NEURONAL PLASTICITY IN THE DENTATE GYRUS
OF A MOUSE MODEL OF FRAGILE-X SYNDROME

by

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Abstract

Fragile-X syndrome (FXS) is the most common form of inherited intellectual disability (ID), representing a considerable burden of health in our society. FXS is caused by repression of the transcription of one gene, \textit{Fmr1}. Normally, expression of the \textit{Fmr1} gene leads to the production of one type of protein, the Fragile-X Mental Retardation Protein (FMRP). At the cellular level, FXS is caused by a lack of FMRP.

The fact that mice and humans possess a nearly identical \textit{Fmr1} gene has permitted the generation of a mouse model of FXS using modern transgenesis techniques (\textit{Fmr1} knockout (KO) mice). The study of the behavior of \textit{Fmr1} KO mice was expected to quickly reveal ID with subsequent elucidation of the syndrome’s neurobiological underpinnings. Unfortunately, the manifestation of presumed ID (defined as significant impairments in intellectual and adaptive functioning) at the behavioral and neurobiological levels in \textit{Fmr1} KO mice has been surprisingly elusive. How repression of \textit{Fmr1} gene expression affects the human brain to produce ID is unclear.

The dentate gyrus (DG) subfield of the hippocampus is a region of the brain that is associated with learning and emotion, exhibits marked structural and functional plasticity, and was unexplored in \textit{Fmr1} KO mice prior to the work presented in this thesis. Our overarching hypothesis is that lack of expression of the \textit{Fmr1} gene deleteriously alters structural and functional plasticity in the mammalian DG, and impairs aspects of learning and emotion associated with this brain region.

Chapter 1 introduces topics such as FXS, the hippocampus, plasticity and the mouse model of FXS. Specific hypotheses are listed at the end of chapter 1. Chapters 2 and 3 are manuscripts
written for publication in peer-reviewed journals. The bulk of the data relating to the testing of
the specific hypotheses are presented in these chapters. Chapter 4 is a general discussion that
seeks to place the results presented in the thesis into context within the literature, and also
identifies important future directions. The thesis concludes with a new model posited for the
pathophysiology of FXS.
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List of Symbols and Abbreviations

A/N - AMPA/NMDA ratio
AAID - American Association of Intellectual and Developmental Disabilities
ABC - Avidin-Biotin-Complex
ADHD - Attention-Deficit Hyperactivity Disorder
AGG - Adenine-Guanine-Guanine
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)
ANOVA - Analysis of Variance
AP - Action Potential
AP-5 or APV - (2R)-amino-5-phosphonopentanoate; (2R)-amino-5-phosphonovaleric acid
APRA - Antibody-Positioned RNA Amplification
ASD - Autism Spectrum Disorder
BDNF - Brain-Derived Neurotrophic Factor
BrdU - 5-bromo-2-deoxyuridine
CA - Cornu Ammonis
Ca²⁺ - Calcium
CaCl₂ - Calcium Chloride
cDNA - Complementary Deoxyribonucleic Acid
CGG - Cytosine-Guanine-Guanine
CIHR - Canadian Institutes of Health Research
CNS - Central Nervous System
CS - Conditioning Stimulus
Cy3 - Cyanine 3
Cy5 - Cyanine 5
dCA1 - Dorsal Cornu Ammonis 1
dCA3 - Dorsal Cornu Ammonis 3
DCX - Doublecortin
dDG - Dorsal Dentate Gyrus
dFmr1 - Drosophila Fragile-X Mental Retardation gene 1
DG - Dentate Gyrus
dHip - Dorsal Hippocampus
DNA - Deoxyribonucleic Acid
dNTP - Deoxyribonucleotide triphosphate
DSM-IV - Diagnostic and Statistical Manual -IV
EC - Entorhinal Cortex
EDR - Electrodermal Reactivity
ANS - Autonomic Nervous System
EDTA - Ethylenediaminetetraacetic acid
EGFP - Enhanced Green Fluorescent Protein
EIA - Enzyme Immuno Assay
ELISA - Enzyme-Linked Immunosorbent Assay
EM - Electron Microscopy
EPM - Elevated Plus Maze
EPSC - Excitatory Postsynaptic Current
ES - Embryonic Stem Cell
EtOH - Ethanol
tEPSP - Field Excitatory Postsynaptic Potential
FITC - Fluorescein isothiocyanate
*Fmr1* - Fragile-X Mental Retardation gene 1
FMR1 - Fragile-X Mental Retardation mRNA 1
*Fmr4* - Fragile-X Mental Retardation gene 4
FMRP - Fragile-X Mental Retardation Protein
FSIQ - Full Scale Intelligence Quotient
*FXR1* - Fragile X mental retardation, autosomal homolog 1
*FXR2* - Fragile X mental retardation, autosomal homolog 2
FXRFC - Fragile-X Research Foundation of Canada
FXS - Fragile-X Syndrome
FXTAS - Fragile-X Tremor Ataxia Syndrome
GABA - γ-Aminobutyric Acid
GABA-A - γ-Aminobutyric Acid-A
GCL - Granule Cell Layer
GFAP - Glial Fibrillary Acidic Protein
GluR1 - Glutamate Receptor 1
H₂O₂ - Hydrogen Peroxide
HFS - High-Frequency Stimulation
HICAP - hilar interneuron with commissural-associational pathway-associated axon terminals
HIPP - hilar interneuron with perforant pathway-associated axon terminals
i.p. - intraperitoneal
ID - Intellectual Disability
ID3 - Inhibitor of DNA Binding protein 3
IF - Intermediate Filament protein
IgG - Immunoglobulin G
IGZ - Inner Granular Zone
IHC - Immunohistochemistry
IO - Input-Output
IQ - Intelligence Quotient
IS - Interneuron-Specific (interneuron)
KCl - Potassium Chloride
KH - K Homology domain
KO - Knockout
Kv3.1 - Voltage-gated Potassium channel 3.1
LFS - Low-Frequency Stimulation
LTD - Long-Term Depression
LTP - Long-Term Potentiation
MAP1B - Microtubule-Associated Protein 1B
Mg²⁺ - Magnesium
MgCl₂ - Magnesium Chloride
mGluR2/3 - Metabotropic Glutamate Receptor 2/3
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>mGluR5</td>
<td>Metabotropic Glutamate Receptor 5</td>
</tr>
<tr>
<td>MOPP</td>
<td>molecular layer perforant path-associated interneuron</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>Munc13-2</td>
<td>Mammalian homologue of unc-18 13-2</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris Water Maze</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>nACSF</td>
<td>Normal Artificial Cerebrospinal Fluid</td>
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<tr>
<td>NaHCO₃</td>
<td>Sodium Bicarbonate</td>
</tr>
<tr>
<td>NaHPO₄</td>
<td>Sodium Phosphate</td>
</tr>
<tr>
<td>NE</td>
<td>North-East</td>
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<tr>
<td>neo</td>
<td>neomycin</td>
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<tr>
<td>NES</td>
<td>Nuclear Exportation Signal</td>
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<tr>
<td>NeuN</td>
<td>Neuronal Nuclei</td>
</tr>
<tr>
<td>NICHD</td>
<td>National Institutes of Child Health and Human Development</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>NR1</td>
<td>N-methyl-D-aspartic acid subunit 1</td>
</tr>
<tr>
<td>NR2B</td>
<td>N-methyl-D-aspartic acid subunit 2B</td>
</tr>
<tr>
<td>NSERC</td>
<td>Natural Sciences and Engineering Research Council</td>
</tr>
<tr>
<td>NW</td>
<td>North-West</td>
</tr>
<tr>
<td>OGZ</td>
<td>Outer Granular Zone</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
<tr>
<td>POF</td>
<td>Premature Ovarian Failure</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Poly-Sialated Neural Cell Adhesion Molecule</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post-Synaptic Density -95</td>
</tr>
<tr>
<td>PVA-DABCO</td>
<td>Polyvinyl 1,4-diazabicyclo[2,2,2]octane</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative Centrifugal Force</td>
</tr>
<tr>
<td>RGG</td>
<td>Arginine-Glycine-Glycine (motif)</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RPM</td>
<td>Rounds Per Minute</td>
</tr>
<tr>
<td>S-</td>
<td>No Shock condition</td>
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<tr>
<td>S+</td>
<td>Shock condition</td>
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<tr>
<td>SAPAP</td>
<td>SAP-90/PSD-95 Associated Protein</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>SE</td>
<td>South-East</td>
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<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
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<tr>
<td>SHIRPA</td>
<td>SmithKline Beecham Pharmaceutical – Harwell MRC Mouse Genome Centre and Mammalian Genetics Unit – Imperial College School of Medicine at St Mary’s – Royal London Hospital – St Bartholomew’s and the Royal London School of Medicine – Phenotype – Assessment</td>
</tr>
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<td>SIDS</td>
<td>Sudden Infant Death Syndrome</td>
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SSC - Saline Sodium Citrate (buffer)
SRCFC - Scottish Rite Charitable Foundation of Canada
SSRI - Selective Serotonin Reuptake Inhibitor
SVZ - Subventricular Zone
SW - South-West
TAE - Tris-Acetic Acid-EDTA
TBS - Tris-Buffered Saline
Tris-HCl - Tris-Buffered Hydrochloric Acid
UBC - University of British Columbia
UTR - Untranslated Region
UVic - University of Victoria
VCHRI - Vancouver Coastal Health Research Institute
vHip - Ventral Hippocampus
VTA - Ventral Tegmental Area
WISC - Wechsler Intelligence Scale for Children
WT - Wild-Type
XLMR - X-Linked Mental Retardation
YAC - Yeast Artificial Chromosome
αCAMKII - alpha calcium/calmodulin-dependent protein kinase II
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I have been fortunate to be influenced by many wonderful and intelligent individuals in the course of my graduate work. I would like to start by thanking my mentor and supervisor Dr. Brian Christie for his patience and unwavering support. I remember the excitement associated with being taught and trusted to conduct electrophysiological experiments as an undergraduate student in the Department of Psychology at the University of British Columbia. Without this opportunity, and blood, sweat and tears from both of us, I wouldn’t be where I am today. Thank you.

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Thank you to my parents, Ms. Susan K. Wilkes and Mr. Frank R. Eadie, for providing me with the basic skills required to function in life. In retrospect, I know that this must not have been an easy task with such a stubborn and spirited child. I cherish your unwavering compassion and support.

We are nothing without the support from those around us. Thank you.
To my family
Co-Authorship Statement

**Manuscript #1 (Chapter 2)**

The work contained in this manuscript stems from ongoing work conducted by myself and colleagues in the Christie Laboratory ([Appendix A](#)). The identification and design of the specific experiments in this manuscript occurred through collaboration in the writing of a grant to the Fragile-X Research Foundation of Canada (FXRFC) with Dr. Brian R. Christie. I performed the majority of the research and analyzed all data within this manuscript. I had considerable assistance with data collection from all other authors. I wrote, and Dr. Christie edited, the manuscript.

**Manuscript #2 (Chapter 3)**

The work contained in this manuscript stems from ongoing work conducted by myself and colleagues in the Christie Laboratory ([Appendix A](#)). Through collaboration in the writing of a grant to the Fragile-X Research Foundation of Canada (FXRFC) with my supervisor, Dr. Brian R. Christie, we identified and designed the electrophysiological work contained in this manuscript. I performed the majority of the electrophysiology research and analyzed all data. I initiated collaboration with Dr. Michael Fanselow and Mr. Jesse Cushman to conduct critical behavioral experiments. I wrote, and Dr. Christie edited, the manuscript.
1. Introduction

1.1 Fragile-X Syndrome

1.1.1 History

For more than a century it has been apparent that more males than females show significant intellectual disability (ID) (“mental retardation” to use the old terminology). For example, U.S. census figures from the end of the 1800s indicated a 24% excess of mental retardation in males relative to females (Johnson, 1897). At that time, the reason for this was unknown. Throughout the first half of the 20th century, the apparent excess of ID in males was assumed to be due to sampling bias in surveys conducted in institutions (Penrose, 1938). The assumption was that these surveys showed more ID in males because more males with ID were found in institutions due to a greater tendency towards aggression. It is now recognized that this sex difference in ID is real; ID is more prevalent and severe in males than females (Drews et al., 1995; Richardson et al., 1986).

In the 1970’s it became apparent that the increased prevalence of ID in males is due to aberrancies on the X sex chromosome. Females have two X sex chromosomes; whereas, males have an X and a Y sex chromosome. This biological fact means that aberrancies to a gene on the X sex chromosome has more severe effects on males because, unlike females, males lack another X sex chromosome that typically harbors a normal copy of the gene that can potentially compensate. In a doctoral thesis from 1969, Lehrke first argued that the preponderance of mental retardation in males is due to genes found on the X sex chromosome (“X-linked genes”), and that X-linked mental retardation (XLMR) may be responsible for a large proportion of mental retardation (Lehrke, 1974). Also in 1969, cytogenetic assessment (visualization of the
chromosomes) of four individuals in a family affected by XLMR revealed physical abnormalities on the X-chromosome (Lubs, 1969). This report was thought to be an isolated finding and largely went unnoticed (Sherman, 2002). A similar report occurred a few years later (1973), but was written in Portugese and published in Brazil and, unfortunately, also largely went unnoticed (Escalante and Frota-Pessoa, 1973; Escalante et al., 1971). However, by the late 1970’s, it was well recognized that cytogenetic testing of individuals with ID inherited in an X-linked manner often revealed a gap at the end of the long arm of the X chromosome (Giraud et al., 1976; Harvey et al., 1977). This gap became known as the fragile-X site (Figure 1.1).

During the 1970’s it was also becoming recognized that macroorchidism (enlarged testes) was associated with some forms of XLMR (Cantu et al., 1976; Escalante et al., 1971; Turner et al., 1975). In 1978, Turner and colleagues had noted an association between macroorchidism and the fragile-X site within the context of XLMR (Turner et al., 1978). Interestingly, Martin and Bell (1943), two British physicians, had published on a pedigree of individuals with ID inherited in an X-linked manner that included individuals who presented with macroorchidism several years earlier (Martin and Bell, 1943). In recognition of this, the emerging syndrome was given the name Martin-Bell syndrome (Richards et al., 1981).

Today, the Martin-Bell syndrome is typically referred to as Fragile-X syndrome (FXS), although some clinicians

1.1 Electron micrograph of a normal and fragile X chromosome.

(a) Normal chromosome shown using electron microscopy. (b) A fragile X chromosome. (c) Location of the centromere. (d) The fragile site on the fragile X chromosome (modified from Wen et al., 1997).
may still describe the phenotype and classic facial features of FXS as the “Martin-Bell phenotype.” A more sophisticated understanding of the genetic abnormalities underlying the FXS had to wait for technical advances in molecular biology. In 1991, it was found that the fragile-X site harbors a particular gene that has lost its normal ability to code for its protein (Brainard et al., 1991; Fu et al., 1991). The gene is now known as *Fmr1* (Fragile X Mental Retardation gene 1) and the protein that *Fmr1* normally codes for is known as FMRP (Fragile-X Mental Retardation Protein).

### 1.1.2 Etiology

In 1991, the gene and its abnormalities causing FXS were identified (Fu et al., 1991; Pieretti et al., 1991; Verkerk et al., 1991). In the majority of cases of FXS, an unstable CGG (cytosine-guanine-guanine) nucleotide repeat expansion occurs in the 5’ untranslated region (UTR) of the *Fmr1* gene (Garber et al., 2008). Normally, there are 6 to 50 CGG repeats in this region with a few AGG (adenine-guanine-guanine) sequences interspersed approximately every 9 bases. Transmission of this sequence from parent to child may become unstable with either a loss of the AGG regions and/or an increase in the number of CGG repeats. Thus, the presence of 55 to 200 repeats is considered to be unstable and is called the “premutation”. A premutation >60 repeats has a high risk of expanding into >200 repeats, a genetic phenomenon known as “anticipation” (Sutherland and Richards, 1995). The genetic phenomenon of anticipation accounts for the classic “Sherman paradox”, which may be defined as the increase in the probability of inheriting FXS with successive generations (Fu et al., 1991). The discovery of the genetic basis of FXS was the first example of a trinucleotide repeat expansion causing a human disorder (Verkerk et al., 1991; Warren and Nelson, 1993). In males, the premutation is associated with a
neurodegenerative condition known as FXTAS (Fragile-X Tremor Ataxia Syndrome) (Willemsen et al., 2005). In females, the premutation has been most strongly linked to premature ovarian failure (POF), a cause of significant morbidity (Sherman, 2000). The full mutation consists of >200, and approximately 800, hypermethylated repeats (Bassell and Warren, 2008). Via a poorly understood mechanism, the consequence is minimal, or no transcription of the \textit{Fmr1} gene (McConkie-Rosell et al., 1993; Pieretti et al., 1991; Sutcliffe et al., 1992). An important caveat is that some individuals may possess a proportion of cells with a full length, \textit{unmethylated} mutation to the \textit{Fmr1} gene leading to a reduction in overall FMRP production. These cases are often referred to as “mosaics” (Coffee et al., 2008). In general, the full length mutation leads to complete transcriptional repression of the \textit{Fmr1} gene which results in a loss of FMR1 mRNA and FMRP (Fragile-X Mental Retardation Protein). This is the genetic cause of the FXS phenotype. Importantly, because the \textit{Fmr1} gene is on the X chromosome, females can be heterozygous for the mutation whereas males cannot. This causes, on average, a more severe physical, cognitive and behavioral phenotype in males with FXS (Rousseau et al., 1994; Wolff et al., 1988).

1.1.3 Prevalence

One of the largest, early studies of XLMR was conducted over a 20-year period in British Columbia, Canada (Herbst and Miller, 1980). These authors examined all types of non-specific mental retardation and included data regarding individuals within families that were dead or alive. They found that approximately 2.44/1000 females were carriers for mental retardation indicating that 1.83/1000 males are affected by XLMR. We now know that loss of normal
expression of \textit{Fmr1} may account for many of these cases, although over 200 forms of XLMR may exist (Neri and Chiurazzi, 1999).

Initial prevalence estimates for FXS were based on cases diagnosed as FXS using cytogenetic testing. Herbst and Miller (and others) had initially estimated that the prevalence of FXS in males is 0.92/1000 based on (1) the assumption that approximately 50\% of non-specific “mental retardation” is caused by FXS, and (2) the estimation of the prevalence of non-specific “mental retardation” at 1.83/1000 males. Subsequent studies attempting to ascertain prevalence estimates (within target populations ranging from Scandinavia to Australia and using slightly different criteria) revealed values ranging from 0.4 to 0.7/1000 males and 0.2 to 0.6/1000 females (Gustavson et al., 1986; Jenkins et al., 1986; Kahkonen et al., 1987; Webb et al., 1986).

In 1992, DNA diagnostic testing for FXS became available, drastically improving the specificity of the diagnosis of FXS. This was primarily due to the fact that cytogenetic testing yields considerable false-positive results because of the expression of other “fragile” sites on the X chromosome (Turner et al., 1996). FXS was then defined as a fragile-site located at Xq27.3. Subsequent studies using DNA testing revealed prevalence estimates ranging from 1/2000 to 1/6000 males with FXS in the general population. The initial prevalence estimates were revised to 1/4000 males with FXS. All data to date suggest that this is consistent across racial/ethnic groups. Extrapolation of these statistics to females, based on the assumptions that approximately 50\% of female carriers show clinical symptoms and that the frequency of the full mutation is equal between the sexes, suggests that approximately 1/8000 females in the general population have FXS. Individuals carrying the premutation may be as high as 1/1000 for males and 1/350 for females. DNA based testing continues to be routinely used in the assessment of individuals with FXS and potential carriers, because it (1) is less expensive and labor intensive than
cytogenetic testing, (2) yields information regarding the size of the mutation/repeat region (to be discussed more fully in the section “Fmr1 and FMRP”), and (3) it is close to 100% specific and 98% sensitive missing only cases with small deletions or point mutations (Sherman, 2002).

In short, as the etiological basis of FXS has become better defined since this syndrome was first recognized, diagnostic testing for FXS has become more specific and sensitive. A review of various studies investigating the prevalence of FXS in different populations suggest that approximately **1/4000 males and 1/8000 females** are directly affected by FXS.

The burden on the health of individuals in our society by FXS is not limited to those who are directly affected. The subsequent section describes the phenotype of individuals with FXS, and it will become clear that these individuals require significant care and support from family, teachers, physicians and others involved in their lives.

### 1.1.4 Phenotype

Single gene disorders are often associated with a plethora of signs and symptoms, and Fragile-X syndrome is no different. This section begins with a brief description of the classic physical signs, symptoms and general medical conditions observed in individuals with FXS. Next, the neurological, neuropathological, and neuroradiological features of the syndrome are described. Finally, and perhaps most pertinent to this thesis, major domains of cognitive and behavioral abnormalities commonly observed in individuals with FXS are reviewed. It will be appreciated that the phenotype of FXS is complex.
1.1.4.1 General Medical Presentation

Physical Appearance

The classic physical appearance of prepubescent children with FXS is a long narrow face and prominent ears (Chudley and Hagerman, 1987; Verma and Elango, 1994) (Figure 1.2). Other facial features may be apparent to the experienced clinician such as puffiness around the eyes, narrow palpebral fissures, increased head/body ratio, epicanthal folds, strabismus and hypotonia (Butler et al., 1988; Hockey and Crowhurst, 1988; Simko et al., 1989). Adults may show an increase in relative jaw size and may be prone to a variety of dental problem (Loesch et al., 1993). Macroorchidism (enlarged testes) occurs in approximately 80% of adult males with FXS (Merenstein et al., 1996; Sutherland and Hecht, 1985); however, males with FXS are fertile and capable of reproduction (Rousseau et al., 1994; Willems et al., 1992). Heterozygous females with FXS show similar facial stigmata and connective tissue problems as males; however, these features are generally less prominent (Fryns, 1986).

General Medical Observations and Conditions

A variety of medical conditions have been associated with FXS. Enlargement of the ovaries have been reported in a small number of cases (Moore et al., 1990). Ophthalmologic abnormalities appear to be more prevalent in males with FXS, including strabismus (approximately 8%) and refractive errors (approximately 17%) (Hatton et al., 1998). Other sensory modalities may also be indirectly affected by loss of Fmr1 expression due to connective
tissue abnormalities. For example, children with FXS are more at risk for ear infections presumably due to the angle and shape of the eustachian tube (Chudley and Hagerman, 1987). Also related to connective tissue abnormalities include orthopedic, dermatologic and cardiac conditions. Orthopedic problems include pes planus (flat feet), excessive joint laxity and scoliosis (Davids et al., 1990). The most common cardiac condition is mitral valve prolapse, and is presumed to be related to connective tissue abnormalities (Loehr et al., 1986). Another important cardiovascular finding is increased reports of hypertension in males with FXS; however, this has often been attributed to patient anxiety during blood pressure measurement (Hagerman, 2002). It is possible however, that aberrant connective tissue lining vessel walls could also play a role in this observation. Despite these observations, longevity is not typically decreased in males with FXS.

