model of FXS (Hou et al., 2006; Kim et al., 2008; Osterweil et al., 2010; Sawicka et al., 2016). Intracellular PD-98059 (40 μ M), a mitogen-activated protein kinase (MAPK)/ERK blocker, reversed mGluR-LTD in WT hippocampal slices: the amount of mGluR-LTD was significantly decreased compared to control conditions (EPSC% 25 min after LTD induction: 68 ± 8 vs. 108 ± 19 , $n = 11/5$; $t_{(14)} = 2.27$; $P = 0.03$; control vs. PD-98059; **Figures 4A,B**). This result is consistent with previous data showing that ERK activation is necessary for mGluR-LTD (Gallagher et al., 2004).

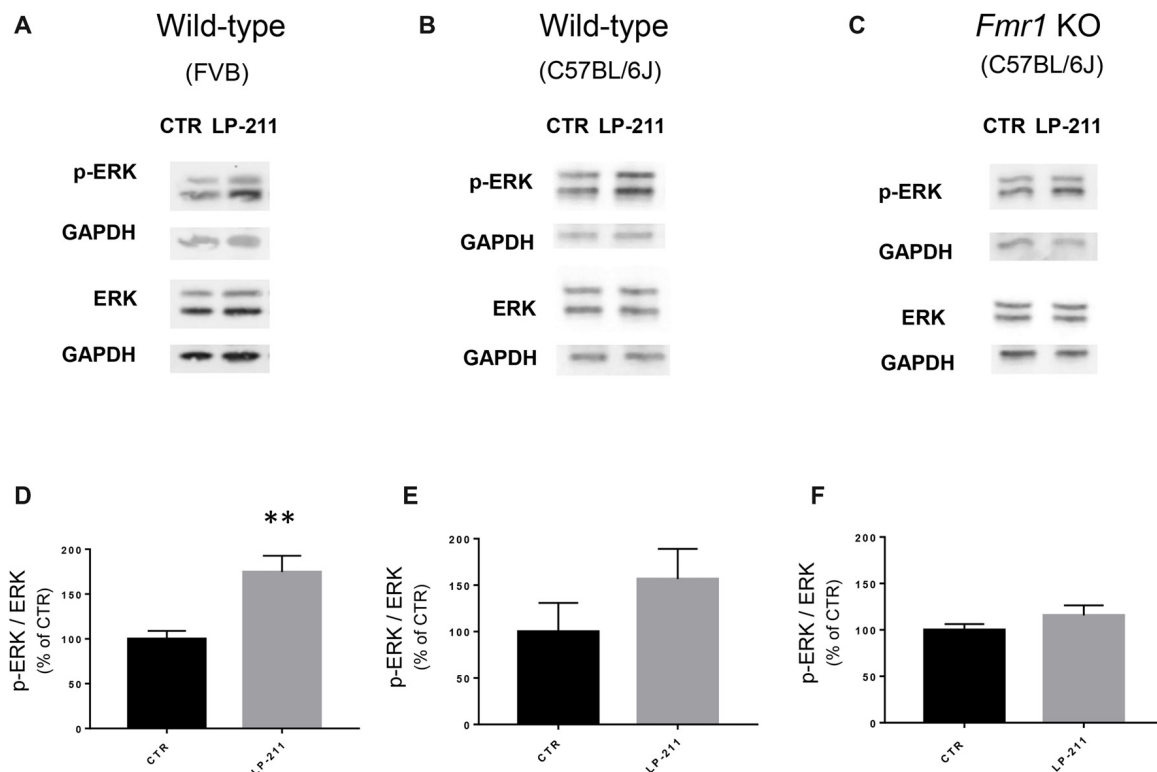


FIGURE 5 | Activation of 5-HT₇ receptors stimulated ERK phosphorylation. **(A–C)** Representative immunoblots showing the levels of phosphorylated and total ERK1/2 in control and LP-211 treated (10 nM, 5 min) hippocampal slices from WT (FVB strain; **A**), WT (C57BL/6J strain; **B**), and *Fmr1* KO (C57BL/6J strain; **C**) mice. **(D–F)** Semi-quantitative analysis of phosphorylated ERK1/2 vs. total ERK1/2 in control and LP-211 treated hippocampal slices from WT (FVB strain; **D**), WT (C57BL/6J strain; **E**), and *Fmr1* KO (C57BL/6J; **F**) mice. GAPDH was used as loading control. Relative optical density is presented as percentage of control. Data represent mean ± SEM of five (**D**), four (**E,F**) separate experiments, each performed on a pool of three mice. ***p* = 0.0064 by unpaired *t*-test. Full length immunoblots are shown in **Supplementary Figures S2, S3**.

mGluR-LTD was equally reversed by LP-211 (10 nM, 5 min) in the presence of PD-98059 (40 μM) and by PD-98059 alone (EPSC % amplitude: 112 ± 20 vs. 96 ± 11; *n* = 5/5; *t*₍₈₎ = 0.66; *P* = 0.52, comparing LP-211+PD-98059 vs. PD-98059; **Figures 4C,D**), indicating that PD-98059 occluded the effect of LP-211. Therefore, using mGluR-LTD as readout of 5-HT₇R-mediated effect, we could not test if 5-HT₇R activation was modulating ERK activity.

To check for a possible coupling of 5-HT₇R to the ERK pathway, we measured ERK phosphorylation levels by Western blotting. Exposure of hippocampal slices to LP-211 (10 nM) for 5 min increased ERK1/2 phosphorylation, showing that 5-HT₇R activation stimulates ERK signaling (**Figures 5A,B,D,E**). Stimulation of ERK phosphorylation by LP-211 was observed in hippocampal slices from WT mice, although it resulted statistically significant only in WT of FVB strain (**Figure 5D**, FVB; CTR: 100 ± 14, LP-211: 182 ± 17, *n* = 5, *P* = 0.0064 by unpaired *t*-test; **Figure 5E**, C57BL/6J; CTR: 100 ± 31, LP-211: 157 ± 32.5, *n* = 4, *P* = 0.254 by unpaired *t*-test). An increased phosphorylation of both ERK1 and ERK2 was detected after LP-211 exposure (**Supplementary Figure S1**). Interestingly, LP-211 caused only a negligible increase of ERK1/2 phosphorylation levels in hippocampal

slices from *Fmr1* KO mice (**Figures 5C,F**; CTR: 100 ± 6; LP-211: 116 ± 11, *n* = 4, *P* = 0.255 by unpaired *t*-test). Overall, our data suggest that LP-211-mediated reversal of mGluR-LTD does not operate through stimulation of ERK signaling.

Acute *in vivo* Administration of a 5-HT₇R Agonist Improved Object Recognition Memory in WT and *Fmr1* KO Mice

Fmr1 KO mice show a cognitive impairment when evaluated by Novel object recognition (NOR) tasks based on the natural tendency of rodents to explore unfamiliar objects (Ventura et al., 2004; King and Jope, 2013; Franklin et al., 2014; Gomis-González et al., 2016). Here we first confirmed that *Fmr1* KO presented a damage of recognition memory, since their *D* index did not significantly differ from zero (*t*₍₁₁₎ = 1.864, *P* = 0.089, *n* = 12; **Figures 6A,B**). Interestingly, *Fmr1* KO spent a higher amount of time exploring the old vs. the new object (55.18 ± 2.78 vs. 44.81 ± 2.78 s of exploration time familiar vs. novel object; paired *t*-test: *t*₍₂₃₎ = 21.446, *P* < 0.0001; **Figures 6A,B**). WT littermates showed a normal recognition memory, as demonstrated by the higher time spent exploring the new object (39.61 ± 3.47 vs.

60.61 ± 3.47 s of exploration time familiar vs. novel object; $n = 11$; paired t -test: $t_{(21)} = 15.039$, $P < 0.0001$; **Figure 6A**) and D index different than zero ($t_{(10)} = 2.99$, $P = 0.014$; **Figure 6B**).

LP-211 is a brain-penetrant molecule, reaching the brain within 30 min after intraperitoneal injection in mice (Hedlund et al., 2010). Intraperitoneal treatment with LP-211 (3 mg/kg) 30 min before training rescued memory in *Fmr1* KO mice (39.74 ± 4.55 vs. 60.25 ± 4.44 s of exploration time familiar vs. novel object; $n = 10$; paired t -test: $t_{(19)} = 12.804$, $P < 0.0001$; **Figure 6A**; $D: t_{(9)} = 2.308$, $P = 0.046$; **Figure 6B**), without modifying cognitive performances in WT animals (34.93 ± 3.39 vs. 65.06 ± 3.39 s of exploration time familiar vs. novel object; $n = 10$; paired t -test: $t_{(19)} = 14.344$, $P < 0.0001$; $D: t_{(9)} = 4.437$, $P = 0.002$; **Figures 6A,B**). These findings were confirmed by the analyses of D among groups (one-way ANOVA with Bonferroni's: $F_{(3,39)} = 6.71$, $P = 0.001$; WT vs. *Fmr1* KO $p = 0.015$; *Fmr1* KO vs. *Fmr1* KO + LP-211 $p = 0.02$). The 4 groups of mice did not show differences in the latency to first approach to the novel object (one-way ANOVA: $F_{(3,39)} = 1.103$, $P = 0.360$; **Figure 6C**) nor in total exploration time (one-way ANOVA: $F_{(3,39)} = 1.394$, $P = 0.259$; **Figure 6D**).

Acute *in vivo* Administration of a 5-HT₇R Agonist Reversed Stereotyped Behavior in *Fmr1* KO Mice

FXS patients present perseverative or stereotypic behaviors that can be studied in rodents through a marble burying task (Thomas et al., 2009). Here, we observed that *Fmr1* KO mice buried a higher number of marbles compared to WT littermates (Bonferroni's $P = 0.043$; **Figure 6E**; **Supplementary Figure S4**), confirming previous data (Veeraragavan et al., 2012; Gholizadeh et al., 2014). An acute treatment with LP-211 (3 mg/kg, 30 min before trial) rescued this stereotypic behavior since it induced a reduction of marble burying in *Fmr1* KO mice (Bonferroni's $P = 0.002$), whereas it did not affect WT behavior (Bonferroni's $P = 1$; **Figure 6E**). ANOVA among all: $F_{(3,39)} = 5.680$, $P = 0.003$.

Then, we performed the OF task, which allows to study locomotor activity, anxiety-like and stereotyped behaviors (Kelley, 2001; Prut and Belzung, 2003). No differences were found in the % time spent in the center ($F_{(3,44)} = 1.144$, $P = 0.342$), the number of crosses ($F_{(3,44)} = 6.656$, $P = 0.308$), the horizontal activity ($F_{(3,44)} = 2.679$, $P = 0.059$), the vertical activity ($F_{(3,44)} = 2.769$, $P = 0.053$), suggesting that locomotor activity and anxiety-like behavior were not influenced by genotype and treatment (**Figures 6F–I**). However, *Fmr1* KO mice showed an increase of grooming (Bonferroni's $P < 0.0001$; **Figure 6J**) and a decrease of freezing (Bonferroni's $P = 0.036$; **Figure 6K**) compared to WT littermates that were rescued by treatment with LP-211 (Bonferroni's $P = 0.245$ and $P = 1$). No differences were detected in defecation (**Figure 6L**), considered as an indirect index of anxiety.

DISCUSSION

We have studied the intracellular action mechanisms underlying 5-HT₇R-mediated reversal of mGluR-LTD in WT and *Fmr1*

KO mice, a model of FXS. mGluR-LTD plays a fundamental role in learning and memory (Luscher and Huber, 2010; Sanderson et al., 2016) and is abnormally enhanced in the hippocampus of *Fmr1* KO mice (Huber et al., 2002). Exaggerated hippocampal mGluR-LTD has been confirmed by several studies (Hou et al., 2006; Zhang et al., 2009; Choi et al., 2011; Costa et al., 2012a; Till et al., 2015), and is considered as a reliable readout of synaptic dysfunction in animal models of FXS and a cause of learning impairment and behavioral alterations (Sanderson et al., 2016). FMRP, the protein lacking in FXS, is most highly expressed at PN 7–12 (Davidovic et al., 2011; Bonaccorso et al., 2015). In the absence of FMRP, dendritic spine morphology and synapse formation are impaired in cortex (Comery et al., 1997) and hippocampus (Grossman et al., 2010) of *Fmr1* KO mice. We have studied the effect of 5-HT₇R activation on mGluR-LTD at PN 14–23, a developmental stage during which physiological synaptogenesis (Semple et al., 2013) and synaptic pruning (Jawaid et al., 2018) reach the highest levels in the brain of rodents.

We have previously shown that activation of 5-HT₇ receptors reverses mGluR-LTD in WT and *Fmr1* KO mouse hippocampus (Costa et al., 2012a). Different effects of the 5-HT₇R agonist LP-211 were observed in mouse cerebellar cortex, where application of LP-211 induced a long-term depression of basal glutamatergic transmission in parallel fibers—Purkinje cells synapses (Lippiello et al., 2016). In the hippocampal CA3-CA1 synapse, instead, we never observed any long-term effect of LP-211 on basal synaptic transmission: application of LP-211 induced a transient enhancement of EPSC amplitude that fully recovered within 20 min (Costa et al., 2012b), indicating that 5-HT₇ receptors activate different mechanisms in distinct brain areas.

Here, we show that the amount of hippocampal mGluR-LTD, which is abnormally enhanced in *Fmr1* KO neurons, is reduced by 5-HT₇ receptors through an increase in intracellular cAMP levels. Our conclusion is supported by data showing that 5-HT₇R-mediated reversal of mGluR-LTD was: (1) mimicked by forskolin, a direct stimulator of adenylate cyclase; (2) mimicked by PACAP, a potent endogenous stimulator of adenylate cyclase; (3) completely abolished by SQ 22536, an adenylate cyclase inhibitor; and (4) fully blocked by an inhibitor of PKA, one of the main cAMP target enzymes.

In the presence of an adenylate cyclase blocker or of a PKA blocker, mGluR-LTD persisted but 5-HT₇R-mediated effect was abolished. These results indicate that the cAMP/PKA pathway is not required for mGluR-LTD induction and/or expression, in agreement with previous studies (Camodeca et al., 1999; Schnabel et al., 2001), but stimulation of this pathway by 5-HT₇ receptors modulates the amount of mGluR-LTD.

