Functional Dysregulation in Stress-Induced Modulation of Synaptic Plasticity in a Mouse Model of Fragile X Syndrome

by

Mohamed Ghilan
B.Sc. University of Victoria, 2010

A Doctoral Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY
in the Department of Biology

Supervisory Committee

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Abstract

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The fragile X mental retardation protein (FMRP) is an important regulator of protein translation, and a lack of FMRP expression leads to a cognitive disorder known as fragile X syndrome (FXS). Clinical symptoms characterizing FXS include learning impairments and heightened anxiety in response to stressful situations. The *Fmr1*$^{-/y}$ mouse has previously been shown to have deficits in context discrimination and novel object recognition tasks, which primarily rely on the dentate gyrus (DG) region of the hippocampal formation, but not in the Morris water maze (MWM) or the elevated plus-maze tasks, which primarily depend on the *Cornu Ammonis* (CA1) region. Furthermore, previous research has demonstrated *N*-methyl-D-aspartate receptor (NMDAR)-associated synaptic plasticity impairments in the DG but not in the CA1. However, the impact of acute stress on synaptic plasticity in the *Fmr1*$^{-/y}$ hippocampus has not been examined. The current study sought to extend previous behavioural investigations in the *Fmr1*$^{-/y}$ mouse, as well as examine the impact of stress on activation of the hypothalamic-pituitary-adrenal (HPA)-axis and on hippocampal synaptic plasticity. To further characterize hippocampus-dependent behaviour in this mouse model, the DG-dependent metric change spatial processing and CA1-dependent temporal order discrimination tasks were evaluated. The results reported here support previous findings and demonstrate that *Fmr1*$^{-/y}$ mice have performance deficits in the DG-dependent task but not in the CA1-dependent task, suggesting that previously reported subregional differences in NMDAR-associated synaptic plasticity deficits in the

hippocampus of the *Fmr1*$^{-/y}$ mouse model may also manifest as selective behavioural deficits in hippocampus-dependent tasks. In addition, following acute stress, mice lacking FMRP showed a faster elevation of the glucocorticoid corticosterone and a more immediate impairment in long-term potentiation (LTP) in the DG. Stress-induced LTP impairments were rescued by administering the glucocorticoid receptor (GR) antagonist RU38486. Administration of RU38486 also enhanced LTP in *Fmr1*$^{-/y}$ mice in the absence of acute stress to wild-type levels, and this enhancement was blocked by application of the NMDAR antagonist 2-amino-5-phosphonopentanoic acid. These results suggest that a loss of FMRP results in enhanced GR signalling that may adversely affect NMDAR-dependent synaptic plasticity in the DG. Finally, synaptic plasticity alterations reported in this work were found to be specific to the DG and were unidirectional, i.e., restricted to LTP, as NMDAR- and metabotropic glutamate receptor (mGluR)-LTD were both unaffected by acute stress in the DG or the CA1 regions. This study offers new insights into synaptic plasticity impairments in the *Fmr1*$^{-/y}$ mouse model, and suggests stress and GRs as important contributors to learning and memory deficits in FXS.

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List of Abbreviations

ACSF	Artificial Cerebrospinal Fluid
AMPAR	α-Amino-3-Hyrdoxy-5-Methyl-4-Isoxazolepropionic Acid Receptor
ANOVA	Analysis of Variance
ATD	Amino Terminal Domain
BDNF	Brain-Derived Neurotrophic Factor
Ca^{2+}	Calcium Ions
CA	Cornu Ammonis
CaMKIIα	Calcium/Calmodulin-Dependent Protein Kinase II Alpha
CAP	Commissural Associational Pathway
CGG	Cytosine-Guanine-Guanine
CTD	C-Terminal Domain
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EC	Entorhinal Cortex
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
fEPSP	Field Excitatory Postsynaptic Potential
Fmr1	Fragile X Mental Retardation Gene 1
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
GABA	γ-Aminobutyric Acid
GR	Glucocorticoid Receptor
HFS	High Frequency Stimulation
HPA	Hypothalamic-Pituitary-Adrenal
I/O	Input/Output
K^+	Potassium Ions
KCl	Potassium Chloride
KH	K Homology
LBD	Ligand-Binding Domain
LFS	Low Frequency Stimulation
LPP	Lateral Perforant Pathway
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
Mg^{2+}	Magnesium Ions
$MgCl_2$	Magnesium Chloride
mGluR	Metabotropic Glutamate Receptor
mRNP	Messenger Ribonucleoprotein
MKP-1	Mitogen Activated Protein Kinase Phosphatase-1
MPP	Medial Perforant Pathway
mRNA	Messenger Ribonucleic Acid
Na^+	Sodium Ions
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NMDAR	*N*-Methyl-D-Aspartic Acid Receptor
PCR	Polymerase Chain Reaction
PLCγ	Phospholipase C Gamma
PSD-95	Postsynaptic Density Protein 95
RCF	Relative Centrifugal Force
RGG	Arginine-Glycine-Glycine
RNA	Ribonucleic Acid
SAM	Sympathetic-Adrenal-Medullary

SEM	Standard Error of Mean	**UTR**	Untranslated Region
TBS	Theta Burst Stimulation	**WT**	Wild-Type
TrkB	Tyrosine Receptor Kinase B		

Acknowledgments

I would like to thank Dr. Christie for the wonderful opportunity to be a member of his group. The laboratory was not just a place to study neuroscience, a subject that I cannot explain how fascinating it is, but also a place where I met some of the most wonderful people I ever have, and where I grew as an individual. Dr. Christie offered a great learning environment and numerous invaluable insights into the method of science. His mentoring style, support, and encouragement allowed me the freedom to be creative, while at the same time providing direction to complete tasks, as they should. I also am very appreciative that he allowed me to pursue extracurricular endeavours that helped shape me into the person who I am today.

Joana, Patrícia, and Anna are a gift from the heavens. I wish that every grad student could have even one of them, let alone all three, as senior lab members. Some of the experimental designs were a direct product of lunchroom discussions with these wonderful researchers. Patrícia was an immense help during the behaviour experiments and taught me how to properly carry out a microdissection of the mouse hippocampus. Anna offered a listening ear as I tried to talk through the scattered thoughts in my mind to explain some unexpected results. As for Joana, nothing can really be said to properly show my gratitude and appreciation. You were the one who got me excited enough about research to pursue it this far.

The rest of the lab members, past and present, have all been amazing to work with. Crystal, Namat, Emily, Sonata, Alicia, Christine, Jason, Ryan, Mariana, Sarah, and Timal: thank you so much for making the lab an exciting place to be. And Brett, I could not be more proud that I was your mentor for your honours project. You all are fantastic and I wish you success in all your endeavours.

Dedication

I would like to dedicate this dissertation to my family. To my parents, Abdulaziz and Howida, you have been a constant source of emotional support and encouragement. I cannot express how much I appreciate your understanding and patience as I complained to you during times of frustration. To my younger brother Mohanned and his wife Sana'a and their daughter, my beautiful niece, Talia, you have managed to make me laugh with the constant pictures and videos you have sent of Talia. To my younger sisters Shima and Lema, your encouragement as I progressed through my research was invaluable. To José, I appreciate your concern over how I was doing.

Sara, here is a whole paragraph just for you my love. No one can really understand what being a graduate student is like more than another graduate student. But even during your times of difficulties and frustration throughout your program, you still managed to offer your support and encouragement, and were patient to listen to my complaints despite having your own. You even sat through to listen to me practice talks even though you were busy and had your own talks to prepare. If I could put your name on this dissertation and give you all the credit, I would. Thank you does not begin to cover how I feel about what you provided for me during this time.

CHAPTER I. General Introduction

1 – FRAGILE X SYNDROME

1.1 History

In 1943 James Purdon Martin and Julia Bell published a pedigree of a family that included 11 males with intellectual impairments of varying degrees from two generations (Martin and Bell, 1943). After extensive observations over seventeen years Martin and Bell described cases of intellectual impairments occurring almost exclusively in males who were sons of unaffected mothers. This led to the initial hypodissertation that a sex-linked gene was involved (Martin and Bell, 1943). It would be another 25 years before descriptive human cytogenetics allowed for the identification of the X chromosome as the marker for this inherited form of intellectual impairment (Lubs, 1969).

1.2 Etiology

Initially named Martin-Bell syndrome, fragile X syndrome (FXS) is now recognized to be the most common form of inherited intellectual impairment and the leading monogenic cause of autism spectrum disorders (Boyle and Kaufmann, 2010). It is estimated that FXS affects 1 in every 4,000-7,000 males (Turner et al., 1996; Hunter et al., 2014). FXS is caused by mutations in the *Fmr1* gene located on the fragile tip of the X chromosome, in which a polymorphic cytosine-guanine-guanine (CGG) repeat in the 5' untranslated promoter region is expanded from its normal count of under 55 repeats to over 200 repeats (Fu et al., 1991; Verkerk et al., 1991). As a

result of this expansion, the CGG repeats are hypermethylated, and this in turn usually leads to silencing of the gene and loss of its protein product, the Fragile X Mental Retardation Protein (FMRP) (Oberlé et al., 1991) (**Figure I.1**)

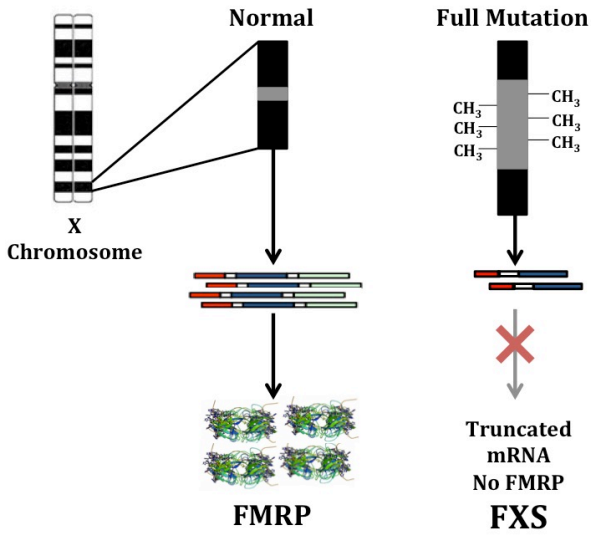

Figure I.1 Fragile X Syndrome Genetics
A polymorphic expansion of the CGG repeats in the 5' untranslated promoter region of the Fmr1 gene from under 55 repeats to more than 200 repeats signals for hypermethylation of the gene. Hypermethylation serves as a silencing tool that stops transcription and thus leads to loss of the protein product, the fragile X mental retardation protein (FMRP).

Male FXS patients present with distinct physical phenotypic features, including an elongated face, prominent ears, smooth skin, and enlarged testes (macroorchidism). Macroorchidism is a phenotypic hallmark of FXS because FMRP is normally highly expressed in the testes and in its absence the proliferation of Sertoli cells found in the seminiferous tubules increases, which in turn increases the number of germs cells in the testes and subsequently their weight (Themmen et al., 1998). FXS patients also exhibit a number of clinical manifestations, including hyperactivity, heightened stress response to novel situations, developmental delay of motor and speech skills, and impaired learning (Oostra and Willemsen, 2003; Till, 2010). Most

of these deficits become noticeable during childhood and seem to be associated with abnormal organization of cortical connections (Till, 2010).

The link between FMRP and learning disabilities in FXS was strengthened by comparative studies between FXS patients who differed in the severity of their symptoms. The highest functioning FXS patients displayed little learning disability, and it was discovered that their expanded CGG repeats were not hypermethylated and they were in fact producing FMRP. On the other hand, low-functioning patients showed hypermethylated CGG repeats and absence of FMRP (Hagerman et al., 1994). Currently, there is no cure for FXS and clinical intervention is greatly limited to symptom management with a combination of psychopharmacological and behavioural support strategies (Garber et al., 2008). Stimulants and anti-depressants rank among most commonly prescribed medications for FXS, as they appear to manage distractibility, hyperactivity, and impulsive behaviour, as well as anxiety and mood dysregulation (Berry-Kravis and Potanos, 2004). On the other hand, behavioural support strategies are usually focused on general recommendations to improve the quality of the home environment to minimize stress, and tailored behavioural interventions in the classroom (Hagerman et al., 2009).

1.3 The Fragile X Mental Retardation Protein

FMRP is translated in neurons at the synapses after activation of group 1 metabotropic glutamate receptors (mGluRs) (Weiler et al., 1997, 2004). It then becomes part of a large messenger ribonucleoprotein (mRNP) complex that has an important role in neuronal mRNA transport and translation (Bagni and Greenough, 2005). Clues about FMRP's function were derived from bioinformatics studies that identified several conserved domains, including two K Homology (KH) domains and an amino terminus that are known to preferentially bind to specific

mRNAs (Bagni and Greenough, 2005). FMRP also contains one RGG* box made up of a cluster of repeating arginine and glycine residues that seem to have an accessory role in mRNA binding; promoting the unfolding of mRNA secondary structure (Bagni and Greenough, 2005). Moreover, although FMRP is primarily found in the cytoplasm, it has been proposed to have a shuttling role between the nucleus and the cytoplasm because it contains both a nuclear localization signal (NLS) and a nuclear export signal (NES) (Eberhart et al., 1996) (**Figure I.2**).

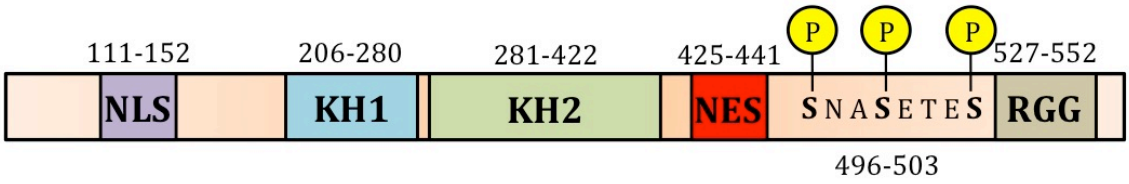

Figure I.2 The Fragile X Mental Retardation Protein
Schematic representation of the FMRP showing the locations of the various domains on the protein sequence and their phosphorylation sites. Numbers indicate amino acid locations. NLS: nuclear localization signal. KH: K homology. NES: nuclear export signal. RGG: arginine and glycine rich cluster. FMRP phosphorylation sites are at serine residues 496, 499, and 503.

FMRP preferentially associates and interacts with actively translating polyribosomes (Corbin et al., 1997). It also has a role in suppressing mRNA translation at the synapse during the absence of a synaptic input (Weiler et al., 1997; Laggerbauer et al., 2001; Li et al., 2001). Furthermore, 60% of the mRNAs interacting with the FMRP complex directly associate with FMRP; the proteins encoded by these mRNAs have been shown to change in abundance and subcellular distribution when FMRP is knocked-out (Miyashiro et al., 2003). The identities of many of these proteins further confirm the importance of FMRP in multiple biological pathways that have roles in synaptic development and maturation in the central nervous system (CNS). The mRNAs regulated by FMRP include those involved in coding for receptor and ion channel

* RGG refers to the one letter designation of arginine (R) and glycine (G)

proteins that are essential for normal synaptic function. Those include mRNAs for *N*-methyl-D-aspartate receptor (NMDAR) subunits GluN1 and GluN2B, and components of the postsynaptic density, such as postsynaptic density protein 95 (PSD-95) and Ca^{2+}/Calmodulin protein kinase IIα (CaMKIIα) (Brown et al., 2001; Darnell et al., 2004; Zalfa et al., 2007; Schütt et al., 2009). In addition, loss of FMRP is associated with elevated mammalian target of rapamycin (mTOR) signalling, which is a vital pathway involved in cell energy metabolism and protein syndissertation (Sharma et al., 2010). Overall, FMRP associates with approximately 4% of the mRNA expressed in the mouse brain, primarily associating with mRNAs encoding proteins involved in neuronal structural development and function (Brown et al., 2001). Hence, it is not surprising that the severity of neurobiological symptoms of FXS are inversely correlated with levels of FMRP (Hagerman et al., 1994).

1.4 From Human to Mouse: Modeling Fragile X Syndrome

Much of the current knowledge about FMRP's role in neurons is derived from studies in different animal models, including the frog, *Drosophila*, zebrafish, rat, and mouse (The Dutch-Belgian Fragile X Consortium, 1994; Tucker et al., 2004; Yan et al., 2004; Huot et al., 2012; McBride et al., 2013; Hamilton et al., 2014). However, a great effort has been focused on the characterization of the *Fmr1*$^{-/y}$ mouse.

The human version of the *Fmr1* gene is 97% homologous to its murine counterpart (Ashley et al., 1993). Moreover, *Fmr1* mRNA and FMRP expression patterns are very similar in both humans and mice (Abitbol et al., 1993; Bächner et al., 1993a, 1993b; Hinds et al., 1993). The first transgenic mouse model was generated by silencing the *Fmr1* gene by inserting a neomycin cassette in Exon 5 in the promoter region (The Dutch-Belgian Fragile X Consortium, 1994). The

antisense orientation of the inserted neomycin cassette causes an abrupt stop to the transcription in the *Fmr1* gene.

Like human FXS patients, *Fmr1*$^{-/y}$ mice express some truncated versions of the *Fmr1* mRNA, do not express FMRP, and show learning deficits and hyperactivity. Aside from sharing the phenotypic feature of macroorchidism present in the human condition, *Fmr1*$^{-/y}$ mice are otherwise physically healthy and have normal structural morphology (The Dutch-Belgian Fragile X Consortium, 1994).

One hallmark feature of FXS is abnormal dendritic spine morphology in the brain. Dendritic spines are small protrusions along neuronal dendrites that serve as sites of excitatory synaptic input containing receptors and signalling molecules required for proper synaptic function and plasticity (Nimchinsky et al., 2002). Post-mortem examination of cortical tissue obtained from FXS patients revealed a higher density of dendritic spines, the majority of which were immature and elongated (Rudelli et al., 1985; Hinton et al., 1991; Wisniewski et al., 1991; Irwin et al., 2001). These findings were paralleled with analogous findings of similar dendritic abnormalities in *Fmr1*$^{-/y}$ mice, including longer and thinner dendrites with greater spine density in the occipital cortex (Comery et al., 1997; Galvez and Greenough, 2005), and increases in spine length and density earlier during cortical synaptogenesis (Nimchinsky et al., 2001; Grossman et al., 2010). More details on the various deficits observed in this mouse model are discussed in Sections 2.6-2.8.

2 – THE HIPPOCAMPUS

The hippocampal formation is a bilateral structure of the limbic system located in the temporal lobe of the mammalian brain. The importance of this brain structure initially became evident after patient H.M.'s surgery to bilaterally remove the hippocampal formation in a radical effort to treat intractable epilepsy after other more conservative forms of treatment failed (Scoville, 1954). Although his perception, abstract thinking, and reasoning abilities remained excellent, and had no changes in personality or general intelligence, H.M. was left with severe global amnesia, unable to remember events subsequent to the surgery (anterograde amnesia), as well as having partial memory loss for events that occurred over the three years leading to the operation (partial retrograde amnesia) (Scoville and Milner, 1957).

It is now well-established that the hippocampal formation plays vital roles in episodic memory, spatial navigation, and in the consolidation of information from short-term to long-term memory (Amaral and Lavenex, 2007). Several studies using hippocampal lesions in rodents have supported the role of the hippocampus in learning and memory, including (but not restricted to) spatial learning in the Morris water maze (MWM) (Morris et al., 1982), recognition memory capacities (Young et al., 1994; Clark et al., 2000), and episodic memory (Fortin et al., 2002).

The role played by the hippocampal formation in learning and memory is thought to be carried out by a number of different classes of neurons whose activity is tuned to position and orientation in space (Wills et al., 2014). These cells include place cells, which fire when the animal is in a specific location in an environment (O'Keefe and Dostrovsky, 1971); head direction cells that encode to where the animal's direction of movement is heading (Taube et al., 1990); grid cells, which are activated in a number of locations in the environment that are laid out in a hexagonal grid and may play a role in calculating distance travelled (Hafting et al.,

2005); and boundary vector/border cells, which respond to boundaries of the environment (Solstad et al., 2008; Lever et al., 2009). A general function for the hippocampal formation in learning and memory is thought to involve the linking of disparate elements across space and time to create a lasting representation, and comparison of current representations with stored ones, or stored representations with one another, in order to guide behaviour (Yassa and Stark, 2011; Olsen et al., 2012).

Based on the available evidence, deficits in hippocampus-dependent learning and memory in human cognitive disorders such as FXS have been proposed to be a manifestation of a series of processing deficiencies in cognitive events that finally lead to observed intellectual impairment (**Figure I.3**). These events begin with deficits in spatial and temporal information processing, i.e., the experience of stimuli in space and time, which lead to poor sensory integration, thus ending with overall intellectual impairment (Simon, 2008). Hence, the modelling of hippocampal cognitive dysfunction observed in human disorders in animals makes use of behavioural tasks that test spatial and temporal processing performance. As mentioned above, the dependency of such tasks on the hippocampal formation is typically established through the use of hippocampal lesions prior to testing and assessing subsequent performance (Morris et al., 1982; Young et al., 1994; Clark et al., 2000; Fortin et al., 2002; Goodrich-Hunsaker et al., 2005).

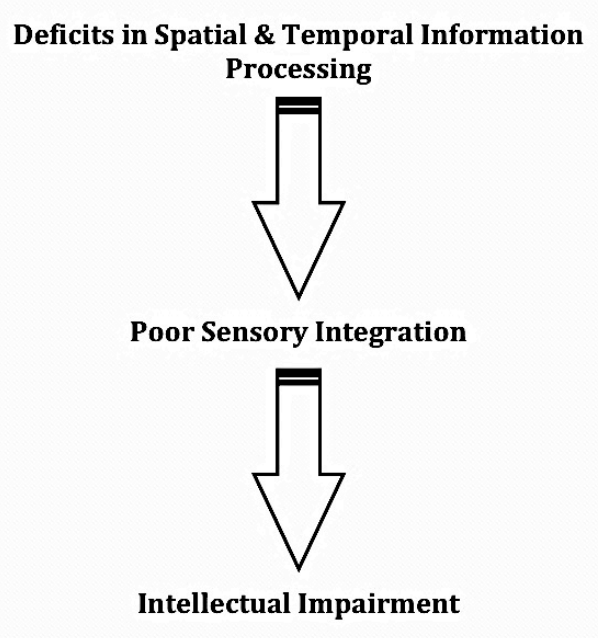

Figure I.3 The Foundations of Hippocampus-Dependent Behavioural Impairment
Intellectual impairment is the end-result of a series of deficits in hippocampus function. Impairments in processing experience of space and time lead to poor sensory integration, the consequence of which is intellectual impairment.

2.1 Hippocampal Neuroanatomy

The hippocampal formation consists of the dentate gyrus (DG), hippocampus, subiculum, presubiculum, parasubiculum, and entorhinal cortex (Andersen et al., 2007). The hippocampus has three subdivisions: *Cornu ammonis* (CA)3, CA2, and CA1 (Amaral and Lavenex, 2007). The neuronal organization of some portions of the hippocampal formation resembles other cortical regions of the brain, including the presence of large pyramid-shaped projection neurons and smaller interneurons. However, what neuroanatomically distinguishes this region of the cortex is the mostly unidirectional passage of information through intrahippocampal circuits and the highly distributed three-dimensional organization of intrinsic associational connections (Amaral and Lavenex, 2007).

2.2 Trisynaptic Circuitry

The predominantly unidirectional passage of functional connections in the hippocampus forms a trisynaptic circuit: three excitatory connections form the major pathways between the DG, CA3, and CA1 (Andersen et al., 1971). Briefly, the angular bundle is a compact structure formed by efferent fibres from the entorhinal cortex (EC) that travels into the hippocampus. These fibres form the perforant pathway, which is the major pathway delivering neocortical information to the hippocampus, through the EC, and into the molecular layer of the DG. The perforant pathway's fibres bifurcate to send projections to the suprapyramidal and infrapyramidal blades of the DG. The DG molecular layer contains three pathways: the medial perforant pathway (MPP), the lateral perforant pathway (LPP), and the commissural associational pathway (CAP). As the names suggest, the MPP originates from the medial aspects of the EC, and the LPP originates from the lateral part of the EC. The CAP originates from efferent connections coming from the contralateral DG. The second pathway of the circuit comes from dentate granule neurons in the DG that project unmyelinated axons, known as mossy fibres, which synapse at the CA3 region. Projections from the CA3 extend the third pathway of the circuit through the Schaffer collaterals towards the CA1 region. Pyramidal neurons from the CA1 project to the subiculum, which in turn projects back to the EC (Amaral and Lavenex, 2007; Deng et al., 2010) (**Figure I.4**).

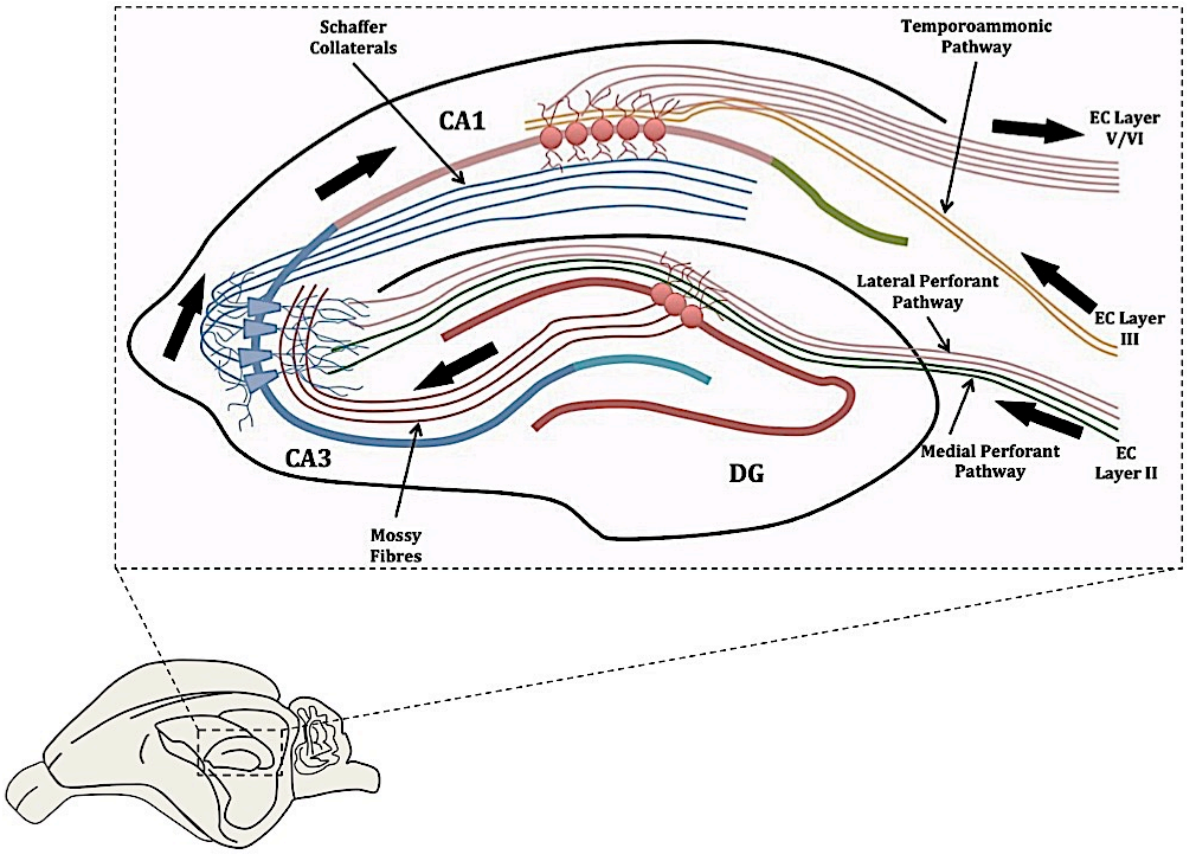

Figure I.4 Hippocampal Trisynaptic Circuitry

Neocortical information arrives at the EC through perforant path that travels from layer II in the EC to the DG. The EC also has a minor projection from layer III through the temporoammonic pathway to the CA1. The DG projects through the mossy fibres to the CA3, which in turn projects through the Schaffer Collaterals to the CA1. The circuit ends with the CA1 projecting back to the EC layers V/VI. EC: entorhinal cortex; DG: dentate gyrus; CA: *Cornu ammonis*. (*Adapted and modified from Deng et al., 2010*)

A unique aspect of DG anatomy is the presence of feedback and feedforward circuits. The DG is formed by a densely packed granule cell layer where axons from layer II of the EC terminate, and an underlying polymorphic cell layer of the hilus, which the mossy fibre axons travel through towards the CA3. The mossy fibre pathway projecting from the granule cells in the DG also synapses onto the hilus, which contains interneuron mossy cells that in turn feedback to innervate the granule cell layer of the DG, forming a feedback excitation loop. In addition, dentate granular neurons also target interneuron basket cells in the hilus, which in

response release the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), creating a feedback inhibitory effect (Amaral et al., 2007) (**Figure I.5**).

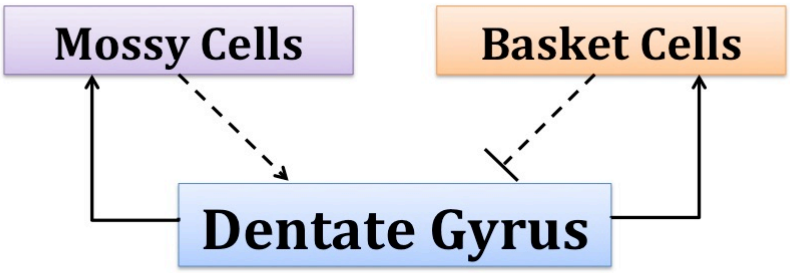

Figure I.5 Feedforward and Feedback Circuits in the DG
In addition to projecting to the CA3, dentate granule cells also project to mossy cells and interneurons in the hilus, which respectively send back excitatory and inhibitory projections to the granule cells.

2.3 Hippocampal Structural Development & Plasticity

Pyramidal neurons in the CA1 are generated between embryonic days (E)10 and E18 in the mouse hippocampus (Angevine, 1965). In contrast, dentate granule cells begin forming on day E10, but continue to generate well into adulthood (Altman and Das, 1965; Fortscher and Seress, 2007). During the course of neuronal maturation, the size and complexity of new neurons increases as they are integrated within existing hippocampal neural networks (Zhao et al., 2008).

The generation of new neurons throughout adulthood is restricted, and mainly occurs in two regions of the mammalian brain: the subventricular zone (SVZ) of the lateral ventricles where newborn interneurons travel the rostral migratory stream to the olfactory bulbs, and the subgranular zone (SGZ) of the DG, where dentate granule cells are generated and incorporated into existing neuronal networks (Zhao et al., 2008). Briefly, adult neurogenesis begins with the slow proliferation of neural progenitor cells, followed by a faster proliferation rate of restricted progenitor cells during the expansion phase. Subsequently, young cells are selected to survive,

differentiate, and mature as they enter the later phases of postmitotic development and are integrated into the pre-existing neuronal network (Ehninger and Kempermann, 2008).

Adult neurogenesis has been proposed to be involved in the mechanisms underlying learning and memory (Zhao et al., 2008; Frankland et al., 2013). However, its functional significance has been difficult to experimentally elucidate. A proposed role based on the available data suggests it to be involved in specific functions of enabling the brain to accommodate continued bouts of novelty, or to prepare the hippocampus for processing greater levels of environmental complexity (Kempermann, 2002).

2.4 Hippocampal Synaptic Plasticity

The mammalian brain is now recognized to retain a degree of plasticity that allows for modification of neural circuitry as a result of experience. Initially proposed by Donald Hebb as a theory explaining how learning and memory occur at the functional level (Hebb, 1949), synaptic plasticity refers to the activity-dependent changes occurring at the synapses within the brain (Howland & Wang, 2008). Several cellular mechanisms for synaptic plasticity have been proposed and studied as models for learning and memory, including short-term and long-term forms. Although long-term synaptic plasticity is the best studied, patterns of cognitive processes are not solely dependent on long-term changes. Therefore, a comprehensive model for synaptic changes that correlate with cognitive process, must include short-term changes on neural activity in response to stimuli and identify the links they may have, if any, to long-term changes.

2.4.1 Short-Term Plasticity

Short-term plasticity is an important component of synaptic function that precedes long-term changes and may influence long-term synaptic responses to various neural stimuli (Zucker

and Regehr, 2002). Short-term plasticity refers to short-term changes that last at most a few minutes after neural stimuli. This form of synaptic plasticity is thought to be the basis for information processing (Fioravante and Regehr, 2011). The various forms of short-term synaptic changes include facilitation, depression, augmentation, and post-tetanic potentiation (Bortolotto et al., 2011). These different forms of short-term plasticity engage in a variety of computational roles that precede long-term responses to stimuli. Facilitation, augmentation, and post-tetanic potentiation lead to enhanced synaptic strength lasting from milliseconds to minutes, whereas depression suppresses transmitter release from milliseconds to tens of seconds (Fioravante and Regehr, 2011). The difference between short-term synaptic enhancement and depression is thought to be due to mechanisms affecting residual levels in presynaptic Ca^{2+} concentration acting on various molecular targets, which appear to be separate from the secretory mechanism responsible for fast transmitter exocytosis and phasic release in response to action potentials (Zucker and Regehr, 2002). More specifically, synaptic depression is thought to result from transmitter vesicle depletion, as well as inactivation of both release sites and Ca^{2+} channels. Mechanisms for synaptic enhancement, on the other hand, include Ca^{2+} channel facilitation, local depletion of Ca^{2+} buffers, increases in the probability of release downstream of Ca^{2+} influx, and altered vesicle pool properties (Fioravante and Regehr, 2011).

The simplest form of short-term plasticity constitutes synaptic changes that occur in response to a pair of stimuli separated by short interstimulus intervals (~50 ms). Such paired-pulse experiments examine the effect of the short-term history of synaptic use on subsequent synaptic response to stimuli, and on the probability of presynaptic neurotransmitter release (P_r). It is well-established that P_r is altered for a short period after stimulation (Mallart and Martin, 1967, 1968). Most major synaptic inputs in the hippocampus exhibit paired-pulse facilitation

(PPF), which is defined as the increase in the size of the synaptic response to the second pulse in comparison to the first. In contrast, the MPP in the DG normally exhibits paired-pulse depression (PPD), which refers to reduction in the size of the second synaptic response to paired-pulse stimuli (Bortolotto et al., 2011). Whether the synapse exhibits PPF (or PPD) is a response that is dependent upon its P_r, both of which were shown to exhibit an inverse relationship to each other (Dobrunz and Stevens, 1997). Moreover, P_r was demonstrated to depend on the size of the readily releasable pool of neurotransmitter filled vesicles at the presynaptic junction (Murthy et al., 2001; Dobrunz, 2002), and this was shown to be the case for individual synapses or populations (Dobrunz, 2002).

Early studies have demonstrated that paired-pulse plasticity is a purely presynaptic phenomenon (Isaac et al., 1998) that displays a linear relationship between Ca^{2+} influx and transmitter release (Wu and Saggau, 1994). In addition to Ca^{2+} influx, residual intraterminal Ca^{2+} is believed to contribute to early synaptic changes before returning to resting concentrations after synaptic use (Magleby, 1987). Indeed, experiments utilizing a Ca^{2+} indicator dye provided evidence that neurotransmitter release can be modulated by residual presynaptic Ca^{2+} concentrations (Connor et al., 1986). It is thought that these early presynaptic events can lead to downstream presynaptic effects that may influence long-term changes in plasticity after stimuli (Wu and Saggau, 1994; Bortolotto et al., 2011). However, the nature of possible influences on long-term changes in plasticity, and whether they confer an advantage remains unclear (Stevens, 2003).

2.4.2 Long-Term Plasticity

The capacity for learning and memory, depends on the ability of neurons to bidirectionally modify the strength of transmission between their synapses beyond the timespan seen in short-term plasticity. Various forms of long-term plasticity have been studied, the most extensively examined of which being long-term potentiation (LTP) and long-term depression (LTD) (Bliss et al., 2014). LTP is the activity-dependent enhancement of synaptic transmission, while LTD is the weakening of such transmission (Dudek and Bear, 1993; Lisman and Hell, 2008).

The first experimental demonstration of LTP was in the DG of the anaesthetized rabbit (Bliss and Lømo, 1973). Several properties of LTP make it an attractive cellular mechanism for how learning and memory occur in the brain, including its rapid induction, as well as exhibition of cooperativity, associativity, and input specificity (Nicoll et al., 1988). These properties refer to LTP's ability to be generated rapidly and be strengthened and prolonged by repetition; induced by coincident activation of a critical number of synapses; ability to potentiate a weak input when it is activated in association with a strong input; and elicited only at activated synapses but not at adjacent inactive ones on the same postsynaptic cell (Citri and Malenka, 2008).