1.1.4.2 Neurological, Neuropathological, and Neuroradiological Presentation

Neurology

Neurologists have observed that young boys with FXS may be hypotonic, and suggest that at least some of the physical signs, such as a long narrow face and joint laxity, may be unrelated to connective tissue abnormalities (Hinton et al., 1991). Generally speaking however, there is a lack of focal or hard neurologic signs upon neurologic examination; common findings are soft neurologic signs indicative of impaired motor coordination. Perhaps the most common neurologic abnormality observed is seizure occurring in approximately 25% of males with FXS (reports vary from 13% to 58% depending on selection bias related to clinical centre) (Bourgeois et al., 2009). Also indicative of significant CNS disturbance is SIDS (Sudden Infant Death Syndrome) which appears to be more prevalent in the context of loss of Fmr1 expression (Fryns et al., 1988).
Neuropathology

Limited post-mortem neuropathologic investigations have been conducted on individuals with FXS. Interestingly, histological analyses of tissue obtained from an 87-year old man with FXS revealed focal Purkinje cell loss in the cerebellum and mild “CA4” cell loss (Sabaratnam, 2000). The CA4 subfield of the hippocampus is subjacent to the dentate gyrus (DG) subfield of the hippocampus, which is the focus of this thesis (neuroanatomy of the hippocampus and DG are discussed in detail in sections 1.3 and 1.4, respectively). Perhaps the most obvious neuropathologic finding in patients with FXS is abnormalities in dendritic spines observed in various regions of the brain (dendritic spine abnormalities are discussed extensively in section 1.5.2).

Neuroradiology

Neuroimaging studies have revealed significantly enlarged cerebral ventricles in approximately 39% of cases and significantly increased intracranial volume in approximately 12% of cases. Interestingly, Reiss and colleagues negatively correlated ventricular size with IQ (Reiss et al., 1995). It is suggested that this may be related to more rapid cell death in periventricular brain regions. Reiss and colleagues have also reported increased hippocampal volume which may increase with age (Reiss et al., 1994). Such findings have led to the hypothesis that FXS is a syndrome of rapid brain aging (Murphy et al., 1999) as normal aging is also associated with increased ventricular volume (LeMay, 1984). In addition, Reiss and colleagues report decreased volumes in other brain regions such as the superior temporal gyrus. Contradictory findings to those of Reiss et al. regarding hippocampal volume exist however. Jakala and colleagues did not find a significant difference between hippocampal volume between individuals with FXS and controls (Jakala et al., 1997). A difference between these studies may
be the age of the subjects. Reiss et al. (1994) studied children whereas Jakala et al. (1997) studied adults. Note that this difference would suggest abnormal brain development rather than increased brain aging. Interestingly, Jakala et al. found non-specific changes to the hippocampus in the majority of patients with the full mutation. Focal hyperintensities in temporal pole white matter, atypical hippocampal morphology and enlargement of perivascular spaces were observed. Alterations in the volumes of other brain regions have also been associated with a variety of symptoms in FXS including the caudate nucleus, cerebellar vermis and amygdala. In short, the volume of the hippocampus and related structures may be altered in FXS, and this may be particularly apparent in children. In adulthood, neuroimaging may only be able to resolve subtle changes such as alterations in hippocampal morphology.

1.1.4.3 Cognitive and Behavioral Abnormalities in Fragile-X Syndrome

The true essence of Fragile-X syndrome (FXS) is perhaps best appreciated from a review of the typical cognitive and behavioral abnormalities. Individuals with FXS often have severe intellectual disability (ID); however, other behavioral abnormalities are also evident (Hagerman, 2002). Some individuals with FXS, particularly males, are hyperactive with attention difficulties. Some individuals with FXS, particularly females, exhibit social anxiety and extreme shyness. These observations may be related to increased reactivity to sensory stimuli (hyperarousal). In addition, the co-occurrence of other neuropsychiatric dysfunctions such as Tourette’s syndrome, aggression and even psychosis may be higher in FXS. These cognitive and behavioral abnormalities make the clinical presentation of individuals with FXS similar to that of other neurodevelopmental disorders, such as Autism Spectrum Disorder (ASD) and Attention Deficit Hyperactivity Disorder (ADHD). A thorough understanding of the cognitive and behavioral domains affected in individuals with FXS can guide the neuroscientist investigating
the neurobiological underpinnings of impairments in FXS, because different cognitive and behavioral abnormalities are associated with different regions and systems in the brain.

Cognition

Intellectual disability (ID) is the prominent feature in the cognitive profile of males with FXS harboring the full mutation (Hessl et al., 2009). ID is defined as disability originating before the age of 18, characterized by significant limitations both in intellectual functioning and in adaptive behavior as expressed in conceptual, social, and practical adaptive skills (AAIDD). The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) classifies ID in the following degrees of severity based on adaptive functioning and IQ (Intelligence Quotient): Mild (50–55 to approximately 70), Moderate (35–40 to 50–55), Severe (20–25 to 35–40), and Profound (below 20 or 25) (American Psychiatric Association., 1994). Intellectual functioning is defined as IQ obtained by assessment with a standardized, individually administered intelligence test such as the Wechsler Intelligence Scales (WISC), the Stanford–Binet, or the Kaufman Assessment Battery. The vast majority of individuals with ID (approximately 85%) fall into the “mild” category (Bourgeois et al., 2009).

The severity of ID in Fragile-X Syndrome (FXS) is apparent from studies assessing children using intelligence tests. In one study using the WISC, all individuals with FXS received an FSIQ (full scale IQ) score of 40 (4 standard deviations below the mean) (Dyer-Friedman et al., 2002; Glaser et al., 2003; Hessl et al., 2001). This is the minimum score that one can receive on this test (commonly referred to as “flooring”). Other assessments of IQ in FXS yield similar results showing “flooring.” Males with FXS fall into the “severe” to “profound” categories of ID. The ability to further resolve degrees of intellectual impairment becomes difficult at this low end of the spectrum. Although many females with FXS show some degree of ID, approximately 50%
of females are found to be borderline or higher for ID as measured using IQ tests. This is presumably related to the fact that some level of *Fmr1* gene expression still exists in females with FXS. Indeed, the level of expression of FMRP has been correlated to IQ in both males and females (Kaufmann et al., 1999; Tassone et al., 1999).

It is difficult to ascertain which intellectual domains are predominantly affected in cases of severe ID (Hessl et al., 2009). Despite this, one study has indicated that individuals with FXS may be particularly impaired in the following cognitive domains: visual-motor coordination, spatial memory, and arithmetic ability (Freund and Reiss, 1991). A more recent study corroborates this conclusion. Roberts et al. (2005) showed that the academic weaknesses of boys with FXS include deficits in visual-spatial processing, writing skills and mathematics (Roberts et al., 2005).

In short, cognition in the majority of males with FXS who possess the full mutation to the *Fmr1* gene is characterized by severe ID. This appears to be related to a complete loss of expression of FMRP based on correlations between IQ and levels of FMRP amongst individuals with decreased expression of FMRP (Loesch et al., 2004). Although some specific cognitive domains may be particularly impaired, it is methodologically difficult to conduct such studies in the context of severe ID.

**Attention and Hyperactivity**

Attention-Deficit Hyperactivity Disorder (ADHD) is a common neuropsychiatric disorder observed in children (affecting approximately 8-12% of children), the diagnosis of which overlaps considerably with FXS (Biederman and Faraone, 2005). In one study of children with FXS, 100% of 14 boys had attention or concentration problems and 71% met the full criteria for
ADHD (Bregman et al., 1988). The authors report that those individuals not meeting full criteria for ADHD may have received that diagnosis at a younger age and may have outgrown these symptoms. In a more recent study, 54-59% of boys with FXS met criteria for diagnosis of ADHD (Sullivan et al., 2006). Similar to attention deficit, hyperactivity is a common clinical observation of boys with FXS, and attention difficulties and hyperactivity often co-occur (Finelli et al., 1985; Largo and Schinzel, 1985). Fortunately, like ADHD, the prevalence of attention-deficit and hyperactivity in FXS appears to decrease with age (Bregman et al., 1988; Simon et al., 2009).

Most of the studies discussed above that showed that the majority of individuals with FXS have symptoms of ADHD were based on males with FXS. In contrast to males, a minority of females (approximately 35%) exhibit symptoms of ADHD (Freund et al., 1993; Hagerman et al., 1992). The reason for this difference is not known.

Considerable overlap exists between FXS and ADHD. A clear difference between these neurodevelopmental disorders is that the cause of FXS is clear, whereas, the cause of ADHD is largely unknown. This fact makes the study of the pathophysiology of FXS pertinent to those investigating ADHD.

**Autism**

Autism Spectrum Disorder (ASD) is a common neuropsychiatric disorder in children in our society (affecting approximately 0.5% of children), and its diagnosis overlaps considerably with the diagnosis of FXS. Brown and colleagues first reported an association between FXS and ASD when 5 of 27 males with FXS were also diagnosed with ASD (18.5%) (Brown et al., 1982a; Brown et al., 1982b). It is now estimated that approximately 30% of children with FXS meet
diagnostic criteria for ASD (Rogers et al., 2001). In addition, many of the children with FXS who do not meet the full criteria for a diagnosis of ASD still exhibit behaviors seen in children with ASD such as poor eye contact, speech perseveration, hand biting and hand flapping. Conversely, one early review suggested that the percentage of males with ASD who also have FXS (as identified by cytogenetics) is 6.5% (Brown et al., 1986; Harris et al., 2008). Using more modern DNA testing, this value has been estimated to be slightly lower, between 2 and 6%. It is clear that considerable overlap exists between FXS and ASD.

More direct comparisons between FXS and Autism suggest that, in general, children with ASD exhibit more echolalia, deviant language or inappropriate responses (Ferrier et al., 1991; Paul et al., 1987; Sudhalter et al., 1990). Also, the lack of eye contact observed in both disorders may have different roots. Cohen and colleagues have noted that children with FXS do not make eye contact when others are looking at them, whereas individuals with ASD do not make eye contact regardless of whether others are looking at them (Cohen et al., 1989; Cohen et al., 1991). This may be suggestive of greater reactive avoidance in children with FXS compared to children with ASD.

Although considerable overlap exists between FXS and ASD, these neurodevelopmental disorders clearly differ in one important way: the cause of FXS is known whereas the cause of ASD is largely unknown. This fact makes the study of the pathophysiology of FXS pertinent to those seeking clues to the pathophysiology of ASD.

**Shyness and Social Anxiety**

Shyness and social anxiety is a common presenting feature in females with FXS and a common complaint of females with the premutation. Although most boys with FXS are
considered “shy”, it has been suggested that the severity of ADHD or intellectual disability overshadows the shyness (Merenstein et al., 1996; Sobesky et al., 1995).

Shyness and anxiety can be debilitating for some individuals with FXS. For example, in one study of 17 females with FXS, 4 were diagnosed with avoidant personality disorder, a disorder characterized in part by extreme shyness (Freund et al., 1993). Others have observed mutism in some females with severe social anxiety. This is particularly apparent in certain situations, such as school, suggesting a link between shyness and hyperarousal in FXS (Hagerman et al., 1999). Although most females with FXS appear to outgrow symptoms of shyness and anxiety, they do appear to persist for many females with the full mutation (Hagerman, 2002; Hull and Hagerman, 1993).

Shyness and anxiety can be severe and debilitating for some individuals with FXS for extended periods of their lives. These symptoms are most apparent in females with FXS, which may relate to decreased, rather than abolished, levels of FMRP.

**Hyperarousal**

Hyperarousal appears to be a common clinical observation of individuals with FXS. Belser and Sudhalter (1995) found that electrodermal reactivity (EDR; palm sweat measurement) is increased in males with FXS in response to direct eye contact, indicative of an increased stimuli-induced sympathetic response (Belser and Sudhalter, 1995). A subsequent study by Miller and colleagues extended on these findings demonstrating enhanced EDR in males and females with FXS in response to repetitive stimuli in visual, auditory, tactile, olfactory and vestibular modalities (Miller et al., 1999). Interestingly, the degree of EDR enhancement is negatively correlated to the degree of FMRP expression. Although the sympathetic response is increased in
response to stimuli, it has been suggested that, under basal conditions, it is actually the parasympathetic nervous system that is affected (Boccia and Roberts, 2000). The autonomic nervous system (ANS) may be dysregulated in individuals with FXS and this may predispose to hyperarousal in response to sensory stimulation from the environment. An imbalance in the ANS in FXS may underlie the manifestation of other cognitive and behavioral abnormalities observed in individuals with FXS.

Other Comorbid Psychiatric Conditions

Significant psychiatric comorbidities, other than presented above, may also occur disproportionately in FXS. These include Tourette’s syndrome, psychosis, and aggression.

A diagnosis of FXS appears to be associated with Tourette’s syndrome (Kerbeshian et al., 1984). Specifically, coprolalia (repetitive bursts of swearing) and palilalia (perseverative, compulsive and pressured speech) are not uncommon in FXS, and are indicative of a pathological language deficit (Hagerman, 2002). Complex stereotypies involving the hands and arms may also be present in FXS, reminiscent of tics observed in Tourette’s syndrome and ASD. Anxiety appears to increase the frequency and intensity of stereotypies dramatically (Kano et al., 1988).

Psychosis is an understudied comorbid condition in FXS despite some evidence to suggest a predisposition to psychotic ideation (Bourgeois et al., 2009). Although difficult to diagnose, it may be important to recognize psychosis in the context of FXS due to the fact that patients with FXS generally respond well to antipsychotics.

Although aggression is not a psychiatric condition per se, it is a behavior that can be a major problem for many males with FXS. In fact, one study reported that approximately half of males
with FXS have a problem with periodic aggressive outbursts (Merenstein et al., 1996). This may worsen with puberty, persist into young adulthood and then decrease in middle and later life, suggesting a potential hormonal basis. Interestingly, aggressive outbursts appear to be precipitated by excessive environmental stimulation (Hagerman, 2002).

A variety of other psychiatric conditions are associated with FXS. For example, Backes and colleagues have documented the frequency of the following conditions in FXS as follows: oppositional defiant disorder (29%), functional enuresis (27%), functional encopresis (20%), separation anxiety disorder (10%) and obsessive-compulsive disorder (2%) (Backes et al., 2000).

Individuals with FXS may be at increased risk of particular psychiatric conditions. These findings speak to the complexity of the presentation of individuals with FXS in the clinic and the breadth of effects that lack of FMRP can have on the nervous system.

1.1.4.4 Summary of the Phenotype of FXS

The cognitive and behavioral phenotype of FXS is complex. Despite this fact, a review of the literature illuminates some important points. First, males with the full mutation typically present with severe intellectual disability. Analyses of individuals with reduced levels of FMRP, such as females with FXS, suggest that a correlation exists between FMRP levels and IQ. Second, males with FXS often exhibit behaviors similar to that observed in individuals with ADHD and ASD. This suggests that studies into the pathophysiology of FXS may provide important information regarding these latter, more prevalent disorders. This is important because the cause of ADHD and ASD are less well-defined than the cause of FXS. Third, anxiety appears to be a common feature observed in humans with FXS. Anxiety appears to be more obvious in females than males suggesting that emotional responses may be more sensitive to a
reduction in FMRP expression. These observations have led to a model (Figure 1.3) relating the spectrum of symptoms observed in FXS to levels of FMRP expression. Fourth, the manifestation of many symptoms in FXS may relate to a state of hyperarousal associated with imbalances in the autonomic nervous system. Fifth, individuals with FXS may be particularly predisposed to some psychiatric conditions, further complicating the clinical presentation of any particular individual with FXS, but also providing insight into the array of potential behavioral effects of loss of FMRP in the brain.

1.1.5 Treatment

The cause of FXS and the phenotype of individuals with FXS have been thoroughly described. Despite this, the development of treatments for FXS has been slow and the possibility of a cure seems several years away. Surveys into pharmacological treatments for FXS suggest that the majority of individuals with the diagnosis currently receive, or have received, psychototropic medications (e.g., antidepressants, stimulants) directed towards the presenting symptoms (e.g., anxiety, hyperactivity, attention deficit) (Hall, 2009; Valdovinos, 2007). Surprisingly, the use of such medications in this manner is supported by reports from parents and physicians rather than evidence from double-blind, randomized, controlled trials. Similarly, the side effect profiles of these medications in individuals with FXS have been poorly documented.
For example, methylphenidate (Ritalin; a psycho-stimulant commonly used to treat ADHD) is the most commonly prescribed pharmacological agent in FXS (Berry-Kravis and Potanos, 2004). Methylphenidate is presumably prescribed by clinicians due to (1) the common presentation of symptoms of ADHD in individuals with FXS and (2) research showing that this drug decreases impulsivity and increases concentration in children with ADHD (Biederman and Faraone, 2005). Only a single, small controlled study has been conducted to date on the efficacy of methylphenidate in the context of FXS (Hagerman et al., 1988). This study appears to suffer from some methodological issues and, in fact, reports small improvements on the majority of outcome measures. Side effects included mood lability and irritability. More research is clearly warranted into the efficacy and side effects of all commonly prescribed psychotropic medications for individuals with FXS.

The paths to the discovery of drugs, such as methylphenidate, currently used to treat FXS have largely started with a serendipitous discovery that a compound affects some aspect of behavior. This can be attributed to a rudimentary understanding of the neurobiological basis of symptoms observed in neuropsychiatric conditions in general.

The 21st century may mark a point in history where drug discovery for neuropsychiatric disorders, such as FXS, move from a dependence on serendipity to a dependence on an understanding of pathophysiology. The latter approach is generally referred to as “rational” drug design because it targets the underlying pathophysiological abnormalities rather than the specific presenting symptoms. The hope is that, assuming the correct target is identified, the manifesting symptom(s) will be alleviated in a more efficacious manner with less side effects and a greater population response rate. Currently, several open-label studies are underway aimed at targeting particular biochemical pathways that may be abnormal in FXS (Hall, 2009). The success of this
approach will depend upon the illumination of the correct pathophysiological targets. Although the ultimate target in FXS is replacement of normal functioning of the \textit{Fmr1} gene, considerable practical barriers currently exist for this approach (Peier et al., 2000; Pfeifer and Verma, 2001; Spencer et al., 2008; Zeier et al., 2009).

1.1.6 Summary

Tremendous advances have occurred in the last century in our understanding of intellectual disability including the recognition, characterization and study of FXS. Prior to this, clinicians and researchers noted that “mental retardation” appears more in males in institutions. By the 1970s it became apparent that this may be related to the presence of genetic mutations on the X sex chromosome. A gap was noted on the X chromosome in some individuals that inherited intellectual disability in an X-linked manner. The phenotype of these individuals was characterized and similarities were noted between individuals meeting these criteria. In 1991, the fragile site on the X chromosome was found to harbor a dysfunctional gene, now known as \textit{Fmr1}. The phenotype of individuals with this genetic abnormality has been well described, and the syndrome is commonly known as FXS. Recent advances in basic research and the effects of repressed \textit{Fmr1} gene expression provide hope for the development of novel, rational therapeutics for FXS and related conditions.
1.2  \textit{Fmr1} and FMRP

1.2.1  \textit{Fmr1} gene

The \textit{Fmr1} gene is extremely similar between mice and humans (Kirkpatrick et al., 2001). The \textit{Fmr1} gene is found at the Xq27.3 locus corresponding to the fragile site observed using cytogenetic methods. The degree of homology in the \textit{Fmr1} gene across mammalian species has been estimated at 97\% (Ashley et al., 1993; Hinds et al., 1993). Both mouse and human \textit{Fmr1} genes possess the same number of exons and introns and, although the size of introns differ, the size of fifteen of seventeen exons are identical between these species (\textbf{Figure 1.4}). The first and the last exon differ in size between mice and humans, but do not appear to correspond to the identified functional motifs of FMRP. This suggests that FMRP is highly orthologous between mice and humans (Kirkpatrick et al., 2001).

The \textit{Fmr1} gene has been conserved across mammals and non-mammalian vertebrate species including chicken (Price et al., 1996), xenopus (Siomi et al., 1995), and zebrafish (Tucker et al., 2004). Considerable divergence in amino acid composition appears to have occurred in invertebrate evolution. This is based on studies showing that only about the first third of the
drosophila Fmr1 gene (dFmr1) is homologous with the mammalian Fmr1 genes (Wan et al., 2000). There is no homologue in C. elegans or yeast. Two mammalian paralogs of Fmr1 exist (FXR1 and FXR2) (Kirkpatrick et al., 2001). Although it has been suggested that the protein products of these genes may compensate for loss of FMRP expression in FXS, little evidence exists to support this hypothesis (Coffee et al., 2010; Darnell et al., 2009; Zhang et al., 2009). The paralogs of Fmr1 will thus not be discussed further here. Importantly for the generation of a mouse model of FXS, the Fmr1 gene appears to be highly homologous, and presumably orthologous, across mammalian species.

1.2.2 FMRP

An obvious question is why is Fmr1 highly conserved across mammals? The answer presumably lies in an understanding of the role of the protein product in cells of the body. Alternative splicing of the Fmr1 gene produces several variants of the Fragile-X Mental Retardation Protein (FMRP) ranging from 70 to 80 kDa (Bardoni et al., 2001; Devys et al., 1993; Sittler et al., 1996). The physiological role of FMRP splice variants is not well understood. These are therefore commonly collectively referred to as FMRP. Important clues to the function of FMRP have been gleaned from experiments assessing the details of the nature of the FMRP molecule and analyses of the cell types that express FMRP.

1.2.2.1 Structure of FMRP

FMRP contains several motifs providing clues to its cellular function, such as RNA-binding motifs and nuclear localization and export signals (Figure 1.5). Early studies of the predicted amino acid sequence of the Fmr1 gene revealed that FMRP harbors several RNA-binding motifs (Ashley et al., 1993; Siomi et al., 1993). These motifs include two tandem KH domains and an
RGG box that confer an ability to bind approximately 4% of mRNA in the mammalian brain (Bassell and Warren, 2008). FMRP has gained most notoriety for its prominent role in the regulation of mRNA.

The observations that FMRP is an RNA-binding protein and is found in the cytoplasm of neurons led to the consideration of the involvement of FMRP in mRNA shuttling and protein translation. In addition, it appears that FMRP is associated with functional polysomes. Feng and colleagues localized FMRP to the cytoplasmic polysome fraction generated from normal rat brains (Azar et al., 1997). This localization of FMRP was lost following treatment of this fraction with agents that cause ribosomes to run-off translated mRNA. Interestingly, a point mutation to a particular amino acid in the second KH domain (I304N) also prevented the localization of FMRP to the polysome fraction (Azar et al., 1997). Two other important domains appear to be the nuclear localization signal (NLS) and nuclear export signal (NES). The

1.4 The Fmr1 gene and FMRP.

The Fmr1 gene showing expansion of CGG repeats from a normal number of repeats to the number of repeats causing the “premutation” and the number of repeats causing the full mutation for FXS (bottom left). The regions of the Fmr1 gene (line with blue boxes representing exons) that correspond to the functional domains of FMRP (line with green boxes) are also shown (top) (modified from Bassell and Warren, 2008).
presence of these domains in FMRP is associated with nucleocytoplasmic shuttling of FMRP (Bardoni et al., 1997; Eberhart et al., 1996). These observations have led to the hypothesis that FMRP may function to translocate mRNA from the nucleus to the cytoplasm for subsequent translation at polyribosomes.