In WT neurons, following blockade of adenylate cyclase the amount of mGluR-LTD became comparable to that observed in *Fmr1* KO slices, suggesting that exaggerated mGluR-LTD in *Fmr1* KO mice might be related to reduced cAMP production. This hypothesis is in line with different studies supporting the view that the cAMP cascade is impaired in FXS. The hypothesis of low cAMP levels in FXS was suggested by

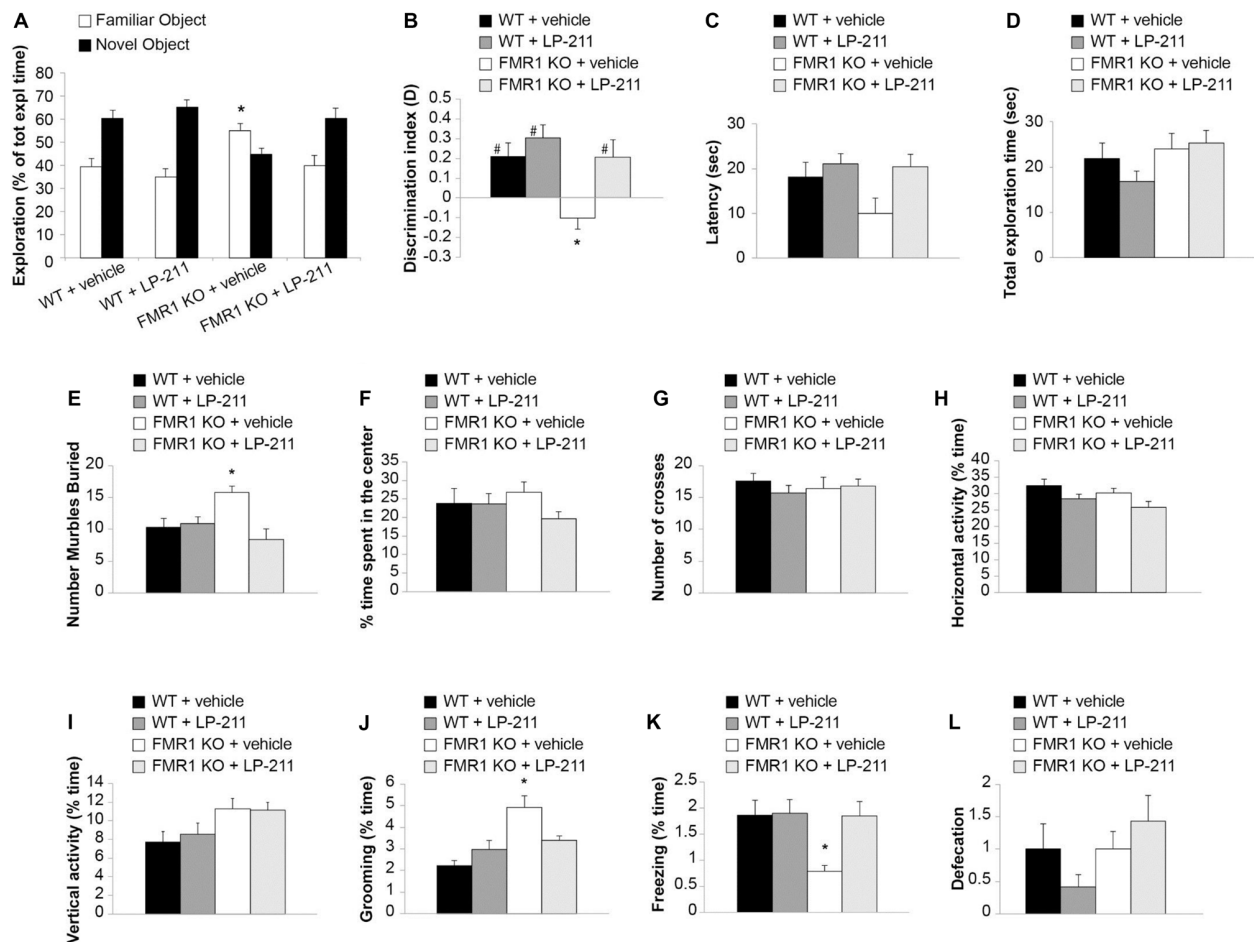


FIGURE 6 | Acute *in vivo* administration of LP-211 improved memory and reduced stereotyped behavior in *Fmr1* KO mice. **(A)** Exploration times of familiar and novel object during T2 (after a 24-h retention interval) show that *Fmr1* KO mice treated with vehicle present an impairment of memory (higher amount of time exploring the familiar vs. the novel object; $p < 0.0001$) that is rescued by treatment with LP-211 (3 mg/kg 30 min before T1). WT + vehicle = 11; *Fmr1* KO + vehicle = 12; WT + LP-211 = 10; *Fmr1* KO + LP-211 = 10. **(B)** Analysis of the discrimination **(D)** index confirms that the impairment of recognition memory in *Fmr1* KO ($P = 0.015$ vs. WT) is rescued by LP-211 ($P = 0.02$ vs. *Fmr1* KO + LP-211). A difference from 0 is depicted with hashes ($^{\#}p < 0.05$). **(C)** Latency to first approach to the novel object and **(D)** total exploration time are comparable in the 4 groups of mice. **(E)** *Fmr1* KO treated with vehicle buried a higher number of marbles compared to WT ($P = 0.002$). This stereotypic behavior was rescued by treatment with LP-211 (3 mg/kg 30 min before test). WT + vehicle = 11; WT + LP-211 = 10; *Fmr1* KO + vehicle = 12; *Fmr1* KO + LP-211 = 10 for novel object recognition (NOR; **A–D**) and Marble Burying **(E)** tasks. **(F)** Time spent in the center of the arena and **(G)** the number of crosses into the center is comparable in the four groups of mice. **(H–I)** General locomotor activity analyzed as horizontal activity and vertical activity (rearing) is not modified by genotype and treatment. **(J)** The increase in stereotyped behavior such as grooming in *Fmr1* KO mice ($P < 0.0001$) is rescued by treatment with LP-211. **(K)** *Fmr1* KO mice show a decrease of spontaneous freezing behavior compared to WT littermates ($P = 0.036$) that is rescued by LP-211. **(L)** Defecation is comparable in the four groups of mice. WT + vehicle = 10; WT + LP-211 = 12; *Fmr1* KO + vehicle = 12; *Fmr1* KO + LP-211 = 14 for Open field (OF) test. Data are expressed as mean $\pm$ SEM. *Significant difference ($p < 0.05$ by one-way ANOVA with Bonferroni's multiple comparisons test); $^{\#}$ difference with zero (one-sample *t*-test).

early studies showing reduced basal cAMP levels and reduced cAMP production in blood platelets from FXS patients (Berry-Kravis and Huttenlocher, 1992; Berry-Kravis and Sklena, 1993). Another study shows that forskolin-induced cAMP production was reduced in blood platelets and brain tissues from different FXS animal models, but basal cAMP levels were unchanged (Kelley et al., 2007). Comparable total cAMP levels were also found in WT and *Fmr1* KO mouse hippocampus homogenates (Sethna et al., 2017). Furthermore, a very recent report reveals that the mRNA encoding phosphodiesterase 2A (PDE2A), the main cAMP degradative enzyme, is a prominent target of FMRP

and the absence of FMRP leads to PDE2A overexpression in cortical and hippocampal *Fmr1* KO neurons (Maurin et al., 2018a). Overall these data strongly support the hypothesis of unbalanced cAMP production/degradation potentially leading to reduced cAMP levels in FXS.

In the present work, we show that activation of 5-HT₇R reversed mGluR-LTD through an increase of intracellular cAMP levels and activation of PKA, one of the main effectors of cAMP.

5-HT₇R activation also stimulated ERK, a subclass of the MAPKs that can interact with the cAMP pathway (Dumaz and Marais, 2005). In the hippocampus, ERK phosphorylation is

stimulated by activation of group I mGluRs and is required for mGluR-LTD (Gallagher et al., 2004; Banko et al., 2006). Our electrophysiology data confirm that mGluR-LTD was completely abolished by intracellular inclusion of a MAPK/ERK blocker, thus required ERK activation. Using Western blotting, we found that activation of 5-HT₇Rs by LP-211 also stimulated ERK phosphorylation in WT mice, with a major effect on the FVB background, in line with previous reports (Errico et al., 2001; Lin et al., 2003; Norum et al., 2003). Interestingly, we detected a much lower and not significant 5-HT₇R-mediated ERK phosphorylation in *Fmr1* KO mice, in line with evidence showing that ERK phosphorylation after receptor activation is reduced or blunted in Fragile X mouse (Hou et al., 2006; Hu et al., 2008; Kim et al., 2008; Shang et al., 2009). Future experiments will be aimed at clarifying the mechanisms underlying the reduced/absent ERK phosphorylation after 5-HT₇ receptor stimulation in *Fmr1* KO mice. For the purpose of the present study, since mGluR-LTD was significantly reversed by 5-HT₇R activation in both WT and *Fmr1* KO mice, the lack of phospho-ERK activation in *Fmr1* KO mice suggests that LP-211-mediated reversal of mGluR-LTD operates through a distinct mechanism that does not involve stimulation of ERK signaling.

Other intracellular proteins are crucially involved in mGluR-LTD among which the activity-regulated cytoskeletal-associated protein (Arc; Arc/Arg3.1). Arc/Arg3.1 belongs to the intracellular signaling machinery that is necessary for mGluR-LTD, as a rapid synthesis of Arc/Arg3.1 is triggered by mGluR activation and induces mGluR-dependent endocytosis of AMPA receptors (Park et al., 2008). Several other intracellular molecules are necessary for mGluR-LTD (Luscher and Huber, 2010), thus modulation of mGluR-LTD may occur at many different steps. We do not exclude that 5-HT₇R activation might modulate translation/transcription of proteins playing a key role in mGluR-LTD; we are currently investigating which “LTD proteins” might be regulated by 5-HT₇ receptors.

Our present data indicate that 5-HT₇ receptors reverse mGluR-LTD through the cAMP/PKA pathway, which is not required for mGluR-LTD induction and/or expression as shown by previous data and confirmed by our results. As a matter of fact, inhibition of adenylate cyclase or PKA did not block mGluR-LTD but completely blocked 5-HT₇-mediated reversal of mGluR-LTD. Thus, the 5-HT₇R-activated pathway is not necessary for mGluR-LTD induction but is able to modulate the final amount of synaptic inhibition.

We have previously speculated that activation of 5-HT₇ receptors may indeed affect AMPA receptor trafficking. Interestingly, 5-HT₇R activation was recently shown to phosphorylate AMPA receptors in rat hippocampal neurons, increasing their membrane insertion and conductance, thus enhancing AMPAR-mediated synaptic currents (Andreotta et al., 2016), consistent with data from our laboratory (Costa et al., 2012b). We have also shown that 5-HT₇ receptor activation prevented DHPG-induced internalization of AMPA receptors in WT and *Fmr1* KO (Costa et al., 2012a). In view of these data, the 5-HT₇R-activated cAMP/PKA pathway might ultimately

phosphorylate AMPA receptors, reducing their internalization and increasing their conductance, thus reducing the amount of mGluR-LTD.

Our result that increasing cAMP levels rescues abnormal synaptic plasticity in *Fmr1* KO hippocampus suggests that other molecules acting on Gs-coupled receptors might be used for FXS therapy. Among these, we show that PACAP is a promising candidate since this neurotrophic peptide modulates hippocampal synaptic transmission (Costa et al., 2009) and plasticity, correcting abnormal mGluR-LTD in *Fmr1* KO neurons (present results).

In accordance with cAMP involvement, exaggerated mGluR-LTD in *Fmr1* KO neurons was reversed by mGluR2 blockade (Choi et al., 2011, 2016) or by PDE4 inhibition (Choi et al., 2015, 2016), both virtually increasing cAMP levels. Altered mechanisms leading to reduced cAMP production in FXS are under investigation. Over-expression of FMRP causes an increased production of cAMP in transfected cell lines, leading to the conclusion that FMRP might directly regulate the translation of mRNA(s) coding for cAMP cascade proteins (Berry-Kravis and Ciurlionis, 1998). Indeed, several mRNAs encoding components of cAMP signaling cascade are target of FMRP (Darnell et al., 2011) among which, as above discussed, PDE2A is a major FMRP target (Maurin et al., 2018a). Importantly, very recent data demonstrate that PDE2A is overactivated in *Fmr1* KO mouse brain, leading to reduced cAMP levels, and pharmacological inhibition of PDE2A reverses exaggerated mGluR-LTD in *Fmr1* KO hippocampus (Maurin et al., 2018b).

Impaired signaling through dopamine Gs-coupled receptors was also evidenced in *Fmr1* KO cultured cortical neurons, where D1 receptor-stimulated cAMP formation was decreased with respect to WT, due to reduced coupling of D1 receptors to adenylate cyclase (Wang et al., 2008). Interestingly, the same work also shows that AS-19, a selective 5-HT₇R agonist, instead stimulated adenylate cyclase comparably in WT and in *Fmr1* KO neurons and we show that 5-HT₇R activation corrects excessive mGluR-LTD in *Fmr1* KO mice. These results have important therapeutic implications indicating that, unlike D1 receptors, 5-HT₇Rs are fully functional in *Fmr1* KO mice, thus 5-HT₇R agonists might become new pharmacological tools.

In this perspective, in this manuscript we also investigated whether systemic administration of LP-211 to *Fmr1* KO might rescue learning and behavioral deficits that mirror cognitive impairment and autistic-like behavior in FXS patients. We used the NOR test to study cortex- and hippocampus-dependent novelty detection ability (Broadbent et al., 2010), which is known to involve hippocampal mGluR-LTD (reviewed by Sanderson et al., 2016). We first confirmed that recognition memory tested by NOR is impaired in *Fmr1* KO mice (Ventura et al., 2004; King and Jope, 2013; Franklin et al., 2014; Gomis-González et al., 2016), since discrimination index is impaired compared to WT littermates and less time is spent exploring the novel object. Interestingly, *Fmr1* KO mice showed a preference for the familiar compared to the novel object,

consistent with previous studies demonstrating alterations of novelty preferences, with stereotyped behavior and restricted interests, in autism spectrum disorders (Jacob et al., 2009). Here we show that an acute systemic administration of LP-211 rescued recognition memory impairment in *Fmr1* KO mice.