Enhancement of synaptic transmission, i.e. LTP, begins with release of glutamate in response to a strong presynaptic depolarizing stimulus. Glutamate diffuses across the synaptic cleft and binds to α-amino-3-hyrdoxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and NMDARs (Jahr and Stevens, 1987). Conformational change in postsynaptic AMPARs opens their ion channels and allows the flow of Na^+ into the intracellular space, which leads to membrane depolarization and thus displacement of the Mg^{2+} blocking NMDARs (Traynelis et al., 2010). The combination of conformational change after glutamate binding and postsynaptic membrane depolarization is required to open the ion channel in the NMDAR, thus allowing the

passage of Ca^{2+} (Mayer et al., 1984; Nowak et al., 1984). Ca^{2+} acts as a second messenger and is required to induce LTP (Lynch et al., 1983). The local rise in Ca^{2+} concentration activates protein kinases, including CaMKII and protein kinase C (PKC), which will phosphorylate AMPARs (Lee et al., 2000). More specifically, LTP induction leads to phosphorylation of the AMPAR on Ser-831 in the GluA1 subunit (Barria et al., 1997), which increases AMPAR conductance (Derkach et al., 1999) and signals for additional AMPARs to be inserted into the postsynaptic membrane (Lu et al., 2001; Pickard et al., 2001) (**Figure I.6**).

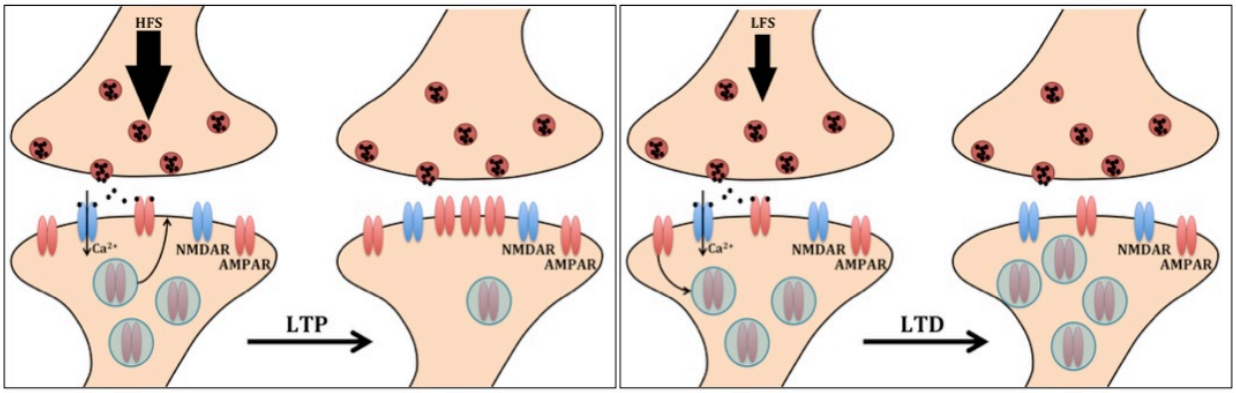

Figure I.6 Schematic Representation of Long-Term Potentiation and Long-Term Depression
In response to a strong depolarizing stimulus, such as high-frequency stimulation (HFS), glutamate is released and binds to AMPARs and NMDARs. Membrane depolarization displaces Mg^{2+} that normally block Ca^{2+} from entering through NMDARs. The activated signalling cascades lead to exocytosis of vesicles containing AMPARs (left) and insertion of additional AMPARs results in strengthened synaptic transmission, i.e., long-term potentiation (LTP). A weak depolarizing stimulus, such as low frequency stimulation (LFS), also leads to Ca^{2+} entering through NMDARs, however, the kinetics of Ca^{2+} rise (i.e., a slower rise in intracellular Ca^{2+} levels) lead to activation of signalling cascades that result in the removal of AMPARs via recycling vesicles (right), which results in weakened synaptic transmission, i.e., long-term depression (LTD).

In addition to neurotransmission enhancement in response to strong depolarization, synapses can also undergo weakening, i.e., LTD, in response to weaker forms of synaptic stimulation (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Fox et al., 2006). The difference in how synapses modulate the strength of transmission lies in the kinetics of Ca^{2+}

influx. In LTD, both AMPARs and NMDARs are activated, however, the rise in postsynaptic Ca^{2+} is smaller and slower (Mulkey and Malenka, 1992; Cummings et al., 1996). This difference in Ca^{2+} influx leads to activation of protein phosphatases (Mulkey et al., 1993), including the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (Mulkey et al., 1994). It is important to note, however, that although LTP and LTD refer to bidirectional modulation of synaptic transmission strength, they are not functional inverses of each other. Rather, the phosphorylation and dephosphorylation associated with LTP and LTD, respectively, takes place on distinct GluA1 sites in the AMPAR. Furthermore, the specific site modulation depends on the stimulation history of the synapse (Lee et al., 2000). Thus, in contrast to phosphorylation of Ser-831 in the GluA1 subunit of the AMPAR, which facilitates LTP, Ser-845 in GluA1 is dephosphorylated by calcineurin to facilitate LTD (Lee et al., 2000). Ser-845 dephosphorylation leads to decreased AMPAR channel open probability, and activates internalization of AMPARs (Banke et al., 2000; Lee et al., 2002) (**Figure I.6**)

2.4.3 The NMDA Receptor

A member of the ligand-gated ionotropic glutamate receptors, the NMDAR is composed of four large subunits that come together to form a central ion channel pore (Traynelis et al., 2010). Each subunit contains four domains: an extracellular amino-terminal domain (ATD), an extracellular ligand-binding domain (LBD), a transmembrane domain (TMD), and an intracellular carboxyl-terminal domain (CTD). NMDAR subunits include GluN1, GluN2A-GluN2D, GluN3A and GluN3B (Traynelis et al., 2010). To form a functional NMDAR two obligatory GluN1 subunits must assemble with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits (Monyer et al., 1992; Schorge and Colquhoun, 2003; Ulbrich and

Isacoff, 2008). Activation of the receptor requires the simultaneous binding of glutamate and glycine (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Lerma et al., 1990). Binding sites for glycine are located in GluN1 and GluN3 subunits, whereas GluN2 subunits provide binding sites for glutamate (Furukawa and Gouaux, 2003; Furukawa et al., 2005; Yao et al., 2008) (**Figure I.7**).

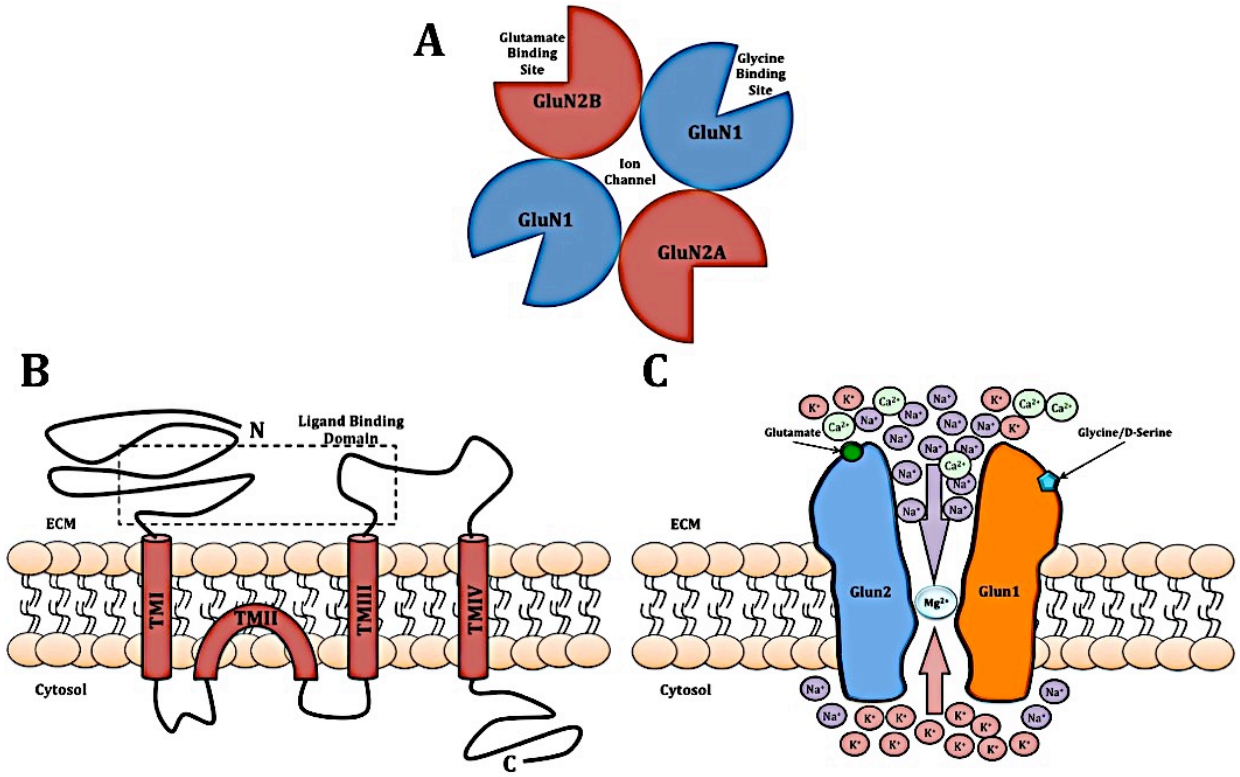

Figure I.7 The *N*-Methyl-D-Aspartate Receptor

(A) The NMDAR is formed from 4 large subunits that come together to form a central canal: 2 obligatory GluN1 subunits that provide glycine binding sites, and any combination of GluN2 or GluN3 subunit subtypes that provide glutamate or glycine binding sites, respectively. **(B)** Each NMDAR subunit has an extracellular N-terminus, an extracellular ligand-binding domain, 4 transmembrane domains, and an intracellular C-terminus. **(C)** When opened; the NMDAR ion channel allows the passage of Na^+ and Ca^{2+} to pass into the cell and of K^+ out of the cell. However, channel opening must also coincide with displacement of a Mg^{2+} that blocks the passage, which takes place during an action potential.

It is now well-established that inducing LTP in the CA1 subregion of the hippocampus requires activation of NMDARs during strong postsynaptic depolarization (Citri and Malenka, 2008). NMDAR activation leads to an increase in postsynaptic Ca^{2+} concentration, which activates signalling cascades necessary for expressing LTP (Malenka, 1991; Impey et al., 1999). As mentioned above, unlike AMPARs, postsynaptic NMDARs require the presynaptic release of glutamate, as well as postsynaptic depolarization due to simultaneous activation of a population of synapses, which will displace the Mg^{2+} blocking the channel (Mayer et al., 1984; Nowak et al., 1984). For this reason, the NMDAR is often referred to as a 'coincidence detector' (Citri and Malenka, 2008). Moreover, the NMDAR shares the basic properties of LTP, which make it a foundational receptor for a neurobiological model of learning and memory; multiple synapses need to be activated simultaneously to generate adequate postsynaptic depolarization to remove the Mg^{2+} block from the NMDAR channel (cooperativity and associativity), and Ca^{2+} increase is compartmentalized to the postsynaptic dendritic spine without affecting adjacent spines (input specificity) (Nicoll et al., 1988).

2.5 Structural Plasticity Dysregulation in the *Fmr1*$^{-/y}$ Hippocampus

FMRP regulates hippocampal neurogenesis through controlling the expression levels of glycogen synthase kinase 3β (GSK3β) (Min et al., 2009; Luo et al., 2010), an important modulator of β-catenin and the canonical Wnt signalling pathway involved in neurogenesis (Hur and Zhou, 2010). Various reports have provided different lines of evidence that hippocampal neurogenesis is altered in *Fmr1*$^{-/y}$ mice. Targeted deletion of FMRP in neural stem and progenitor cells in the hippocampus leads to reduced neurogenesis and impaired performance in the trace conditioning, a task that requires hippocampal neurogenesis (Kitamura et al., 2009; Guo

et al., 2011). A detailed analysis revealed that neurogenesis reductions in *Fmr1*$^{-/y}$ mice were restricted to the ventral DG where cell proliferation and differentiation were not altered, but cell survival was significantly reduced (Eadie et al., 2009). These deficits in cell survival may be explained by reports showing that lack of FMRP in newborn neurons significantly impaired dendritic development (Guo et al., 2011), as well as integration of new neurons into existing neural networks (Krueger et al., 2011). Given the regulatory role FMRP plays on GSK3β, and that in its absence GSK3β levels were found to be elevated (Min et al., 2009), it was hypothesized and shown that treatment with a GSK3β inhibitor rescued the reported deficits in hippocampal neurogenesis (Guo et al., 2012).

In addition to the evidence for deficits in hippocampal neurogenesis in *Fmr1*$^{-/y}$ mice, a number of studies have reported deficits in other forms of structural plasticity, namely in dendritic morphology and synaptic connectivity. Hippocampal neurons lacking FMRP express shorter dendrites, a reduced number of dendritic spines, and fewer functional synaptic connections (Braun and Segal, 2000). Moreover, the size of hippocampal intra- and infrapyramidal fibre terminal fields is reduced in *Fmr1*$^{-/y}$ mice, a deficit that was associated with performance deficits in the radial maze, a hippocampus-dependent task that tests spatial memory (Mineur et al., 2002). Additional work also demonstrated that hippocampal pyramidal and dentate granule *Fmr1*$^{-/y}$ cells exhibit longer, immature dendritic spines, and increased spine density (Grossman et al., 2006, 2010; Levenga et al., 2011a, 2011b). It should be noted, however, that inconsistent findings for detecting dendritic spine morphology changes in the hippocampus of *Fmr1*$^{-/y}$ mice appear to depend on specific regions and/or when they are examined during the developmental time scale and age of the animals, as they may be transient in nature and restricted to the early period during cortical synaptogenesis (Nimchinsky et al.,

2001) (**Table I.1**). These reported deficits in structural plasticity observed in *Fmr1*$^{-/y}$ mice might be a contributing factor to the impairments reported in this FXS mouse model.

Table I.1 Summary of Abnormal Dendritic Spine Phenotypes in the Hippocampus of FXS Mice

Phenotype	Hippocampal Region	Age	References
Increased spine density	CA1	25 weeks	(Levenga et al., 2011a)
	DG	P15-60	(Grossman et al., 2010)
	Whole Hippocampus	P0 + 16div	(Antar et al., 2006)
		E17 + 18 div	(Swanger et al., 2011)
Normal spine density	CA1	P60-P90	(Grossman et al., 2006)
	Whole Hippocampus	P0 + 7div	(Braun and Segal, 2000)
		P0 + 8div	(Su et al., 2011)
		E16 + 14 div	(Levenga et al., 2011b)
		P1 + 14div	(Segal et al., 2003)
		E18 + 21div	(de Vrij et al., 2008)
Immature spines	CA1	P7	(Bilousova et al., 2009)
		P60-P90	(Grossman et al., 2006)
		25 weeks	(Levenga et al., 2011a)
	DG	P15-P60	(Grossman et al., 2010)
	Whole Hippocampus	P0 + 8div	(Antar et al., 2006)
		E18 + 21div	(de Vrij et al., 2008)
		P0 + 8div	(Su et al., 2011)
		E16 + 14div	(Levenga et al., 2011b)
		E15 + 14 div	(Bilousova et al., 2009)
		E17 (mouse) E18 (rat)	(Swanger et al., 2011)

2.6 Functional Plasticity Dysregulation in the *Fmr1*$^{-/y}$ Hippocampus

A number of studies have reported hippocampal synaptic plasticity deficits in *Fmr1*$^{-/y}$ mice. Investigations of LTP in the DG revealed significant impairments in absence of FMRP (Eadie et al., 2010; Yun and Trommer, 2011; Bostrom et al., 2013; Franklin et al., 2014a, 2014b). In addition, significant LTD deficits in the DG have also been noted (Eadie et al., 2010). Impairments of bidirectional synaptic plasticity were shown to be associated with decreased NMDAR-mediated currents (Eadie et al., 2010; Yun and Trommer, 2011), and impaired DG-dependent behavioural performance (Eadie et al., 2010; Franklin et al., 2014a). Work from our laboratory demonstrated that NMDAR-LTP deficits in the DG were associated with significantly

reduced levels of the NMDAR GluN1, GluN2A, and GluN2B subunits, as well as reduced AMPAR GluA1 phosphorylation (Bostrom et al., 2013). Interestingly, we were able to rescue NMDAR-LTP impairment in the DG by treating of *Fmr1*$^{-/y}$ hippocampal slices with the NMDAR co-agonist glycine or D-serine (Bostrom et al., 2013). This finding is quite significant as it suggests the NMDAR hypofunction we and others have previously reported (Eadie et al., 2010; Yun and Trommer, 2011) can be augmented using a co-agonist without leading to toxic effect (Coyle et al., 2003; Papouin et al., 2012). NMDAR-LTP deficits in the DG were also recently rescued using GSK3 inhibitors, which were also effective in preventing a number of hippocampus-dependent behaviour deficits, including novel object recognition, coordinate and categorical spatial processing, and temporal ordering of visual objects (Franklin et al., 2014a).

In the CA1 of *Fmr1*$^{-/y}$ mice, subtle findings of alterations in LTP and LTD have been reported. A number of studies that used high-frequency stimulation (HFS) and theta burst stimulation (TBS) to induce LTP have reported no deficits in LTP in the CA1 (Godfraind et al., 1996; Paradee et al., 1999; Li et al., 2002; Larson et al., 2005; Lauterborn et al., 2007; Zhang et al., 2009; Connor et al., 2011; Bostrom et al., 2013). However, altering the TBS induction stimulus has successfully unmasked LTP deficits, indicating the threshold of LTP induction in the hippocampus of *Fmr1*$^{-/y}$ mice may be elevated (Lauterborn et al., 2007; Lee et al., 2011).

A great deal of attention has been focused on LTD in the CA1 that can be triggered by activation of group 1 mGluRs. Unlike NMDAR-dependent LTD, which depends on activation of postsynaptic NMDARs and protein phosphatases, mGluR-LTD depends on activation of postsynaptic group 1 mGluRs and local translation of dendritic mRNA (Huber et al., 2001; Lüscher and Huber, 2010). Moreover, while NMDAR-LTD results from internalization of postsynaptic AMPARs (Carroll et al., 1999, 2001; Lüscher et al., 1999), activation of mGluRs

leads to the rapid internalization of both AMPARs and NMDARs (Huber et al., 2000; Snyder et al., 2001).

The link between mGluR-LTD and FXS is intriguing. Several groups have reported that FMRP is translated after stimulation of group 1 mGluRs (Weiler et al., 1997, 2004; Todd et al., 2003a, 2003b; Antar et al., 2004). mGluR-LTD was found to be significantly enhanced in the CA1 of *Fmr1*$^{-/y}$ mice in both young (Huber et al., 2002; Hou et al., 2006) and adult animals (Choi et al., 2011). Interestingly, although mGluR-LTD is normally dependent on protein syndissertation, it was found to be protein syndissertation independent in *Fmr1*$^{-/y}$ mice (Nosyreva and Huber, 2006; Zhang et al., 2009; Sharma et al., 2010). Furthermore, there is evidence to suggest that FMRP plays a dynamic role in the regulation of synaptic plasticity in response to mGluR activation, where it is rapidly synthesized and degraded to regulate target proteins involved in expression of mGluR-LTD (Hou et al., 2006). As a result of the converging lines of evidence on the link between mGluRs and FMRP, the mGluR theory of fragile X mental retardation was developed, stating that mGluR activation leads to syndissertation of proteins that contribute to neuronal functions, the translation of which in absence of FMRP is enhanced, leading to the deficits in structural and functional plasticity observed in FXS (Bear et al., 2004).

2.7 Hippocampus-Dependent Behavioural Deficits in *Fmr1*$^{-/y}$ Mice

The morphological changes that take place in the brain in absence of FMRP seem to underlie cognitive deficits observed in children with FXS, including slower learning and suboptimal intellectual growth (Skinner et al., 2005; Hall et al., 2008). A number of studies have sought to model intellectual impairment associated with FXS through characterization of behavioural performance deficits in *Fmr1*$^{-/y}$ mice. MWM is a hippocampus-dependent task in

which rodents are evaluated on their visual-spatial memory to locate a submerged platform using spatial cues (Morris et al., 1982; Morris, 1984). Initial studies reported that *Fmr1*$^{-/y}$ mice have deficits in MWM performance only when the platform position is switched after they learned its original location (i.e., in the reversal version) (The Dutch-Belgian Fragile X Consortium, 1994; D'Hooge et al., 1997). However, this was later found to have been due to strain effects (Paradee et al., 1999), and further testing confirmed no robust learning deficits can be observed when testing *Fmr1*$^{-/y}$ mice on the MWM (Peier et al., 2000; Qin et al., 2002; Eadie et al., 2009).

A recent study challenged these negative findings using *Fmr1*$^{-/y}$ mice bred onto an albino background and found they had significant deficits during place navigation, probe trials, and a serial reversal version of the MWM (Baker et al., 2010). These inconsistent findings on MWM in *Fmr1*$^{-/y}$ mice raise the question whether this test may be producing results that are idiosyncratic to the experimental conditions or background strains of mice used, and hence may not be an appropriate test to derive significant objective and reproducible findings. A recent study made use of the temporal ordering discrimination task, one that tests the animal's ability to recognize the temporal order objects are presented and is dependent on the CA1 region (Goodrich-Hunsaker et al., 2005; Hunsaker et al., 2010). Findings from this task demonstrated that *Fmr1*$^{-/y}$ mice had significant performance impairments (Franklin et al., 2014a).

Another behavioural task that has been employed to assess hippocampal function is context fear discrimination, which is a test for the ability to process pattern separation. Pattern separation refers to the ability where two mostly identical patterns of multi-modal stimuli are separated from each other based on identifying features unique to each one (Gilbert et al., 2001; Kesner et al., 2004; Goodrich-Hunsaker et al., 2005). Pattern separation has been found to depend on NMDARs in the hippocampus, as intrahippocampal injections of an NMDAR antagonist led to

hippocampus-dependent performance deficits (Young et al., 1994). In context fear discrimination the animal is placed in two identical contexts for a period of time, one of which is coupled with a footshock, and freezing behaviour is measured during the time preceding footshock administration on successive trials. An intact ability for pattern separation in this task indicates the animal is able to discern between "safe" and "dangerous" environments that appear similar. As the case has been in studies on MWM performance, inconsistent findings have been reported with some showing deficits in context fear discrimination performance in *Fmr1*$^{-/y}$ mice (Eadie et al., 2010; Auerbach et al., 2011), and others reporting none (Dobkin et al., 2000; Van Dam et al., 2000).

Other pattern separation tasks have yielded performance deficits in *Fmr1*$^{-/y}$ mice. The metric change spatial task is one in which the distance between two objects is altered between the habituation and test periods. *Fmr1*$^{-/y}$ mice displayed performance deficits in this task showing an inability to recognize the change in spatial relationship between the two objects (Franklin et al., 2014a). Similarly, *Fmr1*$^{-/y}$ mice exhibited impairment in the novel object recognition task, which tests the animal's ability to discriminate between a novel and familiar object (Franklin et al., 2014a).

It is interesting to note here that although findings *Fmr1*$^{-/y}$ mice having performance deficits in CA1-dependent tasks have been largely inconsistent, the reported evidence from various pattern separation tasks, which depend on the DG (Gilbert et al., 2001; Kesner et al., 2004), have mostly been consistent in finding impaired performance in *Fmr1*$^{-/y}$ mice. Nevertheless, it remains inconclusive whether the behavioural deficits are necessarily linked with hippocampal synaptic plasticity impairments in this mouse model.

3 – STRESS

"Everybody knows what stress is and nobody knows what it is." - Hans Selye

3.1 The Stress Response

Response to stress involves a two-phase cascade of events (Morris, 2007). Once threat is perceived, the emotional arousal triggers the hypothalamus to activate the sympathetic-adrenal medullary (SAM) system through activation of the autonomic nervous system. This SAM activation is what is known as the 'fight-or-flight' response as it results in rapid release of catecholamines from the adrenal medulla into the bloodstream, the consequences of which are rapid heartbeat, elevated breathing rate, and increased pressure of the blood being sent to the muscles all for the purpose of facilitating escape from threat. The second phase of the stress response involves activation of the hypothalamic-pituitary-adrenal (HPA) axis, which has multi-level effects. HPA-axis activation results in the release of a cascade of hormones in response to each other, which retains a built-in regulatory negative feedback mechanism (Thiel and Dretsch, 2011).

Upon perception of threat, central autonomic neurons in the brainstem and the paraventricular nucleus in the hypothalamus activate* sympathetic preganglionic neurons regulating adrenal medullary function (Jansen et al., 1995). Preganglionic neurons are found within a columnar grouping known as the intermediolateral nucleus running longitudinally through the lateral horn of the spinal gray matter between the first thoracic segment (T1) and the third lumbar segment (L3). Axons of preganglionic neurons exit from the spinal cord segment in which its soma is located and project to targeted postganglionic neurons present in sympathetic

* The term *activate* is being loosely used here. Body systems do not simply shut down or turn on. They are always actively maintaining homeostatic mechanisms. But their activity can increase substantially in response to various triggers.

chain ganglia. Postganglionic neurons will in turn send their axons to join peripheral nerves that innervate target organs (Powley, 2008).

The adrenal gland receives a subset of thoracic postganglionic fibres from the greater thoracic splanchnic nerve, which innervate the adrenal medulla and release acetylcholine triggering the adrenal medulla to release the catecholamine norepinephrine (or noradrenaline) into the bloodstream, thus activating the fight-or-flight response (**Figure I.8**). High levels of norepinephrine lead to elevated heartbeat, increased blood pressure and dilation of blood vessels in skeletal muscles, constriction of blood vessels in the gastrointestinal tract, and dilation of pupils among other effects that are directed at the single goal of escaping eminent threat (Powley, 2008).

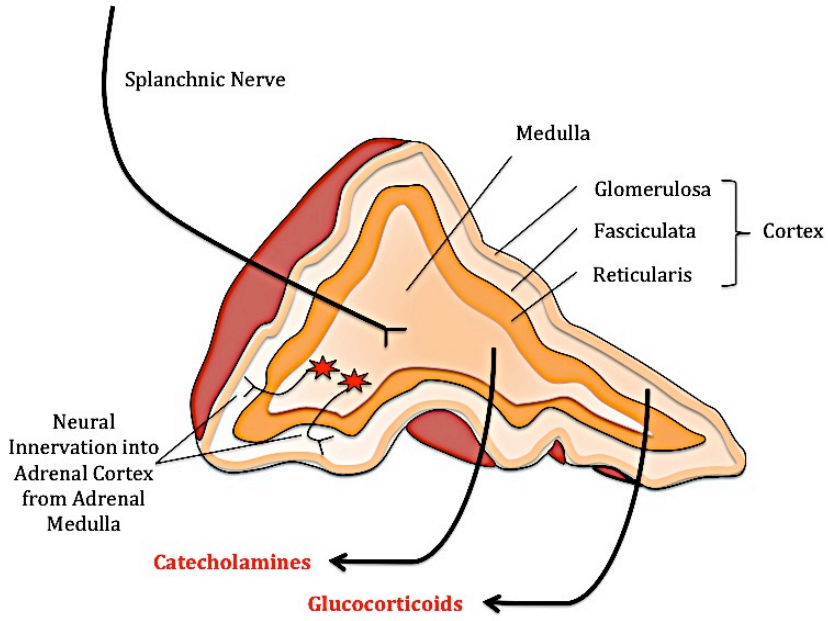

Figure I.8 Adrenal Gland Neural Innervation
The adrenal gland is innervated by postganglionic fibres from the greater thoracic splanchnic nerve, which trigger the adrenal medulla to release the catecholamine norepinephrine (noradrenaline). The adrenal cortex, which releases glucocorticoids (GCs) in response to ACTH, is innervated by projections from neurons in the adrenal medulla that can also trigger it to release GCs.

In addition to inducing the adrenal medulla to produce norepinephrine, the hypothalamus also secretes corticotropin-releasing hormone* (CRH) from its paraventricular nucleus into the portal vein leading to the anterior pituitary gland. CRH binds to receptors in the anterior pituitary gland, thus triggering the secretion of adrenocorticotropic hormone (ACTH) into the bloodstream. Released ACTH eventually binds to receptors in the adrenal zona fasciculata in the adrenal cortex, inducing the release of glucocorticoids (GCs) into circulation (**Figure I.8, I.9**). GCs are downstream products of steroidogenesis. In humans, the main GC is cortisol, while in rodents it is corticosterone (CORT). GCs will in turn bind to receptors in the hypothalamus and anterior pituitary gland, as well as the hippocampus and other higher brain structures, to inactivate the HPA-axis through induction of a classic regulatory negative feedback loop (**Figure I.9**) (Morris, 2007).

* Is it a factor or a hormone? By the rules of endocrinology, a putative hormone is referred to as a "factor" until its structure is confirmed, which happened for CRH back in the mid-1980s.

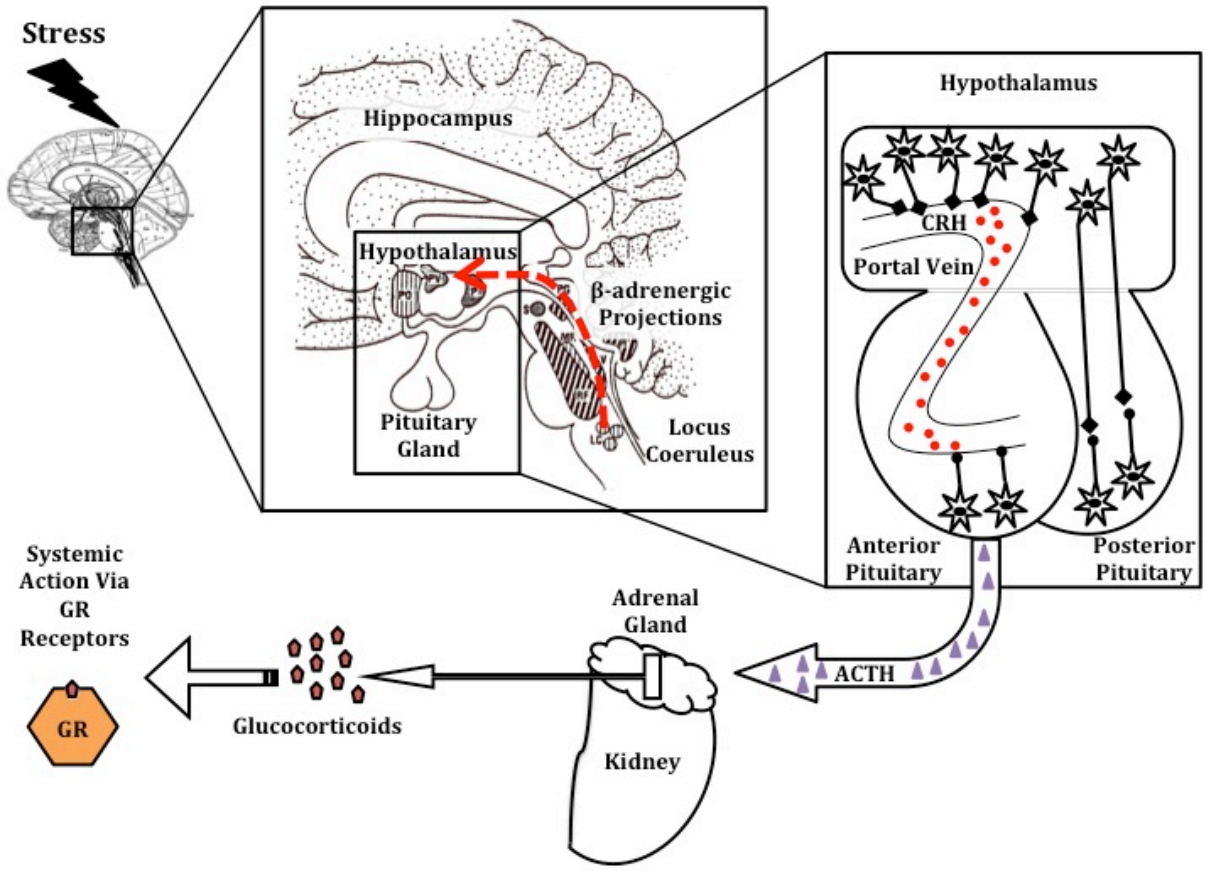

Figure I.9 Schematic of HPA-axis Activation in Response to Stress

Upon perception of stress β-adrenergic projections from the locus coeruleus in the brainstem trigger the hypothalamus to release corticotropin-releasing hormone (CRH) from the paraventricular nucleus to the portal vein. CRH binds to receptors in the anterior pituitary gland, which in response releases adrenocorticotropic hormone (ACTH) to the bloodstream. ACTH binds to receptors in the cortex of the adrenal gland, which releases GCs in response. GCs exert their actions in the body via GC receptors (GRs).

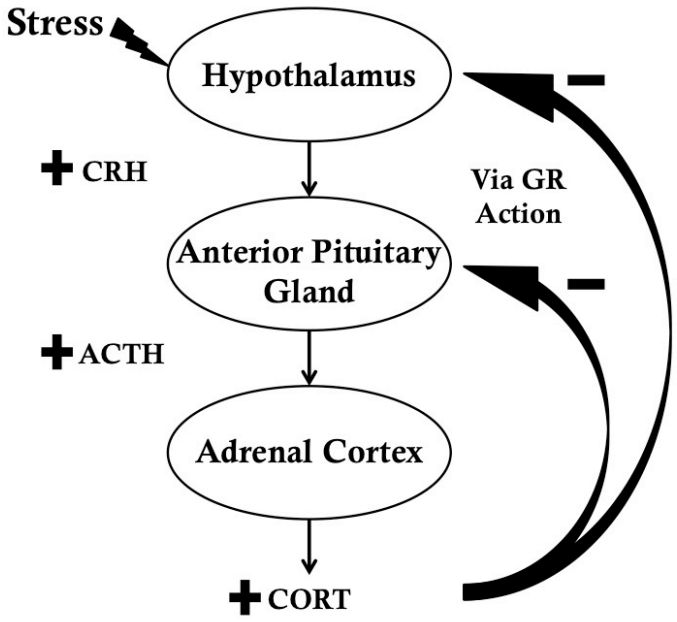

Figure I.10 HPA-Axis Negative Feedback Regulation
Released GCs from the adrenal cortex bind to GC receptors (GRs), which act to repress the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland and corticotropin-releasing hormone (CRH) from the hypothalamus.

The eventual output of HPA-axis activation is the integration of signalling cascades that facilitate coping mechanisms when the body is faced with a real or imagined stressor. The goal of these events is to either escape or adapt to the stressful environment, and store the experience as memory in order to improve the animal's chances of handling and surviving such stress in the future. The functional involvement of GCs in the different aspects of the stress response has led to integrative theories that expanded the roles of GCs to include permissive, stimulative, suppressive, and preparative actions with respect to the stress response (Sapolsky et al., 2000).

Permissive actions of GCs are those enacted to prime the body's defence mechanism in response to stress before any stress is perceived. Suppressive GC actions are those occurring an hour or more after stress, and their role is to prevent the body's systems from exceeding homeostatic limits. Stimulating actions of GCs also occur an hour or so after a stress-induced rise in their concentrations, but those enhance some of the body's initial responses to stress. Rather

than act in direct opposition, both suppressive and stimulatory actions of GCs function in mediating the stress response. Finally, preparative GC actions are long term effects on the body's systems that modulate their responses to future stressors (Sapolsky et al., 2000). These diverse roles of GCs may take place individually or all at once, and they depend on the nature of the stressful event and how it has affected the organism. Much of the early controversies surrounding GC action were due to the attempts of generalizing their effects to a single role. As will be discussed shortly, discoveries of variant forms of GC receptors that exert divergent, and in certain cases opposite functions have resolved this conundrum and provided explanations for the complex effects of GCs.

GCs are normally secreted under basal conditions in hourly pulses, produced within minutes and typically lasting in the bloodstream for approximately 20 min (Walker et al., 2010). This rhythmical activity is shared among many mammals, including humans (Lightman et al., 2008). GCs are secreted in bursts once stress is perceived, and the phase in their ultradian* rhythm determines the magnitude of such bursts; GC secretion is enhanced if stress is perceived during the ascending phase of the pulse and attenuated if experienced during the descending phase (Windle et al., 1998).