### 1.2.2.2 Tissue and Cell Expression of FMR1 mRNA and FMRP

The expression pattern of FMRP in particular cell types of various systems of the body provide information suggestive of the normal function of this protein. Two key studies presented in *Nature Genetics* in 1993 provided considerable insight into this issue. Hinds et al. (1993) assessed normal expression of the *Fmr1* gene in various human and mouse tissues using Northern blot and in situ hybridization approaches (Hinds et al., 1993). The greatest expression of the gene appeared to be in the testes and brain. FMR1 mRNA was detectable in some other organs such as the lungs, kidneys and placenta. *In situ* hybridization for FMR1 mRNA in the adult mouse brain was “most intense” in the granular layer of the hippocampus (Figure 1.6). In addition, these authors noted that expression levels were highest in tissue obtained from young individuals. Intermediate or low levels of signal were evident throughout the cerebral cortex and other structures, with the exception of the cerebellar granule

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Level of expression</th>
</tr>
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<tbody>
<tr>
<td>Brain</td>
<td>cerebellum, granular layer +++</td>
</tr>
<tr>
<td></td>
<td>hippocampus, granular layer +++</td>
</tr>
<tr>
<td></td>
<td>cerebral cortex +</td>
</tr>
<tr>
<td>Testis</td>
<td>seminiferous tubules +++</td>
</tr>
<tr>
<td>Esophagus</td>
<td>epithelium +++</td>
</tr>
<tr>
<td>Thymus</td>
<td>cortex +++</td>
</tr>
<tr>
<td>Spleen</td>
<td>medulla +++</td>
</tr>
<tr>
<td></td>
<td>white pulp +++</td>
</tr>
<tr>
<td></td>
<td>red pulp ++</td>
</tr>
<tr>
<td>Ovary</td>
<td>follicles +++</td>
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<tr>
<td>Eye</td>
<td>retina +++</td>
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<td>iris +++</td>
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<td>lens epithelium +++</td>
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<td>Colon</td>
<td>corneal epithelium +</td>
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<tr>
<td>Uterus</td>
<td>++</td>
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<td>Thyroid</td>
<td>+</td>
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<td>Liver</td>
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Reprint of table indicating normal levels of FMR1 mRNA expression in various tissues of the adult murine body and various regions of the brain. Note the high levels of expression in the brain and the granular layer of the hippocampus (modified from Hinds et al., 1993).
layer. Within the cerebral cortex, the FMR1 mRNA signal was most intense in the piriform and entorhinal cortices, the latter being the major input to the granule neurons in the DG of the hippocampus (anatomy to be discussed more extensively in subsequent sections). Pertinent to this thesis, these data suggest that, under normal conditions, FMRP likely plays an important role in granule neurons of the adult brain. Interestingly, FMR1 mRNA expression levels appeared highest early in development, suggesting that FMRP may play a particularly important role in neuronal development (Ito and Sugie, 1999). It is thus not surprising that expression of FMR1 mRNA was found to be relatively high in the granule cell layer of the hippocampus, as this is one of the only regions in the brain that produces new neurons across the lifespan. In a separate study in 1993, the significant expression of FMRP in the brain and testes was confirmed using western blot and immunohistochemical analyses (Hanzlik et al., 1993). An important observation from the publication by Hinds and colleagues was that FMRP is expressed mainly in neurons rather than glia. These observations have been confirmed using biochemical approaches (Feng et al., 1997). (A caveat is that some recent studies suggest that FMRP may be transiently expressed in young glial cells of the brain (Pacey and Doering, 2007; Wang et al., 2004).) In short, it appears that FMRP expression is found in various organs and brain regions, and expression levels of FMRP may be amongst the highest in granule neurons in the hippocampus of the mammalian brain. It may be that FMRP plays a particularly important role in the shuttling of mRNA from the nucleus to the cytoplasm in these cells.

1.2.3 FMRP and gene expression

Shortly after the identification of the Fmr1 gene and characterization of its protein product FMRP as an RNA-binding protein, experiments aimed at identification of other proteins that may
be affected in FXS appeared. An understanding of which proteins are affected was expected to provide further insight into the specifics of how the neuron may be affected by loss of expression of the \textit{Fmr1} gene to FMRP. In other words, this information would provide important clues into the mechanism by which the genetic mutation that defines FXS deleteriously alters neurons whose functioning presumably underlies key symptoms in this disorder.

Initially, researchers assessed the interaction of FMRP with individual mRNAs. Interestingly, one of the first mRNAs that FMRP was shown to bind to was its own (Ashley et al., 1993). In a separate study, FMRP was shown to bind to the 3’ untranslated region (UTR) of myelin basic protein (Brown et al., 1998). Starting in the year 2000, more sophisticated approaches aimed at obtaining all the mRNA that interact with FMRP started to be employed. Denman and colleagues captured mRNA that associates with FMRP using biotinylated FMRP and subsequent amplification with differential display PCR (Sung et al., 2000). Many of the mRNA identified coded for unknown protein products. Shortly after this, Darnell and colleagues reported that the preferred RNA-binding site on FMRP is in fact the RGG box (Darnell et al., 2001). The RGG box was found to bind with high-affinity to the tertiary RNA structure called a G-quartet (Figure 1.7).

These authors used an \textit{in vitro} RNA selection protocol

![Figure 1.7 G-quartet RNA tertiary structure.](image)

This mRNA structure confers binding to FMRP. (A) Chemical structure of a single G-quartet. (B) The tertiary structure of mRNA showing the predicted conformation associated with G-quartets (green) (modified from Maizels, 2006).
involving a set of iterative experiments passing pools of RNA through FMRP-affinity columns. A bioinformatic screen of a genome database for G-quartet-containing mRNA subsequently revealed possible FMRP targets (Darnell et al., 2004). In 2001, Brown et al. co-immunoprecipitated FMRP with its mRNA from Fmr1 KO and WT mouse brains and generated cDNAs from the isolated mRNA (Brown et al., 2001). The cDNAs were then subjected to analyses using Affymetrix gene chips. The mRNA targets identified were screened for ones that possess G-quartets, yielding 13 candidate mRNA targets for FMRP. A comparison of the presence of these 13 candidate mRNAs in the polysome fraction of lymphoblastoid cell lines from individuals with or without FXS revealed that 10 of these 13 showed alterations in their polysome distribution in the absence of FMRP. Approximately half of these were increased and half decreased. Eleven of the 13 candidate mRNAs initially identified relate to (1) receptors and ion channels (V1a receptor and Kv3.1 potassium channel), (2) proteins involved in synaptic function (munc13-2, NAP-22, sec-7 related guanine nucleotide exchange factor, and rab-6 binding protein), (3) proteins involved in neurite extension and development (MAP1B, semaphorin 3F, and ID3). Greenough, Eberwine and colleagues performed a similar technique with two notable exceptions: the use of primary rat hippocampal cells and a technique called APRA (antibody-positioned RNA amplification) following isolation of mRNAs using the anti-FMRP antibody 1C3 (Miyashiro et al., 2003). The proteins encoded by the identified mRNA fell into several categories including cell signaling and communication, cell structure and motility, secretory system and regulation of transcription and translation. A screen for the presence of G-quartet-like elements suggested that more than a quarter of the identified mRNA possess this complex tertiary structure.
A series of exciting recent articles have shown that the mRNA coding for the postsynaptic scaffolding protein PSD-95 possesses the canonical G-quartet structure within the 3’UTR of its mRNA (Todd et al., 2003). The binding of FMRP to PSD-95 mRNA appears to be important for the stabilization, rather than the localization, of PSD-95 at the synapse (Zalfa et al., 2003). A more recent article has extended upon these findings showing that in addition to PSD-95, the mRNA for other synaptic scaffolding molecules (e.g., Shank1 and SAPAP1-3) bind FMRP (Schutt et al., 2009). These authors also report that the mRNA for the NMDA-type glutamate receptor subunits NR1 and NR2B normally bind FMRP. FMRP appears to have a particularly important role in localization or stabilization of key synaptic molecules involved in the maintenance of synaptic structure and function.

It is possible that loss of FMRP affects the translation of several other proteins in a more indirect manner. For example, Zalfa et al. (2003) provided data suggesting that FMRP associates with BC1 in the cytoplasm of neurons in rodents and BC200 in primates (Zalfa et al., 2003). These proteins in turn anneal to the 3’UTR of important dendritically-localized messages such as MAP1B, αCaMKII and Arc. The fact that the expression level of these proteins is increased in brains of Fmr1 KO mice suggest a model whereby FMRP normally negatively regulates the expression of these messages. FMRP is normally part of a complex of at least five other RNA-binding proteins (Ceman et al., 1999; Ceman et al., 2000; Ohashi et al., 2002) and it is possible that loss of FMRP from this complex alters the function of the whole complex. FMRP may affect the expression of genes that code for mRNA that directly bind to FMRP and genes that code for mRNA that don’t normally bind directly to FMRP but do rely upon the complex normally containing FMRP.
In short, loss of FMRP directly and indirectly affects the expression of a number of genes. Some of these genes code for proteins integral to synaptic structure and function. It appears that loss of Fmr1 can lead to an array of changes in the molecular composition of the neuron.

1.2.4 Summary

The Fmr1 gene appears to be highly homologous across mammalian species, permitting the study of this gene and its associated protein (FMRP) in a variety of systems. Investigations into the normal role of FMRP in the cell have suggested that it binds and shuttles mRNA to the cytoplasm for subsequent translation. The expression profiles of FMRP in the body suggest that it may be particularly important in the testes and brain. In the brain, it appears that FMRP is highly expressed in the granule neurons of the hippocampus. Within the cell, repression of transcription of the Fmr1 gene appears to influence the expression of multiple genes which could potentially alter cellular functioning. The research presented in this thesis assesses the effects of deletion of the Fmr1 gene on granule neurons of the hippocampus in the mammalian brain.
1.3 The Hippocampus

Expression levels of FMRP within the mammalian brain are normally amongst the highest in dentate granule neurons in the hippocampus (Feng et al., 1997; Hinds et al., 1993). Thus, it is reasonable to expect that a lack of production of FMRP may have significant effects on this brain region. Neuroimaging and neuropathology suggest that gross morphological alterations exist in the hippocampus, and that these abnormalities may be most evident in the young brain (Jakala et al., 1997; Reiss et al., 1994). In addition, the hippocampus has been associated with learning (e.g., visuo-spatial learning) and emotion (e.g., anxiety), domains clearly affected in individuals with FXS (Hagerman, 2002). This thesis focuses on the effects of loss of FMRP on the dentate gyrus (DG), the subfield of the hippocampus containing granule neurons. This section provides critical background information on the hippocampus.

The hippocampus is a bilateral, curvilinear structure located in the medial temporal lobe of the mammalian brain (Figure 1.8) (Amaral and Lavenex, 2007). The hippocampus is part of a larger brain system typically referred to as the hippocampal formation which includes the entorhinal cortex, dentate gyrus (DG), hippocampus proper (including the CA1-4 subfields), the presubiculum, subiculum and parasubiculum. Throughout this thesis, the hippocampus is defined as the DG subfield and the subfields of the hippocampus proper. These subfields are clear in cross-sections of the hippocampus (Figure 1.9).
1.8 The hippocampus in the human and rodent brain.

The location of the hippocampus (red) within the human (left panels) and rodent (right panels) brains. Multiple perspectives provide an appreciation of the curvilinear nature of this brain structure. The entorhinal cortex (green) provides the major excitatory input to the hippocampus (modified from Amaral and Lavenex, 2007).

1.9 The hippocampus in cross-section.

The mouse hippocampus in cross-section showing its major subfields: DG (dentate gyrus), CA (cornu ammonis) 3, and CA1. (A) Coronal section obtained from a wild-type mouse processed with a Nissl stain (cresyl violet). (B) Cajal sketch of the hippocampus and its connections (modified from Cajal, 1909 1st Ed.; 1911 2nd Ed.).
1.3.1 Trisynaptic circuit

Ramon y Cajal first illustrated the connections of the hippocampus (Cajal, 1909 1st Ed.; 1911 2nd Ed.; Cajal, 1995). The unidirectional, or non-reciprocal, nature of these connections led to a description of the hippocampal trisynaptic circuit (Andersen et al., 1969), referring to the three major sets of fast, excitatory connections between the following pathways (Figure 1.10).

![Schematic of the trisynaptic circuit of the hippocampus.](image)

**1.10 The trisynaptic circuit of the hippocampus.**

Schematic of the trisynaptic circuit of the hippocampus. The first set of synapses is between the perforant path input fibres from the entorhinal cortex (green dashed line) to dentate granule neurons (red circle). The second set of synapses is between the mossy fibres (red dashed line) and large pyramidal neurons of CA3 (yellow triangle). The third set of synapses is between the Schaffer’s collaterals (yellow dashed line) and pyramidal neurons of CA1 (blue triangle).

The majority of neocortical input to the hippocampus arises from a unidirectional projection from the entorhinal cortex (EC) via the **perforant path** to the molecular layer of the dentate gyrus (DG) (the perforant path also projects to the stratum lacunosum moleculare of the hippocampus proper). This represents the first set of synapses in the trisynaptic circuit, the first point of potential plasticity for information flow into the hippocampus and the focus of this thesis. The perforant path travels from the EC to the hippocampus via the angular bundle (runs between the EC and the subiculum) and the alveus (a thin white sheet of myelinated fibres; runs
adjacent to the CA3 and DG lining the deep, ventricular side of the hippocampus). Generally, the angular bundle carries fibres to the dorsal subregion of the hippocampus whereas the alveus carries fibres to the more ventral subregion (dorsal and ventral subregions of the hippocampus are discussed in detail in section 1.3.3).

Dentate granule neurons of the DG send un-myelinated axons, the mossy fibres, to the CA3 subfield, making a physiologically and structurally unique type of synapse called the mossy fiber synapse. This set of synapses is unique because of the unusually large presynaptic terminals and complex postsynaptic dendritic spine structure of CA3 pyramidal neurons called thorny excrescences. Also, this set of synapses does not exhibit NMDA-dependent synaptic plasticity. The axons of CA3 pyramidal neurons, make significant reciprocal and autaptic connections onto themselves.

The axons of CA3 project to the CA1 (and CA2) subfield of the hippocampus. This pathway is commonly referred to as Schaffer’s collaterals, named after the Hungarian neuroanatomist Karoly Schaffer. This is the last of the three sets of synapses in the classic trisynaptic circuit. The CA1 projects to the subiculum and back to the EC (Figure 1.11). The trisynaptic circuit is best viewed as a conceptual scaffold to which information regarding other neuroanatomical subtleties and complexities can be added.

The hippocampus is a well-organized structure found in the mammalian brain consisting of at least three well-demarcated subfields. The DG subfield is the first subfield along the transverse axis. The DG receives its main excitatory input from the EC and projects its axons to the CA3 subfield of the hippocampus proper. The CA3 subfield projects to the CA1 subfield, completing the unidirectional, trisynaptic circuit.
1.11 Projections along the transverse axis of the hippocampal formation.

The hippocampus is conducive to laboratory experiments for several reasons: single cell layers with strictly laminated inputs, predominant unidirectional connections between series of cortical regions, and axonal axes orthogonal to dendritic axes. The experiments employed in this thesis take advantage of these features to investigate the effects of deletion of the \textit{Fmr1} gene on synaptic communication.

1.3.2 Proposed functions

The hippocampus is arguably the most thoroughly studied structure in the mammalian brain. The implication of the hippocampus in learning, memory, and emotion, and the importance of these processes/experiences to one’s sense of self, may be at least partially responsible for this observation.

Prior to the 1930’s, the prominent view was that the hippocampus was chiefly part of the olfactory system (although neuroanatomists had suggested a variety of functions for the
hippocampus) (Andersen et al., 2007; LeDoux, 2000). This view largely stemmed from the observation that mammals with prominent olfactory sensation and primary olfactory sensory structures also possessed a relatively large hippocampus. In 1947, Brodal generated a comprehensive and highly influential review of this literature that raised significant doubts on the role of the hippocampus in olfaction (Brodal, 1947). Today, it is appreciated that the functions of the hippocampus include the processing of olfactory information; however, other cognitive/behavioral modalities, such as memory and emotion, play a more central role in discussions surrounding hippocampal function.

During roughly this same time period (1930s to 50s), another influential neuroanatomist, James Papez, suggested that the hippocampus is part of a neural system subserving emotion (Papez, 1995). Papez’s view was that the hippocampus collects sensory information from cortical inputs and links this information to an emotive state. Then, Papez suggested, the information is transferred to downstream structures that are important for conscious awareness. Although “Papez’s circuit” has been scrutinized for its suggestion that the processing of emotion is restricted to particular structures in the brain, Papez had shifted discussions regarding hippocampal function from olfaction to more complex processes. By the early 1950s, years of research into the processing of emotion had culminated in MacLean’s “Limbic System” concept (Mac, 1949; Maclean, 1955). The problem of how the brain makes emotion seemed to have been largely solved (LeDoux, 2000). The Limbic System Theory viewed cognition as the business of the neocortex and emotion as the business of the limbic system. Although this dissociation was a gross oversimplification, data remains supporting the notion that limbic structures (e.g., amygdala) play central roles in some aspects of emotion (e.g., fear). Modern researchers investigating the role of the hippocampus in emotion suggest that its special function
may be the coding of contextual cues associated with fearful environments (Engin and Treit, 2007; LeDoux, 2000). The years following the proclamation of the “Limbic System Theory” of emotion, brain research into emotion suffered. This appears to be a function of difficulties defining emotion and measuring emotion in animals, as well as the emergence of the “cognitive” revolution focused on the neural mechanisms of perception and memory.

In 1957, the first influential reports implicating the hippocampus in learning and memory were put forth. Scoville and Milner described the effects of a bilateral temporal lobectomy on the patient H.M. (Scoville and Milner, 1957; Scoville and Milner, 2000) This patient had severe, intractable seizures for which he received this experimental operation. Although the seizures subsided, H.M. was left with severe, anterograde amnesia for explicit information; that is, he could not form new memories for information such as person, place or thing. Following the case of H.M., it was clear that the temporal lobe plays a critical role in learning and memory.

Many important advancements in the study of the functions of the hippocampus occurred in the 1970 and 1980s. For example, new behavioral tasks for rodents and non-human primates, and new techniques to study the activity of single neurons in behaving animals with implanted microelectrodes were developed. In 1971, O'Keefe and Dostrovsky articulated the “Cognitive Map Theory” of the hippocampus which suggested that the hippocampus is dedicated to spatial memory (O'Keefe, 1990; O'Keefe, 1991; O'Keefe and Dostrovsky, 1971). This was largely based on the observation of place cells in the CA1 subfield of the hippocampus – cells that fire when an animal is in a particular place in its environment. Also, new classifications of learning and memory were proposed by Squire (Squire, 1982; Squire, 1998; Squire, 2004). The “cognitive” revolution was gaining prominence in discussions of hippocampal function.
More support for the role of the hippocampus in learning and memory came from the discovery of a physiological phenomenon known as long-term potentiation (LTP) in the hippocampus (Bliss and Lomo, 1973) (synaptic plasticity is discussed in detail in section 1.5.3). Based on earlier theorizations of how learning and memory occur in the brain (i.e., Hebb’s neurophysiological postulate), LTP was hypothesized as a neurobiological model of learning and memory (Hebb, 1949). The initial discovery of LTP in the hippocampus provided ancillary evidence that the primary role of the hippocampus is learning and memory. Today, we know that LTP or related forms of synaptic plasticity can be found in many regions of the brain, and that synaptic plasticity may be associated with different aspects of behavior depending on the region of the nervous system in which it is observed.

Collingridge and Morris provided further evidence to support the relationship between LTP, learning/memory and the hippocampus. Collingridge had noted that LTP is dependent on the activation of the NMDA receptor, a glutamate receptor with unique properties (e.g., rectification and calcium permeability) (Collingridge et al., 1988a; Collingridge et al., 1988b). Morris infused an antagonist of the NMDA receptor into the hippocampus and noted that the animals were impaired in learning a spatial task (i.e., Morris water maze; MWM) (Davis et al., 1992).

Today, the primary function of the hippocampus is considered learning and memory; however, the role of the hippocampus in other processes/experiences such as olfaction and emotion has not been discredited. An emerging view is that the hippocampus functions in multiple capacities, employing distinct mechanisms for specific aspects of a particular process/experience.
1.3.3 Longitudinal axis

The function of the hippocampus may not be homogeneous across the longitudinal axis. By convention, the region of the hippocampus closest to the septal pole of the longitudinal axis is referred to as the dorsal hippocampus (dHip) and the region of the hippocampus closest to the temporal pole is referred to as the ventral hippocampus (vHip) (Figure 1.12) (Moser and Moser, 1998). In humans and non-human primates, these regions correspond to the posterior and anterior hippocampus. In an attempt to adhere to convention, we have adopted the dorsal-ventral terminology. Historically, a small fraction of studies investigating the anatomy, histology and physiology of the hippocampus have made the distinction between the dHip and vHip. Indeed, many studies have investigated only one subregion, typically the dHip. Many of the studies that have made this distinction have found important differences. The first manuscript presented in chapter 2 of this thesis makes this distinction. Functional differences across the longitudinal axis of the hippocampus are reviewed below.

1.12 Dorsal and ventral subregions of the hippocampus.

The rodent hippocampus (red) with the dorsal and ventral subregions demarcated with arrows (modified from Amaral and Lavenex, 2007).
1.3.3.1 Dorsal Hippocampus

The emphasis on the relationship between learning/memory and the hippocampus is largely rooted in O’Keefe’s “Cognitive Map Theory” (Andersen et al., 2007; O’Keefe and Dostrovsky, 1971). This theory is founded on one very important observation: pyramidal neurons in the CA1 subfield of the hippocampus electrically discharge when the animal is located in a *specific* location of an environment. These cells were named “place cells”, and the location of the environment that induce their activity is referred to as the corresponding “place field.” It is often not emphasized that these *in vivo* electrical recordings were conducted in the dHip. This was presumably due to the ease of access to this subregion of the hippocampus. Although place cells appear to exist in both the dHip and vHip (Jung et al., 1994; Poucet et al., 1994), the proportion of cells with spatial correlations is decreased in the ventral subregion (Jung et al., 1994). In addition, Moser and colleagues have shown that place cells in the dHip are associated with a much more specific environmental area (Kjelstrup et al., 2008). These authors recorded from CA1 pyramidal neurons in the dHip or vHip *in vivo* in rats while the animals traversed an 18-meter long track. The size of place fields associated with place cells in the dHip were found to be approximately 1 meter. In contrast, place field sizes associated with place cells in the vHip were approximately 10 meters (approximately 10 x more diffuse than that observed in the dHip). It appears that the dorsal subregion processes spatial information with greater resolution than the ventral subregion.