About one third of FXS patients display autistic behavior, including gaze and touch avoidance and repetitive behavior (Garber et al., 2008). Using the marble burying and the OF tasks, two protocols revealing stereotyped behavior in rodents (Thomas et al., 2009), *Fmr1* KO mice showed increased repetitive behavior, i.e., marble burying and grooming, with respect to WT, as previously demonstrated (Veeraragavan et al., 2012; Gholizadeh et al., 2014; Kazdoba et al., 2014). Interestingly, *Fmr1* KO mice presented less spontaneous freezing behavior compared to WT, consistent with previous studies in GAP43 mice model of autism spectrum disorder (Zaccaria et al., 2010) and probably reflecting some aspects of maladaptive behavior to stress and catatonia in patients. This phenotype was completely rescued by systemic administration of LP-211.

In conclusion, we show that selective activation of 5-HT₇Rs corrects abnormal intracellular signaling and synaptic plasticity in newborn *Fmr1* KO mice and rescues learning and behavior in young adult *Fmr1* KO mice. The latter result has important implications for therapy, indicating that a rescue of FXS phenotypes by pharmacological treatment can also be possible at adult age. Therefore, selective 5-HT₇ receptor agonists might represent a new pharmacological strategy for FXS therapy.

AUTHOR CONTRIBUTIONS

LCosta: electrophysiology data collection, analysis and interpretation; final approval of manuscript. LS: electrophysiology data collection; animal care, final approval of manuscript. MS, CB and SD'A: western blotting data collection, analysis and interpretation; animal care, final approval of manuscript. WG and MT: behavioral data collection, analysis and interpretation; final approval of manuscript. ML and EL: design and synthesis of 5-HT₇R agonists; data interpretation; final approval of manuscript. LCiranna, MVC and DP: conception and design; data analysis and interpretation; manuscript writing; final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2018.00353/full#supplementary-material>

FIGURE S1 | LP-211 treatment increased levels of both phospho-ERK1 and phospho-ERK2 in hippocampal slices of wild-type (WT) mice. **(A)** Semi-quantitative analysis of phosphorylated ERK1 vs. total ERK1 in control and LP-211 (10 nM, 5 min) treated hippocampal slices from WT mice (FVB strain). Relative optical density is presented as percentage of control. Data represent mean ± SEM of four separate experiments, each performed on a pool of three mice. **p* = 0.0286 by Mann-Whitney Rank Sum Test. **(B)** Semi-quantitative analysis of phosphorylated ERK2 vs. total ERK2 in control and LP-211 treated hippocampal slices from WT mice (FVB strain). Relative optical density is presented as percentage of control. Data represent mean ± SEM of four separate experiments, each performed on a pool of three mice. **p* = 0.0412 by unpaired *t*-test.

FIGURE S2 | Original images of immunoblots shown in **Figure 5A**.

FIGURE S3 | **(A)** Original images of immunoblots shown in **Figure 5B**. **(B)** Original images of immunoblots shown in **Figure 5C**.

FIGURE S4 | Experimental procedures for marble burying test. Marbles distribution before testing shows twenty marbles equidistantly distributed. Mice were left in the cage for 20 min and number of buried marbles was analyzed in four different groups (vehicle-treated WT; WT treated with LP-211; vehicle-treated *Fmr1* KO; *Fmr1* KO treated with LP-211).

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Fragile-X Syndrome Is Associated With NMDA Receptor Hypofunction and Reduced Dendritic Complexity in Mature Dentate Granule Cells

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Fragile X syndrome (FXS) is the most common form of inherited intellectual disability. It is caused by the overexpansion of cytosine-guanine-guanine (CGG) trinucleotide in *Fmr1* gene, resulting in complete loss of the fragile X mental retardation protein (FMRP). Previous studies using *Fmr1* knockout (*Fmr1* KO) mice have suggested that a *N*-methyl-D-aspartate receptors (NMDAR) hypofunction in the hippocampal dentate gyrus may partly contribute to cognitive impairments in FXS. Since activation of NMDAR plays an important role in dendritic arborization during neuronal development, we examined whether deficits in NMDAR function are associated with alterations in dendritic complexity in the hippocampal dentate region. The dentate granule cell layer (GCL) presents active postnatal neurogenesis, and consists of a heterogenous neuronal population with gradient ages from the superficial to its deep layer. Here, we show that neurons with multiple primary dendrites that reside in the outer GCL of *Fmr1* KO mice display significantly smaller NMDAR excitatory post-synaptic currents (EPSCs) and a higher α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) to NMDA ratio in comparison to their wild-type counterparts. These deficits were associated with a significant decrease in dendritic complexity, with both dendritic length and number of intersections being significantly reduced. In contrast, although neurons with a single primary dendrite resided in the inner GCL of *Fmr1* KO mice had a trend toward a reduction in NMDAR EPSCs and a higher AMPA/NMDA ratio, no alterations were found in dendritic complexity at this developmental stage. Our data indicate that the loss of FMRP causes NMDAR deficits and reduced dendritic complexity in granule neurons with multiple primary dendrites which are thought to be more mature in the GCL.

Keywords: fragile X syndrome (FXS), NMDA (*N*-methyl-D-aspartate receptor), dendrite complexity, neurogenesis, dentate gyrus, AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)

INTRODUCTION

Cognitive impairment is a frequently reported issue for patients with fragile X syndrome (FXS), which is the most common form of inherited intellectual disability, and the leading single gene cause of autism spectrum disorder (Rousseau et al., 1991; Kabakus et al., 2006; Alanay et al., 2007). This syndrome is caused by the transcriptional silencing of the fragile X mental retardation 1 (*Fmr1*)

gene, resulting in the loss or mutation of its product, fragile X mental retardation protein (FMRP), a RNA-binding protein that associates with polyribosomes and regulates translation (Brown et al., 2001; Zalfa et al., 2003). FMRP has been shown to repress the translation of several targets, including proteins critical for synaptic function (Zalfa et al., 2003; Darnell et al., 2011). Of particular interest for the current study is the identification of interaction partners that play roles in signaling pathways related to synaptic plasticity (Tcherkezian et al., 2010) and dendritic structure (Schenck et al., 2003; De Rubeis et al., 2013).

Using *Fmr1* knockout (*Fmr1* KO) mice, we and others have demonstrated that the cognitive impairments in FXS may be linked to a disruption in *N*-methyl-D-aspartate receptor (NMDAR)-dependent synaptic plasticity in the hippocampal dentate gyrus (DG) (Yun and Trommer, 2011; Eadie et al., 2012; Franklin et al., 2014; Bostrom et al., 2015, 2016). The NMDA receptor forms a heterotetramer between two obligatory GluN1 subunits and two GluN2 subunits. The GluN2 subunits are differentially expressed during development, with GluN2B subunits initially being more highly expressed than GluN2A subunits early in neuronal development (Wenzel et al., 1997). The impact of NMDAR on dendritic structure can also have functional implications, as neuronal models indicate that dendritic morphology can have a significant impact on neuronal firing patterns (Mainen and Sejnowski, 1996).

Activation of NMDARs is known to play an important role in dendritic arborization and spine morphogenesis during neuronal development (Tolias et al., 2005). NMDARs appear to contribute to spine and dendrite formation; however their exact role remains controversial. Using specific GluN2 antagonists in cultured cells, GluN2A subunits have been associated with dendritic arborization, while GluN2B subunits were associated with dendritic spine formation (Henle et al., 2012). In contrast, introducing the GluN2B subunits into ventral spinal neurons in culture enhanced dendritic arborization, an effect not observed with GluN2A subunits (Sepulveda et al., 2010). Recently we have shown that genetic deletion of the GluN2A subunit significantly decreases dendritic growth in maturing dentate granule cells (Kannangara et al., 2014), suggesting that NMDA hypofunction in the DG may affect dendritic arborization in this brain region that exhibits developmental regulated changes in neurogenic activity (Gil-Mohapel et al., 2013).

In the current study we sought to determine if the reduction in NMDAR function observed in *Fmr1* KO animals was associated with developmental deficits in dendritic arborization of neurons in the hippocampal dentate granule cell layer (GCL) (Cameron and McKay, 2001). The adult-born neurons of the DG have been shown to express primarily GluN2B subunits early on (Spampanato et al., 2012), but are also known to undergo extensive dendritic arborization as they migrate into an already extensively populated GCL (van Praag et al., 2002). Indeed, dendritic arborization and cell body positioning have been used to identify young and old neurons in the DG (Wang et al., 2000; Eadie et al., 2005). Newly generated neurons tend to be preferentially located in the inner layer of the GCL (Overstreet et al., 2004; Espósito et al., 2005; Redila and Christie, 2006), whereas more mature granule cells appear to be

located in the outer GCL (Wang et al., 2000; Overstreet et al., 2004; Redila and Christie, 2006). Combined morphological and electrophysiological analyses also indicate that neurons in the outer GCL are morphologically more complex and thus have a lower series resistance than neurons in the inner GCL (Wang et al., 2000; van Praag et al., 2002; Kannangara et al., 2014). Using the location of neurons in the GCL as a means to select neurons for whole-cell patch clamp analyses, we investigated how the loss of FMRP affects NMDAR function and dendritic arborization of both younger and more mature hippocampal DG neurons.

MATERIALS AND METHODS

Animals

Adult male *Fmr1* KO mice with a C57BL/6 genetic background (Bakker et al., 1994) and their wild-type (WT) littermates at the age of 4- to 6-week month old were used for the experiments. All mice housed with food and water available *ad libitum* on a 12 h light/dark cycle. All experiments were performed in accordance with the guidelines set out by the Canadian Council on Animal Care and approved by the University of Victoria Animal Care Committee.

Electrophysiology

Electrophysiological Preparation

Adult mice were anesthetized with isoflurane, their brains removed, and transverse hippocampal slices were prepared as previously described (Vasuta et al., 2007). Briefly, hippocampal slices (350 μ m) were acquired using a Vibratome 1500 (Ted Pella, Inc., Redding, CA, United States). The brain was immersed in oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 3 KCl, 1.25 NaHPO₄, 25 NaHCO₃, 1 CaCl₂, 6 MgCl₂, and 25 Glucose at 4°C. After sectioning, slices were transferred to a holding chamber containing warm (30°C) oxygenated normal ACSF (nACSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 NaHPO₄, 25 NaHCO₃, 2 CaCl₂, 1.3 MgCl₂, and 10 dextrose for 1 h before being used for electrophysiological recordings.

Whole Cell Recording

Cells were patched using a borosilicate glass recording electrode (5–7 M Ω) and the formation of a gigaseal (2 G Ω) was required prior to break-in. Recordings with a series resistance higher than 30 M Ω or presenting a variation of more than 10% were excluded from the analyses. The intracellular solution consisted of (in mM) 20 KCl, 120 κ -gluconate, 4 NaCl, 0.1 EGTA, 4 ATP, 0.3 GTP, 14 Phosphocreatine (Osmolarity 270 mOsm/kg, pH 7.2) when action potentials were measured in current clamp mode. To examine NMDA/AMPA receptor mediated excitatory post-synaptic currents (EPSCs) in Voltage-Clamp mode, the internal solution was composed of (in mM) 135 Cesium methanesulfonate, 8 NaCl, 10 HEPES, 2 ATP, 0.3 GTP, 7 Phosphocreatine, 10 QX-314 (Osmolarity 280 mOsm/kg, pH 7.3) and biocytin (0.2–0.4%). In all cells, Alexa Fluor 488 (40 mM)

was included in the intracellular solution to assist with the visualization and classification of granule cells. EPSCs were evoked with bipolar stimulating electrodes placed in the medial perforant pathway and recorded using Axopatch 200B amplifier and pClamp10 software (Axon Instruments). AMPAR-mediated EPSCs were measured at a holding voltage of -70 mV, while NMDA EPSCs were measured by applying a $+40$ mV holding potential in the presence of picrotoxin (100 μ M) in nACSF. Some granule cells located in the inner cell layer did not display NMDA receptor currents; however, only granules cells showing both NMDAR and AMPAR EPSCs were included in the analyses.

Intracellular Filling and Immunostaining of Biocytin-Filled Cells

Cells were filled with Alexa Fluor 488 together with biocytin for immediate visualization with fluorescence microscopy (Olympus Fluoview 1000). After each recording, the electrode was quickly retracted from the cell to help maintain cell integrity for histology. Slices were then fixed with 4% paraformaldehyde and left overnight at 4°C . The following day they were washed with 0.01 M PBS repeatedly and then incubated in 3% H_2O_2 for 45 min to block any endogenous peroxidase activity. Slices were then washed with 0.01 M PBS, before being incubated in an avidin-biotinylated HRP complex (ABC) solution containing 0.1% Triton-X for 48 h at room temperature. Biocytin-filled cells were visualized using a diaminobenzidine (DAB; Sigma-Aldrich) solution. Slices were then mounted onto gelatin-coated glass slides and allowed to dry at room temperature for 2 days before being dehydrated in graded ethanol and cover-slipped with Permount.

Co-labeling of Biocytin-Filled Cells With Neuronal Markers

Following antigen retrieval in citric acid buffer for 15 min (pH 6.8 at 60°C), brain slices were washed thoroughly with 0.01 M PBS and then incubated with primary antibodies: rabbit anti-doublecortin (Abcam, 1: 200, Cat No.: ab18723) or mouse anti-NeuN (Millipore, 1: 200, Cat No.: MAB377) at 4°C for 3 days. Brain slices were then washed with PBS and incubated in Streptavidin-conjugated with Cy3 (Sigma, 1: 400 Cat No.: 6402) and Alexa Fluor 488 conjugated donkey anti-rabbit or donkey anti-mouse IgG antibodies (Life Technologies, 1: 200, Cat No.: S-11223) for 4 h at room temperature. Following washes in PBS, slides were coverslipped using Fluoromount (Thermo Fisher Scientific).

Selection and Classification of Granule Cells

We and others have previously shown that granule cells located in the outer GCL tend to have several primary dendrites and more dendritic branching, while granule cells located in the inner GCL are more likely to have only one primary dendrite and less dendritic branching (Desmond and Levy, 1985; Green and Juraska, 1985; Claiborne et al., 1990; Wang et al., 2000; Kannangara et al., 2014). In accordance with our prior work,

young and mature dentate granule neurons were selected based on their position in the inner and outer GCL, as well as their morphology, respectively. These cells typically had either single (inner cells) or multiple (outer cells) primary dendrites extending from the cell body, as previously reported (Wang et al., 2000; Bartesaghi and Serrai, 2001; Kannangara et al., 2014; Yau et al., 2016b).