Although GC production is primarily driven by HPA-axis activity through ACTH binding to receptors in the adrenal cortex, a dissociation of GC and ACTH levels can occur under normal physiological conditions and disease (Bornstein et al., 2008). For example, the diurnal variations of GCs are modulated by variations in the adrenal responsiveness to ACTH (Dallman et al., 1978; Kaneko et al., 1981). These variations were shown to be dependent upon splanchnic nerve activity and integrity (Jasper and Engeland, 1994; Dijkstra et al., 1996; Ulrich-

* Ultradian is the term used to describe biological processes that have an oscillation of less than 24 hours, and typically it refers to 90-120 min cycles during sleep and awake states. Circadian is the term used for processes that have an oscillation period of 24 h.

lai et al., 2006). Furthermore, the adrenal zona fasciculata layer in the adrenal cortex can be stimulated by various immune-derived cytokines and adipose-derived factors to produce GCs. Moreover, the adrenal cortex is innervated by nerve endings that originate from two different locations. Some nerve endings originate from cell bodies located outside the adrenal gland and reach the adrenal cortex with the blood vessels independently from the splanchnic nerve. Others originate from cell bodies within the adrenal medulla, which are regulated by splanchnic nerve activity (**Figure I.8**) (Bornstein and Chrousos, 1999). Indeed, it appears that catecholamines and co-stored neuropeptides released from the adrenal medulla in large amounts may account for most of the observed splanchnic nerve-dependent GC release. It has been demonstrated that catecholamines can stimulate adrenocortical function *in vitro* in primary cultures (Bornstein et al., 1990; Ehrhart-Bornstem et al., 1994), as well as *in situ* in perfused adrenals (Ehrhart-Bornstein et al., 1991; Güse-Behling et al., 1992). Combined with the morphological characterization of the adrenal gland revealing that adrenomedullary chromaffin cells are found dispersed in all zones of the adrenal cortex, fully surrounded by steroid-producing cells, these findings provide an explanation for how adrenomedullary secretions can reach the adrenal cortex and stimulate steroidogenesis independently from ACTH (Fortak and Kmieć, 1968; Nussdorfer, 1986; Gallo-Payet et al., 1987).

Available evidence indicates that the number of non-ACTH pathways converging on GC secretion plays a significant role in different stress responses (**Figure I.11**). For example, long-term voluntary exercise has been shown to change GC response to different stressors independently of ACTH. On the other hand, chronic stress was demonstrated to lead to enhanced release of GC to a given level of ACTH, which suggests that receptor sensitivity to ACTH is enhanced after chronic stress (Droste et al., 2003, 2006, 2007; Ulrich-Lai et al., 2006). It is

interesting to note here that the impact of stress on GC production observed in these studies was type specific. Anxiety-producing stressors, i.e., those considered of having a psychological nature, resulted in reduced GC secretion from the adrenal cortex, whereas physically demanding stressors, i.e. exercise, resulted in enhanced adrenocortical secretion of GCs (Bornstein et al., 2008).

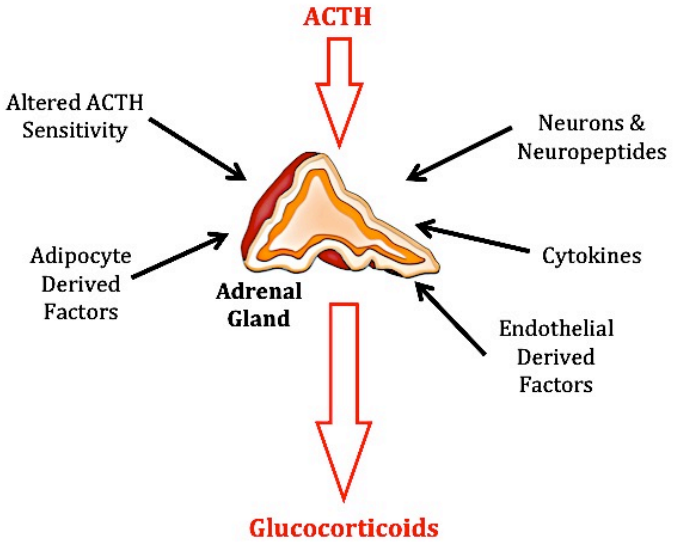

Figure I.11 Dissociation Between GCs and ACTH

ACTH and GC levels typically correlate with each other. However, additional contributing factors may lead to dissociation between ACTH and GCs. Altered ACTH receptor sensitivity in the adrenal cortex may trigger it to release additional GCs in response to the same levels of ACTH. Factors derived from adipose and endothelial tissues, as well as circulating neuropeptides and cytokines may also trigger release of GCs.

3.2 Glucocorticoid Receptors

Receptors for GCs were first proposed in the 1960s after studies of ion regulation in the toad bladder (Porter and Edelman, 1964). The receptors were subsequently described and identified as Type I mineralocorticoid receptors (MRs) and Type II GC receptors (GRs) (Marver et al., 1974). Later biochemical and functional studies have shown that both MRs and GRs are present in the brain (Beaumont and Fanestil, 1983; Reul and de Kloet, 1985). Although normally

acting as receptors for aldosterone in the kidneys, MRs have 10-times higher affinity for GCs in hippocampal and septal neurons, where they are most densely localized. GRs are more universally distributed in the brain and require much higher concentrations of GC to become significantly activated, at which point their role overtakes that of MRs (Reul and de Kloet, 1985).

MRs and GRs are members of the nuclear receptor superfamily and belong to the steroid receptor subfamily (Pascual-Le Tallec and Lombès, 2005). The gene for MR is found on chromosome 4 in the q31.1 region, whereas the gene for GR is located on chromosome 5 in the q31-32 region. MR is composed of 10 exons, the first two of which are untranslated and provide alternative splicing sites to produce different MR mRNA isoforms, hMRα and hMRβ. The remaining eight exons encode the full MR protein that is composed of 984 amino acids (Viengchareun et al., 2007). GR is composed of 9 exons, the first of which contains three transcription initiation sites to produce three alternative first exons to be fused onto exon 2 after splicing. The remaining exons code for the 777 amino acids-long GR protein (**Figure I.12**) (Duma et al., 2006).

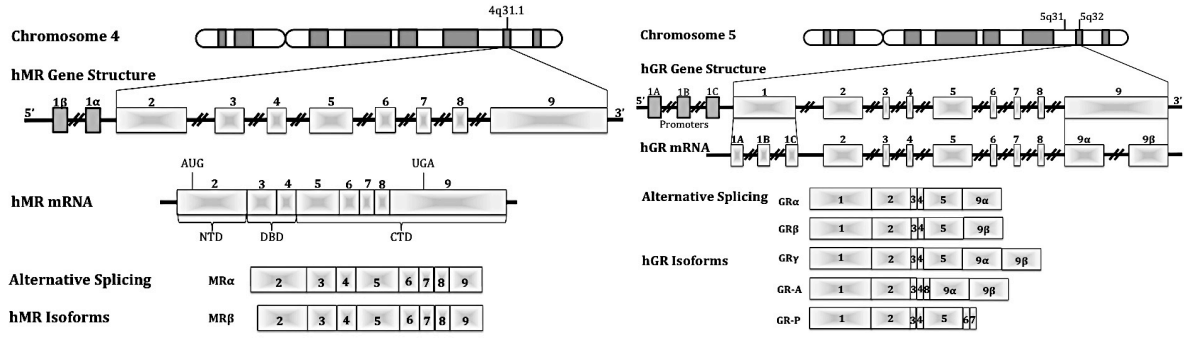

Figure I.12 The Mineralocorticoid and Glucocorticoid Receptors: Gene to Protein
(Left) The gene for the mineralocorticoid receptor (MR) is located on chromosome 4 in the q31.1 region. MR is composed of 10 exons, the first two of which provide alternative splicing sites to produce different MR mRNA isoforms, hMRα and hMRβ. The remaining eight exons encode the 984 amino acids-long MR protein. (Right) The gene for the glucocorticoid receptor (GR) is located on chromosome 5 in the q31-32 region. GR is composed of 9 exons, the first of which contains three transcription initiation sites to produce three alternative first exons to be fused onto exon 2 after splicing. The remaining exons encode the 777 amino acids-long GR protein.

Similar to other members of the nuclear receptor superfamily, MR and GR proteins have three distinct functional domains: an N-terminal domain (NTD), followed by a DNA-binding domain (DBD), then a hinge region linking them to a LBD (Zhou and Cidlowski, 2005; Viengchareun et al., 2007). In MR, exon 2 encodes the NTD, which includes two activation function 1 (AF1) domains, exons 3 and 4 encode the two zinc fingers of the DBD, and exons 5-9 code for the LBD and the 3'-untranslated region (Viengchareun et al., 2007). In the case of GR, Exon 2 codes for the NTD that includes AF1. Similar to MR, exons 3 and 4 in GR also code for the zinc fingers of the DBD. Exons 5-9 code for the LBD and the 3'-untranslated region (Encío and Detera-Wadleigh, 1991; Duma et al., 2006).

When unbound from its ligand, MR becomes part of a hetero-oligomer protein complex in the cytoplasm (Rafestin-Oblin et al., 1989). This complex includes heat shock proteins hsp90 and hsp70, p23 and p48 proteins, and the FKBP-59 immunophilins or CYP40 cyclophillin (Binart et al., 1995; Bruner et al., 1997; Pratt and Toft, 1997). The role of these chaperones is to maintain the MR in the highest affinity conformation to bind its ligand when it becomes available. In addition to chaperones, MRs also interacts with actin, which may serve a function in nuclear translocation (Jalaguier et al., 1996). Once bound, MRs dissociate from the hetero-oligomer complex and translocate to the nucleus where they interact with different molecular partners and engage in transcription regulation (Viengchareun et al., 2007).

Similarly to MRs, unbound GRs also reside in the cytoplasm in a large multiprotein complex containing a similar set of chaperone proteins as with MRs (Pratt and Toft, 1997, 2003). Once a GR is bound with its ligand it also undergoes a series of events that include a conformational change that exposes its nuclear localization signals, dissociation from the

chaperone protein complex, and translocation to the nucleus where it readily recognizes GC response elements (GREs) and influences transcription (**Figure I.13**).

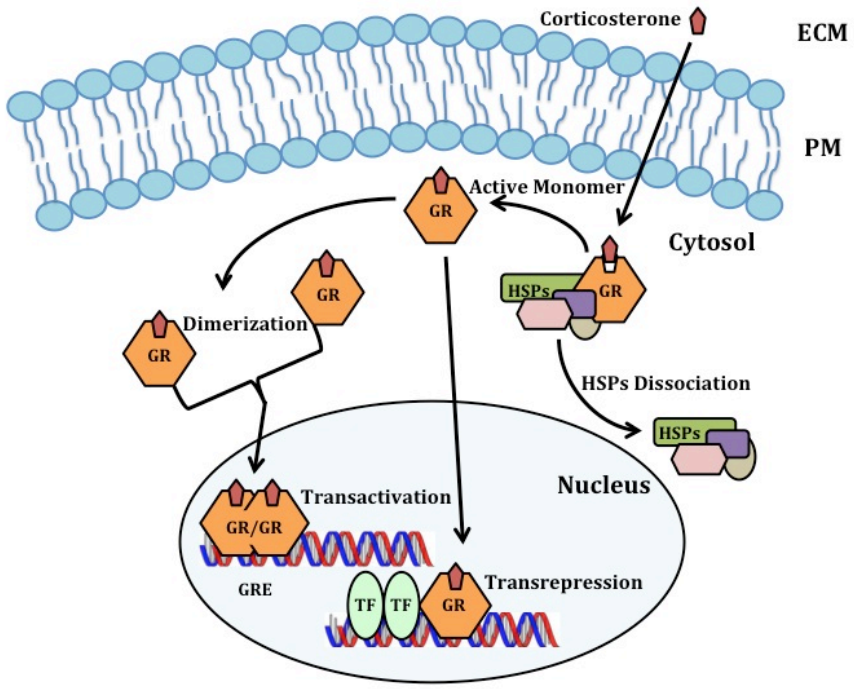

Figure I.13 Activation of GRs
Glucocorticoids are lipid soluble compounds that can readily pass across the plasma membrane. In the unbound state GRs are present in the cytosol in association with a complex of heat shock proteins (HSPs). Upon binding of corticosterone to GRs, HSPs dissociate and GRs can either come together to make homodimers before translocating to the nucleus or they can translocate as monomers. In the nucleus GRs can act as transcription factors and bind to glucocorticoid response elements (GREs), activating expression of genes that mediate the stress response, or they can bind to other transcription factors and repress activation of other genes.

Although both MRs and GRs act as gene transcription factors, they have also been shown to undertake rapid nongenomic actions when activated, such as enhancing the frequency of miniature postsynaptic potentials and reducing paired-pulse facilitation in CA1 pyramidal neurons (Karst et al., 2005; de Kloet et al., 2011). The interplay between the roles of MRs and GRs as transcription factors, as well as membrane-associated receptors that mediate rapid effects to influence synaptic transmission is proposed to be a major contributor to the modulation of basal neural function (Olijslagers et al., 2008; Sousa et al., 2008; Groeneweg et al., 2012).

As mentioned, GC binding results in a change in MR and GR conformation leading to their translocation from the cytoplasm to the nucleus and exposure of their DNA binding domains (Bodwell et al., 1991). Because of the 10-times higher affinity of MRs for GCs, this receptor is usually maintained under resting levels in an occupied state within the nucleus. The lower affinity GRs become occupied when GC levels rise during peaks of their ultradian rhythm or in response to stress (Conway-Campbell et al., 2007). Hence, GRs can be considered to act as a type of "stress detector".

Although both MRs and GRs can bind the same GREs and facilitate transcription in a complementary pattern, their receptor dynamics and kinetics are very distinct (Datson et al., 2008). For example, unlike MRs' requirement for dimerization to bind DNA, GRs can act as monomers and bind to other transcription factors to impede their activity, thus acting as transrepressors (de Kloet et al., 2011). MRs, on the other hand, recruit different coregulators and display patterns of sumoylation and proteasomal activity that are distinct from GRs (Meijer et al., 2006; Tirard et al., 2007). Analyses of MRs and GRs have revealed that their differential pattern of activity and regulation serve important roles in basal HPA-axis activity, initial responsiveness to stress, and the subsequent recovery and adaptation to stress (de Kloet et al., 2011).

The complementary roles of MRs and GRs can be further appreciated in light of the two-phases of the stress response. As stated above (see Section 3.1), the SAM system mediates the initial phase through norepinephrine released from the adrenal medulla, and the HPA-axis mediates the second phase through the eventual release of GCs. However, as GCs begin exerting their effects, they do so in two stages. The first stage enhances some of the effects observed in the first phase of the stress response by promoting arousal, motivation, and improving cognitive performance. This initial enhancement by GCs is later dampened in order to prevent the various

physiological systems of the body from overshooting (Sapolsky et al., 2000). It is now recognized that the immediate effects of GCs are mediated through nongenomic actions of the MRs and GRs, some variants of which appear to be membrane associated in contrast to the more common nuclear receptor forms (Di et al., 2003; Karst et al., 2005; Tasker et al., 2006; Joëls et al., 2008, 2011)

Gene transcription regulation can occur through several means, including the activation or repression of GC-responsive genes (Zilliacus et al., 1995), repression of gene transcription factors (Hayashi et al., 2004), as well as crosstalk with other nuclear receptors and transcription factors (Patchev and Almeida, 1996). Disruption of the relationship between the roles of MRs and GRs via an imbalance of their relative levels can lead to pathological hippocampal functioning and alterations in hippocampus-dependent behaviours (Sousa et al., 2008).

Most of what is known about the effects of GCs on gene expression is from studies examining GRs. In humans, two alternative GR C-termini can be coded, which give rise to two variant isoforms of this receptor: hGR-α and hGR-β (Scoltock and Cidlowski, 2011). The hGR-α is most universally expressed and considered to be the dominant functional isoform. The hGR-β isoform is not as widely expressed and cannot bind the GC hormone. However, some evidence suggests that it functions as an inhibitor of hGR-α, thus offering a source of regulation of GR activity (Bamberger et al., 1995; Oakley et al., 1996, 1997).

GC responsive genes can either be activated or repressed by GRs. After entry into the nucleus, active GR monomers pair with each other to form homodimers (Freedman and Yamamoto, 2004; Hager et al., 2004) that can bind to specific promoter sequences on the DNA. GR homodimers bind GREs of genes destined for transactivation by enhancing the basal transcription machinery's ability to express them (Scoltock and Cidlowski, 2011). GR

homodimers also bind negative GREs (nGREs), which are DNA sequences in the promoter region of genes repressed by GCs. This includes genes involved in the negative regulation of the HPA-axis such as the CRH gene, as well as the proopiomelanocortin (POMC) gene, which codes for the precursor of ACTH (Scoltock and Cidlowski, 2011).

GR activity is not restricted to direct influence over GREs or nGREs on DNA. For example, it has long been established that GCs suppress inflammatory responses, which strongly indicate a repressive role for GRs. However, no GC-regulated inflammatory genes were found to utilize nGREs (Scoltock and Cidlowski, 2011). Instead, GRs were found to bind and inhibit other cytokine inducing transcription factors, thus effectively repressing cytokine production (Almawi and Melemedjian, 2002). GRs can achieve this indirect form of gene repression by physical interaction of active GR monomers with other transcription factors, sequestering them in the cytoplasm and inhibiting their translocation to the nucleus. Alternatively, GR monomers can interact with transcription factors bound to the DNA and inhibit activation of the basal transcription machinery, or compete for mutual cofactors. Another method of indirect gene repression that has been shown is the GR-mediated obstruction of histone acetyltransferase activity, which renders the DNA inaccessible to the transcription machinery (Ito et al., 2000, 2006).

3.3 Stress & the Hippocampus

As described in Section 3.1, the SAM system induces the adrenal medulla to secrete catecholamines during the first phase, and the HPA-axis induces the adrenal cortex to release GCs for the second phase of the stress response. Nevertheless, multiple lines of evidence have also implicated the hippocampus in the regulation of the stress response (**Table I.2**). Early

characterization studies have revealed that GC receptors are highly expressed in the hippocampus (Reul and de Kloet, 1985). It is now established that the rate of receptor activation in this region has a modulatory effect on cellular activity, neuronal excitability, and network function in the brain (de Kloet et al., 1999).

In addition, there seems to be an inverted U-shaped correlation between the severity of acute stress and hippocampus-dependent cognitive function, as has been demonstrated in a number of animal studies (**Figure I.14**). A small amount of stress and exogenous administration of low concentrations of GCs can facilitate spatial memory (Sandi et al., 1997; Akirav et al., 2004), passive avoidance learning (Sandi and Rose, 1994, 1997; Liu et al., 1999), and contextual fear conditioning (Cordero et al., 2003). On the other hand, high stress levels or high concentrations of administered GCs have been shown to impair spatial memory (Diamond et al., 1996; de Quervain et al., 1998; Stillman et al., 1998; Conrad et al., 1999; Diamond and Park, 2000), recognition memory (Baker and Kim, 2002), and contextual fear conditioning (Pugh et al., 1997; Rudy et al., 1999).

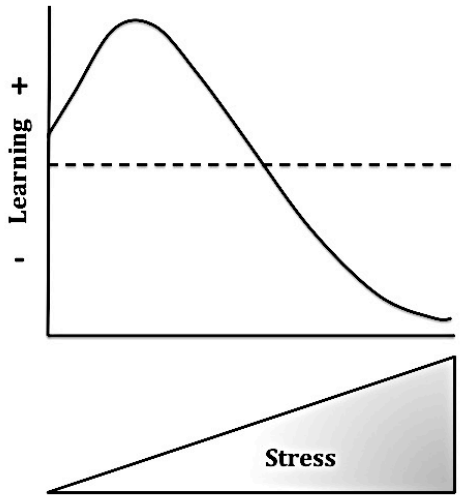

Figure I.14 An Inverted U-shaped Relationship Between Cognitive Function and Stress Levels
A small amount of stress that results in moderate levels of released GCs is necessary to facilitate cognitive function. However, as the severity of stress and GC levels increase, cognitive performance is impaired.

An additional line of evidence implicating hippocampal involvement in stress is the modulation of intrinsic hippocampal excitability and activity-dependent synaptic plasticity after stress exposure. Such a modulation has in fact been shown to mirror the two-phase cascade of events in response to stress involving activation of the SAM system and the HPA-axis. At the immediate onset of a stressor adrenaline engages the amygdala, which in turn affects the hippocampus through neuroanatomical projections that terminate in EC, the CA3 and CA1 subfields in the ventral hippocampus (Pitkänen et al., 2000; Morris, 2007). Furthermore, initial GC receptor activation results in immediate responses within neurons that in turn change their excitability through molecular reaggregation and remodelling of receptors in the cytoplasm (de Kloet, 2003). The second phase effects of HPA-axis activation and GC actions on the hippocampus involve gene transcription as has been shown from in vitro intracellular studies. These effects include gene-dependent signalling, which results in smaller Ca^{2+} currents through voltage dependent Ca^{2+} channels, weakened responses to serotonin, and reduced turnover of dentate granule cells (Joëls, 2001). More specifically, acute exposure to high GC levels leads to upregulation $GABA_A$ receptor and downregulation of Ca^{2+} channel subunit transcript expression (Qin et al., 2004). Moreover, high GC levels increase phosphorylation of histone H3 in adult dentate granule neurons in the rat and mouse, providing evidence for transcriptional activation of silent genes (Bilang-Bleuel et al., 2005).

Chronic exposure to high levels of GCs has also been shown to have a structural impact on the hippocampus. Indeed, high levels of GCs were shown to result in atrophy of apical dendrites in CA3 pyramidal cells and DG neurons (Woolley et al., 1990; Magariños and McEwen, 1995; Magariños et al., 1996), alterations in synaptic terminal structure (Magariños et al., 1997), and loss of hippocampal CA1 and CA3 neurons. Hippocampal atrophy in response to

high levels of GCs has also been seen in human functional magnetic resonance imaging (fMRI) studies as well. Patients afflicted with Cushing's disease, a condition in which the pituitary gland releases too much ACTH due to hyperplasia or presence of a tumour, revealed a significant reduction in hippocampal volume. The observed hippocampal atrophy in these patients was correlated to the magnitude of hypercortisolemia and to the extent of cognitive impairment (Starkman et al., 1992).

Stress and GCs have also been shown to impair hippocampal neurogenesis. Acute and chronic stress, as well as administration of GCs have all been demonstrated to inhibit neurogenesis in the DG (Cameron and Gould, 1994; Tanapat et al., 1998). Given that granule cell precursors do not express GRs, this inhibition is thought to occur indirectly. Although the details are still under investigation, NMDAR activation is recognized to play an important role in how stress impacts hippocampal neurogenesis by altering the balance of neuronal excitation and inhibition (Gould et al., 1997; Cameron et al., 1998). Recent studies provided evidence that in newborn neurons where GR expression is knocked down, a faster rate of neuronal differentiation and migration, an increased number of dendritic spines, and elevated basal excitability was exhibited (Fitzsimons et al., 2013).

In addition to possibly affecting NMDAR activation, GCs could be exerting their influence on neurogenesis through GR-expressing cells in the surrounding environment. Neurogenesis occurs in the DG in what is known as a "neurogenic niche", which is a specialized microenvironment that modulates neural stem cell activity and consists of numerous cell types that express GRs, including astrocytes, interneurons, and oligodendrocytes among others (Saaltink and Vreugdenhil, 2014). These cells could modulate newborn neuronal development in response to elevated GCs.

Lastly, the most significant link between the hippocampus and stress is that GC-sensitive hippocampal neurons were shown to be involved in terminating HPA-axis activity after stress. Studies of rat strains having a selective depletion or a complete loss of hippocampal GRs without cell loss were shown to double their secretion of GCs in response to stress and were unable to return to basal levels during the same period of time as their control littermates (Sapolsky et al., 1984). This deficit in GC regulation after stress exposure has also been observed in aged rats. These animals showed a 30% decline in GRs in the hippocampus, which was associated with elevations in basal GC levels and prolonged latency to return to basal levels after stress-induced elevations in comparison to their younger counterparts (Sapolsky et al., 1983).

Table I.2 Summary of Evidence Demonstrating a Relationship Between the Hippocampus and the Stress Response

Evidence	References
GC receptors are more densely concentrated in the hippocampus than in other brain regions	(Reul and de Kloet, 1985)
Dentate granule cells undergo a change in transcription profile in response to stress	(Qin et al., 2004; Bilang-Bleuel et al., 2005)
Intrinsic neuronal excitability and synaptic plasticity is modulated by stress exposure	(de Kloet et al., 1999)
Chronic stress exposure results in hippocampal dendritic atrophy, alterations in synaptic structure, and loss of neurons	(Woolley et al., 1990; Magariños and McEwen, 1995; Magariños et al., 1996)
Hippocampal neurogenesis is inhibited by stress and GC exposure	(Cameron and Gould, 1994; Gould et al., 1997; Cameron et al., 1998; Tanapat et al., 1998; Fitzsimons et al., 2013)
HPA axis activity is dysregulated after loss of hippocampal GC-sensitive neurons	(Sapolsky et al., 1983, 1984)

3.4 Stress & Synaptic Plasticity

It is now well established that acute stress impairs NMDAR-LTP in the CA1 region of the rat hippocampus (Foy et al., 1987; Diamond and Rose, 1994; Kim et al., 1996; Yang et al., 2004, 2006). Findings in the rat DG have been scarce and inconsistent. One early report suggests that the DG is not impacted by acute stress (Bramham et al., 1998), but a more recent one provided evidence for stress-induced NMDAR-LTP impairment in the rat DG (Avital et al., 2006) (**Table I.3**). This may be due to the various types of stressors being employed in these studies and lack of standardized procedure to replicate findings. Chronic stress was also shown to impair NMDAR-LTP (Shors et al., 1989). These effects were shown to depend on the rat's perception of control over the stressful stimulus (Shors et al., 1989), and were dependent on activation of the amygdala (Kim et al., 2005). In addition, stress was also demonstrated to enhance NMDAR-LTD in the CA1 (Kim et al., 1996; de Kloet, 2004; Xiong et al., 2004; Saxe et al., 2006; Yang et al., 2006), as well as mGluR-LTD (Chaouloff et al., 2007).

Less is known about the effects of stress on hippocampal LTP in mice. It is generally assumed that findings in in the rat hippocampus are likely to apply in the mouse. Indeed, one study examining the effect of acute stress in the mouse CA1 reported impaired NMDAR-LTP (Garcia et al., 1997). Another reported impaired NMDAR-LTP in the CA3 region (Chen et al., 2010) (**Table I.4**).

Table I.3 Effects of Different Acute Stress Paradigms on Hippocampal LTP in Rats

Paradigm	Hippocampal Subfield	Induction Protocol	Pre-stress LTP%	Post-stress LTP%	References
30-min Restraint	CA1 (*in vitro*)	100 Hz for 1 s	105%	21%	(Foy et al., 1987)
30-min Restraint + Tail Shock				13%	
Novel Environment Exposure	CA1 (*in vitro*)	1X200 Hz	100%	0%	(Diamond and Rose, 1994)
30-min Restraint + Tail Shock	CA1 (*in vitro*)	5X100 Hz	41.1±6.4%	7.9±4.9%	(Kim et al., 1996)
60-min Restraint + Tail Shock	CA1 (*in vivo*)	TBS	~20%	~8%	(Shors et al., 1997)
90-min 4^{o}C Cold Stress	MPP-DG (*in vivo*)	8X400 Hz	17.4±5.3%	13±5.4%	(Bramham et al., 1998)
60-min Restraint + Tail Shock	CA1 (*in vitro*)	2X100 Hz	56.2±7.8%	5.8±7.6%	(Yang et al., 2004)
30-min Elevated Platform	CA1 (*in vitro*)	10X200 Hz	24.49±3.55%	0.75±5.66%	(Xiong et al., 2004)
60-min Restraint + Tail Shock	CA1 (*in vitro*)	5X100 Hz	138.6±5.2%	104.5±5.2%	(Kim et al., 2005)
15-min Swim Stress	PP-DG (*in vivo*)	5X400 Hz	63%	0%	(Avital et al., 2006)
10-min Restraint + Foot Shock	CA1 (*in vivo*)	10X200 Hz	126.3±5.3%	100.0±1.7%	(Wang et al., 2006)
60-min Restraint + Tail Shock	CA1 (*in vivo*)	2X100 Hz	~60%	20.2±8.5%	(Yang et al., 2006)

Table I.4 Effects of Different Acute Stress Paradigms on Hippocampal LTP in Mice

Paradigm	Hippocampal Subfield	Induction Protocol	Pre-stress LTP%	Post-stress LTP%	References
60-min Restraint + Tail Shock	CA1 (*in vitro*)	4X100 Hz	64±6%	15±3%	(Garcia et al., 1997)
	CA3 (*in vitro*)	2X100 Hz	70.8±6.5%	30.4±9.8%	(Chen et al., 2010)

Multiple studies have examined the modulation of synaptic plasticity by GCs and GC receptors. For example, LTP is observed when GCs levels are held at minimum basal levels (Diamond et al., 1992) whereas high GC levels result in the facilitation of LTD in the hippocampus (Diamond et al., 1992; Pavlides and McEwen, 1999; Krugers et al., 2005). These divergent effects of GCs were suggested to be exerted through MRs and GRs having opposite

effects on hippocampal synaptic plasticity: MR activation at low CORT levels results in the preservation of excitability and stability of neural networks, and GR activation at increased CORT levels leads to the suppression of these networks (de Kloet et al., 2005).

Using receptor antagonists, Avital and colleagues provided evidence for the critical involvement of MRs and GRs in modulating the stress-induced impairments in synaptic plasticity. Their findings demonstrated that MRs facilitate LTP, and in contrast, LTD is facilitated though GR activity (Avital et al., 2006; Maggio and Segal, 2007). Recent studies examining the role of MRs and GRs on LTP after stress provided an interesting set of findings. The initial response to stress involves the elevation of both MR and GR protein levels in the hippocampus, which was correlated with impaired LTP. As stress is repeated, LTP is restored back to its pre-stress levels, and this restoration is coupled with a return of GRs to pre-stress levels and a sustained elevation of MR levels (Spyrka and Hess, 2010).

An imbalance between MR and GR activity can be aggravated by stress, which in turn can lead to increased deficits in hippocampal synaptic plasticity and hippocampus-dependent behaviour (Sousa et al., 2008). This was indeed ascertained in several studies. For example, transgenic mice having a point mutation in GRs that prevents them from binding DNA when activated were shown to exhibit a selective impairment of spatial memory in the MWM (Oitzl et al., 2001). The current evidence suggests that initial MR-activation in response to stress works to amplify attention, vigilance and appraisal processes, and this is followed by GR-activation to initiate a negative feedback mechanism that works to enhance recovery from the stressor, storage of the experience as memory and elimination of irrelevant behaviours. Ultimately, it is the action of GRs that restores GCs back to basal levels after cessation of stress through a negative feedback loop that acts on the HPA-axis.

Given that much of synaptic plasticity is mediated by glutamate receptor activity, it is likely that GCs mediate their effects through an eventual modulation of glutamate receptor levels and function. Indeed, application of CORT to hippocampal neurons results in a selective increase of GluA1- and GluA2-AMPAR surface diffusion (Groc et al., 2008). This effect was shown to be mediated by membrane-associated MRs and to result in an increase in GluA2-AMPAR density at the synapse after synaptic potentiation (Groc et al., 2008).

The evidence indicates that GCs are involved in stress-induced modulation of learning and memory. However, it has been demonstrated that an increase in GC levels is neither sufficient nor necessary to impair hippocampal function. For example, rats exposed to predator stress then tested on the radial arm water maze, a hippocampus-dependent spatial memory task, had memory impairments that were associated with elevated CORT levels. To test the causal relationship between elevated CORT and memory impairments, the rats received CORT injections to artificially raise their levels but were not exposed to predator stress. In this case the rats did not have memory impairments (Park et al., 2006). Other studies have demonstrated an indispensible role for the amygdala in modulating hippocampal synaptic plasticity and dependent learning and memory after stress, regardless of the increases in serum CORT levels (Kim et al., 2005). Indeed, the negative impact of stress and GCs on hippocampal synaptic plasticity were shown to depend on β-adrenergic receptor activity in the amygdala (Roozendaal et al., 2006).

3.5 Stress & Fragile X Syndrome

In addition to cognitive impairment, FXS is characterized by abnormal "stress-related" behaviours, such as increased anxiety and heightened response to novel situations, and children with FXS have an abnormal HPA-axis function (Hessl et al., 2006). Although they have similar

diurnal patterns, male children with FXS have higher salivary cortisol levels at two time points during a day where they are exposed to a social stressor task in the morning: first, 30 min after the stressor, and subsequently at bedtime (Wisbeck et al., 2000). Children with FXS also respond to social challenge with higher salivary cortisol levels than their unaffected siblings (Hessl et al., 2002). In response to a social challenge task at home, boys and girls with FXS demonstrated more gaze aversion, task avoidance, behavioural signs of stress, and poorer vocal quality. The most gaze-aversive children with FXS had cortisol reductions, whereas those with more eye contact demonstrated the most cortisol activity (Hessl et al., 2006).

Fmr1$^{-/y}$ mice exhibit abnormal anxiety and social behaviour interactions (Spencer et al., 2005; McNaughton et al., 2008; Eadie et al., 2009). Also, *Fmr1*$^{-/y}$ mice exhibit biochemical alterations following stress. After exposure to acute stress, *Fmr1*$^{-/y}$ mice have a significantly enhanced activation of hippocampal *c-fos* mRNA (Lauterborn, 2004), and a delayed return of stress-induced elevated CORT back to basal levels (Markham et al., 2006). These data suggest that HPA-axis function is altered in the absence of FMRP. However, there is some controversy in the literature regarding HPA-axis activation in *Fmr1*$^{-/y}$ mice. Although some reports indicate altered plasma CORT level regulation in response to stress (Lauterborn, 2004; Markham et al., 2006; Eadie et al., 2009), others have not found significant differences in either plasma CORT or ACTH levels after acute stress (Qin and Smith, 2008).

The inconsistencies in reports for HPA-axis activation in *Fmr1*$^{-/y}$ mice may be a product of mouse strain differences. Restraint stress for a period of 30 min resulted in normal serum CORT increases but a protracted return to baseline in *Fmr1*$^{-/y}$ mice on the C57Bl/6 background strain (Markham et al., 2006). On the other hand, *Fmr1*$^{-/y}$ mice on the FVB/N background strain showed similar increases and post-stress recovery of plasma CORT and ACTH levels after 30

and 120 min of restraint (Qin and Smith, 2008). Therefore, it is plausible that the lack of consistency between the various reports could be due to the use of different mouse strains, as well as the different periods of stress to which the animals are subjected.

Studies characterizing anxiety-related behaviours in *Fmr1*$^{-/y}$ mice have also yielded inconsistent findings. Analysis of mouse behaviour in the open field revealed *Fmr1*$^{-/y}$ mice spend significantly greater time travelling in the centre area of the arena (Peier et al., 2000; Yan et al., 2004; Spencer et al., 2005; Eadie et al., 2009; Yuskaitis et al., 2010). This is proposed to be an indicator for reduced anxiety. This interpretation is supported by findings that *Fmr1*$^{-/y}$ mice defecate far less in the open field, and that their plasma CORT levels after 3 h of restraint were significantly reduced in comparison to WT (Eadie et al., 2009). Furthermore, it was reported that in the elevated plus-maze, a test that exploits a mouse's preference to be in the dark by measuring the number of entries and amount of time it spends in the dark, enclosed arms in comparison to open arms of the maze, *Fmr1*$^{-/y}$ mice spent more time in the open arms than in the closed ones (Peier et al., 2000; Eadie et al., 2009; Yuskaitis et al., 2010; Liu et al., 2011; Heulens et al., 2012). However, in these studies, *Fmr1*$^{-/y}$ mice travelled more distance in the open field and the elevated plus-maze. This increased level of activity (i.e., hyperactivity) is a confounding factor in interpreting such results, because they may be a byproduct of the higher level of activity displayed by these mice. Indeed, others have reported increased anxiety in these mice as assessed with the mirrored chamber task, and avoidance of the centre of the open field (Spencer et al., 2005; Bilousova et al., 2009). Similar to the situation with studies evaluating HPA-axis activation and stress responses in *Fmr1*$^{-/y}$ mice, anxiety-related behaviour studies may also be confounded by experimental conditions, including strain differences, and the animal's age at the time of testing (Kazdoba et al., 2014).

