Hippocampal Synaptic Plasticity in a Murine Knock-Out Model of Fragile X Syndrome

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General Abstract

The dissertation is divided into two separate experiments that explore the effects of visual-spatial learning on PSD-95 dorsal hippocampal expression. Specifically, the aim of these studies was to explore the effect of learning an assay, the Hebb-Williams mazes, on the protein expression of PSD-95 in *Fmr1* KO mice. PSD-95 is an important scaffolding protein hypothesized to be involved in learning and memory. In cellular models of Fragile X Syndrome it has been shown to be dysregulated but it has never been measured following behavioural learning. Establishment of a deficit using an ecologically valid behavioural assay could lead to the development of novel interventions.

Study one employed a subset of the Hebb-Williams mazes of various levels of difficulty to evaluate PSD-95 protein expression in Fmrp intact and *Fmr1* KO mice following learning. The results revealed significant increases in PSD-95 protein expression in control runners when compared to *Fmr1* KO mice. There was a negative correlation between PSD-95 protein levels and mean total errors on the mazes meaning that as expression was increased, errors were decreased.

The goals of study two were to reverse the molecular and behavioural deficits using pharmacological antagonist treatment shown to be effective in cellular models of Fragile X Syndrome. *Fmr1* KO mice were treated with either saline or 20 mg/kg of a metabotropic glutamate receptor antagonist, 2-Methyl-6-(phenylethynyl) pyridine (MPEP). Relative to saline treated controls, drug treated *Fmr1* KO mice made fewer errors on the same subset of Hebb-Williams mazes used in study one. Latency to complete these mazes did not differ between groups, indicating that MPEP treatment does not adversely affect motor functioning. Protein assessment revealed that PSD-95 was selectively rescued in MPEP treated mice and not saline

controls. Similar to study one, a negative correlation between PSD-95 protein levels and mean total errors was observed.

When taken together, these studies indicate that protein deficits are associated with a deficit of learning that can be reversed with a selective glutamate receptor antagonist. One of the strengths of the Hebb-Williams mazes is that performance is measurable without floor or ceiling effects, which plague other common behavioural assays. These data further suggest that pharmacological antagonist treatments may be promising in correcting the learning deficits in human Fragile X Syndrome patients.

Contributions of Authors

Two manuscripts comprise the doctoral thesis. Both papers have been accepted and published in peer reviewed journals. The following is a statement regarding the contributions of the author and co-authors of the work described.

Réno Gandhi was the first author on the manuscripts that comprise the thesis. He contributed to the conceptualization and design of the experiments described, tested murine participants in experiment one and two, completed all molecular assays, planned statistical analyses, analyzed and interpreted all the data. Manuscripts presented were written by him.

Manuscripts were co-authored by Dr. Cary Kogan, in keeping with his role as thesis supervisor and mentor. Dr. Kogan provided guidance and assistance in all aspects of the process, especially in the reviewing and revising of the manuscripts.

Dr. Claude Messier contributed to the conceptualization and methodological design for both animal experiments. He reviewed the manuscripts. He also provided the necessary laboratory space to conduct the experiments.

Dr. Lindsey MacLeod performed the behavioural testing in experiment one. She contributed to the methodological design in experiment one. She provided advice on behavioural and statistical analyses for experiment two.

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List of Abbreviations

ANOVA: Analysis of variance
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
CGG: Cytosine, Guanine, Guanine
CA1: *Cornu Ammonis* area 1
DHPG: (*S*)-3,5-Dihydroxyphenylglycine
ERK: Extracellular signal-related kinase
EPSC: Excitatory post-synaptic current
FISH: Fluorescent *in situ* hybridization
Fmr1 KO mice: *Fmr1* knock-out mice
FMRP/Fmrp: Fragile X mental retardation protein
FXS: Fragile X syndrome
FMR1: Fragile X mental retardation 1
HFS: High frequency stimulation
H-W mazes: Hebb-Williams mazes
IQ: Intelligence quotient
LFS: Low frequency stimulation
LTD: Long term depression
LTP: Long term potentiation
mGluR: Metabotropic Glutamate Receptor
MPEP: 2-Methyl-6-(phenylethynyl) pyridine
mRNA: Messenger RNA
mRNP: Messenger ribonucleoprotein particle
MWM: Morris Water Maze
NMDAR: N-methyl-D-aspartate receptor
PSD: Post-synaptic density
PSD-95: Post-synaptic density protein 95
PP2A: Protein phosphatase 2A
S6K1:Protein S6 kinase

Chapter One: General Introduction

Fragile X syndrome (FXS) is a debilitating condition characterized by physical and behavioural abnormalities. It is the most frequent cause of inherited mental retardation (Tuner, Webb, Wake & Robinson, 1996) and autism (Hagerman, Ono & Hagerman, 2005) with estimated prevalence rates of 1:5000 in males and approximately 1:7500 females (Coffee et al., 2009; Hagerman, 2008). FXS occurs due to loss of function mutation on the fragile X mental retardation 1 (*FMR1*) gene on the X chromosome (reviewed in O'Donnell & Warren, 2002; Santoro, Bray, & Warren, 2012) producing an excess of Cytosine, Guanine, Guanine (CGG) repeats in the promoter region of *FMR1* (Verkerk et al., 1991). In unaffected individuals, the trinucleotide CGG repeats typically number between 5-50, however in the full mutational state of FXS, the number of CGG repeats is > 200 (Verkerk et al., 1991). Excessive repeats produce transcriptional silencing of *FMR1* and loss of expression of the fragile X mental retardation protein (FMRP) (Fu et al., 1991; Pieretti et al., 1991). In addition to the full mutation, a premutation state has been identified that is characterized by smaller expansions between 55-200 CGG repeats and is associated with an increase of *FMR1* mRNA as well as symptoms that are not typically found in full-mutation carriers such as ovarian insufficiency and fragile X-associated tremor/ataxia syndrome (FXTAS) (Allingham-Hawkins et al., 1999; Jacquemont et al., 2003).

FMR1 mRNA is predominately expressed in the developing human fetus as well as adult somatic cells in the CNS and PNS (Abitbol et al., 1993; Devys, Lutz, Rouyer, Bellocq & Mandel, 1993). It is also found in a number of brain regions including the hippocampus (Abitbol et al., 1993). Development of the brain without the expression of FMRP results in a behavioural phenotype that is characterized by several of the following: attention deficits, impulsivity, hyperactivity, anxiety disorders including social and specific phobias, seizure activity, obsessive

compulsive and autistic behaviours, stereotypy, avoidance of eye contact, social isolation, tactile defensiveness and pronounced physical features such as an elongated face, large ears and macroorchidism (Baumgardner, Reiss, Freund, & Abrams, 1995; Cordeiro, Ballinger, Hagerman, & Hessl, 2011; Cornish et al., 2004; Hagerman, 1996; Hagerman, 2002; Hatton et al., 2006; Hessl et al., 2001; Kau et al., 2004; Scerif, Cornish, Wilding, Driver, & Karmiloff-Smith, 2007; Sullivan et al., 2006; Turk, 1998). Ultimately, the severity of symptoms in FXS varies as a function of FMRP expression level which may be affected by mosaicism or X inactivation in females (DeBoulle et al., 1993; Kaufmann, Abrams, Chen, & Reiss, 1999; Loesch, Huggins, Petrovic, & Slater, 1995). In addition to FXS, low levels of FMRP have been found in other disorders such as autism, schizophrenia, bipolar and major depressive disorders (Fatemi & Folsom, 2010).

FXS patients present with intelligence quotients (IQ) in the moderate to severely retarded range although females, who are hemizygous for the X chromosome, are less severely impaired than males (Bennetto & Pennington, 2002; Cornish, Munir & Cross, 1998). Despite IQs in the moderate to severely retarded range, studies of FXS patients have documented a cognitive profile that consists of relative strengths and weaknesses (Van der Molen et al., 2010). Areas of strength include vocabulary, verbal expressive (Maes, Fryns, Van Walleghem, & Van den Berghe, 1994) and receptive language (Philofsky, Hepburn, Hayes, Hagerman, & Rogers, 2004) long term memory for significant information (Maes et al., 1994; Freund & Reiss, 1991) and object recognition memory (Kogan et al., 2009). Areas of weakness include deficits of inhibition, working memory, cognitive flexibility, attentional set-shifting, and planning (reviewed in Hooper et al., 2008), visual-spatial working memory (Kogan et al., 2009; Baker et al., 2011), visuo- construction and visuo-motor skills (Cornish, Munir, & Cross 1998; Cornish, Munir, &

Cross, 1999) visual-perceptual processing (Kogan et al., 2004) and spatial navigation and learning (MacLeod, Kogan, Collin, Berry-Kravis, Messier, & Gandhi, 2010). Taken together, these findings indicate that FXS patients have considerable challenges in executive functioning and visual-spatial cognitive abilities.

Over the last decade much progress has been made in understanding the functions of Fmrp, an mRNA binding protein (Ashley, Wilkinson, Reines & Warren, 1993). Frmp is involved in dendritic transport and translational regulation of hundreds of mRNAs, many of which have established roles in synaptic plasticity and dendrite morphology (Bagni & Greenough, 2005; Bassell & Warren, 2008; Darnell et al., 2001, 2011). Post-synaptic modifications of synapses are believed to be the molecular mechanism underlying learning and memory (Bliss & Collingridge, 1993; Malenka & Bear, 2004; Scholz et al., 2010). In the absence of Fmrp, a distinct type of glutamatergic-based signalling known as Long Term Depression (LTD) is exaggerated and downstream of this, mRNA translation is dysregulated (Bear, Huber, & Warren, 2004; Huber, Gallagher, Warren, & Bear, 2002; Hou, Antion, Hu, Spencer, Paylor, & Klann, 2006; Lu et al., 2004; Niere, Wilkerson, & Huber, 2012; Nosyreva & Huber, 2006; Osterweil, Krueger, Reinhold, & Bear, 2010; Todd, Mack, & Malter, 2003).

Dendritic spine abnormalities are most commonly associated with disorders of mental retardation such as Down syndrome, Rett's syndrome and FXS (Kaufmann & Moser, 2000). Dendritic spines are important because they are the primary area where synaptic plasticity takes place in neurons (Ronesi & Huber, 2008; Segal, 2005; Yuste & Bonhoeffer, 2004). Post-mortem analyses of the temporal and visual cortices of human FXS patients revealed that in the absence of FMRP, increased numbers of dendritic spines with abnormally long, thin and immature appearances were present reflecting atypical synaptic development and/or gross impairments in

function (Hinton, Brown, Wisniewski, & Rudelli, 1991; Irwin et al., 2001; Wisniewski, Segan, Miezejeske, Sersen, & Rudelli, 1991).

Commensurate with the human autopsy data, animal models of FXS that are Fmrp-null have also revealed the presence of long, thin and immature appearing dendritic spines in the visual, primary somatosensory and occipital cortices in *Fmr1* knock-out (KO) mice (Galvaz & Greenough, 2005; Irwin et al., 2001; McKinney, Grossman, Elisseou, & Greenough, 2005). In comparison to the neocortex, fewer long and thin spines and increased numbers of short and stubby spines were reported in the dentate gyrus and *Cornu Ammonis* area 1 (CA1) of hippocampal neurons of *Fmr1* KO mice (Bilousova et al., 2009; Grossman, Elisseou, McKinney, & Greenough, 2006; Grossman et al., 2010; Levenga et al., 2011a; Levenga et al., 2011b). However, the appearance of spines from the *Fmr1* KO mice in the CA1 were morphologically more immature relative to controls. Combined, these data suggest that dendritic spine morphology varies by brain region in the normal brain and in the absence of FMRP/Fmrp, spine maturation and function is compromised and could underlie some of the cognitive impairments that typically occur in FXS.

As a result of a common etiology which encompasses a single gene defect, FXS represents a potentially useful model to convert basic neuroscience findings into clinically relevant treatments for affected human patients (Berry-Kravis, Knox, & Hervey, 2011; Krueger & Bear, 2011). Much progress has been made with the use of the *Fmr1* KO mouse, a murine model of FXS.

***Fmr1* KO Mice**

The *FMR1* gene is fairly conserved across species, and the murine *Fmr1* gene shares 97% percent comparable amino acid sequence with humans (Ashley et al., 1993). To determine

whether the loss of Fmrp could produce comparable phenotypes in species of mouse and human, an *Fmr1* KO mouse was created by adding a neomycin cassette into exon 5 of the *Fmr1* murine gene (The Dutch-Belgian Fragile X Consortium, 1994). While the mutation in *Fmr1* KO mice does not increase the number of CGG trinucleotide repeats as in the human condition, it nevertheless results in a loss of Fmrp production (The Dutch-Belgian Fragile X Consortium, 1994; Kooy, 2003). Notwithstanding the challenges in modelling human diseases using animal models, the murine phenotype recapitulates multiple aspects of the human condition (The Dutch-Belgian Fragile X Consortium, 1994; Kooy, 2003).

Aligned with some of the characteristics of fragile X patients, *Fmr1* KO mice display macroorchidism (The Dutch-Belgian Fragile X Consortium, 1994), increased susceptibility to audiogenic seizures (Michalon et al., 2012; Pacey, Heximer, & Hampson, 2009; Thomas, Bui, Graham, Perkins, Yuva-Paylor, & Paylor, 2011), heightened sensitivity to auditory stimuli (Chen & Toth, 2001; Rotschafer & Razak, 2013), hyperactivity as assessed by performance on the Open Field Test (Mineur, Sluyter, deWit, Oostra, & Crusio, 2002; Spencer et al., 2011; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005), increased stereotypic activity (repeated patterns of beam breaks on the Open Field Test; Spencer et al., 2011) significantly fewer social interactions with novel conspecifics and social anxiety (Mineur, Huynh, & Crusio, 2006; Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005), impairments in perceptual discrimination tasks reflecting deficits of attention (Casten, Gray, & Burwell, 2011) impaired cognitive flexibility and inhibitory control (Krueger, Osterweil, Chen, Tye, & Bear, 2011; Dickson et al., 2013) and deficits of fear conditioning reflecting maladaptive learning (Paradee, Melikian, Rasmussen, Kenneson, Conn, & Warren, 1999; Zhao, Toyoda, Ko, Ding, Wu, & Zhuo, 2005). In addition to the murine model, many of these phenotypic features have also been

documented in the drosophila (fruit fly) and zebrafish models of FXS (Mcbride, Bell, & Jongens, 2012; Tessier & Broadie, 2012; Tucker Richards, & Lardelli, 2006).

In situ hybridization of healthy fetuses and adults revealed that *FMR1* mRNAs are expressed in the human brain with high levels reported in neurons of the hippocampus (Abitbol et al., 1993; Devys et al., 1993). Likewise, in mouse wild- type embryonic and adult tissue, *Fmr1* mRNA was localized throughout the brain, and expressed within the hippocampus (Hinds et al., 1993). These comparable findings suggest that FMRP/Fmrp is important in the hippocampus and in the underlying functions sub-served by this brain region.

In humans (Bartsch et al., 2010; Ekstrom et al., 2003; Iaria, Petrides, Dagher, Pike & Bohbot, 2003) and animals (McDaniel, Compton & Smith, 1994; Muller & Kubie, 1987; O'Keefe, & Dostrovsky, 1971), spatial navigation and learning is primarily reliant on the dorsal hippocampus (Bannerman et al., 2004; Engin & Treit, 2007; Moser & Moser, 1998). A "benchmark" task for assessing spatial navigation, learning and memory is the Morris Water Maze (MWM) (Burger et al., 2007; Clark, Broadbent & Squire, 2005; Garthe & Kemperman, 2013; Morris, Garrud, Rawlins, & O'Keefe, 1982). In this task, rodents must learn the initial location of an escape platform by using only distal, allocentric navigation cues. Initially, a naive rodent will swim around until it bumps into this platform and climbs onto it, ending the trial. On subsequent trials, the animals will swim directly to this platform via the quickest route possible (Morris, 1981; Morris et al., 1982). Studies that have produced hippocampal lesions in animals have resulted in impaired performance on the MWM (Clark et al. 2005; Miyoshi et al., 2012; Morris, 1982; Okada & Okaichi, 2009; Sutherland, Kolb & Whishaw, 1982). Likewise, hippocampal lesions impair spatial navigation and learning in other tasks such as the Radial Arm

maze and T-maze, which are also hippocampal dependent (Goodrich-Hunsaker & Hopkins, 2010; Hock & Bunsey, 1998; Jarrard, 1993; Lee & Kesner, 2003).

Inconsistencies in Assessment of Spatial Navigation and Learning using *Fmr1* KO mice

Tests of spatial navigation and learning in *Fmr1* KO mice have produced inconsistent results. Some research has noted deficits in *Fmr1* KO mice during the reversal phase of the MWM (Baker, Wray, Ritter, Mason, Lanthorn, & Savelieva, 2010; D'Hooge et al., 1997; Kooy et al., 1996; The Dutch-Belgian Fragile X Consortium, 1994) and plus-shaped water maze (Van Dam et al., 2000), which requires mice to employ a novel spatial navigation strategy after the learned location of an escape platform has been moved to an alternative quadrant. By comparison, other studies have not found corroborating results during the reversal phase (Eadie et al., 2009; Paradee et al., 1999; Peier et al., 2000; Yan et al., 2004). Additional inconsistencies were reported on the cross-shaped water maze as *Fmr1* KO mice of a FVB/N-129/OlaHsd hybrid background but not those of a C57BL/6 background demonstrated deficiencies in learning the location of a hidden escape platform (Dobkin et al., 2000). Finally, in the Radial Arm Maze, Mineur et al., (2002) and Yan et al., (2004) observed discrepant performances in two different strains of *Fmr1* KO mice.

The inconsistencies in the literature can be attributed to the lack of a common genetic background used (Spencer et al., 2005) or the number of generational backcrossing of the rodents (Gu et al, 2002). The most common genetic backgrounds used in animal models of FXS have been the C57BL/6 and the FVB strains (Kooy, 2003). Typically, better results have been achieved using mice of a C57BL/6 background on the MWM and the Cross-Shaped Water Maze; a finding attributed to the innately enhanced spatial abilities of this strain (Dobkin et al., 2000; Paradee et al., 1999) Regarding the FVB background, it has traditionally been used for the

generation of transgenic animals as a result of oocytes that have larger and stronger pronuclei (which are amenable to microinjection) and because they tend to breed at a faster pace producing more experimental animals (Auerbach et al., 2003; Errijgers et al., 2007). However, there is a retinal degradation within this strain which may compromise results on behavioural tests requiring intact visual abilities. Recently, a sighted version of the FVB strain pigmented (Tyrc-ch/c-ch) and free of retinal degeneration (Pde6bþ/þ) was bred and shown to have ameliorated performance on the MWM (FVB.129P2-Pde6bþ Tyrc-ch/Ant; Errijgers et al., 2007). Using this sighted FVB background, MacLeod et al. (2010) reported that the latency for *Fmr1* KO mice to complete a set of spatial navigation and learning problems did not significantly differ from wild-type controls. Further, all mice were able to complete the trials by successfully navigating through the mazes of their own volition. This suggests that basic visual-spatial and perceptual abilities are intact in this sighted strain.

The Hebb-Williams Mazes as an Alternative to Assess Spatial Navigation and Learning

The lack of ecological validity of the behavioral assay could also account for some of the variability reported in the animal literature. The Hebb-Williams Mazes have garnered empirical support as a viable alternative to the spatial navigation and learning tasks discussed thus far. They were originally designed by Donald Hebb and Kenneth Williams (1946) as a measure of rat intelligence and were subsequently standardized into 12 mazes of increasing difficulty by Rabinovich and Rosvold (1951). With respect to the layout, a square field is divided into 36 equal areas to delineate error zones and on diagonally opposite corners are start and goal areas. Subjects must navigate their way through various configurations of problems of ascending difficulty and are assessed on outcome measures of latency to complete and number of errors committed on each maze (Rabinovich & Rosvold, 1951). A corollary of having multiple mazes

of increasing difficulty is a richer pattern of results and greater sensitivity to detecting hippocampal deficits in spatial navigation and learning (Shore, Stanford, MacInnes, Klein, & Brown, 2001). The Hebb-Williams mazes were reported to be sensitive in detecting alterations of hippocampal dependent spatial functioning (Churchwell, Morris, Musso & Kesner, 2010; Rogers & Kesner, 2006; Winocur & Moscovitch, 1990) more so than other measures of spatial navigation and learning such as the Radial Arm Maze or MWM (Marques Pereira, Cosquer, Schimchowitsch, & Cassel, 2005). Further, the Hebb-Williams mazes were reported to be sensitive to the detection of subtle learning deficits induced by partial lesions (Marques Pereira et al., 2005), and animals with learning deficits tended to perform more poorly as the problems became increasingly complex (Standford & Brown, 2003). As a result, the Hebb-Williams mazes may be more capable of detecting learning differences that would otherwise not show up in behavioural measures that consist of easier problems or single assessments (Standford & Brown, 2003).

Interestingly, virtual Hebb-Williams mazes have been developed to measure the performance of humans navigating comparable mazes as those used during murine testing. Such a procedure can theoretically provide greater insight into the nature of spatial navigation and learning processes across species. Using this procedure and an outcome measure combining latency and number of errors, Shore et al. (2001) reported comparable human and murine performance on Hebb-Williams mazes # 6 - 12. They suggested that performance discrepancies on some of the other mazes could be accounted for by species specific tendencies drawn out by particular maze configurations. For instance, some maze configurations require passage through the centre of the maze for successful completion, an area which mice but not human have an aversion to. Shore et al. (2001) also found a gender difference consisting of poorer performance

on the Hebb-Williams mazes in females of both species, replicating the spatial learning differences that exist between genders documented in many human studies. MacLeod et al. (2010) employed virtual and physical versions of the Hebb-Williams mazes to investigate commonalities in spatial navigation and learning deficits using a human FXS population and *Fmr1* KO mice on a sighted FVB background. Counterintuitively, they did not note any performance differences on mazes deemed more difficult (Shore et al., 2001). However, humans and *Fmr1* KO mice had a comparable pattern of deficits committing more errors than controls on the same mazes, a finding which suggests commonalities in maze navigational performance and that the spatial learning deficits were attributable to a lack of FMRP/Fmrp (MacLeod et al., 2010).

The comparable findings by Shore et al. (2001) and MacLeod et al. (2010) suggest that cross-species comparisons may represent a fruitful means to investigation spatial navigation and learning processes. Moreover, the establishment of a valid behavioural assay such as the Hebb-Williams mazes would be important to establish the effectiveness of pharmacological or behavioural interventions to ameliorate typical symptoms of FXS.

Cellular Models of Learning

Principles of Long Term Potentiation and Long Term Depression

Long Term Potentiation (LTP) and Long Term Depression (LTD) are molecular mechanisms proposed to underlie learning and hypothesized to occur at every CNS synapse in the mammalian brain (Malenka & Bear, 2004). LTP and LTD involve alterations of synaptic strength at dendritic spines in response to the excitatory neurotransmitter, glutamate (Bear, Connors, & Paradiso, 2007; Cooke & Bliss, 2006). Although many receptors are implicated in plasticity, arguably the most important glutamate receptors for hippocampal-dependent

alterations related to learning are NMDA, AMPA and metabotropic glutamate receptors (mGluRs) (Cooke & Bliss, 2006; Malenka & Bear, 2004). LTP is the process that underlies the strengthening of synaptic connections (Malenka & Bear, 2004). To provide a simplified example of a complex phenomenon, a synapse would be potentiated when its activity is correlated with that of many other converging inputs on the same postsynaptic neuron which are of sufficient strength (by virtue of temporal and spatial EPSPs) to produce a postsynaptic depolarization. As a result of being correlated with strong postsynaptic activation by other inputs, in the future, this pre-synaptic axon would be capable of depolarizing the postsynaptic neuron even in the absence of those previously required converging inputs (Bear et al., 2007). Conversely, if a pre-synaptic axon is activated and correlated with weak postsynaptic activation by other converging inputs on a postsynaptic neuron, this synapse would undergo LTD. Therefore, future stimulation of this depressed pre-synaptic axon would result in EPSPs of lower magnitudes than the initial stimulation (Bear et al., 2007). Part of the intrigue surrounding these forms of plasticity involves their longevity and robustness. For example, high frequency electrophysiological stimulation (HFS) applied to the rat dentate gyrus was reported to elicit stable LTP lasting months and in some cases up to one year (Abraham, Logan, Greenwood & Dragunow, 2002).