Moser et al. (1993) were amongst the first to note that the dHip may be preferentially involved in spatial learning and memory (Moser et al., 1993). These researchers made various sized aspiration lesions to the dorsal *or* ventral subregion of the hippocampus of rats, and subsequently assessed their performance on the MWM. A relatively small lesion to the dHip
(~20% of the hippocampus) was sufficient to impair the rate and precision of acquisition of the MWM. In contrast, only lesions that covered the entire vHip could impair the rate and precision of acquisition of the MWM. These results suggested that the dHip may play a disproportionate role in spatial learning and memory. Also, these data begged the question: What is the role of the vHip?

1.3.3.2 The Ventral Hippocampus

The dHip/vHip functional dissociation has led to a re-emergence of the concept of the ‘emotional’ hippocampus. This is primarily based on neuroanatomical data showing that the vHip is more clearly integrated into neural systems involving subcortical structures such as the amygdala (to be discussed further below). For example, the infusion of the NMDA-antagonist AP-5 into the vHip produces an anxiolytic effect; whereas, infusions of AP-5 into the dHip does not appear to affect anxiety (Nascimento Hackl and Carobrez, 2007; Padovan et al., 2000). However, approximately half of several recent studies employing GABA-A agonists suggest that the dHip does in fact play a role in anxiety (Engin and Treit, 2007). Further work is required to assess the efficacy of GABA-A agonists on anxiolysis in the vHip. Further work is also required on the role of glutamate receptor subtypes in anxiety across the longitudinal axis of the hippocampus to clarify this putative dissociation.

1.3.3.3 Neuroanatomical Evidence

Corroborating evidence for a functional dissociation between the dHip and vHip lies in the simple fact that these subregions show differences in their connections to other brain regions. Two general neuroanatomical differences are generally agreed upon: (1) regions of the entorhinal cortex more strongly associated with visuo-spatial information preferentially innervate the dHip,
and (2) subcortical structures are more clearly connected to the vHip (Amaral and Lavenex, 2007).

The caudolateral entorhinal cortex (EC) projects to the dorsal 50% of the hippocampus; the intermediate EC projects to the adjacent 25% in the ventral hippocampus; the rostromedial zone of the EC projects to the most ventral 25% (Dolorfo and Amaral, 1998). Information from the sensory association cortices channel through the caudolateral and intermediate EC, carrying visual and somatosensory, that innervate the most dorsal 75% of the longitudinal axis of the hippocampus (Burwell and Amaral, 1998; Moser and Moser, 1998; Witter et al., 1989). The EC contains “grid cells”, which are akin to “place cells” in the hippocampus. Grid cells differ from place cells in that grid cells fire in multiple fields in an environment, typically generating a triangular or hexagonal pattern (Brun et al., 2008). Interestingly, the size of the “grid fields” varies in a dorsal/ventral manner within the EC. The grid cells with the most spatially restricted grid fields preferentially innervate the dHip; whereas, grid cells with the most spatially diffuse grid fields preferentially innervate the vHip. This parallels observations of place field sizes across the longitudinal axis of the hippocampus (Kjelstrup et al., 2008). In short, subregions of the EC that are more clearly associated with visuo-spatial information preferentially innervate the dHip.

The vHip possesses strong and specific connections to several subcortical brain regions putatively implicated in the processing of components of emotion. Canteras and Swanson (1992) have shown that major projections exist from the vHip to the amygdala (and adjacent structures) and the hypothalamus via the ventral subiculum (Canteras and Swanson, 1992). The vHip appears to specifically project to the basal and accessory basal subregions of the amygdala. Damage to either of these areas impairs context fear conditioning, suggesting that the vHip plays
an important role in a neural system involving emotion (Maren and Fanselow, 1995). The vHip also has significant projections to neuroendocrine and preautonomic cell groups in the periventricular zone and various hypothalamic nuclei. These pathways are thought to be involved in the control of neuroendocrine and autonomic responses. Although few studies have investigated the effects of lesions to the hippocampal subregions on neuroendocrine and autonomic responses, studies do exist supporting a prominent role for the vHip (Moser and Moser, 1998). For example, one study supporting this postulation showed that vHip, but not dHip, lesions exacerbate restraint-induced gastric erosion (Henke, 1990). In addition, the vHip is more strongly linked into neural networks with the cingulate cortex (Amaral and Lavenex, 2007). The cingulate cortex may play an important role in the attending to information made salient through emotion or sympathetic activity (Allman et al., 2001; Bush et al., 2000).

1.3.3.4 Where to draw the line?

Different authors have suggested that the hippocampus be conceptually subdivided along the longitudinal axis in different ways. For example, a dorsal 2/3 and ventral 1/3 grouping has been suggested based on the longitudinal extent of axon collaterals of CA3 pyramidal neurons and DG mossy cells (Amaral and Witter, 1989). Others have suggested a grouping based on projections from the caudolateral, intermediate and rostromedial EC that would split the longitudinal axis into a dorsal ½ and two ventral quarters. In the absence of a sound a priori rationale to break the hippocampus down into multiple subregions across the longitudinal axis, we believe that a 50/50 distinction is most appropriate.

1.3.3.5 Summary

The vHip may be functionally dissociated from the dHip by a more prominent role in the processing of fear and/or anxiety. At the very least, it appears that the vHip adds contextual
information to neural networks involved in the processing of emotionally charged information (Maren and Fanselow, 1995). Neuroanatomical data appear to support this postulation. Amaral and Lavenex have synthesized findings across the longitudinal axis of the hippocampus as follows (Amaral and Lavenex, 2007). The dHip is more strongly embedded in an information loop with neocortical regions associated with sensory information and therefore may be more “highly involved” in the processing of exteroceptive information. In contrast, the vHip is more strongly embedded in an information loop with subcortical regions associated with emotional information and therefore may “preferentially deal with” the processing of interoceptive information.

1.3.4 Transverse axis

Early studies into functional differences along the transverse axis of the hippocampus suggested that the hippocampus functions as a single circuit, with interruption to any level attenuating an animal’s ability to acquire a novel spatial task. For example, Sutherland et al. (1983) administered colchicine, a mitotic poison, to the dentate gyrus (DG) of rats and found that this caused impaired acquisition of spatial learning (Sutherland et al., 1983). Similar learning impairments were found for kainic acid lesions to CA3 (Handelmann and Olton, 1981; Sutherland et al., 1983) and ischemic lesions to CA1 (Olsen et al., 1994; Volpe et al., 1992; Volpe et al., 1984). Importantly, these findings do not necessarily imply that the hippocampal subfields are homogeneous in function. The formation of a memory trace is likely a complex process, involving distinct and interdependent computations which may take place in hippocampal subfields connected in series (Buzsaki, 1989; Moser and Moser, 1998; Treves and
It is also possible that within each subfield, multiple neuronal ensembles are responsible for different aspects or types of learning and memory (Moser and Moser, 1998).

Kesner and colleagues have recently contributed significantly to the dissociation of the functions of the hippocampal subfields (Goodrich-Hunsaker et al., 2008; Hunsaker and Kesner, 2008; Rolls and Kesner, 2006). Importantly, these authors have typically reported on the effects of lesions to the dorsal subregion of the hippocampus in rats – a fact appropriately emphasized by these authors. These authors have emphasized that the dorsal DG (dDG) produces sparse representations of the spatial environment and forces these representations on the dorsal CA3 (dCA3) subfield via the mossy fibre pathway. This led to the suggestion that the dDG is involved in “pattern separation.” The dCA3 subfield then acts as an auto-associative network based on observations of its importance in learning arbitrary associations where space is an important component. This led to the suggestion that the dCA3 is involved in “pattern completion.” These authors have conceptualized the dorsal CA1 (dCA1) as a subfield that recodes spatial information from the dCA3 subfield and generates associatively learned backprojections to neocortex. This led to the suggestion that the dCA1 subfield is involved in “consolidation.”

In the last decade, Tonegawa and colleagues have spearheaded an investigation of this issue using new genetic technologies in mice producing similar findings as those from Kesner’s group. These authors have deleted the obligatory NMDA receptor subunit, NR1, from specific hippocampal subfields (not limited to the dorsal subregion). Consistent with the above findings, deletion of NMDA receptor expression specifically in the CA1 subfield induced significant impairments in acquisition of the Morris water maze (MWM) (Rondi-Reig et al., 2001). This is consistent with early findings from Moser and colleagues showing that lesions restricted to the...
The hippocampus proper is sufficient to impair learning on the MWM (Moser et al., 1993). When NR1 was deleted specifically in the CA3 subfield, a novel finding emerged. Performance on the MWM was not impaired (Nakazawa et al., 2002). However, a version of the MWM involving fewer extra-maze cues revealed a learning impairment suggesting that NMDA receptors in the CA3 subfield are involved in pattern completion. Mice lacking functional NMDA receptors in the DG performed as well as controls on the MWM (McHugh et al., 2007). Interestingly, these mice were impaired on a context discrimination task, where the mice had to learn to dissociate between a context where they received a shock and a context where they have not received a shock. Thus, the emerging view is that memory acquisition is indeed a complex process that may consist of components associated with the different hippocampal subfields. In chapter 2, we describe our assessment of Fmr1 KO mice on two forms of the water maze. In chapter 3, we describe our assessment of Fmr1 KO mice on a context discrimination task similar to that performed on the DG-specific NR1 KO mice, described by McHugh et al. (2007), to assess learning associated with the DG.

1.3.5 Summary

The hippocampus is amongst the best-studied regions of the brain. Its well-defined anatomical connections are conducive to cellular and molecular analyses in vitro and in vivo. Further, the proposed behavioral functions of the hippocampus are beginning to be resolved. Subregional and subfield analyses have been particularly informative in delineating the cellular basis of the multiple, overlapping behavioral functions that have historically been associated with the hippocampus. It appears that the hippocampus is involved in multiple aspects of cognition and emotion. Dissociations across the longitudinal axis suggest that the ventral
subregion may be particularly involved in emotions such as anxiety and the linkage of these emotions to the environmental context. The application of this dissociation to the study of the hippocampus of \textit{Fmr1} KO mice is emphasized in the manuscript presented in chapter 2. Dissociations across the transverse axis of the hippocampus suggest that the DG subfield may play a particular role in pattern separation. The application of this dissociation to the study of the hippocampus of \textit{Fmr1} KO mice is emphasized in the manuscript presented in chapter 3. Both manuscripts show that the DG subfield of the hippocampus may be severely affected in the mouse model of FXS. The next section provides important background information regarding the DG subfield of the hippocampus.
1.4 Dentate Gyrus

Early studies of normal expression of FMRP in the tissues of the body and regions of the brain suggested that FMRP is normally produced at disproportionately high levels in granule neurons in the hippocampus (i.e., the dentate gyrus; DG) (Hinds et al., 1993). Despite this, the DG was a previously unexplored subfield of the brain in the mouse model of FXS. Thus, the overarching hypothesis (as stated in the abstract of this thesis) is that repressed expression of the Fmr1 gene deleteriously alters structural and functional plasticity in the mammalian dentate gyrus (DG), and impairs aspects of learning and emotion associated with this brain region. In order to understand structural and functional plasticity in the DG, a basic understanding of the cellular anatomy of the DG is required.

The DG is unique because it possesses the ability to continually generate new, excitatory neurons across the lifespan (commonly referred to as “adult neurogenesis”; discussed in detail in section 1.5.1) (Altman and Das, 1965; Christie and Cameron, 2006; Gage, 2002). The cells produced are predominantly principal neurons of the DG subfield, although glial cells also appear to be generated through this process. The DG consists of neurons, glia, progenitor cells, endothelial cells and perhaps the occasional peripheral immune cell.

1.4.1 Cell types

1.4.1.1 Neurons

Excitatory and inhibitory neurons exist in the DG (Amaral and Lavenex, 2007; Spruston and McBain, 2007). The main excitatory and principal neuron of the DG is the granule neuron. The other excitatory neuron is the mossy cell which is situated just deep to the granule cell layer.
Granule neurons extend their axons to other regions of the brain, making them the principal neurons of the DG. In the mouse hippocampus, there are approximately 200,000 to 300,000 granule cells (rat = 1 million; monkey = 5 million; human = 10 million). In the DG subfield of the hippocampus, granule cells are organized into a single, relatively densely-packed layer of cells (4-8 cell bodies thick). A typical granule cell body is elliptical (approximately 10 x 18 µm), and possesses a characteristic cone-shaped dendritic arborization that extends into the molecular layer towards the hippocampal fissure. The granule cell layer plus the molecular layer is called the “fascia dentata.” The region subjacent to the granule cell layer is the polymorphic layer, sometimes referred to as CA4. The input to dentate granule cell dendrites is from the entorhinal cortex via the unidirectional perforant path, forming the first set of synapses within the classic trisynaptic circuit. Intrinsic and extrinsic connections make numerous en passant contacts with target neuronal dendrites that run orthogonal to the dendritic axes of dentate granule neurons.

The most dorsal blade of the ‘C’-shaped granule cell layer is referred to as the suprapyramidal blade; whereas, the ventral blade is referred to as the infrapyramidal blade (not to be confused with the dorsal and ventral subregions of the longitudinal axis of the hippocampus). Some differences exist between the two blades such as the cells of the infrapyramidal blade being smaller with a lower density of spines. We are unaware of significant dissociations in the functions of these blades.

In short, granule neurons are the principal neurons of the DG, which are packed into a dense layer of cells called the granule cell layer. The DG harbors the first set of unidirectional synaptic
connections in the hippocampus. It also possesses a relatively simple and well-defined cyto-
architecture. For these reasons, the DG is an ideal region to study structural and functional
plasticity.

1.4.1.2 Interneurons

Inhibitory neurons of the DG are the interneurons (Freund and Buzsaki, 1996). Inhibition is
achieved by DG interneurons releasing the neurotransmitter GABA onto postsynaptic, ionotropic
GABA receptors that permit an influx of chloride anions to hyperpolarize the postsynaptic
neuron. There are many different types of interneurons that can be classified based on
morphology, neurochemistry and electrophysiological properties. Interneurons in the DG are
morphologically classified into the following groups: pyramidal basket interneurons, chandelier
or axo-axonic interneurons, MOPP interneurons, HICAP interneurons, HIPP interneurons,
interneuron-specific (IS) interneurons, hilar neurons with projections to CA1 and subiculum and
hilar neurons with unknown projections. Classification based on neurochemistry uses markers in
the general categories of classic neurotransmitters, neurotransmitter receptors, calcium-binding
proteins and neuropeptides. Classification based on electrophysiological properties is primarily
based on the rate of spiking (e.g., fast or regular). A vast continuum likely exists regarding the
characteristics of interneurons in the DG. (Interneurons are not investigated in this thesis and
will not be discussed further.) The effect of interneurons on synaptic communication in the DG
is often eliminated by blocking GABA-A receptors pharmacologically.

1.4.1.3 Glia

Glia are classically thought of as supporting cells; but recent evidence suggests that they play
a much more active role in the nervous system than originally appreciated. Glia are abundant in
the hippocampus, particularly during development (Kimoto et al., 2009). For example, radial
glia cells appear to provide an indispensible scaffold for the migration of neuroprogenitor cells during development (Gotz and Barde, 2005; Rakic, 2003). Even within the adult brain, glia are thought to be far more abundant than neurons (Kimelberg and Norenberg, 1989). Astrocytes, microglia and oligodendrocytes all appear in the DG subfield of the hippocampus (Kimoto et al., 2009). The research in this thesis has not focused on glia due to observations that FMRP is not normally expressed in glia.

1.4.1.4 Progenitor Cells

One of the most interesting features of the DG is its ability to continually produce new neurons from a pool of progenitor cells located in the subgranular zone. Progenitor cells have been classified into several types, each being associated with the expression of a different constellation of endogenous markers and developmental time points. Kempermann (2004) has proposed 6 developmental milestones of neurogenesis from putative stem cells expressing nestin and GFAP to the mature postmitotic granule neurons (Kempermann et al., 2004). The different types of progenitor cells are not investigated in this thesis.

1.4.1.5 Endothelial Cells

Endothelial cells line blood vessels throughout the body and brain. An important observation has been that a large proportion of dividing cells in the DG are found in clusters close to the vasculature (referred to as the “vascular niche”) (Palmer et al., 2000). The endothelial cells associated with groups of dividing cells in the DG disappear over weeks, suggesting that the process of neurogenesis involves active vascular recruitment and subsequent remodeling. Endothelial cells are not investigated in this thesis and are not discussed further here.
1.4.2 Afferents and efferents

1.4.2.1 Dentate Gyrus Afferents

The major inputs to the dentate gyrus include the (1) entorhinal cortex, (2) basal forebrain, (3) hypothalamus and (4) brain stem (Amaral and Lavenex, 2007; Amaral et al., 2007; Spruston and McBain, 2007). These important pathways are discussed in turn.

Entorhinal Cortex Projection to the Dentate Gyrus

The main input to the dentate gyrus (DG) is from the entorhinal cortex (EC). The perforant path carries axons from layer II pyramidal and stellate cells to the molecular layer of the DG (cortical layer II cells also target CA3 in the mouse; layer III cells target CA1 and the subiculum). The perforant path terminals are primarily (>85%) axo-spinous and asymmetrical on spines of dentate granule neurons. The perforant path terminals are periodic varicosities (0.5-1.0 μm in diameter) on thin axonal branches (0.1 μm in diameter). A single EC afferent projects to 20 - 50% of the longitudinal axis of the hippocampus (sheet-like axonal arborization). Cells in the DG without dendrites in the molecular layer do not receive EC input.

The EC can be conceptually subdivided into medial and lateral regions in the rodent. This has been associated with laminar innervation of the outer 2/3 of the molecular layer (medial EC → middle 1/3 of the molecular layer; lateral EC → outer 1/3 of the molecular layer). Timm’s stain has been used to illustrate these layers (dense staining in outer 1/3 and inner 1/3). The “lateral” and “medial” perforant paths demonstrate a number of distinguishing features. For example, the medial, and not the lateral, perforant path fibres are immunoreactive for mGluR2/3. Conversely, the lateral, and not the medial, perforant path fibres are immunoreactive for dynorphin. As was described in section 1.3.3 above, differences also exist in the projections from subregions of the EC to the dorsal and ventral subregions of the hippocampus.
**Basal Forebrain (Septal Nuclei) Projection to the Dentate Gyrus**

The basal forebrain is a collection of nuclei, including the septal nuclei, representing the major source of cholinergic fibers in the brain. Cholinergic neurons from the medial septal nucleus and diagonal band of Broca innervate the DG in a narrow region superjacent to the granule cell layer, but septal fibers can be found innervating all areas of the DG *albeit* more heavily in the polymorphic layer than the molecular layer. The cholinergic fibres thus primarily make excitatory synapses onto granule cell dendrites in the inner third of the molecular layer. Although the cholinergic component of this projection has historically received more attention, in fact, a large proportion of this projection is GABAergic. Most GABAergic neurons in the basal forebrain project onto GABAergic interneurons in the polymorphic layer of the DG. Thus, it has been suggested that isolating hippocampal sections leads to increased inhibition on granule neurons via removal of the septal GABAergic input onto GABAergic interneurons in the DG. Researchers have commonly included a GABA-A receptor antagonist in the extracellular solution in an attempt to counteract this effect.

**Hypothalamic Projections to the DG**

A glutamatergic population of cells in the supramammillary area projects heavily to a narrow zone just superficial to the granule cell layer (and lightly to other areas of the DG) mainly making excitatory contacts with proximal dendrites of dentate granule neurons. Some of these projection neurons may co-localize calretinin and substance P. There is a second sizeable input to the DG that comes from cells scattered throughout the hypothalamus that terminate in a diffuse pattern in the DG.
Brain Stem Projections to Dentate Gyrus

The connections from the brain stem to the DG are primarily thought of as neuromodulatory in function and include noradrenergic, dopaminergic and serotonergic projections. The DG receives a prominent noradrenergic input from the pontine nucleus locus coeruleus and the termination is mainly in the polymorphic layer and stratum lucidum of CA3. The dopaminergic input is relatively minor, originating in the ventral tegmental area (VTA) and terminating mainly in the polymorphic layer. The serotonergic input to the DG is from raphe nuclei and the termination is in the polymorphic layer (mainly the subgranular zone) preferentially innervating interneurons.

Commissural projections from the contralateral hippocampus are almost entirely absent in mice, monkeys and humans (they are prominent in rats). In the rat, these projections from the contralateral hippocampus are to the inner 1/3 of the molecular layer and to the stratum radiatum of the hippocampus proper. All experiments in this thesis were conducted in mice and, as such, the commissural projections will not be discussed further here.

1.4.2.2 Dentate Gyrus Efferents

The only region that the DG directly connects to is the CA3 subfield of the hippocampus proper; it does not project to the CA1. The axons from dentate granule cells (i.e., the mossy fibres; not to be confused with the axons of dentate mossy cells) are unmyelinated and terminate in a relatively narrow zone mainly located just above the CA3 pyramidal cell layer (the stratum lucidum) where they make en passant connections with thorny excrescences on dendrites of CA3 pyramidal neurons. The transparency of the stratum lucidum is due to the lack of myelination of mossy fibres. There is no indication that any other neuron type in the DG (mossy or interneurons) sends axons to the CA3 subfield of the hippocampus. The border of CA3 to CA2
is in part demarcated by the fact that DG mossy fibres do not innervate CA2. The axon trajectory of the mossy fibres is in part determined by the location of the granule cells: (generally) granule neurons in the suprapyramidal blade project via the suprapyramidal bundle, granule neurons in the crest project via the intrapyramidal bundle, and granule neurons in the infrapyramidal blade project via the infrapyramidal bundle. Axons from the infrapyramidal bundle project deep (basal side) to the CA3 pyramidal cell layer but ultimately terminate in the stratum lucidum. Axons from neurons in the crest of the granule cell layer project via the intrapyramidal bundle into the CA3 pyramidal cell layer but also ultimately terminate in the stratum lucidum. Axons from neurons in the suprapyramidal blade of the granule cell layer project via the suprapyramidal bundle into the superficial aspect of the stratum lucidum.

Mossy fibres give rise to unique, complex *en passant* presynaptic terminals called: mossy fibre expansions. These can be as large as 8 µm but are typically 3-5 µm. A single mossy fiber expansion can form as many as 37 synaptic contacts. A single mossy fiber expansion usually contacts several spines on a single dendrite. These special, central synapses are observed approximately 15 times per granule cell axon (approximately every 135 µm). Thus, each granule cell communicates with only 15 CA3 pyramidal cells. Each CA3 cell however receives input from an estimated 72 granule cells. This pattern is unique in the CNS and computational neuroscientists suggest that this system is designed for reliable depolarization of the postsynaptic CA3 neurons.
1.4.3 Development

The development of the DG begins in late embryogenesis and continues through the second postnatal week in rodents, peaking near the end of the first postnatal week (Frotscher et al., 2007; Rahimi and Claiborne, 2007). The first granule cells of the DG are generated in the ventricular zone, the “primary proliferative zone”, and migrate to form the suprapyramidal blade of the DG. Later, the “secondary proliferative zone” emerges in the hilus subjacent to the newly forming granule cell layer. The majority of granule neurons are produced postnatally in the rodent. In fact, the entire infrapyramidal blade is thought to be generated from the secondary proliferative zone. In the transverse axis, the granule cell layer becomes populated starting at the tip of the suprapyramidal blade moving towards the crest and finally to the infrapyramidal blade. Across the longitudinal axis, the granule cell layer becomes populated in a dorsal to ventral direction.