4 – Objectives

Our laboratory has recently shown that *Fmr1*$^{-/y}$ mice have performance deficits in context discrimination, a pattern separation task that is dependent on the DG, which was associated with deficits in NMDAR-dependent LTP and LTD (Eadie et al., 2010). The aim of the present work was to further characterize hippocampus-dependent behavioural changes in this mouse model of FXS. In addition, although some work has been published by other groups on the impact of stress on HPA-axis activation in *Fmr1*$^{-/y}$ mice (Lauterborn, 2004; Markham et al., 2006; Qin and Smith, 2008; Eadie et al., 2009), the data available is inconsistent. Moreover, to date no study has characterized the impact of acute stress on synaptic plasticity in absence of FMRP. The objectives of this dissertation were to:

1. Characterize spatial and temporal processing performance in *Fmr1*$^{-/y}$ mice on a C57Bl/6 background using the metric change spatial processing and temporal order discrimination tasks.
2. Characterize the impact of acute restraint stress on CORT response in *Fmr1*$^{-/y}$ mice, and on synaptic plasticity in the DG and CA1 regions of the hippocampus
3. Investigate the roles of GC receptors in stress-induced modulation of hippocampal synaptic plasticity in *Fmr1*$^{-/y}$ mice

The results presented in the following chapters provide evidence for performance deficits in a pattern separation behavioural task but not in a temporal order discrimination task. These findings lend support for selective behavioural impairments in tasks that rely on the neural networks in the DG. Evaluation of HPA-axis activation revealed a faster-to-peak elevation of CORT in *Fmr1*$^{-/y}$ mice after acute stress, a change that was associated with a more immediate impact on LTP in the DG. Pharmacological experiments provided evidence that the impact of

stress on *Fmr1*$^{-/y}$ mice was due to functional dysregulation in MRs and GRs, which exert their effects on synaptic plasticity earlier following stress in absence of FMRP. In addition, LTP deficits in the DG of *Fmr1*$^{-/y}$ mice were rescued using the GR antagonist RU38486, and the results suggest that GRs may be exerting an inhibitory role on NMDARs in this mouse model. Finally, synaptic plasticity alterations reported in this work were found to be specific to the DG and were unidirectional, i.e., restricted to LTP, as both NMDAR- and mGluR-LTD were unaffected by acute stress in the DG or the CA1 regions.

CHAPTER II. Impaired Spatial Processing in a Mouse Model of Fragile X Syndrome

A version of this chapter is in prepared manuscript form for submission to PLoS ONE

Introduction

In humans, FXS cognitive and behavioural phenotypes include reduced IQ and attention deficit/hyperactivity (Jacquemont et al., 2007). It is hypothesized that cognitive deficits observed in neurodevelopmental disorders such as FXS are a result of poor spatial and temporal information processing, which lead to poor sensory integration and subsequently impaired cognitive function (Simon, 2008). In other words, measured intellectual impairments in FXS are the end result of a cascade of cognitive events that rest on a foundation of deficits in spatial and temporal information processing. Therefore, behavioural tests involving tasks requiring spatial and/or temporal processing can serve as important neurodevelopmental characterization tools in mouse models of FXS.

When rodents are placed in a new environment they use exploration as a means to gather spatial knowledge about their context (Wilz and Bolton, 1971; Poucet et al., 1986). Previous studies of hippocampus-dependent behaviour experiments report that rodents with hippocampal lesions have impaired performance in spatial tasks (Galani et al., 1998; Goodrich-Hunsaker et al., 2005). Furthermore, a dissociation in the function of the DG and CA1 subregions has been observed, providing evidence that the DG supports spatial pattern separation, whereas the CA1 supports temporal pattern learning (Gilbert et al., 2001; Kesner et al., 2004; McHugh et al., 2007; Lee et al., 2008).

Spatial pattern separation refers to the ability to separate partially overlapping patterns of activation so that they are viewed as distinct (Kesner et al., 2004). One form of spatial pattern separation is metric spatial processing, in which the exact distances separating objects in the environment are determined irrespective of the objects' identities (Gallistel, 1993; Goodrich-Hunsaker et al., 2005, 2008). This capacity was found to be dependent on a neural network in the DG (Gilbert et al., 2001; Goodrich-Hunsaker et al., 2005), and is dependent on NMDAR function (McHugh et al., 2007). Temporal order discrimination on the other hand, relies more heavily on the CA1, as well as the prefrontal cortex, and involves the separation of chunks of information over time to provide it with a temporal structure to be remembered (Gilbert et al., 2001; Kesner et al., 2004).

The study presented in this chapter sought to characterize *Fmr1*$^{-/y}$ mice performance in spatial processing and temporal ordering tasks.

Materials and Methods

Animals

All experiments were carried out in accordance with national standards on animal welfare and guidelines set by the Canadian Council on Animal Care and the Animal Care Committee at the University of Victoria. Adult male C57Bl/6 *Fmr1*$^{-/y}$ and wild-type (WT) littermate mice were generated in our facility by crossing female heterozygous *Fmr1*$^{+/-}$ mice with either a WT or *Fmr1*$^{-/y}$ male mouse from our established breeding colony as described previously (Eadie et al., 2010; Bostrom et al., 2013). Experimenters were blinded to genotypes during the course of experimentation.

Genotyping

To identify WT and *Fmr1*$^{-/y}$ mice DNA was extracted from ear punch tissue stored at -20 °C as described previously (Eadie et al., 2009). Briefly, tissue was placed in 150 µL digestion buffer (100 nM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS and 0.1 mg/mL proteinase K; pH 8.0) in a sterile 1.5 mL Eppendorf tube and incubated overnight at 55 °C. The sample was centrifuged at 15,800 RCF for 2 min and the supernatant was transferred into a new sterile tube. 15 µL of 3.0 M potassium acetate was added to the tube and mixed. Phenol/chloroform/isoamyl alcohol (165 µL) was added, mixed and centrifuged at 13,000 RPM for 10 min. The top layer was then transferred to a new sterile tube, and 80 µL of isopropanol was added, mixed and incubated for 30-60 min at room temperature. The supernatant was centrifuged at 13,000 RPM for 10 min. The clear supernatant was then discarded, and 100 µL of 70% ethanol was added and centrifuged at 13,000 RPM for 2 min. The ethanol was then removed carefully, the tube was covered and the clean pellet was left to dry at room temperature. Finally, the DNA pellet was re-suspended in 10 µL re-suspension buffer (10 mM Tris-HCl; pH 8.0) and stored at 4 °C until PCR.

The PCR reaction was performed by mixing 13 µL PCR-grade H_2O, 2.5 µL 10X PE Buffer II, 2.5 µL (25 mM) $MgCl_2$, 2.0 µL (2.5 mM) dNTP, 1.25 µL of each forward and reverse primer, 2 µL DNA and 0.5 µL Taq DNA polymerase (Invitrogen Canada; Burlington, Ontario, Canada). The cycling parameters employed were as follows: first cycle of 5 min at 94 °C, 90 s at 65 °C and 150 s at 72 °C. Primers M2 = 5’ ATCTAGTCATGCTATGGATATCAGC 3’ and N2 = 5’ GTGGGCTCTATGGCTTCTGAGG 3’ were used to test for the *Fmr1*$^{-/y}$ allele (amplified fragments of 800 base pairs). S2 = 5’ CAGGTTTGTTGGGATTAACAGATC 3’ and S2 = 5’ CAGGTTTGTTGGGATTAACAGATC 3’ were used to test for the WT mouse allele amplifying

a fragment of 465 base pairs. PCR products were run on a 1.5% agarose gel with ethidium bromide or SYBR-safe and visualized under a conventional trans-illuminator.

Behavioural Apparatus

For both the metric change and temporal discrimination tasks, a white plastic floor circular arena measuring 23 inches in diameter surrounded with black walls measuring 15.5 inches high was used in a dimly illuminated (20 ± 0.5 lx) testing room. The arena was raised 30 inches above the floor. Objects measuring between 1-2.5 inches at the base and 3-3.5 inches tall were used as stimuli in these tasks (Lego pieces, overturned coffee cups, glass bottles). The ANY-maze video tracking system (Stoeling Co.) was used to record the behavioural tasks on a laptop.

Behavioural Methods

All animals underwent a 5-day period of habituation to handling and to the testing apparatus before having their performance tested on the behavioural tasks (**Figure II.1**). In the first 3 days the mice were handled and placed in the arena without the objects and allowed to freely explore for a period of 5 min. On days 3 and 4 the animals were introduced into the arena with identical pairs of objects different from those used in the tasks and allowed to freely explore for a period of 5 min.

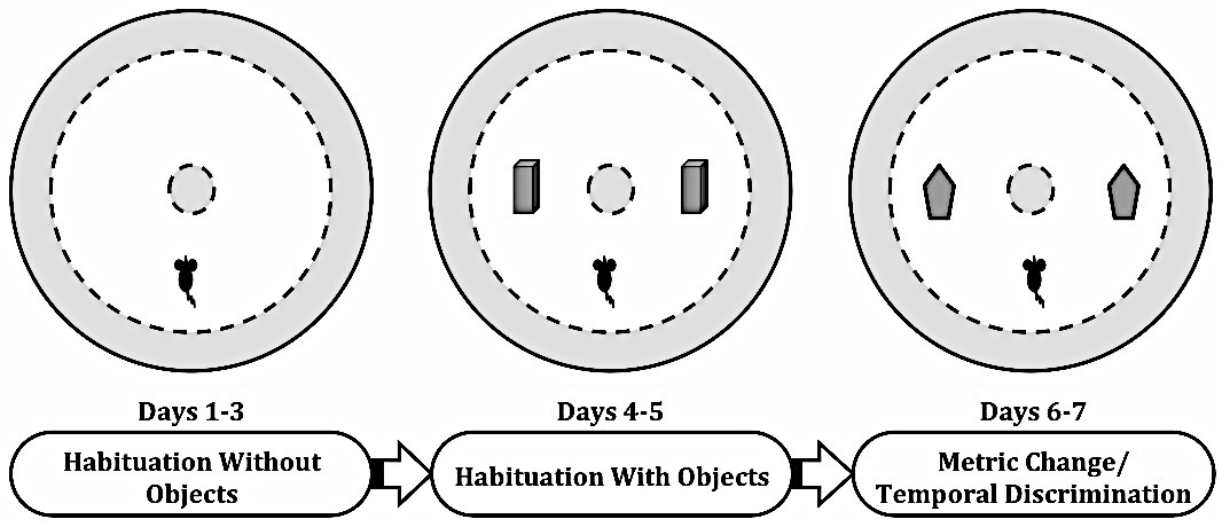

Figure II.1 General Experimental Design

Mice were habituated to the testing environment for a total of 5 days before behavioural testing. On days 1-3 the animals were handled then placed in the arena for a total of 5 min. On days 4 and 5 they were exposed to objects for 5 min in the arena. On days 6 and 7 behavioural testing was carried out using different objects than those used on habituation days 4 and 5. The shaded area near the walls represents the perimeter. The white area and shaded circle in the center represent the open field. The mouse must cross the center circle to count as a crossing.

Locomotor activity. Total distance travelled, thigmotaxis and the number of center crossings were measured and calculated by the computer every minute across a 5-min period in the circular arena without the presence of objects.

Metric spatial processing task. The experimental arrangement for the arena and objects in the metric spatial processing task is shown in **Figure II.2A** and is as described previously with slight modifications (Goodrich-Hunsaker et al., 2005; Hunsaker et al., 2009). The mouse was placed in the arena, facing the wall, halfway between a set of two identical objects placed 40 cm apart. It was then allowed 15 min to freely explore the environment, objects, and distal environmental cues, including large markings pasted on four sides of the walls. As the animals habituated their exploration decreased over the 15-min period. At the end of the 15-min habituation session the mouse was removed and placed in a holding cage for a 5-min

intercession. During this time the objects were moved closer to each other so that the distance separating them was reduced by half to 20 cm. The mouse was then reintroduced to the arena and allowed to freely explore for a 5-min test session. Given the change in spatial relationship between objects, it was expected that exploration would increase. The same set of objects was used for all mice tested on this task. The arena and objects were cleaned with 70% isopropyl alcohol during the intercession periods and between mice.

Temporal order discrimination task. The paradigm employed for this task is shown in **Figure II.2B**. Three sets of pairs of identical objects different from those used in the metric task were used as stimuli and were always placed 40 cm apart in the arena. The mouse was placed in the arena between the first pair of objects and allowed to freely explore for 5 min, and then removed for a 5-min intercession during which the second pair of objects were exchanged in place of the first pair. The mouse was brought back for a second 5-min habituation session then removed for a 5-min intercession, and the third pair of objects were exchanged in place of the second pair. At the end of the third habituation session the mouse was removed for a final 5-min intercession, and one of the third identical pair of objects was removed and exchanged with one from the first identical pair. The mouse was then reintroduced to the arena for a test session and was allowed to freely explore for a 5-min period. The arena and objects were cleaned with 70% isopropyl alcohol during the intercession periods and between mice. The order of the metric spatial and temporal order processing tasks was randomized so that half of the WT and *Fmr1*$^{-/y}$ mice received each task first.

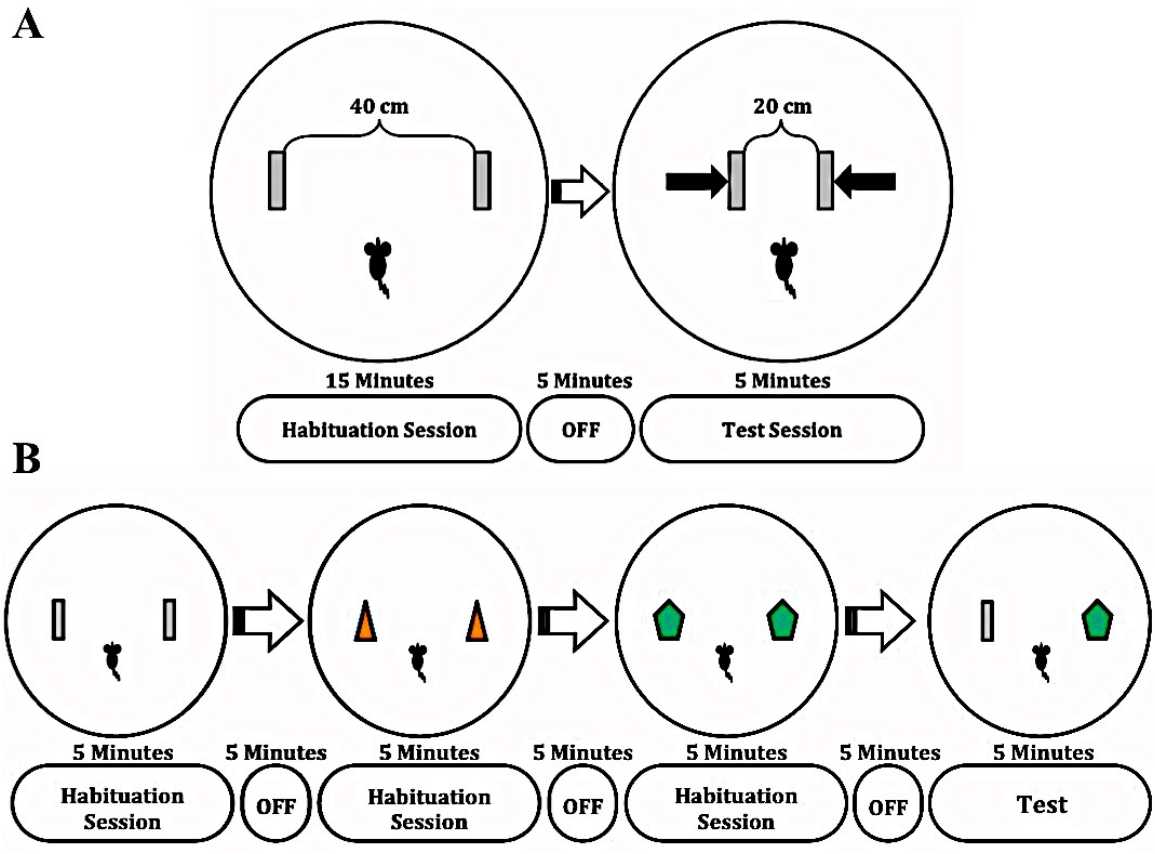

Figure II.2 The Metric Spatial Processing and Temporal Order Discrimination Tasks
(A) In the metric change task a mouse is allowed to freely explore the environment for 15 min with an identical pair of objects separated by 40 cm. The mouse is then removed for a 5 min intercession, during which the distance separating the objects is reduced by one half to 20 cm. Following the intercession period the mouse is brought back for a 5-min test session and allowed to freely explore the environment. **(B)** In the temporal discrimination task the mouse is introduced to 3 different identical pairs of objects and allowed to explore each for a period of 5 min followed by a 5-min intercession before the next pair. The second pair of objects serves as a distractor. For the test session one of the third pair of objects is exchanged for one of the first pair, and the mouse is allowed to freely explore the objects for 5 min.

Dependent Measures and Statistical Analyses

The metric change task assesses the animal's ability to recognize a change in the spatial relationship between objects in the environment. Hence, the dependent variables recorded from this task were the total distance travelled by the mice, and times spent in the open field vs. the perimeter. Distance travelled and times spent in the open field vs. the perimeter were

summarized in 5-min periods during the 15-min habituation period to enable comparison with the 5-min test session (Goodrich-Hunsaker et al., 2005, 2008; Hunsaker et al., 2009). A 2-way (genotype X session) repeated measures analysis of variance (ANOVA) was performed on the exploration data during the habituation session. An exploration ratio was calculated to compare between the test session and the last 5 min of the habituation session (Goodrich-Hunsaker et al., 2005). The ratio was calculated as: [(exploration distance during the 5-min test session)/(exploration distance during the 5-min test session + exploration distance during the last 5 min of the habituation session)]. A ratio >0.5 indicates increased exploration during the 5-min test session, whereas a ratio ≤0.5 indicates decreased exploration (or continued habituation).

The temporal discrimination task assesses the animal's recall of objects over time. Hence, the time spent exploring each object was recorded as the dependent variable. Object exploration was recorded in 0.5 s increments and was defined as the mouse actively sniffing or touching the object with its nose, vibrissae, or forepaws. Being located near the objects without active interaction with them was not considered exploration. For this task only animals not showing a preference for objects during the habituation sessions were included in the analysis. An exploration ratio was calculated to compare between the time spent exploring object 1 and object 3 during the test session. The ratio was calculated as: [(time spent exploring object 1 – time spent exploring object 3)/(time spent exploring object 1 + time spent exploring object 3)]. Mice typically show a preference in exploring object 1 compared to object 3. A score close to +1 indicates more time spent exploring object 1. A score close to -1 indicates more time spent exploring object 3. A score near 0 indicates equal exploration of both objects and a failure to detect the temporal order in which the objects were presented (Hunsaker et al., 2010). To

confirm the ratio was statistically significant from zero, a one-sample *t*-Test was calculated for each genotype.

For pair-wise comparisons, a Student's *t*-Test was employed. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using the Statistica 7.1 analytical software (StatSoft Inc., Tulsa, OK, USA). All data are presented as mean ± S.E.M.

Results

Fmr1$^{-/y}$ Mice Exhibit Hyperactivity and Decreased Thigmotaxis in the Open Field

A number of behaviours in the open field were examined to assess motor function, including total distance travelled, number of times the mouse crossed the centre, and time spent in the centre of the arena (open field) vs. the perimeter. As we have previously reported (Eadie et al., 2009), *Fmr1*$^{-/y}$ mice travelled significantly more during the open field test than their WT littermates (WT: 14 ± 0.32 m; *Fmr1*$^{-/y}$: 17.98 ± 0.22 m; $p < .001$) (**Figure II.3A**). In addition, the number of times *Fmr1*$^{-/y}$ mice crossed the centre was also significantly higher than WT (WT: 72.5 ± 1.25; *Fmr1*$^{-/y}$: 170.5 ± 8.50; $p < .001$) (**Figure II.3B**). Furthermore, *Fmr1*$^{-/y}$ mice spent more time in the centre of the open field (WT: 109.30 ± 2.36 s; *Fmr1*$^{-/y}$: 147.15 ± 1.22 s; $p < .001$) and less time in the perimeter (WT: 185.36 ± 2.36 s; *Fmr1*$^{-/y}$: 146.55 ± 1.22 s; $p < .001$), indicating decreased thigmotaxis (**Figure II.3C**). These results confirm previous findings of hyperactivity in *Fmr1*$^{-/y}$ mice.

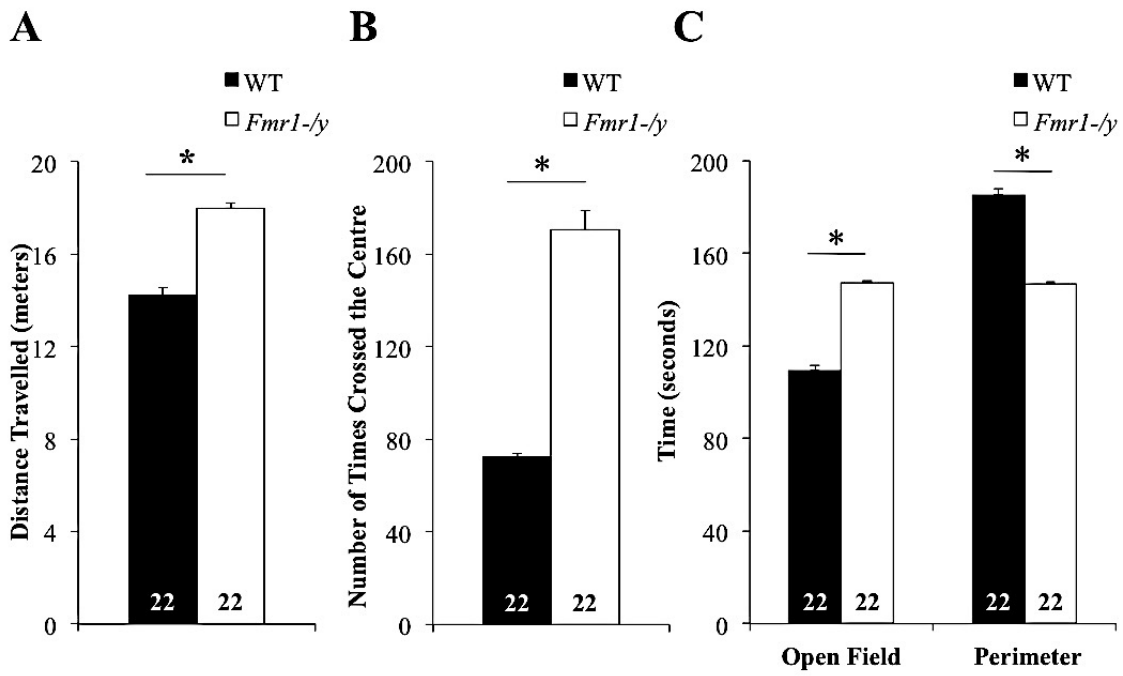

Figure II.3 *Fmr1*$^{-/y}$ Mice Show Hyperactivity and Reduced Thigmotaxis
Locomotor activity was measured and collected by a computer every minute across a 5-min period in the testing arena without the presence of objects **(A)** *Fmr1*$^{-/y}$ mice travel more distance in the open field than WT. **(B)** *Fmr1*$^{-/y}$ mice cross the centre of the open field more than WT. **(C)** *Fmr1*$^{-/y}$ mice spend more time in the open field and less time in the perimeter (decreased thigmotaxis) than WT. * indicates significant difference set at $p < 0.05$.

Fmr1$^{-/y}$ Mice Present with Impaired Performance in Metric Spatial Processing Task

The metric spatial processing task was performed following five days of habituation to the behavioural apparatus. In this task the distance between the objects was decreased between the habituation and the test sessions. To analyze the 15-min habituation period a two-way, repeated measures ANOVA with genotype as the between-group factor and distance travelled (in m) during the three 5-min time periods as the repeated within-group factor was used. A main effect of genotype was revealed ($F_{(1,23)} = 11.77$, $p < 0.001$), reflecting the hyperactivity observed in *Fmr1*$^{-/y}$ mice. However, environment exploration as measured by distance travelled in the arena was significantly reduced over the three time periods in both groups ($F_{(2,46)} = 32.90$, $p < 0.001$),

indicating habituation to the arena. This data suggests that despite the hyperactivity observed in *Fmr1*$^{-/y}$ mice, they show a similar habituation pattern across the 15-min habituation period.

Changes in exploratory behaviour between the final 5-min period during habituation and the 5-min test session were expressed as exploration ratio scores. As expected, WT animals showed increased environment exploration during the 5-min test session in comparison to the final 5-min period during the habituation session after the objects were repositioned (0.62 ± 0.05), indicating recognition of change in the spatial relationship between the objects. In contrast, the ratio calculated for *Fmr1*$^{-/y}$ mice was significantly reduced in comparison to WT as they presented with similar exploration activity during the 5-min test session as during the final 5-min period during the habituation session (0.52 ± 0.02; $p < 0.05$). This data suggests either the mice were unable to detect the change, or they were continuing to habituate to the environment (**Figure II.4A**).

To examine the exploratory strategy employed by the mice in assessing the spatial relationship between objects the time spent between the open field and the perimeter was analyzed as a proxy measure. Interestingly, although *Fmr1*$^{-/y}$ mice did not increase their exploratory activity as measured by distance travelled during the test session, they did present with a significant change in how much time they spent between the open field and the perimeter. While WT mice spent an equal amount of time between the open field and the perimeter, *Fmr1*$^{-/y}$ mice spent a significantly greater amount of time in the open field during the 5-min test session where the objects were located (Student's t-Test $p < 0.001$) (**FigureII.4B**). Overall, these results suggest that *Fmr1*$^{-/y}$ mice may have recognized a change in the environment, but the deficit was in the exploration strategy.

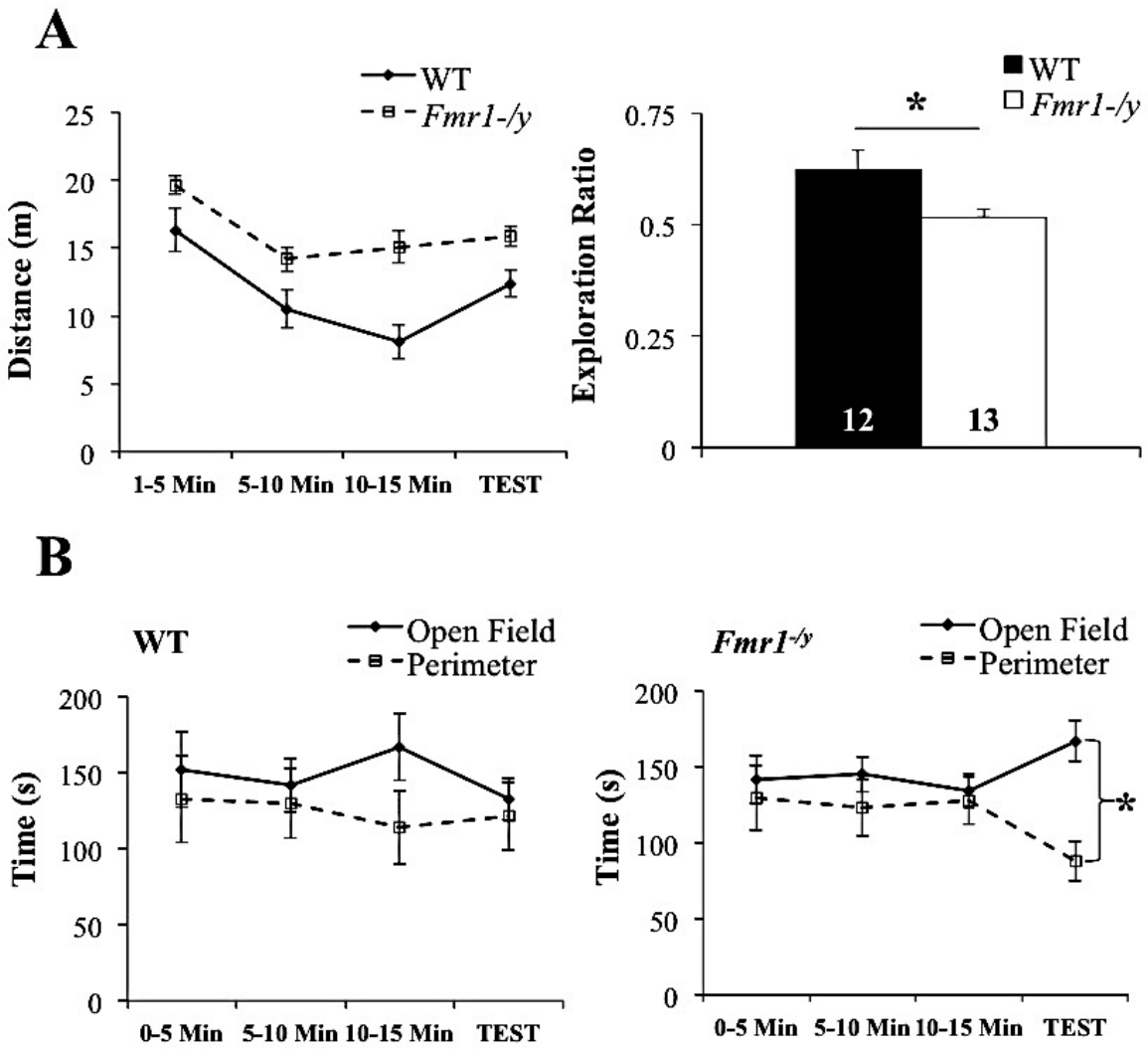

Figure II.4 *Fmr1*$^{-/y}$ Mice Exhibit Performance Deficits in the Metric Spatial Processing Task
(A) WT mice progressively travel less during the 15-min habituation period reflecting familiarity with the environment and engage in increased exploration during the test session reflecting recognition of the metric change. In contrast to WT, *Fmr1*$^{-/y}$ mice travel less during the habituation session but do not engage in re-exploration during the test session, indicating lack of recognition of the metric change (or continued habituation). **(B)** As an exploratory strategy, WT mice spend an equal amount of time between the open field where the objects are located and the perimeter. In contrast, *Fmr1*$^{-/y}$ mice on spend an equal amount of time between the open field and the perimeter during the habituation session, but significantly spend more time in the open field near the objects than the perimeter after the metric change takes place. * indicates significant difference set at $p < 0.05$.

Fmr1$^{-/y}$ Mice Perform Similar to WT in the Temporal Order Discrimination Task

In order to control for the possible confounding factor of object preference in interpretation of exploration ratios, individual animals that showed preference for either object 1 or object 3 as

indicated by comparison of how much time was spent exploring the object during the habituation sessions were eliminated. As shown in **Figure II.5A**, when only animals showing equal preference for both objects were included, it was noted that *Fmr1*$^{-/y}$ mice spent significantly less time exploring the objects than their WT littermates for object 1 (WT: 88.48 ± 15.53 s; *Fmr1*$^{-/y}$: 49.40 ± 5.87 s; $p < 0.05$) and object 3 (WT: 89.83 ± 13.57 s; *Fmr1*$^{-/y}$: 54.87 ± 8.81 s; $p < 0.05$). Performance during the test session indicated that WT mice preferred to explore object 1 (Object 1: 59.50 ± 12.40 s; Object 3: 26.53 ± 6.33 s, $p < 0.05$), as expected. Despite the noted differences observed in exploration activity during the habituation sessions, *Fmr1*$^{-/y}$ mice also preferred to explore object 1 (Object 1: 34.14 ± 5.09 s; Object 3: 19.26 ± 2.89 s, $p < 0.05$). The exploration ratio scores obtained from the test session indicate that despite the difference in time spent exploring the objects between genotypes, both WT and *Fmr1*$^{-/y}$ mice display similar performance in that they prefer to explore the object 1 than object 3 (WT: 0.34 ± 0.08; *Fmr1*$^{-/y}$: 0.26 ± 0.09; $p = 0.22$) (**Figure II.5C**). A one-sample *t*-test for WT and *Fmr1*$^{-/y}$ mice revealed that both genotypes had exploration ratios that were significantly greater than zero (WT: $p < 0.01$; *Fmr1*$^{-/y}$:$p < 0.05$), confirming that *Fmr1*$^{-/y}$ mice did not have performance deficits in this task.

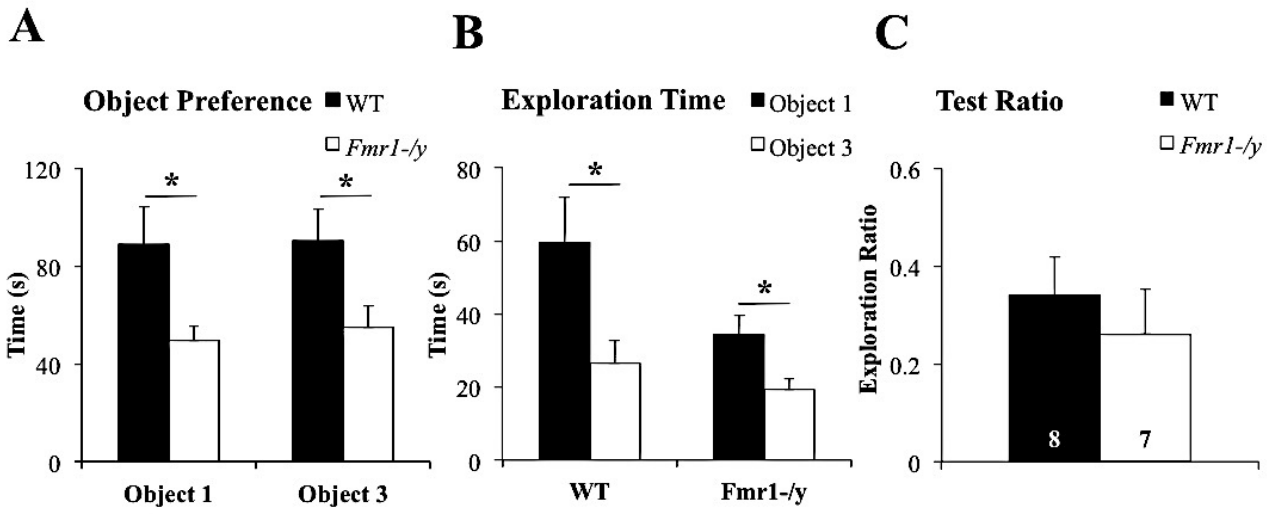

Figure II.5 *Fmr1*$^{-/y}$ Mice Perform Similar to WT in the Temporal Order Discrimination Task
(A) Animals included in the analysis did not have an object preference during the habituation sessions for object 1 or object 3. However, *Fmr1*$^{-/y}$ mice spent less time exploring objects 1 and 3 than their WT littermates. **(B)** WT and *Fmr1*$^{-/y}$ mice spend more time exploring object 1 than object 3 during the test session. **(C)** Ratio of exploration between object 1 and object 3 indicates both WT and *Fmr1*$^{-/y}$ mice prefer to explore object 1 than 3. * indicates significant difference set at $p < 0.05$.

Discussion

The FXS mouse model has previously been shown to exhibit deficits in the MWM, a task that examines visual/spatial capacities to identify a hidden platform (D'Hooge et al., 1997; Paradee et al., 1999; Gantois et al., 2001; Eadie et al., 2009). Mice lacking the *Fmr1* gene show deficits in hippocampal synaptic plasticity, the model for learning and memory. Published studies from our group and others report NMDAR-dependent LTP deficits in the DG (Eadie et al., 2010; Yun and Trommer, 2011; Bostrom et al., 2013; Franklin et al., 2014a, 2014b), but not in the CA1 subregion of the hippocampus (Bostrom et al., 2013). These LTP deficits in the DG correlate with deficits in context discrimination tasks that require a normal capacity for pattern separation (McHugh et al., 2007; Eadie et al., 2010; Franklin et al., 2014a).

The results presented in this study provide evidence for selective hippocampus-dependent behavioural deficits in the *Fmr1*$^{-/y}$ mouse model. The metric spatial processing task is DG-dependent where the animal is examined for their ability to detect a change in the relationship between objects in the environment (Goodrich-Hunsaker et al., 2005; Hunsaker et al., 2009). When rodents are placed in a new environment they begin with an active bout of exploration to learn about the objects and their placement in relation to each other, after which exploration decreases as the animals become familiar with their setting. If the objects are rearranged in a way such that their relationships to each other changes (i.e., in distance or angles), the animals will re-explore the environment once again as they did before (Wilz and Bolton, 1971; Poucet et al., 1986).