The translation machinery (i.e., polyribosomes) and localization of mRNAs to synapses allows for independent control of synaptic strength through the local synthesis of proteins. In fact, the longevity of synaptic alterations requires protein synthesis (Steward & Schuman, 2001). Postsynaptic LTP and LTD induction is regulated by the amount of calcium influx (Malenka & Bear, 2004). HFS consisting of a tetanus of 100 stimuli at 100 Hz induces LTP leading to a large influx of calcium and the activation of a protein kinase, calcium/calmodulin-dependent protein kinase (CaMKII). On the other hand, low frequency electrophysiological stimulation (LFS) of

approximately 900 stimuli at 1-5 Hz induces LTD leading to minimal calcium influx, activation of protein phosphatases and the dephosphorylation of several protein kinases (Cooke & Bliss, 2006; Malenka & Bear, 2004). Following the induction phase of LTP or LTD, the expression and maintenance of each are dependent on the post-translational modifications and alterations of the number of AMPA receptors (AMPARs) via their activity dependent trafficking (Bredt & Nicoll, 2003; Malinow, 2003). In addition to modification and trafficking of AMPARs, the persistence of LTP and LTD ("late phases") which can last hours, days or months requires transcription factors, gene transcription and protein synthesis (Abrahams & Williams, 2003).

While NMDA receptor (NMDAR) LTP and LTD in the CA1 region of the hippocampus is the most well studied and prototypical examples of plasticity (Malenka & Bear, 2004), mGluRs are also important in hippocampal dependent LTD. Group I mGluRs consist of receptor subtypes mGluR1 and mGluR5. Both NMDAR- and mGluR-LTD can be induced by identical patterns of synaptic stimulation (Oliet, Malenka, & Nicoll, 1997), maintained by decreased surface expression of AMPARs and each rely on distinct intracellular signalling whereby activation of one pathway does not preclude or disrupt the other (Oliet et al., 1997; Snyder et al., 2001). Despite sharing many commonalties, the activation of mGluRs causes a form of LTD in CA1 neurons that is functionally distinct from that of NMDAR-LTD (Huber, Kayser & Bear, 2000; Huber, Roder & Bear, 2001; Oliet et al., 1997). In this regard, the induction of mGluR dependent AMPAR endocytosis requires protein synthesis whereas the NMDAR-mediated losses of AMPARs does not (Huber et al., 2000; Synder et al, 2001) suggesting that the mGluR alterations are longer lasting and more stable owing to the requirement of protein synthesis. Moreover, mGluR mediated LTD can lead to the internalization of NMDA receptors in hippocampal neurons (Snyder et al., 2001).

With respect to FXS, an abnormal type of plasticity has been documented which is characterized by exaggerated mGluR-LTD and normal NMDAR-LTD in *Fmr 1* KO mice (Hou et al, 2006; Huber, Gallagher, Warren & Bear, 2002). The data concerning LTP in FXS are inconsistent and ostensibly NMDAR-mediated. In several studies, LTP induction was impaired in CA1 neurons in *Fmr1* KO mice and reduced by as much as 50% (Lauterborn et al., 2007; Hu et al., 2008) yet in others, normal LTP was reported 1-2 hours after LTP induction in the CA1 area of the hippocampal neurons (Godfraind et al., 1996; Paradee et al., 1999).

AMPAR Modification following LTP and LTD

In the adult hippocampus, AMPAR subtypes of GluR1, GluR2 and GluR3 are present and form four-subunit receptors made up of either the GluR1/2 or GluR 2/3 configurations (Rosenmund, Stern-Bach, & Stevens, 1998; Wenthold, Petralia, Blahos & Niedzielski, 1996). Each AMPAR contains either long or short cytoplasmic carboxy-terminal tails (c-tails), and interacts with cytoplasmic proteins within the postsynaptic density (PSD) (Kessels & Malinow, 2009; Malinow, 2003). The PSD consists of glutamate receptors, associated signaling proteins, and cytoskeletal elements and is involved in organizing and holding together signaling complexes by stabilizing transmembrane proteins to the cells cytoskeleton and by improving channel response specificity. These functions are largely attributed to a number of important scaffolding proteins (Tsunoda et al., 1997; Kim & Sheng 2004). Synaptic strengthening is believed to result from the addition of long-tailed AMPARs (such as GluR1, GluR4, GluR2L) to synapses as a result of synaptic activity (Kessels & Malinow, 2009) and the majority of the research has focused on GluR1-containing receptors.

GluR1 is a target of Fmrp (Muddashetty, Kelic, Gross, Xu, & Bassell, 2007) and when tagged with green florescent protein (GFP) to explore mobility, it was reported to be immobile in

the absence of stimulation but moved from the dendritic shaft to dendritic spines and synapses following HFS that generated LTP (Shi et al., 1999; Shi, Hayashi, Esteban & Malinow, 2001). Likewise, LTP induced by HFS of spinal nociceptive transmission was associated with increased densities of GluR1-containing AMPA receptors, as well as an increased synaptic ratio of GluR1 to GluR2/3 subunits (Larsson & Broman, 2008). Zamanillo et al., (1999) generated a mouse model that lacked the gene encoding for the GluR1 and reported that in adult GluR1-/- mice, LTP induction was absent in CA3 to CA1 synapses. Regarding FXS, in hippocampal slice cultures the synaptic delivery of GluR1-, but not GluR2L- or GluR4-containing AMPARs was impaired in *Fmr1* KO mice, resulting in a loss of GluR1-dependent LTP (Hu et al., 2008).

Experience dependent forms of plasticity have also been reported in the literature. In this regard, *in vivo* gene delivery combined with *in vitro* recordings showed that sensory experience (whisker stimulation) drives recombinant GluR1 into synapses in barrel cortex pyramidal neurons (Takahashi, Svoboda, & Malinow, 2003). The C terminal of the AMPAR GluR1 subunit undergoes modifications including the phosphorylation of Serine 831 after LTP and the dephosphorylation of Serine 845 after LTD (Lee, Barbarosie, Kameyama, Bear, & Huganir, 2000). In rats that underwent a one-trial inhibitory avoidance (IA) learning paradigm, Whitlock, Heyen, Shuler and Bear (2006) reported an increase in phosphorylation of Serine 831 specific to the hippocampus 30 minutes after learning, relative to controls. Additionally, there were increased GluR1 protein levels in hippocampal synaptoneurosomes (a separation of the synapses from dendritic spines; Whitlock et al., 2006). Similarly, Matsuo, Reijmers and Mayford (2008) reported the recruitment of newly synthesized GFP-GluR1 selectively to dendritic spines in adult hippocampal CA1 neurons within 2 hours and maintained for 24 hours following footshock conditioning. In contrast, GluR1-deficient mice were noted to have normal performance on

paradigms requiring protracted learning across many days, such as on spatial reference memory tasks (Schmitt, Deacon, Seeburg, Rawlins, & Bannerman 2003), suggesting that GluR1-containing receptors are most important during initial LTP induction and likely maintained by other factors thereafter (Kessels & Malinow, 2009).

Synaptic weakening is hypothesized to involve the removal of long (GluR1, GluR2L, GluR4) AMPARs from synapses (Kessels & Malinow, 2009). Specifically, LTD is expressed as the activity dependent endocytosis of AMPARs including the selective removal of GluR1 and GluR2 containing receptors from the synapse (Seidenman, Stenberg, Huganir, & Malinow, 2003; Lee et al., 2000). An additional role for short-tailed AMPARs (GluR2, GluR3, GluR4c) involves their localization to synapses in the absence of neural activity and without altering synaptic strength. Specifically, the synaptic removal of GluR1-, GluR2L-, or GluR4-containing AMPARs that were localized to the synapse as a result of LTP were replaced with GluR2-containing AMPARs at hippocampal and cortical synapses *in vitro* and in intact brains (McCormack, Stornetta, & Zhu, 2006). It has been proposed that the replacement of synaptic AMPARs may be important in stabilizing synaptic strength and maintaining gains made during neuroplasticity (Kessels & Malinow, 2009).

The literature reviewed on AMPAR trafficking supports the neural activity-dependent addition or removal of AMPARs at excitatory synapses as the primary mechanisms mediating LTP and LTD. At a functional level, increases in the number of AMPARs following LTP induction was found to be correlated with morphological increases in dendritic spine enlargement (Matsuzaki, Honkura, Ellis-Davies, & Kasai 2004; Yang, Wang, Frerking & Zhou, 2008) whereas LTD induction was associated with spine shrinkage in hippocampal neurons (Zhou, Homma & Poo, 2004).

A Putative Role for PSD-95 in Synaptic Plasticity

Relative to the relationship between surface AMPARs and LTP/LTD, much less is known about the molecular mechanisms governing AMPARs (Xu et al., 2008). One hypothesis is that ‘‘slot’’ proteins maintain the long-term transmission efficiency of a synapse because they can remain in the synapse after AMPARs are removed and can be subsequently refilled by other AMPARs later on (Malinow, 2003; McCormack et al., 2006; Ehrlich & Malinow, 2004). As such, slot proteins would serve as placeholders for AMPA receptors (Malinow, 2003). Postsynaptic density protein of 95 kDa (PSD-95) has been proposed to possess many qualities of a slot protein (Schnell et al., 2002). PSD-95 binds to Stargazin, a protein that shuttles cytoplasmic AMPARs to the synapse (Schnell et al., 2002) and the over-expression of PSD-95 selectively enhanced the frequency of AMPAR excitatory post-synaptic currents (EPSCs), increased the AMPAR/NMDAR ratio of EPSCs while having minimal effects on NMDARs EPSCs, thus reflecting an increased number of synapses expressing functional AMPARs (Schnell et al., 2002, Béïque & Andrade, 2003; Ehrlich & Malinow, 2004; Xu et al., 2008; Steiner et al., 2008). Moreover, knockdown of PSD-95 by expression of short hairpin RNAs (shRNAs) led to a selective decrease in AMPAR-EPSCs and not NMDAR-EPSCs as well as impairments in the early (one minute) and late phases (30 minutes) of spine growth; effects which were rescued by the re-introduction of PSD-95 (Ehrlich, Klein, Rumpel & Malinow, 2007; Steiner et al., 2008). Consistent with these findings, El-Husseini, Schnell, Chetkovich, Nicoll and Bredt (2000) reported that PSD-95 over expression enhanced postsynaptic clustering, activity of glutamate receptors as well as the size and number of dendritic spines. Taken together then, the over expression of PSD-95, increased AMPAR mediated-EPSCs and enhancement of spine morphology suggests that PSD-95 levels could be altered within the PSD to accommodate

increases in AMPARs which occurs during the course of LTP (Malinow & Malenka, 2002). Following LTD, PSD-95 was ubiquitinated and transfection of neurons with the mutant, PSD-95Δ PEST, defective in its ability to be ubiqitinated, blocked the synaptic surface loss of AMPARs (Colledge et al., 2003).

The phosphorylation of PSD-95 on serine 295 (ser-295) was reported to promote the synaptic accumulation of PSD-95 and mutants incapable of phosphorylation resulted in less synaptic expression of PSD-95 and GluR1 (Kim, Futai, Jo, Hayashi, Cho & Sheng, 2007). As opposed to HFS, a chemical protocol used to induce LTP in the hippocampus increased PSD-95 ser-295 phosphorylation and GluR1 phosphorylation, whereas chemically induced LTD rapidly decreased this phosphorylation. Furthermore, a ser-295 phosphorylation mimicking mutant, S295D-PSD-95, blocked AMPA receptor internalization and LTD (Kim et al., 2007). Thus, PSD-95 may play a role in bi-directional plasticity, by altering the numbers of AMPARs at the synapse.

Fmrp has been implicated in the PSD insofar as it is involved in the transport of mRNAs and dynamic regulations of proteins involved in plasticity (Pfeiffer & Huber, 2009). In a murine model of FXS, characterized by dendritic spine abnormalities, PSD-95 mRNA is dysregulated such that there is increased association with translating polyribosomes and elevated protein production at basal levels (Muddashetty et al., 2007) and PSD-95 protein synthesis following synaptic activation using mGluR agonists is deficient (Todd, Mack & Malter, 2003). In the absence of Fmrp, PSD-95 mRNA transcripts across adolescence and adulthood in *Fmr1* KO mice were less stable over time, selectively deteriorating in the hippocampus but not the cortex or cerebellum (Zhu, Xu, Zhao, Gu & Wu, 2011; Zalfa et al., 2007). Specific to FXS, these data imply that the dysregulated protein synthesis of PSD-95 may contribute to the abnormally thin

dendritic spines and on a molecular level could represent one of reasons for the cognitive impairments in FXS.

The Molecular Functions of Fmrp

Fmrp Binds and Regulates mRNA

Fmrp is an mRNA binding protein (Ashley et al., 1993), highly conserved across vertebrate species (Price, Zhang, Ashley, & Warren 1996; Ashley et al., 1993), and regulates a substantial number of mRNAs, many of which are important in neuronal development and synaptic plasticity (Brown et al., 2001; Darnell et al., 2011). Fmrp binds approximately 4% of mammalian total mRNA, representing more than 400 potential targets including its own transcript, *FMR1* mRNA (Ashley et al., 1993; Brown et al., 2001). Whereas Fmrp is found throughout many cells of the body, it is most strongly expressed in neurons, in particular those of the hippocampus and cerebellum (Hinds et al., 1993; Ashley et al., 1993; Abitbol et al., 1993). In comparison to glial cells, where expression is minimal, the majority of Fmrp has been identified in the cytoplasm of neurons (Eberhart, Malter, Feng & Warren, 1996; Feng, Gutekunst, Eberhart, Warren & Hersch, 1997a). In the cytoplasm, Fmrp associates most abundantly with translating polyribosomes, and with rough endoplasmic reticulum (RER)-associated ribosomes and not with other organelles such as mitochondria, Golgi apparati or the plasma membrane (Feng et al., 1997a). Further removed from the soma, Fmrp was also found to associate with polyribosomes engaged in translation in post-synaptic areas (Stefani, Fraser, Darnell & Darnell, 2004). As a result, Fmrp has been implicated in protein translation in neurons (Eberhart et al., 1996; Feng et al, 1997a; Stefani et al., 2004). Fmrp is also localized along with its mRNA cargo in the form of motile granules to dendrites, dendritic spines and synapses (Antar, Afroz, Dictenberg, Carrol & Bassell, 2004; Zalfa, et al, 2003). The presence of mRNA, polyribosomes and translational

factors in dendrites and spines indicates that synapses are capable of modification by local protein synthesis (Sutton & Schuman, 2006). The association with polyribosomes along with identified roles in mRNA transport and translational regulation provides evidence that Fmrp participates in mechanisms that regulate neuroplasticity (Bagni & Greenough, 2005).

FMRP contains a number of known sequence motifs that are characteristic of RNA proteins including two heterogeneous nuclear ribonucleoprotein (hnRNP)-K-homology domains (KH 1 and 2) and a C-terminal arginine-glycine-glycine (RGG) box (Siomi, Siomi, Nussbaum & Dreyfuss,1993). Alterations of RNA bindings, in particular those involving KH domain-RNA interactions, can result in FXS. For example, DeBoulle et al., (1993) reported that a point mutation to the *FMR1* gene resulted in a change to a highly conserved residue of the KH2 motif, (an isoleucine to asparagine substitution at residue 304; I304N) culminating in severe FXS in one patient. As with the KH domains, the C-terminal RGG box also appears to be important in Fmrp-RNA interactions. Schaffer et al., (2001) reported that a G-quartet loop in RNA is recognized by the Fmrp-RGG box. Subsequent research found that many mRNAs possessed G-quartet like structures that were recognizable by the RGG box in the Fmrp. Approximately 70% of the several hundred mRNA transcripts that bind with Fmrp contain G-quartet like structures (Brown et al., 2001). Although many of details remain to be worked out, it has been suggested that translational inhibition of target mRNAs might occur through Fmrp-G-quartet recognition (Darnell et al., 2001). PSD-95 mRNA does not form a G-quartet structure nor does it interact specifically with the RGG box, per se. Instead, PSD-95 interacts with the C-terminal domain, a more elaborate area of the Fmrp encompassing the RGG box. Interestingly, Fmrp is involved in increasing the message stability of this mRNA (Zalfa et al., 2007). Taken together, this literature suggests that proper Fmrp-mRNA interaction is required for normal cellular functioning.

Fmrp and mRNA Transportation: From the Nucleus to Dendritic Spines

In neurons, the majority of the mRNAs present in the cell are localized in the soma, however their transportation to synapses is believed to be an important mechanism underlying synaptic plasticity (Steward & Schuman, 2003). The current consensus is that mRNAs are transported into dendrites and synapses as part of messenger ribonucleoprotein particles (mRNPs; reviewed in Kiebler & Bassell, 2006), motile transport granules that contain a mixture of molecular elements such as mRNA, mRNA-binding proteins, motor proteins and small non-coding micro RNA. Florescence imagi ng studies in cultured neurons have demonstrated that Fmrp traffics in the form of RNA granules and these are believed to be translationally arrested mRNP complexes (Kiebler & Bassell, 2006). It is of interest to ascertain how certain mRNAs are selected from the total pool in the nucleus and localized to post-synaptic dendrites and spines in response to presynaptic and other sources of stimulation (Ferrari et al., 2007). Bramham and Wells (2007) have outlined a composite model for mRNA translation in dendrites based on outcome studies involving multiple mRNAs. This model stipulates that binding proteins that are capable of inhibiting translation allow for nucleic mRNAs to be bound and consequently sequestered from the protein-synthesis machinery in the cell. Thereafter, these repressed mRNAs are packaged in motile granules (i.e., mRNPs) and transported into the dendrites by kinesin motors on microtubules. In response to synaptic activity, the granules become dispersed and the cargo mRNAs are localized to spines via actin-based myosin mechanisms where they undergo protein translation by dendritic polyribosomes.

The functions of Fmrp complement most features of this model nicely. In this regard, microarray analyses have revealed that Fmrp preferentially selects target mRNAs that encode proteins important for plasticity and neuronal development (Brown et al., 2001; Miyashiro et al.,

2003). Furthermore, Fmrp has been shown to repress the translation of mRNA transcripts *in vitro*, *in vivo* and in transfected cells (Laggerbauer, Ostareck, Keidel, Ostareck-Lederer & Fischer, 2001; Qin, Kang, Burlin, Jiang, & Smith, 2005; Wang et al., 2004). Both a nuclear localization signal (NLS) and nuclear export signal (NES) have been identified meaning that Fmrp can shuttle into the nucleus and associate with mRNAs (Eberhart et al., 1996; Feng et al., 1997b). These findings along with the fact that most mRNPs are formed in the nucleus (Dreyfuss, 1986), suggest that nascent Fmrp can enter into the nucleus, interact with specific mRNAs and become incorporated into mRNPs prior to direct export out of the nucleus (Eberhart et al., 1996). In addition, Fmrp appears to be an integral component of the mRNPs as a mutation in Fmrp produced mRNPs that were of smaller size and did not associate with translating polyribosomes, an otherwise viable and required function for this protein in the non-mutated form (Feng et al., 1997b). Until recently, it was not well understood whether Fmrp travelled on microtubules after it was incorporated into polyribosomes as a result of translation initiation or whether transport occurred in translationally dormant granules. The latter would imply that Fmrp and its mRNA targets come into contact with polyribosomes in the dendrites and synapses, post transport. Wang et al. (2008) outlined a dual role for Fmrp with regards to polyribosome interactions and granule transport. Whereas the majority of Fmrp was incorporated into elongating polyribosomes in the soluble cytoplasm, inhibiting translation initiation caused a significant redistribution and incorporation of Fmrp from polyribosomes to translationally dormant, polyribosome free mRNPs. Mutant Fmrp that failed to be incorporated into polyribosomes was nonetheless incorporated into mRNPs and transported to dendrites on microtubules in a similar neuronal stimulation-dependent manner as that observed with wild-type cells. Thus, these data confirm that dendritic transport of Fmrp on microtubules occurs in the

form of polyribosome free, translationally dormant mRNPs sequestered from translation initiation and further that Fmrp interacts with dendritic polyribosomes following synaptic stimulation and translational de-repression.

Other research has focused on understanding the rates of granule transport that occur under basal and stimulated conditions. With respect to the basal transport levels of Fmrp and *Fmr1* mRNA, fluorescent in situ hybridization (FISH) and immunofluorescence confirmed the presence of *Fmr*1 mRNA and Fmrp granules in dendrites and spine synapses (Antar, Aforz, Dictenberg, Carroll, & Bassell., 2004). Additional, activity dependent stimulation experiments performed using various agonists and antagonists demonstrated the dendritic localization of granules containing *Fmr*1 mRNA and Fmrp were specific to mGluR-5 receptor activation. In a subsequent experiment, Antar, Dictenberg, Plociniak, Afroz and Bassell (2005) replicated the mGluR activity dependent localizations of Fmrp and showed that microtubules were required for the mGluR-dependent translocation of Fmrp into dendrites. While these data outlined a role for the mGluR-driven pathway in Fmrp granule localization in dendrites (Antar et al., 2004; Antar et al., 2005), they have not clearly addressed the *function* of Fmrp at the synapse within the dendritic spines. Ferrari et al. (2007) attempted to clarify the role of the localization of Fmrp at the synapse in dendritic spines, beginning by further characterizing the composition of the mRNP complexes. They reported that the FMRP-mRNPs contained α-CaMKII mRNA, an important synaptic plasticity protein and Fmrp ligand (Zalfa et al., 2003), the translational repressor *BC1* RNA, as well as Staufen, one of the best characterized proteins involved in neuronal transport. Double immunostaining for Fmrp/PSD-95 and Fmrp/Shank in control and DHPG-treated neurons revealed that Fmrp was present along dendrites but mainly absent from post-synaptic sites (i.e., spines) under basal conditions. Following DHPG stimulation (an agonist

of mGluR1/5), Fmrp moved into the spines, near the postsynaptic density where it co-localized with PSD-95 and Shank proteins. The majority of the Fmrp that moved into the spine after synaptic stimulation came from *de novo* protein synthesis of *Fmr1* mRNA. These data indicate that Fmrp-mRNP complex relocate into dendritic spines following synaptic activity, reinforcing the idea that Fmrp might be a mechanism linking mRNA transport in dendrites to synaptic modification.

Although Fmrp is a component of granules that traffics into dendrites along microtubules, its direct role in mRNA transport is unclear. In this regard, Fmrp could be a component of the mRNPs that controls translation but lacks influence on the regulation of mRNA transport (Bassell & Warren, 2008). If Fmrp is directly involved in mRNA localization then there should be noticeable differences in the dendritic mRNA levels in *Fmr1* KOs relative to wild-type phenotypes. However, Steward, Bakker, Willems, and Oostra (1998) reported no differences in the mRNA dendritic levels of α-CaMKII, ARC (both Fmrp ligands) or MAP2 mRNA in KOs compared to wild-type brains. Similarly, Muddashetty et al. (2007) did not observe reductions of *in vivo* levels of AMPA receptor subunits (GluR1, GluR2), or PSD-95 mRNAs in the dendrites of *Fmr1* KOs in the hippocampus, dentate gyrus or cerebral cortex. Additional quantitative *in vitro* analyses of the mRNA distributions of α-CaMKII, PSD-95 and GluR1 demonstrated similar patterns of dendritic localization in *Fmr1* KO and wild-type mice. Another paper reported that PSD-95 largely co-localized with Fmrp (confirming that it is part of an Fmrp-mRNP complex) but immunofluorescence indicated the expression of a comparable punctate pattern in dendrites from *Fmr1* KO and wild-type hippocampal cultures, again reflective of similar patterns of expression in both phenotypes (Zalfa et al., 2007). Taken together, the comparable levels of dendritic mRNAs in *Fmr 1* KO and wild-type mice suggest that Fmrp likely does not play a role

in mRNA transport under basal conditions (i.e., under conditions lacking synaptic activity). Instead, this function may be regulated by another mRNA binding protein (Bassell & Warren, 2008). Relevant to the present investigation, PSD-95 mRNA was reported to be controlled by Fmrp in a regionally specific manner. In this regard, *total* PSD-95 mRNA abundance (as opposed to dendritic abundance) under basal conditions was significantly reduced in the hippocampus in the absence of Fmrp whereas the stability of PSD-95 mRNA was unaffected in *Fmr1* KO cortical cultures. Not surprisingly then, in *Fmr*1 KO mice, PSD-95 basal protein levels were selectively reduced in the hippocampus and not in other brain regions such as the cerebral cortex (Zalfa et al., 2007). Collectively, these findings suggest that a role for Fmrp in controlling the total mRNA/protein abundance in a regional specific area, such as the hippocampus.

Fmrp and Synaptic Plasticity

Given the theorized importance of Fmrp in synaptic plasticity, dynamic assays are important to investigate how target mRNAs respond to neuronal activity in the presence or absence of Fmrp. Dictenberg, Swanger, Antar, Singer and Bassell (2008) used FISH and quantitative digital imaging of live neurons to assess the response of dendritic mRNAs from *Fmr1* KO and wild-type hippocampal neurons treated with DHPG (a mGluR agonist). Consistent with the literature, no differences in the localization of Fmrp target mRNAs were found under basal conditions across the two phenotypes. However, following DHPG stimulation, mRNAs believed to be involved in synaptic plasticity such as MAP-1B, α-CAMKII, SAPAP4 and RGS5 were significantly elevated in wild-type mice but showed markedly reduced trafficking in *Fmr1* KOs, showcasing the important function of Fmrp under conditions that invoke synaptic activity. Fmrp was also demonstrated to act as an adaptor for kinesin, (a protein involved in anterograde transport using microtubules) and the lack of stimulation-induced mRNA transport in the *Fmr1*

KO was attributed to a widespread uncoupling of target mRNAs from kinesin in the KO brains (Dictenberg et al. 2008). Importantly, it was also shown that transfected neurons, which produced acute suppression of Fmrp for 36 hours in the presence of synaptic activity (and therefore reduced mRNA localizations) in wild-type neurons, resulted in an increased length and number of dendritic filopodia-spine protrusions. That the effect of a temporary suppression of Fmrp negatively affected dendritic morphology in a similar manner as that seen with the loss of Fmrp is suggestive of a link between decreases of mRNA trafficking important in synaptic function and the pathogenesis of FXS (Dictenberg et al., 2008).