Sholl Analysis of Dendritic Complexity

Only those granule cells that exhibited intact dendrites with no cut branches were used in these analyses. Dendritic tracing was performed using Neurolucida software (MBF Bioscience, Williston, VT, United States) with a 40X objective lens. Sholl analysis was used to measure dendritic length and dendritic branching with a concentric 10 - μm interval as previously reported (Kannangara et al., 2014; Yau et al., 2016b).

Statistical Analysis

Repeated measure ANOVA for dendritic branching and dendritic length analysis were performed using SPSS 14.0 (SPSS, Inc., Chicago, IL, United States). In some instances, two group comparisons between WT and *Fmr1* KO were performed using Student's *t*-test. Data are presented as mean $\pm$ SEM. Statistical significance was indicated by a probability (*P*) value less than 0.05.

RESULTS

Morphological Difference of Biocytin-Filled Granule Cells Located in the Inner and Outer Granule Cell Layers

The neurons co-labeled with the immature neuronal marker doublecortin (DCX) displayed as the ones with a single primary dendrite were located in the inner cell layer (Figures 1A–C), while neurons co-labeled with the mature neuronal marker NeuN displayed multiple dendrites and were located in the outer cell layer (Figures 1D–F). Representative images showing differences in morphology and location of granule neurons in the GCL are demonstrated in Figures 1G–I.

Action Potential and Membrane Properties of Dentate Granule Cells in *Fmr1* KO Mice

Figure 2A shows a representation of action potential trains in neurons with a single primary dendrite or multiple primary dendrites from WT mice. There were no significant differences in action potential frequency when comparing granule cells from WT to *Fmr1* KO animals (single primary dendrite: $F_{1,20} = 0.007$, $P = 0.933$, Figure 2B; multiple primary dendrites: $F_{1,24} = 1.158$, $P = 0.293$, Figure 2C). However, granule neurons with multiple primary dendrites from *Fmr1* KO mice displayed a trend toward a higher maximum frequency of action potential (Student's *t*-test, WT: $P = 0.156$; *Fmr1* KO: $P = 0.05$, Figure 2D), and a trend toward a lower input resistance when compared with the ones with a single primary dendrite (Table 1).

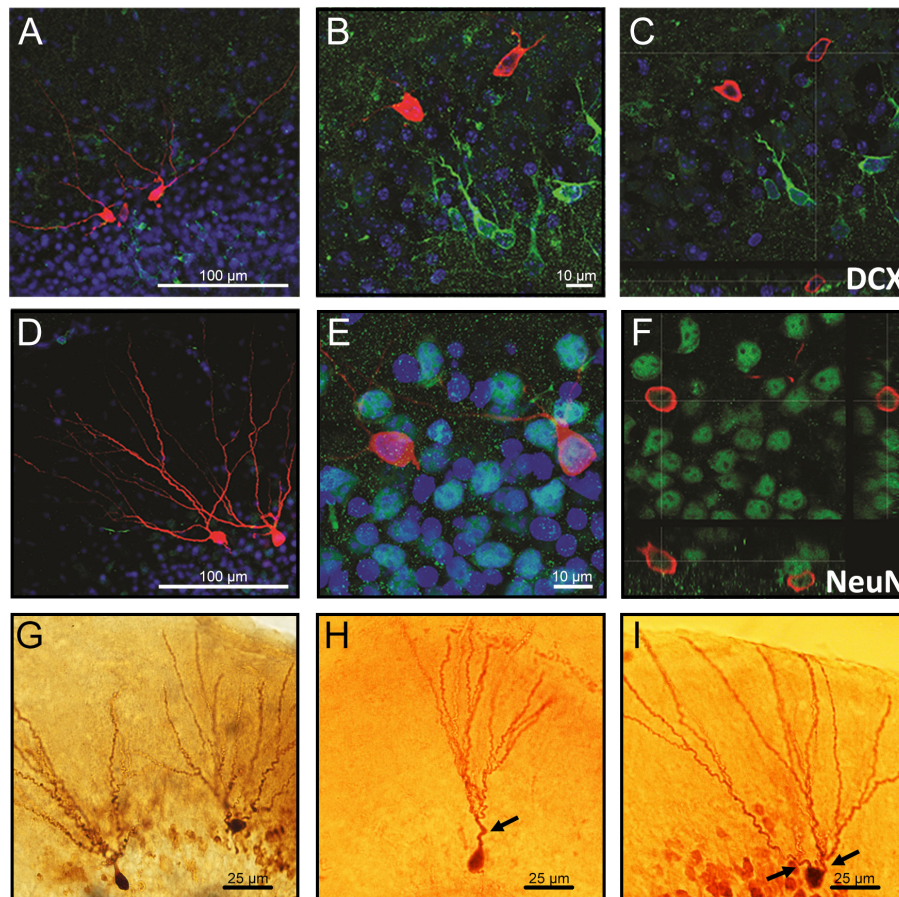


FIGURE 1 | Confocal images of biocytin-filled cells in the granule cell layer (GCL) of the hippocampal dentate gyrus. **(A–C)** Biocytin-filled cells presenting multiple dendrites located in the outer GCL, while doublecortin-positive (DCX; immature neurons) cells located in the inner GCL layer. **(D–F)** Co-labeling of granule neurons projecting multiple dendrites from the soma with NeuN confirms that these cells are mature neurons. **(G)** Representative images of biocytin-filled neurons in the GCL of the hippocampal DG showing **(H)** an immature neuron localized in the inner layer and **(I)** a mature neuron localized in the outer cell layer. Red: biocytin-filled cells; Green: DCX **(A–C)** and NeuN **(D–F)**; Blue: Dapi nucleus staining; Arrows: primary dendrites. Slice thickness: 350 μm .

Decreased NMDA EPSCs in Hippocampal Granule Cells From *Fmr1* KO Mice

For all recordings, AMPAR and NMDAR EPSCs were evoked using increasing stimulation intensity to construct I/O curves and determine the maximum response size. Representative traces acquired from granule neurons are depicted in **Figure 3A**. Similar levels of AMPAR-mediated EPSCs were recorded in younger granule cells from both experimental groups ($F_{1,25} = 1.36$, $P = 0.254$, **Figure 3B**), while a trend toward a reduction in NMDAR response size was observed in the *Fmr1* KO mice when compared to their WT littermates ($F_{1,25} = 3.69$, $P = 0.067$, **Figure 3C**). No alterations were found in AMPAR-mediated EPSCs of mature cells ($F_{1,20} = 1.82$, $P = 0.194$, **Figure 3D**), but a significant reduction in NMDAR-mediated EPSCs was observed in mature granule cells from *Fmr1* KO mice ($F_{1,20} = 14.843$, $P = 0.001$, **Figure 3E**).

The analyses of the maximum amplitude of AMPAR-mediated EPSCs in neurons with a single primary dendrite did not reveal

a significant difference between genotypes ($P = 0.89$), but a trend toward a reduction in NMDAR EPSCs was observed in cells from *Fmr1* KO mice in comparison with WT mice ($P = 0.09$, **Figure 4A**). This trend was also observed in the AMPAR/NMDAR ratio ($P = 0.07$, **Figure 4B**). A significant decrease in absolute maximum NMDAR-mediated EPSCs was found in mature neurons from *Fmr1* KO mice ($P < 0.01$, **Figure 4C**), as well as an increase in the AMPAR/NMDAR ratio ($P < 0.05$, **Figure 4D**) when compared to their WT counterparts. The amplitude of individual AMPAR- and NMDAR-mediated responses from hippocampal neurons at distinct developmental stages is depicted in **Figure 4E**.

Fmr1 KO Mice Present Decreased Dendritic Complexity in More Mature Granule Cells, but Not Younger Granule Cells

Examples of biocytin filled cells used for the analysis of dendritic complexity are shown in **Figures 5A,B**. Sholl analysis

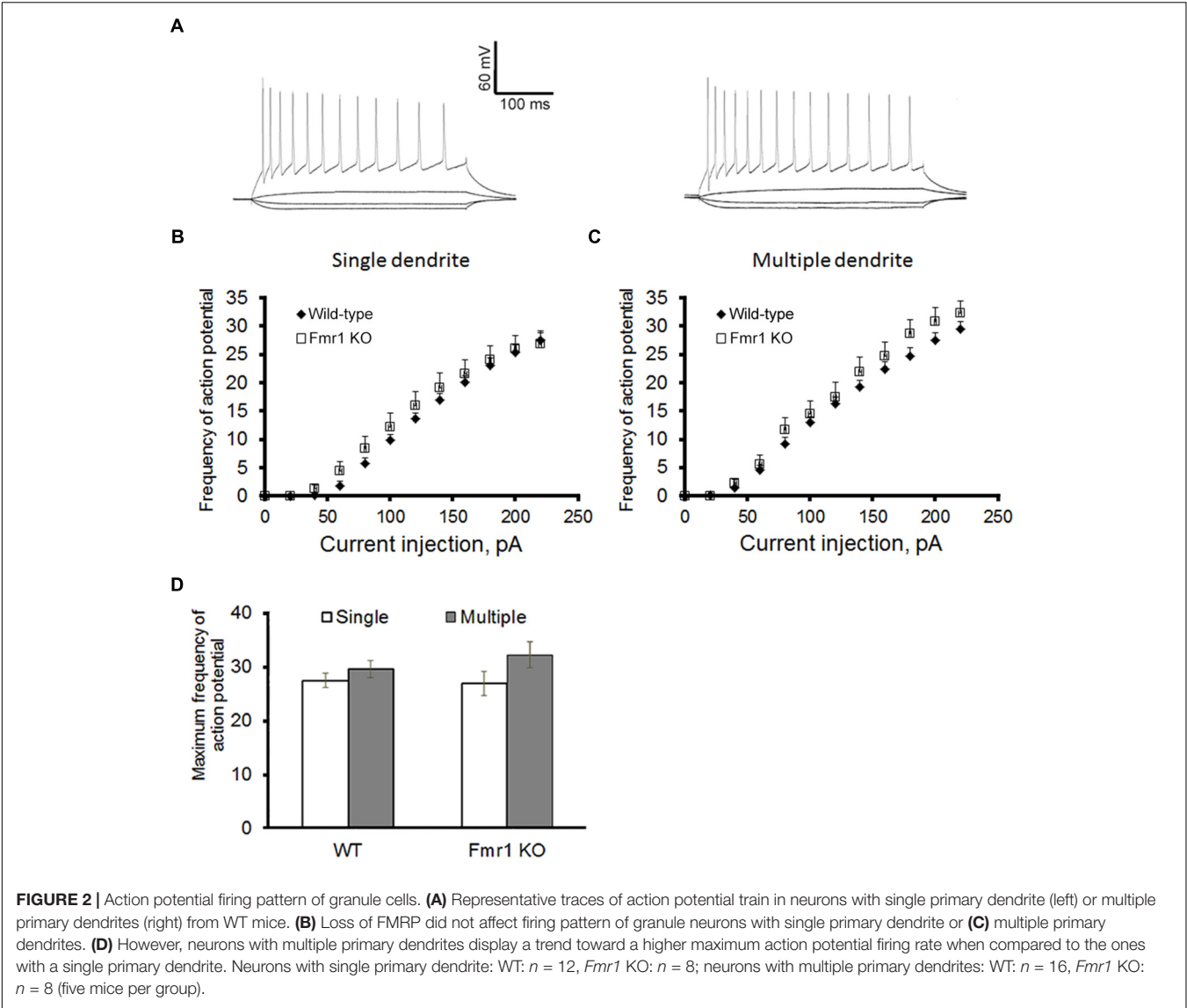


TABLE 1 | Membrane properties of granule cells with single and multiple primary dendrites that display both AMPAR and NMDAR-EPSCs.

	WT		Fmr1 KO		P-value
	Single	Multiple	Single	Multiple	
Ri (mΩ)	344.99 ± 15.1	223.45 ± 8.92	299.21 ± 32.4	211.09 ± 16.7	>0.05
RMP (mV)	−74.06 ± 1.03	−75.9 ± 0.60	−72.44 ± 3.73	−72.68 ± 1.12	>0.05

revealed that WT and *Fmr1* KO mice presented similar dendritic measures (dendritic length: $F_{1,18} = 0.185$, $P = 0.673$, **Figure 5C**; dendritic branching: $F_{1,18} = 0.473$, $P = 0.501$, **Figure 5D**). Conversely, cells with multiple primary dendrites from *Fmr1* KO mice displayed a drastic reduction in both dendritic length ($F_{1,22} = 26.291$, $P < 0.005$, **Figure 5E**) and number of intersections ($F_{1,22} = 15.301$, $P = 0.001$, **Figure 5F**) when compared to their WT littermates. These findings indicate that the loss of FMRP may lead to a significant decrease in the development of dendritic complexity in more mature neurons.