A particularly important developmental pattern of the granule cell layer can be exploited for studies designed to evaluate neurons of different cellular ages. Within the granule cell layer, the neurons generated earliest are located closest to the molecular layer; whereas, the younger neurons are located closest to the hilus. This is in contrast to the “inside-out” pattern observed in many neocortical regions where the newer neurons migrate past older neurons to more superficial regions. This means that, at any given time point, the most mature neurons can be found closest to the molecular layer and the youngest closest to the hilus.

In the rat, the width of the molecular layer approximately doubles between postnatal days 4 and 14 reflecting the associated developmental increase in dendritic arborization. Lubbers and Frotscher (1988) were amongst the first to exploit these features of the DG to study the morphology of neurons at different stages in development in an animal of a specified age.
(Lubbers and Frotscher, 1987). Neurons in the earliest stages of their development had only rudimentary dendritic trees while the most mature neurons had elaborate dendritic trees and longer dendrites. A similar pattern has emerged studying the morphology of neurons in rodents at different ages during development. Dendritic spines appear only on the most mature neurons during development; slightly younger neurons appear to have increased numbers of filopodia. The most immature neurons may have vast regions of smooth dendrites. The density of dendritic spines appears to increase during development as well. Average spine densities from rodents indicate at least a 2-fold increase between postnatal weeks 1 and 2. This may be correlated with an even faster increase in synaptogenesis. Electron microscopy (EM) studies indicate a 16-fold increase in the number of synapses between P5 and P10. It should be kept in mind however, that many new synapses are found on dendritic shafts and may be symmetrical, inhibitory synapses. By postnatal day 14, most granule neurons have obtained a mature morphological phenotype. Amazingly, neurogenesis in the DG continues across the lifespan albeit at a continually declining rate. It appears that this same developmental sequence is generally maintained.

The innervation of the DG by EC afferents actually precedes the development of the granule cell layer, suggesting that granule neurons are not responsible for the development of this pathway (Frotscher et al., 2007). It is thought that Cajal-Retzius cells and specific extracellular proteins are largely responsible for the generation of the perforant path.

In short, the DG is predominantly generated from a secondary proliferative pool located within the hilus. The oldest neurons can be found closest to the molecular layer and the youngest can be found closest to the hilus. This provides a unique system to compare the structure and function of neurons of different cellular ages across the lifespan of an animal.
1.4.4 Summary

The DG normally exhibits considerable expression of the \textit{Fmr1} gene, which when mutated causes FXS. Prior to this thesis, the effects of deletion of \textit{Fmr1} on the DG were unexplored. The DG is unique in its ability to continually produce new neurons, lending itself to experiments assessing neural development. The predominant excitatory input to granule neurons occurs in distal dendritic regions from afferents originating in the EC. One important developmental feature is the relatively simple migration pattern of granule neurons from the subgranular zone towards the molecular layer, permitting a classification of neurons by cellular age using location within the granule cell layer as a proxy indicator.
1.5 Plasticity in the Dentate Gyrus

The dentate gyrus (DG) is arguably one of the most plastic regions in the mammalian brain. The following sections describe the inherent ability of the DG to continually add new neurons to its pre-existing cytoarchitecture across the lifespan. A second form of structural plasticity occurs at the level of granule cell dendrites. A variety of factors appear to possess the capacity to both increase or decrease dendritic arborizations and the morphology and density of dendritic spines. Third, the DG possesses the capacity to exhibit robust physiological/functional plasticity; the efficacy of synaptic transmission can either increase or decrease in response to patterned electrical activity. These forms of plasticity may be related. For example, the primary sites of fast, excitatory functional plasticity in the DG are the dendritic spines that possess the ability to exhibit structural plasticity. Also, emerging evidence suggests that new neurons may disproportionately contribute to plasticity in the DG (Ge et al., 2006; Ge et al., 2008; Ge et al., 2007; Snyder et al., 2001; Wang et al., 2000) and behaviors subserved by the DG (Gould et al., 1999a; Gould et al., 1999b; Kee et al., 2007). This suggests that neurogenesis may be related to spine and synaptic plasticity. Normal structural and functional plasticity in the DG are presumed to maintain normal behaviors dependent on the DG. Conversely, aberrancies in DG plasticity may underlie abnormal behaviors dependent on the DG.

1.5.1 Neurogenesis

Prior to the latter half of the 20th century, it was almost universally believed that all of the principal cells of the brain are generated prior to the onset of adulthood (Gage, 2002; Kempermann, 2006). Following the commencement of adulthood, it was thought that neurons could not be added, just lost. By the end of the 20th century this perspective, what some have
called “dogma”, has been turned on its head. It is now almost universally appreciated that neurogenesis does occur across the lifespan of adults in at least two main regions of the brain: the subventricular zone and the DG. The implication of the DG, and the hippocampus in general, in learning and memory caught the attention of researchers who wondered if new neurons could play a role in such cognitive processes.

Much has been learned in the past several decades regarding neurogenesis, although the exact function of this process is still somewhat controversial (Aimone et al., 2006; Kempermann et al., 2008; Leuner et al., 2006; Sahay and Hen, 2007). We know that neuroprogenitor cells reside in the subgranular zone where they can proliferate, and produce daughter cells that progress through various developmental stages (Kempermann et al., 2004; Seri et al., 2001). Daughter cells can obtain a neuronal phenotype, extend axons to CA3 (Toni et al., 2008) and dendrites towards the hippocampal fissure (Zhao et al., 2006), and survive to a structural and physiological mature neuronal age (Ge et al., 2008; Schmidt-Hieber et al., 2004; van Praag et al., 2002; Zhao et al., 2006). As these cells continue to mature, it appears that they gradually migrate through the granule cell layer towards the molecular layer, recapitulating what is observed in the primary neurodevelopment of the DG. The major stages of neurogenesis commonly observed to be altered by a variety of factors include cell proliferation, cell survival and neuronal differentiation. An understanding of all three stages is important for a complete appreciation of how any condition affects adult hippocampal neurogenesis.

Neurogenesis in the young adult rat DG leads to the addition of an estimated 6% of the total granule cell population each month (Cameron and McKay, 2001). In mice, these values may even be higher based on the fact that the duration of the cell cycle is nearly 50% shorter (24.7 h versus 13 h) (Hayes and Nowakowski, 2002). Significant mouse strain differences appear to
exist as well. For example, the proliferative population of BALB/c mice is approximately 50% less than that for C57BL/6 mice. In this thesis, we chose to employ C57BL/6 mice (Hayes and Nowakowski, 2002). Neurogenesis leads to the production of a relatively small number of new neurons each month in the adult DG; however, accumulating evidence suggests that the physiological properties of young neurons in the DG establishes themselves as disproportionate contributors to the overall functioning of this brain region (Ge et al., 2007; Kee et al., 2007).

The behaviors associated with hippocampal neurogenesis overlap greatly with the proposed functions of the hippocampus in general. Research into the function of neurogenesis has primarily emphasized correlations to spatial learning and memory ability (Epp et al., 2009; Gould et al., 1999a; Gould et al., 1999b; Leuner et al., 2006). Other recent research has begun to emphasize that adult neurogenesis may also be associated with emotions such as depression and anxiety (Sahay et al., 2007; Sahay and Hen, 2007). One review has suggested that these aspects of neurogenesis may relate to the subregion of the hippocampus. In other words, the neurogenesis in the dHip may be more associated with spatial learning and memory; whereas, neurogenesis in the vHip may be more associated with emotion. Little data currently exist to support this dissociation; however, data is emerging that appears to support this generalization under some conditions (Banasr et al., 2006; Eadie et al., 2009; Jayatissa et al., 2006).

1.5.2  Neuromorphology

The vast majority of fast, excitatory synaptic transmission impinging upon dentate granule neurons is located at synapses on dendritic spines of dendrites extending from the granule cell layer into the molecular layer of the DG (Amaral et al., 2007). Adult neurogenesis appears to recapitulate development, albeit at a somewhat slower rate, with regard to the development of
mature dendritic spines (Rahimi and Claiborne, 2007; Zhao et al., 2006). Dendrites start out smooth, gradually increase the density of filopodia and dendritic spines, and increase the proportion of stubby and mushroom spines relative to thin spines and filopodia. By approximately 3 weeks into a neuron’s development, a relatively mature morphology has been obtained, permitting their integration into the pre-existing cyto-architecture. However, these neurons are still young and are not as morphologically mature compared to pre-existing neurons in the DG. This may confer a disproportionate ability for these neurons to participate in the overall function of the DG (Ge et al., 2008; Kee et al., 2007). Curiously, manipulations that affect adult neurogenesis can also influence dendritic spine characteristics in the DG (Eadie et al., 2005; Olson et al., 2006). For example, voluntary exercise appears to nearly double the number of proliferating cells and increases the density of dendritic spines in the young adult DG. Neurogenesis and neuromorphology are related in the DG.

The notion that the structure of dendrites and their spines could change in response to environmental conditions, has only emerged in the last several decades (Rosenzweig, 2007). In the early 1960s Rosenzweig and colleagues (1962) exposed rats to one of the following three conditions: (1) environmental enrichment (EE; or “complexity” as it was originally referred), (2) social isolation and stimuli-deprived, or (3) an intermediate condition (Rosenzweig et al., 1962). These authors were surprised to discover that the weight of the cerebral cortex of these rats was greatest in the EE group, least in the social isolation/deprived condition and intermediate for the intermediate environmental condition. Subsequent studies revealed that this was associated with a statistically significant 5% increase in the thickness of the cortex (Diamond et al., 1964). In addition, this effect was associated with an increase in the expression (Grouse et al., 1978) and amount of RNA produced (Ferchmin et al., 1970) as well as the amount of protein produced in
the cerebral cortex (Bennett et al., 1964). These data suggested an increase in cellular growth in the cerebral cortex of mammals following EE. The cellular basis for this appeared to be related to (i) an increase in the size of pyramidal neuron cell bodies (approximate 13% increase in cell body area) (Diamond et al., 1975), (ii) an increase in the number of glia per unit of volume of cortex (approximate 14% increase), (iii) an increase in the number of dendritic spines on pyramidal neurons (Globus et al., 1973), and (iv) an increase in the size of individual synapses (West and Greenough, 1972). In short, seminal research from the 1960s and 1970s revealed that environmental factors (e.g., enrichment) can significantly alter dendritic morphology in the neocortex of the mammalian brain.

Plasticity of dendrites and dendritic spines has not been limited to effects of environmental complexity/enrichment or the cerebral cortex. For example, cerebral ischemia appears to induce rapid alterations in dendritic spines (Brown et al., 2009; Brown et al., 2008; Zhang et al., 2005). Another clear example is the well-documented effect of stress on dendritic spines in the hippocampus (Galea et al., 1997; McEwen and Magarinos, 1997; Watanabe et al., 1992). We have observed that voluntary exercise can increase the density of dendritic spines in the DG (Eadie et al., 2005). Finally, abnormalities in dendritic spines have also been apparent in a number of neurodevelopmental disorders. Specifically, analyses of dendritic spines from post-mortem tissue from individuals with Fragile X syndrome (FXS) and the mouse model of FXS show abnormalities in dendritic morphology (Beckel-Mitchener and Greenough, 2004).
1.5.3 Synaptic plasticity

It has long been believed that the ultimate functionality of a particular brain region rests in the ability of its constituent neurons to process or integrate information in the form of electrical signals. The application of this to cognition led to the pervasive “neurophysiological postulate” articulated by Dr. Donald O. Hebb (1949) which states (Hebb, 1949):

“When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased.”

The neurophysiological postulate gained considerable credence following Bliss and Lomo’s discovery of long-term potentiation (LTP) in 1973 (Bliss and Lomo, 1973; Lomo, 2003). These authors placed electrodes into the DG of anesthetized rabbits and evoked excitatory postsynaptic potentials (EPSPs). They noted that the magnitude of the postsynaptic signal could be increased by administering high-frequency stimulation (HFS), and that this increase could persist for several minutes. This is now called LTP and it may be defined as a persistent increase in synaptic efficacy following high-frequency stimulation of presynaptic afferents. The hypothesis that LTP is a neurobiological model for learning and memory has led to the production of several thousands of publications on LTP (Shors and Matzel, 1997). Approximately a decade following the discovery of LTP, the discovery of the inverse of LTP was made (Christie and Abraham, 1992a; Christie and Abraham, 1992b; Dudek and Bear, 1992; Lynch et al., 1977). The new form of synaptic plasticity was called long-term depression (LTD). LTD may be defined as a persistent decrease in synaptic efficacy following low-frequency stimulation (LFS) of
presynaptic afferents. Together, LTP and LTD are referred to as bidirectional synaptic plasticity. In the CA1 and DG subfields of the hippocampus, HFS-induced LTP and LFS-induced LTD are dependent on functional NMDA receptors (Collingridge et al., 1988a; Collingridge et al., 1988b; Dudek and Bear, 1992; Vasuta et al., 2007) (personal observations; chapter 3). Early experiments using infusion of the NMDA-antagonist APV (Davis et al., 1992) and more recent experiments genetically removing functional NMDA-receptors from the CA1 subfield (Rondi-Reig et al., 2001) have been shown to cause impairments in rodents on the classic Morris water maze (MWM). These data support the association between bidirectional synaptic plasticity and learning and memory ability. Despite this, considerable controversy exists regarding the details of the relationship between LTP and LTD and learning and memory. Nonetheless, long-term synaptic plasticity is unarguably the leading neurobiological hypothesis of memory formation in the mammalian brain (Kessels and Malinow, 2009; Shors and Matzel, 1997).

1.5.4 Summary

The dentate gyrus (DG) of the mammalian brain is a highly plastic region capable of changing (i) the number of new neurons added, (ii) the morphology of dendritic processes and the spines on residing neurons, and (iii) the efficacy of synaptic transmission that occurs at the dendritic spines of the neurons in this region. This thesis explores all of these forms of plasticity in the DG of the mouse model of the most common inherited form of intellectual disability, Fragile-X syndrome.
1.6 The Mouse Model of Fragile-X Syndrome

In 1994, the Dutch-Belgian Fragile X Consortium, led by Dr. Oostra (Netherlands) and Dr. Willems (Belgium), produced the first mouse model of Fragile-X syndrome (FXS) (1994). These authors generated a targeting vector containing 5.7 kb of Fmr1 genomic DNA with exon 5 interrupted with the bacterial neomycin (neo) gene. Embryonic stem cell (ES) technology was used for homologous recombination of this targeting vector into the mouse germline (E14 ES). A positive clone was injected into blastocysts and transferred to pseudo-pregnant females. Male offspring chimeras were crossed with wild-type (WT) female mice to determine germline transmission. Female offspring from a male parent showing 100% germline transmission were crossed with male WT C57BL/6 mice yielding some male Fmr1 knockout (KO) offspring. The mice used in our experiments (and many others) could be traced back to these founder mice. (Genetic drift can occur within colonies, an effect curbed by continually reintroducing WT C57BL/6 breeders into individual colonies. We obtain new WT male C57BL/6 mice from Jackson Laboratories annually in support of this effort.)

Initial evaluation of the newly generated mouse model of FXS revealed some differences suggesting that it does indeed mimic FXS in some respects (1994). For example, macroorchidism is a common feature in FXS (Hagerman, 2002; Nielsen, 1983; Nielsen et al., 1982), and as such, testicular weight was compared between the newly generated Fmr1 KO and WT mice. Testicular weight was significantly increased in young adult Fmr1 KO mice, while comparisons of the weights of other organs were not significantly different.

Gross histological evaluations of the brains revealed largely non-significant results (1994). Brain weights were not significantly different between genotypes. A cursory investigation of major brain regions, including the hippocampus, did not reveal any obvious differences. A
normal number of pyramidal neurons were observed in the cerebral cortex, which also appeared normally laminated. A normal number of GFAP+ astrocytes were also observed in the glia limitans and white matter.

General behavioral observations indicated that *Fmr1* KO mice appear to have normal social interaction with littermates and human investigators (consistent with our personal observations) (1994). Initial investigations of basic behaviors such as gait, grooming, feeding, swimming, circadian activity and mating activity all appeared normal in *Fmr1* KO mice. The authors suggested that this is consistent with the lack of hard neurologic signs in patients with FXS.

Assessment of the mice in a light-dark exploration task revealed that the *Fmr1* KO mice actually travelled a greater distance in the lit compartment (consistent with our findings presented in chapter 2 regarding assessment of *Fmr1* KO mice in the open field and elevated plus maze). In addition, the newly generated *Fmr1* KO mice showed significantly more activity in the open field. No differences were observed in a passive-avoidance task. Assessment of *Fmr1* KO mice on the classic hippocampal-dependent learning task, the Morris water maze (MWM), revealed that the mice performed as well as controls in acquiring the task. A reversal phase did however reveal an impairment in the *Fmr1* KO mice (1994). (In chapter 2, we present our data on the acquisition and reversal phases of the classic MWM and the Plus-shaped water maze.)

1.6.1 Behavior

A few years following the generation and initial assessments of the *Fmr1* KO mice, a second Belgian-Netherlands collaboration replicated their initial finding of impaired reversal learning in the MWM (D’Hooge et al., 1997). With the exception of a single study (Van Dam et al., 2000),
subsequent researchers have had difficulty replicating this finding and it has been suggested that this may be related to alterations in the genetic background of the mice employed in the various experiments (Eadie et al., 2009; Paradee et al., 1999). For example, the mice used in the initial studies may have had a disproportionate contribution of the 129 genetic background (commonly used as ES cells due to their robustness) and this may have permitted the manifestation of deficits on the MWM. This is supported by a study by Paradee et al (1999) showing a lack of deficits using the C57BL/6 background and minor deficits when these mice were backcrossed on the 129 background. The single paper by Van Dam et al. (2000) replicating the initial reversal deficit in the MWM also showed a similar impairment using a Plus-shaped water maze. (In chapter 2 we describe our findings using both the classic MWM and the Plus-shaped water maze with reversal learning components.)

A second learning behavior investigated in Fmr1 KO mice was fear conditioning. Paradee et al. (1999) reported significantly less freezing 24 hours after training (associating a tone with a foot shock in a context defined by shape, texture and smell) (Paradee et al., 1999). A difference in responsiveness to the foot shock was not apparent between the genotypes. In contrast, Van Dam et al. (2000) employed a similar protocol and were unable to find a significant difference in contextual fear conditioning (Van Dam et al., 2000). Similar to this latter result, Peier et al. (2000) did not observe a significant difference in fear conditioning when studying Fmr1 KO mice expressing human FMRP from a Yeast Artificial Chromosome (Peier et al., 2000). (In chapter 3 we describe contextual fear conditioning in experiments conducted leading up to an assessment of context discrimination.)
1.6.2 Neurogenesis

The hypothesis that neurogenesis in the dentate gyrus (DG) subserves a function is controversial; however, correlative evidence appears to support the view that neurogenesis may be influenced by learning (Epp et al., 2009; Gould et al., 1999a; Kee et al., 2007) and that neurogenesis may influence learning (Shors et al., 2001; van Praag et al., 1999; Zhao et al., 2003). Emerging evidence also exists to support the postulate that neurogenesis is related to emotions, notably anxiety (Kempermann et al., 2008; Sahay et al., 2007; Sahay and Hen, 2007). For example, selective serotonin reuptake inhibitors (SSRIs) have been shown to increase neurogenesis and reverse stress-induced reductions in neurogenesis (Hitoshi et al., 2007; Malberg and Duman, 2003; Malberg et al., 2000; Santarelli et al., 2003). Interestingly, one study even suggests that the beneficial effect of SSRIs on anxiety and depression may require on-going neurogenesis in the DG (Santarelli et al., 2003). Interestingly, a recent study indicates that these changes may be related to synaptic plasticity in the DG (Wang et al., 2008). These observations beg the question, how is neurogenesis altered in the mouse model of FXS, a disorder characterized by both intellectual impairment and emotional dysregulation?

Prior to the experiments described in chapter 3, very little was known about neurogenesis in general in FXS or Fmr1 KO mice, and literally nothing was known about adult hippocampal neurogenesis. Potential clues from other systems do exist however. Studies conducted by Castren and colleagues from the Netherlands provide the most pertinent information. These authors generated neurospheres from both Fmr1 KO mice and a fetus carrying the FXS mutation (Castren et al., 2005). Surprisingly, neurospheres from these groups showed a 3-5 fold increase in neuronal differentiation. The generated neurons were also morphologically abnormal with shorter neurites and a smaller cell body volume. A concomitant decrease in gliogenesis was observed which correlated to increases in apoptosis. More of the cells lacking FMRP showed
intense oscillatory Ca\textsuperscript{2+} spikes in response to neurotransmitters. An analysis of cell proliferation in the developing cortex of postnatal \textit{Fmr1} KO mice did not reveal significant differences. However, more recent analyses by this group have suggested that there may be an increase in the proportion of glutamatergic neurons produced in the subventricular zone in a FXS fetus and the mouse model of FXS (Tervonen et al., 2009). In a recent study by Bhattacharyya et al. (2008) isolated human neural progenitors from a FXS fetal cortex containing cells possessing the \textit{Fmr1} mutation to study their ability to proliferate and differentiate \textit{in vitro} (Bhattacharyya et al., 2008). Although these authors reported alterations in gene expression profiles, alterations in neurogenesis were not apparent. Although data exist to suggest alterations in neurogenesis, such as increased neuronal differentiation, not all studies have consistently found this effect.

The effects of repression of \textit{Fmr1} expression on cytogenesis can be found in systems other than the nervous system. For example, the finding of macrooordism in FXS and \textit{Fmr1} KO mice provided impetus to investigate cell proliferation in the testes. For example, Slegtenhorst-Eegdeman et al. (1998) showed an increase in the rate of Sertoli cell proliferation between embryonic days 12 to 15 in \textit{Fmr1} KO mice (Slegtenhorst-Eegdeman et al., 1998). Other gonadal cell types have been suggested to be involved as well. Bachner et al. (1993) reported increased expression of the \textit{Fmr1} gene during germ cell proliferation (Bachner et al., 1993). In the ovaries of drosophila with the ortholog/paralog of \textit{Fmr1} deleted (dFmr1), a reduction in the number of germ cells was observed. This was related to alterations in key molecules associated with proper cell cycle progression (Epstein et al., 2009). In addition, it was recently shown that a gene adjacent to the \textit{Fmr1} gene, \textit{Fmr4}, is also silenced in FXS by the trinucleotide repeat expansion (not necessarily in the mouse model of FXS), and that the product of this gene has antiapoptotic actions (Khalil et al., 2008). This array of results yields a confusing picture and limits our ability
to generalize to neurogenesis in the DG. (Our laboratory’s results regarding adult neurogenesis in the hippocampus of *Fmr1* KO mice are described in detail in chapter 2.)