Another commonly reported and perhaps related characteristic in *Fmr1* KO mice is the dysregulation of protein synthesis that occurs in the absence of Fmrp. In isolated dendritic spine synapses from *Fmr1* KO mice (called synaptoneurosomes), several groups have reported reduced protein synthesis following synaptic stimulation as well as increased protein expression under basal conditions (Weiler et al., 2004; Zalfa et al. 2003; Lu et al., 2004). Interestingly, this dual profile of deficient *and* excessive protein translation is specific to only the *Fmr1* KO phenotype. In wild-type mice, quantitative real-time PCR analysis showed mRNAs such as PSD-95, α-CaMKII and AMPA receptor subunits GluR1 and 2 were actively incorporated into translating polyribosome fractions derived from synaptoneurosomes following DHPG stimulation. Analyses of synaptoneurosomes using radioimmunoprecipitations (to quantify rapid protein synthesis) showed that following 5 minutes of DHPG stimulation, protein levels for PSD-95 and α-CAMKII were markedly increased. By comparison, mRNAs from *Fmr1* KO mice synaptoneurosomes preparations were elevated in polyribosomes at basal levels and were not incorporated into polyribosomes during synaptic stimulation in a similar manner as that observed with the wild-type mice and in fact, DHPG induced protein synthesis of PSD-95 and α-CAMKII

was absent (Muddashetty et al., 2007). Thus, excess basal translation and a lack of stimulation induced protein synthesis can co-occur in the *Fmr1* KO depending on the activity state of the cell. In effect, the *Fmr1* KO polyribosomal and protein profile at baseline mirrors that of the wild-type profile when undergoing synaptic stimulation. This loss of control over mGluR induced translation is likely to have deleterious consequences on the ability of the neural circuitry to undergo synaptic modifications.

Despite the identified roles for Fmrp thus far, the findings reveal little about the regulation of Fmrp itself. Post-translational modifications, in particular, phosphorylation of existing proteins are important in the regulation of activity dependent protein synthesis (Ceman et al., 2003). Ribosomal run-off assays demonstrated that unphosphorylated Fmrp was associated with actively translating polyribosomes whereas phosphorylated Fmrp was associated with stalled polyribosomes (Ceman et al., 2003). This suggests that phosphorylated Fmrp is not actively involved in translation. Protein phosphatase 2A (PP2A) was subsequently identified as a phosphatase for Fmrp and it was shown that DHPG induced mGluR activation of less than a minute resulted in dephosphorylation of dendritic Fmrp, an effect which was attributed to the enhanced PP2A enzyme activity (Narayanan et al. 2007). Temporally, the dephosphorylation of Fmrp corresponded with the translation of SAPAP3, a post-synaptic scaffolding protein and Fmrp target mRNA, closely associated with PSD-95. By comparison, extended mGluR activation (1-5 minutes) resulted in mammalian target of rapamycin (mTOR)-mediated PP2A suppression and a rephosphorylation of Fmrp along with a reduction of SAPAP3 protein expression (Narayanan et al., 2007). A follow up paper by these authors identified ribosomal protein S6 kinase (S6K1) as a major Fmrp protein kinase in the mouse hippocampus and that mGluR phosphorylation of Fmrp by S6K1 required inputs from mTOR, ERK 1/2 and PP2A.

Moreover, because SK61 KO mice hippocampal lysates showed a lack of Fmrp phosphorylation as well as elevated SAPAP3 expression, this suggested that the translational responses of Fmrp were regulated by a S6K1-PP2A signaling molecule (Narayanan et al. 2008). Taken together, these data suggest that following the stimulation of mGluRs, PP2A is activated and dephosphorylates Fmrp allowing for the translation of its previously repressed mRNAs, presumably by regional polyribosomes. By five minutes, mTOR-mediated PP2A suppression occurs followed by mTOR- activated S6K1 leading to Fmrp phosphorylation and the translational repression of Fmrp targets (Narayanan et al., 2007; Narayanan et al., 2008). These data are consistent with the involvement of Fmrp in regulating translation at the level of elongation (Bassell & Warren, 2008; Bhakar et al., 2012) whereas earlier findings of Fmrp regulation in mRNP granules outlined the role of Fmrp regulation on translation initiation, or the process by which repressed mRNAs in mRNP granules are unrepressed and translation is commenced following synaptic stimulation. Thus, at this juncture it appears that Fmrp is an important component in regulating translation initiation and elongation and this may differ depending on the particular mRNA that is a target of Fmrp (Bassell & Warren, 2008).

Several working models exist that attempt to explain how Fmrp is involved in mGluR stimulated translation, localized protein expression and ultimately synaptic plasticity (Bassell & Warren, 2008; Pfeiffer & Huber, 2009; Ronesi & Huber, 2008; Wijetunge, Chattarji, Wyllie, & Kind, 2013). Fmrp acts as translational suppressor of target mRNAs either in a RNA granule, mRNP, or stalled polyribosome (Ronesi & Huber, 2008; Aschrafi, Cunningham, Edelman & Vanderklish, 2005). Activation of group I mGluRs allows for the local translation of proteins that are involved in AMPAR trafficking such as MAP 1B, Arc/Arg 3.1, PSD-95 and α-CAMKII (Bassell & Warren, 2008; Dictenberg et al., 2008; Park et al., 2008; Ronesi & Huber, 2008). In

stalled polyribosomes, translational elongation occurs due to the release of translational suppression of Fmrp, in response to synaptic stimulation, which involves the dephosphorylation of Fmrp by increased PP2A activity (Narayanan et al., 2007). Following mGluR activation, Fmrp is rephosphorylated by S6K1 leading to translational suppression (Narayanan et al., 2008), although it is unclear if the same Fmrp molecules are de- and subsequently re-phosphorylated. mGluR stimulation also results in Fmrp being degraded by the ubiquitin-proteasome pathway (Hou et al., 2006) which may also serve to de-repress mRNA targets of Fmrp. Indeed, proteasome inhibitors abrogate mGluR-LTD suggesting that the degradation of Fmrp is necessary for this type of plasticity (Hou et al., 2006). In order to restore the translational repression, it was suggested that new Fmrp synthesis occurs to off-set that which is lost during stimulation (Bassell & Warren, 2008). In support of this notion, Fmrp is synthesized upon mGluR stimulation in synaptoneurosomes and hippocampal cultures (Weiler et al, 1997; Weiler & Greenough, 1999; Antar et al., 2004; Figure 1).

In *Fmr1* KO mice, some of the mRNA targets known to be regulated by Fmrp may associate with actively translating polyribosomes, resulting in increased basal protein expression (Ronesi & Huber, 2008). Because a distinct type of plasticity exists in the *Fmr1* KO characterized by exaggerated LTD (Huber et al., 2002), some have suggested that the prematurely translated proteins are "LTD proteins" (Hou et al., 2006; Nosyreva & Huber, 2006). LTD may persist in the absence of Fmrp as a result of the loss of translational control and the accumulation of steady-state levels of proteins. Consequently, LTD in the *Fmr1* KO continues despite the presence of protein inhibitors and thus does not require *de novo* synthesis, an effect that is phenotype specific (Nosyreva & Huber, 2006). The exaggerated mGluR-LTD in the *Fmr1* KO mice results in significant decreases in AMPAR surface expression and has been

hypothesized to contribute to some of the pathological cognitive symptoms in FXS (Kruger & Bear, 2011).

mRNAs may shuttle between various types of granules and polyribosomes (Kiebler & Bassell, 2006) but the molecular logistics of how this occurs following synaptic stimulation is unknown. Fmrp is hypothesized to be an important protein in this regard. The reported deficits of mGluR-induced trafficking of synaptic mRNAs under stimulated but not basal conditions in the *Fmr1* KO has lead to the idea that constitutively expressed mRNAs are transported to the synapse in both phenotypes (KOs and wild-types) via alternative mRNA binding proteins under conditions of synaptic inactivity (Bassell & Warren, 2008; Dictenberg et al., 2008). In wild-type mice, proteins are repressed by Fmrp at the synapse and accumulated until enhanced mGluR stimulation in neurons stimulates de-repression and their translation. Somatic Fmrp is subsequently involved by increasing rates of mRNA transport and over time continued glutamatergic stimulation of classes of neurons involved in synaptic modifications results in the recruitment and translation of specific mRNAs at certain synapses (Dictenberg et al., 2008). Without Fmrp, the ability to augment mRNAs required for synaptic modifications is lost and culminates in net reductions of these synaptic proteins and wide spread dysregulation of signaling in response to synaptic stimulation (Dictenberg et al., 2008).

mGluR Antagonists

The loss of Fmrp, a translational repressor of mRNAs, produces some of the clinical manifestations of FXS such as cognitive dysfunction and audiogenic seizures (Beckel-Mitchener & Greenough, 2004; Bear et al., 2004 Bear, Dolen, Osterweil, & Nagarajan, 2008; Kruger & Bear, 2011). Pharmacological treatment with antagonists that selectively target mGluR-5 have been promising in that they can block this receptor and correct some of the downstream

signalling that occur in the absence of Fmrp. Consistent with group I mGluR-signaling as mediating prolonged LTD in *Fmr1* KO mice, Nakamoto et al., (2007) used small interfering RNA (siRNA) specific to the *Fmr1* gene sequence (and not sharing homology with paralogs *Fxr1* and *Fxr2*) to demonstrate that reductions of Fmrp in dendrites of hippocampal neurons led to an increase in the internalization of the AMPAR subunit, GluR1. Treatment with 2-methyl-6-phenylethynyl-pyridine (MPEP), an mGluR-5 antagonist, rescued the abnormal AMPAR trafficking as evidenced by the relative increase in the quantification of immunofluorescence signal intensities of GluR1 in Fmrp-deficient dendrites (Nakamoto et al., 2007). This process did not involve NMDAR-driven AMPAR internalization because APV, an NMDAR antagonist, did not rescue the internalizations (Nakamoto et al., 2007). In the absence of Fmrp and following 20 days of culturation *in vitro*, neurons from adult *Fmr1* KO mice were classified as having an excess of filopodia (spines with a long and thin appearance) relative to wild-type cultured neurons (deVrij et al., 2008). Treatment of *Fmr1* KO neurons with two different mGluR-5 antagonists (200μM MPEP and 300 μM fenobam) for 4 hours, rescued the protrusion phenotype, restoring the spine/filopodia ratio in *Fmr1* KO neurons to the levels observed in wild-type neurons (deVrij et al, 2008). In another study, daily one week administration of 20 mg/kg of MPEP ameliorated average spine length and density in adult cortical neurons from *Fmr1* KO mice without producing significant tolerance or toxicity effects (Su et al., 2011).

Behaviorally, *Fmr1* KO mice of the hybrid strain C57Bl/6J X FVB/NJ displayed increased centre square entries and duration during open field testing. Single intraperitoneal (i.p.) injection of either 10 or 30 mg/kg of MPEP to one and three month old mice rescued these deficits such that open field performance 30 minutes after injection was statistically

indistinguishable from wild-type mice (Yan et al., 2005). In addition, the administration of MPEP corrected the increased sensitivity to audiogenic seizures in these *Fmr1* KO mice.

Perhaps as a reflection of the evolutionary conservation of the function of mGluRs and Fmrp, FXS models in other species have documented similar findings as those observed in the intervention studies with *Fmr1* KO mouse. In a drosophila model of FXS, *dfmr1* KOs display excess protein synthesis (Bolduc, Bell, Cox, Broadie, & Tully, 2008) deficits of learning and memory and courtship behaviours all of which can be rescued with MPEP treatment (Bolduc et al., 2008; Choi, McBride, Schoenfeld, Liebelt, Ferreiro, & Ferrick, 2010; McBride, Choi, Wang, Liebelt, Braunstein, & Ferreiro, 2005). Mushroom body abnormalities have also been corrected with MPEP treatment (Pan, Woodruff, Liang, & Broadie, 2008) a noteworthy outcome given their relevance in olfactory learning and memory in drosophila. Using zebra fish embryos as a model for *Fmr1* loss of function analysis, Tucker, Richards and Lardelli (2006) noted neurite morphogenesis deficits in embryonic cells and the subsequent rescue of these deficits with MPEP treatment.

Perhaps the strongest support for targeting mGluR- signaling comes from research that cross-bred *Fmr1* KO mice with Grm5 mutant mice that have 50% reduction of mGluR-5 expression (rather than a complete KO which would negatively impact brain function) and which rescued several phenotypic aspects of the FXS mouse model (Dolen et al., 2007). In this regard, reducing mGluR5 expression in *Fmr1* KO mice significantly reduced LTD; rescued the increased density of long and thin spines; reduced the elevated basal protein synthesis rates typically observed in *Fmr1* KO mice; and reduced audiogenic seizures (Dolen et al, 2007).

The Proposed Experiments

FXS is the most common cause of inherited mental retardation as well as autistic behaviours and occurs as a result of a mutation leading to silencing of the *FMR1* gene and the subsequent lack of FMRP expression. The lack of FMRP during brain development results in a constellation of symptoms ranging from moderate to severe mental retardation, attention deficits and problems of executive functioning, anxiety, affective illnesses, and increased risk of epilepsy (Hagerman et al, 2009; Hooper et al., 2008; O'Donnell & Warren, 2002; Scerif et al., 2007; Tuner et al., 1996).

Hippocampal synaptic plasticity and development have long been the focal points of research aimed at delineating the mechanisms of learning and memory in both normal and diseased brains (Bird & Burgess, 2008). The literature suggests that much progress has been made using genetic KO murine models of FXS. In this regard, the cognitive impairments observed in *Fmr1* KO models have been attributed to pathophysiological disturbances of synaptic remodelling. However, a criticism and potential confound in previous experiments has been the over reliance on artificial means of stimulating neural circuits involved in learning as opposed to employing more ecologically valid methods (i.e., high frequency electrophysiological stimulation (HFS) has been used rather than maze learning). Despite much speculation, there is scant data concerning the expression (i.e., upregulation/downregulation) of synaptic proteins believed to mediate LTP or LTD beyond the initial induction stages. Thus, artificial means of stimulating neural circuitry and a lack of data on the expression of synaptic proteins past the induction stage could result in misleading conclusions regarding the precise mechanisms underlying the learning impairments that occur in FXS. The Hebb-Williams mazes are well-established measures of spatial navigation and learning that allow for the assessment of humans and animals under comparable conditions (Shore et al., 2001). Previous research using the Hebb-

Williams mazes indicated a comparable spatial navigation and learning deficit in humans and mice (Shore et al., 2001) and in FXS these deficits were attributed to a lack of FMRP/Fmrp across species (MacLeod et al., 2010). These data suggest that the Hebb-Williams mazes are a valid behavioural assay which can accurately model the spatial navigation and learning deficits. Additionally, the Hebb-Williams mazes could be used to test pharmacological interventions designed to mitigate the protein deficits and ultimately improve some of the core symptoms of FXS.

In experiment one, Fmrp-deficient mice (*Fmr1* KO) and wild-type control mice will be run through a set of spatial navigation and learning problems. Protein of interest will be PSD-95 as it has been implicated in the morphology and etiology of FXS and known to be important in synaptic plasticity in the hippocampus following experimental models of learning. It is hypothesized that there will be an upregulation of PSD-95 in wild-type mice following the completion of the Hebb-Williams mazes but not in the *Fmr1* KO mice.

It is of interest to ascertain whether there is a relationship between a behavioural criterion reflecting learning and molecular substrates. It is hypothesized that hippocampal PSD-95 will be correlated with reduced performance on the Hebb-Williams mazes as reflected by a greater number of total errors in the *Fmr1* KO mice. The protein levels of PSD-95 are hypothesized to be correlated with a lower number of total errors in wild-type mice who ran the Hebb-Williams mazes.

Pharmacological treatment with antagonists that selectively target group I mGluR receptors have been promising insofar as they can correct some of the downstream effects that occur in the absence of Fmrp (deVrij et al., 2008; McBride et al., 2005; Su et al., 2011). The second experiment will focus on attenuating the hypothesized protein deficits in the *Fmr1* KO

mice by administration of the mGluR-5 antagonists, MPEP. During the testing phase of the Hebb- Williams mazes, *Fmr1* KO mice will be administered a dose of 20 mg/kg intraperitoneal (i.p.) injection of MPEP prior to navigating the test mazes. It is hypothesized that relative to those saline treated *Fmr1* KO mice, MPEP administration will correct the PSD-95 deficits in *Fmr1* KO mice. It is hypothesized that the correction of PSD-95 protein deficits will be correlated with a reduced number of mean total errors compared to the number of mean total errors committed by *Fmr1* KO mice treated with saline.

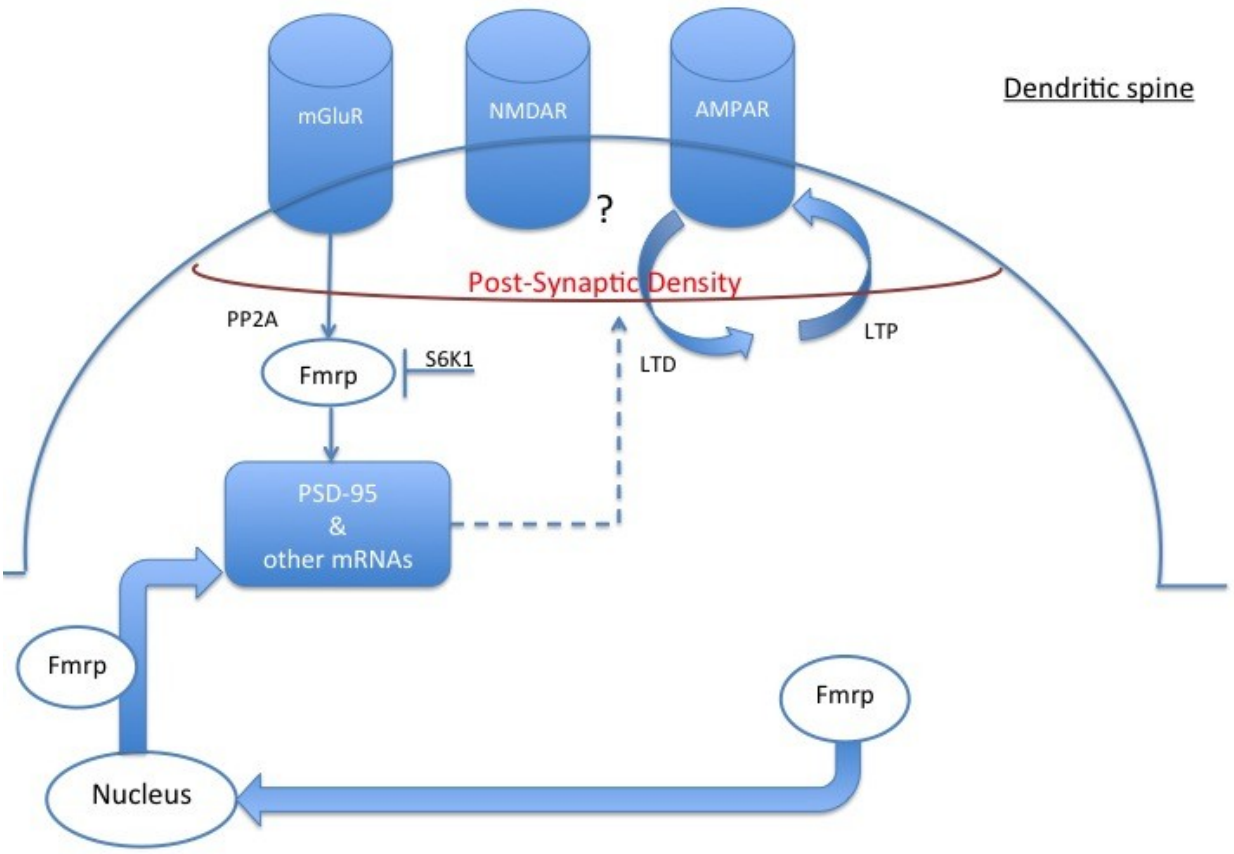

Figure 1: Synaptic activity triggers distinct types of plasticity known as LTP or LTD which are necessary for the modification of synapses following learning. Magnitude of learning is hypothesized to be expressed by endo- or exocytosis of AMPARs and protein translation. Fmrp is believed to be in a mRNP complex which consists of translationally arrested mRNAs, motor proteins and micro RNAs. Following activation of mGluRs, Fmrp mRNA targets are rapidly de-repressed by PP2A and Fmrp is dephosphorylated allowing for translation of proteins that affect AMPAR trafficking. Within minutes, Fmrp is rephosphorylated by S6K1 and protein translation is halted. Fmrp is ubiquitinated following mGluR-5 activation but can also be synthesized as well in synaptoneurosomes- functions which may allow for strict regulation of this mRNA binding protein at the dendritic spine (actions not shown in diagram). Fmrp may travel from the synapse to the cell nucleus where it can shuttle into the nucleus and associate with nascent mRNAs. Fmrp is necessary for the transport of mRNAs into dendrites following mGluR stimulation. This model speculates that Fmrp is phosphorylated. While the molecular interactions between NMDARs and Fmrp remain to be determined, these receptors are hypothesized to be important in LTP and LTD given their well defined roles in learning and memory in the hippocampus and elsewhere in the CNS.

Chapter Two: Manuscript I

Manuscript I

Visual–spatial learning impairments are associated with hippocampal PSD-95 protein dysregulation in a mouse model of fragile X syndrome.

Gandhi, R.M., Kogan, C.S., Messier, C., & MacLeod, L.S. (2014). *NeuroReport*, *25*, 255-261.

Abstract

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability and is caused by lack of fragile X mental retardation protein (FMRP) expression. *In vitro* findings in mice and *post mortem* autopsies in humans are characterized by dendritic spine abnormalities in the absence of Fmrp/FMRP. Biochemical and electrophysiological studies have identified post synaptic density protein (PSD)-95 as having an established role in dendrite morphology as well as a molecular target of Fmrp. How Fmrp affects the expression of PSD-95 following behavioral learning is unknown. In the current study, wild type controls and *Fmr1* knockout (KO) mice were trained in a subset of the Hebb-Williams (H-W) mazes. Dorsal hippocampal PSD-95 protein levels relative to a stable cytoskeleton protein (β-tubulin) were measured. We report a significant upregulation of PSD-95 protein levels in wild type mice whereas training-related protein increases were blunted in *Fmr1* KO mice. In addition, there was a significant negative correlation between mean total errors on the mazes and PSD-95 protein levels. The coefficient of determination indicated that the mean total errors on the H-W mazes accounted for 35% of the variance in PSD-95 protein levels. These novel findings suggest that reduced PSD-95 associated post-synaptic plasticity may contribute to the learning and memory deficits observed in human FXS patients.

Keywords: Hebb-Williams mazes, Fragile X Syndrome, Visual-spatial learning and memory, Hippocampus, Western blot.

Visual–spatial learning impairments are associated with hippocampal PSD-95 protein dysregulation in a mouse model of fragile X syndrome.

FXS is the most common cause of inherited intellectual disability and occurs due to loss of function mutation on the fragile X mental retardation 1 (*FMR1*) gene on the X chromosome resulting in a lack of fragile X mental retardation protein (FMRP) expression (reviewed in (O'Donnell & Warren, 2002). Relevant to the present investigation, FXS patients display poorer performances as compared to developmentally-matched participants on a number of different visual-spatial dependant tasks (Cornish, Munir, & Cross, 1998, 1999; Kogan et al., 2004; Kogan et al., 2009; MacLeod et al., 2010).

Fmr1 knock-out (KO) mice ("Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium," 1994) show several behavioural deficits found in human patients (Casten, Gray, & Burwell, 2011; Chen & Toth, 2001; "Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium," 1994; Kooy, 2003; Mineur, Huynh, & Crusio, 2006; Mineur, Sluyter, de Wit, Oostra, & Crusio, 2002; Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005; Zhao et al., 2005). In humans and mice, *FMR1*/*Fmr1* mRNA is highly expressed in a number of brain regions including the hippocampus (Abitbol et al., 1993; Hinds et al., 1993), suggesting that FMRP/Fmrp may be important in the underlying functions sub-served by this region. Spatial navigation and learning measures are dependent on the hippocampus, however tests using *Fmr1* KO mice have generated inconsistent results that may be a function of the tasks employed (Baker et al., 2010; D'Hooge et al., 1997; Dobkin et al., 2000; "Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian

Fragile X Consortium," 1994; Kooy et al., 1996; Mineur et al., 2002; Paradee et al., 1999; Peier et al., 2000; Van Dam et al., 2000; Yan et al., 2005).