Previous studies used object exploration as the dependent measure, with reduced exploration during the habituation phase indicating familiarity with the objects, and increased exploration during the test phase indicating recognition of change in object distance (Goodrich-Hunsaker et al., 2005; Hunsaker et al., 2009). However, spatial processing is done through environment exploration and movement between the perimeter and the open field where the objects are located to assess distances between the objects, as well as the distance between the wall and the objects, and this ability is independent of the objects' identities (Gallistel, 1993; Kesner et al., 2004). The use of object exploration as a proxy may not be appropriate for testing the animal's processing of spatial relationships between objects in the environment. Familiarity with the environment leads to reduced exploration during the habituation phase, especially in the last 5 min of the session, and recognition of change in the spatial relationship between objects would be indicated with increased exploration of the environment. Hence the use of distance travelled as the chosen proxy to assess detection of change.

WT mice tested on the spatial processing task reduced their levels of exploration progressively during the habituation session, reflecting increased familiarity with the environment, as expected. This was followed by higher levels of exploration during the test session after the objects were brought closer to each other, reflecting recognition of change in the spatial relationships within the testing arena. In contrast, *Fmr1*$^{-/y}$ mice reduced their levels of exploration during the habituation period, but did not elevate their exploration activity after the metric change, which could indicate continued habituation or lack of recognition that a change had occurred (**Figure II.4A**).

The finding that WT mice spend an equal amount of time between the perimeter and the open field during the habituation and the test sessions could reflect a strategy the animals employ as they explore the spatial relationship between objects in the testing environment. To identify spatial relationships between objects in a setting the animal needs to move back and forth between the objects in the open field and between the objects and the walls at the perimeter. The results reported here showed that during the test period WT mice increased their exploratory activity and utilized a similar strategy as when they were introduced to the arena during the habituation period, in which they spend an equal amount of time moving between the open field and the perimeter. In contrast, *Fmr1*$^{-/y}$ mice may have sensed a change in the environment as evident by their change in where they distribute their exploration time (i.e., more in the open field where the objects were repositioned), but may be unable to engage in re-exploration due to deficits in the relevant neural networks in the DG that the metric change task is dependent on.

In contrast to the deficits observed in the DG-dependent spatial processing task, *Fmr1*$^{-/y}$ mice performed at WT levels in the CA1-dependent temporal order discrimination task. This finding is inconsistent with one recent report providing evidence for impaired performance by

Fmr1$^{-/y}$ mice in the same task (Franklin et al., 2014a). Despite the hyperactivity of *Fmr1*$^{-/y}$ mice tested in this study, they did spend significantly less time exploring the objects in comparison to their WT littermates. Hyperactivity and reduction of object exploration time in comparison to WT, combined with possible methodological differences (e.g., the number days the animals were habituated before testing) may contribute to this inconsistency between studies.

Furthermore, although a number of studies have reported a significant, albeit small, enhancement of type I metabotropic glutamate receptor (mGluR)-mediated LTD in the CA1 of the *Fmr1*$^{-/y}$ hippocampus (Huber et al., 2002; Hou et al., 2006; Nosyreva and Huber, 2006; Lüscher and Huber, 2010; Choi et al., 2011; Westmark et al., 2011) behavioural studies of CA1-dependent tasks such as the Morris Water Maze (MWM) have been conflicted (D'Hooge et al., 1997; Paradee et al., 1999; Eadie et al., 2009). Indeed, the deficits observed in *Fmr1*$^{-/y}$ mice in the MWM were subtle and reported evidence suggests they may be more due to strain effects than to loss of FMRP (Paradee et al., 1999). The relevance of enhanced mGluR-LTD in the CA1 region to behaviour remains to be uncovered.

The data reported in the present study lend correlative support to the available evidence of selectively robust NMDAR-dependent synaptic plasticity deficits in the DG in absence of FMRP (Paradee et al., 1999; Eadie et al., 2010; Yun and Trommer, 2011; Bostrom et al., 2013; Franklin et al., 2014a, 2014b). *Fmr1*$^{-/y}$ mice displayed deficits in a DG-dependent task involved in detecting changes in spatial relationships, but were performing at WT levels in the CA1-dependent temporal order discrimination task. The intellectual impairments observed in FXS patients are thought to arise from deficits in spatial and temporal processing (Simon, 2008). The findings reported here provide insight into the extent of these deficits in the *Fmr1*$^{-/y}$ mouse model and where they arise in mouse behaviour.

Experimental Limitations and Pitfalls

Hyperactivity in *Fmr1*$^{-/y}$ mice has previously been observed in behavioural tasks performed by our group (Eadie et al., 2009) and is a prominent feature of this mouse model (The Dutch-Belgian Fragile X Consortium, 1994). Thus, dependent measures utilized to interpret performance in hippocampus-dependent tasks such as those presented in this chapter must account for locomotor activity to possibly confound interpretations. Moreover, decreased thigmotaxis may also influence mouse performance and obscure possible deficits or lead to false-positive results.

The dependent measures employed in the metric change spatial processing task were distance travelled and time spent in the perimeter vs. the open field as proxies for detecting spatial change and the strategy for exploration, respectively. When rodents sense a change in the context of their environment they increase their exploration activity (Wilz and Bolton, 1971; Poucet et al., 1986). Hyperactivity in *Fmr1*$^{-/y}$ mice may pose a challenge for the interpretation of the reported findings here. However, this is limited by restricting the analysis of exploratory behaviour within each genotype, comparing exploration during the last 5 min of the 15-min habituation session to that during the 5-min test session. The results suggest that *Fmr1*$^{-/y}$ mice either did not recognize the spatial change or were still continuing to habituate.

A question that presents itself here is whether hyperactivity in *Fmr1*$^{-/y}$ mice makes the design of this task appropriate to identify performance deficits. If the animals are not able to become familiar with their environment during the habituation session due to their hyperactivity, performance during the test session does not necessarily allow for a definitive conclusion with regards to the mice's spatial processing abilities. Although a valid concern, analysis of exploratory performance of *Fmr1*$^{-/y}$ mice during the 15-min habituation session provides

evidence that the animals significantly reduced their movement over time in a similar fashion to WT ($p < 0.001$). Furthermore, the finding that *Fmr1*$^{-/y}$ mice altered their exploratory strategy during the 5-min test session, spending more time in the open field where the object are located, as opposed to spending an equal amount of time between the open field and the perimeter as they did during the 15-min habituation session, suggests the mice did sense a change in the environment but were unable to initiate re-exploration as a plausible interpretation of the results.

Moreover, *Fmr1*$^{-/y}$ mice explored the arena during the habituation session in a similar fashion as WT animals. This suggests that exploration at the initial introduction to an environment begins with spending an equivalent amount of time between the open field where the objects are located and the perimeter, and that the change observed in *Fmr1*$^{-/y}$ mice during the testing session may be due to their recognition of a change, but an inability to identify what *type* of change it was, i.e., spatial, and therefore an inability to reinitiate the appropriate exploration strategy to process it.

An issue that may be relevant to the behavioural tests presented in this chapter relates to the experimenter. A recent study reported findings that exposure of mice and rats to male experimenters, or clothing worn by men, induced a quantifiable stress response that was not evident when the animals were exposed to female experimenters (Sorge et al., 2014). This may be a confound, especially in the case of *Fmr1*$^{-/y}$ mice as there is evidence suggesting that the stress response is altered in *Fmr1*$^{-/y}$ mice (Lauterborn, 2004; Markham et al., 2006). More strikingly, the 'male observer' stress effect was demonstrated to lead to plasma CORT increases that were equivalent to those occurring after 15-min of restraint stress (Sorge et al., 2014). As will be shown in the next chapter, in comparison to WT littermates *Fmr1*$^{-/y}$ mice have a heightened response to 15-min of acute stress, which poses a significant challenge to the

experiments reported here. However, the male observer effect was shown to depend on the exclusive presence of male scent, and the simultaneous presence of a female scent abolished this effect (Sorge et al., 2014). A male and a female experimenter, which theoretically should ameliorate the male observer effect, conducted the behavioural experiments presented in this chapter. However, this may need to be confirmed by replicating the experiments with the exclusive presence of a female experimenter and assessing whether this is a contributing factor to how the animals perform.

Despite this being a possible pitfall, it should be noted that WT mice performed in the metric change spatial processing and the temporal discrimination tasks as expected (Goodrich-Hunsaker et al., 2005, 2008; Hunsaker et al., 2009, 2010). Hence, replication of the behavioural deficits observed in *Fmr1*$^{-/y}$ mice by exclusively male and exclusively female experimenters may provide insight into the possibility they may be due to either an elevated stress response from *Fmr1*$^{-/y}$ mice or due to neural network communication impairments in the hippocampus, both of which would nevertheless be consequential to absence of FMRP.

Behavioural studies are challenging due to the number of variables that could influence experimental results. However, if conducted and analyzed carefully and replicated successfully, they offer a valuable characterization tool for translational studies and testing of therapeutic strategies.

CHAPTER III. Enhanced Corticosteroid Signalling Alters Synaptic Plasticity in the Dentate Gyrus in Mice Lacking the Fragile X Mental Retardation Protein

A version of this chapter was accepted for publication in the Journal Neurobiology of Disease

Introduction

Clinical symptoms of FXS include heightened anxiety and response to novel situations, and male patients with FXS have been reported to present with a prolonged elevation of salivary cortisol levels after exposure to social stress, indicating that the HPA-axis is not well regulated in these individuals (Wisbeck et al., 2000; Hessl et al., 2006). *Fmr1*$^{-/y}$ mice mimic some facets of the behaviours observed in the clinical FXS population. In particular, these mice present with abnormal anxiety responses and impaired social interactions (Spencer et al., 2005; McNaughton et al., 2008; Eadie et al., 2009). In part, these behavioural changes may reflect that *Fmr1*$^{-/y}$ mice exhibit a prolonged elevation in CORT following exposure to stress (Lauterborn, 2004; Markham et al., 2006). In addition, this altered stress response could further impair learning and memory function in these animals. The hippocampus, a structure critical for learning and memory, is particularly vulnerable to the effects of stress (Kim and Diamond, 2002).

Memory retrieval is impaired at both very low and very high levels of GCs, and the relationship between memory and GC levels may originate from the different roles played by MRs and GRs (Joëls, 2001). Indeed, MRs and GRs play contrasting roles in modulating NMDAR-LTP, the main biological model of learning and memory processes. MR activation facilitates NMDAR-LTP while, conversely, GR activation impairs NMDAR-LTP (Avital et al., 2006; Spyrka and Hess, 2010). Previous studies have shown that FMRP associates with GR

mRNA (Miyashiro et al., 2003) indicating that this protein may be integrally linked with the stress response. The goal of the present chapter is to determine if a change in the stress response in *Fmr1*$^{-/y}$ mice may contribute to impaired hippocampal synaptic plasticity.

Materials and Methods

Animals

Adult male C57Bl/6 *Fmr1*$^{-/y}$ and WT littermate mice (55-65 days) were randomly assigned to control or an acute restraint stress condition. All experiments were initiated between 9:00 and 11:00 AM to minimize the impact of the diurnal variation in circulating CORT. To obtain hippocampal slices, mice were briefly anaesthetized with isoflurane immediately when brought to the lab (control) or following acute stress then quickly decapitated (**Figure III.1**). Their brains were removed directly into oxygenated (95% O_2/5% CO_2), ice-cold normal artificial cerebrospinal fluid (nACSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 $NaHPO_4$, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, and 10 dextrose at a pH of 7.3. A Vibratome 1500 (Ted Pella, Inc., Redding, CA, USA) was used to obtain 350 μm transverse hippocampal slices. The slices were obtained in continuously oxygenated nACSF maintained at 2-4 °C then incubated in oxygenated nACSF at 30 °C and allowed to recover for a minimum of 1 hour prior to conducting electrophysiology experiments. All experiments were carried out in accordance with international standards on animal welfare and guidelines set out by the Canadian Council on Animal Care and the University of Victoria.

Acute Stress Paradigm & Drug Treatments

Experimental animals in the stress conditions were individually placed in a Plexiglas restrain tube (10 cm length, 3 cm diameter; Braintree Scientific Inc., Braintree, MA) situated under a 60-W bulb (File and Peet, 1980; Godsil and Fanselow, 2004) for either 15, 30, or 60 min. Immediately following this period mice were sacrificed for electrophysiology experiments (**Figure III.1**).

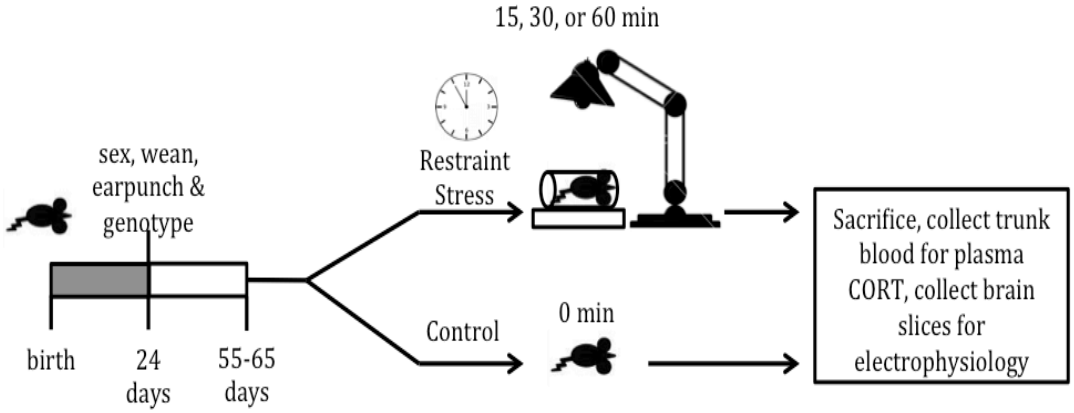

Figure III.1 Timeline for Experimental Protocol
In each experiment animals were raised in litters until day 24 when they were sexed, weaned and placed in group housing with same-sex littermates. At PND 55-65 animals were randomly assigned to one of 4 experimental stress protocols (No acute stress; control, 15-min, 30-min, or 60-min acute restraint stress). Following the acute stress period, animals were immediately sacrificed, trunk blood was collected for serum CORT measurements, and brain slices were used for electrophysiology.

In some experiments mice received subcutaneous injections of either the GR antagonist RU38486 (Sigma, St. Louis, MO) dissolved in 1% Tween 80 (20 mg/kg, volume 2 ml/kg) or the MR antagonist spironolactone (Sigma, St. Louis, MO) dissolved in 0.9% saline (20 mg/kg, volume 2 ml/kg) 1 hour prior to removal from the animal care facility. Previous studies have shown GRs and MRs to be effectively blocked with these concentrations (Avital et al., 2006; Spyrka and Hess, 2010).

Serum Collection & Corticosterone Analysis

Immediately following decapitation, trunk blood was collected from all animals and allowed to clot at room temperature for 15 min. The blood was then centrifuged at 14,000 RCF and the serum portion was collected and stored at -20 °C until CORT analysis was conducted. Serum CORT was measured using an ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) as per the manufacturer's instructions. A coefficient of variance (CV) value of less than 10% from the ELISA results for each set of triplicate measures was required to be included in the final results.

Field Electrophysiology

Field recordings of excitatory post-synaptic potentials (fEPSPs) were made from slices submerged in nACSF using an Axon MultiClamp 700B amplifier connected to a PC running Clampex 10.3 software (Molecular Devices, CA, USA). As described previously (Eadie et al., 2010; Bostrom et al., 2013) fEPSPs were elicited by delivering a 120-µs (10-40 µA) current pulse to the MPP of the DG via a concentric bipolar stimulating electrode (FHC, Bowdoin, ME). fEPSPs were recorded using a borosilicate glass recording electrode (1-2 MΩ) filled with nACSF, and placed in the MPP about 200 µm from the stimulating electrode. In some experiments, fEPSP recordings were also made from pyramidal neurons of the CA1 subfield by stimulating the Schaeffer collateral-commissural fibres and recording from the *stratum radiatum* region. In all cases, an input/output (I/O) function was first constructed by sequentially increasing the magnitude of the current pulse applied and measuring the resulting fEPSP. Following this, the stimulation magnitude was set to elicit a response of ~50% of the amplitude maximum obtained.

fEPSPs elicited every 15 s were recorded until a stable 20-min baseline was established, and then a high frequency stimulus (HFS; 4 trains of 50 pulses at 100 Hz, 30 s apart) was administered in the presence of the $GABA_A$ receptor antagonist bicuculline methiodide (5 μM washed for a minimum of 10 min; Sigma-Aldrich, Oakville, ON, Canada) to reduce tonic inhibition in the DG (Hanse and Gustafsson, 1992; Chapman et al., 1998) and isolate the excitatory component of synaptic transmission in order to induce LTP. Bicuculline methiodide was not required to induce LTP in the CA1 subregion. Following the application of the conditioning train, fEPSPs were recorded every 15 s for 60 min in nACSF to evaluate long-term changes in synaptic efficacy. All waveform analyses were conducted using Axon ClampFit 10.3 software (Molecular Devices, CA, USA).

Immunohistochemistry

A separate cohort of WT and *Fmr1*$^{-/y}$ mice was sacrificed and a series of free-floating brain slices were obtained from them to be processed for GR immunohistochemistry. Mice were brought to the lab from the animal care facility and deeply anaesthetized with urethane (2.5%, i.p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). The brains were removed and left in 4% PFA at 4 °C for 24 hours then transferred to 30% sucrose. Following saturation of the brains in sucrose a Leica VT1000S Vibratome (Nusslock, Germany) was used to obtain 30 μm coronal slices that were collected and stored in a cryoprotectant solution (0.04 M TBS, 30% ethylene glycerol, 30% glycerol) at -20 °C.

For the staining procedure, the slices were thoroughly rinsed in 0.1 M PBS then quenched in 3% H_2O_2 0.1 M PBS for 15 min. They were subsequently thoroughly rinsed again then pre-incubated for 1 hour in 5% normal goat serum (NGS) and 0.25% Triton X-100 in 0.1 M PBS at

room temperature and then incubated for 48 hours at 4 °C with a rabbit polyclonal antibody against GR (1:50; Santa Cruz Biotechnology, Dallas, TX, USA) in 0.1 M PBS containing 5% NGS and 0.25% Triton X-100. After incubation with a biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) for 2 hours, the bound antibodies were visualized using an avidin-biotin-peroxidase complex system (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB; Vectro Laboratories) as a chromogen. The slices were mounted onto 2% gelatin-coated microscope slides, dehydrated in a series of ethanol solutions of increasing concentrations, and cover-slipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

Molecular Studies

A separate cohort of WT and *Fmr1*$^{-/y}$ mice was sacrificed and had their brains microdissected as previously described (Bostrom et al., 2013) to examine hippocampal MR and GR levels. Mice were anaesthetized with isoflurane (Abbott Laboratories, North Chicago, IL, USA), immediately decapitated, and the brain was then rapidly removed, cooled, and placed in cold TBS (0.1M). The DG was isolated as described previously (Farmer et al., 2004; Hagihara et al., 2009; Patten et al., 2013) and frozen in liquid N_2 in a microcentrifuge tube and stored at -80 °C until sonication. Sonication was performed in lysis buffer at 4 °C (20 mM Tris pH8, 137 mM NaCl, 0.1% NP-40, 10% Glycerol, 2 mM EDTA, 1X Halt™ phosphatase and protease inhibitors [ThermoScience, Rockford, IL, USA]) at 10 mL buffer/1 g of tissue. The samples were centrifuged at 14,000 g for 15 min at 4 °C, and supernatants were eluted and stored at -80 °C until processing.

A bicinchoninic acid (BCA) assay (BCA Protein Assay Kit, Pierce, Rockford, IL, USA) was used to determine sample protein concentrations. Equal amounts of protein were used to measure MR and GR levels using an ELISA kit (MyBioSource Inc., San Diego, CA, USA) as per the manufacturer's instructions. A coefficient of variance (CV) value of less than 10% from the ELISA results for each set of triplicate measures was required to be included in the final results.

Statistical Methods

All statistical analyses were performed using Statistica 7.0 software (StatSoft, Tulsa, OK, USA). Group data are presented as mean ± standard error of the mean (SEM). An analysis of variance (ANOVA) was carried out to assess the pattern of response in WT and *Fmr1*$^{-/y}$ mice to different stress periods, followed by a Tukey *post-hoc* test where significant. For pair-wise comparisons, a Student's *t*-Test was employed. Statistical significance was set at $p < 0.05$

Results

Enhanced Corticosterone Response in *Fmr1*$^{-/y}$ Mice Following Acute Stress

Stressors normally trigger activation of the HPA-axis, leading to elevation of GCs in the blood that peak approximately 30 min after the onset of stress in normal animals (de Kloet et al., 2005). To examine HPA-axis activation in *Fmr1*$^{-/y}$ mice we performed ELISA for CORT levels in serum collected immediately following 15-, 30- or 60-min of acute restraint stress. The acute stress paradigm produced a significant elevation of serum CORT concentration in WT ($F_{(3,50)}$ = 11.4, $p < .001$) and *Fmr1*$^{-/y}$ ($F_{(3,36)}$ = 7.3, $p < .001$) mice (**Figure III.2**). *Post-hoc* analyses revealed a significant CORT elevation in WT mice after 30 ($p < .001$) and 60 min ($p < .001$) of restraint stress, but not after 15 min ($p = .22$). In contrast, *Fmr1*$^{-/y}$ mice showed significant

elevations in CORT at all time points tested (15- and 30-min: $p < .001$; 60-min: $p = .02$). Pair-wise comparisons revealed that *Fmr1*$^{-/y}$ mice had significantly higher serum CORT levels after 15 min of restraint stress than their WT littermates ($p = .002$). These data indicate that even short exposure to stressors can produce significant increases in CORT in the *Fmr1*$^{-/y}$ mice.

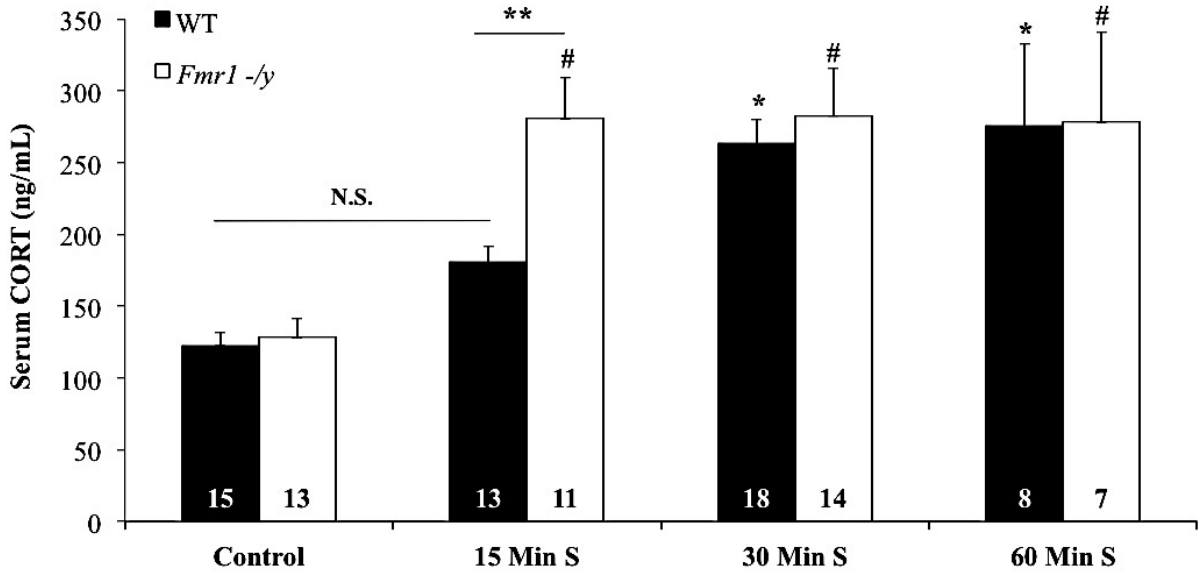

Figure III.2 Animals Lacking FMRP Show Enhanced CORT Levels Earlier Following Acute Stress

CORT analyses were performed using an ELISA on serum isolated from trunk blood. A one-way ANOVA revealed there was a significant elevation in serum CORT after stress in WT and in *Fmr1*$^{-/y}$ mice. Tukey *post hoc* tests revealed that there was only a significant elevation in CORT for the WT animals following 30 and 60 min of acute restraint stress. In contrast, there was a significant increase in CORT levels for all periods of acute stress in *Fmr1*$^{-/y}$ mice. Independent comparisons in each condition revealed a significant difference between genotypes only in the 15-min stress condition. Significance was set at $p < 0.05$. * indicates significant difference from control WT mice. # indicates significant difference from control *Fmr1*$^{-/y}$ mice. ** indicates significant difference between genotypes.

Loss of FMRP Promotes Stress-Induced LTP Deficits in the DG But Not in the CA1

Synaptic transmission in *Fmr1*$^{-/y}$ animals was evaluated by constructing a fEPSP I/O curve in response to a series of ascending stimulus intensities. The slope of the fEPSPs in the DG significantly increased with increasing stimulation [repeated measures ANOVA; $F_{(8,840)} = 292.52$, $p < 0.001$], and there were no significant main effects of genotype ($F_{(1,60)} = 2.06$, $p =$

0.16) (Figure III.3). Similarly, CA1 fEPSPs significantly increased with increasing stimulation ($F_{(8,232)}$ = 320.59, $p < 0.001$), and no significant main effect of genotype was observed ($F_{(1,29)}$ = 0.36, p = 0.55). This data indicates that basal synaptic transmission is not significantly altered in the DG or CA1 of *Fmr1*$^{-/y}$ mice, and that they retain a normal capacity to exhibit single evoked responses in response to synaptic stimulation.

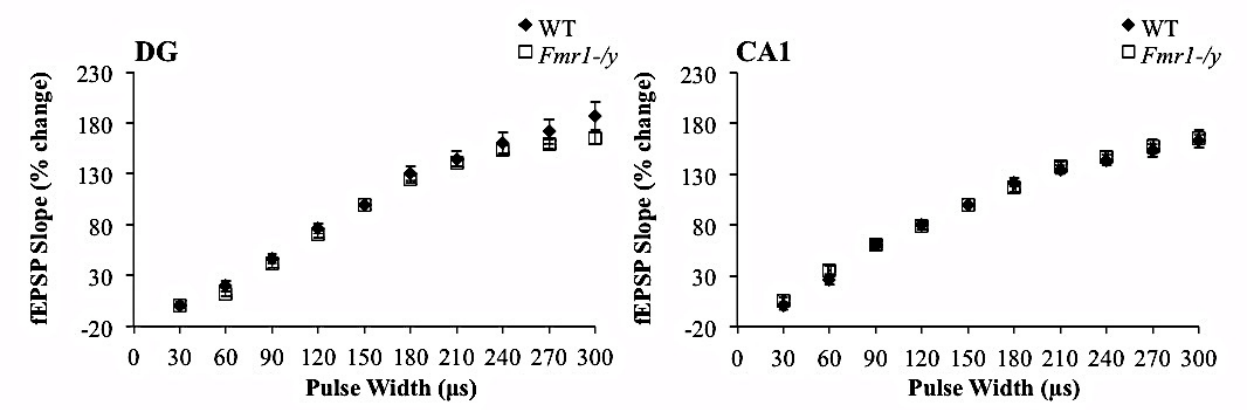

Figure III.3 Normal Basal Synaptic Transmission in the DG and CA1 of *Fmr1*$^{-/y}$ Mice
A stimulus response curve was generated to examine fEPSPs by increasing the pulse width in successive stimulations in the DG and CA1. The slope of fEPSPs significantly increased with increasing stimulation in the DG (left) and the CA1 regions (right). No significant differences were observed between WT and *Fmr1*$^{-/y}$ mice in the DG or the CA1 regions. Significance was set at $p < 0.05$

Acute stress can inhibit hippocampal LTP by reducing NMDA receptor function (Kim et al., 1996; Wang et al., 2006). To determine whether the rapid CORT response in the *Fmr1*$^{-/y}$ mice would impact LTP more severely we examined NMDAR-LTP in both the DG and CA1 regions of these animals following acute stress. As we and others have reported previously (Eadie et al., 2010; Yun and Trommer, 2011; Bostrom et al., 2013; Franklin et al., 2014a, 2014b), *Fmr1*$^{-/y}$ mice normally show significantly lower levels of LTP than their WT littermates (WT: 69.39 ± 8.35%; *Fmr1*$^{-/y}$: 32.77 ± 9.96%; $p < 0.001$) (**Figure III.4**). Acute stress significantly impaired LTP in the DG of both WT ($F_{(3,40)}$ = 13.35, $p < .001$) and *Fmr1*$^{-/y}$ mice ($F_{(3,36)}$ = 7.93, $p < .001$). However, the effect of stress has a different time course between

genotypes. WT mice stressed for 15 min had a non-significant reduction in DG LTP (43.67 ± 7.93%; $p = 0.19$), but as expected, LTP was blocked in WT mice following 30-min of acute stress (-1.52 ± 6.15%; $p < .001$). Extending the stress period to 60 min did not exacerbate deficits in LTP for WT. Instead, LTP levels in those mice were equivalent to levels observed in their control littermates (57.62 ± 12.87%, $p = 0.78$).

In accordance with the more rapid onset of high CORT levels we observed in the *Fmr1*$^{-/y}$ mice, the application of restraint stress for 15 min completely blocked LTP in the *Fmr1*$^{-/y}$ DG (-13.79 ± 8.77%; $p < .01$). *Fmr1*$^{-/y}$ mice that were stressed for 30 min no longer showed a significant LTP impairment (50.32 ± 11.20%; $p = 0.59$), and *Fmr1*$^{-/y}$ mice stressed for 60 min also did not show a significant LTP impairment (40.05 ± 9.97%, $p = 0.96$). These data indicate that the loss of FMRP in the *Fmr1*$^{-/y}$ mice results in acute stress having a more immediate effect on LTP than is normally seen in WT mice (**Figure III.4**).

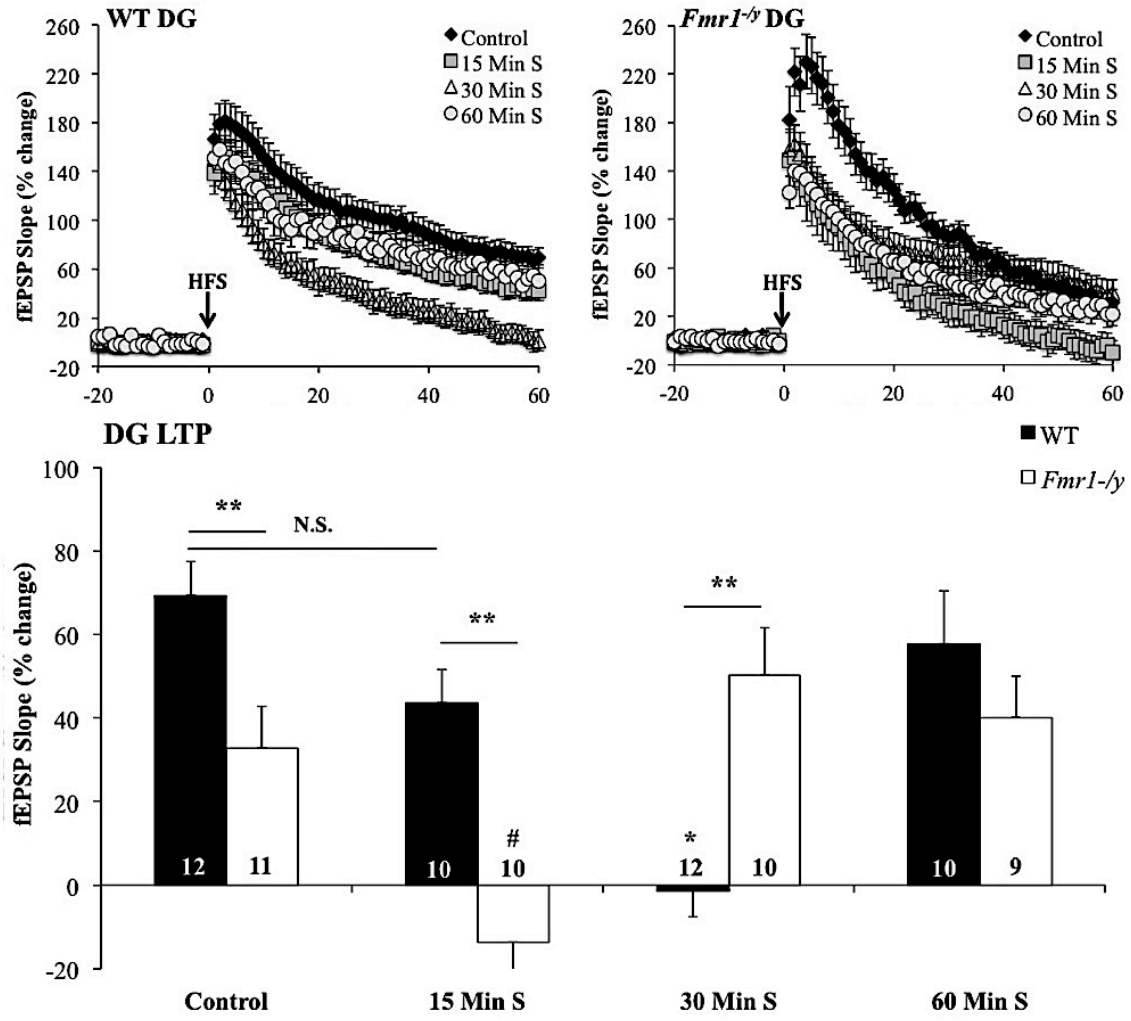

Figure III.4 Loss of FMRP Leads to Shifted Impairment of LTP in the DG Following Acute Stress

A stable 20-min baseline of synaptic response to stimulation was established prior to delivering of the conditioning stimulus (HFS). Control WT animals showed significantly higher levels of LTP than their *Fmr1*$^{-/y}$ littermates as measured 60-min post-HFS. WT mice stressed for 15 min had a reduction of LTP in comparison to the control group, but it was not significant. 30-min stressed mice had a complete impairment of LTP. After 15-min stress, LTP was completely impaired in the DG of *Fmr1*$^{-/y}$ slices. Slices from *Fmr1*$^{-/y}$ mice stressed for 30- and 60-min had equivalent levels of LTP as the *Fmr1*$^{-/y}$ control group, whereas 30- and 60-min of restraint stress had not. Significance was set at $p < 0.05$. * indicates significant difference from control WT mice. # indicates significant difference from control *Fmr1*$^{-/y}$ mice. ** indicates significant difference between genotypes.

In contrast to the dynamic changes in LTP expression in the DG, LTP in the CA1 hippocampal subfield was not significantly impacted by acute stress. WT and *Fmr1*$^{-/y}$ mice had equivalent levels of LTP in in the absence of stress (WT: 45.56 ± 8.43%; *Fmr1*$^{-/y}$: 37.36 ±

10.07%; $p = 0.15$). Exposure to acute stress did not impact LTP in the CA1 subfield in either the 15-min (WT: 44.20 ± 9.71%; *Fmr1*$^{-/y}$: 39.67 ± 5.70%; $p = 0.35$); 30-min (WT: 40.26 ± 6.15%; *Fmr1*$^{-/y}$: 40.36 ± 10.34%; $p = 0.98$) or 60-min (WT: 66.85 ± 19.34%; *Fmr1*$^{-/y}$: 70.01 ± 12.76%; $p = 0.81$) acute stress groups. A one-way ANOVA did not reveal a main effect of stress on either genotype (WT: $F_{(3,31)} = 0.98$, $p = 0.41$; *Fmr1*$^{-/y}$: $F_{(3,23)} = 1.28$, $p = 0.31$). These data suggest that acute stress differentially impacts synaptic plasticity in the mouse hippocampus when immediately examined following stress, regulating LTP in the DG, but not in the CA1 subfield (**Figure III.5**).