### 1.6.3 Neuromorphology

The majority of fast, glutamatergic input to principal neurons occurs at synapses located on spines of dendritic arbors (Andersen et al., 1966; Megias et al., 2001) and the complexity and morphology of dendritic arbors and spines has been suggested to reflect the capacity of a neuron to integrate information (Bourne and Harris, 2008; Chklovskii et al., 2004; Magee, 2000; Magee and Johnston, 2005). In addition to representing an important measure of plasticity, such alterations in the ‘wiring’ of neural circuits may represent an important link between synaptic plasticity and neurogenesis: new neurons appear to have relatively more thin spiny protrusions and may participate in synaptic plasticity more readily (Ge et al., 2008). The size and flexibility of the synaptic inputs appears to be related to the shape of dendritic spines (Bourne and Harris, 2007). To date, there are no published studies on the complexity or morphology of dendritic arbors and spines in the DG of *Fmr1* KO mice.

Dendritic spines appear to be abnormal in neocortical regions of both patients with FXS and *Fmr1* KO mice. Histological analyses of brain tissue obtained post-mortem from patients with FXS reveals abnormally long, thin dendritic spines with prominent heads on neocortical (parietal, temporal and occipital cortices) principal neurons (Hinton et al., 1991; Irwin et al., 2001; Rudelli et al., 1985; Wisniewski et al., 1991). Similar analyses performed on tissue from *Fmr1* KO mice appear to mimic the spine abnormalities in certain preparations and brain regions. Greenough’s group has contributed significantly to this field, and although some inconsistencies appear to exist, dendritic spines on layer V pyramidal neurons in visual cortex
appear to be longer and more immature in \textit{Fmr1} KO mice (Comery et al., 1997; Grossman et al., 2006; Irwin et al., 2002). These results showing that repression of \textit{Fmr1} gene expression increases the proportion of long, thin dendritic spines have been the most frequently articulated.

Several results exist suggesting that repression of \textit{Fmr1} gene expression can severely affect dendritic morphology in a manner much different than that described above. For example, Grossman et al. (2006) conducted dendritic spine analyses in the CA1 subfield of the hippocampus of \textit{Fmr1} KO mice and reported small, but significant differences essentially opposite to that previously observed in neocortex (Grossman et al., 2006). The reason for this disparity is unclear. Another example comes from analyses of neurons cultured from the hippocampus that show substantial morphological abnormalities that appear at odds with results from adult neocortical neurons. Braun and Segal analyzed 7 and 21-day old cultured hippocampal neurons and observed shorter dendrites, fewer spines and no significant differences in spine length (Braun and Segal, 2000). Similarly, (Castren et al., 2005) reported less neurites and decreased dendritic length on cells generated from mouse and human FMRP-deficient neurospheres. Svoboda and colleagues (2001) analyzed the dynamic nature of spines, in addition to morphology, using two-photon laser scanning microscopy in layer V neurons of barrel cortex (Nimchinsky et al., 2001). Spine length and density was significantly increased at postnatal week 1 and reached marginal or undetectable differences by 4 weeks of age. It appears that the morphology of mammalian FMRP-deficient neurons depends on both brain region and neuronal age. These findings beg the question: How is spine maturation affected in the DG, a brain region continually adding new neurons? (This issue is explored in chapter 3 of this thesis.)
1.6.4 Synaptic plasticity

An increase or decrease in synaptic efficacy can last milliseconds to months (Abraham, 2003; Fox et al., 2006; Liu et al., 2004; Zucker and Regehr, 2002). Considerable attention has been given to longer forms of enhancement of synaptic plasticity, namely long-term potentiation (LTP), presumably because of its intuitive appeal as a substrate for the formation of new memories. Indeed, long-term synaptic plasticity in the hippocampus is currently the leading neurobiological model of learning and memory. Support for this theory comes from studies finding impaired synaptic plasticity in several models of cognitive impairment including models of Alzheimer’s disease, Schizophrenia and Down syndrome (Belichenko et al., 2007; Jay et al., 2004; Rowan et al., 2003).

LTP in the CA1 subfield of the hippocampus appears normal in Fmr1 KO mice (Godfraind et al., 1996; Larson et al., 2005), consistent with apparent normal acquisition of the MWM (1994; D'Hooge et al., 1997; Davis et al., 1992; Eadie et al., 2009; Morris et al., 1986; Paradee et al., 1999). These findings left most researchers wondering if Fmr1 KO mice are simply a poor model of FXS and some researchers searching for alternative forms of impaired plasticity. Researchers have had more success discovering impairments in more subtle forms of synaptic plasticity. For example, Bear and colleagues have demonstrated that metabotropic glutamate receptor-5 (mGluR5)-dependent LTD is increased in the CA1 of the hippocampus proper (Huber et al., 2002). This study also demonstrated that classic low-frequency-induced LTD is normal in the CA1 subfield of the hippocampus. The behavioural significance of increased mGluR-LTD is not clear. More recently, it has been shown that the threshold for inducing LTP in the CA1 of the hippocampus proper may be abnormal in Fmr1 KO mice, although this change was not reported in another study performed in a similar manner (Larson et al., 2005; Lauterborn et al.,
Other subtle alterations in synaptic plasticity appear to occur in other regions of neocortex of Fmr1 KO mice, such as differences in the threshold of spike-timing dependent plasticity and mGluR5-mediated LTP (McHugh et al., 2007; Meredith et al., 2007; Wilson and Cox, 2007). How abnormalities in these brain regions relate to behavioral abnormalities observed in the Fmr1 KO mice are again unknown.

In contrast to the above studies, classic forms of long-term synaptic plasticity have been observed in brain regions other than the CA1 subfield of the hippocampus or neocortex. Robust impairments in LTP appear to occur in the cingulate, amygdala and anterior piriform cortex. These abnormalities may in fact be linked to behavioral abnormalities observed in Fmr1 KO mice such as impaired fear conditioning, hyperactivity and anxiety (Larson et al., 2005; Zhao et al., 2005). These brain regions may be part of an information-processing loop involving the ventral hippocampus (i.e., the interoceptive loop) (Amaral and Lavenex, 2007). To date, there are no published studies on synaptic plasticity specifically in the DG of Fmr1 KO mice. (Our laboratory’s results regarding LTP and LTD in the DG of Fmr1 KO mice are described in chapter 3.)

1.6.5 Glutamate receptors

Although the majority of research into the receptor-dependence of aberrant synaptic plasticity in the mouse model of FXS has focused on metabotropic glutamate receptors, emerging evidence implicates abnormalities in the AMPA-type glutamate receptors (workhorses of basal glutamatergic synaptic transmission) and the NMDA-type glutamate receptors (the classic glutamate receptor involved in synaptic plasticity) during neuronal development in the Fmr1 KO mouse. Seeburg’s group has recently reported that early postnatal Fmr1 KO mice exhibit a
significant decrease in AMPA-mediated EPSCs relative to controls in the hippocampal CA1 subfield, and that this difference is abolished when assessed in adulthood (Pilpel et al., 2009). This is consistent with one largely ignored study showing impaired LTP and GluR1 expression in the prefrontal cortex of Fmr1 KO mice (Li et al., 2002). Interestingly, Hu et al. (2008) have recently shown robust impairments in LTP in the CA1 subfield of young (postnatal day 14) Fmr1 KO mice that is associated with decreased GluR1 expression (Hu et al., 2008). In addition, Shutt et al. (2009) have recently shown, using cultured hippocampal and neocortical neurons, that FMRP is associated with mRNA for key synaptic proteins (e.g., PSD-95, SAP-97, and SAPAP1, 2 and 3) and glutamate receptor subtypes (e.g., NR1 and NR2B-containing NMDA receptors, and GluR1-containing AMPA receptors) (Schutt et al., 2009). It appears that the expression of glutamate receptors, particularly the GluR1-containing AMPA receptor, may be deleteriously altered by loss of Fmr1 expression, and that young neurons may be particularly affected. The expression or properties of glutamate receptors in the DG have not been analyzed in Fmr1 KO mice.

1.6.6 Summary

The mouse model of Fragile-X syndrome (Fmr1 KO mice) was first generated 16 years ago (1994) by the Dutch-Belgian Consortium on Fragile-X syndrome. Initial studies indicated that the mice may indeed be a good mouse model of the most common form of inherited intellectual disability. For example, macroorchidism is apparent in the transgenic mice. In addition, like many children with FXS, the Fmr1 KO mice are generally found to be hyperactive. Other striking resemblances between the Fmr1 KO mice and humans with FXS are the long, thin “tortuous” dendritic spines observed in neocortex of adults. One major gap in the literature has
been a lack of discovery of a robust impairment in learning in *Fmr1* KO mice. This is a major problem for a model of a syndrome characterized by severe intellectual disability. In addition, and perhaps as a consequence, robust abnormalities in synaptic plasticity in a brain region clearly associated with learning impairment in the mouse model have been elusive.

Previous research suggests that the process of neuronal development appears to be abnormal in *Fmr1* KO mice. In addition, alterations in dendritic morphology appear to be more severe in developing neurons as a consequence of loss of *Fmr1* transcription. Finally, robust alterations in synaptic plasticity, such as LTP, are apparent in developing neurons. These observations beg the important question: How is plasticity in the DG, a region that continually adds young neurons across the lifespan, affected by loss of *Fmr1* expression? We investigate neurogenesis, dendritic morphology and synaptic plasticity in the DG of young, adult, male *Fmr1* KO mice in this thesis.
1.7 Summary

In the last century, considerable strides have been made in both the classification of cognitive impairment in individuals in our societies and in our understanding of the neurobiology of cognition in the mammalian brain. The main challenge in coming decades will be to translate our basic knowledge to practical, efficacious treatment of individuals with cognitive impairment in our societies. The key step in this knowledge translation is the identification and characterization of aberrant changes in the brain of individuals with specific classes of cognitive impairment. Unarguably, currently the most etiologically well-defined intellectual impairment is Fragile-X syndrome (FXS), a disorder caused by loss of expression of the Fmr1 gene. Despite the considerable advances into the neurobiology of cognition, the treatment of FXS is still based on the targeting of symptoms, rather than pathophysiological abnormalities. The generation of mouse models of genetic diseases in recent decades has revealed new opportunities to bridge this gap.

We have reviewed findings ranging from the behavior of individuals with FXS to the behavior of mice with loss of a functional Fmr1 gene. We have discussed the data that can be gleaned from human FXS studies (i.e., neuroimaging, neuropathology, etc.) as well as the key findings from studies using the mouse model of FXS. It appears that although many brain regions may be affected, converging evidence suggests that the DG may be particularly affected in FXS. First, FMRP is normally expressed at relatively high levels in the hippocampus, especially the DG. Second, the hippocampus is theorized to be involved in the key neuropsychological domains affected in FXS, namely intellectual ability and emotional regulation. The neural circuits subserving these domains may be polarized along the dorsal-ventral axis of the hippocampus. Third, the DG subfield of the hippocampus may be particularly
involved in the pathogenesis of FXS due to (1) accumulating research suggesting that young neurons may be particularly affected by loss of Fmr1 expression and (2) the well-established observation that the DG subfield of the hippocampus possesses the unique ability to continually produce young neurons across the lifespan.
1.8 Hypotheses

Our overarching hypothesis is:

A lack of expression of the Fmr1 gene deleteriously alters structural and functional plasticity in the mammalian DG, and impairs aspects of learning and emotion associated with this brain region.

Our specific hypotheses relating to the behavior of Fmr1 KO mice were:

1. Fmr1 KO mice show decreased anxiety as assessed using the open field and elevated-plus maze.
2. Fmr1 KO mice show decreased basal corticosterone and stress-induced corticosterone response.
3. Fmr1 KO mice are impaired on the reversal, but not acquisition phases, of the Morris water maze and Plus-shaped water maze.
4. Fmr1 KO mice show impaired fear conditioning and context discrimination.

Our specific hypotheses relating to adult neurogenesis in Fmr1 KO mice were:

1. Fmr1 KO mice show decreased cell proliferation.
2. Fmr1 KO mice show decreased cell survival.
3. Fmr1 KO mice show increased neuronal differentiation.
Our specific hypotheses relating to dendritic morphology in Fmr1 KO mice were:

(1) Fmr1 KO mice show decreased dendrite length.

(2) Fmr1 KO mice show long, thin dendritic spines.

(3) Fmr1 KO mice show no change in dendritic spine densities.

Our specific hypotheses relating to synaptic plasticity in Fmr1 KO mice were:

(1) Fmr1 KO mice show impaired long-term potentiation (LTP).

(2) Fmr1 KO mice show no change in long-term depression (LTD).

Our specific hypotheses relating to AMPA and NMDA receptors were:

(1) Fmr1 KO mice show decreased AMPA-mediated currents.

(2) Fmr1 KO mice show no change in NMDA-mediated currents.
1.9 References

AAIDD. The American Association of Intellectual and Developmental Disabilities.


Eadie BD, Zhang WN, Boehme F, Gil-Mohapel J, Kainer L, Simpson JM, Christie BR. 2009. Fmr1 knockout mice show reduced anxiety and alterations in neurogenesis that are specific to the ventral dentate gyrus. Neurobiol Dis.


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2. *Fmr1* Knockout Mice Show Reduced Anxiety and Alterations in Neurogenesis that are Specific to the Ventral Dentate Gyrus

2.1 Introduction

Fragile X syndrome (FXS) is the most common form of inherited mental retardation (Bagni and Greenough, 2005) affecting an estimated 1 in 4000 males and 1 in 8000 females (Turner et al., 1996). The loss of transcription of the *Fmr1* gene on the fragile tip of the X chromosome leads to loss of production of the fragile X mental retardation protein (FMRP) (Fu et al., 1991; Verkerk et al., 1991). Normally, FMRP is highly expressed in principal cells in the brain, particularly in hippocampal neurons. It remains unclear if the loss of expression of this gene in the hippocampus results in the same behavioral abnormalities that are characteristic of FXS in humans (*i.e.*, intellectual impairment and emotional dysfunction).

The cognitive and behavioral symptoms of FXS in humans include reduced IQ, increased anxiety, attention deficit/hyperactivity and autistic behaviors (Jacquemont et al., 2007). Curiously, mice lacking the *Fmr1* gene show decreased anxiety (Qin et al., 2002; Qin et al., 2005; Restivo et al., 2005; Yan et al., 2005) and show no robust deficits in learning when tested on the Morris water maze (MWM) (1994; D'Hooge et al., 1997; Gantois et al., 2001; Paradee et al., 1999; Peier et al., 2000; Qin et al., 2002). Based on these behavioral observations, we hypothesize that the neural circuitry associated with anxiety is altered by a loss of *Fmr1* expression.

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1 A version of this chapter has been published. Eadie B.D., Zhang W.N., Boehme F., Gil-Mohapel J., Kainer L., Simpson J.M., Christie B.R. (2009) *Fmr1* knockout mice show reduced anxiety and alterations in neurogenesis that are specific to the ventral dentate gyrus, Neurobiol Dis. 36:361-373.
The ventral hippocampus is a brain region strongly linked to anxiety-related behaviors (Bannerman et al., 2004; Bannerman et al., 1999; Degroot and Treit, 2004; Engin and Treit, 2007), and the dorsal hippocampus appears to be subservient to spatial learning and memory (Engin and Treit, 2007; Maurer et al., 2005; Moser et al., 1993). There are notable differences in the afferent and efferent projections to other limbic structures along the longitudinal axis of the hippocampus that appear to support this hypothesis (Bannerman et al., 1999; Dolorfo and Amaral, 1998b; Pikkarainen et al., 1999; Pitkanen et al., 2000; van Groen et al., 2003). In addition, the different subfields of the hippocampus (i.e., the dentate gyrus (DG), CA3 and CA1) have been associated with different aspects of spatial learning behavior. For example, lesions of the CA1 of the dorsal hippocampus can disrupt spatial memory (Duva et al., 1997), and the selective deletion of the NMDA receptor subunit NR1 in the DG impairs pattern separation in a contextual fear-conditioning task without impairing performance in the classic MWM (Goodrich-Hunsaker et al., 2008; Hunsaker et al., 2008; McHugh et al., 2007).

A relatively unique phenomenon observed across the longitudinal axis of the hippocampus, specifically within the DG, is adult neurogenesis: the production of new neurons into adulthood (for reviews see Christie and Cameron, 2006; Gage, 2002). Prevailing theories regarding the function of adult neurogenesis typically involve learning and memory (van Praag et al., 1999), but there has been recent speculation that adult neurogenesis may also play important roles in emotional disorders such as depression and anxiety (Sahay et al., 2007; Sahay and Hen, 2007; Santarelli et al., 2003; Wang et al., 2008). In addition, several laboratories have also suggested that the dorsal and ventral aspects of the hippocampus may play different roles in spatial and emotional learning (Quinn et al., 2005; Rogers and Kesner, 2006; Snyder et al., 2009). Furthermore, neurogenesis in these subregions may also be specifically involved in different
forms of learning (Snyder et al., 2009). In the current study, we examined whether there were region-specific abnormalities in adult neurogenesis in the dorsal and ventral DG, and whether these abnormalities were correlated with altered performance in spatial memory-related and anxiety-related behavioral tasks in Fmr1 KO mice.
2.2 Materials and Methods

2.2.1 Animals

Sixty-nine C57BL/6 male *Fmr1* knockout (KO) (*n* = 37) and *Fmr1* wild-type (WT) (*n* = 32) littermate mice were generated by breeding WT male C57BL/6 (obtained from Jackson Laboratories) with female C57BL/6 mice heterozygous for the *Fmr1* gene (*Fmr1* KO mice were produced from founders originally provided by Dr. Mark Bear, MIT). The C57BL/6 background strain was employed because they perform well on spatial learning and memory tasks and show substantial neurogenesis (Holmes et al., 2002; van Praag et al., 1999). All animals were sexed, weaned and ear-punched at post-natal day 24 and housed with minimal enrichment (tubes and/or nestlets). All experiments were conducted on mice between the age of 2 to 4 months. Animals lacking the FXS gene were identified using a standard genotyping protocol (see below), and the experimenter was blinded to the identity of all animals during the course of the experiments.

Separate cohorts of mice were employed for behavioral and histological experiments due to the fact that behavioral experiments can alter histological parameters addressed in this study (Leuner et al., 2006). Male animals were used because there is an increased prevalence of FXS in males and because females with a full mutation show a less severe and more varied phenotype (Jacquemont et al., 2007). Experiments were carried out in accordance with international standards on animal welfare and guidelines set out by the Canadian Council on Animal Care, the University of British Columbia and the University of Victoria. All efforts were made to minimize pain and discomfort for all animals (Appendix B).
2.2.2 Genotyping

2.2.2.1 DNA isolation

DNA extraction was performed on ear punch or tail snip tissue stored at −20°C. Briefly, tissue was placed in 150 μL digestion buffer (100 mM 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. Fifteen microliters 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.

2.2.2.2 PCR assay

The PCR reaction was performed by mixing 13 μL PCR-grade H₂O, 2.5 μL 10× PE Buffer II, 2.5 μL (25 mM) MgCl₂, 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 then 30 cycles of 60 s 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 KO allele (amplified fragments of 800 base pairs). Primers S1 = 5′ GTGGTTAGCTAAAGTGAGGATGAT 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.

2.2.3 Behavior

A single cohort of mice (6 WT and 9 KO mice) was initially assessed in an open-field arena, then tested on an elevated plus maze and finally examined in the MWM. All tasks were separated by at least 1 week. A separate cohort of mice was also used to assess spatial learning and memory in the Plus-shaped version of the water maze (7 WT and 9 KO mice). A video camera, connected to an image analysis system (HVS Image, England) attached to a microcomputer running the HVS maze software, was mounted above the center of each behavioral apparatus. This was used to track, digitize and store behavioral data for subsequent analyses.

To examine anxiety-related behaviors, animals were tested in the open-field and elevated plus maze. These assays do not involve confounding factors such as conditioning, learning, social interaction, fear induced by a predator or other major stressors and give a good indication of how anxious animals are normally. This is based upon decades of research assessing the construct validity of these assays (Hall, 1934; Handley and Mithani, 1984; Prut and Belzung, 2003; Walf and Frye, 2007).
2.2.3.1 Open-field arena

Locomotor activity, thigmotaxis and the number of fecal boli (defecations) were measured across a 15-min period in a circular arena (diameter = 70 cm, height = 20 cm) positioned in the middle of a dimly illuminated (approximately 20 ± 0.5 lx) testing room. The floor of the maze was raised 75 cm above the floor. The experimenter was not in the room during the testing period. The total distance the mouse traveled in the complete arena and the time spent in the center of the arena were measured and calculated by the computer for each minute of testing. Fecal boli were counted for each animal at the end of the 15-min testing period. To control for the potential confounding effect of alterations in basal metabolic or gastrointestinal function, we also counted fecal boli from home cages of Fmr1 KO mice and wild-type controls. This was accomplished by individually housing a separate cohort of mice from each genotype for a 24 hour period followed by manually counting fecal boli within a random 100 ml sample of bedding from each cage.

2.2.3.2 Elevated plus maze

Open arm entries in this task have been used as an index of anxiety-related behavior. Locomotor activity and the number of entries into the arms were measured across a 5-min period in an elevated plus maze (two open and two closed arms opposite each other) (arm length = 30 cm, wall height = 15 cm). The maze was positioned in the middle of a dimly illuminated (approximately 20 ± 0.5 lx) testing room. The floor of the maze was raised 75 cm above the floor. The experimenter was out of the room for the entirety of the experimental testing. The total distance the mouse traveled in the complete maze, the total number of arm entries and the total number of open arm entries were measured and calculated for each minute of testing.
2.2.3.3 Morris water maze

The MWM consisted of a white, circular, fiberglass tank (diameter = 100 cm) (Morris, 1984; Van Dam et al., 2006). The water was made opaque using non-toxic, white paint and the temperature was held constant at 24 ± 1 °C. The floor of the maze was raised 45 cm above the room floor. A variety of small objects were fixed around the tub as distal visual cues. A hidden escape platform was located 1 cm under the water surface. Mice were trained individually and each session consisted of 2 blocks of 3 trials. The blocks were separated by approximately 20 min. Each trial was started and ended manually by the experimenter who was blind to the genotype of the mice. On the first day, the animals were pre-trained using a cued (i.e., visible) platform positioned in the center of the maze in order to familiarize them to the apparatus and to swimming in the pool. In the hidden platform training phase, the platform was positioned at 30 cm from the centre in the S direction. Each animal underwent three consecutive trials. The starting position varied randomly among three possible release points (N, E and W). To begin a trial, the subject was gently released from the start point facing the wall of the maze and was allowed to locate the escape platform within 60 s. Path length for each mouse on each trial was recorded. Upon reaching the platform, the subject spent an inter-trial interval of 15 s on it before the second trial commenced. If an animal failed to locate the platform within the time limit of 60 s, a maximal escape latency of 60 s was recorded, and it was guided to the platform by the experimenter and allowed to stay on it for 15 s. After each trial, the mouse was removed from the platform and placed in the holding cage for 15 s before being released for the next trial. After 6 days of acquisition, the escape platform was changed to a novel position opposite the location used for the acquisition trials and the animals had to learn this new location during three reversal training days.