The Hebb-Williams (H-W) mazes are a viable test alternative as they are sensitive in detecting hippocampal deficits in spatial navigation and learning (Churchwell, Morris, Musso, & Kesner, 2010; Rogers & Kesner, 2006; Shore, Stanford, MacInnes, Klein, & Brown, 2001; Winocur & Moscovitch, 1990). We previously reported that human FXS patients and *Fmr1* KO mice had a comparable pattern of deficits whereby they committed more errors than controls on the same H-W mazes. These results suggested commonalities in maze navigational performance and that the spatial learning deficits were attributable to a lack of FMRP/Fmrp (MacLeod et al., 2010).

Post-synaptic modifications of AMPA receptors (AMPARs) by long term potentiation (LTP) and/or long term depression (LTD) are believed to be the neural correlates of learning and memory (Bliss & Collingridge, 1993; Malenka & Bear, 2004; Scholz et al., 2010). One candidate protein that may be involved in both AMPAR regulation and dendritic spine structure is post-synaptic density protein of 95 kDa (PSD-95). PSD-95 has been implicated as a scaffolding protein, which can indirectly bind to AMPARs (Schnell et al., 2002), modulate AMPAR synaptic numbers and synaptic strength (Beique & Andrade, 2003; Colledge et al., 2003; Ehrlich & Malinow, 2004; Keith & El-Husseini, 2008; Xu et al., 2008). Regarding structure, over expression of PSD-95 enhanced postsynaptic clustering, size and number of dendritic spines (El-Husseini, Schnell, Chetkovich, Nicoll, & Bredt, 2000) whereas knockdown or mutation of PSD-95 N or C termini impaired spine growth (Steiner et al., 2008).

The murine model of FXS is characterized by dendritic spine abnormalities (Bilousova et al., 2009; Galvez & Greenough, 2005; Grossman, Elisseou, McKinney, & Greenough, 2006;

Levenga et al., 2011; McKinney, Grossman, Elisseou, & Greenough, 2005). Fmrp has an established role in regulating plasticity-associated proteins such as PSD-95 [17]. Regarding PSD-95, increased translational levels were observed during basal states in *Fmr1* KO as compared to wild type mice [18] as well as relatively low protein levels following stimulus induction in this genotype [19]. Furthermore, PSD-95 mRNA transcripts were found to selectively deteriorate in the hippocampus but not in the cortex or cerebellum of *Fmr1* KO mice (Zalfa et al., 2007; Zhu, Xu, Zhao, Gu, & Wu, 2011). These data suggest that some of the cognitive impairments in FXS could be attributable to a lack of dynamic regulation of PSD-95 following synaptic activity due to the lack of Fmrp.

In an effort to address inconsistencies in the animal literature and to better understand protein dynamics in an *in vivo* learning model of FXS, we examined PSD-95 protein levels using the H-W mazes. We hypothesized there would be an upregulation of PSD-95 in wild type mice and this response would be reduced in *Fmr1* KO mice. Protein levels from both genotypes were hypothesized to correlate negatively with total errors on the H-W mazes.

Methods

Animals

A total of 36, male, naïve mice of a FVB background strain were obtained from Jackson Laboratories (Bar Harbor, ME, USA) that included 18 wild type control (FVB.129P2-*Pde6b*$^{+}$*Tyr*$^{c\text{-}ch}$/AntJ; JAX Stock # 004828) and 18 *Fmr1* KO mice (FVB.129P2-*Fmr1*^{tm1Cgr}/J; JAX Stock # 004624). The *Fmr1* KO mice were bred from homozygote mating pairs and backcrossed for 11 generations. The FVB genetic background was chosen in view of the documented modest visual-spatial abilities. Mice of both genotypes were shipped at 4 weeks of age and were approximately 12 weeks old when they began experimental procedures**.** Mice were given two weeks acclimation and were housed in groups of four in standard (27 x 21 x 14 cm)

polypropylene cages. Eight days prior to testing, all mice were housed in individual cages. To ensure high levels of motivation during the study, mice were maintained at approximately 85-90% of their original body weight and fed a food ration approximately 30 minutes after daily testing procedures ended. The study received ethical approval from the University of Ottawa and efforts were made to minimize pain and suffering as outlined by the Canadian Council of Animal Care.

Apparatus

The H-W test apparatus was constructed according to the specifications outlined by the developers, (Rabinovitch & Rosvold, 1951). The apparatus, made of black plexiglass (Plastics of Ottawa Ltd., Ottawa, ON, Canada), consisted of a large open area, square in shape (60 x 60 x 10 cm), with diagonally opposing start and goal box areas (20 x 10 x 10 cm). The start and goal box areas were equipped with sliding, removable plexiglass doors to control entry and confinement, covered by clear plexiglass lids. In the goal box, a recessed food cup (2.5 cm diameter) was placed in the centre and baited with a piece of food (Harlan Global Rodent Chow, 20 mg) during the latter phases of the experiment. The floor of the square open area was delineated by 36 equal squares and these were used for manually placing barriers that defined different maze problems and error zones (Rabinovitch & Rosvold, 1951). The barriers (10 cm high) were constructed with black opaque plexiglass.

Procedure

Wild type control (n=12) and *Fmr1* KO (n=12) mice underwent behavioral testing. During the acclimation period, one wild type mouse was euthanized due to poor health (of an unknown cause) and two KO mice were euthanized due to progressive and continuous seizures resulting in a smaller sample size (control n=11; *Fmr1* KO n=10). Twelve additional mice (6

wild type; 6 *Fmr1* KO) were used as an untrained control group. These mice did not participate in maze learning, however underwent daily handling, food restrictions procedures and exposure to the H-W mazes similar to those animals running the mazes.

The experiment was conducted in three phases: habituation, acquisition and testing. During the habituation phase, the H-W apparatus was cleared of all barriers and each mouse was allowed 20 minutes/day on four consecutive days to explore. During the last two days, the goal box area was baited with a small piece of food (20 mg) and each mouse had *ad lib* access to the food for the duration of the session.

The acquisition phase consisted of training mice on six practice mazes ([3]; Fig. 1). Specifically, each mouse was trained for two sessions per day, the first starting at 0800 and the second at 1300. Each session consisted of one different practice maze (five trials per maze) commencing with maze A. A trial was considered complete when the mouse entered the goal area and took a bite of food or 180 seconds had elapsed. Mice completed all six acquisition mazes in sequence (A-F) as many times as necessary for them to reach criterion; that of two consecutive sessions completed in less than 30 seconds each.

Following acquisition, mice were given a selection of the standard test mazes ([3]; Fig 1B) based on the same procedures used during acquisition. Mice were tested on a different maze in each session (five trials per maze) in the same order (i.e., 2, 4, 5, 8, 9, 11, 12) until all seven were completed, spanning 3.5 days/animal. The dependent measures of interest were latency and number of errors. Latency was recorded from the moment the barrier in the start box was raised until the animal took its first bite of food. An error was registered each time a mouse crossed its two front paws into an error zone ([3]; Fig 1B). Data from the testing phase were recorded using an overhead SONY camcorder and Media Cruise software (Thomson Canopus Co. Ltd., Kobe,

Japan) on a standard desktop computer. The experimenter was blind to the genotype of the mice and never visible to the mice during the runs.

Western Blot

Immediately after finishing the H-W mazes, mice were euthanized (100µl intraperitoneal (i.p.) injection of euthasol), their brain removed and tissue blocks were cut using a stainless steel brain matrix (1 x 1.5 x .75 inches). Both dorsal hippocampi were dissected according to the mouse atlas of (Paxinos, 2001) and frozen on dry ice. Briefly, hippocampi were homogenized over ice in a homogenate buffer/protease inhibitor cocktail (Sigma Aldrich, Oakville, ON, Canada). The homogenates were centrifuged, protein content was quantified using a standard BSA kit (Pierce, Rockford, IL, USA) and samples were frozen at -80°C until further analysis. Proteins were loaded at a concentration of 300 µg/ml and samples in quadruplicate (12 µg/lane) were resolved by SDS-PAGE. Proteins were then transferred to pure nitrocellulose membranes and blocked for 1 hour in 5% skim milk and 10 M phosphate buffered saline (PBS) solution at room temperature. Antibody specificity was determined prior to commencing western blot analyses on experimental animals by confirming a single band of binding of the proteins of interest at the appropriate molecular weight, 95 kDa (PSD-95) and 55 kDA (β-tubulin). Optimal concentrations of primary/secondary antibody were then confirmed by serial dilutions. Membranes were then incubated in 5% skim milk and TBST (20mM Tris/HCl, 137mM NaCl, 0.4% Tween 20, pH 7.6) solution with monoclonal anti-PSD-95 antibody (1:2000; Millipore Corporation, Burlington, ON, Canada) and monoclonal anti-ß-tubulin antibody (1:10,000; Sigma Aldrich, Oakville, ON, Canada) at 4°C overnight. After 3 x 10 minute washes in TBST, fluorescent Alexa 680-linked antibody (1:10,000, Molecular Probes, Burlington, ON, Canada) and IR 800 antibody (1:10,000; LI-COR Biosciences, Lincoln, NE, USA) in 5% skim milk and

TBST solution were applied for one hour at 4°C. After 3 x 10 minute washes in TBST, western blots were scanned using the Odyssey infra-red system (LI-COR Biosciences, Lincoln, NE, USA) in 700 nm and 800 nm channels in a single scan at 169 µm resolution. Simultaneous detection of two fluorescent antibodies (i.e., Alexa 680 and IR 800) allowed for the measurement of PSD-95 and ß-tubulin proteins within each sample. The density of each protein band of interest was measured, background subtracted and normalized to ß-tubulin by the LI-COR analysis software. Similar western blot procedures were carried out on the untrained control group who were euthanized after 15.5 days, the average time it took for the maze learning mice to complete all phases of the H-W mazes.

Immunohistochemistry

To visualize PSD-95 antibody staining in CA1 region of the hippocampus, one mouse from each genotype that completed the H-W mazes was prepared for immunohistochemistry. Briefly, immediately following maze learning, mice were administered a 100 µl i.p. injection of euthasol prior to intracardiac perfusions consisting of brains being flushed with 20 ml of saline followed with 20 ml of 4% paraformaldehyde, 0.2% picric acid in 0.16 M sodium phosphate buffer, pH 7.1. After a 3.5 hour post-fixation at 4°C, brains were transferred to 10% sucrose in 0.1M sodium phosphate buffer, pH 7.1 for cyroprotection. Fourteen micron saggital sections were then cut using a cryostat (Leica Microsystems Inc., Concord, ON, Canada) and stored at -80°C. Antibody specificity was confirmed by visualizing section staining consisting of primary/secondary antibody; secondary antibody only; or antifade only on a Zeiss Axioplan fluorescence microscope. Optimal concentrations of primary/secondary antibody were then confirmed by serial dilutions. Tissue sections were later washed briefly in .01 M PBS and incubated in monoclonal anti PSD-95 antibody (1:100, Cell Signaling Technology, Boston, MA,

USA) diluted with 0.3% Triton X-100 containing 4% normal donkey serum in PBS for three hours at room temperature. After 3 x 5 minute washes in PBS, sections were incubated in Alexa Fluor 488 secondary (1:1000, Cell Signaling Technology, Boston, MA, USA) with 0.3% Triton X-100 containing 4% normal donkey serum in PBS for 30 minutes at 37°C. After the secondary antibody, a NeuroTrace (530/615) red fluorescent Nissl stain (1:50, Molecular Probes, Life Technologies, Burlington, ON, Canada) was applied according to the manufacturer's protocol. Sections were then cover slipped using a standard antifade medium (Fisher Scientific, Ottawa, ON, Canada). Fluorescence staining of the CA1 region of the hippocampus was then visualized with a Zeiss LSM 510 Axiolmager. M1 confocal microscope at a magnification of 40 X, according to the stereotaxic coordinates outlined by (Paxinos, 2001). Single optical planes were captured.

Statistical Analyses

The behavioral data (latency and errors) were previously published and are presented in (MacLeod et al., 2010). Briefly, whereas latency to complete the H-W mazes did not differ between wild type and *Fmr1* KO runners, significantly fewer errors were made by Fmrp intact mice (MacLeod et al., 2010). In order to examine whether KO mice were able to perform the basic, non-specific behaviours necessary for maze navigation, an independent samples *t*–test was conducted with genotype as independent variable and days to reach criterion as dependent variable. Because wild type as compared to KO mice spent significantly more days in the acquisition mazes (see Results section), this nuisance variable was used as a covariate in subsequent analyses of PSD-95 levels of H-W maze runners.

To test the hypothesis that hippocampal PSD-95 is upregulated only in the wild type runners following successful completion of test mazes, a 2 X 2 ANOVA was performed (SPSS

19) with genotype (wild type; *Fmr1* KO mice) and task (H-W mazes; untrained control) as the independent variables and the protein ratio of PSD-95 normalized to a control protein, ß-tubulin, as the dependent variable. An additional 2 X 2 ANOVA was performed to determine if levels of ß-tubulin significantly differed within the independent variables.

In order to examine the potential relationship between PSD-95 levels and errors committed on the test mazes, three separate bivariate correlational analyses (Pearson's *r*) were conducted. These analyses used relative PSD-95 protein levels (normalized to ß-tubulin) and mean total errors on the H-W mazes, defined as aggregate errors divided by the total number of learning trials (Maze X Trials = 35). Specific correlations focused on the relationship between PSD-95 protein levels and mean errors from: (1) H-W maze runners of both genotypes (2) wild type runners and (3) *Fmr1* KO runners. As a control, correlations were also performed between β-tubulin protein levels and mean errors from 1, 2, and 3 as listed above.

Results

Whereas western blots were used to quantify hippocampal PSD-95 protein expression levels, confocal images of the hippocampal distribution of PSD-95 in the CA1 region in wild type and *Fmr1* KO mice shown in Figure 2 are provided for visual depiction purposes only.

Fmr1 KO mice reached criterion in the acquisition phase significantly faster than wild type controls ($t = 3.01$, $p = .006$). The mean time to complete the acquisition phase was 5.35 days for the *Fmr1* KO group, 95% CI [4.00, 6.86] and 10.5 days for wild type mice, 95% CI [7.61, 13.21]. Despite these differences, the one way ANCOVA was significant for genotype, $F(1,18) = 7.19$, $p = .01$, partial $\eta^2 = .29$, indicating higher PSD-95 levels in wild type runners relative to

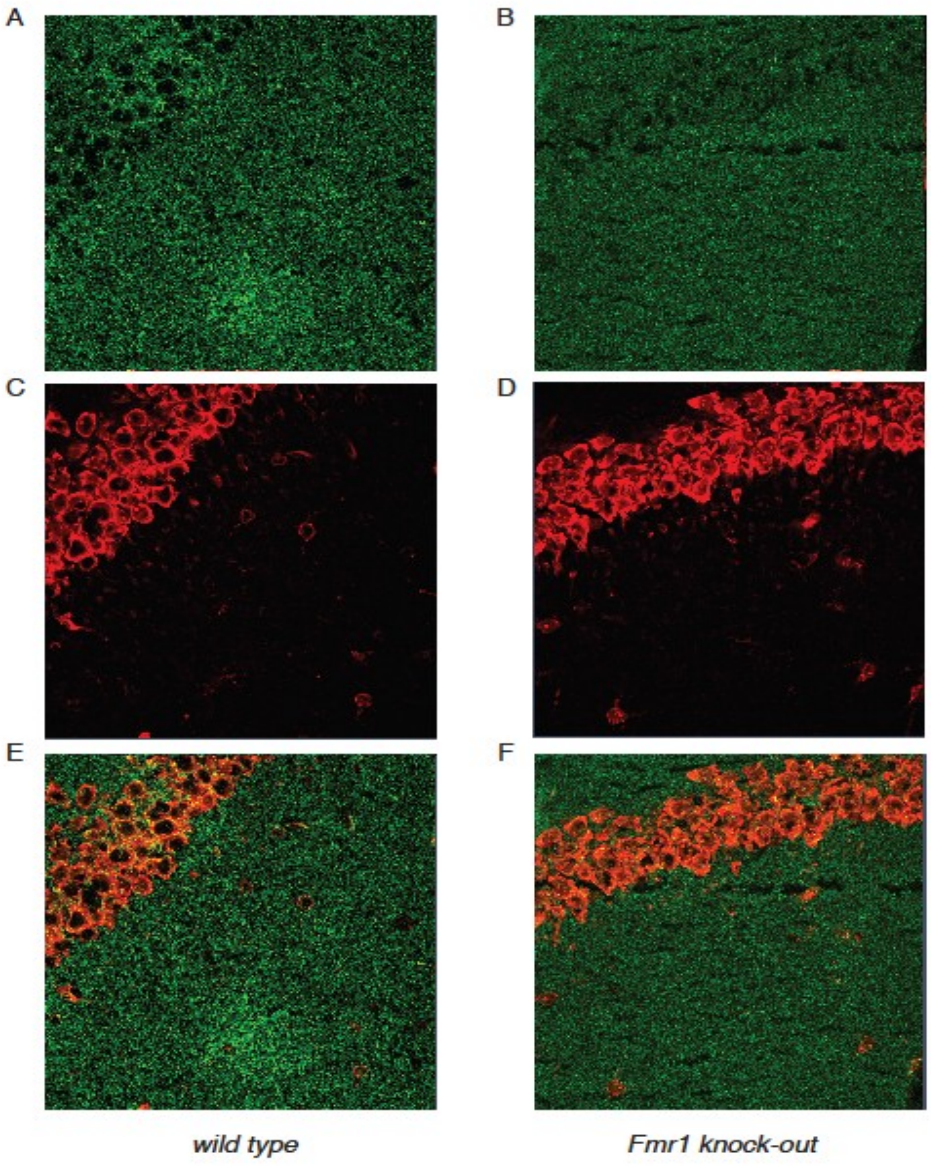

Figure 2: x 40 confocal image of the CA1 region of the dorsal hippocampus. (A, B) depicts a representative raw image of postsynaptic density protein-95 punctate staining, (C, D) of Nissl fluorescent staining, and (E, F) a merge of both stainings in wild-type (n=1; left) and *Fmr1* knockout (n=1; right) mice.

KO runners, when statistically controlling for number of days required to reach acquisition criterion.

The results of the 2 X 2 ANOVA conducted to evaluate the effects of H-W maze learning on PSD-95 protein levels as measured by optical density of western blots in wild type and *Fmr1* KO mice indicated a significant main effect for genotype, $F(1,29) = 14.31$, $p = .001$, partial $\eta^2 = .33$, and for task, $F(1,29) = 10.01$, $p = .004$, partial $\eta^2 = .26$. There was also a significant interaction between genotype and task, $F(1,29) = 4.07$, $p = .05$, partial $\eta^2 = .12$. With respect to β-tubulin protein levels, there was no main effect of genotype, $F(1,29) = 2.44$, $p = .13$, partial $\eta^2 = .08$, or task, $F(1, 29) = .05$, $p = .82$, partial $\eta^2 = .002$, and the genotype by task interaction was not significant, $F(1,29) = .10$, $p = .75$, partial $\eta^2 = .003$ (Figure 3).

Bonferroni corrections were made to the α level of .05 before performing simple main effect analyses resulting in .0125 (.05/4 = .0125). Simple main effects of task within genotype indicated that wild type mice that ran the H-W mazes had significantly higher PSD-95 levels than wild type mice in the untrained control group, $F(1,29) = 13.71$, $p = .001$, partial $\eta^2 = .32$. This difference was not found in *Fmr1* KO mice when runners were compared with non-runners, $F(1,29) = .66$, $p = .42$, partial $\eta^2 = .02$. Examination of genotype within each task (H-W runners; naïve control group) revealed that wild type runners had significantly higher PSD-95 levels than *Fmr1* KO runners following completion of the mazes, $F(1, 29) = 23.10$, $p = .0001$, partial $\eta^2 = .44$. By comparison, there were no PSD-95 protein differences between wild type and KO mice in the naive, untrained control group, $F(1, 29) = 1.23$, $p = .28$, partial $\eta^2 = .04$. Thus, PSD-95 protein upregulation occurs in wild type mice that ran the H-W mazes and this response is blunted in *Fmr1* KO mice (Figure 3).

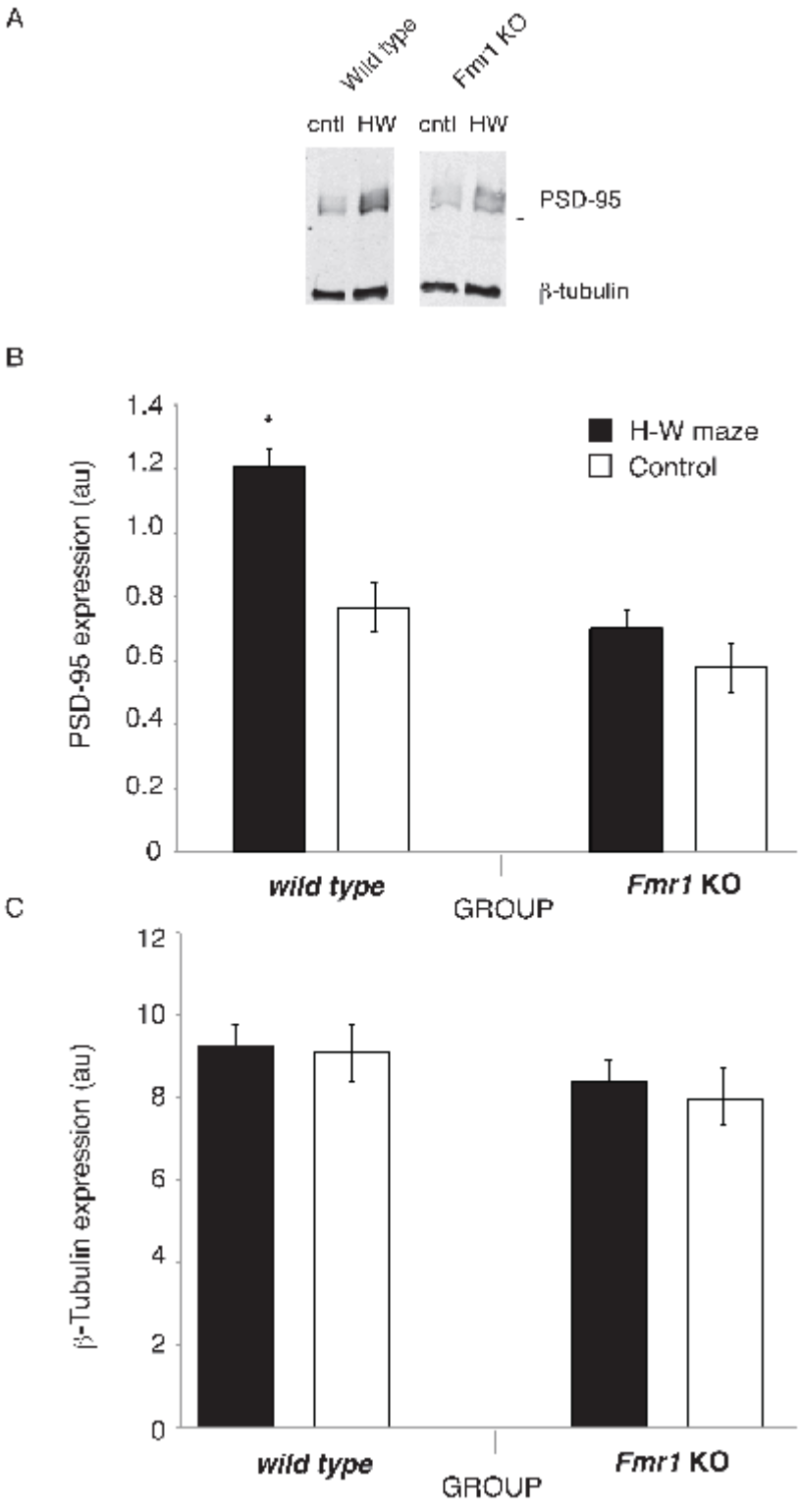

Figure 3: Postsynaptic density protein-95 (PSD-95) expression is increased in wild- type but not *Fmr1* KO mice following a learning paradigm. (A) Representative western blots from dorsal hippocampi of wild-type and KO mice for protein expression of PSD-95 and β-

tubulin. PSD-95 is found around the expected molecular weight of 95 kDa and β-tubulin at 55 kDa. Control represents animals in the untrained group, whereas Hebb–Williams (H–W) refers to trained animals from each genotype. (b) Quantification of protein levels for PSD-95 normalized to β-tubulin, and (c) β-tubulin in wild-type (n =17) and KO (n=16) mice. Error bars represent the SEM; *p<0.025.