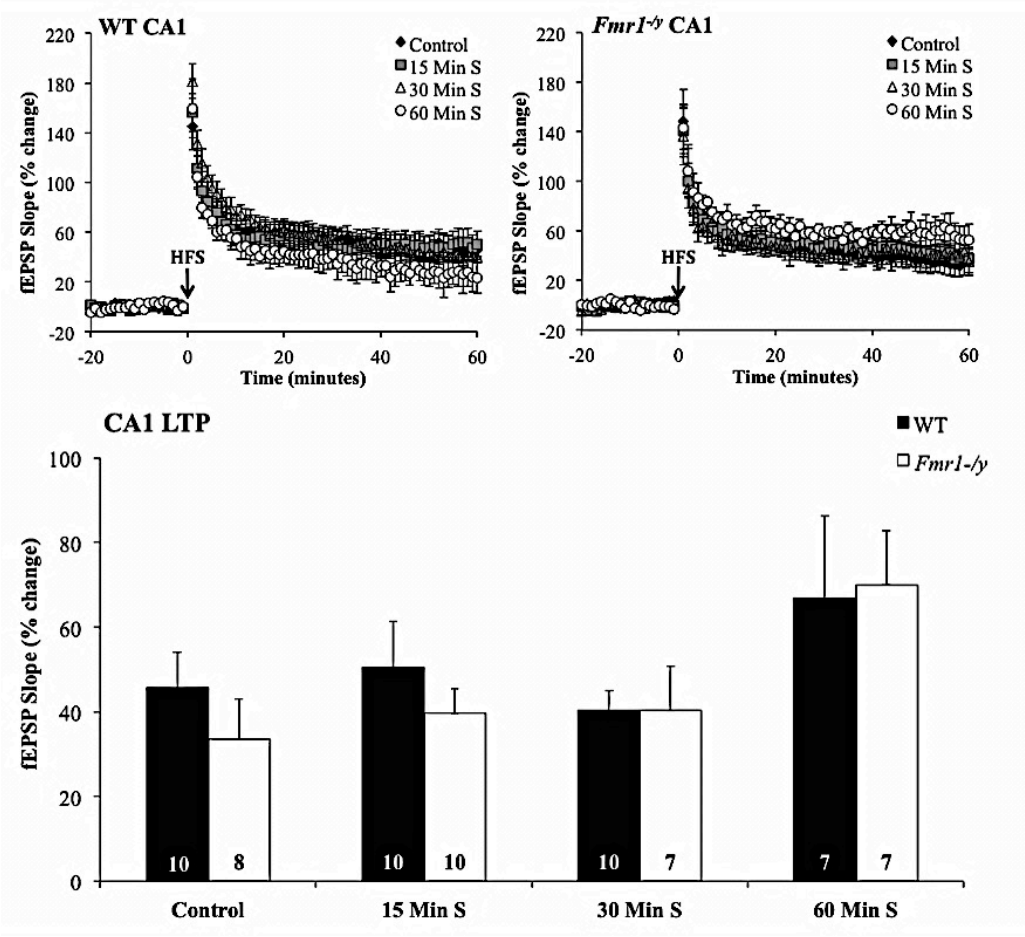

Figure III.5 Acute Stress Does Not Impact LTP in the CA1 of WT or *Fmr1*$^{-/y}$ Mice
A stable 20-min baseline of synaptic response to stimulation was established prior to delivering of the conditioning stimulus (HFS). Control WT and *Fmr1*$^{-/y}$ mice exhibited equivalent LTP levels in the

CA1 as measured 60-min post-HFS. Application of acute stress for 15, 30, or 60 min did not impact LTP levels in either genotype. Significance was set at $p < 0.05$

The GR Antagonist RU38486 Rescues LTP Deficits in the DG of *Fmr1*$^{-/y}$ Mice

Acute periods of stress can negatively impact LTP by activating GR's (Avital et al., 2006; Spyrka and Hess, 2010) and there is a potential role for FMRP in this process as GR mRNA has been shown to associate with FMRP (Miyashiro et al., 2003). To determine whether GRs play a role in the observed effects of acute stress on LTP in the DG, a separate cohort of WT and *Fmr1*$^{-/y}$ mice received subcutaneous injections of the GR antagonist RU38486 1 hour prior to removal from the animal care facility. In WT mice that were not stressed, RU38486 treatment did not impact LTP expression (No Drug: 69.39 ± 8.35% vs. RU38486: 75.99 ± 16.35%). RU38486 treatment alleviated the stress-induced impact on LTP levels in the DG of WT mice stressed for 15 min (No Drug: 43.67 ± 7.93% vs. RU38486: 59.79 ± 9.86%); 30 min (No Drug: -1.52 ± 6.15% vs. RU38486: 64.30 ± 8.91%) or 60 min (No Drug: 57.62 ± 12.87% vs. RU38486: 58.82 ± 16.21%).

Surprisingly, in *Fmr1*$^{-/y}$ mice that were not stressed, RU38486 administration was associated with a significant increase in LTP (No Drug: 32.77 ± 9.96% vs. RU38486: 73.92 ± 5.89%; $p < 0.001$). This "rescue" of LTP expression in *Fmr1*$^{-/y}$ mice was to a level that was indistinguishable to that observed in WT mice in the absence of stress (WT vs. *Fmr1*$^{-/y}$ $p = 0.74$). Administration of the GR antagonist also rescued LTP in *Fmr1*$^{-/y}$ mice following 15 min of stress (62.60 ± 9.17%; $p = 0.56$). However, RU38486 did not have an effect on *Fmr1*$^{-/y}$ mice stressed for 30 min (*Fmr1*$^{-/y}$ No Drug: 32.77 ± 9.96% vs. RU38486: 38.28 ± 11.39%; $p = 0.63$). Interestingly, RU38486 enhanced LTP in the DG of *Fmr1*$^{-/y}$ mice stressed for 60 min (*Fmr1*$^{-/y}$ No Drug: 32.77 ± 9.96% vs. RU38486: 54.97 ± 9.12%; $p < 0.001$) (**Figure III.6**). Overall, these data indicate that LTP deficits in non-stressed *Fmr1*$^{-/y}$ mice, as well as the shift towards earlier

stress induced-impairment of LTP, may reflect enhanced GR activity that occurs in the absence of FMRP.

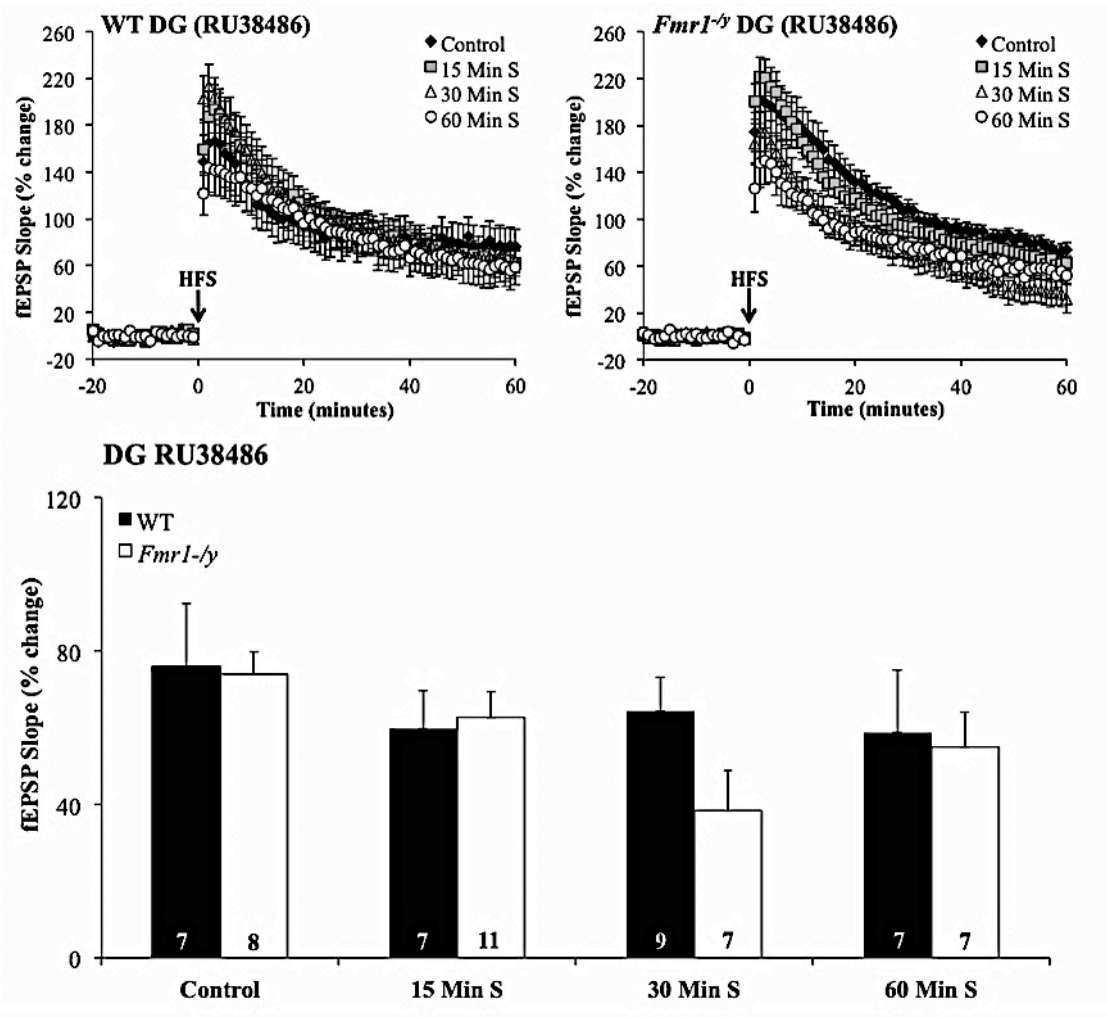

Figure III.6 The GR Antagonist RU38486 Rescues LTP Deficits in the DG of *Fmr1*$^{-/y}$ Mice
A separate cohort of WT and *Fmr1*$^{-/y}$ mice received subcutaneous injections of the GR antagonist RU38486 1 hour prior to removal from the animal care facility. LTP deficits after acute stress WT and *Fmr1*$^{-/y}$ DG slices were rescued by RU38486. LTP under control conditions did not change in WT mice after RU38486 treatment, but was enhanced in *Fmr1*$^{-/y}$ mice to WT levels, thus rescuing deficits in LTP observed in *Fmr1*$^{-/y}$ mice under control conditions. In addition, RU38486 prevented stress-induced block of LTP in *Fmr1*$^{-/y}$ mice stressed for 15 min and enhanced it to WT levels. RU38486 rescued LTP in WT mice stressed for 30 min but did not have an effect on LTP in *Fmr1*$^{-/y}$ mice in this stress group. RU38486 had no effect on LTP in WT mice stressed for 60 min but enhanced LTP in *Fmr1*$^{-/y}$ mice.

LTP Rescue in the DG of Non-Stressed *Fmr1*$^{-/y}$ Mice with the GR Antagonist RU38486 is NMDAR-Dependent

Our group, and others, have previously shown that a reduction in the contribution of NMDARs contributes to the deficits in LTP observed in the DG of *Fmr1*$^{-/y}$ mice (Eadie et al., 2010; Yun and Trommer, 2011) and that NMDAR co-agonists glycine or D-serine can restore LTP in the DG of these mice (Bostrom et al., 2013). Interestingly, NMDARs can also play a role in regulating the effects of acute stress on synaptic plasticity in the hippocampus (Wang et al., 2006). To examine whether blocking GRs is restoring an NMDAR-dependent form of LTP in *Fmr1*$^{-/y}$ mice to WT levels, we bath applied the NMDAR antagonist 2-amino-5-phosphonopentanoic acid (APV; 50 μM) for a minimum of 5 min before and during HFS on slices obtained from *Fmr1*$^{-/y}$ mice that were injected with RU38486 but were not subjected to acute stress. In these animals, LTP was completely blocked, as measured 60 min after HFS (1.67 ± 18.42%; **Figure III.7**). These data suggest that the reduced LTP normally observed in the DG of *Fmr1*$^{-/y}$ mice may be the result of enhanced GR function in the absence of FMRP, leading to reduced NMDAR function.

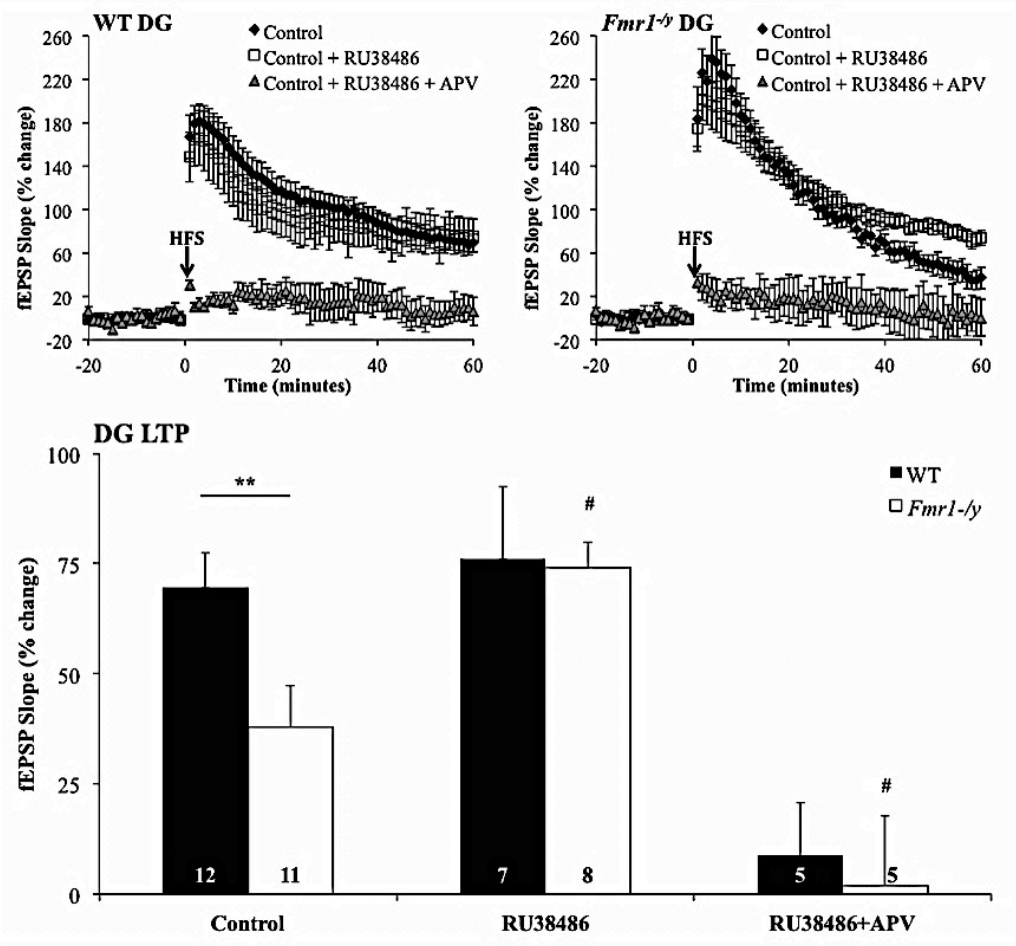

Figure III.7 LTP Rescue in the DG of Non-Stressed *Fmr1*$^{-/y}$ Mice Using the GR Antagonist is NMDAR-Dependent

Bath application of APV (50 μM) 5 min before and during HFS completely blocks LTP in the DG of WT and *Fmr1*$^{-/y}$ mice that were injected with RU38486. Significance was set at $p < 0.05$. * indicates significant difference from control WT mice. # indicates significant difference from control *Fmr1*$^{-/y}$ mice. ** indicates significant difference between genotypes.

GRs Have Equal Density in the WT and *Fmr1*$^{-/y}$ Hippocampus and Have Higher Presence in the DG than the CA1 Hippocampal Subfield

Given the findings for LTP rescue in the DG of *Fmr1*$^{-/y}$ mice and that acute stress impacted LTP in the DG but not the CA1 subfield, we sought to examine whether this was a consequence from differences in GR distribution between the DG and the CA1 regions, and if GR levels are altered in mice lacking FMRP. A separate cohort of WT and *Fmr1*$^{-/y}$ littermate mice was sacrificed, perfused, and their brains were extracted and sliced for GR immunohistochemistry

staining. Pixel intensity ratio measurements between the cell layer and the neuropil revealed that GRs had equal levels in the hippocampus of WT and *Fmr1*$^{-/y}$ mice, but had greater distribution density in the DG than in the CA1 region in WT (DG: 0.74 ± 0.03 vs. CA1: 0.57 ± 0.02; $p < 0.001$) and *Fmr1*$^{-/y}$ mice (DG: 0.77 ± 0.03 vs. CA1: 0.62 ± 0.02; $p < 0.001$) (**Figure III.8**). These data suggest that the differences observed in how stress impacts LTP in the DG and CA1 may be due to higher GR levels in the DG, which make it more sensitive to acute stress. However, the more rapid impact of stress on DG LTP in *Fmr1*$^{-/y}$ mice than their WT littermates may be due to loss of regulation on GR function as opposed to GR levels in the absence of FMRP.

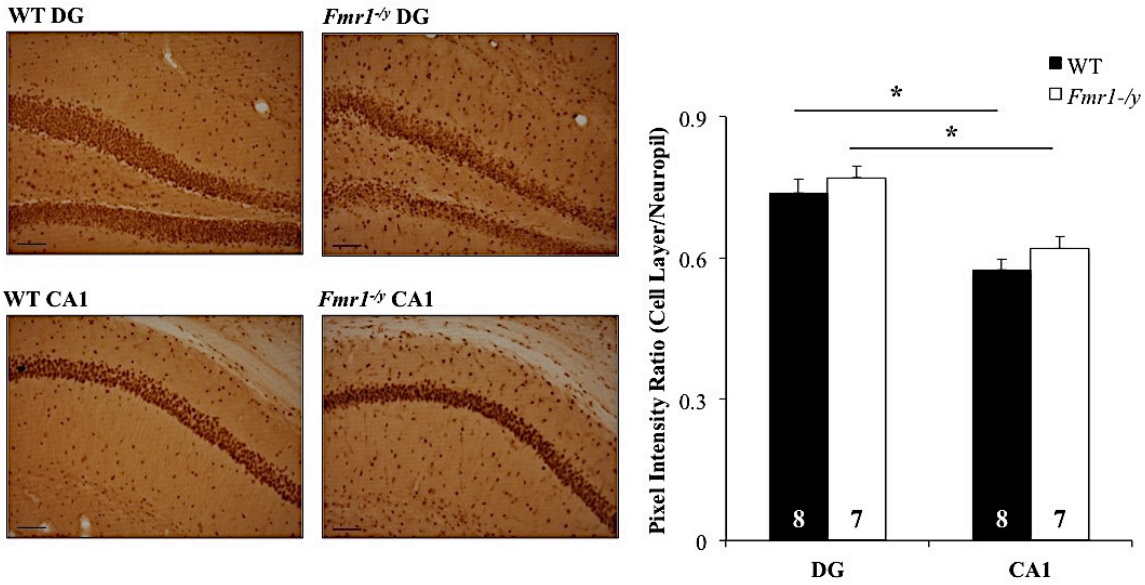

Figure III.8 GRs are Present in Equal Densities in the Hippocampus of WT and *Fmr1*$^{-/y}$ Mice and Have a Higher Density in the DG than in the CA1

GR levels and distribution in the hippocampus were examined by immunohistochemistry. Both WT and *Fmr1*$^{-/y}$ mice show equal GR levels in the hippocampus and similar distribution. Pixel intensity measurements of the ratio between the cell layer and the neuropil show higher density of GRs present in the DG than in the CA1 region of WT (DG: 0.74 ± 0.03 vs. CA1: 0.57 ± 0.02; $P < 0.001$) and *Fmr1*$^{-/y}$ mice (DG: 0.77 ± 0.03 vs. CA1: 0.62 ± 0.02; $P < 0.001$). Scale bars = 20 µm. Significance was set at $p < 0.05$. * indicates significant difference from control WT mice. # indicates significant difference from control *Fmr1*$^{-/y}$ mice.

Loss of FMRP Does Not Impact MR and GR Expression Levels in the DG

Balance between MR and GR levels is important for regulating hippocampal response to stress, the loss of which leads to dysregulation of functional plasticity (Sousa et al., 2008). FMRP regulates protein translation (Bagni and Greenough, 2005) and was previously reported to associate with the mRNA of GRs (Miyashiro et al., 2003). Hence, the observed differences in the impact of acute stress on LTP in the DG of WT and *Fmr1*$^{-/y}$ mice could be a result of an imbalance between MR and GR levels in the DG in absence of FMRP. A separate cohort of WT and *Fmr1*$^{-/y}$ littermate mice was sacrificed and their brains were microdissected to isolate the DG. ELISA measurement of the amount of MR and GR levels to total protein revealed no significant differences of MR (WT: 0.016 ± 0.0015 ng/mg; *Fmr1*$^{-/y}$: 0.021 ± 0.0027 ng/mg; p = 0.18) or GR levels (WT: 13.40 ± 1.10 ng/mg; *Fmr1*$^{-/y}$: 13.82 ± 1.11ng/mg; p = 0.79) in the DG (**Figure III.9**). These results suggest that the shift in stress-induced impairment on LTP in the DG of *Fmr1*$^{-/y}$ mice is likely due to a functional dysregulation of the receptors due to loss of FMRP rather than a structural alteration of their levels.

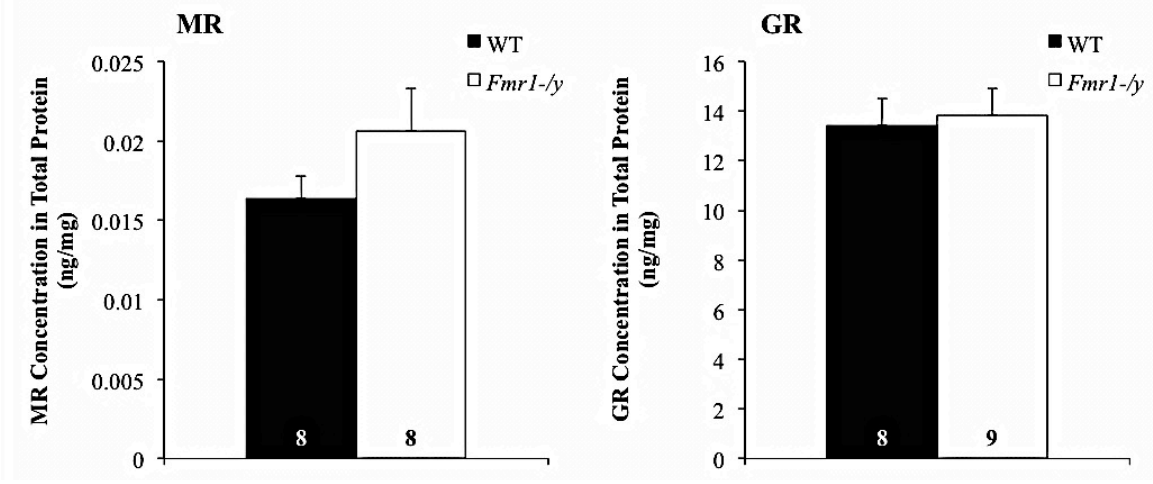

Figure III.9 MRs and GRs are Present in Equal Levels in the DG of WT and *Fmr1*$^{-/y}$ Mice
WT and *Fmr1*$^{-/y}$ littermate mice were sacrificed and their brains were microdissected to isolate the DG. ELISA measurement of the amount of MR and GR levels to total protein revealed no significant differences of MR or GR levels. Significance was set at $p < 0.05$.

Activation of MRs Can Enhance LTP Following Prolonged Stress

Our findings indicate that GR-antagonist administration can restore LTP when acute stress is applied for short periods. However, the increase in LTP observed in mice exposed to acute stress for longer periods suggests that MRs may also play a role. Previously, prolonged stress has been shown to enhance LTP by activating MRs (Spyrka and Hess, 2010), suggesting contrasting roles for MRs and GRs in the stress response. To examine this possibility, MRs were selectively blocked with the antagonist spironolactone. In contrast to the robust LTP observed when only GRs were blocked following 60 min of acute stress, in the presence of spironolactone LTP was completely blocked in WT mice following 60 min of acute stress (6.79 ± 10.18%), indicating a role for MRs in facilitating synaptic plasticity following longer periods of exposure to stress in WT animals. In addition, LTP was reduced in control WT mice treated with spironolactone (No Drug: 69.39 ± 8.35% vs. Spironolactone: 38.92 ± 8.53%) but saw no effect for the drug on LTP in WT mice stressed for 15 min when compared to their non-treated counterparts (No Drug: 43.67 ± 7.93% vs. Spironolactone: 39.11 ± 9.10%), indicating that MRs may be playing a role in facilitating LTP under control conditions. Surprisingly, similar to when treated with the GR antagonist RU38486, WT mice administered spironolactone and then stressed for 30 min showed normal LTP (No Drug: 69.39 ± 8.35% vs. Spironolactone: 67.03 ± 12.25%).

Spironolactone administration prior to stress led to significant LTP reduction in *Fmr1*$^{-/y}$ mice following 30 min (No Drug: 50.32 ± 11.20% vs. Spironolactone: 17.16 ± 11.33%) and 60 min (No Drug: 40.05 ± 9.97% vs. Spironolactone: 20.14 ± 8.32%) of acute stress, suggesting a role for MRs in enhancing LTP under these stress conditions in these animals. Furthermore, *Fmr1*$^{-/y}$ mice in the 15-min stress group that were administered spironolactone did not have stress-induced LTP deficits as their non-administered counterparts (No Drug: -13.79 ± 8.77% vs.

Spironolactone: 34.71 ± 12.48%). In addition, blocking MRs did not have an effect on LTP levels in the DG of control *Fmr1*$^{-/y}$ mice (No Drug: 32.77 ± 9.96% vs. spironolactone: 33.67 ± 9.33%) (**Figure III.10**). Overall, these data indicate acute stress leads to the activation of both MRs and GRs in the DG, but that these receptors have opposing and temporally distinct effects on LTP that manifest earlier in the stress time course observed in *Fmr1*$^{-/y}$ mice.

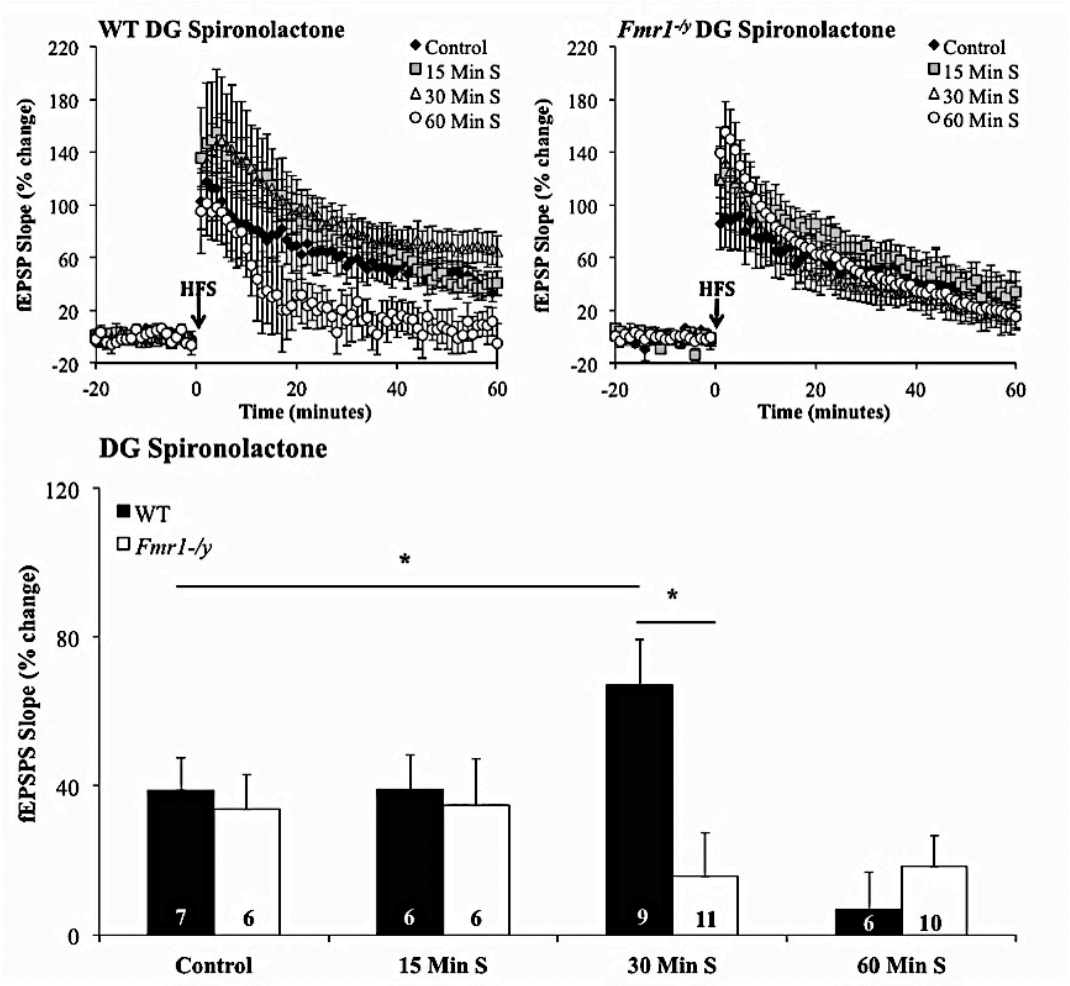

Figure III.10 The MR Antagonist Spironolactone Impairs LTP Enhancement in the DG of WT and *Fmr1*$^{-/y}$ Mice Under Longer Stress Periods

Treatment with spironolactone 1 hour before stress led to reduce LTP levels in WT mice but not in *Fmr1*$^{-/y}$ mice. Spironolactone had no effect on LTP in WT mice stressed for 15 min but it led to recovered LTP in *Fmr1*$^{-/y}$ mice back to pre-stress levels, albeit it did not enhance LTP as RU38486 did. Spironolactone led to recovery of LTP in WT mice stressed for 30 min, but it blocked LTP in WT mice stressed for 60 min. *Fmr1*$^{-/y}$ mice treated with spironolactone prior to 30 or 60 min acute stress had significantly reduced LTP levels. Significance was set at $P < 0.05$. * indicates significant difference from control WT mice.

Table III.1 LTP levels obtained from the CA1 and DG hippocampal subfields
LTP presented as the average of the last 5 min 60-min post-HFS in the CA1 and DG of WT and *Fmr1-/y* mice. Values are average ± SEM. * indicates significant difference from control WT mice. # indicates significant difference from control *Fmr1-/y* mice. ** indicates significant difference between genotypes.

DG	Control	15 Min S	30 Min S	60 Min S
WT	69.39±8.35	43.67±7.93	-1.52±6.15*	57.62.12.87
Fmr1-/y	32.77±9.96**	-13.79±8.77#	50.32±11.20**	40.05±9.97**
CA1				
WT	45.56±8.43	44.20±9.71	40.26±6.15	66.85.19.34
Fmr1-/y	37.36±10.07	39.67±5.70	40.36±10.34	70.01±12.76
DG RU38486				
WT	75.99±16.35	59.79±9.86	64.30±8.91	58.82±16.21
Fmr1-/y	73.92±5.89	62.60±9.17	38.28±11.39	54.97±9.12
DG Spironolactone				
WT	38.92±8.53	39.11±9.10	67.03±12.25*	6.79±10.18
Fmr1-/y	33.67±9.33	34.71±12.48	17.16±11.33**	20.14±8.32

Discussion

Individuals with FXS are reported to have a loss of HPA-axis regulation (Wisbeck et al., 2000; Hessl et al., 2006), which may lead to the observed heightened anxiety and learning impairments associated with this condition (Garber et al., 2008). The described experiments indicate that *Fmr1-/y* mice exhibit a rapid elevation in plasma CORT levels in response to even short periods of acute restraint stress. This in turn leads to the activation of GRs and MRs by CORT, and these receptors exert time-dependent, and opposing, effects on synaptic plasticity. These findings replicate human clinical data where an exaggerated stress response has been reported in individuals with FXS (Wisbeck et al., 2000; Hessl et al., 2002, 2006). Indeed, altered HPA-axis function has been reported in *Fmr1-/y* mice previously (Beckel-Mitchener et al., 2003;

Lauterborn, 2004; Markham et al., 2006), and here we show for the first time how this can impact the functioning of learning and memory related synaptic signalling.

The effects of acute stress on long-term synaptic plasticity in the DG appear to involve contributions from both GRs and MRs. Normally GR activation is believed to lead to a reduction in LTP, while MR activation can facilitate it (Avital et al., 2006). In the present study, the GR antagonist RU38486 enhanced DG LTP in naïve *Fmr1*$^{-/y}$ mice, and animals stressed for a brief period of time. Conversely, the LTP observed after more prolonged periods of stress was not impacted by the GR antagonist. Rather, administration of the MR antagonist, prior to undergoing prolonged acute stress, revealed that MR activation was facilitating LTP under these conditions. These results are in line with models predicting that it is the balance of activation between GRs and MRs that determines how stress will impact synaptic plasticity (Sousa et al., 2008).

The rescue of NMDAR-LTP by blocking GR signalling in control non-stressed *Fmr1*$^{-/y}$ mice is an exciting result. We have previously reported NMDAR hypofunction in the DG of this mouse model, which was associated with significant reductions in LTP levels, as well as deficits in context fear discrimination, a hippocampus-dependent task that relies on NMDARs (Young et al., 1994; Eadie et al., 2010). Furthermore, we recently demonstrated that LTP deficits in the *Fmr1*$^{-/y}$ DG can be rescued using the NMDAR co-agonists glycine or D-serine (Bostrom et al., 2013). Recent studies revealed a distinct modulation of NMDARs after hippocampal slices with stress levels of CORT, which lead to a transient potentiation of evoked NMDAR-mediated synaptic responses that subsided as expression of GluN2A-containing NMDAR levels increased (Tse et al., 2011). The combination of these findings suggest that in absence of FMRP, enhanced GR signalling may be exerting tonic inhibition on NMDARs in the DG of *Fmr1*$^{-/y}$ mice, possibly through either a membrane-associated form of GRs that modulate NMDAR function, or through

downstream signalling that leads to modulation of NMDAR levels or specific subunit ratios, or both. Future experiments can investigate these possibilities.

The finding that both GR and MR antagonists equally rescued the blocked LTP in the DG of WT mice stressed for 30 min and *Fmr1*$^{-/y}$ mice stressed for 15 min is interesting. MRs normally have a 10-fold higher affinity for CORT than GRs and are normally thought to be predominantly occupied at resting levels of CORT, whereas GRs are more likely to be occupied when CORT levels increase (Conway-Campbell et al., 2007). In the present study, the findings may indicate that when MRs are blocked, Overactivation of GRs may lead to activation of some distinct signalling pathway that also facilitates LTP induction in the DG following shorter periods of stress. While a number of studies have reported that MR activation facilitates hippocampal LTP and GR activation impairs it (Pavlides et al., 1995; Pavlides and McEwen, 1999; Spyrka and Hess, 2010) to date we have found no studies showing that blocking MRs can lead to enhanced LTP due to GR activation. However, a previous report suggests that MRs and GRs are only part of the story, and that coinciding the timing of CORT application with synaptic stimulation can serve as a critical component of synaptic potentiation in the mouse hippocampus (Wiegert et al., 2006). The specific effects of MR and GR activation on LTP may depend on the length of the stress period and when LTP is measured.

The fact that synaptic plasticity in the CA1 subfield of either WT or *Fmr1*$^{-/y}$ mice was not affected by acute stress is interesting. This work shows that restraint stress periods of 15, 30 and even 60 min failed to impair LTP in the CA1 region of both WT and *Fmr1*$^{-/y}$ mice. Indeed, to our knowledge, only one study has reliably shown that acute stress can impact LTP in the CA1 subfield of the mouse hippocampus (Garcia et al., 1997). However, in this study a reduction in LTP was only observed for LTP induced with TBS when animals were administered acute stress

in conjunction with a tail shock and then sacrificed 60 min later. In contrast, the conditioning stimulus used in this study was HFS, the acute stress did not involve electric tail shocks, and the mice were sacrificed immediately following the end of their stress period. This may imply that more significant stressors are needed to impact this CA1 region in mice and that the effect may be a delayed one, whereas the DG is a more sensitive indicator of stress.

The heightened sensitivity of the DG to acute stress may also be due to the observed differences in GR levels between the hippocampal subfields. Indeed, the immunohistochemistry stains for GR reported here show that the CA1 has significantly less density of GRs than the DG. Early studies characterizing GR distribution in the rat hippocampus reported equal levels of the receptor higher levels of the receptor in the CA1 subfield (Herman et al., 1989). However, others have reported equal levels in the CA1 and DG (Eekelen et al., 1988; Morimoto et al., 1996). While a number of studies have reported the susceptibility of synaptic plasticity in the CA1 subfield of the rat hippocampus following acute stress (Foy et al., 1987; Kim et al., 1996; Kim and Diamond, 2002), the results reported in here show the DG subfield of the mouse hippocampus to be more readily affected by acute stress than the CA1. Differences in species' susceptibility to acute stress in the different hippocampal subfields may be explained by differences observed in GR distribution. Future studies in mice can examine LTP in the CA1 following a delayed period after acute stress before sacrificing the animals to assess whether the effect of stress on the CA1 is evident later than is in the DG.