DISCUSSION

The current study indicates that a loss of FMRP results in a significant reduction in NMDAR-mediated EPSCs and a concomitant decrease in dendritic complexity in hippocampal granule cells. Our findings also indicate that these deficits are primarily observed in the mature granule neurons which are with multiple primary dendritic processes arising from the soma, and are primarily located in the outer GCL. On the other hand, younger granule neurons that reside in the inner GCL, did

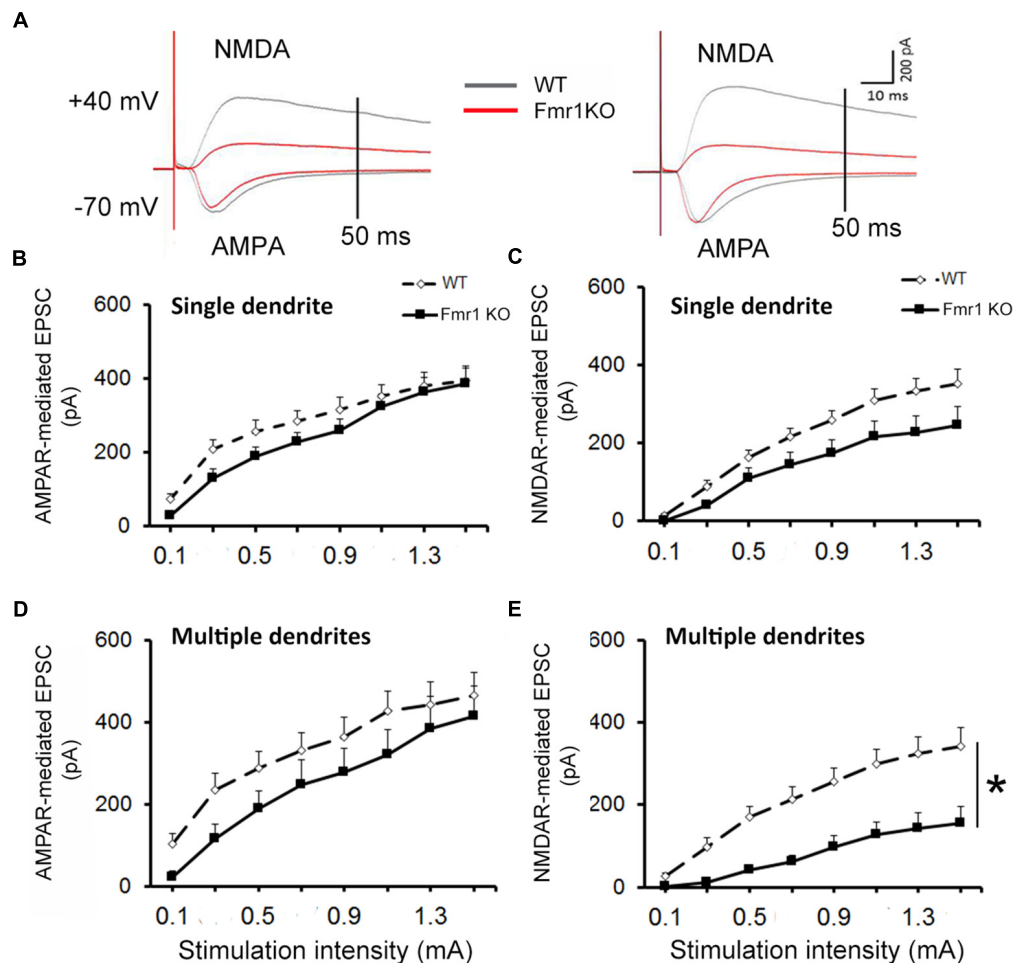


FIGURE 3 | Decreased NMDAR-mediated EPSC in dentate granule cells of *Fmr1* KO mice. **(A)** Representative traces of granule neurons. Neurons with a single primary dendrite (left panel) and neurons with multiple dendrites (right panel) at maximal amplitude were shown. **(B)** No significant difference in AMPAR-mediated EPSC was observed between *Fmr1* KO and WT mice. **(C)** A trend toward a lower NMDAR-mediated EPSC was found in *Fmr1* KO mice when compared to WT mice ($P = 0.067$). **(D)** No significant difference in AMPAR-mediated EPSCs of older granule neurons was observed between *Fmr1* KO and WT mice; **(E)** but a significant decrease in NMDAR-mediated EPSC was observed in *Fmr1* KO mice when compared to WT mice. * $P < 0.005$ ($n = 9-14$; five mice per group; ANOVA, repeated measures).

not show a significant change in dendritic complexity, although they did show a trend toward a reduction in NMDAR EPSCs induced by the lack of FMRP. These data further support our previous findings indicating that *Fmr1* KO mice present a dysfunction in NMDAR in the hippocampal DG (Bostrom et al., 2015; Yau et al., 2016a), and show for the first time that these deficits are preferentially associated with a reduction in dendritic arborization in a specific population of granule cells in the outer GCL.

Dendritic morphology can affect synaptic plasticity by modulating neuronal firing rates, as well as altering the propagation of EPSPs and action potentials (Mainen and Sejnowski, 1996; Magee, 2000; Vetter et al., 2001). Loss of FMRP has been shown to reduce pruning of dendrites in the somatosensory cortex (Galvez et al., 2003) and in mitral cells of the olfactory bulb (Galvez et al., 2005), suggesting that this protein plays an important role in the development of dendritic

processes. In the current study, we found that the loss of FMRP leads to a significant decrease in dendritic complexity in a specific subpopulation of granule neurons located in the outer GCL. This population of newborn neurons present multiple primary dendrites arising from the cell body and has been previously classified as being “mature neurons” based on morphological and electrophysiological analyses (Liu et al., 1996; Wang et al., 2000; Bartesaghi and Serrai, 2001; Kannangara et al., 2014). Our findings are in line with previous studies showing that the lack of FMRP leads to significant impairments in neurite extension of primary neural progenitor cells derived from the hippocampal DG (Guo et al., 2011). In addition, an *in vivo* study using retroviral labeling with green fluorescent protein found that mature newborn neurons from *Fmr1* KO mice present a decrease in dendritic complexity when compared to their WT counterparts (Guo et al., 2012). Our study corroborate these findings and provide further evidence indicating that the lack of

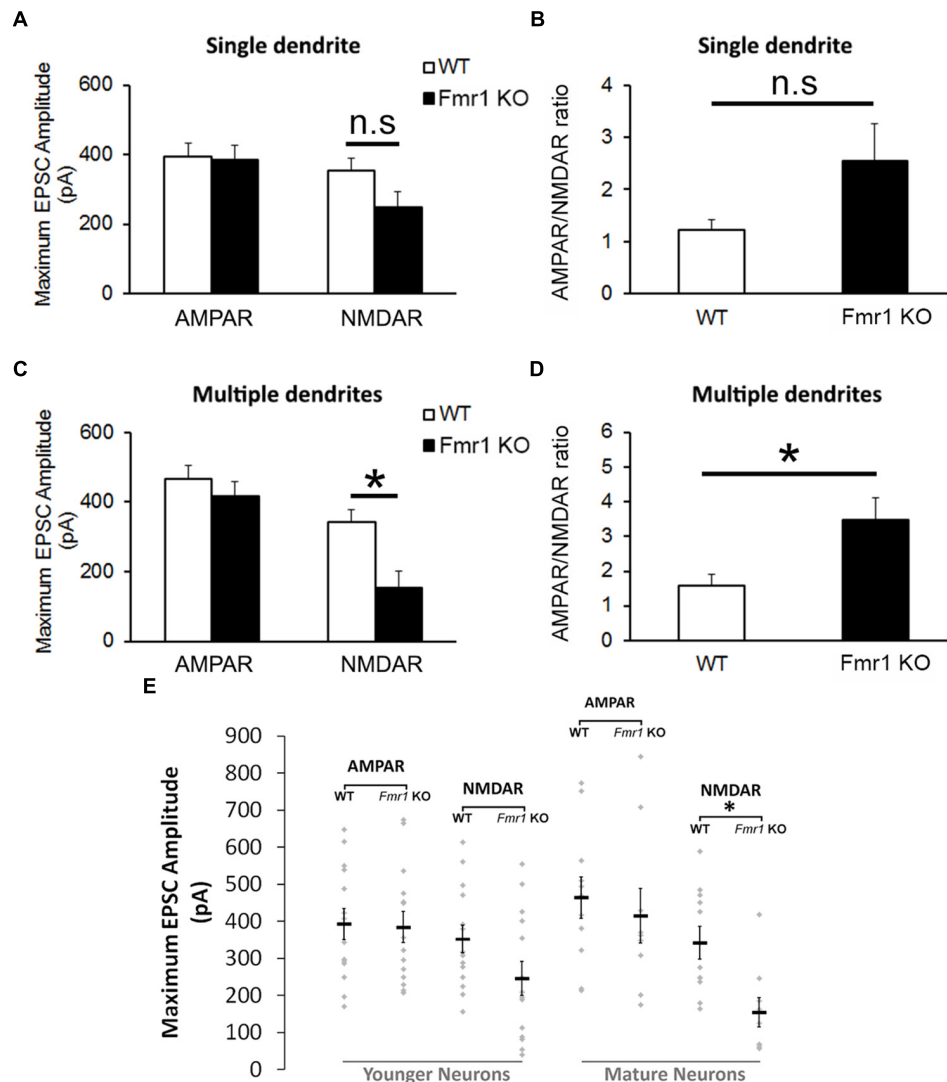


FIGURE 4 | Altered NMDA currents and AMPA/NMDA ratio in *Fmr1* KO mice. **(A)** No difference was found in AMPAR-mediated EPSCs from neurons with a single primary dendrite, but a trend toward a reduction in NMDAR-mediated EPSCs ($P = 0.09$) and **(B)** an increase in AMPAR/NMDA ratio ($P = 0.07$) were observed in *Fmr1* KO mice. **(C)** Although neurons with multiple primary dendrites from both groups demonstrated similar responses of AMPAR-mediated EPSCs, a significant decrease in NMDAR-mediated EPSCs **(D)** and a significant higher AMPAR/NMDA ratio were found in *Fmr1* KO mice when compared to WT mice. **(E)** Scatterplot showing the amplitude of AMPAR- and NMDAR-mediated responses of individual neurons at distinct developmental stages in the hippocampal dentate gyrus of WT and *Fmr1* KO mice. * $P < 0.05$; n.s., non-significant ($n = 9-14$; five mice per group; Student's *t*-test).

FMRP affects hippocampal newborn cells in an age-dependent manner.

The regulation of neuronal morphology and synaptic modifications depends on the remodeling of the actin cytoskeleton, which in turn has been previously shown to be regulated by FMRP (Dichtenberg et al., 2008; Nolze et al., 2013). Thus, it is possible that the deficits in dendritic length and branching observed in the present study are related to alterations in the organization of actin filaments induced by the lack of FMRP in the hippocampal DG. These changes may be related to the NMDAR hypofunction we observed, since these receptors are known to play a critical role in activity-dependent development of dendritic arbors and synaptogenesis

(Nikonenko et al., 2002; Wong and Ghosh, 2002). It may be that the deficits in NMDAR function observed in our study in turn affect several NMDAR-dependent proteins who exert an influence on dendritic morphology. These may include extracellular signal-regulated kinases (Vaillant et al., 2002), Ca^{2+} /calmodulin-dependent protein kinase II (Shi and Ethell, 2006), Rho family GTPases (Tolias et al., 2005) and glycogen synthase kinase 3 β (GSK-3 β) (Rui et al., 2013). These proteins are interesting targets for future studies, particularly GSK-3 β , since alterations in the translation of this protein seem to play a major role in the impaired differentiation of hippocampal neurons in FXS (Luo et al., 2010; Guo et al., 2012).

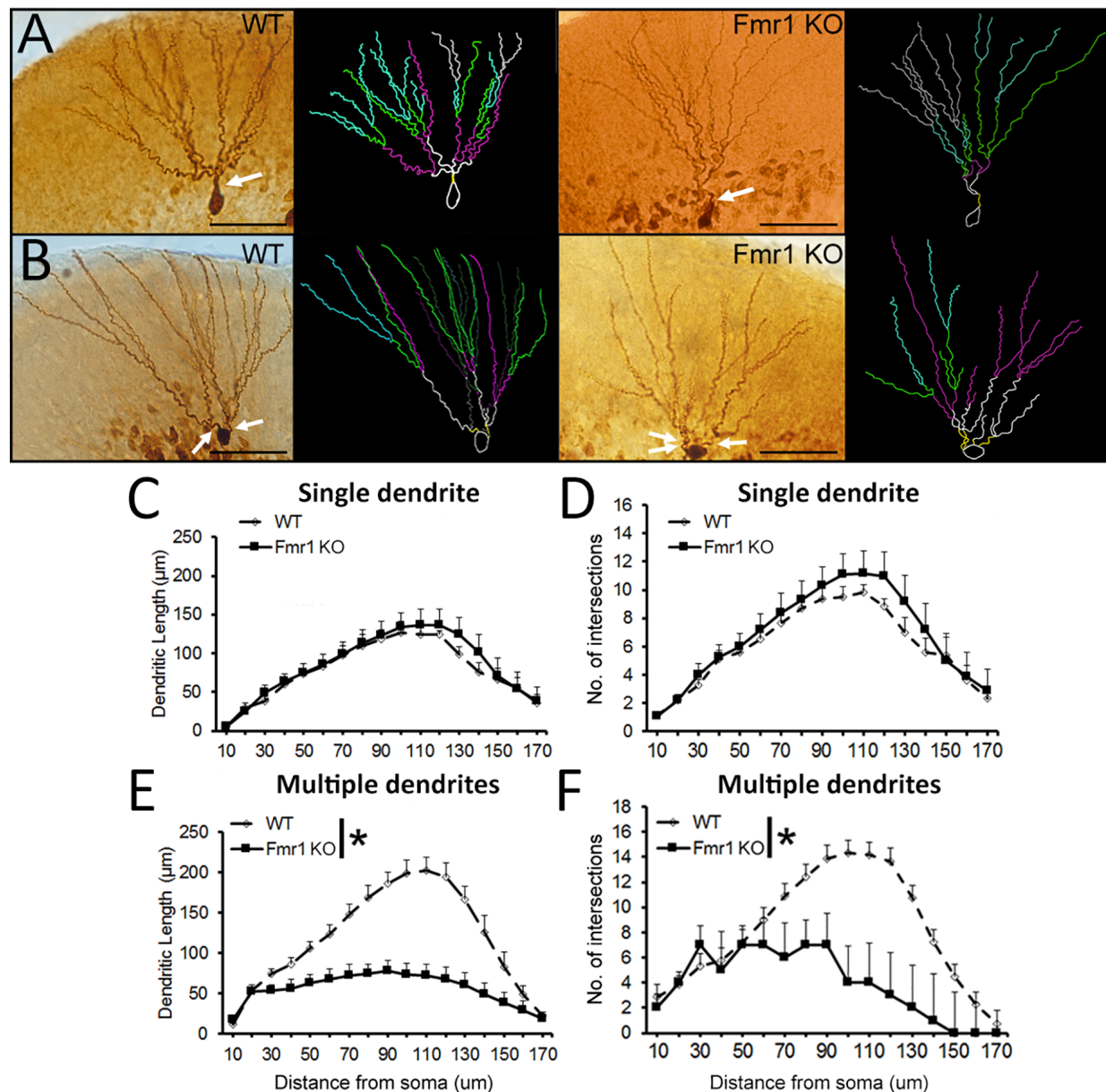


FIGURE 5 | Sholl analysis of biocytin-filled granule cells. **(A)** Representative images of younger and **(B)** mature granule neurons from WT and *Fmr1* KO mice. Scale bar: 50 μ m. **(C,D)** There were no differences in dendritic length and number of intersections in younger granule cells between WT ($n = 10$) and *Fmr1* KO mice ($n = 8$). **(E,F)** Dendritic length and number of intersections in more mature granule cells were significantly decreased in *Fmr1* KO mice ($n = 10$) when compared to WT mice ($n = 12$). Arrows: primary dendrites (yellow traces). * $P < 0.005$ (five animals per group; ANOVA, repeated measures).