The first correlational analysis revealed that for H-W maze runners of both genotypes, there was a significant, negative correlation between PSD-95 protein levels and mean total errors on the H-W mazes, $r(19) = -.59$, $p = .002$, $r^2 = .35$ (Figure 4). Two further analyses within genotype were also completed and revealed correlations that trended towards significance (after Bonferroni adjustments to α). Specifically, in wild type mice, there was a negative correlation between PSD-95 protein levels and mean total errors, $r(9) = -.44$, $p = .09$, $r^2 = 0.18$. In *Fmr1* KO mice, there was a negative correlation between PSD-95 protein levels and mean total errors, $r(8) = -.54$, $p = .05$, $r^2 = 0.29$. As a control, β-tubulin protein levels were also correlated with mean total maze errors. For runners of both genotypes, there was no correlation between β-tubulin protein levels and mean total errors on the H-W mazes, $r(19) = -.078$, $p = .37$, $r^2 = .006$. Within genotype, there was no correlation between β-tubulin protein levels and mean total errors for wild type runners, $r(9) = -.20$, $p = .27$, $r^2 = .04$ nor for *Fmr1* KO mice, $r(8) = .31$, $p = .19$, $r^2 = .09$. Thus, these results highlight a relationship of covariance, specific to PSD-95, between hippocampal protein levels and mean errors on the H-W mazes.

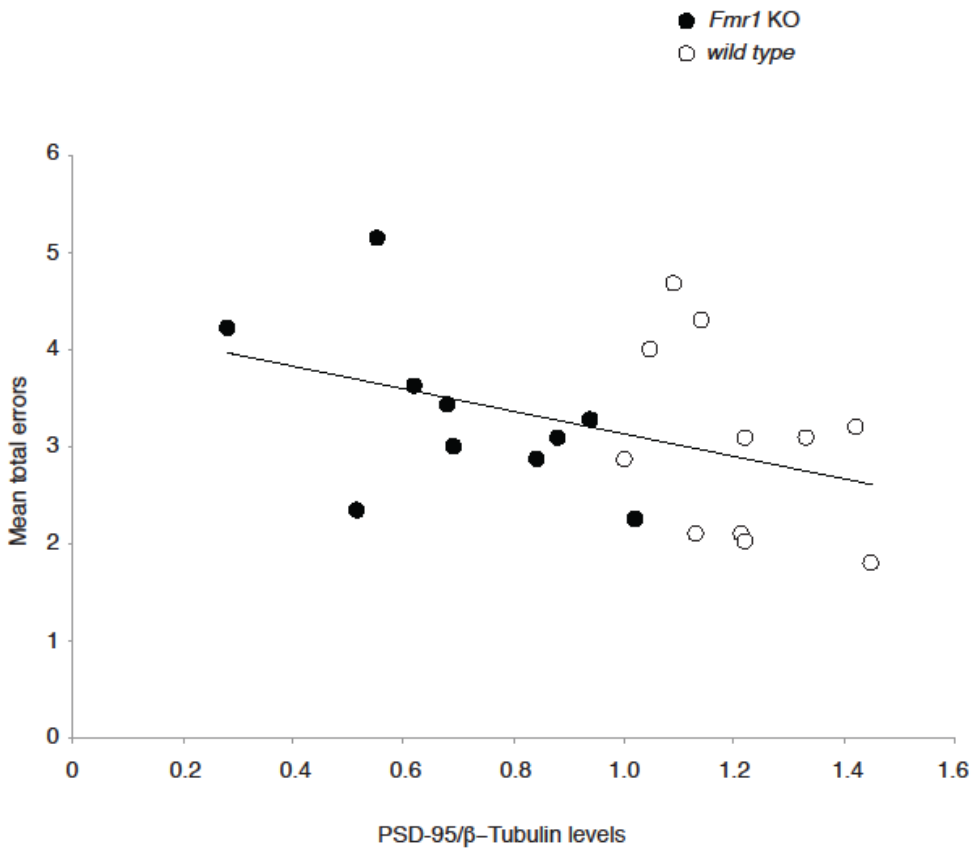

Figure 4: Levels of hippocampal postsynaptic density protein-95 (PSD-95) are correlated with behavioral performance. Negative correlation between mean maze errors on the Hebb–Williams mazes and PSD-95 protein levels were normalized to β-tubulin in wild- type (n=11) and knockout (KO; n=10) mice. A significant negative correlation was observed [$r(19) = -0.59$, $p=0.003$, r2 =0.35].

Discussion

We examined the spatial navigation and learning abilities in wild type and *Fmr1* KO mice in order to better understand the protein changes that accompany learning of a visual-spatial navigation measure, the H-W mazes. FXS patients as well as *Fmr1* KO mice completing the H-W mazes were previously shown to have comparable maze navigational performances such that they committed more errors than controls, results which were attributable to a lack of FMRP/Fmrp (MacLeod et al., 2010). We add to this literature by demonstrating that runners completing the H-W mazes exhibited an upregulation of an important scaffolding protein normally under the control of Fmrp in the hippocampi of wild type mice and that this response was blunted in *Fmr1* KO mice who lack Fmrp. Moreover, protein upregulation was specific to PSD-95, as evidenced by stable levels of a control protein (β-tubulin) across genotypes and condition. Given the commonalities in visual-spatial learning impairments in humans and mice (MacLeod et al., 2010; Shore et al., 2001) our results suggest that human FXS patients may display poorer performance on visual-spatial dependant tasks as a result of dysregulation of PSD-95 protein levels. Further evidence that strict regulation of PSD-95 may contribute to normal cognitive functioning in humans is supported by findings of decreased PSD-95 expression in conditions with neurocognitive impairment such as Alzheimer's disease, schizophrenia and mood disorder (Gylys et al., 2004; Toro & Deakin, 2005).

One of our goals was to further validate the H-W mazes as a behavioral assay for reliably documenting visual-spatial learning deficits in *Fmr1* KO mice. This would in turn allow for future studies to evaluate the effectiveness of pharmacological or behavioral interventions intended to mitigate symptoms of FXS. Interestingly, *Fmr1* KO mice reached criterion in the acquisition phase significantly faster than wild type controls. This was likely due to the

combination of the ease of the acquisition mazes (e.g., requiring a single response to correctly reach the goal box) and the known pattern of hyperactivity in *Fmr1* KO mice of the FVB background strain [26], which makes them more likely to achieve the <30 second latency criterion faster than their wild type counterparts. Although KO mice reached criterion earlier, the pattern of errors on acquisition mazes was similar between genotypes. The acquisition data, taken together with the finding that *Fmr1* KO mice commit more errors than controls [3], suggest that when presented with more challenging visual-spatial tasks such as the test mazes, *Fmr1* KO mice evidence a poorer learning strategy. Moreover, impaired performance of *Fmr1* KO mice on the test mazes alongside superior performance on the acquisition mazes serves to strengthen the notion that observed deficits and concomitant blunted PSD-95 expression are meaningful rather than being attributable to a generalized impairment of the KO mice in being able to perform the basic, non-specific, behaviors necessary for maze navigation.

We observed a significant negative correlation between mean maze errors and PSD-95 expression. That is, mice that committed fewer mean errors on the mazes exhibited greater PSD-95 protein levels and *vice versa*. In an attempt to further characterize this relationship, we performed additional correlation analyses within each genotype, which were found to trend towards significance, and may have achieved significance with larger sample sizes.

The deficits of PSD-95 protein upregulation in *Fmr1* KO mice is consistent with a lack of and an inability to dynamically regulate protein synthesis during maze learning. It has been suggested that pharmacological treatments stabilizing basal protein translation levels may ameliorate some of the core symptoms in FXS by restoring normal protein synaptic synthesis thereby allowing for improved regulation during periods of synaptic plasticity(Bassell & Warren, 2008). However, the identification of specific proteins responsible for the morphological changes

in FXS has to date remained speculative. Additional studies investigating the use of pharmacological agents are needed to ascertain if spatial navigation and learning deficits and protein correlates such as PSD-95 are amenable to treatment in *Fmr1* KO mice. Such studies may provide valuable insight into the neurobiological basis of and treatment for the FXS phenotype.

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Conflicts of interest

There are no conflicts of interest.

Chapter 3: Manuscript II

Manuscript II

2-Methyl-6-(phenylethynyl) pyridine (MPEP) reverses maze learning and PSD-95 deficits in *Fmr1* knock-out mice.

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Abstract

The etiology of Fragile X syndrome (FXS) is the lack of expression of the fragile X mental retardation protein (FMRP), which results in intellectual disability and other debilitating symptoms including impairment of visual-spatial functioning. FXS is the only single-gene disorder associated with a high co-morbidity with autism and can therefore provide insight into the pathophysiology of this disorder. At the cellular level, the lack of FMRP results in altered group I metabotropic glutamate receptor (mGluR) signalling and, proposed treatments have targeted the mGluR-5. One way to measure learning and memory is with Hebb-Williams mazes, which are a set of increasingly complex spatial navigation problems that depend on intact hippocampal and thus mGluR-5 functioning. In the present investigation we examined whether an antagonist of the mGluR-5 would reverse the behavioural deficits in *Fmr1* KO mice. Thus, mice were trained on a subset of the Hebb-Williams mazes and then treated with either 20 mg/kg of an mGluR-5 antagonist, 2-Methyl-6-(phenylethynyl) pyridine (MPEP; n = 11) or an equivalent dose of saline (n = 11) prior to running test mazes. Latency to complete the mazes as well as errors made across maze trials were recorded during the test phase. Immediately after completing each test, marble burying behavior was assessed to ensure that the drug treatment was pharmacologically active during maze learning. The results indicate that MPEP remained active during the full extent of testing. Although latency was not statistically different between the groups, MPEP treated *Fmr1* KO mice made significantly fewer errors on mazes deemed more difficult suggesting a reversal of the behavioural deficit. Collapsed across mazes and trials, MPEP treated mice were also less perseverative and impulsive when navigating maze problems. Furthermore, MPEP treatment reversed PSD-95 protein deficits in *Fmr1* KO treated mice whereas levels of a control protein (β-tubulin) were unchanged by treatment. These data further

validate MPEP as a potentially beneficial treatment for FXS. Given the extreme levels of variability observed using alternative cognitive measures in human FXS studies, our findings also suggest that adapted Hebb-Williams mazes may be a useful tool to document alterations in behavioural functioning following pharmacological intervention in FXS patients.

Key words: Fragile X syndrome, Hebb-Williams mazes, 2-Methyl-6-(phenylethynyl) pyridine, Post-Synaptic Density (PSD)-95, Western Blot

2-Methyl-6-(phenylethynyl) pyridine (MPEP) reverses maze learning and PSD-95 deficits in *Fmr1* knock-out mice

Fragile X Syndrome is a neurodevelopmental disorder that is caused by the loss of function mutation of the fragile X mental retardation 1 (*FMR1*) gene on the X chromosome (reviewed in (O'Donnell & Warren, 2002; Santoro, Bray, & Warren, 2012); Online Mendelian Inheritance in Man ® [OMIM] 309550) resulting in lack of fragile X mental retardation protein (FMRP) expression (Fu et al., 1991; Pieretti et al., 1991). In turn, lack of FMRP results in a number of symptoms including cognitive dysfunction, attention deficit and hyperactivity, anxiety, epilepsy, as well as particular physical features such as an elongated face and macroorchidism (R. J. Hagerman, 1996; Hatton et al., 2006; O'Donnell & Warren, 2002; Scerif, Cornish, Wilding, Driver, & Karmiloff-Smith, 2007; Sullivan et al., 2006; Turner, Webb, Wake, & Robinson, 1996). Importantly, a large proportion of individuals (25-47%) affected by FXS display autistic behaviors or a co-morbid diagnosis of autism (Hatton et al., 2006; Kaufmann et al., 2004), making FXS the only clear genetically associated form of autism. Relevant to the present investigation, FXS patients display poorer performances as compared to developmentally matched participants on a number of different visual-spatial dependent tasks (Cornish et al., 1998, 1999; Kogan et al., 2004; Kogan et al., 2009; MacLeod et al., 2010; Van der Molen et al., 2010).

In *Fmr1* KO mice an exaggerated form of mGluR mediated long term depression (LTD) has been documented in hippocampal neurons (Huber, Gallagher, Warren, & Bear, 2002) evidenced by elevated levels of "LTD" proteins at basal states (Nosyreva & Huber, 2006; Osterweil, Krueger, Reinhold, & Bear, 2010) and by the internalization of AMPA receptors (Snyder et al., 2001). Following the identification of, and much research on, LTD in *Fmr1* KO

mice, the prevailing opinion is that Fmrp, which binds to approximately 4% of total brain mRNA (Brown et al., 2001; Darnell et al., 2011), acts as a translational suppressor of proteins *in vivo*, many of which are implicated in synaptic plasticity (Bassell & Warren, 2008; Bhakar, Dolen, & Bear, 2012; Darnell et al., 2011).

It has been hypothesized that in the absence of the translational suppression functions of Fmrp, abnormally elongated spines are responsible for some of the clinical manifestations of FXS such as cognitive dysfunction and audiogenic seizures (Bear, Huber, & Warren, 2004; Krueger & Bear, 2011). Thus, intervention with antagonists that selectively target mGluR-5 has been promising in that they can mitigate signaling and as a result correct some of the downstream effects that occur in the absence of Fmrp. Consistent with group I mGluR-signaling as mediating prolonged LTD in *Fmr1* KO mice, one study employed small interfering RNA (siRNA) specific to the *Fmr1* gene sequence to demonstrate that reductions of Fmrp in dendrites of hippocampal neurons led to an increase in the internalization of the AMPAR subunit, GluR1 (Nakamoto et al., 2007). Treatment with 2-methyl-6-phenylethynyl-pyridine (MPEP), an mGluR5-antagonist, rescued the abnormal AMPAR trafficking, an effect not found with NMDA receptors (NMDARs). In the absence of Frmp and following 20 days of *in vitro* culturing, neurons from adult *Fmr1* KO mice were classified as having excess filopodia (spines with a long and thin appearance) relative to wild-type cultured neurons that had a mushroom shaped appearance with a large spine head (de Vrij et al., 2008). Treatment of *Fmr1* KO neurons with two different mGluR-5 antagonists (200µM MPEP and 300µM fenobam) for 4 hours rescued the protrusion phenotype, restoring the spine/filopodia ratio in *Fmr1* KO neurons to the levels observed in wild-type neurons (de Vrij et al., 2008). Consistent with this, other researchers have reversed hippocampal spine elongations by using alternative mGluR-5 antagonists such as

AFQ056 (Levenga et al., 2011). Regarding cortical neurons, in one study, daily administration of 20 mg/kg of MPEP over the course of a week ameliorated average spine length and density in adult *Fmr1* KO mice without producing significant tolerance or toxicity effects (Su et al., 2011).

Arguably the strongest support for targeting mGluR- signaling with antagonists comes from research that cross-bred *Fmr1* KO mice with Grm5 mutant mice that have 50% reduction of mGluR-5 expression (rather than a complete KO which would negatively impact brain function). This procedure rescued several phenotypic aspects of the FXS mouse model. In this regard, reduction of mGluR-5 expression in *Fmr1* KO mice significantly reduced hippocampal LTD, rescued the increased density of long and thin spines, reduced the elevated basal protein synthesis rates and finally, reduced audiogenic seizures (Dolen et al., 2007).

Behaviorally, *Fmr1* KO mice of the hybrid strain C57Bl/6J X FVB/NJ displayed increased centre square entries and duration during open field testing indicative of impulsivity and disinhibiton. Single intraperitoneal (i.p.) injection of either 10 or 30 mg/kg of MPEP rescued these deficits such that open field performance 30 minutes after injection was statistically indistinguishable from control mice (Yan et al., 2005).

Despite much progress with antagonism interventions, there remains a need for reliable and valid means of assessing improvement in patients receiving treatments, which are comparable to those used in animal studies. Typically human FXS studies attempting to assess progress in various cognitive domains have produced inconsistent findings as a result of outcome measures that are confounded by floor and ceiling effects (Berry-Kravis et al., 2006). We previously showed that Hebb-Williams mazes are a viable visual-spatial assay for use with both human and murine populations with FXS participants and KO mice exhibiting similar behavioural impairments (i.e., more errors than controls) (MacLeod et al., 2010). More recently,

we demonstrated that Fmrp intact mice evidenced upregulations of PSD-95 following completion of the Hebb-Williams mazes. This pattern of upregulation was blunted in *Fmr1* KO maze learners (Gandhi, Kogan, Messier, & Macleod, 2013). As PSD-95 has been hypothesized as a key protein ostensibly involved in both AMPAR regulation and dendritic spine structure (Keith & El-Husseini, 2008), our data suggested that PSD-95 would be a good candidate protein to examine the effects of antagonism treatment in *Fmr1* KO mice.

Therefore, for the present study, we hypothesized that MPEP treatment of *Fmr1* KO mice would result in reversal of the previously described deficit (i.e., significantly fewer errors) on the Hebb-Williams mazes as well as a reversal of the PSD-95 protein deficit relative to saline treated controls. We also report results from a manipulation check (i.e., a marble burying assay) experiment that confirms that the MPEP treatment remains active throughout maze testing. Specifically, when MPEP is pharmacologically active, marble burying (a repetitive behavior) is significantly reduced without a corresponding decline in locomotor activity (A. M. Thomas, Bui, Perkins, Yuva-Paylor, & Paylor, 2012).

Method

Animals

A total of 22, male, naïve *Fmr1* knock-out (KO) mice with a FVB background, bred from homozygote mating pairs that had been backcrossed for 11 generations, were obtained from Jackson Laboratories (FVB.129P2-Fmr1^{tm1Cgr}/J; JAX Stock # 004624; Bar Harbor, ME, USA). These mice do not carry the rd1 mutation and consequently, do not develop retinal degeneration. The FVB genetic background was chosen in view of the documented modest visual-spatial abilities (Dobkin et al., 2000; Van Dam et al., 2000). Mice were shipped at 4 weeks of age and were approximately 12 weeks old when they began experimental procedures. Mice were given

two weeks to acclimate to the vivarium. During that time, they were housed in groups of four in standard (27 x 21 x 14 cm) polypropylene cages. All mice were kept on a 12 hour light-dark cycle (light 0700-1900) in a temperature controlled environment (21°C) and fed Rodent Chow (Harlan Global, City, Canada) and tap water. Eight days prior to testing, all mice were housed in individual cages. Behavioural testing took place during 0800-1500 to reduce variability associated with diurnal rhythms. To ensure high levels of motivation during the study, mice were maintained at approximately 85-90% of their original body weight and were fed a food ration approximately 30 minutes after daily testing procedures ended. The ethics protocol was approved by the University of Ottawa Animal Care Committee (UOACC) and precautions were taken to minimize any pain or discomfort according to the guidelines of the Canadian Council on Animal Care (CCAC).

Apparatus

The Hebb-Williams test apparatus was constructed according to the specifications outlined by the developers, Rabinovitch and Rosvold, (1951) (Rabinovitch & Rosvold, 1951). Specifically, the maze was built using black opaque plexiglass and fitted with a translucent plexiglass cover top (Plastics of Ottawa Ltd., Ottawa, ON, Canada). The apparatus consisted of a large open area, square in shape (60 x 60 x 10 cm), with diagonally opposing start and goal box areas (20 x 10 x 10 cm). The start and goal box areas were equipped with sliding, removable plexiglass doors to control entry and confinement, covered by clear plexiglass lids. In the goal box, a recessed food cup (2.5 cm diameter) was placed in the centre and baited with a 20 mg of Rodent Chow, during the latter phases of the experiment. The floor of the square open area was delineated by 36 equally sized squares. The squares were used as markers for manually placing barriers that defined different maze problems and error zones (Rabinovich & Rosvold, 1951). The barriers

(10 cm high) were constructed with black opaque plexiglass. Extra-maze cues were minimized by placing the apparatus on a desk table (100 x 75 cm) and by enclosing it within white wall coverings hanging from the ceiling.

Drug treatment

Gq-coupled, group I metabotropic glutamate receptors (mGluR) consist of two different types of receptors. Treatments for Fragile X Syndrome targeting mGluR-5 receptors have been favoured over mGluR-1 receptors given that the latter produces motor deficits in animals (Berry-Kravis, Knox, & Hervey, 2011). As such, the mGluR-5 antagonist, 2-methyl-6-(phenylethynyl) pyridine hydrochloride, MW 229.70, (MPEP; Sigma Aldrich, Oakville, ON, Canada) was used in the current investigation. MPEP is a potent and selective antagonist of mGluR-5 that is able to cross the blood-brain barrier readily (Gasparini et al., 1999). Regarding preparation, drug powder was dissolved into a vehicle (saline) and aliquots containing 5mg/ml of stock solution were stored at -20°C. Thereafter single aliquots were allowed to warm to room temperature, briefly centrifuged, and MPEP treated mice received an intraperitoneal (i.p.) injection of 20 mg/kg based on their body weight on the day of testing. Similarly, aged matched control mice were administered an equivalent dose of saline without the drug based on their body weight. MPEP was previously reported to be biologically active from 15 to 75 minutes following i.p. injections (Yan et al., 2005). Based on this data and to allow sufficient time for the drug to take effect, mice in both groups were tested 30 minutes following drug or vehicle-only administration. The mg/kg dosage was determined based on a pilot study prior to experimentation (see data below).

Marble Burying

A marble burying assay (A. Thomas et al., 2009) was used to ensure that MPEP doses were biologically active before and after maze testing. This assay reflects repetitive digging

behaviour without habituation effects to burying even if marble presentations are repeated multiple times during the same day or across several days (A. Thomas et al., 2009). The number of marbles buried decreases following the administration of Grp I mGluR antagonists (A. M. Thomas et al., 2012) and MPEP treatment does not significantly reduce voluntary locomotor. Concerning the assay itself, 20 marbles of varying colour were arranged (15 mm in diameter) in a 4 x 5 pattern on top of approximately three and a half cm of bedding (SANI-CHIP) using clean (27 x 21 x 14 cm) polypropylene cages. Approximately 4 cm of open space, clear of marbles was left at one end of each cage in order to place a single mouse into the apparatus. Each mouse was allotted 20 minutes to bury as many marbles as possible. Marbles were considered buried if they were covered by >50% of SANI-CHIP bedding.

Pilot study – Dose Response Determination

Studies using *Fmr1* KO mice and MPEP treatments (via an i.p. route of administration) followed by behavioural testing have attempted to optimize effective dose ranges from 0.05 mg/kg to 40 mg/kg (Su et al., 2011; A. M. Thomas et al., 2012; Yan et al., 2005). Based on the results from these studies, an initial pilot study was conducted with *Fmr1* KO mice (N=6; Jackson Laboratories, Bar Harbor, ME, USA; FVB.129P2-Fmr1^{tm1Cgr}/J; JAX Stock # 004624). Mice (n=2) received vehicle, 20 mg/kg, or 30 mg/kg of MPEP treatments over consecutive 4 days. Vehicle or MPEP treatments were administered twice per day (0800 and 1300) and mice were allowed 30 minutes to allow sufficient time for the drug to take effect prior to participating in the marble burying assay. The total number of marbles buried collapsed across four days and eight marble burying trials was measured. Bonferroni adjusted independent sample *t*-tests (α .05/3 = .017) indicated there was significantly less marbles buried by 20 mg/kg treated mice relative to vehicle treated mice ($t = 9.40$, $p = .011$); and by 30 mg/kg treated mice relative to

controls (t = 12.80, p = .006). However, there were no differences in aggregated marbles buried between the two doses of MPEP treatment (t = .949, p = .433). As such, the dose used for MPEP treated mice in the current investigation was set at 20 mg/kg to avoid potential unwanted side effects from the higher dose.

Procedure

All 22 *Fmr1* KO mice underwent behavioral testing with half of the animals (n =11) receiving 20 mg/kg treatment of MPEP and the others an equivalent dose of vehicle only (n =11). The experiment was conducted in three phases: habituation, acquisition and testing. During the habituation phase, the Hebb-Williams apparatus was cleared of all barriers and each mouse was allowed 20 minutes/day on four consecutive days to explore the maze including the start and goal box areas. During the last two days, the goal box area was baited with 20 mg of Rodent Chow and each mouse had *ad lib* access to the food for the duration of the session.

The acquisition phase consisted of training mice on six practice mazes (Figure 5A). Specifically, each mouse was trained for two sessions per day, the first starting at 0800 and the second at 1300. Each session consisted of one of six possible practice mazes (five trials per maze) commencing with maze A. A trial was considered complete when the mouse entered the goal area and took a bite of food or 180 seconds had elapsed. Mice completed all six acquisition mazes in sequence (A-F) as many times as necessary for them to reach criterion; that of two consecutive sessions completed in less than 30 seconds each. The mean time to complete the acquisition phase was 11.6 days. Mice that were assigned to either MPEP or vehicle treatment during the subsequent phase (i.e., testing) did not differ in the number of days required to reach criterion in the acquisition phase (t =.18, p = .86).