Balance between MR and GR levels is critical for proper hippocampal response to stress and in turn learning and memory (Sousa et al., 2008). Given that FMRP regulates protein translation (Bagni and Greenough, 2005) and was previously reported to bind GR mRNA (Miyashiro et al., 2003), it is plausible to hypothesize that loss of FMRP would lead to loss of

balance in expression levels of GRs in relationship to MRs, thus leading to change in temporal dynamics of receptor roles in the stress response. However, as the results reported in this chapter indicate, MR and GR levels in the DG were observed to be comparable between WT and *Fmr1*$^{-/y}$ mice. This finding suggests that LTP deficits in the DG of *Fmr1*$^{-/y}$ mice under control conditions, as well as the temporal shift in the impact of acute stress on LTP may be due to a functional dysregulation of the receptors rather than a structural deficit. It is possible that FMRP could be playing a role in regulating downstream gene expression products of MRs and GRs, and its absence leads to dysregulation of signalling cascades initiated by MR- and GR-mediated gene transcription.

The intellectual impairments observed in FXS are generally thought to depend on a combination of genetic and environmental factors. The results reported in this chapter demonstrate that loss of FMRP leads to a more robust elevation of CORT after acute stress and a temporal shift in synaptic plasticity deficits leading to an earlier block of LTP in the DG. Furthermore, stress-induced modulation of LTP in the DG is dependent on a temporal activation of GRs and MRs, where GR activation leads to an initial impairment of LTP after a short period of acute stress, and MR activation enhanced LTP after a longer period of the same stress conditions. Moreover, under non-stress conditions, enhanced GR signalling may be an underlying factor in the reduced NMDA receptor function observed in the DG of *Fmr1*$^{-/y}$ mice. These experiments improve our understanding of the intellectual impairments and how heightened anxiety observed in FXS may directly impair memory performance.

Experimental Limitations and Pitfalls

A challenge that remains unresolved in stress research is the lack of standardized stress protocols to assess the various physiological responses to stress. Animal housing, animal transport from the animal care facility, and the stress paradigm employed are a few of the factors that may contribute to a masking effect. Various research groups take an approach in which the animals are brought to the laboratory in their home cages and allowed to acclimate for a few days before the stress procedures are initiated (Adlard and Cotman, 2004). Others simply transfer the animals to the laboratory and immediately begin the stress procedures, assigning a group to serve as a control group that is sacrificed immediately upon arrival (Xiong et al., 2004). This lack of consistency can prove to be a challenge in replicating findings.

Previous studies have investigated the effects of common laboratory procedures in rodents. Cage change, restraint and subcutaneous injection, restraint and tail-vein injection, exposure to the odour of urine and feces from stressed rats, and exposure to the odour of dried rat blood were all shown to initiate stress-like responses in rats, all of which were reduced by group housing (Sharp et al., 2002). These factors are common in animal research and therefore raise a possibility that some of the findings reported in this chapter may be idiosyncratic to the experimental conditions rather than the acute stress paradigm used. Part of the procedures conducted here required the transportation of mice between buildings and exposure of the animals to changes in environments prior to experimentation. In order to assess whether this transport had an effect on serum CORT levels and synaptic plasticity, a separate cohort of WT (n = 5) and *Fmr1*$^{-/y}$ (n = 5) littermate mice were transported to the laboratory and allowed to habituate for one week before sacrifice. Serum CORT concentrations obtained from the lab-habituated cohort revealed no significant difference in levels between WT and *Fmr1*$^{-/y}$ mice

(**Figure III.11A**). However, overall levels were significantly lower than those reported from transported WT (Lab-habituated: 26.78 ± 14.32 ng/mL; Transported: 122.30 ± 9.16 ng/mL; $p < 0.001$) and *Fmr1*$^{-/y}$ mice (Lab-habituated: 32.01 ± 18.69 ng/mL; Transported; 128.49 ± 13.18 ng/mL; $p < 0.001$).

To examine whether the transport process impacts LTP levels in the DG, hippocampal LTP recordings from the MPP in the DG were obtained. Despite the significantly lower levels of serum CORT in lab-habituated mice, LTP levels in the DG were comparable to those observed in transported WT (Laboratory-habituated: 79.39 ± 21.73%; Transported: 69.39 ± 8.35%; $p = 0.60$) and *Fmr1*$^{-/y}$ mice (Laboratory-habituated: 45.64 ± 8.21%; Transported: 32.77 ± 9.96%; $p = 0.55$) (**Figure III.11B**). These results provide evidence that the transport procedure from the animal housing unit to the laboratory activates the HPA-axis in mice as measured by serum CORT levels, but this does not impact LTP in the DG, thus supporting the findings reported in this chapter as being consequential from exposure to the acute restraint stress paradigm used.

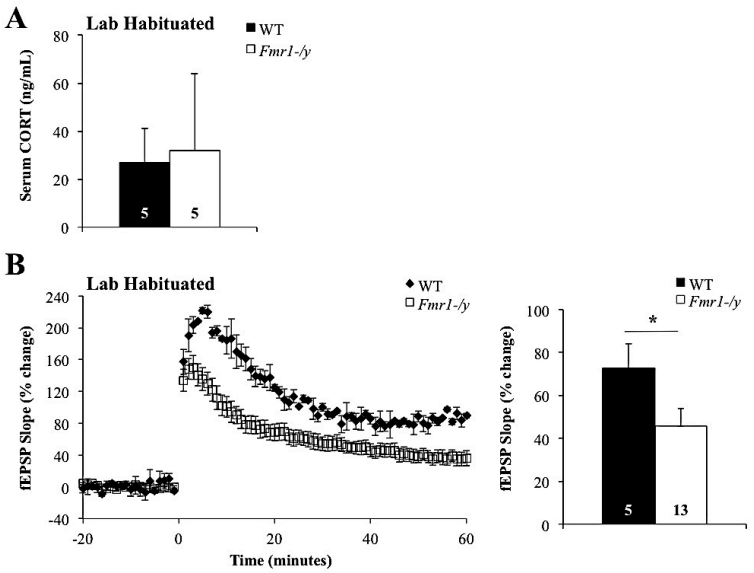

Figure III.11 CORT and DG LTP from Mice that were Habituated to the Laboratory for 7 Days
(A) ELISA measurements of plasma CORT levels obtained from WT and *Fmr1*$^{-/y}$ mice after 7 days of habituation in the laboratory were equivalent between genotypes. **(B)** LTP in the DG region of hippocampal slices obtained from *Fmr1*$^{-/y}$ mice was significantly reduced in comparison to WT. Significance was set at $p < 0.05$. * indicates difference from WT.

The injection procedure is considered a stressor for rodents (Sharp et al., 2002). Combined with an episode of acute restraint, additional signalling pathways could be acting in concert to produce the effects reported in the experiments using GR and MR antagonists. Hence, it was important to conduct control experiments using vehicle injections to confirm the role of MRs and GRs in the dynamic changes observed on DG LTP. A separate cohort of WT (n = 8) and *Fmr1*$^{-/y}$ (n = 8) littermate mice were assigned to the different stress conditions and received subcutaneous vehicle injections an hour prior to removal from the animal housing facility. Vehicle injections did not alter the impact of acute stress on DG LTP (**Figure III.12**). Overall, these results provide evidence that the reported effects of GR and MR antagonists on DG LTP were not due to the injection procedure, and that the observed deficits in LTP were not induced by animal transport from the animal housing facility.

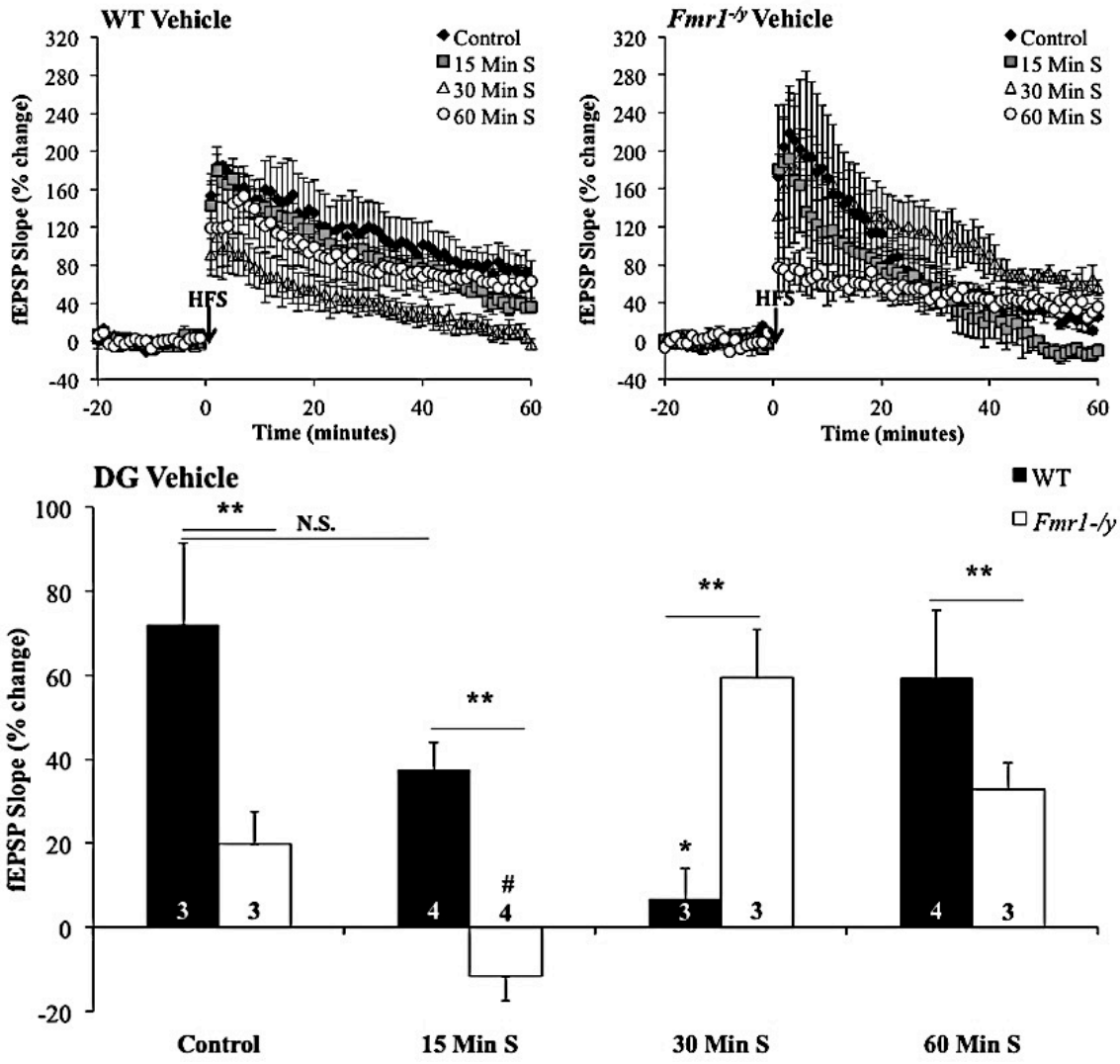

Figure III.12 Vehicle Injections Do Not Alter the Effect of Acute Stress on LTP in the DG of WT and *Fmr1*$^{-/y}$ Mice

A separate cohort of WT and *Fmr1*$^{/y}$ mice received subcutaneous injections of saline and then assigned to control or an acute stress condition. Significance was set at $P < 0.05$. * indicates significant difference from control WT mice. # indicates significant difference from control *Fmr1*$^{-/y}$ mice. ** indicates significant difference between genotypes.

A challenge can be posed to the interpretation offered from the GR immunohistochemistry and ELISA data. Although both WT and *Fmr1*$^{-/y}$ mice had similar levels of MRs and GRs in the DG, the analysis was conducted on the full region, which does not differentiate between expression levels in the cell and the synapse. While total protein levels may not be altered, differences may become evident at the synapses. Indeed, the GR mRNA was identified as a

target for FMRP (Miyashiro et al., 2003), and GRs were recently shown to be present in dendritic spines and necks (Jafari et al., 2012). Hence, while total protein levels in neurons may be similar, the specific distribution and how this changes in response to stress may be different in absence of FMRP. Future experiments should examine synaptoneurosomes to identify localized differences in GR levels in the DG of WT and *Fmr1*$^{-/y}$ mice.

CHAPTER IV. Acute Stress Does Not Alter Homosynaptic Hippocampal Long-Term Depression in Mice

Introduction

The presented findings in the previous chapter raised the question whether the impact of acute stress on hippocampal synaptic plasticity is bidirectional. Our group has reported significant reduction in NMDAR-LTD in the DG of *Fmr1*$^{-/y}$ mice (Eadie et al., 2010). In addition, acute stress has previously been shown to to facilitate NMDAR-LTD (Kim et al., 1996; de Kloet, 2004; Xiong et al., 2004; Saxe et al., 2006; Yang et al., 2006), an effect that was shown to be mediated by GR activation (Avital et al., 2006; Maggio and Segal, 2007). The data presented thus far suggests that enhanced GR activity in absence of FMRP leads to selective deficits in LTP in the naïve *Fmr1*$^{-/y}$ DG, and a shift towards earlier stress-induced LTP impairment. However, it remains to be seen whether acute stress impacts LTD in the CA1 or the DG.

In contrast to NMDAR- LTD, mGluR-LTD depends on activation of postsynaptic group 1 mGluRs and local translation of dendritic mRNA (Huber et al., 2001; Lüscher and Huber, 2010). Furthermore, while NMDAR-LTD results from internalization of postsynaptic AMPARs (Carroll et al., 1999, 2001; Lüscher et al., 1999), mGluR-LTD induction leads to internalization of both AMPARs and NMDARs (Huber et al., 2000; Snyder et al., 2001). Interestingly, although mGluR-LTD is normally dependent on protein syndissertation, it was found to be protein syndissertation independent in *Fmr1*$^{-/y}$ mice (Nosyreva and Huber, 2006; Zhang et al., 2009; Sharma et al., 2010). Similar to NMDAR-LTD, mGluR-LTD may also be facilitated after acute stress, in a GR-mediated manner (Chaouloff et al., 2007). A recent study provided evidence that

mGluR activation regulates GR levels at glutamatergic synapses, increasing their levels in response to mGluR stimulation (Jafari et al., 2012).

Several studies have reported significantly enhanced mGluR-LTD in the CA1 of *Fmr1*$^{-/y}$ mice (Huber et al., 2002; Hou et al., 2006; Choi et al., 2011). However, our laboratory has been unable to replicate these findings (Bostrom, 2012). Given the data presented thus far indicating a heightened sensitivity of *Fmr1*$^{-/y}$ mice to acute stress, we aimed to determine whether the reported enhancement of mGluR-LTD in absence of FMRP is due to the animals experiencing stress prior to sacrifice as part of the experimental protocols employed by other laboratories. The objectives of the following experiments are to determine whether NMDAR- and mGluR-LTD in the hippocampus of *Fmr1*$^{-/y}$ mice are modulated by acute stress.

Materials and Methods

Animal generation, stress procedure, and field electrophysiology protocols for the DG and CA1 subfields with the exception of the conditioning stimulus were conducted as described in Chapter III of this dissertation.

LTD Induction Protocols

For NMDAR-LTD, fEPSPs elicited every 15 s were recorded in nACSF until a stable 20-min baseline was established, and then a low frequency stimulus (LFS; 900 single pulses at 1 Hz) was administered. Following the application of the conditioning train, fEPSPs were recorded every 15 s for 60 min in nACSF to evaluate long-term changes in synaptic depression.

mGluR-LTD was induced by adding 100 μM 3,5-dihydroxphenylglycine (DHPG) with 50 μM D-APV to the nACSF bath for 5 min after a 20-min stable baseline was established in nACSF. Fresh stocks of DHPG were prepared every week as a 100X stock in millique H_2O,

aliquoted, and stored at -20 °C. D-APV was prepared as a 10X stock in millique H_2O, aliquoted, and stored at -20 °C. Stocks were diluted in nACSF to achieve the required final concentrations. At the end of the 5-min bath application of the drugs the slices were washed in oxygenated nACSF and recording of fEPSPs continued for 60 min. Waveform and statistical analysis was conducted as described in Chapter III.

Results

Significant Differences in NMDAR-LTD Between WT and *Fmr1*$^{-/y}$ Mice in the DG Disappear Following Acute Stress

The delivery of LFS conditioning stimulus produced marked levels of NMDAR-LTD in the DG of WT and *Fmr1*$^{-/y}$ mice in the control non-stress group, with *Fmr1*$^{-/y}$ mice showing significantly reduced NMDAR-LTD in comparison to their WT littermates (WT: -32.41 ± 4.78%; *Fmr1*$^{-/y}$: -14.44 ± 4.88%; $p < 0.001$). One-way ANOVA revealed that exposure to acute stress did not significantly impact NMDAR-LTD in the DG of WT ($F_{(3,30)} = 0.28$, $p = 0.84$) or *Fmr1*$^{-/y}$ mice ($F_{(3,24)} = 0.54$, $p = 0.66$) (**Figure IV.1**). However, individual pairwise comparisons between genotypes revealed that deficits in NMDAR-LTD noted in control *Fmr1*$^{-/y}$ mice in comparison to their WT littermates were abolished in mice that were stressed for 15 min (WT: -19.43 ± 5.59%; *Fmr1*$^{-/y}$; -19.40 ± 3.15%; $p = 0.99$), 30 min (WT: -33.09 ± 6.69%; *Fmr1*$^{-/y}$; -25.70 ± 8.56%; $p = 0.13$), or 60 min (WT: -19.09 ± 10.24%; *Fmr1*$^{-/y}$; -27.19 ± 10.11%; $p = 0.55$).

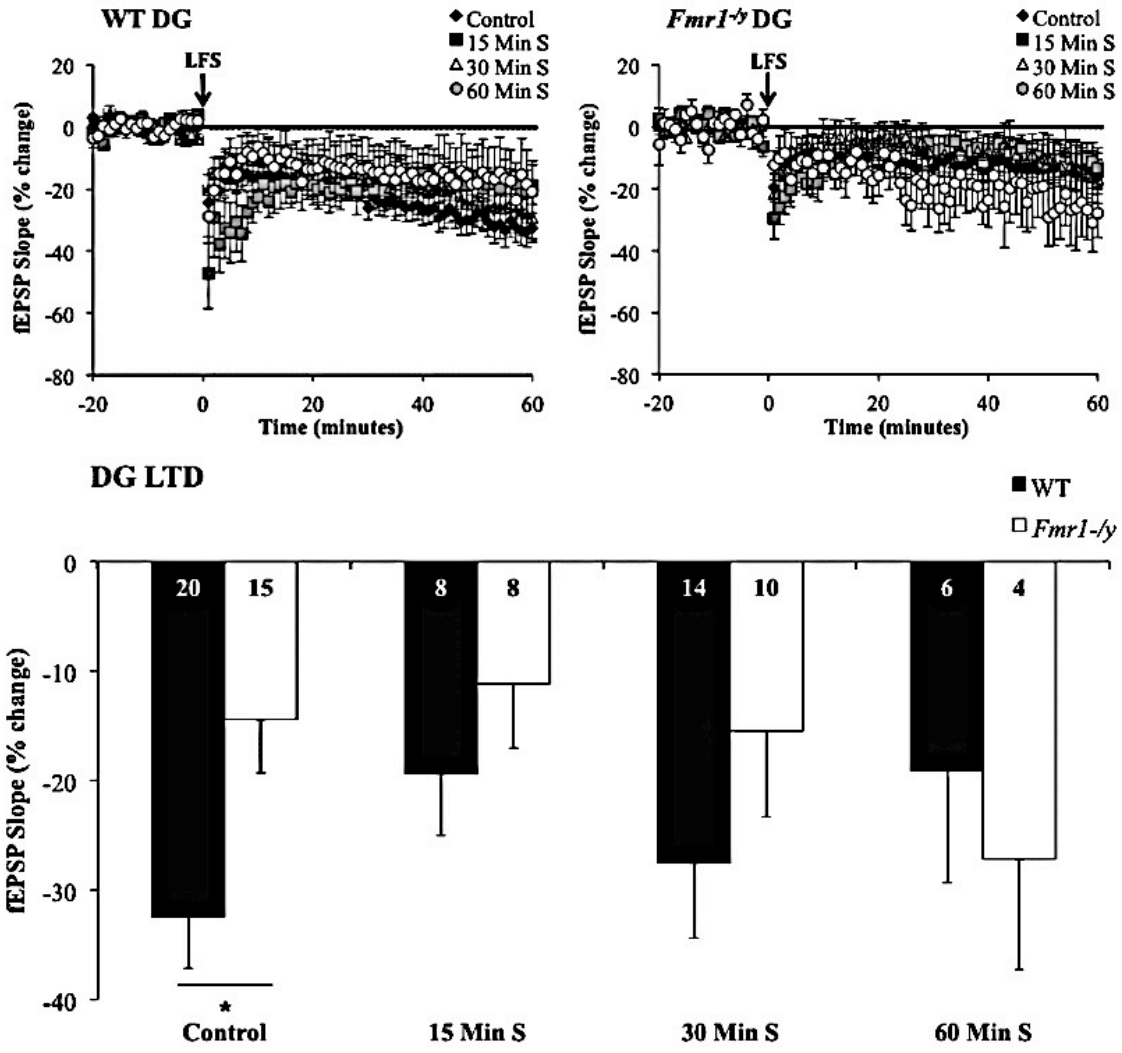

Figure IV.1 Acute Stress Abolished Significant NMDAR-LTD Differences in the DG between WT and *Fmr1*$^{-/y}$ Mice

Acute stress did not significantly impact NMDAR-LTD levels in the DG of WT or *Fmr1*$^{-/y}$ mice in comparison to control. However, under control conditions *Fmr1*$^{-/y}$ mice exhibit significantly reduced levels of NMDAR-LTD following LFS delivered to the MPP in the DG in comparison to WT, and this difference disappears after application of acute stress. Significance was set at $p < 0.05$. * indicates significance between genotypes.

Acute Stress Does Not Impact NMDAR-LTD in the CA1 of WT or *Fmr1*$^{-/y}$ Mice

In contrast to the DG, NMDAR-LTD in the CA1 was not affected in absence of FMRP (WT: -16.37 ± 4.80%; *Fmr1*$^{-/y}$: -12.39 ± 2.64%; $p = 0.48$). But similar to the DG, a one-way ANOVA revealed that exposure to acute stress did not significantly impact NMDAR-LTD in the

CA1 of WT ($F_{(3,22)}$ = 1.14, p = 0.36) or *Fmr1*$^{-/y}$ mice ($F_{(3,18)}$ = 2.519, p = 0.12) (**Figure IV.2**). Furthermore, individual pairwise comparisons between genotypes did not reveal any differences between genotypes in the 15 min (WT: -12.33 ± 8.21%; *Fmr1*$^{-/y}$; -11.40 ± 8.31%; p = 0.94), 30 min (WT: -28.95 ± 15.70%; *Fmr1*$^{-/y}$; -18.83 ± 3.50%; p = 0.23), or 60 min acute stress groups (WT: -19.95 ± 12.43%; *Fmr1*$^{-/y}$; -34.98 ± 12.71%; p = 0.48).

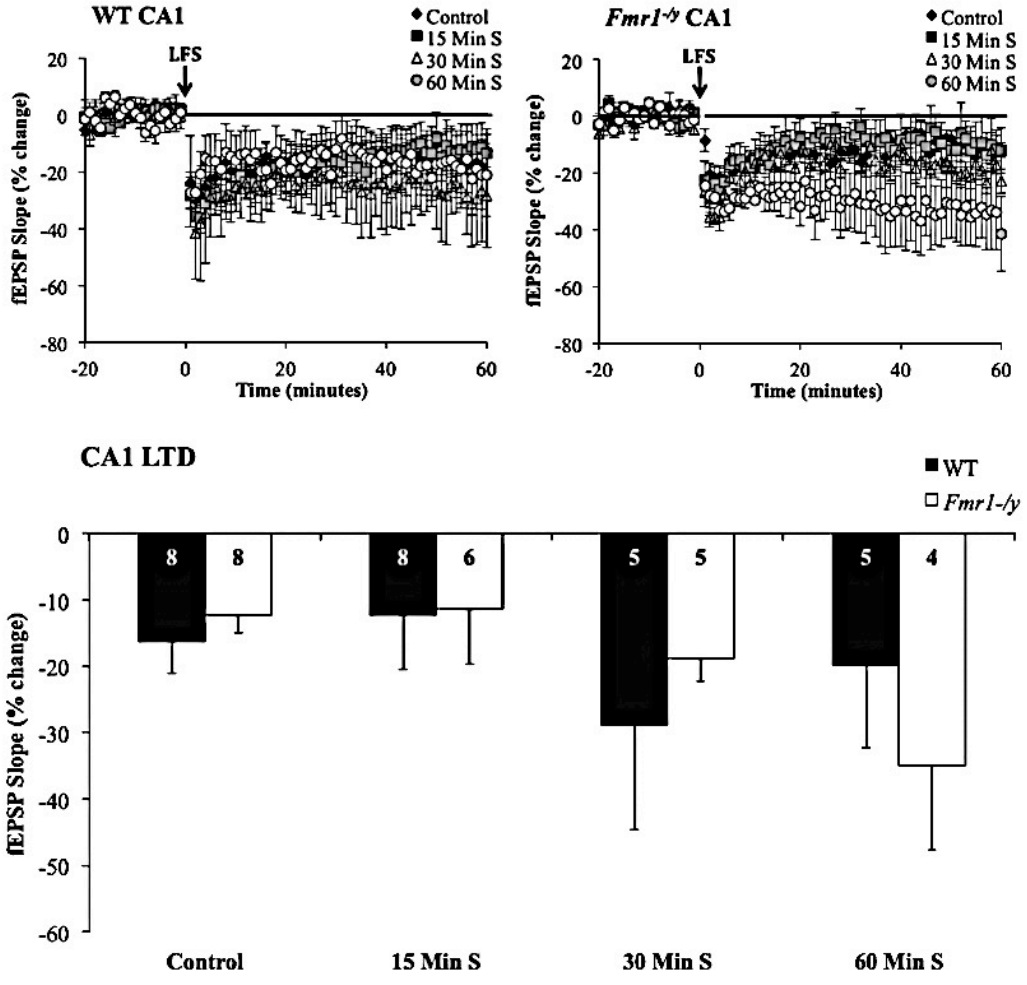

Figure IV.2 Acute Stress Does Not Impact NMDAR-LTD in the CA1

Fmr1$^{-/y}$ mice exhibit equal levels of NMDAR-LTD in the CA1 in response to LFS as WT, and application of increasingly lengthier periods of acute stress did not have an impact on either genotype. Significance was set at $p < 0.05$.

mGluR-LTD is Not Impacted by Loss of FMRP or by Acute Stress in the DG or the CA1

To test the hypodissertation that hippocampal mGluR-LTD may be enhanced in *Fmr1*$^{-/y}$ mice due to stress, slices obtained from control and stressed animals were subject to chemical induction of m-GluR LTD by applying the mGluR agonist DHPG (100 μM) combined with the NMDAR antagonist D-APV (50 μM) for 5 min after establishment of a 20-min stable baseline of fEPSP responses to stimulation. mGluR-LTD in the DG measured 60-min post chemical stimulation was similar between WT and *Fmr1*$^{-/y}$ mice (WT: -16.37 ± 4.80%; *Fmr1*$^{-/y}$: -12.39 ± 2.64%; $p = 0.90$). Acute stress did not have a significant impact on mGluR-LTD in the DG of WT ($F_{(3,30)} = 0.28$, $p = 0.84$) or *Fmr1*$^{-/y}$ mice ($F_{(3,24)} = 0.54$, $p = 0.66$) (**Figure IV.3**).

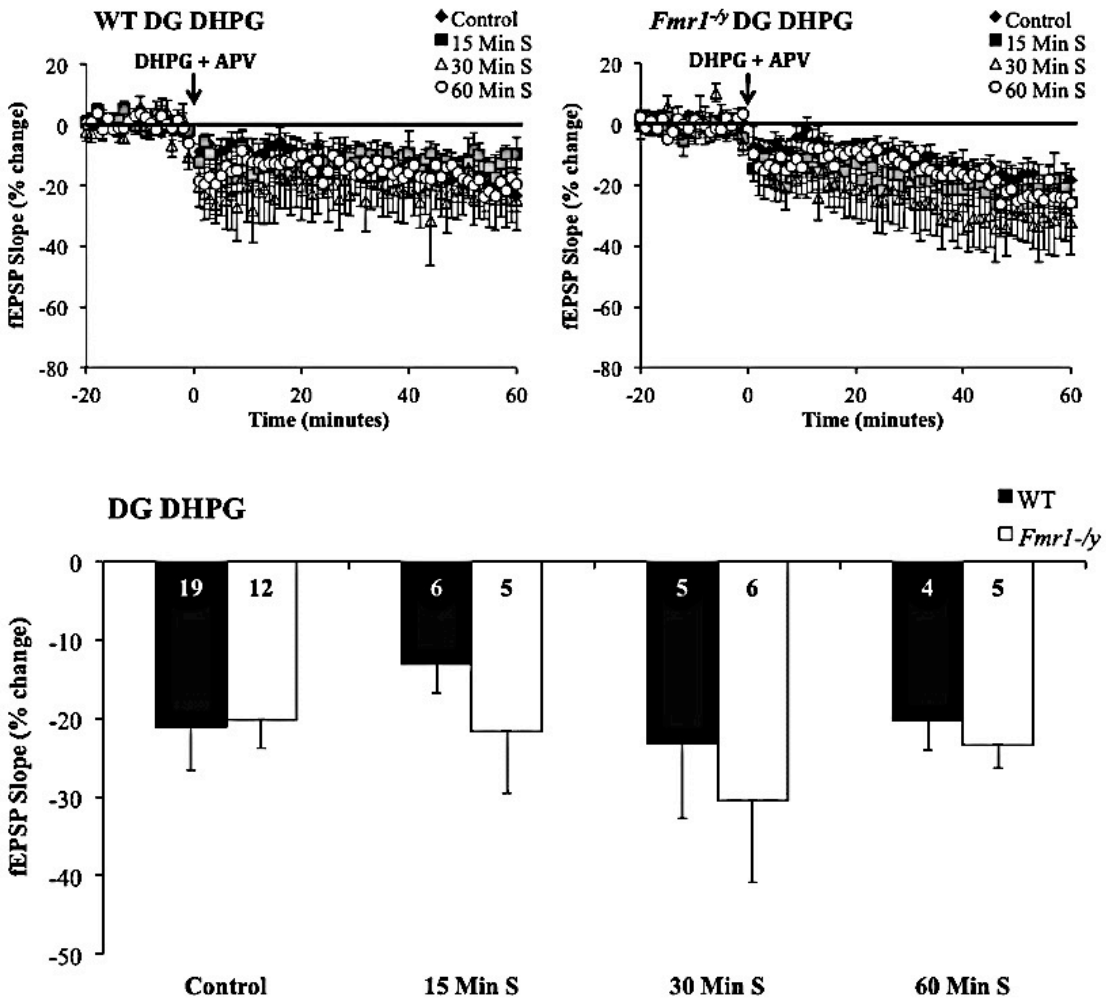

Figure IV.3 mGluR-LTD is Not Altered in the DG in Absence of FMRP and Acute Stress Does Not Impact Its Levels in WT or *Fmr1*$^{-/y}$ Mice

The mGluR agonist DHPG (100 μM) and the NMDAR antagonist D-APV (50 μM) were applied to the nACSF bath for 5 min after establishment of a stable 20-min baseline of fEPSP responses to stimulation. mGluR-LTD measured 60-min post chemical induction in the DG was not altered in in *Fmr1*$^{-/y}$ mice and was not impacted by varying periods of acute restraint stress. Significance was set at $p < 0.05$.

Similarly, WT and *Fmr1*$^{-/y}$ mice exhibited equal levels of mGluR-LTD in the CA1 (WT: -16.37 ± 4.80%; *Fmr1*$^{-/y}$: -12.39 ± 2.64%; $p = 0.94$), and acute stress did not have an impact on mGluR-LTD in either genotype (WT: $F_{(3,33)} = 0.75$, $p = 0.53$; *Fmr1*$^{-/y}$: $F_{(3,31)} = 0.47$, $p = 0.71$) (**Figure IV.4**).

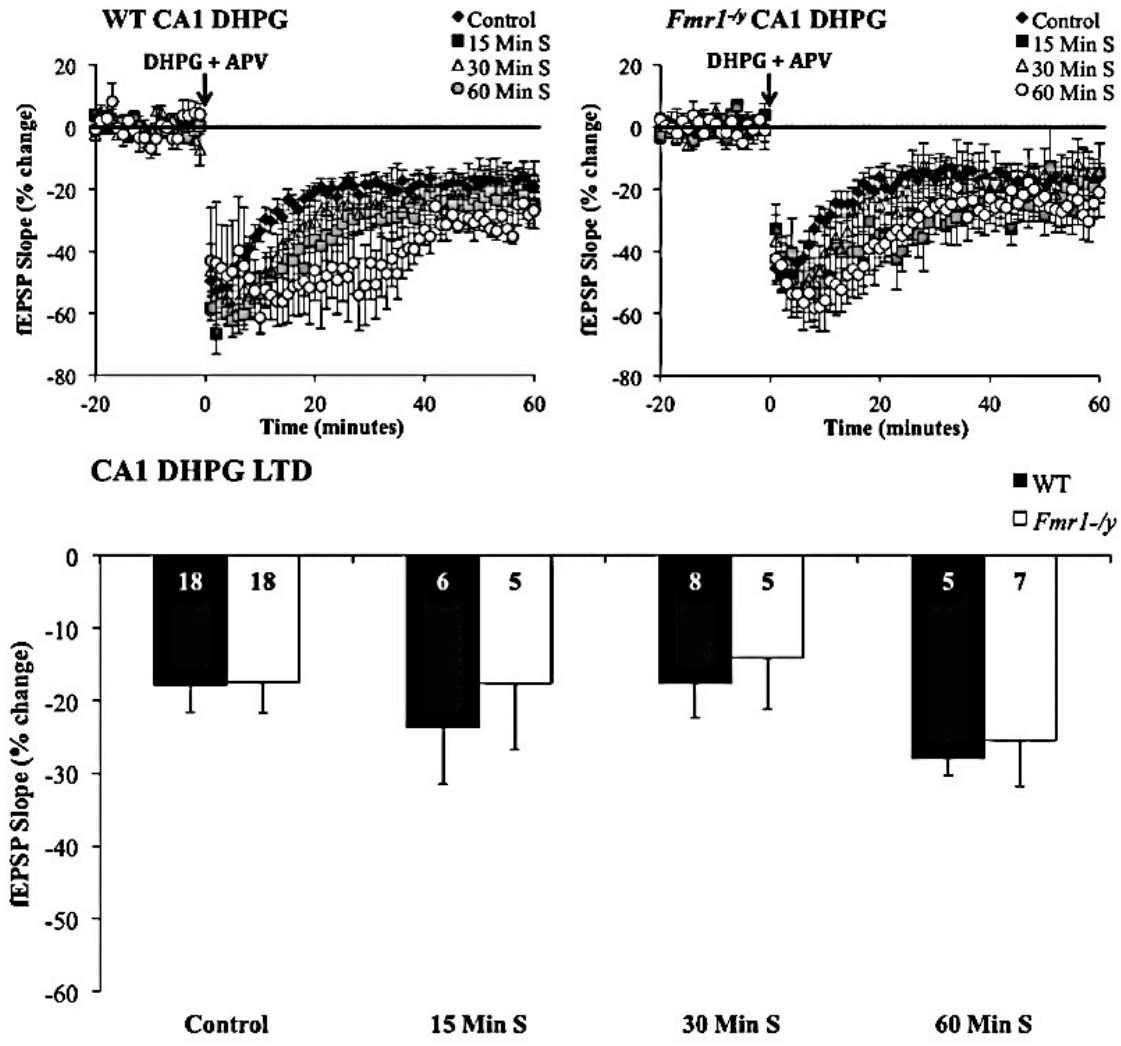

Figure IV.4 mGluR-LTD is Not Altered in the CA1 in Absence of FMRP and Acute Stress Does Not Impact Its Levels in WT or *Fmr1*$^{-/y}$ Mice

Application of the mGluR agonist DHPG (100 μM) and the NMDAR antagonist D-APV (50 μM) to the nACSF bath for 5 min produced significant depression. mGluR-LTD measured 60-min post chemical induction in the CA1 was not altered in in *Fmr1*$^{-/y}$ mice and was not impacted by varying periods of acute restraint stress. Significance was set at $p < 0.05$.