Prior research indicates that GluN2A subunit-containing NMDARs are important for dendritic arborization (Kannangara et al., 2014), while GluN2B may be more critical in regulating spine formation (Henle et al., 2012). Interestingly, we have previously shown that NMDAR hypofunction is associated with a significant decrease in the expression of both GluN2A and GluN2B subunits in the DG of *Fmr1* KO mice (Bostrom et al., 2015). However, because FMRP is a translational repressor, it is still not clear how its deletion leads to a reduction in the expression of NMDAR subunits, as well as to what extent it affects

temporal changes in GluN2A and GluN2B expression in younger and more mature dentate granule cells. One factor that could be contributing to this deficit is the dysregulation of translational responses induced by excessive mGluR activation (Muddashetty et al., 2007), disruption of PSD-95 and CAMKII activities (Zalfa et al., 2007) and/or by the abnormal morphology of dendritic spines seen in FXS (Comery et al., 1997; Bilousova et al., 2009). On the other hand, since the loss of FMRP has an impact in the activity of a vast number of proteins, we cannot rule out the possibility that NMDAR-independent mechanisms are also

contributing to the alterations in dendritic development found in our study. Regardless, the fact that we did not observe significant reductions in dendritic complexity and NMDAR function in the younger population of granule cells, suggests that the effects of FMRP on NMDAR function and dendritic complexity may occur over time. The DG offers a unique opportunity to study developmental changes, as it is one of the brain areas that exhibits continual neurogenesis throughout the lifespan. Indeed, the loss of FMRP also alters hippocampal neurogenesis in adult animals, which show increased cell proliferation, but impaired neuronal differentiation (Luo et al., 2010; Guo et al., 2012). Together these findings may suggest that the system is trying to compensate for reduced synaptic signaling by enhancing the production of new cells, but that loss of FMRP also negatively impacts the development of these cells. Our data indicate that a loss of FMRP causes significant deficits in both NMDAR function and dendritic arborization in mature neurons, and this could contribute to the aberrant neurogenic process and reduced cognitive performance that has been observed with the loss of FMRP.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work. Drafting the work or revising it critically for important intellectual content. Provide approval for publication of the content. Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Structural and Functional Abnormalities in the Olfactory System of Fragile X Syndrome Models

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Fragile X Syndrome (FXS) is the most common inherited form of intellectual disability. It is produced by mutation of the *Fmr1* gene that encodes for the Fragile Mental Retardation Protein (FMRP), an important RNA-binding protein that regulates the expression of multiple proteins located in neuronal synapses. Individuals with FXS exhibit abnormal sensory information processing frequently leading to hypersensitivity across sensory modalities and consequently a wide array of behavioral symptoms. Insects and mammals engage primarily their sense of smell to create proper representations of the external world and guide adequate decision-making processes. This feature in combination with the exquisitely organized neuronal circuits found throughout the olfactory system (OS) and the wide expression of FMRP in brain regions that process olfactory information makes it an ideal model to study sensory alterations in FXS models. In the last decade several groups have taken advantage of these features and have used the OS of fruit fly and rodents to understand neuronal alteration giving rise to sensory perception issues. In this review article, we will discuss molecular, morphological and physiological aspects of the olfactory information processing in FXS models. We will highlight the decreased inhibitory/excitatory synaptic balance and the diminished synaptic plasticity found in this system resulting in behavioral alteration of individuals in the presence of odorant stimuli.

Keywords: olfactory coding, olfactory behavior, *Fmr1*-KO, FMRP, *dfmr1*, structural plasticity, excitation/inhibition balance

INTRODUCTION

Fragile X syndrome (FXS) is one of the most common causes of inherited intellectual disability and the most common monogenetic cause of autism. It is estimated that the syndrome prevalence is 1 in 5,000–7,000 in males, while in females is 1 in 8,000–11,000. It is produced by the repeat expansion of the CGG trinucleotide in the promoter region of the human *FMR1* gene located on chromosome X which leads to hypermethylation

and transcriptional silencing of the gen. Individuals with more than 200 CGG repetitions exhibit the full mutation and FXS (Hagerman et al., 2017; Nelson et al., 2017; Sherman and Hunter, 2017).

The Fragile Mental Retardation Protein (FMRP) is a selective RNA-binding protein that regulates the transcription of 4% of the total proteins found in the mammalian brain (Ashley et al., 1993), where its primary function is to repress local protein translation of specific mRNAs at dendrites in an activity-dependent manner, down-regulating the synthesis of proteins involved in synaptic plasticity and function (Brown et al., 2001; Darnell and Klann, 2013; Sidorov et al., 2013; Suhl and Hoeffler, 2017; Bagni and Zukin, 2019). Recently, however, evidence supports additional roles for FMRP, positively regulating mRNA translations and modulating protein activity or molecules stability by direct interaction (Davis and Broadie, 2017; Bagni and Zukin, 2019). It is important to highlight that FMRP interacts with a broad range of coding mRNAs, regulating its translation in both murine and human brain (Ascano et al., 2012; Maurin et al., 2018) and in adult neuronal stem cells (Liu et al., 2018). Moreover, FMRP binds to the coding region of transcripts encoding both pre- and post-synaptic proteins (Darnell et al., 2011), hence playing a major role in learning, memory, adaptation and sensory perception. FXS individual's symptoms include learning disabilities, attention deficit and behavioral and social alterations such as hyperactivity, impaired social communication, hyperarousal and extreme sensitivity to sensory stimuli, among others. Some of all abnormal social behavior in FXS might be secondary to inappropriate filtering to daily life sensory stimulus and a consequent altered sensitivity across sensory modalities including olfaction (Hagerman et al., 1996; Miller et al., 1999; Christie et al., 2009; Rotschafer and Razak, 2013; Arnett et al., 2014; Juczewski et al., 2016; Goel et al., 2018), yet compared to cognitive and social functioning how sensory information is processed in FXS has been largely understudied.

To gain insights into the pathology, physiology and molecular processes of FXS, researchers have predominately used rodents and *Drosophila melanogaster* as experimental animal models. These models have been genetically manipulated to emulate the genotype of FXS, knocking out or down the *Fmr1* gene in mice and *Drosophila*. In both models, the olfactory system (OS) is the most conserved sensory system and critical for the species survival and reproduction, an ideal candidate to study sensory information processing issues found in the absence of FMRP. Moreover, the OS has several traits that make it a highly attractive system to study the neuromorphological and neurophysiological aspects of sensory perception in FXS: (1) in humans, FMRP expression has been confirmed in olfactory neuroblasts, harvested through a nasal biopsy of control individuals and its absence corroborated in patients with FXS (Abrams et al., 1999). Similarly, in rodents and flies FMRP is expressed in high levels in the adult and developing rodent OS (Zhang et al., 2001; Schenck et al., 2002; Christie et al., 2009; Akins et al., 2012; Sudhakaran et al., 2014); (2) the OS has an exquisitely organized neuronal circuitry with a layered anatomy where the input and output information can be easily identified (Farbman, 1992; Murthy, 2011); (3) experimental models exhibit

stereotyped olfactory-mediated behaviors, making it an ideal model to pair physiology with behavior, and (4) together with the hippocampus, the OS is the only brain region that exhibits adult neurogenesis in rodents (Lois and Alvarez-Buylla, 1993; Gheusi et al., 2012), which allows to study the dynamics of neuronal proliferation, migration, maturation, synaptic integration and ultimately experience-mediated plasticity in the post-natal brain.

In this review article, we will discuss molecular, morphological and physiological aspects of olfactory information processing in *Drosophila* and rodents in the absence of FMRP.

OLFACTORY DISCRIMINATION IN FXS MODELS

Mice where the *Fmr1* gene has been knocked out (*Fmr1*-KO; The Dutch-Belgian Fragile X Consortium et al., 1994) exhibit some phenotypic features similar to humans with FXS such as hypersensitivity, hyperactivity, diminished attentive capacity and anxiety (Kooy et al., 2017). FMRP is widely expressed in the rodent brain in the somatodendritic domain of virtually all neurons. In the OSs it is expressed throughout the brain regions that process olfactory information, from the peripheral olfactory sensory neurons (OSNs) in the Olfactory epithelium (OE), to all the neuronal types found in the olfactory bulb (OB) and olfactory cortex (OC, **Box 1**; Hinds et al., 1993; Christie et al., 2009; Akins et al., 2012; Brackett et al., 2013). FMRP is also localized at pre-synaptic terminals, in discrete granules called Fragile X Granules (FXG). FXG are structures that comprise proteins, ribosomes and mRNA and can be found only in a subset of brain regions including the axons of OSNs and in the glomeruli neuropil in the OB, suggesting that FMRP could not only have a post-synaptic role regulating post-synaptic protein translation but also be involved in plastic pre-synaptic olfactory processes (Christie et al., 2009; Akins et al., 2012; Korsak et al., 2017) as have been shown in hippocampal pyramidal neurons (Deng et al., 2013; Myrick et al., 2015). The ubiquitous expression of FMRP in the OS suggests that it might play a role in odorant sensing and processing, as well as in higher order bulbar and cortical computations such as olfactory discrimination, learning and memory. Indeed, some groups have reported that *Fmr1*-KO mice exhibit olfactory dysfunctions, such as a decreased olfactory sensitivity, which refers to the ability of the animal to detect an odorant, when tested in a spontaneous cross-habitation task (Schilit Nitenson et al., 2015). In this task, an animal is presented with an odor consecutively for brief periods of time to induce habituation, which is reflected in a progressive reduction of the time the animal spends investigating the sample. After the habituation period a novel odor, or the same odor at a different concentration, is presented. Failure to increase the investigation time indicates cross-habitation or incapacity to discriminate between the two odorants (Cleland et al., 2002). *Fmr1*-KO, albeit they are able to discriminate odorants, increase the investigation time to a sample one log of concentration higher than WT controls (Schilit Nitenson et al., 2015), reflecting a decreased sensitivity for processing olfactory information. *Fmr1*-KO mice olfactory sensitivity has

also been tested in a two-alternative olfactory discrimination forced choice task with contradictory results. In this task water deprived animals learn to poke with their nose one of two odor ports randomly delivering water as a reward. Under these circumstances, there is not any difference in olfactory sensitivity between *Fmr1*-KO and WT. The difference between these two studies could arise on the difference between the two behavioral paradigms chosen. The two-alternative forced choice, as opposed to the cross-habituation task, requires operant conditioning and the consolidation of a stimulus-reward association. This learning process engages top-down neuronal circuits such as cholinergic nuclei in the basal forebrain (Richardson and DeLong, 1991; Lin and Nicolelis, 2008) modifying how the bulbar neurons respond to a stimulus through context-dependent plasticity (Doucette and Restrepo, 2008), which may by itself regulated the perceptual threshold of an odor. Even though most *Fmr1*-KO mice displayed similar learning curves than control, during the shaping learning curves (where the animal learns the behavioral task itself) *Fmr1*-KO made more errors during the learning process with some *Fmr1*-KO not being able to reach criteria at all (Larson et al., 2008), emulating the intellectual deficits found in the majority of individuals with FXS (Hall et al., 2008). Thus, depending on the environmental conditions and decision-making requirements bottom up and top-down processes that regulate olfactory information computations could be altered in *Fmr1*-KO.

Impaired olfactory performance has also been observed in the fly model of FXS (*dfmr1*) where the absence of dFMRP (the human homolog of FMRP) resulted in reduced olfactory attraction and aversion. In the behavioral experiment, starved flies were presented with an attractive (ethyl acetate) or aversive (benzaldehyde) odorant in an arena with two chambers. The number of flies in the odorized and non-odorized section of a behavioral arena was then counted. They found that *dfmr1*⁻ flies spent less time exploring the quadrant with the attractive odor and that they were less repelled with the aversive odor compared to controls. The same behavioral phenotype was observed when dFMRP was selectively downregulated in the antennal lobe (AL) projection neurons (Franco et al., 2017), suggesting that the behavioral alteration could originate in a somehow dysregulated olfactory projection neuron (PN, **Box 1**)

activity. Flies *dfmr1*³ heterozygous also show defects in the olfactory associative learning test (OAT) or negatively reinforced paradigm, where an odor (CS) is delivered to a chamber in parallel with foot shocks and later the number of flies that prefer a chamber with the CS are counted (Kanellopoulos et al., 2012). These results suggest that FMRP expression is required to adequately process olfactory information and generate context-dependent memories. The role that FMRP plays in olfactory sensing (or sensory processing in general) is not yet understood, but some evidence started to shed light on its function regulating structural plasticity and neuronal excitability.

ANATOMICAL ALTERATIONS IN THE OLFACTORY SYSTEMS OF FXS MODELS

In agreement with a role that FMRP plays regulating neuronal branching (Morales et al., 2002; Galvez et al., 2005), anatomical defects have been found in the olfactory system of FXS models. For instance, mitral cells (MCs) from *Fmr1*-KO exhibit altered architecture when compared to WT controls. As described in **Box 1**, MCs usually project only one dendrite to a unique glomerulus (GL; Malun and Brunjes, 1996) transmitting information in parallel columns downstream the OB and into the olfactory cortex (OC). *Fmr1*-KO OB, however, has on average two apical dendrites per MC (Galvez et al., 2005). Whether the apical dendrites project to one or multiple GL in the *Fmr1*-KO has not been explored yet, but it can be hypothesized that this aberrant morphology would distort olfactory processing. If MCs project to multiple GL, a single MC will be activated by different types of OSNs expressing different Olfactory receptors (ORs), forcing the system to an early integration of information that would otherwise had occurred in the OC. On the other hand, in the case that both apical dendrites project to a single GL, the same glutamatergic release from the OSN will have an augmented effect in the MC and produce an artificially elevated perception of the stimulus as has been described in mice auditory and somatosensory cortex (Arnett et al., 2014) and in humans with FXS (Miller et al., 1999; Christie et al., 2009).

Structural deficits have also been found in the olfactory learning and memory center of the *dfmr1*⁻ flies. Specifically,

BOX 1 | Olfactory System in Rodents and *Drosophila*.