Following acquisition, mice were given a selection of the standard test mazes (Figure 5B;

(Rabinovitch & Rosvold, 1951)) based on the same procedures used during acquisition. Thirty minutes prior to maze running mice were administered a either a dose of 20 mg/kg of MPEP or an equivalent volume of vehicle. Mice were then tested on a different maze in each session (five trials per maze) in the same order (i.e., #2, #4, #5, #8, #9, #11, #12) until all seven were completed, spanning 3.5 days/animal. The dependent measures of interest were latency and number of errors. Latency was recorded from the moment the barrier in the start box was raised until the animal took its first bite of food. An error was registered each time a mouse crossed its two front paws into a defined error zone (Figure 4B). Data from the testing phase were recorded using an overhead SONY camcorder and Media Cruise software (Thomson Canopus Co. Ltd., Kobe, Japan) on a standard desktop computer. Immediately after each maze, individual mice were placed in separate marble burying assays for 20 minutes each, following which the number of marbles buried was recorded. Over all phases of the study, the experimenter was never visible during the runs. To reduce odors from conspecifics, the maze was thoroughly cleaned between trials with diluted ethanol.

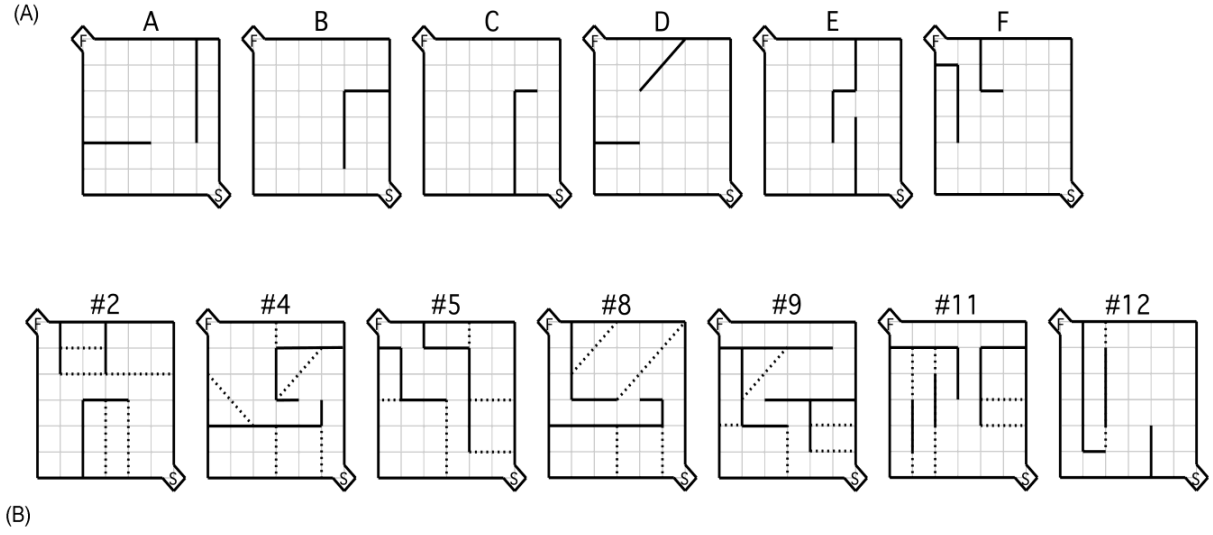


Figure 5: Maze configurations (A) Testing was conducted during the acquisition phase using the six practice mazes labeled (A–F) and (B) the seven test mazes depicted, each of which was used

during the testing phase. For each maze configuration, the (S) indicated in the bottom right hand corner represents the start box, whereas the (F) in the top left corner represents the goal box. Error zones are delineated by the dotted lines.

Western Blot

Immediately after finishing the Hebb-Williams mazes, mice were euthanized (100µl i.p. injection of euthasol; Sigma Aldrich, Oakville, ON, Canada), their brains removed and tissue blocks were cut using a stainless steel brain matrix (1 x 1.5 x .75 inches). Both dorsal hippocampi were dissected according to the mouse atlas and frozen on dry ice (Paxinos, 2001). Western blots were then prepared as described previously (Choeiri et al., 2006). Briefly, hippocampi were homogenized over ice in a homogenate buffer/protease inhibitor cocktail (Sigma Aldrich, Oakville, ON, Canada). The homogenates were centrifuged, protein content was quantified using a standard BSA kit (Pierce, Rockford, IL, USA) and samples were frozen at -80°C until further analysis. Proteins were loaded at a concentration of 300 µg/ml and samples in quadruplicate (12 µg/lane) were resolved by SDS-PAGE. Proteins were then transferred to pure nitrocellulose membranes and blocked for one hour in 5% skim milk and 10 M phosphate buffered saline (PBS) solution at room temperature. Antibody specificity was determined prior to commencing western blot analyses on experimental animals by confirming a single band of binding of the protein of interest at the appropriate molecular weight. Optimal concentrations of primary/secondary antibody were then confirmed by serial dilutions. Membranes were incubated in 5% skim milk and TBST (20mM Tris/HCl, 137mM NaCl, 0.4% Tween 20, pH 7.6) solution with monoclonal anti-PSD-95 antibody (1:2000; Millipore Corporation, Burlington, ON, Canada) and monoclonal anti-ß-tubulin antibody (1:10,000; Sigma Aldrich, Oakville, ON, Canada) at 4°C overnight. After 3 x 10 minute washes in TBST, fluorescent Alexa 680-linked antibody (1:10,000, Molecular Probes, Burlington, ON, Canada) and IR 800 antibody (1:10,000;

LI-COR Biosciences, Lincoln, NE, USA) in 5% skim milk and TBST solution were applied for one hour at 4°C. After 3 x 10 minute washes in TBST, western blots were scanned using the Odyssey infra-red system (LI-COR Biosciences, Lincoln, NE, USA) in 700 nm and 800 nm channels in a single scan at 169 µm resolution. Simultaneous detection of two fluorescent antibodies (i.e., Alexa 680 and IR 800) allowed for the measurement of PSD-95 and ß-tubulin proteins within each sample. The density of each protein band of interest was measured, background subtracted and normalized to ß-tubulin by the LI-COR analysis software.

Statistical Analyses

Latency to complete the Hebb-Williams mazes, number of errors, as well as hippocampal PSD-95 levels in MPEP treated mice compared with saline treated controls were the variables of interest in this study. Using SPSS 19 (IBM Canada Ltd., Markham, Canada), latency was analyzed by a 2 X 7 X 5 mixed-design ANOVA with treatment (MPEP; saline) as the between-subjects variable and both maze (seven levels) and trial (five levels) as the repeated measures variables. Similarly, the number of errors made on the Hebb-Williams mazes was analyzed by a separate 2 X 7 X 5 mixed-design ANOVA. Prior to analyses, data were evaluated to ensure that assumptions underlying mixed-design ANOVA were met. These preliminary analyses indicated that the majority of the latency as well as error data were skewed, and consequently, these variables were subjected to $\log_{10}$ transformations in order to normalize the distributions of the data. Following $\log_{10}$ transformation, neither latency nor error data were identified as outliers (> four SDs from the group mean; (Van Selst, 1994)). There were no missing data in this study.

In order to confirm the effectiveness of MPEP treatment, each mouse underwent the marble burying assay immediately after each test maze. These data remained skewed following square root, $\log_{10}$, and inverse transformations and therefore were not amenable to a 2 X 7

ANOVA analysis. As such, data were analysed using several non-parametric two independent sample, Mann-Whitney *U* tests. Specifically, analyses focused on the number of marbles buried following completion of each maze as a function of treatment (MPEP; saline). Assumptions underlying the Mann-Whitney *U* tests were met prior to running the analyses.

To examine protein levels following mGluR-5 antagonist treatment, an independent samples *t*-test was performed with treatment (MPEP; saline) as the independent variable and the protein ratio of PSD-95 normalized to a control protein, ß-tubulin, as the dependent variable. To ensure equal loading of protein samples across groups, an additional *t*-test was conducted with treatment as the independent variable (MPEP; saline) and ß-tubulin as the dependent variable. Prior to analyses, data were evaluated to ensure that assumptions underlying independent samples *t*-test were met.

In order to examine the association between protein expression and behavioural performance, three separate bivariate correlations (Pearson's *r*) were conducted. The correlational analyses were based on relative PSD-95 protein levels (normalized to ß-tubulin) and mean total errors on the Hebb-Williams mazes, defined as aggregate errors divided by the total number of learning trials (maze X trials = 35). Specific correlations focused on the relationship between PSD-95 protein levels and mean errors from: (1) *Fmr1* KO maze runners of both treatments, (2) MPEP treated runners only, and (3) saline treated runners only. As a control, correlations were also performed between β-tubulin protein levels and mean total errors from # 1.

Given the *a priori* hypotheses that specified the direction of the effect in each of the aforementioned correlations, one-tailed tests of significance for the correlational coefficients were conducted.

Results

A 2 X 7 X 5 mixed measures ANOVA was conducted to evaluate the effects of treatment (MPEP; saline) as the between-groups measure and repeated measures of both maze (seven levels) and trial (five levels) on the latency to complete the Hebb-Williams mazes. There was a main effect for maze, $F(5, 94) = 3.01$, $p = .01$, partial $\eta^2 = .13$, and for trial, $F(3, 67) = 60.12$, $p < .001$, partial $\eta^2 = .75$, but not for treatment, $F(1, 20) = 1.45$, $p = .24$, partial $\eta^2 = .07$, indicating that the latency to complete the mazes did not differ between MPEP and saline treated mice. There was also a significant interaction between treatment and maze, $F(5, 94) = 4.06$, $p = .003$, partial $\eta^2 = .17$, as well as maze and trial $F(9, 189) = 1.89$, $p = .05$, partial $\eta^2 = .08$. However, the interaction between treatment and trial $F(3, 67) = 1.07$, $p = .37$, partial $\eta^2 = .05$ was not significant. Likewise, the three-way interaction between treatment, maze, and trial was not significant $F(9, 189) = 1.01$, $p = .44$, partial $\eta^2 = .05$.

Bonferroni corrections were made to the α level of .05 before exploring simple main effect analyses of treatment within maze, resulting in $p < .007$ (.05/7 = .007) for significance. These analyses indicated that there were differences in the latencies between MPEP and saline treated mice on maze # 9, $F(1, 20) = 5.08$, $p = .04$, partial $\eta^2 = .20$, maze # 11 $F(1, 20) = 5.36$, $p = .03$, partial $\eta^2 = .21$ and maze #12, $F(1, 20) = 6.08$, $p = .02$, partial $\eta^2 = .23$. However, given the adjustment to guard against Type I error, these differences were not statistically significant. Given the similar latencies to complete maze running between drug and vehicle groups, these findings are consistent with previous research indicating that MPEP treatment does not significantly reduce locomotor activity (Figure 6).

Bonferroni corrections were made to the α level of .05 before exploring simple main effect analyses of trial within maze, resulting in $p < .007$ (.05/7 = .007) for significance. These

analyses indicated that there were differences in the latencies between trials on maze # 2, $F(4, 17) = 7.64$, $p = .001$, partial $\eta^2 = .64$, maze # 4 $F(4, 17) = 23.36$, $p = .000001$, partial $\eta^2 = .85$, maze #5, $F(4, 17) = 6.22$, $p = .003$, partial $\eta^2 = .59$, maze # 8, $F(4, 17) = 11.05$, $p = .0001$, partial $\eta^2 = .72$, maze # 9 $F(4, 17) = 11.26$, $p = .0001$, partial $\eta^2 = .73$, maze #11, $F(4, 17) = 12.54$, $p = .0001$, partial $\eta^2 = .75$, but not on maze #12 $F(4, 17) = 4.68$, $p = .01$, partial $\eta^2 = .52$.

Pairwise comparisons on the latency data adjusted to control for the effects of comparing mean trial differences within each maze ($\alpha = .05/60 = .0008$) showed that on maze #2, *Fmr1* KO mice were significantly slower on trial one relative to completion times on trials three and four. In addition they were significantly slower on trial two compared to trial four. On maze #4, mice were slower on trial one compared to their completion times on trials three, four and five

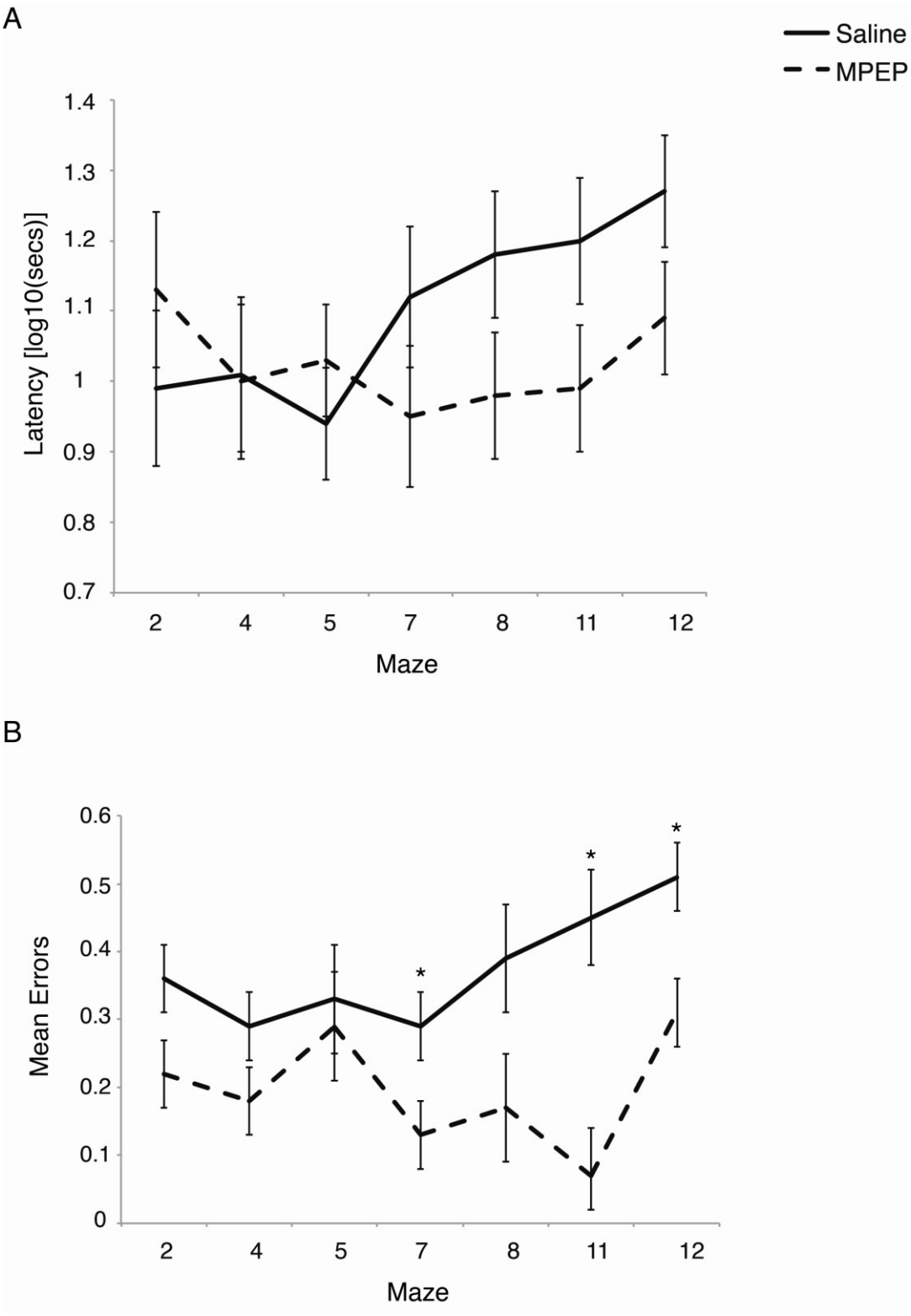


Figure 6: (A) Latency to complete each H-W test maze for *Fmr1* KO mice treated with saline or MPEP. Drug treatment did not statistically affect completion times between groups between groups. (B) Mean errors collapsed across trials for each H-W test maze for *Fmr1* KO mice

treated with saline or MPEP. Mice treated with MPEP made significantly fewer errors on mazes #8, 11 and 12. Error bars represent the S.E.M;* $p < 0.007$.

whereas trial two took longer to complete than trial four. On maze #5, mice took longer to complete trial one compared with run times on trials four and five. Subsequently, mice completed maze #8 slower on trial one compared to trials two, three, four and five, whereas trial four was completed faster than trial two. During maze #9, latencies were again slower on trial one compared with trials three, four and five. Finally on maze #11, run times were quicker on trials two, three and five relative to trial one. Thus, despite some variability in the trial by maze interaction data, pairwise comparisons indicated latencies were longest for trial one and in general, tended to decrease with increased repetition, as would be expected if mice were learning the maze configuration and motivated to obtain the food reward.

Regarding error data, a 2 X 7 X 5 mixed measures ANOVA was conducted to evaluate the effects of treatment (MPEP; saline) as the between-groups measure and repeated measures of both maze (seven levels) and trial (five levels) on the number of errors committed on the Hebb-Williams mazes. There was a main effect for treatment $F(1, 20) = 63.71$, $p < .001$, partial $\eta^2 = .76$, for maze, $F(4, 84) = 4.13$, $p = .004$, partial $\eta^2 = .17$, indicating that the number of errors made on the mazes differed between MPEP and saline treated mice. There was also a main effect for trial, $F(3, 65) = 24.43$, $p < .001$, partial $\eta^2 = .55$. There was a significant interaction between treatment and maze, $F(4, 84) = 2.82$, $p = .03$, partial $\eta^2 = .12$, whereas the interaction between treatment and trial $F(3, 65) = 2.22$, $p = .09$, partial $\eta^2 = .10$ approached significance. However, the interaction between maze and trial $F(9, 188) = 1.32$, $p = .23$, partial $\eta^2 = .06$ was not significant. Likewise, the three-way interaction between treatment, maze, and trial was not significant $F(9, 188) = .94$, $p = .50$, partial $\eta^2 = .04$.

Bonferroni corrections were made to the α level of .05 before exploring simple main effect analyses of treatment within maze, resulting in $p < .007$ (.05/7 = .007) for significance. These analyses indicated that there were significantly less errors committed by MPEP treated mice on maze #2, $F(1, 20) = 6.21$, $p = .02$, partial $\eta^2 = .24$, maze #4 $F(1, 20) = 5.94$, $p = .02$, partial $\eta^2 = .23$, maze #8, $F(1, 20) = 8.67$, $p = .007$, partial $\eta^2 = .30$, maze #9, $F(1, 20) = 7.53$, $p = .01$, partial $\eta^2 = .27$, maze #11, $F(1, 20) = 30.80$, $p = .00002$, partial $\eta^2 = .61$, and maze #12, $F(1, 20) = 17.28$, $p = .0005$, partial $\eta^2 = .46$, However, when adjustments were made to guard against Type I error, MPEP treated mice committed significantly fewer errors on only three mazes relative to saline controls (mazes #8, 11, 12). Combined, these data indicate that on several of the Hebb-Williams mazes, MPEP administration results in significantly less errors than in *Fmr1* KO mice treated with saline only (Figure 6).

Bonferroni corrections were made to the α level of .05 before exploring simple main effect analyses of treatment within trial, resulting in $p < .01$ (.05/5 = .01) for significance. MPEP administration resulted in significantly fewer errors on trial one, $F(1, 20) = 54.95$, $p = .0000004$, partial $\eta^2 = .73$, trial two, $F(1, 20) = 17.16$, $p = .001$, partial $\eta^2 = .46$, trial four, $F(1, 20) = 21.62$, $p = .0002$, partial $\eta^2 = .52$, and trial five, $F(1, 20) = 25.97$, $p = .0001$, partial $\eta^2 = .57$. Unexpectedly, treatment had no effect on the mean errors observed on trial three, $F(1, 20) = 4.43$, $p = .05$, partial $\eta^2 = .18$. Thus, MPEP treatment reduces errors committed on four out of five trials with the biggest impact (as reflected by effect size) occurring on the first trial (Figure 7).

Several Mann-Whitney *U* tests were conducted to evaluate whether MPEP remained at physiologically active levels during the experiments. Bonferroni corrections were made to the α level of .05 before performing these tests, resulting in $p < .007$ (.05/7 = .007) for significance.

The results of the Mann-Whitney *U* tests were in the expected direction and significant such that MPEP treated mice were found to bury significantly less marbles than saline treated controls

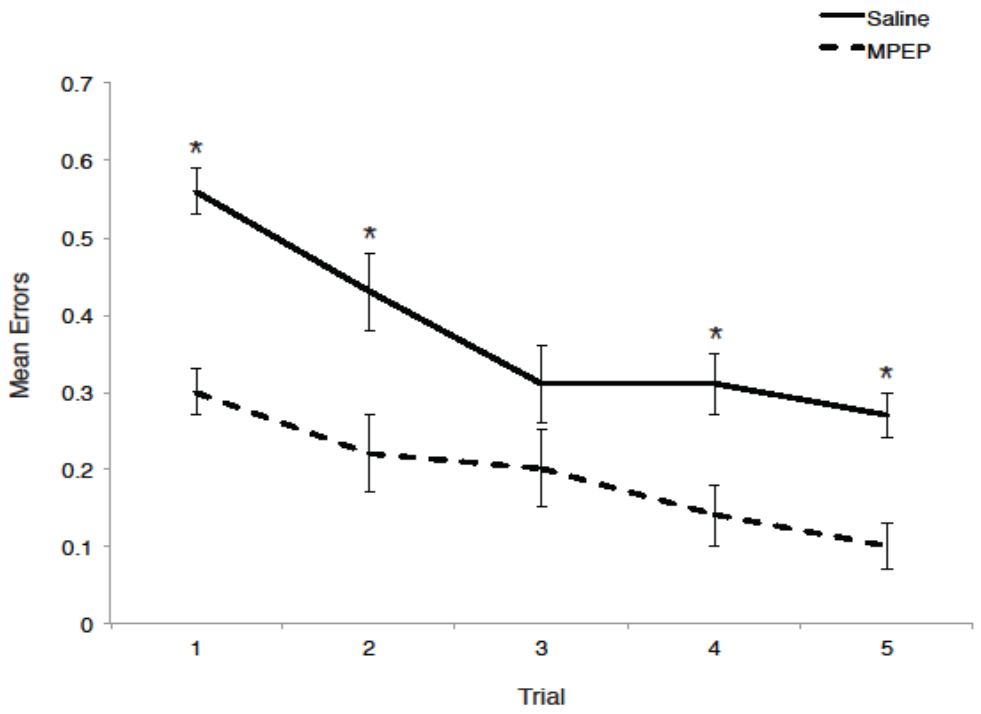


Figure 7: Mean errors on H-W test mazes for *Fmr1* KO mice treated with saline or MPEP. MPEP treated KO mice made significantly fewer errors on trials 1, 2, 4, 5. * $p < 0.005$.

for maze # 2, z = -3.306, p = .001 (MPEP average rank = 6.95; Saline = 16.95), maze # 4, z = -3.31, p = .001 (MPEP average rank = 6.95; Saline = 16.05), maze # 8, z = -3.30, p = .001 (MPEP average rank = 6.95; Saline = 16.05), maze # 9, z = -2.74, p = .006 (MPEP average rank = 7.73; Saline = 15.27), maze # 11, z = -3.34, p = .001 (MPEP average rank = 6.95; Saline = 16.05), and maze # 12, z = -3.56, $p < .001$ (MPEP average rank = 6.59; Saline = 16.41). With the adjusted level of α, the number of marbles buried was not statistically different between MPEP and saline treated mice for maze # 5, z = -2.58, p = .01 (MPEP average rank = 7.95; Saline = 15.05). Taken together, the marble burying assay following the completion of each of the Hebb-Williams test mazes confirmed that the MPEP treatment was physiologically active during the test phases (Figure 8).

Independent sample *t*-tests were performed to evaluate the hypothesis that mGluR-5 antagonist treatment could selectively rescue hippocampal PSD-95 protein levels. Hippocampal β-tubulin levels were also measured because this housekeeping protein was not expected to vary with treatment condition. Bonferroni corrections were made to the α level of .05 before performing these tests, resulting in $p < .025$ (.05/ = .025) for significance. The *t*-tests indicated that PSD-95 levels were significantly higher in MPEP treated mice compared with vehicle condition, $t(20) = 3.00$, p = .007, 95% CI [.064, 3.56], whereas there were no differences in β-tubulin levels between MPEP and vehicle treated mice, $t(20) = .851$, p = .40, 95% CI[-.80, 1.89]. The effect size as reflected by, η^2, indicated that 31 % of the variance in PSD-95 levels was accounted for by whether or not mice received MPEP/vehicle treatment whereas only .03% of the variance in β-tubulin levels was accounted for the treatment. These data suggest that mGluR-5 antagonism has an augmenting affect on the levels of the scaffolding protein PSD-95 (Figure 9).