2.2.3.4 Plus-shaped water maze

The Plus-shaped water maze consisted of a transparent Plexiglas, Plus-shaped swimming maze (arm width = 20 cm, arm length = 26 cm, arm height = 25 cm) filled with water made opaque using white, non-toxic paint (held at a temperature of 25 °C) and placed in a circular tub (diameter = 1 m) (Van Dam et al., 2000). The floor of the maze was raised 0.45 m above the room floor. A variety of small objects were fixed around the tub as distal visual cues. A hidden escape platform was located 1 cm under the water surface in one of the four arms. A video camera, connected to an image analysis system (HVS Image, England) attached to a microcomputer running the HVS maze software, was mounted above the center of the water maze. The swim path of the animal was tracked, digitized and stored for subsequent behavioral analysis.

Mice were trained in squads of four. Each daily session consisted of 2 blocks of three trials. The blocks were separated by approximately 20-min intervals. Each trial was started and ended manually by the experimenter who was blind to the genotype of the mice. Mice were subsequently released from the other three arms not containing a platform and allowed to swim for 1 min. Swimming into any of the three non-target arms was recorded as an error. Swimming directly to the platform arm without entering any of other three arms was recorded as a correct response. A choice was considered “correct” when the animal turned directly to the arm containing the platform at the intersection of the maze and successfully escaped. The numbers of entries in the three arms not containing a platform and in the target arm were counted, and the latency to reach the platform was measured. When the mouse found the escape platform within the 60 s, it was allowed to remain there for 20 s. If the mouse did not find the escape platform within 60 s, it was gently guided onto the platform and left there for 20 s. After each trial, the
mouse was removed from the platform and placed in the holding cage for 15 s before being released in another arm for the next trial. Because the walls of the Plus-shaped water maze are transparent, the animals were able to use distal cues in the environment of the Plus-shaped water maze to locate the platform. The spatial reference memory water maze task lasted 13 days and consisted of five stages organized sequentially as follows: One day of visible platform testing (three trials and the escape platform was made “visible” by mounting a visual cue on top of it with the starting point constant across trials, but the locations of the escape platform different for the three consecutive trials). Six days of acquisition training (one training session per day with a submerged, fixed platform in the SW arm with the start positions distributed in a predetermined, pseudorandom order so that no two consecutive start points were the same, and the first and second blocks of three trials each consisted of the three different start points from NW, NE and SE arms). One day of probe testing without the platform (where the mouse was allowed to swim for 60 s in a pool without an escape platform before being removed from the maze). Four days of reversal training (with the hidden platform in the opposite arm (NE)) and one final day of probe testing without the platform 24 h after the reversal training (where the mouse was allowed to swim for 60 s in a pool without an escape platform before being removed from the maze) (protocol illustrated in Figure 2.4C).

2.2.4 Restraint stress and corticosterone ELISA

A separate cohort of Fmr1 KO (n = 4) and WT (n = 4) mice were subjected to 3 h of restraint in a closed, ventilated 50-ml plastic centrifuge tube, which permitted some movement but prevented the mouse from fully turning. Animals were removed from the restraint tubes and immediately sacrificed. Non-stress control mice were moved to the testing room but remained in
the home cage undisturbed. Testing took place between approximately 9:00 a.m. and 1:00 p.m. (12:12-h light/dark cycle) prior to the late diurnal rise in circulating glucocorticoids. Trunk blood was collected following rapid decapitation and permitted to clot for 2 h at room temperature. Following centrifugation for 10 min (RCF = 6238), the serum fraction of each sample was collected and stored at − 20 °C until analysis. Corticosterone levels were assayed using a competitive Correlate-EIA (TM) Corticosterone Enzyme Immunoassay Kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions.

2.2.5 Histology

One cohort of mice (5 WT and 5 KO mice) was used to assess cell proliferation and neurogenesis using the intrinsic markers PCNA and Ki67 (proliferation) and doublecortin (neurogenesis). In a separate cohort of animals, bromodeoxyuridine (BrdU; Sigma, St. Louis, MO; 200 mg/kg; 20 mg/ml) was administered at post-natal day 60 (Cameron and McKay, 2001; Eadie et al., 2005), and animals were sacrificed either 3 h later to assess proliferation (6 WT; 6 KO) or 4 weeks later to assess neurogenesis (4 WT; 4 KO).

All animals were sacrificed and given a transcardial perfusion with 30 ml of 0.9% saline followed by 60 ml of 4% paraformaldehyde. Brains were then stored in 4% paraformaldehyde for 24 h and then transferred into 30% sucrose. After saturation in sucrose, 30-μm coronal sections were cut with a vibratome (Leica VT1000, Nussloch, Germany) to obtain a series of sections throughout the dorsal–ventral axis of the hippocampus. The hippocampus was segregated into two halves, with the dorsal hippocampus defined as the anterior half (− 1.34 to − 2.30 bregma) and the ventral hippocampus defined as the posterior half (− 2.46 to − 3.40 bregma; Figure 2.1B). This arbitrary division was used because we did not have an a priori
hypothesis to justify further divisions in the initial experiments. Due to a discrepancy in results obtained using the two endogenous markers of cell proliferation, a careful assessment of cell proliferation was also conducted by administering a single BrdU injection (200 mg/kg; 20 mg/ml). In these experiments, sections were subdivided into quartiles across the dorsal–ventral axis prior to immunohistochemistry and quantification.

2.1 Timeline of experimental procedures.

Timeline of experimental procedures and illustration of the curvilinear shape of the dentate gyrus (DG) of the hippocampus as it extends from its dorsal (rostral; septal) to ventral (caudal; temporal) aspects. (A) Male Fmr1 knockout (KO) or wild-type (WT) littermate mice were generated, weaned and ear-punched at post-natal day 24. (B) On the left, an outline of a sagittal section of a mouse brain with approximate location of the DG (red) illustrating its shape and demarcating the dorsal and ventral subregions (arrows) (modified from Paxinos and Franklin, 2001).
2.2.5.1 Antibodies

All antibodies were diluted in TBS+ containing 0.1% Triton X-100 and 3% donkey serum as previously described (Eadie et al., 2005). The primary antibodies used for immunohistochemistry in this study were biotinylated monoclonal mouse anti-BrdU (1:100; Chemicon, Temecula, CA), monoclonal rat anti-BrdU (1:500; Harlan Seralab, Indianapolis, IN), monoclonal mouse anti-NeuN (1:100; Chemicon), polyclonal rabbit anti-S100β (1:2000; SWant, Bellinzona, Switzerland), polyclonal rabbit anti-Ki67 (1:500; Vector Laboratories, Burlingame, CA), polyclonal rabbit anti-proliferating cell nuclear antigen (PCNA) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-doublecortin (DCX) antibody (1:400; Santa Cruz Biotechnology). The secondary antibody used for Ki67 and PCNA staining was biotin-conjugated goat anti-rabbit IgG (1:200; Vector Laboratories). For indirect immunofluorescence, the following secondary antibodies with minimal cross-reactivity with each other were used (all at 1:250; Jackson ImmunoResearch, West Grove, PA): FITC-conjugated donkey anti-rat IgG, Cy3-conjugated donkey anti-mouse IgG and Cy5-conjugated donkey anti-rabbit IgG (Burlingame, CA).

2.2.5.2 BrdU, Ki67 and PCNA pre-treatment

Tissue to be stained for BrdU was rinsed in TBS then submerged in 0.6% H2O2 for 30 min to quench endogenous peroxidase activity. The tissue was then rinsed in TBS, submerged in a formamide solution (50% formamide, 10% 20× SSC, 40% dH2O) at 65 °C, rinsed in 2× SSC for 5 min, then submerged in 2 N HCl for 30 min at 37 °C and rinsed in 0.1 M borate buffer (pH 8.5) for 10 min to unravel the DNA to expose the BrdU. Slices were thoroughly rinsed in TBS. Separate tissue to be stained for Ki67 and PCNA was rinsed in TBS then submerged in 10 mM sodium citrate buffer (in PBS, pH 6.0) at 95 °C for 5 min. This step was repeated twice in order
to unmask the antigens. Endogenous peroxidase activity was then quenched with 3% H₂O₂/10% methanol in 0.1 M TBS for 15 min. Prior to incubation with the Ki67 primary antibody (48 h at 4 °C), tissue was submerged in 5% donkey serum for 1 h.

2.2.5.3 Immunohistochemistry (IHC)

Briefly, following the appropriate pre-treatment (see above), tissue was blocked with TBS+ for 30 min followed by incubation in the appropriate primary antibody (against BrdU, DCX, Ki67 and PCNA) in TBS+ overnight at 4 ºC. Tissue was then washed in TBS, blocked with TBS+ for 30 min and incubated in biotinylated secondary antibody for 2-4 h at room temperature. Following TBS washes, the tissue was then submerged in ABC Vectastain Elite reagent (Vector Laboratories) for 2 h to form the avidin–biotin–peroxidase complex and placed in diaminobenzidine (chromogen) solution (Vector Laboratories) for approximately 10 min, separated with thorough rinses with TBS. Finally, tissue was thoroughly washed, dehydrated and cover-slipped with permount (Sigma).

2.2.5.4 Immunofluorescence (IF)

To phenotype newly generated cells in the DG, the tissue was blocked with TBS+ for 30 min followed by incubation in a cocktail of primary antibodies against BrdU, NeuN and S100β for 36 h at 4 ºC. After washing with TBS and blocking with TBS+ for 30 min, the tissue was incubated in a cocktail of secondary antibodies against rat and mouse for 24 h at room temperature. Finally, tissue was thoroughly washed, mounted and cover-slipped in polyvinyl alcohol with diazabicyclo-octane (PVA-DABCO) (Sigma) as an anti-fading agent.
2.2.5.5 Quantification of cell types

For cell survival and differentiation experiments (BrdU IHC and IF, respectively), we stained randomly chosen groups of at least five dorsal and ventral serial sections to ensure random, unbiased sampling of both the dorsal and ventral hippocampus for all staining procedures. Thirty-micrometer-thick serial sections were randomly selected from the anterior or posterior half of sections through the hippocampus and used for histology as described above. Five complete serial sections with intact granule cell layers (GCL) and clear staining, which were all estimated to be located either greater than −2.3 relative to bregma for dorsal sections or less than 2.46 relative to bregma for ventral sections, were included in analyses (Paxinos and Franklin, 2001). To ensure unbiased sampling methods, the volume of the DG was estimated in all samples as follows: First, the area of the GCL was obtained using ImagePro Plus software (version 5.0 for Windows™, Media Cybernetic, Inc., Silver Spring, MD) and an Olympus BX51 microscope (Olympus, Center Valley, PA). The area was measured three times and the average was taken and converted to a volume using the anterior–posterior (AP) thickness of the group of five sections (150 μm) as the third dimension. The average volumes of each GCL within each subregion and genotype were used for statistical analyses. Due to the lack of volumetric difference, we assessed histological differences as densities per reference volume consistent with accepted histological practices. In addition, to avoid overestimation of the number of cells per region, we were careful not to count cells that appeared to be located in the top 5 μm of each section, as doing so can lead to counting half cells twice. Differentiation analyses were conducted by random sampling of at least 50 BrdU+ cells and determination of co-localization with the phenotypic markers NeuN and S100β, and thus expressed as a percentage of BrdU+ cells. Co-localization of markers was considered positive if nuclear co-localization was observed in x–y, x–z and y–z cross-sections produced by orthogonal reconstructions from z-series.
(example shown in Figure 2.6E). Fluorescent images were collected using a confocal laser scanning microscope (Nikon Eclipse TE 2000-U) connected to a PC running EZ-C1 Nikon Confocal Microscope Software (Version 3.5). For each BrdU+ cell analyzed for co-labeling with NeuN or S100β, at least 20 consecutive scans with a step size of 0.6 μm (> 11.4 μm total) was performed to create a rotatable 3D image.

2.2.6 Statistical methods

Differences are presented as mean ± standard error of the mean (SEM). Differences between mean values of experimental groups were compared using Student's t-test or analysis of variance (ANOVA) followed by Tukey's post hoc tests as appropriate using Statistica 7.0 software (StatSoft, Tulsa, OK). Differences were considered statistically significant when \( p < 0.05 \).

2.3 Results

2.3.1 Anxiety in Fmr1 KO mice

Several specific behaviors in the open field were assessed, including the time spent in the center of the arena, the rate of movement and the number of defecations. In comparison to WT littermates, Fmr1 KO mice traveled significantly more in the center of the open field (WT: 10.13 ± 0.77%; Fmr1 KO: 13.65 ± 1.14%; \( t(13) = 2.43, p = 0.03; \) Figure 2.2A), indicating they were less anxious about being exposed to the open environment. Similarly, the inverse of this measure, percentage of time spent in the periphery of the arena, was also significantly lower. These data suggest that Fmr1 KO mice appear less anxious than WT littermates when exposed to
the open environment. These differences were not a function of any obvious motor deficits, as there were not any significant differences in the rate of movement in the open field between the groups (WT: 316.14 ± 12.51 cm/min; *Fmr1* KO: 347.92 ± 16.74 cm/min; *t*(13) = 1.47, *p* = 0.17; Figure 2.2B). In addition, *Fmr1* KO mice defecated far less than WT littermates (WT: 7.17 ± 1.40 fecal boli; *Fmr1* KO: 1.78 ± 0.75 fecal boli; *t*(13) = 4.01, *p* = 0.002; Figure 2.2C), providing support for the interpretation that these data reflect decreased anxiety in *Fmr1* KO mice (Commissaris et al., 1986). In separate groups of animals, we found no difference in estimations of home cage fecal boli (*p* = 0.734).

2.2 Open field and elevated plus maze assays for anxiety in *Fmr1* KO mice.

*Fmr1* KO mice exhibit behaviors suggesting decreased anxiety. (A) *Fmr1* KO mice travel a greater distance in the centre of the open field (decreased thigmotaxis). (B) *Fmr1* KO mice do not appear to show any change in overall locomotion. (C) *Fmr1* KO mice defeate substantially less in the open field. (D) *Fmr1* KO mice enter the open arms of the elevated plus maze more than WT mice during the first minute of testing. (E) *Fmr1* KO mice do not appear to show any change in activity on the elevated plus maze. (F) Total number of arm entries was not significantly different between genotypes. (*p* < 0.05).
Anxiety-like behavior was also assessed using a second independent task, the elevated plus maze. *Fmr1* KO mice were not significantly different from WT littermates on measures of total number of arm entries across a 5-min session (WT: 22.1 ± 4.7 total arm entries; *Fmr1* KO: 21.1 ± 4.8 total arm entries; t(13) = 0.288, p = 0.777; Figure 2.2F). However, during the first minute of testing, *Fmr1* KO mice showed significantly more entries into the open arms compared to WT littermates (WT: 1.83 ± 0.34 open arm entries; *Fmr1* KO: 3.89 ± 0.74 open arm entries; t(13) = 2.29, p = 0.0394; Figure 2.2D). There was no difference in total arm entries after the first minute of testing (WT: 6.0 ± 0.6; *Fmr1* KO: 7.6 ± 0.9 total arm entries; t(13) = 1.395, p = 0.186). These data, like that from the open field, suggest that *Fmr1* KO have decreased anxiety to the open environment compared to WT littermates. Also, similar to data from the open field, we did not observe statistically significant differences in the distance traveled in this task (WT: 1181.6 ± 145.2 cm total arm entries; *Fmr1* KO: 1363.8 ± 112.0 total arm entries; t(13) = 1.553, p = 0.144; Figure 2.2E), indicating that these differences were not due to an obvious change in motor behavior.

### 2.3.2 Corticosterone response to acute stress in *Fmr1* KO mice

To assess whether *Fmr1* KO mice exhibit a decreased corticosterone response to an acute stressor, we conducted ELISA on trunk blood serum obtained from mice immediately following a 3-h restraint procedure. No differences were found in corticosterone levels between genotypes in the non-stress condition (WT: 32.00 ± 10.55; KO: 22.60 ± 5.50; t(6) = 0.821, p = 0.449; Figure 2.3). However, following acute stress, the *Fmr1* KO mice had significantly lower corticosterone levels (WT: 208.818 ± 11.385; KO: 155.463 ± 20.960; t(6) = 3.096, p = 0.021).
Thus, in addition to showing lower anxiety levels behaviorally, *Fmr1* KO mice also show a blunted corticosteroid response following acute stress.

![Graph showing corticosterone levels in WT and Fmr1 KO mice before and after restraint stress](image)

### 2.3 Blunted corticosterone response to acute stress in *Fmr1* KO mice.

*Fmr1* KO mice show similar basal corticosterone levels and a significantly decreased corticosterone response to a 3-h restraint stress relative to WT controls. ELISA for corticosterone was conducted on serum obtained from a cohort of male *Fmr1* KO and WT mice. The acute stressor induced a significantly smaller corticosterone response in *Fmr1* KO mice, supporting the behavioral data presented in figure 2, suggesting decreased anxiety responses to exposure to open environments (*p* < 0.05).

### 2.3.3 Spatial learning and memory in *Fmr1* KO mice

Two different versions of the water maze, the classic MWM and the Plus-shaped water maze, were used to assess spatial learning and memory in *Fmr1* KO and WT littermate mice. Both tasks included a reversal phase following an acquisition phase.

Assessment of spatial learning and memory using the classic Morris water maze revealed that *Fmr1* KO mice appear to learn the location of the hidden platform equally well as WT mice. The exception was a single KO mouse that showed inconsistent behavior over the entire course of testing and was therefore excluded from the data analyses. The remaining animals all showed a progressive reduction in the distance traveled to the hidden platform across the 6 days of testing (WT: Day 1 = 352 ± 100 cm, Day 6 = 127 ± 30 cm, *F*(5,30) = 2.648, *p* = 0.043; KO: Day
1 = 364 ± 54 cm, Day 6 = 147 ± 40 cm, $F(5, 42) = 2.450, p = 0.049$) that was not significantly
different between genotypes ($F(1,12) = 0.259, p = 0.620; \textbf{Figure 2.4A}$). On the subsequent
3 days, reversal learning was assessed by moving the hidden platform to an adjacent quadrant.
Both genotypes were able to learn the new location of the hidden platform (WT: Day
7 = 216 ± 44 cm, Day 9 = 87 ± 30 cm, $F(2, 15) = 5.614, p = 0.016$; KO: Day 7 = 180 ± 26 cm,
Day 9 = 90 ± 16 cm; $F(2, 21) = 7.281, p = 0.004$), and there was no significant difference
between the genotypes ($F(1,12) = 0.017, p = 0.898$). Both groups of mice exhibited similar
swim speeds during both the acquisition (WT: Day 1 = 12.3 ± 1.2 cm/s (average swim speed
prior to finding the hidden platform), Day 6 = 6.3 ± 0.9 cm/s; KO: Day 1 = 11.5 ± 0.5 cm/s, Day
6 = 5.7 ± 0.9 cm/s; $F(1, 12) = 0.355, p = 0.562; \textbf{Figure 2.4B}$) and reversal phases (WT: Day
7 = 8.7 ± 0.9 cm/s, Day 9 = 4.2 ± 0.8 cm/s; KO: Day 7 = 8.7 ± 0.9 cm/s, Day 9 = 4.9 ± 0.8 cm/s;
$F(1, 12) = 0.638, p = 0.440$) of this task. Thus, WT and $Fmr1$ KO animals were not significantly
different in any aspect of the classic MWM.
2.4 *Fmr1* KO mice exhibit normal learning ability in the water maze.

*Fmr1* KO mice and WT littermates performed equally well on acquisition and reversal learning of both the classic Morris water maze (MWM) and the Plus-shaped water maze. Separation of acquisition and reversal phases is indicated by a vertical dashed line. (A) All mice learned the classic Morris water maze over 6 days. All mice learned to find the hidden platform during subsequent reversal learning testing on this task as well. (B) There were no significant differences in swimming speed between genotypes during testing in the MWM. (C) Outline of the experimental procedure for the Plus-shaped water maze. (D) *Fmr1* KO mice learned the acquisition and reversal phase of the Plus-shaped water maze, as measured by % entries into the correct arm per trial, and was not significantly different from controls. (E) There were no significant differences in swimming speed between genotypes during testing in the Plus-shaped water maze. (F) Probe trials in the Plus-shaped water maze showed an equivalent preference between genotypes for the target arm after the acquisition and reversal phases.
The Plus-shaped water maze was also used to provide convergent evidence for lack of an impairment in spatial learning and memory in Fmr1 KO mice (Van Dam et al., 2000). In the Plus-shaped water maze, both groups of mice appeared to learn the location of the hidden platform, as evident by a progressive increase in the percentage of entries into the correct arm per trial (WT: Day 1 = 21.43 ± 5.14, Day 6 = 95.24 ± 5.14, F(5,36) = 13.945, p = 0.000; KO: Day 1 = 14.81 ± 5.47, Day 6 = 87.04 ± 4.91, F(5,48) = 18.900, p = 0.000). Furthermore, there was not a significant difference between Fmr1 KO and WT mice during the initial acquisition phase of this task (F(1, 14) = 2.705, p = 0.122; Figure 2.4D). On the subsequent 4 days, reversal learning was assessed by moving the hidden platform to the opposite arm. During the reversal phase of testing, the Fmr1 KO mice (Day 7 = 37.04 ± 5.73, Day 10 = 70.37 ± 8.73) and the WT mice (Day 7 = 38.09 ± 5.14, Day 10 = 69.05 ± 9.15) did not show any significant differences in their re-acquisition of the task (F(1,14) = 0.052, p = 0.824). Both groups of mice exhibited similar swim speeds during both the acquisition (WT: Day 1 = 11.1 ± 1.9 cm/s, Day 6 = 6.3 ± 0.4 cm/s; KO: Day 1 = 9.9 ± 0.7 cm/s, Day 6 = 6.3 ± 0.4 cm/s; F(1, 14) = 0.05, p = 0.826; Figure 2.4E) and reversal phases (WT: Day 7 = 8.5 ± 0.5 cm/s, Day 10 = 8.4 ± 0.6 cm/s; KO: Day 7 = 8.9 ± 0.5 cm/s, Day 10 = 7.1 ± 0.4 cm/s; F(1, 14) = 0.004, p = 0.949) of this task. In the first probe trial (between acquisition and reversal phases), both genotypes spent a greater percentage of time in the target arm relative to non-target arms (WT: target = 57.09 ± 5.21, F(3,24) = 39.231, p = 0.000; KO: target = 53.43 ± 5.31, F(3,32) = 25.933, p = 0.000; Figure 2.4F). No significant difference between genotypes was found in the first probe trial (F(1,56) = 0.000, p = 0.988). In the second probe trial (following the reversal phase), WT mice spent the largest percentage of time in the target and opposite arms, which was significantly greater than the time spent in one of the other arms (target = 31.4 ± 4.03,
opposite = 31.0 ± 2.72, Arm 1 = 17.0 ± 4.0, Arm 3 = 20.5 ± 4.1; $F(3,24) = 4.493$, $p = 0.012$).