Odorant molecules enter the nose through inhaled air and interact with olfactory receptors of sensory neurons (OSN) located in the nasal olfactory epithelium. To process the diverse and vast number of odors found in the environment, a combinatorial approach is used by the olfactory system. From a ~1,000 olfactory receptors (OR) found in rodents, each OSN in the olfactory epithelium expresses only one of them (Buck and Axel, 1991). An odor can activate different types of OR and each of these OR gets activated by different odorants exhibiting different tuning properties (Araneda et al., 2004). This property of the OR will produce a distributed pattern of activation of OSNs for each odor mixture. Interestingly, olfactory neurons expressing the same receptor project to only one or two glomeruli in the olfactory bulb (OB; Mombaerts et al., 1996), creating a two-dimensional distributed map of odor information across the glomerular layer (Mori et al., 1999). Mitral cells (MC) in the OB receive the upcoming information by extending their apical dendrite to only one glomerulus maintaining this map downstream and projecting their axon to pyramidal cells (Pyr) in the olfactory cortex (OC). Olfactory information is therefore coded in the OB by changes in MC activity that creates a spatio-temporal code, information that is later integrated and ultimately decoded by the cortex (**Figure 1A**). In flies, similarly, OSNs from the antenna and maxillary palp also express a single OR and project to a single glomerulus in the antennal lobe (AL) where they synapse onto the projection neurons (PN). PN also extend their dendrite to only one glomerulus, analogous to the rodent OB and MCs. PN, then send their axons to the Kenyon cells (KCs) in the mushroom body (MB), the learning and memory center of the fly and to the lateral horn (LH) in the protocerebrum (Su et al., 2009; Semaniuk, 2015; **Figure 1B**). Importantly, in both species, inhibitory neurons strictly regulate olfactory processing. In mice, periglomerular neurons (PG) in the glomerulus regulate the influx of information into the brain, while granule cells (GCs) regulate the efflux of information to the OC by making inhibitory synaptic contacts onto the dendrites of MCs. In *Drosophila*, GABAergic local neurons inhibit the pre-synaptic activity at the axon terminals of OSNs and excitatory cholinergic neurons mediate interglomerular excitation.

Kenyon cells (KCs) in the mushroom body (MB) exhibit defects in axonal outputs and dendritic arborization (Pan et al., 2004, 2008; Doll and Broadie, 2015), while PN neurons show reduced neuronal branching, enlargement of the synaptic boutons and reduced connectivity with the post-synaptic KC (Doll et al., 2017). Structural abnormalities have also been observed in GABAergic neurons in flies lacking FMRP, displaying morphological alteration during development with early underdevelopment and later overcompensation (Gatto et al., 2014).

One of the most relevant histological features in neurons of individuals with FXS is the increased abundance of immature and elongated dendritic spines (Altman and Das, 1965; Comery et al., 1997; He and Portera-Cailliau, 2013). Inadequate size and morphology of spines are linked to altered neuronal connectivity, synaptic function and synaptic plasticity (Sala and Segal, 2014) suggesting an underlying role of spine abnormality in some of the symptoms observed in FXS. The classic methodological approximation used to measure spine density and morphology had been performed mostly in the postnatal brain of whole *Fmr1*-KO models, where the effect of FMRP absence in single neurons cannot be dissected from the potential large-scale synaptic effect of knocking down this protein in the whole system (He and Portera-Cailliau, 2013). To solve this problem, the rodent olfactory system exhibits a unique feature, only shared with the hippocampus: the ability of inhibitory neurons—granule cells (GCs) and periglomerular cells (PGs) to proliferate in the postnatal brain. Adult neurogenesis is a widespread process occurring in several organisms from insects to rodents, but it is very limited in *Drosophila* (von Trotha et al., 2009; Simões and Rhiner, 2017) subscribing scarcely only to the optical lobes (Fernández-Hernández et al., 2013). Rodent adult-born neurons originate from progenitor cells located in the subventricular zone (SVZ) of the brain, from where they migrate for about 2 mm to the OB and become functionally integrated within the OB network in a process that takes between 21 and 30 days (Altman and Das, 1965; Lois and Alvarez-Buylla, 1993, 1994). This spatial segregation allows for genetic manipulations to be performed exclusively in the neuronal progenitors and to study the effects of those manipulations at later time points in the OB after the neurons have migrated. Adult-generated GC, in which the *Fmr1* gene was knocked-down by injecting RNA-interference in SVZ, had denser and longer dendritic spines compared to control when evaluated 21 days post injection (d.p.i; Scotto-Lomassese et al., 2011), once the neurons have already reached the OB (Petreanu and Alvarez-Buylla, 2002). Healthy adult-generated GCs when are functionally integrated into the OB neuronal network (Bardy et al., 2010) form glutamatergic synapses with the lateral dendrites of the MCs (Belluzzi et al., 2003). Interestingly, knockdown *Fmr1* GC had more mature glutamatergic synaptic sites and accordingly received more glutamatergic inputs than control GCs (Scotto-Lomassese et al., 2011), which recapitulates the hyperexcitability phenotype found in other brain regions in FXS (Contractor et al., 2015; Ethridge et al., 2017). Interestingly, at 28 d.p.i, when new born GCs are fully mature, the number of dendritic spines in GC lacking FMRP was no different to control GCs (Scotto-Lomassese et al., 2011), suggesting that neurons

without FMRP exhibited an accelerated rate of spinogenesis, that could homeostatically counterbalanced during development in an attempt to re-establish similar rates of connectivity in the FXS network. Taking together this evidence suggests that the absence of FMRP could interfere with normal neuronal architecture and synaptogenesis leading to olfactory dysfunctions in FXS.

STRUCTURAL PLASTICITY ALTERATIONS IN THE OLFACTORY SYSTEM OF FXS MODELS

It has been suggested that changes in the number or morphology of dendritic spines and dendritic arborizations, contributes in the regulation of the physiological changes of synaptic transmission underlying learning and memory (Lamprecht and LeDoux, 2004; Caroni et al., 2012). This process, known as structural plasticity, also occurs in healthy adult-born GCs in response to changes in the environment and olfactory input, but fail to occur in knockdown *Fmr1* GCs. For instance, in response to reduced sensory input a decrease in the complexity of the dendritic arborization is observed in WT GCs (Saghatelyan et al., 2005), process that does not occur in GCs lacking FMRP after animals were deprived of olfactory stimuli occluding one nostril unilaterally (Scotto-Lomassese et al., 2011). In addition, perceptual learning, the improved ability of the sensory system to discriminate stimuli based on experience, also induces profound morphological changes in rodent adult-born neurons. WT mice cannot naturally discriminate between the two perceptually similar odorants limonene+ (Lim+) and limonene− (Lim−), but when WT mice are pre-exposed to the odors for 10 days, they acquire the ability to discriminate between them. *Fmr1*-KO, however, cannot learn to discriminate between Lim+ and Lim−. This learning process in WT was accompanied with increase in the length and complexity of the dendritic branching and spine density in adult-born neurons, changes that were not observed in new neurons lacking FMRP (Daroles et al., 2016). In *Drosophila*, dFMRP has also been shown to play an important role in activity-dependent synaptic remodeling of the olfactory system, during the critical period. During this time, AL projection neurons (specifically AL-mPN2) of WT flies normally reduce their neuronal branch length after a passive exposure to pyrrolidine (Doll et al., 2017). Pyrrolidine is a natural aversive odorant to flies (Schlieff and Wilson, 2007) that promotes structural plastic changes in AL-mPN2 neurons, which have a strong and highly specific response to it (Silbering et al., 2011). In the mutant *dfmr1* fly, the branch length is already diminished in basal conditions and the pyrrolidine-induced structural change reduction is not observed (Doll et al., 2017). Taken together, these results suggest that FMRP plays an important role in synapse formation and that the deficits in activity-dependent structural plasticity observed in GCs could mediate in part the cognitive defects found in the experimental FXS models.

At the neurophysiological level, long-term potentiation (LTP), a long-term change in synaptic strength involving morphological changes in dendritic spines (Nicoll, 2017) was studied *in vitro* in mice brain slices of the piriform cortex.

The piriform cortex is a paleocortical three-layer structure that exhibits excitatory association fibers in layer 1b and is thought to play a major role in the formation of olfactory memories and olfactory discrimination (Bekkers and Suzuki, 2013). LTP induction by theta burst stimulation (TBS) in cortical layer 1b showed that *Fmr1*-KO mice had a substantial reduction of the LTP compared to controls in animals older than 6 months old. In flies, studies have shown that dFMRP directly interacts with Staufen and AGO1, two proteins that play a key role during long term memory (LTM) formation (Bolduc et al., 2008). These findings give a hint on the plastic processes that could be altered in the FXS, specifically in a brain region in charge of integrating sensory information and crucial to generate olfactory memories.

METABOTROPIC GLUTAMATE RECEPTOR (mGluR) THEORY AND OLFACTION

Recent studies have suggested that the *Fmr1*-KO mouse exhibit an imbalance between LTP and long-term depression (LTD; Contractor et al., 2015). LTD is another mechanism contributing to learning and memory and has been widely studied in FXS models giving rise to the “mGluR theory.” In this theory, the absence of FMRP has been associated to an increment of non-regulated protein synthesis mediated by the post-synaptic metabotropic glutamate receptor (mGluR). Once activated, mGluR stimulates the rapid translation of specific preexisting mRNAs in the dendritic spines which are involved in the internalization of AMPA receptors and in the generation of mGluR-mediated LTD in the synapse. This type of LTD occurs and is enhanced in *Fmr1*-KO mice, suggesting that FMRP is required to inhibit the translation of mRNAs involved in LTD stabilization (Snyder et al., 2001; Bear et al., 2004). Importantly, odorant-gated behavior could be rescued just by inhibiting mGluR activation in the conditional *Fmr1*-KO mouse, since injection of MPEP (a mGluR type 1 antagonist), rescued the learning deficits of these animal in a cross-habitation task and the dendritic arbor structural plasticity in bulbar adult-born GCs (Daroles et al., 2016). Similarly, *dfmr1*³ heterozygous flies fed with MPEP eliminated the behavioral alterations these flies exhibited in the olfactory associative test, where an odor is paired with an electric shock and the flies are later tasted for their preference to the odor (Kanellopoulos et al., 2012). Consistent with the ability of FMRP to act as negative regulator of mRNA translation (Laggerbauer et al., 2001; Mazroui et al., 2002), feeding *dfmr1*[−] flies with low concentrations of the protein synthesis inhibitors cycloheximide and puromycin ameliorated olfactory-LTM deficits (Bolduc et al., 2008). Thus, modifying and decreasing protein synthesis directly by activating mGluR or unspecifically by protein synthesis inhibitors, could rescue olfactory behavioral phenotypes in FXS models.

INHIBITORY CIRCUIT DYSFUNCTION IN THE OLFACTORY SYSTEM OF FXS

Sensory processing alterations in FXS are believed to occur by an unregulated inhibitory/excitatory synaptic balance that could

promote the observed hyperexcited state of neuronal circuit and ultimately underpin the increase number of seizures and hypersensitivities found in FXS (Contractor et al., 2015; Davis and Broadie, 2017). For instance, in the *Fmr1*-KO mouse, an hyperexcitable phenotype has been described in the auditory cortex (Rotschafer and Razak, 2013), where neurons exhibit an elevated responsiveness to auditory stimuli, which could lead to altered auditory processing and also a lower threshold for audiogenic seizures (Yan et al., 2005). Furthermore, in the somatosensory cortex of *Fmr1*-KO mice cellular deficits to adapt to repetitive whisker stimulation could account for sensory perception deficits and, more specifically, the tactile defensiveness phenotype seen in the syndrome (He et al., 2017). Moreover, *Fmr1*-KO mice exhibit larger sensory maps in the barrel and visual cortex impairing learning in a whisker- or visual-dependent behavioral task (Arnett et al., 2014).

In addition to the described expression of FMRP in excitatory neurons, FMRP is also present in inhibitory neurons (Olmos-Serrano et al., 2010; Gatto et al., 2014) suggesting that hyperexcitation is mediated in part by a faulty inhibitory system (Cea-Del Rio and Huntsman, 2014; Huntsman and Kooy, 2017).

In the olfactory system of the fly, albeit the general GABAergic neuron number is normal in *dfmr1*[−], the GABA-synthetizing enzyme GAD is strongly reduced in the MB compared to WT individuals. GABA receptors are also downregulated in *dfmr1*[−] AL PN and there is a decrease synaptic connectivity between interneurons and PN, suggesting the dFMRP might be required for proper inhibitory synaptic control (Gatto et al., 2014).

Appropriate inhibitory lateral interactions among GL on the AL are critical for odor information processing, especially for odor mixtures (Figure 1B, inset). In flies, lateral interactions narrows GL odor tuning (Olsen and Wilson, 2008) to enhance contrast and generate adequate olfactory representations downstream. The functional consequences of the faulty GABAergic system observed in *dfmr1*[−] were studied measuring the changes in calcium dynamics using the fluorescent calcium sensor GCaMP3 in the GL. Indeed, when the fly glomeruli activity was measured in response to an odor, the response profile was broader in *dfmr1*[−] flies, suggesting a decrease odor selectivity and contrast (Franco et al., 2017). In addition, GABAergic neurons from mutant flies innervating the MB have an augmented response to stimulation which was also observed by an increase in the fluorescence mediated by GCaMP3 (Gatto et al., 2014).

This evidence indicates that alterations in the inhibitory circuit is abnormal in FXS, which could in part promotes an excitatory/inhibitory imbalance and explain the altered olfactory information processing and olfactory-guided behavior observed in *dfmr1*[−] flies.