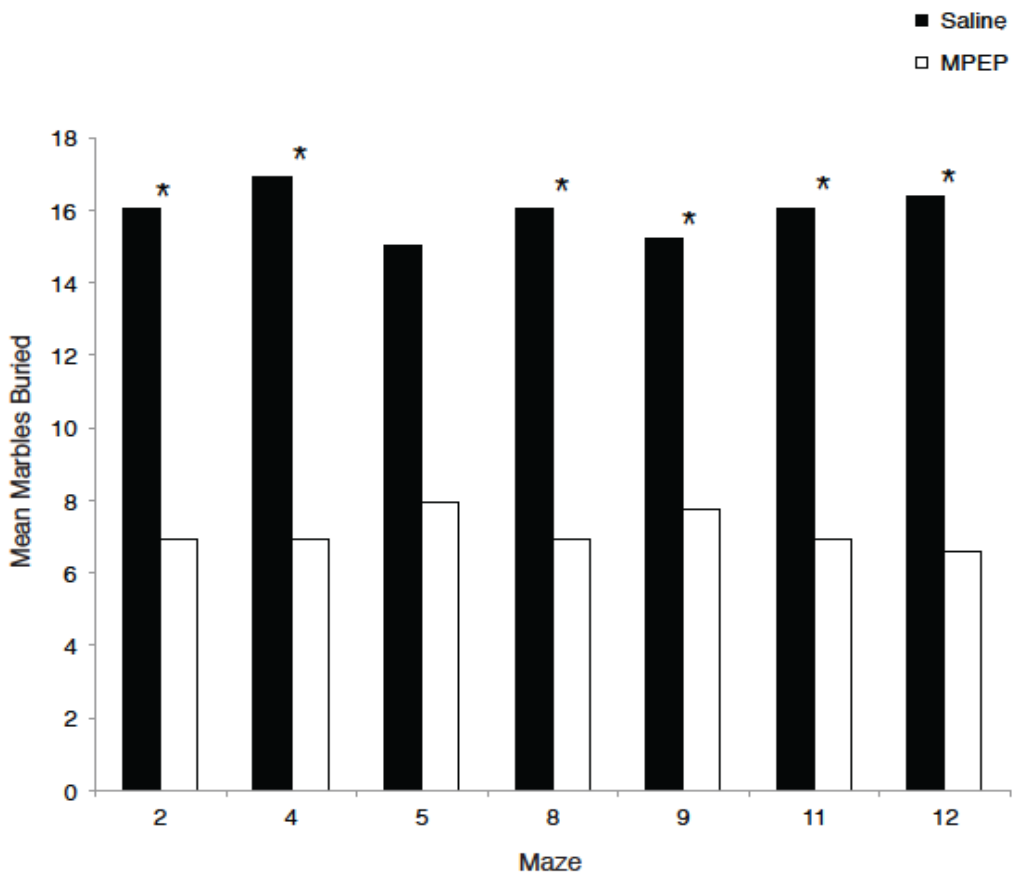


Figure 8: Mean marbles buried across each H-W test maze for *Fmr1* KO mice treated with saline or MPEP. MPEP treated mice buried significantly less marbles following each maze except for maze 5. Data were analyzed by non-parametric, Bonferroni corrected Mann Whitney *U*-tests; * $p < 0.007$.

Similar to the group data from Gandhi et al. (2014); that comprised the entire sample of animals) a correlation of all *Fmr1* KO mice, irrespective of treatment, indicated there was a negative association between PSD-95 levels and mean total errors on the Hebb-Williams mazes, $r(20) = -.40$, $p = .03$, $r^2 = 0.16$ (Figure 9). This association was not evident when examining the correlation between β-tubulin levels and mean total errors from *Fmr1* KO mice, $r(20) = -.26$, $p = .12$, $r^2 = 0.06$. Within treatment groups, there were no relationships between the PSD-95 levels of MPEP treated mice and mean total errors, $r(9) = -.042$, $p = .45$, $r^2 = 0.01$, nor saline treated mice and mean total errors, $r(9) = .28$, $p = .21$, $r^2 = 0.08$. In addition, after adjusting the α levels to control for repeated tests, (.05/4 = .012) only the initial correlation consisting of the entire sample of *Fmr1* KO mice trended towards significance (Figure 10). Collectively, these data confirm that as PSD-95 levels increase, mean errors on the Hebb-Williams mazes decrease and *vice versa*.

Discussion

Fragile X syndrome is a debilitating mental, physical, and behavioral condition that occurs due to lack of expression of the Fragile X Mental Retardation 1 protein (FMRP; reviewed in (Santoro et al., 2012)). The altered expression results in a number of characteristic symptoms including cognitive dysfunction. Despite a common finding in the research literature that hippocampal lesions impair performance on tasks of spatial navigation and learning (Clark, Broadbent, & Squire, 2005; Hock & Bunsey, 1998; Jarrard, 1993; Lee & Kesner, 2003; Morris, Garrud, Rawlins, & O'Keefe, 1982; Okada & Okaichi, 2009; Sutherland, Kolb, & Whishaw, 1982), inconsistent results have been reported when testing *Fmr1* KO mice. These differences may be a function of variability in the background strain used or the assays employed. Inconsistencies in the literature were addressed in the present investigation by choosing an

animal background (FVB) with modest spatial abilities, arguably more aligned with the human visual FXS phenotype. The assay of choice was the Hebb-Williams mazes given the literature demonstrating this measure is sensitive to detecting dorsal hippocampal deficits (Rogers & Kesner, 2006; Shore et al., 2001) and in some cases better than "benchmark" tasks

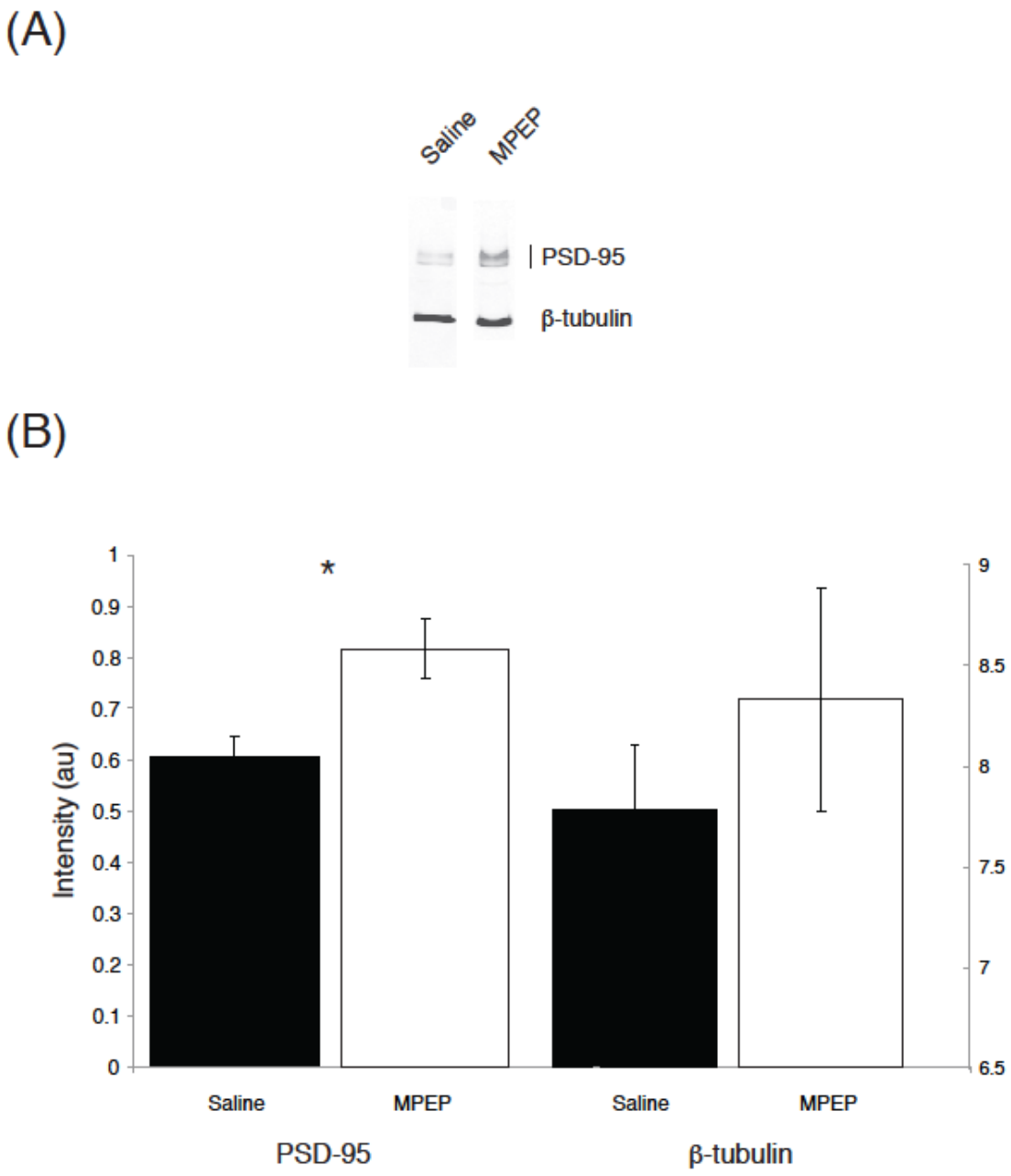


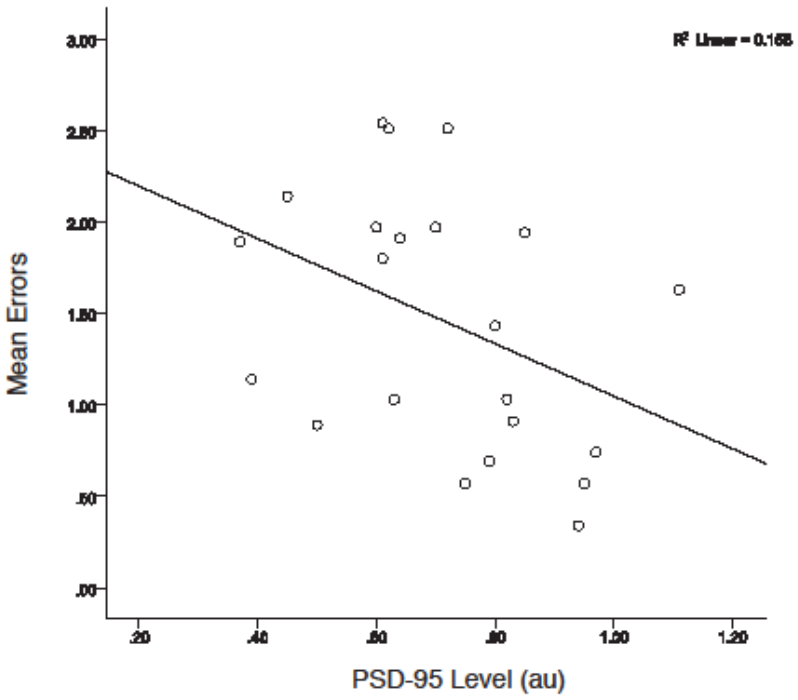
Figure 9: Representative Western blots from dorsal hippocampi of *Fmr1* KO mice treated with saline or MPEP for protein expression of PSD-95 and β-tubulin**.** PSD-95 is found around the expected molecular weight of 95kDa and β-tubulin is found at 55kDa. PSD-95 levels are rescued in MPEP treated *Fmr1* KO mice only. Error bars represent the S.E.M;* $p < 0.025$.

such as the Morris Water Maze (Marques Pereira, Cosquer, Schimchowitsch, & Cassel, 2005). Unlike other measures, the Hebb-Williams mazes consist of problems of increasing difficulty and animals with learning deficits were noted to have poorer performances during more complex items. Specific to FXS, the Hebb-Williams mazes were shown to reliably differentiate performance between wild-type control and *Fmr1* KO mice as evidenced by a greater number of total errors committed by KO animals (MacLeod et al., 2010) and wild type maze learners demonstrated upregulations of a synaptic plasticity protein, PSD-95, an effect that was blunted in *Fmr1* KO mice (Gandhi et al., 2013). Combined, these findings suggest that the Hebb-Williams mazes are a reliable assay to use for pharmacological intervention studies.

The molecular mechanisms of synapse modifications at dendritic spines are unknown. One perspective is that certain scaffolding proteins maintain the long-term transmission efficiency of a synapse (Ehrlich & Malinow, 2004; McCormack, Stornetta, & Zhu, 2006). As such, scaffolding proteins would serve as placeholders or slot proteins for receptors such as AMPARs. PSD-95 has been proposed to possess many qualities of a slot protein (Schnell et al., 2002). It is more stable than other PSD proteins such as CaMKII α, CaMKII ß, GluR2 or Stargazin, consistent with a role in regulating the PSD (Sturgill, Steiner, Czervionke, & Sabatini, 2009). Levels of PSD-95 were reported to be redistributed to dendrites in the visual cortex following eye opening in litters of rodents, and these changes lasted upwards of six hours and were contingent on sustained environmental experience (Yoshii, Sheng, & Constantine-Paton, 2003). Moreover, changes in the sizes of individual PSDs over days were associated with changes in PSD-95 retention times and PSD-95 increased with developmental age and dropped sharply following sensory deprivation (Gray, Weimer, Bureau, & Svoboda, 2006). In FXS, there is evidence that PSD-95 is dysregulated. Specifically, increased translational levels were

observed during basal states in *Fmr1* KO as compared to wild- type mice as well as relatively low protein

(A)



(B)

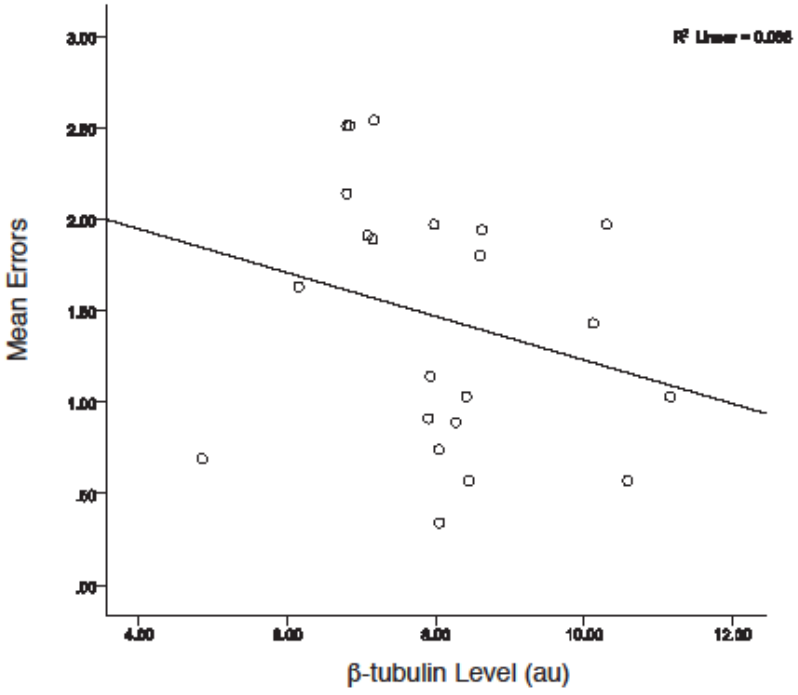


Figure 10: Levels of hippocampal PSD-95 but not control (β-tubulin) are correlated with behavioural performance. A negative correlation between mean errors on the H-W mazes and PSD-95 protein levels was observed. r (20) = -0.40, p = 0.03, $r2$ = 0.16.

levels following stimulus induction in this genotype. PSD-95 mRNA transcripts were also found to selectively deteriorate in the hippocampus but not in the cortex or cerebellum of *Fmr1* KO mice (Muddashetty, Kelic, Gross, Xu, & Bassell, 2007; Todd, Mack, & Malter, 2003; Zhu et al., 2011).

Pharmacological treatments blocking mGluR-5 receptors can stabilize basal protein translation levels and this approach has been hypothesized as a means of ameliorating some of the core symptoms of FXS, including cognitive dysfunction (Bhakar et al., 2012; Dolen & Bear, 2005). In studies using drosophila KO (*dfmr1*) and *Fmr1* KO murine models, the use of mGluR-5 antagonists has been successful in correcting many features of FXS including elevated and inappropriately expressed protein levels at basal states, decreasing frequency of audiogenic seizures, reversing excessive AMPA internalization, reducing the number of abnormally thin dendritic spines, and reversing behavioural/learning deficits (Choi et al., 2010; de Vrij et al., 2008; Levenga et al., 2011; McBride et al., 2005; Nakamoto et al., 2007; Osterweil et al., 2010; Pan, Woodruff, Liang, & Broadie, 2008; Su et al., 2011; Tauber, Vanlandingham, & Zhang, 2011; Yan et al., 2005). Despite the rescue of many phenotypic features of FXS, the identification of the specific proteins underlying these functions remains to be elucidated. Theoretically, the stabilization of PSD-95 protein in *Fmr1* KO mice would allow for improved local regulation during periods of synaptic plasticity while learning the Hebb-Williams mazes.

Our western blot analyses following completion of the Hebb-Williams mazes revealed that MPEP treated mice had statistically higher PSD-95 protein levels. This effect was specific to PSD-95 as levels of the control protein (β-tubulin) were unchanged across treatments. Thus, our

finding suggests that PSD-95 protein deficits can be rescued by targeting mGluR-5 receptors. An additional implication pertains to the broader question of "when" it is appropriate to intervene with pharmacological treatment. As FXS is a developmental disorder, the vast majority of animal model studies have targeted intervention at the embryonic stages or very early in post-natal life. Conceptually, it is of great interest to determine if the FXS phenotype can be corrected after symptom onset. If not, it would suggest that a critical therapeutic window has been missed and argue against the idea that the symptoms of FXS are caused by ongoing irregularities of synaptic signalling (Michalon et al., 2012). This question was addressed in a study examining *Fmr1* KO mice aged 4-5 weeks with anatomically developed and highly plastic brains, corresponding to young adults. Specifically, treatment with an mGluR-5 inhibitor corrected learning and memory deficits in an inhibitory avoidance paradigm, improved dendritic spine abnormalities, and ameliorated elevated ERK and mTOR kinase activation (pathways previously shown to underlie the pathophysiology of FXS). Our data, which suggest reversal of molecular and behavioral deficits, are consistent with these findings (Michalon et al., 2012). In addition, since the *Fmr1* KO mice in our study were 12 weeks or older before beginning behavioural testing, our findings further demonstrate that a model of the FXS phenotype can be corrected in aged mice roughly corresponding to adulthood.

The behavioural data from the Hebb-Williams mazes were analyzed according to two dependent variables of interest, latency and error. Regarding the former, analyses of the treatment by maze interaction indicated that there were differences in the latency between MPEP and saline treated mice on several mazes. Owing to high levels of variability in the runs times, faster completion times by MPEP treated mice were not statistically different from controls. However, the similar latency to complete mazes between drug and vehicle groups indicates that

our data are consistent with previous research demonstrating that MPEP treatment does not adversely affect locomotor activity (Mehta, Gandal, & Siegel, 2011; Silverman, Tolu, Barkan, & Crawley, 2010; A. M. Thomas et al., 2012; Yan et al., 2005). Collapsed across treatment, we also observed that latency of maze completion was longest for trial one and generalizing across mazes, tended to decrease with increased repetition. Thus, both groups of mice were capable of improving their latency performance with increased exposure to the mazes.

Consistent with our hypothesis, on more mazes deemed more challenging (# 8, 9, 11, 12; (Shore et al., 2001)), MPEP treated mice made significantly fewer errors (i.e., # 8, 11, 12). When examining the behavioural performance of the mazes deemed more difficult, on maze #8, saline treated mice continued to explore previously unsuccessful routes towards the goal box whereas MPEP treated mice demonstrated a reduction in errors over trials. Counterintuitively, there were no differences between drug and saline treated mice on maze #9, which may represent variability in the data set or a lack of difficulty of this maze for this background strain. Qualitatively, on mazes #11 and #12, saline treated controls committed more perseverative errors, circling isolated and removed barriers from the goal box, thereby getting stuck in unsuccessful "loops". That MPEP treated mice did not commit a likewise response suggests that MPEP treatment may correct perseveration, a common cognitive feature of FXS (Hooper et al., 2008).

Finally, the treatment by trial interaction data revealed that MPEP treated mice made significantly fewer errors on trials #1, 2, 4, and 5 relative to controls. Given that the largest effect size occurred on the first trial, this suggests that MPEP may also have corrected impulsive responding, which is another feature that is commonly observed in FXS (R. J. Hagerman, 2002).

The pharmacological efficacy of MPEP was confirmed with a marble burying assay immediately following the test phase in order to validate our findings. Marble burying, a

repetitive behaviour, has been shown to be decreased following the administration of Grp I mGluR antagonists (Spooren et al., 2000; A. M. Thomas et al., 2012). In the present investigation, MPEP treated mice buried significantly fewer marbles than controls after the completion of all mazes (thus confirming efficacy), with the exception of # 5. It is unclear why fewer marbles relative to controls were buried here, however as there were no error differences between treatment groups for this maze; interpretation of our findings is not affected by this result.

Overall, our correlational findings are inconclusive and merit further investigation. Although we replicated a negative correlation between PSD-95 levels and mean errors for the entire sample of mice, as found in our previous study (Gandhi et al., 2013), we did not demonstrate a statistically significant relationship within the treatment groups. We suspect that larger sample sizes of mice will provide the necessary power to allow us to characterize this relationship appropriately.

Whether pharmacological studies of mGluR-5 antagonists in mouse models of FXS will translate into effective treatments for human patients remains to be determined. To date, only two studies have been completed in patients affected by FXS. A pilot study was conducted to determine pharmacokinetics and side effects of a single dose trial of the mGluR-5 antagonist, fenobam, to 12 male and female FXS patients (Berry-Kravis et al., 2009). Pre/post outcome measures included prepulse inhibition (PPI) and the continuous performance test (CPT) to assess sensory gating, attention and inhibition. The results indicated there were no adverse reactions to the fenobam administration and PPI improved by at least 20% in half of the sample relative to baseline. By comparison, performance on the CPT did not improve although this finding was attributable to ceiling effects. The other study employing an mGluR-5 antagonist was conducted

using AFQ056 in 30 male FXS patients ranging in age from 18-35 (Jacquemont et al., 2011). These researchers initially did not find any improvement in behavioural symptoms of FXS following treatment as assessed by the Aberrant Behavior Checklist-Community Edition (ABC-C). However, a subset of the patients who had the full *FMR1* promoter methylation and no detectable *FMR1* mRNA improved significantly more on the ABC-C and the Repetitive Behaviour Scale following treatment compared with placebo. Since those patients with partial promoter methylation did not show behavioural improvement following AFQ056 treatment, the authors posited that mGluR-5 antagonism might be better suited for FXS patients with full methylation at the *FMR1* promoter. mGluR-5 antagonists are not the only receptor mechanism/molecular target under investigation. FXS is a complex neurodevelopmental disorder and Fmrp regulates signalling by other receptors as well. Therefore, antagonism of Group I mGluR signalling is not likely to produce beneficial therapeutic effects for every patient. Moreover, there are other aspects of the Fragile X phenotype that are unrelated to mGluR function. Other research has focused on other targets such as GABA-A and B receptors, ampakines, brain derived neurotrophic factor (BDNF), aripiprazole, lithium and intracellular signalling pathways via phosphatase and kinase inhibitors. In all likelihood, patients will display varied outcomes to different targeted treatments based on interplay between genetics, intracellular neuronal pathways and synaptic function (Gross, Berry-Kravis, & Bassell, 2012).

FXS is the most common single gene disorder associated with autism (R. Hagerman, Hoem, & Hagerman, 2010) and there are numerous commonalities between FXS and autistic disorder. Similar to FXS, autism patients suffer from seizure disorder and cognitive impairment (Canitano, 2007). There is also delayed language acquisition and repetitive behaviours (R. J. Hagerman, 1996) and 25-47% of FXS patients have a diagnosis of autism (Hatton et al., 2006;

Kaufmann et al., 2004). Models of FXS are potentially advantageous to autism because Fmrp controls the translation of plasticity proteins implicated in autism such as neuroligins and SHANK proteins (Darnell et al., 2011). Moreover, low levels of FMRP relative to controls have been reported in autistic disorder (Fatemi & Folsom, 2011). Using a BTBR murine model of autism, one study reported that MPEP treatment ameliorated repetitive self-grooming behaviour without significant sedating side-effects (Silverman et al., 2010). Likewise, in a valproic acid (VPA) murine model of autism, MPEP reduced excessive self grooming as well as marble burying behaviour (Mehta et al., 2011). Although further study is needed, preliminarily, MPEP appears to be a suitable pharmacological intervention for both FXS and autistic disorder. Our findings indicate that MPEP treatment can reverse PSD-95 protein deficits and errors on more complicated Hebb-Williams test mazes. Given the significant phenotypic overlap between FXS and autism as well as the lack of a viable behavioural assay to test symptoms improvement in the autism field, the use of the Hebb-Williams mazes with autism murine models appears promising and warrants further investigation.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter IV: General Discussion

General Discussion

Fragile X syndrome is a debilitating mental, physical and behavioral condition that occurs due to loss of the FMRP (reviewed in O'Donnell & Warren, 2002; Santoro, Bray & Warren, 2012). This loss results in a number of characteristic symptoms including: cognitive dysfunction, attention deficit and hyperactivity, anxiety, epilepsy, autistic behaviors as well as pronounced physical features such as an elongated face and macroorchidism (Hagerman, 1996; Hatton et al., 2006; O'Donnell and Warren, 2002; Turner et al., 1996; Scerif et al., 2007; Sullivan et al., 2006). Cognitively, visual-spatial deficits are a common weakness in FXS.