Overall, these results suggest that the effects of stress on LTP are unidirectional as neither forms of NMDAR-LTD or mGluR-LTD were affected in the DG or the CA1 by acute stress (**Table IV.1**).

Table IV.1 LTD levels obtained from the CA1 and DG hippocampal subfields
LTD presented as the average of the last 5 min 60-min post-LFS or DHPG treatment in the CA1 and DG of WT and *Fmr1*$^{-/y}$ mice. Values are average ± SEM. * indicates significant difference between genotypes.

DG	Control	15 Min S	30 Min S	60 Min S
WT	-32.41±4.78	-19.43±5.59	-33.09±6.69	-19.09±10.24
Fmr1-/y	-14.44±4.88*	-19.40±3.15	-25.70±8.56	-27.19±10.11
CA1				
WT	-16.37±4.80	-12.33±8.21	-28.95±15.70	-19.95±12.43
***Fmr1*$^{-/y}$**	-12.39±2.64	-11.40±8.31	-18.83±3.50	-34.98±12.71
DHPG DG				
WT	-21.11±5.53	-13.16±3.67	-23.22±9.56	-20.32±3.79
Fmr1-/y	-20.23±3.56	-21.71±7.85	-30.45±10.49	-23.41±2.94
DHPG CA1				
WT	-17.92±3.79	-23.74±7.80	-17.68±4.77	-27.96±2.38
Fmr1-/y	-17.50±4.23	-17.68±9.07	-14.20±7.03	-25.41±6.43

Discussion

The experiments outlined in this chapter addressed whether the impact of stress on LTP in the DG of WT and *Fmr1*$^{-/y}$ mice is also observed on NMDAR-LTD, and whether reports of enhanced mGluR-LTD in absence of FMRP can be attributed to a heightened response to a short period of acute stress. The present data suggests that acute stress does not impact either form of LTD in the DG or CA1 hippocampal subfields of WT or *Fmr1*$^{-/y}$ mice.

We have previously reported NMDAR-LTD deficits in the DG of *Fmr1*$^{-/y}$ mice (Eadie et al., 2010). This finding was replicated here as demonstrated in the control non-stressed mice (**Figure IV.1**). However, although acute stress did not produce a significant difference in LTD levels in either WT or *Fmr1*$^{-/y}$ mice, LTD deficits observed in the naïve *Fmr1*$^{-/y}$ DG were no longer significant in the stress conditions. These results suggest that significant NMDAR-LTD deficits in non-stressed *Fmr1*$^{-/y}$ mice may reflect an elevated stimulative or preparative state for a

stress response (Sapolsky et al., 2000), which results in subtle deficits in NMDAR-LTD that can be revealed under non-stress conditions but disappear once stress is applied. Reported evidence in Chapter III suggests that LTP deficits in the DG of *Fmr1*$^{-/y}$ mice may be due to enhanced GR signalling through possible inhibitory effects on NMDAR function. Expression of NMDAR-LTD involves the dephosphorylation of Ser-845 on the AMPAR GluA1 subunit by calcineurin (Lee et al., 2000), which decreases AMPAR channel open probability and activates internalization of AMPARs (Banke et al., 2000; Lee et al., 2002). Future experiments can examine the effects that bath application of CORT has on NMDAR and AMPAR currents, as well as the effect that incubation of hippocampal slices in CORT has on AMPAR GluA1 subunit phosphorylation and calcineurin expression in the DG.

Previous work in the rat CA1 region has shown that NMDAR-LTD is facilitated after restraint stress periods lasting 10 min (Wang et al., 2006), 30 min (Kim et al., 1996), and 60 min (Yang et al., 2004). Placing rats on an elevated platform in the middle of a bright room was also demonstrated to facilitate NMDAR-LTD in the CA1 (Xiong et al., 2004). In addition to facilitating NMDAR-LTD, restraint stress also enhances mGluR-LTD through a GR-mediated mechanism (Chaouloff et al., 2007). It is therefore surprising that neither form of LTD was altered by acute stress. However, this may be a product of strain, species, and stress procedure differences.

The current study was conducted in the mouse hippocampus, which although it shares many features with the rat hippocampus, it also has distinctive properties. For example, neuron-specific protein expression during axonal growth during development and regeneration is distinct between the rat and mouse hippocampus (McNamara et al., 1996). Furthermore, the mice used in this study were sacrificed immediately after cessation of restraint stress. In contrast, the rats used

in other reports either received tail shocks during the restraint period (Kim et al., 1996), or were allowed a recovery period post-stress before they were sacrificed (Chaouloff et al., 2007). These findings suggest the need for a physical component to the stressor (i.e., tailshocks), or the inclusion of a recovery period after stress to allow for cellular processes to have an effect on synaptic plasticity. Future experiments should modify the stress protocol to include a recovery period before the animal is sacrificed, as this may reveal long-term changes that are outside the current experimental procedures.

Experimental Limitations and Pitfalls

The results reported in this chapter for mGluR-LTD in the CA1 of *Fmr1*$^{-/y}$ mice are inconsistent with independent groups providing evidence for significantly enhanced mGluR-LTD in this region (Huber et al., 2002; Hou et al., 2006; Nosyreva and Huber, 2006; Zhang et al., 2009; Sharma et al., 2010; Choi et al., 2011). The strain and age of the mice used in the reported experiments of this chapter were consistent with other studies. However, our laboratory has been unsuccessful in replicating these findings (Bostrom, 2012), and the hypodissertation that a short period of acute stress may enhance mGluR-LTD in this mouse model was not confirmed.

It is worthy to note that mGluR-LTD enhancement that is reported in this mouse model was subtle, with ~11% (Huber et al., 2002), 12% (Nosyreva and Huber, 2006), 14% (Choi et al., 2011), 16% (Hou et al., 2006), 10% (Sharma et al., 2010), and 5% (Zhang et al., 2009). Furthermore, it appears that this subtle level of enhancement only becomes statistically significant using a relatively high number of hippocampal slices in most studies (21-27 slices). Although the experiments presented here utilized a high number of hippocampal slices in the control conditions, the same was not done for the stress conditions. Increasing the number of

hippocampal slices from the stress groups may reveal subtle, yet significant differences between WT and *Fmr1*$^{-/y}$ mice.

CHAPTER V. General Discussion

Summary of Findings

The purpose of this dissertation was to further characterize the *Fmr1*$^{-/y}$ mouse model using hippocampus-dependent behavioural tests, and to investigate the impact of acute stress on HPA-axis activation and hippocampal synaptic plasticity. The experiments described provide further evidence that *Fmr1*$^{-/y}$ mice present with performance deficits in a DG-dependent spatial processing task but not in a CA1-dependent temporal ordering task. This dissociation between hippocampal subfields in performance deficits has been noted in previous findings from our group showing performance deficits in DG-dependent, but not in CA1-dependent behaviour (Eadie et al., 2009, 2010). In addition, *Fmr1*$^{-/y}$ mice were shown to have an elevated CORT response to short periods of acute stress, an effect that was associated with earlier stress-induced impairment of LTP in the DG. The impact of stress was mediated by GRs, and absence of FMRP leds to functional dysregulation of MR and GR function, resulting in enhanced corticosteroid signaling that reduces LTP in the DG of naïve *Fmr1*$^{-/y}$ mice. Furthermore, the effects of acute stress on hippocampal synaptic plasticity were demonstrated to be unidirectional and specific to the DG. This work adds to the available evidence for hippocampus-dependent behavioural deficits in *Fmr1*$^{-/y}$ mice, and presents for the first time the impact of acute stress on synaptic plasticity in this mouse model and the unique alterations in GR and MR function that result in absence of FMRP. Moreover, given that most available evidence on the impact of stress on the hippocampus is obtained from rats, the data provided here also contributes valuable information about how stress impacts hippocampal synaptic plasticity in mice.

The impact of stress on the hippocampus

Dissociation Between the DG and CA1

Synaptic plasticity findings presented in this work demonstrate dissociation in the impact of stress between the DG and the CA1. Acute stress applied for varying lengths of time resulted in modulation of LTP levels in the DG, causing impairment in LTP after shorter periods of stress, and an enhancement after longer periods. However, none of the stress periods utilized had an impact on LTP in CA1 region at the time it was assessed. This may be explained by neuroanatomical and structural differences between the DG and CA1 in the hippocampus.

Granule cells in the DG project to mossy cells and interneurons in the hilus, which send excitatory and inhibitory projections, respectively, back to the granular cell layer (Amaral et al., 2007) (**Figure I.4**). Such feedback circuits that can influence neuronal function and response to stimulation are not present in the CA1. Furthermore, unlike pyramidal cells in the CA1 which are formed between E10 and E18 in the mouse hippocampus (Angevine, 1965), dentate granule cells continue to generate well into adulthood (Altman and Das, 1965; Fortscher and Seress, 2007). The functional significance of this difference with regards to synaptic transmission mechanisms underlying LTP lies in the properties of NMDAR expression during development. NMDARs containing the GluN2B subunits predominate in new neurons, and during the course of maturation, GluN2A levels increase relative to GluNB (Sheng et al., 1994; Liu et al., 2004). This distinction between subunit expression influences NMDAR properties. For instance, the GluN2B-NMDAR has a high open probability and peak current, as well as a fast rise, decay, and deactivation times. The GluN2A-NMDAR on the other hand has a low open probability and peak current, as well as a slow rise, decay, and deactivation times (Yashiro and Philpot, 2008).

Previous studies have demonstrated that bidirectional plasticity is supported by different subunit containing NMDARs, where LTP is supported by Glu2A-containing NMDARs, and Glu2B-containing NMDARs facilitate LTD (Fox et al., 2006, 2007). Overall, the differences in neuroanatomical connections (i.e., feedback excitation and inhibition loops in the DG but not in the CA1), structural properties (i.e., the capacity for neurogenesis in the DG but not the CA1), and NMDAR specific subunit ratios can all contribute to dissociation in functional plasticity between the DG and CA1 in response to acute stress. Confirmation of whether these differences are significant requires further investigation.

Inconsistency with Available Literature on the Impact of Stress on the CA1

In the context of this dissertation, it was hypothesized based on well-established findings in the rat hippocampus that restraint stress would impact bidirectional synaptic plasticity in the CA1, decreasing LTP (Foy et al., 1987; Kim et al., 1996; Shors et al., 1997; Yang et al., 2004), and enhancing NMDAR-LTD (Kim et al., 1996; Xiong et al., 2004; Yang et al., 2004, 2006; Wang et al., 2006), as well mGluR-LTD (Chaouloff et al., 2007). However, none of the acute stress groups tested in this dissertation showed alterations in bidirectional synaptic plasticity in the CA1, and the dearth of available data to make direct comparisons with mouse models makes it difficult to formulate definitive conclusions beyond stating that the acute stress paradigm employed in this work (**Figure III.1**) did not impact the CA1 region of the mouse hippocampus.

A plausible hypodissertation that can be made is that the effect of stress on the CA1 needs a longer time to manifest. There are only two studies to our knowledge that examined the impact of stress on the CA1 in the mouse. In the first study, mice were restrained for 60 min but were returned afterwards to their home cage and sacrificed at the earliest 1 h after cessation of stress.

This paradigm, in contrast to the one employed in this dissertation where the mouse was sacrificed immediately after stress, produced significant LTP deficits in the CA1, suggesting that critical cellular events need to occur before the animal is sacrificed (Garcia et al., 1997). In the second study, mice were held in 72-h social isolation, leading to impaired LTP in the CA1 (Kamal et al., 2014). The need for additional time for the CA1 to manifest stress-induced alterations in LTP may be consequential to having relatively reduced GR density in comparison to the DG, as was demonstrated in Chapter III.

Future work can adjust the experimental design applied in this dissertation by adding recovery periods after cessation of restraint stress before the mouse is sacrificed, which may uncover a temporal pattern in how LTP is modulated in the CA1 following stress that could be altered in absence of FMRP. This may also reveal an effect of stress on NMDAR- and mGluR-LTD in the DG and CA1, which was not evident in the present work. In addition, to support the immunohistochemistry results presented in this dissertation and test the hypodissertation that differences in GR levels may be associated with a delayed effect of stress on the CA1, an ELISA measurement of GR levels from microdissected CA would provide more quantifiable evidence for differences between the hippocampal regions.

Stress and HPA-axis Activation in *Fmr1*$^{-/y}$ Mice

HPA-axis dysregulation in *Fmr1*$^{-/y}$ mice in response to stress was previously reported by three independent groups (Lauterborn, 2004; Markham et al., 2006; Eadie et al., 2009). However, the results reported in this work demonstrate, for the first time, the faster elevation of plasma CORT in *Fmr1*$^{-/y}$ mice following acute stress. In addition to evidence of a protracted return of CORT to basal levels after cessation of stress (Markham et al., 2006), a picture emerges that in

response to stress, *Fmr1*$^{-/y}$ mice sustain higher levels of CORT for a longer period of time in comparison to WT (**Figure V.1**). To fully characterize the HPA-axis, however, plasma ACTH and brain CRH levels should be assessed. Based on previous findings of elevated *c-fos* mRNA expression in the hypothalamic paraventricular nucleus following stress, it can be hypothesized that CRH levels, and subsequently ACTH levels, would also undergo a similar pattern of elevation in *Fmr1*$^{-/y}$ mice.

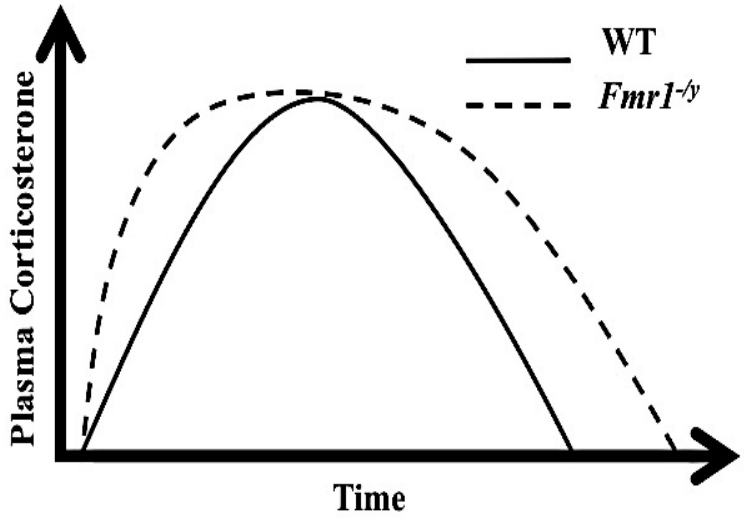

Figure V.1 Loss of FMRP Leads to Faster Rise in CORT and Slower Recovery After Stress
In absence of FMRP, plasma CORT levels rise to peak levels after acute stress in a shorter time period. Following cessation of stress, plasma CORT levels take longer to return to resting levels. The increased time of exposure to CORT due to loss of FMRP may underlie a number of hippocampal synaptic plasticity and behavioural deficits in FXS.

The emerging evidence supports the conclusion that in response to stress, *Fmr1*$^{-/y}$ mice have higher levels of plasma CORT for a longer period of time. An interesting question this poses for future experiments is how the negative feedback regulation of HPA-axis function is altered in absence of FMRP. One of the actions of activated GRs in response to stress is to repress transcription of the pro-opiomelanocortin (POMC) gene, which produces the precursor

for ACTH (Drouin et al., 1989). The available evidence that FMRP associates with GR mRNA (Miyashiro et al., 2003), and FMRP's role as a translation repressor (Bagni and Greenough, 2005), suggest that loss of FMRP should lead to elevated GR levels, which would lead to enhancing GR's repression effects on POMC gene transcription. The expectation from this for hormone levels would be a faster return of plasma CORT to basal levels. However, GR levels were demonstrated using two different experimental techniques in this dissertation (immunohistochemistry stain and ELISA) to be unaltered in absence of FMRP, and plasma CORT levels were shown to have a delayed return to basal levels in this mouse model (Markham et al., 2006). This raises the alternative hypodissertation that FMRP regulates the function of GRs rather than their levels, and that loss of FMRP leads to dissociation between ACTH and CORT elevations in response to stress where GRs may have a heightened sensitivity for CORT in absence of FMRP. Dissociation between ACTH and CORT is a phenomenon that is increasingly reported in disease conditions (Vermes and Beishuizen, 2001; Rubin et al., 2006; Bornstein et al., 2008), thus warranting further investigation in FXS.

Although the presentation of a predominant negative feedback regulation within the HPA-axis is generally correct, it can be somewhat simplistic. Activation of the HPA-axis and its function does involve multiple regulatory mechanisms that act in concert (Makino et al., 2002; Watts, 2005). Nevertheless, as the main hormones involved in stress, future work characterizing how ACTH and CORT are changed in the *Fmr1*$^{-/y}$ mouse model in relation to each other would provide valuable insight into how the hormonal response is altered in absence of FMRP.

Synaptic Plasticity Changes in *Fmr1*$^{-/y}$ Mice: The Link Between FMRP & GR

As explained in Chapter I of this dissertation, an inverted U-shaped function between the severity of acute stress and cognitive function has been observed in a number of animal studies.

The impact of acute stress on synaptic plasticity on the *Fmr1*$^{-/y}$ DG was to shift stress-induced impairment to an earlier time point (**Figure V.2**), which was shown to be due to enhanced GR signalling. The rescue of LTP in the DG to WT levels using the GR antagonist RU38486 suggests the possibility that in absence of FMRP, GRs are involved in tonic inhibition of NMDARs. Given the available evidence that one of the identified targets of FMRP is the GR mRNA (Miyashiro et al., 2003), and that FMRP regulates protein translation (Bagni and Greenough, 2005), a possibility arises that GRs may be translating at a higher rate in *Fmr1*$^{-/y}$ mice. However, this is challenged by the immunohistochemistry and ELISA data reported in Chapter III, demonstrating that GR levels are not altered in the DG by loss of FMRP.

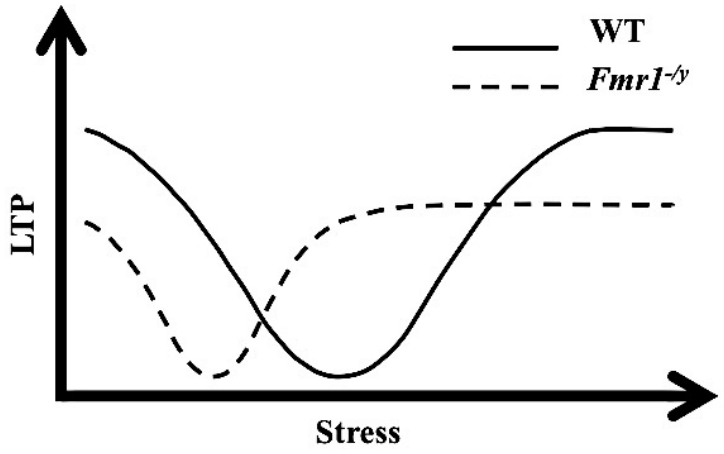

Figure V.2 Shifted Stress-Induced LTP Modulation in the DG of *Fmr1*$^{-/y}$ Mice
LTP levels in the DG of *Fmr1*$^{-/y}$ are reduced prior to application of acute stress. Absence of FMRP leads to a shift in stress-induced modulation of LTP to an earlier time point, due to dysregulation in GC receptor function.

An alternative possibility for how GRs may be impeding NMDAR function could be through an indirect pathway. As described in Chapter I, GRs are nuclear receptors that act as transcription factors when activated, either binding to GREs to transcribe genes, or repressing other factors from transcription. In addition, there is growing evidence indicating that GRs, as

well as MRs, may also be involved in nongenomic-based modulation of synaptic plasticity, possibly through membrane-associated isoforms (Di et al., 2003; Xiao et al., 2010; Joëls et al., 2011; Groeneweg et al., 2012; Zhang et al., 2012). Indeed, GRs are present at dendritic spines (Jafari et al., 2012) and GCs can rapidly suppress the activity of NMDARs in cultured hippocampal neurons, an effect that is dependent on PKA activity and a G-protein-mediated pathway (Zhang et al., 2012). Moreover, activating GRs can impede NMDAR-dependent plasticity without affecting other forms of LTP involving Ca^{2+} channels and other complex mechanisms (Wiegert et al., 2005). This effect on NMDAR-dependent plasticity was shown to be mediated via membrane-associated GRs, and was dependent on PKA activity (Liu et al., 2007). Furthermore, GluN2A-containing NMDAR-induced activation of extracellular signal-regulated kinases (ERK)1/2 signalling can be attenuated by GR activation, without having an affect on the GluN2B-containing NMDAR-induced activation of the p38-mitogen-activated protein kinase (MAPK) signalling pathway (Xiao et al., 2010). GRs facilitate this attenuation via transcription of MAPK phosphatase 1 (MKP-1), which dephosphorylates ERK1/2 (Kassel et al., 2001). In addition, GRs also inhibit glutamate uptake in hippocampal astrocytes and CA1 pyramidal synaptosomes (Virgin et al., 1991; Yang et al., 2005), thus leading glutamate levels to "spill over" and activate extrasynaptic GluN2B-containing NMDARs (Massey et al., 2004; Fox et al., 2006; Papouin et al., 2012). Interestingly, prolonged GR activation was also implicated in facilitating membrane trafficking of GluA2-containing AMPARs, which can lead to endocytosis of AMPARs and suppression of LTP (Martin et al., 2009).

In addition to their effects on NMDAR activity and downstream signalling, acute stress and GRs have also been shown to impact brain-derived neurotrophic factor (BDNF) signalling

via its tyrosine regulated kinase receptor B* (TrkB). Stress can lead to rapid induction of BDNF expression in the hippocampus (Marmigère et al., 2003). There is evidence that under low CORT levels, GRs interact with TrkB to facilitate the BDNF- phospholipase γ (PLCy) downstream signalling pathway, which is required for glutamate release in response to an action potential, but this interaction and facilitation of the PLCγ cascade are impaired after chronic activation of GRs (Numakawa et al., 2009). Interestingly, it has been reported that BDNF signalling via TrkB decreases FMRP expression in cultured hippocampal neurons (Castrén et al., 2002), and a target for FMRP is the GR mRNA (Miyashiro et al., 2003).

Overall, the available evidence suggests a model for feedback regulation that influences expression of LTP in the DG, in which the loss of FMRP leads to a dysregulation of activity, leading to enhanced GR signalling and impaired LTP in the DG of *Fmr1*$^{-/y}$. Normally, under low stress conditions CORT levels are low and mainly occupy MRs, which contribute facilitative effects for LTP in the DG, possibly through enhancing GluN2A-containing NMDARs via membrane-associated MR isoforms. Furthermore, at low levels of CORT, membrane-associated GRs interact with TrkB to facilitate the BDNF-TrkB-PLCγ signalling cascade, which is necessary for glutamate release. In addition, BDNF-TrkB signalling includes the Ras- mitogen-activated protein kinase kinase (MEK)-ERK cascade that promotes LTP. Moreover, at these low stress conditions BDNF/TrkB signalling is at a lower level, which maintains high FMRP regulatory activity over GRs, thus inhibiting GR-induced attenuation of LTP. Hence, GluN2B-containing NMDARs' activity, glutamate uptake, GluA2-containing AMPARs' recruitment, and MKP-1 activity are all kept at a minimum while GluN2A-containing NMDARs' activity is

* Also known as tropomyosin receptor kinase B

potentiated via membrane-associated MRs, and membrane-associated GRs facilitate the BDNF-TrkB-PLCγ signalling cascade, all of which promote LTP (**Figure V.3**).

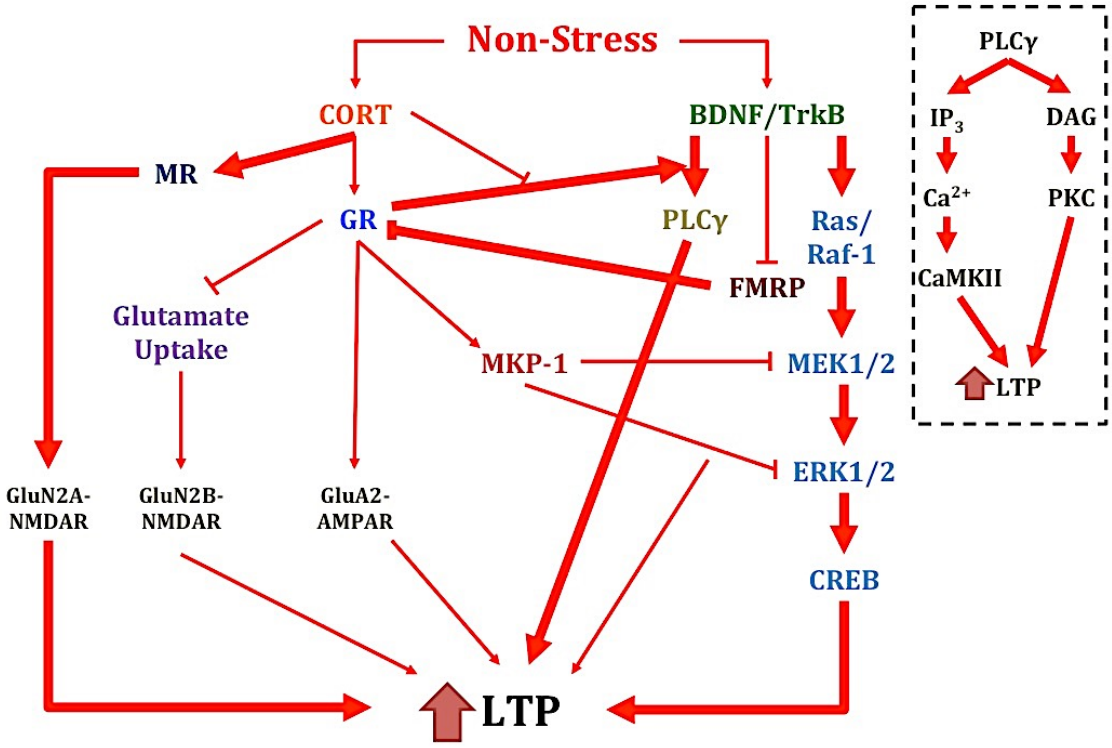

Figure V.3 Active Signalling Pathways Facilitating LTP Under Non-Stress Conditions
Under non-stress conditions CORT levels are low and MR activity predominates over that of GRs, leading to enhanced GluN2A-containing NMDAR activity. GRs interact with TrkB under low CORT levels and promote its interaction with PLCγ, which facilitates the downstream signalling cascade (inset) to release glutamate. In addition, BDNF/TrkB signalling activates the Ras-MEK-ERK pathway and maintains elevated FMRP levels, keeping GR activity low. The combination of these events facilitates LTP.

In response to stress, CORT levels elevate and GRs become occupied and their actions overtake those of MRs. As a consequence, GRs block glutamate uptake into hippocampal astrocytes and neurons, which facilitates the activation of extrasynaptic GluN2B-containing NMDARs. GR activity also controls trafficking of GluA2-AMPARs and leads to transcription of MKP-1, promoting endocytosis of AMPARs and attenuation of the Ras-MEK-ERK cascade,

respectively. Furthermore, membrane-associated forms of GR no longer interact with TrkB, which weakens TrkB-PLCγ interaction and reduces downstream signalling needed to release glutamate. In addition, high stress levels increase BDNF-TrkB signalling, which reduces FMRP levels, and hence reduces its regulation over GRs. Under these conditions LTP is suppressed (**Figure V.4**).

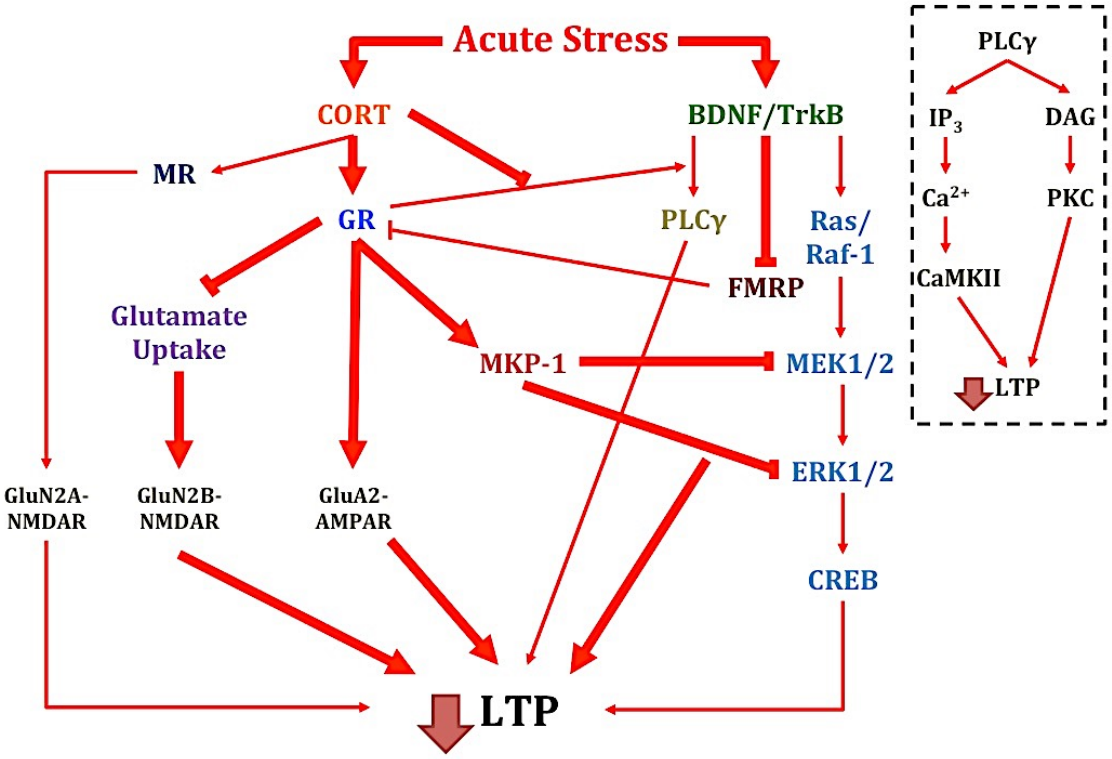

Figure V.4 Active Signalling Pathways in Stress-Induced Suppression of LTP
Acute stress elevates CORT levels, allowing GR activity to predominate over that of MRs. GRs inhibit glutamate uptake, which leads to activation of GluN2B-containing NMDARs; promote recruitment of GluA2-containing AMPARs; and increase MKP-1 levels and activity. In addition, high CORT levels prevent GR interaction with TrkB, which reduces TrkB-PLCγ interaction, leading to reduced downstream signalling (inset) to release glutamate. Acute stress also elevates BDNF signalling, which reduces FMRP levels, thus enhancing GR activity. The combination of these events suppresses LTP.

According to this model, loss of FMRP leads to enhanced GR signalling under low stress levels, which would mimic the downstream effects observed normally under high stress levels (**Figure V.5**). The predictions of this model in this condition include in absence of GR regulation

by FMRP leads to reduced membrane-associated MR potentiation of NMDARs. The results reported in Chapter III provide some evidence for this conclusion, as the use of the MR antagonist reduced LTP levels in the DG of WT, but not in *Fmr1*$^{-/y}$ mice (**Figure III.10**). On the other hand, the GR antagonist did not enhance LTP in the DG of WT, but it rescued it to WT levels in *Fmr1*$^{-/y}$ mice (**Figure III.7**). Experimental evidence provided here suggests LTP facilitation was NMDAR-dependent. However, to answer whether the facilitative effect may have been due to MR activity modulation of NMDAR function, future work should utilize both MR and GR antagonists, as this would address whether MRs are involved in the observed enhancement of LTP in the *Fmr1*$^{-/y}$ DG.

Another prediction of this model is that association between GRs and TrkB would be minimal, and in turn TrkB-PLCγ interaction and downstream signalling would be reduced. In addition, enhanced GR signalling would lead to elevated MKP-1, resulting impaired Ras-MEK-ERK signalling downstream from BDNF/TrkB. Furthermore, the model predicts that glutamate uptake and GluN2A-containing NMDAR activity are reduced due to GR activation, and GluA2-containing AMPAR trafficking would increase, which would eventually facilitate AMPAR endocytosis and in turn suppression of LTP. The model also assumes that GR function is negatively regulated through its own activity (i.e., repressing transcription of the genes for CRH and POMC), as well as through FMRP regulation. Hence, it is predicted that loss of negative regulation by FMRP would lead to enhance LTP-suppressive effects of GRs once activated. The results reported in Chapter III provide support for this prediction. Stress-induced modulation of LTP in the DG was shifted towards an earlier effect (**Figure V.2**).

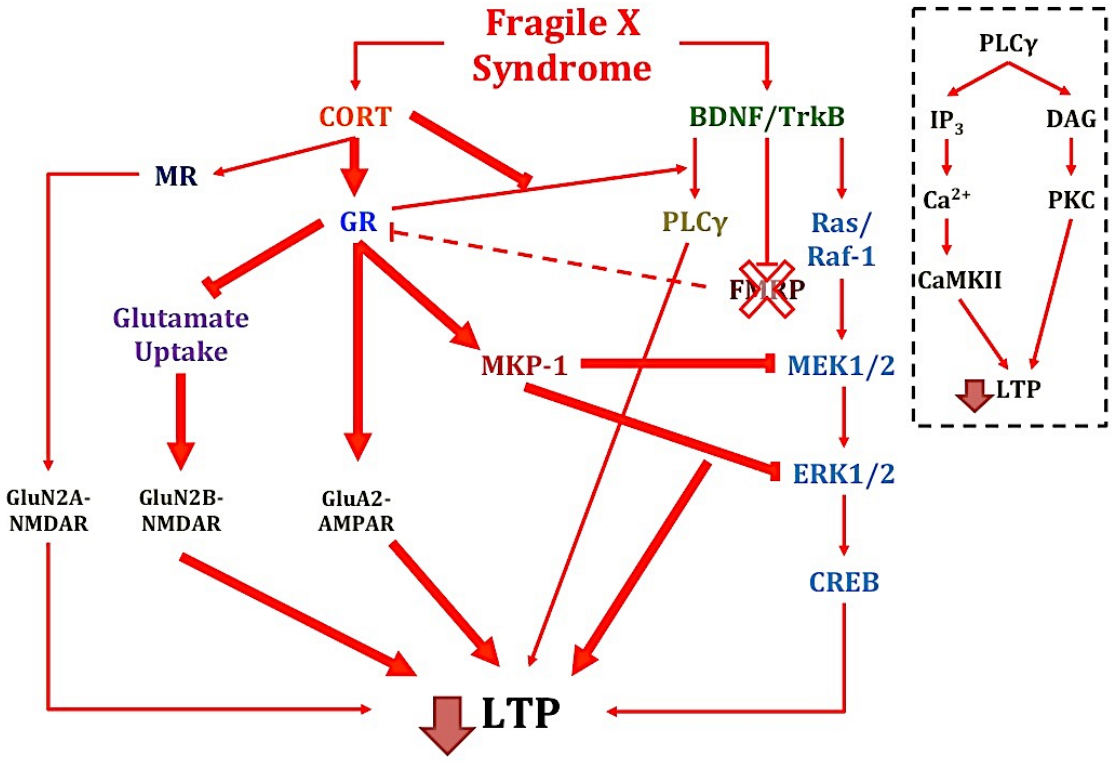

Figure V.5 Loss of FMRP Leads to Enhanced GR Signalling that Results in Suppression of LTP
Loss of FMRP as a regulator of GR function leads to enhanced GR activity under low stress conditions, resulting in reduced MR-mediated potentiation of GluN2A-containing NMDARs, and reduced glutamate uptake, as well as reduced GR interaction with TrkB, which in turn reduces TrkB-PLCγ interaction and downstream signalling required for glutamate release. In addition, enhanced GR activity facilitates trafficking of GluA2-containing AMPARs, as well as transcription of MKP-1, which in turn dephosphorylates kinases in the MAPK pathway initiated by BDNF/TrkB signalling. As a result of enhanced of GR activity in absence of FMRP, the combination of dysregulation in these signalling events leads to impaired LTP in the DG of the *Fmr1*$^{-/y}$ mouse.

FXS is a condition in which the loss of a single protein, FMRP, has devastating consequences. The most effective pharmaceutical treatment must target a central receptor involved in multiple signalling pathways that mediate essential, yet diverse functions. Although further work is needed to elucidate the significance of GR dysregulation to the neurobiology of FXS, the results presented in this dissertation suggest GRs as a promising candidate.

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