FMRP INTERACTORS AND REGULATION OF THE FMRP FUNCTION IN THE FLY OLFACTORY SYSTEM

One of the best-characterized function of FMRP is its role acting as a selective RNA-binding protein regulating the translation

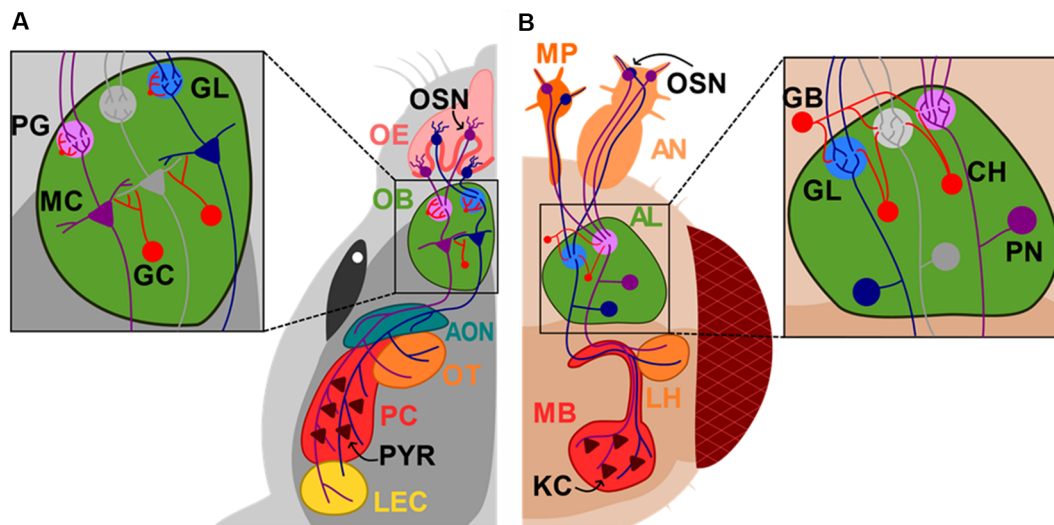


FIGURE 1 | Diagram of the mouse and *Drosophila* olfactory systems. Sensory neurons (OSN) in the nose or antennae (AN) and maxillary palp (MP) project to the superior centers of olfactory sensory processing, olfactory bulb (OB) and antennal lobe (AL) in mice (A) and *Drosophila* (B), respectively. Mitral cells (MC) send their axons directly to the accessory olfactory nucleus (AON), olfactory tubercle (OT), piriform cortex (PC) and lateral entorhinal cortex (LEC), while projection neurons (PN) project to the mushroom body (MB) and lateral horns (LH). Olfactory epithelium (OE); glomerulus layer (GL); periglomerular cell (PG); granule cell (GC); GABAergic neurons (GB); cholinergic neurons (CH); kenyon cells (KCs). Neurons that are synaptically connected are depicted in the same colors.

of multiple proteins related to synaptic physiology (Ashley et al., 1993). Important efforts have been made to study the FMRP molecular function underpinning the sensory alterations found in FXS using the olfactory system of *Drosophila* as the experimental model.

Futsch is a *Drosophila* protein orthologous to the mammalian microtubule-associated protein 1B (MAP1B) that plays crucial roles in dendritic and axonal growth during embryogenesis (Hummel et al., 2000; Roos et al., 2000) and regulates the synaptic microtubule cytoskeleton organization at the neuromuscular junction (NMJ; Bodaleo and Gonzalez-Billault, 2016). It has been shown that dFMRP physically interacts with *futsch* mRNA, acting as a negative translational regulator of Futsch (Zhang et al., 2001). Of note, both *futsch* overexpression and *dfmr1*-null mutants show a synaptic overgrowth phenotype at NMJ characterized by an increased number of synaptic boutons and enhanced levels of neurotransmission. Interestingly, double mutants of *dfmr1*- and *futsch* restore the altered synaptic phenotype to wild-type levels at NMJ (Zhang et al., 2001). In the fly adult olfactory system, *futsch* mutants show a progressive neuronal degeneration, accompanied by deficits in learning and olfactory memory. These detrimental phenotypes are partially suppressed by a *dfmr1* deletion (Bettencourt da Cruz et al., 2005). It has been shown that dFMRP is highly expressed in the larval MB (Schenck et al., 2002) and in PN (Bettencourt da Cruz et al., 2005), making it coherent that its misregulation could lead to defects in the olfactory sensory system. It is important to highlight that the mRNA of the mammal orthologous of *futsch*, *MAP1B*, and for *CAMKII* have been widely reported to interact with FMRP in the mammal brain (Davis and Broadie, 2017; Bagni and Zukin, 2019), opening the possibility that the impairment

of these dFMRP-mRNA interactions may be recapitulated in the olfactory system of FXS mammal models.

Another mRNA regulated by dFMRP is *shrub* (human Chmp4). *Shrub* is a core member, the endosomal sorting complexes required for transport (ESCRT; Schmidt and Teis, 2012). Interestingly, dFMRP binds *shrub* mRNA to negatively regulate *Shrub* protein expression levels in whole brain lysates from newly eclosed animals during the disease state early-use critical period. Both dFMRP loss-of-function and *Shrub* overexpression increase PN innervation, PN synaptic endosomes and PN synaptic area (Vita and Broadie, 2017). Moreover, it has been suggested that *Shrub* controls neuronal morphogenesis in *Drosophila*, since *Shrub*-null animals display abnormal distribution of endosomal markers, and an altered axonal and dendritic branching pattern (Sweeney et al., 2006). These antecedents strongly suggest that membrane trafficking impairments at synapses could be a novel causative mechanism in the FXS disease (Vita and Broadie, 2017) which could negatively affect the structure and synaptic physiology in the olfactory network of FXS models.

In addition of acting as a translational regulator, FMRP functions themselves are regulated by its interaction with other proteins, post-translational modifications and alternative splicing (Pasciuto and Bagni, 2014a; Bonaccorso et al., 2015). For instance, the *Drosophila* dNab2 protein (the ortholog of the human ZC3H14), is a polyadenosin RNA-binding protein that has been related to autosomal recessive intellectual disability (Pak et al., 2011). In the olfactory system of the fly, MB axons lacking dNab2 exhibit disrupted development, projecting aberrantly across the brain midline and show defective branching, which leads to short-term memory impairment (Kelly et al., 2016).

Interestingly, dNab2 interacts with dFMRP in cultured brain neurons and co-distribute in the different neuronal compartments. In the olfactory system, dNab2 and dFMRP are strongly expressed in the PN, where dNab2 can interact with and regulate *CaMKII* mRNA, a dFMRP target. Furthermore, flies carrying mutations in both *dNab2* and *dfmrp* genes show an impaired aversive-odor induced suppression of phototaxis indicating that the dNab2 and dFMRP interaction are required for olfactory memory (Bienkowski et al., 2017). Another protein that interacts with dFMRP is Ataxin-2 (Atx-2). Atx-2 is an RNA-binding protein related to neurodegeneration since mutations resulting in the expansion of a polyglutamine tract in the gene encoding ataxin-2 give rise to the neurodegenerative disorders spinocerebellar ataxia type 2 and Parkinson's disease (Satterfield and Pallanck, 2006). It has been shown that dFMRP and Atx-2 proteins physically associates and bind with the *CaMKII* mRNA. In this context, it has been suggested that dFMRP is required for long-term olfactory habituation (LTH), a phenomenon dependent on Atx2-dependent potentiation of inhibitory transmission from LNs to PNs in the AL (Sudhakaran et al., 2014). Dff related protein-2 (Drep-2), another protein regulating FMRP function, was first described as an apoptosis regulator in fly (Park and Park, 2012). However, it was later determined that Drep-2 expression is highly enriched at post-synaptic densities of MB input synapses in flies, where it plays a role in normal olfactory short- and intermediate-term memory, by forming a protein complex with FMRP (Andlauer et al., 2014). Interestingly, Drep-2 ablation functionally compensates for the loss a FMRP, following a proposed mechanism where Drep-2 is required downstream of mGluR signaling to counteract the translational repression executed by FMRP (Andlauer et al., 2014), thereby recapitulating the rescued phenotype of mutants lacking FMRP induced by pharmacological inhibition of mGluRs (McBride et al., 2005).

Taking together this evidence suggests that the detrimental olfactory sensing observed in FXS models may be partially explained by the altered interaction between FMRP and RNA-binding proteins, such as dNab2 and Atx-2 and other interactors such as Drep-2.

Furthermore, other molecular modifications such as post-translational modifications and alternative splicing also regulate FMRP function in the olfactory system. Of note,

FMRP is phosphorylated on a specific serine residue (human S500; murine S499; *Drosophila* S406), and such phosphorylation influences the translational state of FMRP-associated polysomes (Ceman et al., 2003; Coffee et al., 2012). It has been shown that in murine models FMRP regulates post-synaptic physiology in a mechanism where activation of metabotropic glutamate receptors (mGluR) induces a rapid FMRP dephosphorylation, mediated by PP2A (Nalavadi et al., 2012) promoting then a burst in translation FMRP-bound mRNAs including the one encoding for post-synaptic density-95 protein (PSD-95; Narayanan et al., 2007; Muddashetty et al., 2011). The ectopic expression of human FMRP into a *Drosophila* model for FXS fully rescues the molecular and cellular defects at NMJ, demonstrating functional conservation among species (Coffee et al., 2010). To analyze the effect of the FMRP phosphorylation state on neuronal physiology, human FMRP dephosphomimetic (S500A-*hFMR1*) and phosphomimic (S500D-*hFMR1*) transgenes were transformed into an FXS *Drosophila* model. Interestingly, only S500D-*hFMR1* restore normal synaptic architecture in *dfmr1* null neurons and successfully rescues learning performance back to wild-type levels in a Pavlovian olfactory learning assay, while the dephosphomimetic transgene is unable to rescue learning deficits observed in the FXS model. These results demonstrate that the phosphorylation at S500 residue within human FMRP is necessary for proper olfactory sensory learning (Coffee et al., 2012).

Finally, the FMR1 gene undergoes extensive alternative splicing, and several FMR1 mRNA and FMRP isoforms have been observed in both human and mouse brain tissue. The distribution and abundance of these isoforms may be associated to differential expression and functional properties of FMRP (Pretto et al., 2015). It has been suggested that the FMRP pre-mRNA can be alternatively spliced into as many as 20 different mature transcript isoforms (Brackett et al., 2013). The expression of 12 isoforms was analyzed in different mice brain regions (isoforms 1–6 containing exon 12, and isoforms 7–12 lacking exon 12). Of note, levels of isoforms 1–6 are the highest in the hippocampus and OB. Considering that exon 12 encodes for an extended loop in the RNA-binding KH2 domain of the FRMP protein, the presence of this protein motif in the isoforms preferentially expressed in the OB may define some specific subset of RNA molecules bound to FMRP

TABLE 1 | Summary of dFMRP proteins and mRNAs interactors on the fly olfactory system.

Interactor partner	Interactor function	Relevance in olfactory function	Reference
Protein			
dNab2	RNA-binding protein	Long-term olfactory habituation	Bienkowski et al. (2017)
Ataxin-2	RNA-binding protein	Learning and olfactory memory	Sudhakaran et al. (2014)
Dff related protein-2 (Drep-2)	Synaptic regulator	Olfactory short- and intermediate-term memory	Andlauer et al. (2014)
mRNA			
<i>Futsch</i> (mammal ortholog: <i>MAP1B</i>)	Synaptic regulator/microtubule binding protein	Learning and olfactory memory	Zhang et al. (2001)
<i>Shrub</i> (mammal ortholog: <i>Chmp4</i>)	Endosomal sorting	Regulation of PN innervation on central brain MB	Vita and Broadie (2017)
<i>CaMKII</i> (mammal ortholog: <i>CaMKII</i>)	Synaptic regulator	Olfactory memory	Sudhakaran et al. (2014) and Bienkowski et al. (2017)

in the region involved in olfactory perception in mice (Brackett et al., 2013) and explain the wide array of anatomical alterations and odor-mediated behavioral deficits found in FXS models. The FMRP interactors studied in the fly olfactory system are summarized in **Table 1**. Up to date, no molecular FMRP interactors have been described in the olfactory system of rodents, however, a list of proteins and mRNA targets in other rodent brain regions have been reviewed in Davis and Broadie (2017); Suhl and Hoeffer (2017); Bagni and Zukin (2019) and nicely depicted in Pasciuto and Bagni (2014a,b).

CONCLUDING REMARKS

The *Fmr1*-KO mouse and the *dfmr1*- fly are the most widely used model organisms in the field of FXS. Both rely on olfactory information to survive, reproduce and make context-adequate decisions. Hence, in the last years the olfactory circuitry had emerged as a very interesting experimental model to study the neurobiological basis of behavioral and neuronal alterations in FXS.

FXS individuals exhibit an hyperexcitable phenotype and a high network synchronization that translate into hyperresponsiveness to stimuli (Contractor et al., 2015). Experimental models have revealed that the putative causes for this neuronal hyperexcitation are very diverse and occur at the molecular, synaptic and circuit level. It involves the excitatory and inhibitory systems producing an alteration in synaptic excitatory/inhibitory balance that ultimately translates in cognitive and behavioral impairments. In the olfactory system, the neuronal hyperexcitation is reflected in part into neurons that are broader tuned and are less selective to odors diminishing the discrimination capacity in *dfmr*- flies. Moreover, the inhibitory system in the OB and AL is greatly disturbed in mice and flies not expressing FMRP. Any or all these findings could explain the olfactory deficits and decrease learning capacity observed in FXS.

Another key symptom that appears to be greatly disturbed in mice and flies lacking FMRP is a diminished capacity for activity-dependent structural plasticity. In the olfactory system, GCs lacking FMRP that were born in the adult brain do not regulate their dendritic branch length and number of dendritic spines accordingly with the environmental condition. The proliferation and proper integration of new-born GCs

greatly enhance the plastic capacity of the olfactory systems increasing the inhibitory inputs of targeted MCs selectively playing a fundamental role in olfactory discrimination and learning. GCs in the *Fmr1*-KO mouse exhibit an unregulated increase in dendritic spines and synaptic contact reaching the same number WT mice at early time points during development. These suggest that the critical period could be reduced in FXS and that the accelerated synaptic contact formation could generate aberrant neuronal communication. Structural plasticity and the generation and loss of synaptic contacts underpin the learning ability of the brain and the rigidity observed in mice and flies without FMRP could also be central in their diminished sensory discrimination capacity.

This evidence suggests that inappropriate filtering of information is impaired in FXS, which could translate in aberrant decision-making and behavior and that disruption in the FMRP molecular interactions would explain, at least partially, the synaptic phenotype observed in the FXS models. More evidence, however, needs to be gathered *in vivo*, in freely moving animals that are actively behaving and learning to truly uncover how information processing computations are altered in FXS and advance the field to find potential therapeutic solutions.

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AN-P, FB, CG-B and CC-DR reviewed the literature and wrote the manuscript. CT-M helped to review the literature.

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