Fmrp has a variety of features that suggest an important role in regulating multiple aspects of synaptic plasticity. It is present in dendritic spines (Ferrari et al., 2007; Weiler et al., 1997) which is an area involved in plasticity induction and maintenance. Fmrp also has identified roles in dendritic transport and translational regulation of hundreds of mRNAs, many of which are important in plasticity and dendrite morphology (Darnell et al., 2001; 2011; Bagni & Greenough, 2005).

Using the Hebb-Williams mazes as a behavioural assay, experiment one identified a significant upregulation of PSD-95 protein levels in Fmrp-intact mice whereas training-related protein increases were blunted in *Fmr1* KO mice. In addition, there was a significant negative correlation between mean total errors on the mazes and PSD-95 protein levels. The coefficient of determination indicated that the mean total errors on the Hebb-Williams mazes accounted for 35% of the variance in PSD-95 protein levels, suggesting that errors are a valid outcome measure of learning.

Experiment two examined whether an antagonist of the mGluR-5 could reverse the behavioural and PSD-95 protein deficits in *Fmr1* KO mice. Thus, mice were trained on the

Hebb-Williams mazes and then treated with either 20 mg/kg of an mGluR-5 antagonist, MPEP, or an equivalent dose of saline prior to running test mazes. Latency to complete as well as errors made across maze trials were recorded during the test phase. Although latency was not statistically different between the groups, MPEP treated *Fmr1* KO mice made significantly fewer errors on mazes deemed more difficult, suggesting a reversal of the behavioural deficit. PSD-95 levels were rescued in these MPEP treated mice.

The spatial navigation and learning abilities in Fmrp-intact and *Fmr1* KO mice were examined in order to better understand the protein changes that accompany learning during an ecologically valid visual-spatial measure, the Hebb-Williams mazes. *Fmr1* KO mice and human FXS patients that completed the Hebb-Williams mazes were previously shown to have comparable maze navigational performances, committing more errors than controls, results which were attributable to a lack of Fmrp/FMRP (Macleod et al., 2010). As PSD-95 has been hypothesized to be an important protein in maintaining the long-term transmission efficiency of synapses (Ehrlich & Malinow, 2004; McCormack et al., 2006; Elias & Nicoll, 2007; Keith & El-Hussein, 2008) the results from experiment one provide support that Fmrp has an important role in PSD-95 regulation in response to spatial navigation and learning. That is, wild-type control mice exhibited elevations of PSD-95 protein levels following learning of the mazes whereas comparable responses were not observed in genotypic non-runners. In contrast, *Fmr1* KO mice failed to demonstrate increased expression of this scaffolding protein whether or not they participated in maze learning. Examination of the data within conditions (i.e., Hebb-Williams maze runners and mice in the resting control condition) evidenced further support for upregulation of PSD-95 in Fmrp-intact runners only. In this regard, wild-type runners had higher levels of PSD-95 expression than *Fmr1* KO runners after completion of the Hebb-Williams

mazes whereas there were no statistical differences between wild-type mice and *Fmr1* KO mice in the untrained resting control group.

According to the mGluR theory of FXS, many of the neurological and psychological aspects of FXS are caused by the loss of FMRP because of its important role in translational repression (Bear et al., 2004; Dolen & Bear, 2005; Dolen, Carpenter, Ocain & Bear, 2010). In the absence of Fmrp, a number of diverse neuronal functions are exaggerated in response to Group I (Gp1) mGluR activation at basal (resting) states, including the loss of synaptic AMPA and NMDA receptors, reversal of hippocampal LTP, and dendritic spine reductions (Bear et al., 2004; Kruger & Bear, 2011). The mGluR theory also predicts higher basal levels of proteins in the absence of Fmrp, including PSD-95. However, in experiment one, PSD-95 protein levels were found to be lower in *Fmr1* KO mice who completed the Hebb-Williams mazes. The reasons for such a discrepancy can likely be attributed to changes in the state of PSD-95 that occurs following loss of Fmrp. In Fmrp-intact controls, PSD-95 is translationally controlled by Fmrp (Zalfa et al., 2007), translated in response to mGluR signalling (Todd et al., 2003) and involved in dendritic spine morphology (El-Husseini et al., 2000; Steiner et al., 2008). In *Fmr1* KO mice there is a loss of translational control of PSD-95 resulting in elevations of this protein at resting states. In the prolonged absence of Fmrp, PSD-95 mRNA is a less stable and selectively degrades in the hippocampus as opposed to other brain areas (Zhu et al., 2011), which would engender a net loss of this protein at resting state as animals mature. Consequently, it is likely that dynamic regulation of PSD-95 (i.e., increased translation) that would be expected to take place during learning would be deficient in *Fmr1* KO mice due to degradation of mRNA.

One of the goals in experiment one was to further validate the Hebb-Williams mazes as a behavioural assay for reliably documenting visual-spatial learning deficits in *Fmr1* KO mice that

could improve upon the limitations of commonly employed measures which are confounded by either ceiling or floor effects (Berry-Kravis et al., 2006). This would in turn allow for future studies to evaluate the effectiveness of pharmacological or behavioural interventions intended to mitigate symptoms of FXS. A significant negative correlation between mean maze errors and PSD-95 expression was observed in experiment one. That is, mice that committed fewer mean errors on the mazes exhibited greater PSD-95 protein levels and *vice versa,* independent of genotype. In an attempt to further characterize this relationship, additional correlations were performed within each genotype, which were found to trend towards significance and may have achieved significance with larger sample sizes. These results suggest that intervention studies may benefit from using mean total errors as a reliable dependent variable to assess learning or lack thereof on the Hebb-Williams mazes. The correlation between mean total errors and PSD-95 was strong, given that the proportion of variance in PSD-95 levels accounted for by Hebb-Williams mean total errors was 35%. In addition to *Fmr1* KO mice, this assay may be useful for other animal models that are characterized by visual-spatial learning dysfunction.

Pharmacological treatments blocking mGluR-5 can stabilize basal protein translation levels and this approach has been hypothesized as a means of ameliorating some of the core symptoms of FXS, including cognitive dysfunction (Dolen & Bear, 2005; Bhakar et al., 2012). In studies using drosophila KO (*dfmr1*) and *Fmr1* KO murine models, the use of mGluR-5 antagonists has been successful in correcting many features of FXS including: increased protein synthesis at basal states, increased frequency of audiogenic seizures, excessive AMPA receptor internalization, abnormally thin dendritic spines, and behavioural/learning deficits (Choi et al., 2010; deVrij et al., 2008; Levenga et al., 2011; Nakamoto et al., 2007; Osterweil et al., 2010; McBride et al., 2005; Min et al., 2009; Pan, Woodruff, Liang, & Broadie, 2008; Su et al., 2011;

Tauber, Vanlandingham, & Zhang, 2011; Veloz et al., 2012; Yan et al., 2005). Despite the rescue of many phenotypic features of FXS, the identification of the specific proteins involved remains to be elucidated.

The upregulation of PSD-95 in Fmrp-intact mice observed in experiment one suggested that PSD-95 was a good candidate protein to target for pharmacological intervention. Theoretically, the stabilization of this protein in *Fmr1* KO mice would allow for improved local regulation during periods of synaptic plasticity while learning the Hebb-Williams mazes. In order to test this hypothesis, in experiment two, groups of *Fmr1* KO mice were run in the Hebb-Williams assay with one group receiving the mGluR-5 antagonist MPEP before the test phases, and the other animals receiving saline administration only.

Western blot analyses following completion of the Hebb-Williams mazes revealed that MPEP treated mice had higher PSD-95 protein levels. This effect was specific to PSD-95 as levels of the control protein (β-tubulin) were unchanged across treatments. This finding suggests that PSD-95 protein deficits can be rescued by targeting mGluR-5. An additional implication based on this result pertains to the broader question of "when" it is appropriate to intervene with pharmacological treatment. Because FXS is a developmental disorder, the vast majority of animal model studies have targeted intervention at the embryonic stages or very early in post-natal life. Conceptually, it is of great interest to determine if the FXS phenotype can be corrected after symptom onset. If not, it would suggest that a critical therapeutic window has been missed and argue against the idea that the symptoms of FXS are caused by ongoing irregularities of synaptic signalling (Michalon et al., 2012). This question was addressed by Michalon et al. in *Fmr1* KO mice aged 4-5 weeks with anatomically developed and highly plastic brains, corresponding to young adults. They reported that chronic four week treatment with an mGluR-5

inhibitor corrected learning and memory deficits in an inhibitory avoidance paradigm, improved dendritic spine abnormalities, and ameliorated elevated ERK and mTOR kinase activation (pathways previously shown to be part of the pathophysiology of FXS). The data from experiment two presented here, which indicate reversal of molecular and behavioral deficits are consistent with these findings. In addition, since the *Fmr1* KO mice in the present studies were 12 weeks or older before beginning behavioural testing, the findings further demonstrate that a model of the FXS phenotype can be corrected in aged mice roughly corresponding to adulthood.

The behavioural data were analyzed according to two dependent variables of interest, latency and errors. Regarding the former, analyses of the treatment by maze interaction indicated that there were differences in the latency between MPEP and saline treated mice on several mazes. Owing to high levels of variability in the runs times, faster completion times by MPEP treated mice were not statistically different from controls. However, the similar latency to complete mazes between drug and vehicle groups indicates that this data is consistent with previous research demonstrating that MPEP treatment does not adversely affect locomotor activity (Mehta et al., 2011; Silverman et al., 2010; Thomas et al., 2012; Yan et al., 2005). This is a notable outcome, because drug-induced motor impairments would have represented a serious threat to the validity of the findings. Collapsed across treatment, the latency of maze completion was longest for trial one and tended to decrease with increased repetition. Thus, both groups of mice were capable of improving their latency performance with increased exposure to the mazes.

Consistent with the hypothesis that mGluR blockade would reverse *Fmr1* KO pathology, on more mazes deemed more challenging (# 8, 9, 11, 12), MPEP treated mice made significantly fewer errors on mazes # 8, 11, 12. When examining the behavioural performance on maze #8, saline treated mice continued to explore previously unsuccessful routes towards the goal box

whereas MPEP treated mice appeared to improve upon past errors of navigation. Counterintuitively, there were no differences between drug and saline treated mice on maze #9, which may represent variability in the data set or insufficient difficulty for this background strain. Qualitatively, on mazes #11 and #12, saline treated controls committed more perseverative errors, circling isolated and removed barriers from the goal box, thereby getting stuck in unsuccessful "loops". That MPEP treated mice did not make a likewise response suggests that treatment may correct perseveration, a common cognitive feature of FXS (Hooper et al., 2008)

Finally, the treatment by trial interaction data revealed that MPEP treated mice made significantly fewer errors on trials #1, 2, 4, and 5 relative to controls. Given that the largest effect size occurred on the first trial, this suggests an improvement of impulsivity, another common feature of FXS (Hagerman et al., 2002).

The pharmacological efficacy of the MPEP was confirmed with a marble burying assay immediately following test learning in order to validate the findings. Marbles burying, a repetitive behaviour, has been shown to be decreased following the administration of Grp I mGluR antagonists (Spooren et al., 2000; Thomas et al., 2012). MPEP treated mice buried significantly fewer marbles than controls after the completion of all mazes (thus confirming efficacy), with the exception of # 5. It is unclear why fewer marbles relative to controls were buried here, however since there were no error differences between treatment groups for this maze, interpretation of the findings is not affected by this result.

Overall, the correlational findings are inconclusive and merit further investigation. Although a negative correlation between PSD-95 levels and mean errors for the entire sample of mice was found as in a previous study (Gandhi et al., 2014), there was no statistically significant

relationship within the treatment groups. It is possible that larger sample sizes of mice will provide the necessary power to allow for an appropriate characterization of this relationship.

At the molecular level, learning is expressed by changes in protein expression. However, the precise mechanisms remain unknown. As opposed to the translation of pre-existing dendritic mRNAs, it has been suggested that stable protein upregulation accompanying learning occur through NMDA receptors (NMDARs). This protein upregulation is argued to be somatically mediated, meaning there is a requirement for gene transcription prior to mRNA translation (Sidorov, Auerbach, & Bear, 2013). NMDARs have importance in learning and memory formation within the hippocampus as reflected by their involvement in fear conditioning (Xu et al., 2001), working memory (May-Simera & Levin, 2003), olfactory memory (Si et al., 2004) and spatial memory (Morris et al., 1986; Shimizu et al., 2000). In transgenic mice lacking NMDARs in the CA1 region of the hippocampus, there are spatial learning deficits (Shimizu et al., 2000; Tsien et al., 1996) whereas genetic alterations producing augmentation of NMDARs culminates in improved learning and memory (Tang, Wang, Feng, Kyin, & Tsien, 2001; Wong, Setou, Teng, Takei, & Hirokawa, 2002). Moreover, administration of NMDAR antagonists such as dizocilpine impairs learning and memory (May–Simera & Levin, 2003; de Lima et al., 2005). Consistent with the animal data, ketamine, an NMDAR antagonist, negatively impacts cognitive function, resulting in learning and memory problems in humans (Honey et al., 2005; Newcomer, & Krystal, 2001).

As opposed to mGluRs, which have a clearer role in FXS, under the hypothetical scenario where PSD-95 upregulation is conjectured to be NMDAR mediated, Fmrp would be involved in maintaining translational control of local dendritic mRNAs and not involved in somatic translation. However, because Fmrp is believed to be involved in the transport of mRNAs from

the nucleus to dendritic spine (Bagni & Greenough, 2005; Dictenberg et al., 2008) it is tempting to speculate that the loss of Fmrp may detrimentally impact synaptic plasticity by failing to deliver necessary mRNAs for new protein synthesis and post LTP (Sidorov et al., 2013).

The findings of reduced PSD-95 protein expression in *Fmr1* KO mice when contrasted with their wild-type counterparts in experiment one could be attributable to a disturbance of mRNA transport of "plasticity proteins" during the learning phases of the Hebb-Williams mazes. A caveat regarding this explanation is that whereas there is research literature demonstrating mGluR-5 activation and increased Fmrp mRNA granule transport, it is unknown whether NMDAR activation can regulate the dynamic trafficking of Fmrp and its cargo mRNA in a similar fashion as mGluRs. It is also unknown whether NMDARs and mGluRs regulate a common or different pool of mRNAs. It is also conceivable that mRNA binding proteins other than Fmrp are involved in synaptic plasticity and are responsible for delivering proteins to the synapse during periods of synaptic plasticity. Therefore, at this juncture it appears that the loss of Fmrp affects the requirements for new dendritic protein synthesis in some way that remains to be determined. Notably, electrophysiological alterations as well as lesion studies indicate that NMDARs are involved in induction but not memory maintenance (Constantine-Paton, 1994; Lozano et al., 2001) highlighting the importance of other factors in long term memory consolidation, such as scaffolding proteins.

Consistent with a role for NMDARs in synaptic plasticity, the reversal of PSD-95 protein levels in MPEP treated *Fmr1* KO mice in experiment two suggests another receptor mechanism since mGluRs were blocked during the test phases of maze learning. One plausible explanation is that blocking mGluRs stabilized local, basal levels of PSD-95 mRNA which were then available for translation following learning of the Hebb-Williams mazes by NMDARs. Under this

scenario, following translation of dendritic PSD-95 mRNAs, there would be a plateau of protein expression given the absence of Fmrp to transport additional mRNAs to the site of plasticity. Additional research is needed to clarify how mGluRs and NMDARs signals affect the transport/regulation of mRNAs that bind to Fmrp and the how these molecular pathways function together during learning.

Whether pharmacological intervention studies with mGluR-5 antagonists in mouse models of FXS will translate into effective treatments for human patients remains to be determined. MPEP is not used with human patients due to preclinical findings of organ toxicity and impractical dosing due to a very short half-life. To date, only two studies have been completed in human FXS populations with other mGluR-5 antagonists. Berry-Kravis et al. (2009) conducted a pilot study to determine pharmacokinetics and side effects of a single dose trial of the mGluR-5 antagonist, fenobam, to 12 male and female FXS patients. Pre/post outcome measures included prepulse inhibition (PPI) and the continuous performance test (CPT) to assess sensory gating, attention and inhibition. The results indicated there were no adverse reactions to the fenobam administration and PPI improved by at least 20% in half of the sample relative to baseline. By comparison, performance on the CPT did not improve although this finding was attributable to ceiling effects. The other study employing an mGluR-5 antagonist was conducted using AFQ056 in 30 male FXS patients ranging in age from 18-35 (Jacquemont et al., 2011). These researchers initially did not find any improvement in behavioural symptoms of FXS following treatment as assessed by the Aberrant Behavior Checklist-Community Edition (ABC-C). However, a subset of the patients who had the full *FMR1* promoter methylation and no detectable *FMR1* mRNA improved significantly more on the ABC-C and the Repetitive Behaviour Scale following treatment compared with placebo. As those patients with partial

promoter methylation did not show behavioural improvement following AFQ056 treatment, Jacquemont et al. (2011) posited that mGluR-5 antagonism may be better suited for FXS patients with full methylation at the *FMR1* promoter. Currently, additional human mGluR-5 antagonist studies are underway, one of which is a large US multi-centre clinical trial (RO4917523; Roche Pharmaceuticals). The other study involves the mGluR-5 antagonist AFQ056 (Novartis), and according to the FRAXA Research Foundation, has recently completed recruitment of patients with full mutation fragile X syndrome (aged 12-45).

Limitations

There are several limitations of the experiments reported in this thesis that are worthy of mention. Regarding experiment one, the results may have been affected by the matching procedure employed to form the control group. Typically, human studies employ mental age matching to avoid ceiling level performances between control and FXS groups (Burack, Iarocci, & Flanagan, 2004). In the current investigation chronological age matching was employed given the lack of other accepted methods for mental age matching in mice. In fact, all the animal literature surveyed which tested *Fmr1* KO mice appeared to be based on chronological age matching. The consequence of such a procedure is that the experiments may have inadvertently increased the likelihood of obtaining significant protein differences between groups of wild type and *Fmr1* KO mice. An additional limitation for both experiment one and two pertains to the timing of animal euthanasia. As a result of euthanizing mice immediately following their last learning trial on their final test day, it is unknown how hippocampal PSD-95 protein expression may change over the course of learning the Hebb-Williams mazes. A more complete picture and richer pattern of results may have been obtained by euthanizing and assaying PSD-95 protein

levels at various time points during maze learning (i.e., after habituation, acquisition and each testing day).

It is challenging to reconcile some of the electrophysiological literature with the current findings from both experiment one and two. In studies focusing on the induction phases of LTP or LTD in hippocampal cultures, there is some doubt in the literature over the validity of PSD-95 as a bidirectional regulator of synaptic plasticity (Sun, & Turrigiano, 2011; Xu et al., 2008). The data from experiment one and two, which are far removed from the induction phases of an electrophysiological experiment, are interpreted as consistent with a role of PSD-95 in the "late phases" of LTP or LTD which may last hours, days or months following synaptic stimulation and which requires protein synthesis (Abraham, & Williams, 2003). Typically, electrophysiological studies gather data within minutes or an hour from tetanus protocols whereas results from experiments one and two represent protein changes following a protracted period of learning. Furthermore, alterations of PSD-95 protein levels may not appear during LTP/LTD induction phases because structural remodeling accommodating synaptic weight alterations may require several hours or days to consolidate (Malenka & Bear, 2004). Alternatively, the possibility exists that *in vivo* learning stimulates the production of different intracellular pathways and proteins than those triggered during electrophysiological recordings.

Although all phases comprising the Hebb-Williams mazes (habituation, acquisition and testing) are reflective of learning, treatment was selectively administered prior to the testing phase, spanning a total of three and a half days. This was based on the notion that the testing phase represents the most rigorous aspect of the assay itself. In experiment one, *Fmr1* KO mice were found to complete acquisition but not test mazes faster than wild-type controls. This was interpreted as evidence that the acquisition phase may not represent the most challenging

component of learning on the Hebb-Williams mazes and hence, a less than optimal time point for intervention. Despite a well reasoned rationale for the timing of the MPEP treatment, the consequence of assessing the intervention during the testing phase is that results from experiment two cannot address any behavioural disturbances (i.e. tolerance towards MPEP) or specify the stability of PSD-95 protein reversal had assessment continued over longer periods of time. However, data from other murine protocols that previously employed daily MPEP i.p. injections over a protracted length of time (14 days) did not report any tolerance or adverse effects in mice of an FVB background at a equivalent dosage as used in experiment two (Su et al., 2011). Regarding any concerns about the efficacy of brief MPEP interventions, even single dose administrations of MPEP and other mGluR-5 antagonists have been shown to be beneficial in reversing audiogenic seizures, behavioural deficits, and cultured hippocampal spine abnormalities in murine models of FXS (de Vrij et al., 2008; Michalon et al., 2012; Pacey, Tharmalingam, & Hampson, 2011; Yan et al., 2005).

Future Directions

One aim of translational neuroscience is to identify and treat deficits in animal models which may lead to valuable insights into human pathologies. mGluR-5 antagonists represent only one mechanism under investigation as treatments for FXS. Current research is also focused on a number of other receptors and intracellular targeted treatments for FXS. Lower numbers of GABA-A receptors have been documented in FXS (Gantois et al., 2006) and there has been some progress using agonists of GABA-A in murine models in terms of reducing seizures and improving learning. Other work has focused on agonists of GABA- B receptors as these drugs work by blocking pre-synaptic release of glutamate, thus reducing mGluR-5 activation and downstream signalling (El Idrissi, Boukarrou, Dokin, & Brown, 2009). Ampakines are positive

AMPAR modulators that increase endogenous brain derived neurotrophic factor (BDNF). In murine models, increasing BDNF was reported to facilitate hippocampal LTP and memory and rescued LTP hippocampal deficits (Simmons et al., 2009). FXS is also characterized by dopaminergic system dysfunction and stimulants to correct hyperactivity and AD/HD have targeted frontal-subcortical circuits with some success (Ventura, Pascucci, Catania, Musumeci, & Puglisi-Allegra, 2004). Aripiprazole, an antipsychotic, acts as a dopamine agonist at low doses, and there is evidence of its efficacy in FXS (Wang et al., 2008). Lithium has also been used in murine models of FXS. It alters serotonin and dopamine levels, increases BDNF and inhibits GSK3 beta (a protein kinase) and has been shown to reverse open field hyperactivity and learning deficits (Liu, Chuang, & Smith, 2011; Yuskaitis et al., 2010). Minocycline has traditionally been prescribed as an antibiotic but additional research shows it to be effective in promoting maturation of hippocampal dendritic spines by regulating matrix metalloproteins which would otherwise degrade proteins (Bilousova et al., 2009). Research on targeting intracellular pathways such as kinase inhibitors of ERK—MAPK and PI3--mTOR pathways which are dysregulated in FXS syndrome has begun; and phosphatase inhibitors that target PP2A have been shown to fully restore ERK activation in *Fmr1* KO synaptoneurosomes (Kim, Markham, Weiler, & Greenough, 2008; Sharma et al., 2010). Unlike many other treatments, kinase and phosphatase inhibitors have not been trialed in humans as of yet.

Thus, many ostensible therapeutics are available to treat FXS. All of these agents require further study and validation. It would be interesting to understand how these treatments impact learning and behaviour relative to a treatment such as MPEP, which has thus far represented the "gold standard" in FXS animal model interventions. It would also be of interest to determine if these newer treatments are able to rescue PSD-95 protein deficits and behaviours using the

Hebb-Williams mazes or other assays. Exploration of the roles of other plasticity agents such as ARC or the SHANK family of proteins would further increase our understanding of their functions in learning and memory.

Concluding remarks

Progress in the development of treatments in murine models of FXS may result in the possibility of interventions to reverse clinical manifestations of FXS, a once unimaginable course of action. The present investigation reveals that the Hebb-Williams mazes represent a reliable and valid assay to assess spatial navigation and learning which on a molecular level is associated with increases of dorsal hippocampal PSD-95 protein expression in wild-type controls. Fmrp deficient mice fail to display proportional protein upregulation at levels consistent their wild-type runner counterparts and overall commit a greater number of errors. The use of an mGluR-5 antagonist, MPEP, resulted in a correction of PSD-95 protein deficit and learning problems in *Fmr1* KO mice without considerable negative side-effects (i.e., motor-disturbance). These studies have thus provided insight into the neurobiological basis of and treatment for the FXS phenotype and represent a valuable contribution in the search for an efficacious pharmacological intervention for FXS.

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