Similarly, the KO mice spent significantly more time in the target quadrant than the other arms (KO: target = 42.7 ± 4.1, $F(3, 32) = 20.827$, $p = 0.000$). No significant difference between genotypes was found in the second probe trial ($F(1,56) = 0.000$, $p = 1.000$). Consistent with the results from the classic MWM, WT and Fmr1 KO animals were not significantly different regarding acquisition, reversal learning or swim speed in the Plus-shaped water maze.

### 2.3.4 Cell proliferation in the DG of Fmr1 KO mice

To assess cell proliferation, we conducted immunohistochemistry for two endogenous markers of cell division (Ki67 and PCNA) in addition to performing BrdU immunohistochemistry. The density of Ki67+ cells (per reference volume) was not significantly different between genotypes in the dorsal (WT: 6.798 ± 0.823; KO: 8.899 ± 1.703; $t(8) = 1.241$, $p = 0.250$; **Figure 2.5A**) or ventral (WT: 7.925 ± 0.664; KO: 8.619 ± 1.322; $t(8) = 0.525$, $p = 0.614$) DG. Reference volumes and their locations relative to bregma were again obtained and found to be not significantly different between genotypes. Thus, a loss of Fmr1 expression does not appear to hinder cell proliferation in the adult DG as measured by Ki67+ cell densities. The density of PCNA-positive cells showed a trend towards a significant decrease in the ventral DG of Fmr1 KO mice (WT: 8.22 ± 0.40; KO: 6.23 ± 0.79; $t(8) = 2.048$, $p = 0.086$; **Figure 2.5B**) and was significantly decreased in the dorsal DG of Fmr1 KO mice (WT: 9.32 ± 0.49; KO: 6.62 ± 0.44; $t(8) = 4.519$, $p = 0.003$).
2.5 Cell proliferation in the dentate gyrus of Fmr1 KO mice.

Fmr1 KO mice did not appear to exhibit alterations in cell proliferation. (A) There was no significant difference in the density of cells staining for the endogenous cell proliferation marker Ki67 between genotypes in the dorsal or ventral dentate gyrus (DG). (C) Surprisingly, the density of cells expressing a second endogenous cell proliferation marker, PCNA, showed a significant decrease in the dorsal DG of Fmr1 KO mice. (E) In an attempt to resolve this discrepancy, cell proliferation was assessed with a new cohort of mice by administering a single injection of BrdU and sacrificing the mice 3 h later. The density of BrdU+ cells was carefully assessed across quartiles of the DG, and no significant differences were observed between the genotypes. Taken together, it appears the cell proliferation is not altered in Fmr1 KO mice. (B, D, F) Sample images of Ki67, PCNA and BrdU immunohistochemistry taken at 10× magnification. Arrows demarcate clusters of DAB-stained cells (scale bar = 200 μm).
Cell proliferation was also assessed with BrdU using a 3-h interval between injection and sacrifice. In addition, to more rigorously investigate cell proliferation, we collected and analyzed data from quartiles across the dorsal–ventral axis. We found no significant difference in BrdU+ cells across all quartiles ($F(1,24) = 0.432, p = 0.517$; **Figure 2.5C**). The bulk of data suggest that no significant difference exists in cell proliferation in the *Fmr1* KO mice relative to WT littermate mice.

### 2.3.5 Neurogenesis is altered in the ventral DG of *Fmr1* KO mice

The survival of new cells was assessed by injecting a saturating dose of BrdU (200 mg/kg) 4 weeks prior to the animals being sacrificed for immunohistochemistry. The density of BrdU-positive cells in the dorsal DG subregion was not significantly different between genotypes (WT: 625 ± 228; KO: 685 ± 129; $t(6) = 0.241, p = 0.816$; **Figure 2.6A**). There were significantly fewer BrdU-positive cells in the ventral DG of *Fmr1* KO relative to WT littermates (WT: 1098 ± 197; KO: 419 ± 158; $t(6) = 3.111, p = 0.021$). This effect was not a function of a difference in the volume of the DG for either the dorsal (WT: 0.0158 ± 0.0012 mm$^3$; KO: 0.0130 ± 0.0017 mm$^3$; $t(6) = 1.493, p = 0.179$; **Figure 2.6B**) or ventral (WT: 0.0184 ± 0.0042 mm$^3$; KO: 0.0194 ± 0.0029 mm$^3$; $t(6) = 0.222, p = 0.831$) subregions. These data indicate that less cells survive to maturity in the ventral DG of *Fmr1* KO mice. To minimize the possibility of sampling error skewing our results, we determined the location of the sampled reference volumes relative to bregma and found no significant differences between genotypes in the dorsal (WT: −2.082 ± 0.044; KO: −2.085 ± 0.12; $t(6) = 0.030, p = 0.977$) or ventral (WT: −2.533 ± 0.032; KO: −2.493 ± 0.026; $t(6) = 0.118, p = 0.306$) DG (Paxinos and...
Franklin, 2001). Thus, decreased cell survival was observed specifically in the ventral, and not dorsal, DG.

To examine neurogenesis, BrdU-positive cells were randomly sampled and assessed for co-expression of either neuronal (NeuN) or glial (S100β) markers yielding a relative rather than an absolute measure of cell differentiation. Surprisingly, we observed a significant increase in the proportion of BrdU+ cells that co-labeled with NeuN in the ventral DG of Fmr1 KO mice (WT: 35.2 ± 2.4% BrdU+ and NeuN+/BrdU+; KO: 51.9 ± 4.3% BrdU+ and NeuN+/BrdU+; \( t(4) = 4.088, p = 0.0095 \); Figure 2.6C). We did not observe a significant difference in neuronal differentiation in the dorsal DG between genotypes (WT: 50.2 ± 8.3% BrdU+ and NeuN+/BrdU+; KO: 47.9 ± 7.5% BrdU+ and NeuN+/BrdU+; \( t(4) = 0.206, p = 0.843 \)). There was not a concomitant decrease in BrdU+ cells co-labeling for the glial marker S100β. Interestingly, an estimation of the overall quantity of neurogenesis produced by multiplying the cell survival values by the proportion of BrdU+ cells co-labeling for NeuN+ did not yield a significant difference between genotypes (WT: 311.2 ± 34.39; KO: 278.62 ± 88.80, \( t(4) = 0.342, p = 0.749 \); Figures 2.6D and E). Although the quantity of neurogenesis does not appear abnormal, the process of cell survival and neurogenesis is clearly aberrant in the ventral DG of Fmr1 KO mice.
2.6 Neurogenesis in the DG of Fmr1 KO mice.

Fmr1 KO mice exhibit abnormal neurogenesis confined to the ventral dentate gyrus (DG). (A) Significantly fewer BrdU+ cells were observed in the ventral DG of Fmr1 KO mice when compared to WT mice. (B) There were no significant differences in reference volume between genotypes in the dorsal or ventral DG. (C) A larger percentage of the BrdU+ cells that survived to 4 weeks acquired a neuronal phenotype in the ventral DG of Fmr1 KO mice. (D) Estimated quantification of overall neurogenesis based on total number of BrdU+ cells multiplied by the proportion of BrdU+ cells that acquire a neuronal phenotype suggesting a lack of overall difference in the number of new neurons produced. Although overall numbers are estimated to be similar, the process of neurogenesis is abnormal in the ventral DG of Fmr1 KO mice. (E) An example of a three-dimensional, confocal image of a cell in the subgranular zone of the DG co-labeled for BrdU (green) and NeuN (blue) (Scale bar = 4 μm; *p < 0.05).
To further examine this issue, we used the endogenous marker doublecortin to determine if there was an overall difference in new cell numbers between the two genotypes. Similar to the results obtained using BrdU, there was no significant difference in the quantity of new neurons produced in the dorsal (WT: 6.798 ± 0.823; KO: 8.899 ± 1.703; \( t(8) = 1.241, p = 0.250 \); Figure 2.7) or ventral (WT: 7.925 ± 0.664; KO: 8.619 ± 1.322; \( t(8) = 0.525, p = 0.614 \)) DG.

**2.7 Immature neurons in the DG of Fmr1 KO mice.**

(A) *Fmr1* KO mice exhibit a similar number of new immature neurons in both the dorsal and ventral dentate gyrus (DG) as measured by immunohistochemistry for doublecortin (DCX). This supports the data presented in figure 2.6D, estimating no overall difference in the number of new neurons produced. (B) Sample images of DCX+ cells in the DG (scale bars = 200 µm on 4x image and 50 µm on 10x image).
2.4 Discussion

We have observed selective abnormalities in a unique form of plasticity, neurogenesis, in young adult *Fmr1* KO mice, an animal model for FXS. The results are intriguing considering that neurogenesis has been associated with both learning and anxiety in rodents (Sahay et al., 2007; Sahay and Hen, 2007; Santarelli et al., 2003; Wang et al., 2008), domains clearly impaired in patients with FXS (Jacquemont et al., 2007). Our results show that *Fmr1* KO mice (1) exhibit decreased anxiety on two separate behavioral assays (open-field and elevated plus maze) and (2) do not exhibit a learning impairment on acquisition or reversal learning in two separate forms of the MWM task. In addition, we show that *Fmr1* KO mice have a decreased corticosterone response to an acute stressor, providing physiological support for behavioral data suggesting decreased anxiety to open environments. In general, anxiety has been more closely associated with the ventral than the dorsal hippocampus, whereas spatial learning has been more closely associated with the dorsal than the ventral hippocampus (Bannerman et al., 2003; Bannerman et al., 2004; Bannerman et al., 1999; Degroot and Treit, 2004; Engin and Treit, 2007; Jung et al., 1994; Maurer et al., 2005; Moser et al., 1993). In support of this dissociation, we show that neurogenesis is qualitatively different in the ventral DG of *Fmr1* KO mice.

The Dutch-Belgian Consortium produced the *Fmr1* knockout mouse in 1994, accelerating our ability to investigate the neurobiology of the most common form of inherited human mental retardation (1994). Although initial studies suggested that mild learning and memory impairments could be observed in reversal learning in the classic MWM (D’Hooge et al., 1997), subsequent studies were unable to replicate this result, perhaps due to differences in background strain or pool size (Gantois et al., 2001; Paradee et al., 1999; Peier et al., 2000; Qin et al., 2002). Using a mouse-sized MWM pool, we were also unable to observe a deficit in spatial learning and
memory. Based on our observations in the open-field and elevated plus mazes, it is possible that, in the previous study by D’Hooge et al. (1997), that used the large MWM pool, the Fmr1 KO mice employed a search strategy traveling less near the walls and therefore missing the hidden platform, leading to the interpretation of impaired learning and memory. To test this, we assessed spatial learning and memory using the Plus-shaped water maze, where the mice are always near a wall, and found no significant deficit in the Fmr1 KO mice. Another possibility is that the larger maze is more difficult, and therefore resolves a difference more easily. Arguing against this hypothesis is our observation that KO mice were able to quickly learn the reversal phase of the Plus-shaped water maze. Clearly, a spatial learning deficit is at least not a robust phenotype of the Fmr1 knockout mice. In contrast to studies assessing spatial learning and memory, evidence is accumulating suggesting that emotional abnormalities may be a more prominent behavioral phenotype of the Fmr1 KO mouse model (McNaughton et al., 2008; Spencer et al., 2008). This is interesting since the neuropsychological symptoms in FXS (i.e., attention deficit hyperactivity, anxiety, repetitive behaviors) may be related to abnormalities in neural systems subservient to emotion (Jacquemont et al., 2007). Perhaps the most robust phenotypes of the Fmr1 KO mouse are decreased anxiety to open environments (Hayashi et al., 2007; McNaughton et al., 2008; Peier et al., 2000; Qin et al., 2002; Qin et al., 2005; Spencer et al., 2005; Spencer et al., 2006) and increased social anxiety (McNaughton et al., 2008; Mineur et al., 2006; Spencer et al., 2005; Spencer et al., 2008). Fmr1 KO mice have consistently shown an increase in the time spent in the centre of an open-field arena across several studies including the current one, with less consistent observations regarding hyperactivity. Here we support the view that a change in exploratory activity does not account for this effect with the observation that Fmr1 KO mice also show a marked decrease in defecations in the open field. It was important to
assess for possible differences in home cage defecations because a common complaint in FXS is bowel issues such as loose stools or constipation. We did not observe a significant difference in home cage defecations, arguing against a metabolic or gastrointestinal disturbance that could confound the interpretation of decreased anxiety in Fmr1 KO mice in the open field. Further, we report a significant increase in entries into the open arms of the elevated plus maze, a separate behavioral assay of anxiety, during the first minute of testing. Mineur et al. (2002) have also reported an increase into the open arms of the elevated plus maze (Mineur et al., 2002). However, these authors report an increase in overall arm entries, something we were not able to observe in our studies. Differences in hyperactivity, or conditions influencing hyperactivity, may account for these differences between the two studies. Although this abnormal decrease in anxiety may at first glance seem inconsistent with the phenotype of patients with FXS, it may in fact be related to abnormal responsiveness to stressful or arousing stimuli. In fact, it has been reported that Fmr1 KO mice exhibit impaired arousal regulation that may underlie attention deficits and inhibitory control (Moon et al., 2006). Interestingly, we found that Fmr1 KO mice have a decreased corticosterone response to 3 h of acute restraint stress, despite no differences in their basal corticosterone levels when compared to WT littermate controls. Similarly, it has been shown that immediately after 30 min of restraint stress Fmr1 KO mice exhibit a blunted corticosterone response and no differences in basal corticosterone levels (Markham et al., 2006). This relationship between genotype and corticosterone level reverses if the time between the end of the stressor and blood sampling is increased. Thus, male Fmr1 KO mice exhibit an abnormal responsiveness to stressful or arousing stimuli following exposure to environment stress (Hessl et al., 2002; Markham et al., 2006; Wisbeck et al., 2000).
The observation of a robust abnormality within the domain of anxiety, in the absence of an obvious deficit in spatial learning in Fmr1 KO mice, begged the question: What brain regions are correlated with anxiety and spatial learning, and how are they affected by loss of Fmr1 expression? A critical brain structure to both anxiety and spatial learning (as measured by the classic MWM in rodents) appears to be the hippocampus. However, the dorsal subregion of the hippocampus may preferentially subserve spatial learning whereas that ventral subregion may preferentially subserve anxiety (Bannerman et al., 2004). Neuroanatomical studies suggest that a neural system involving the lateral entorhinal cortex (EC) and dorsal DG may be preferentially involved in the processing of exteroceptive information important for spatial learning and memory. In contrast, a neural system involving the medial EC, ventral DG and other limbic structures may be preferentially involved in the processing of interoceptive information subserving emotion-related behaviors (Dolorfo and Amaral, 1998a). Behavioral support for this dissociation comes primarily from studies assessing changes in performance on tasks such as the MWM and elevated plus maze conducted before and after lesions or microinfusion of various agents across the dorsal–ventral axis of the hippocampus (Bannerman et al., 2004; Engin and Treit, 2007).

In the current study, we investigated neurogenesis, a form of neuronal plasticity that may impact learning and memory performance (Christie and Cameron, 2006; Gage, 2002). This phenomenon occurs in the DG, one of the first structures to receive cortical information that is then conveyed to the other subfields of the hippocampus (Andersen et al., 1969). Although the behavioral significance of neurogenesis is highly controversial, it appears that it may play a role in both learning and emotion (Balu and Lucki, 2009; Becker and Wojtowicz, 2007; Eisch et al., 2008; Gould et al., 1999a; Gould et al., 1999b; Kempermann and Kronenberg, 2003; Leuner et
al., 2006; Sahay and Hen, 2007). Most research on neurogenesis has not made the distinction between dorsal and ventral subregions, making this theoretical distinction largely speculative at present. Sahay et al. (2007) have recently reviewed this subject and highlight the fact that SSRIs (selective serotonin reuptake inhibitors) may preferentially increase neurogenesis in the ventral DG (Sahay et al., 2007; Sahay and Hen, 2007). It is interesting that these pharmaceutical agents, commonly employed to alleviate both anxiety and depression, would have a greater impact on neurogenesis in an area of the hippocampus that seems altered in the Fmr1 KO animals.

Neurogenesis is a complex process that can be affected by alterations in a number of phases such as size of the pool of progenitor cells, proliferative activity, neuronal differentiation, cellular survival and neuronal integration (Kempermann et al., 2004). Although neurogenesis has not been studied in the adult mammalian hippocampus of patients with FXS or Fmr1 KO mice prior to this study, data from in vitro studies of neural stem cells from both Fmr1 KO mice and a FXS fetus suggest that neuronal differentiation may be three- to five-fold higher in cells harboring the Fmr1 mutation (Castren, 2006; Castren et al., 2005). This is consistent with our observation of an increase in neuronal differentiation in the DG of young, adult Fmr1 KO mice. These authors also observed an increase in the number of newborn cells in the subventricular zone of the embryo, whereas we have observed a decrease in the number of newborn cells in the DG. A number of differences could account for this effect including brain region, developmental stage of the animal and delay between BrdU injection and sacrifice.

The endpoint of neurogenesis can be estimated by multiplying the density of BrdU+ cells by the proportion of BrdU+ cells that acquire a neuronal phenotype. This type of analysis suggests that there is no significant difference in the overall number of new neurons added to the pre-
existing cytoarchitecture. Because this is only an estimate, overall neurogenesis was assessed more directly by immunostaining for a protein expressed only in immature neurons in the DG, doublecortin. Consistent with the previous estimates, there was no significant difference in the number of new neurons added to the DG. It appears that in the ventral DG, there may be a homeostatic process occurring that attempts to maintain the number of new neurons in the DG. Importantly, however, the process of neurogenesis is clearly abnormal in the ventral DG. The functional capacity of the new neurons across the dorsal–ventral axis of the DG should be assessed using whole-cell electrophysiology experiments in future studies.

We hypothesized that a decrease in proliferative activity could account for the decrease in the number of 4-week-old BrdU+ cells observed in the ventral DG of Fmr1 KO mice. We did not observe a difference in the number of Ki67+ cells, suggesting that a decrease in cell proliferation does not account for this difference and that attrition in cell numbers must be higher in the ventral DG of Fmr1 KO mice. Surprisingly, we observed a significant decrease in the density of cells expressing another endogenous marker of cell proliferation, PCNA, in the dorsal DG and a trend towards a decrease in the ventral DG. This finding is inconsistent with the majority of our results. To rigorously assess cell proliferation, we conducted an experiment where mice were sacrificed 3 h following an injection of BrdU. The tissue was obtained and analyzed for each quartile across the dorsal–ventral axis. This experiment also more directly assessed possible differences in BrdU bioavailability that could potentially confound the cell survival and differentiation results. We did not observe significant differences in BrdU+ cell densities across the dorsal–ventral axis providing strong support for a lack of an effect on cell proliferation in the Fmr1 KO mice, arguing against any issues associated with BrdU bioavailability and suggesting that the decreased BrdU+ cell densities (observed at 4 weeks post-BrdU injection) in the ventral
DG is indeed a function of attrition. As all methods to assess neurogenesis harbor their own confounds, the use of biomarkers such as PCNA to assess neurogenesis may be confounded by an effect of loss of FMRP on the expression of PCNA (Bhattacharyya et al., 2008; Brown et al., 2001; D'Agata et al., 2002). Future studies should investigate a possible relationship between FMRP and PCNA in the dentate gyrus. In addition, PCNA may be labeling cells that are undergoing DNA repair or even cells that have left the cell cycle. In contrast, Ki67 appears to more specifically mark cells in all phases of the cell cycle with the exception of Go (Taupin, 2007).

The data presented in these experiments indicate that the process of neurogenesis is abnormal in the ventral hippocampus of Fmr1 KO mice. We have observed a decrease in the number of cells that survive to 4 weeks post-mitosis and a concomitant increase in the percentage of remaining cells that acquire a neuronal phenotype. These effects were confined to the ventral hippocampus, an area more closely associated with anxiety than spatial learning, paralleling our behavioral observation in Fmr1 KO mice. These results illuminate a novel abnormality induced in the mammalian brain by loss of expression of the Fmr1 gene which may manifest as alterations in anxiety and anxiety-related behaviors in FXS.
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3. Reduced NMDA–dependent Synaptic Plasticity and Decreased Dendritic Complexity in the Dentate Gyrus of Fmr1 Knockout Mice is Associated with Impaired Context Discrimination\(^2\)

3.1 Introduction

Fragile-X syndrome (FXS) is associated with significant morbidity affecting approximately 1/4000 males and 1/8000 females (Turner et al., 1996). Despite our growing knowledge and characterization of FXS as the most common form of inherited intellectual disability caused by loss of expression of the gene \(Fmr1\), how loss of \(Fmr1\) expression affects neuronal circuits causing intellectual impairment remains unclear. Tremendous hope for clarity into this issue was associated with the generation of the mouse model of FXS by the Dutch-Belgian Fragile-X Consortium (\(Fmr1\) KO mice) (1994). Unfortunately, a robust learning impairment in the mouse model of FXS has been elusive, significantly limiting our understanding of the pathophysiology of FXS and the neurobiological basis of intellectual impairment in general.

Initial studies investigating learning impairment in \(Fmr1\) KO mice employed the classic Morris water maze (MWM) (1994; D'Hooge et al., 1997). Surprisingly, \(Fmr1\) KO mice are not impaired on the acquisition of this task. Perhaps consistent with this finding is the observation of normal NMDA-dependent long-term potentiation and depression (LTP and LTD, respectively) in the CA1 subfield of the hippocampus of \(Fmr1\) KO mice (Godfraind et al., 1996; Huber et al., 2002; Larson et al., 2005). These observations beg the following important questions: Are we

\(^2\) A version of this chapter will be submitted for publication. Eadie B.D., Cushman J., Zhang W.N., Kannangara T.S., Shin J.D., Fanselow M.S., Christie B.R.. (2010) Reduced NMDA-dependent Synaptic Plasticity and Decreased Dendritic Complexity in the Dentate Gyrus of \(Fmr1\) Knockout Mice is Associated with Impaired Context Discrimination.
investigating intellectual impairment appropriately in Fmr1 KO mice? Are we focusing our neurobiological investigations on the correct brain region?

The dentate gyrus (DG) subfield of the hippocampus possesses the relatively unique ability to perpetually add new neurons that may play a particularly important role in learning and memory (Kee et al., 2007). We have recently shown that the process of neurogenesis in the DG is abnormal in young, adult Fmr1 KO mice (Eadie et al., 2009) and hypothesize that young neurons in the DG are structurally and functionally abnormal as a consequence. The mouse model of FXS has consistently reflected the human condition in one important manner: an increase in the density and length of dendritic spines evident in the adult neocortex (Comery et al., 1997; Irwin et al., 2001). We hypothesized that young neurons in the DG of Fmr1 KO mice possess similar aberrancies in dendritic spines. Emerging evidence indicates that newly generated neurons in the DG disproportionately contribute to the synaptic plasticity in this brain region (Ge et al., 2008), perhaps due to more immature-appearing dendritic spines (Ge et al., 2008; Zhao et al., 2006). Therefore, we further hypothesized that Fmr1 KO mice exhibit impairments in NMDA-dependent, long-term synaptic plasticity in the DG.

If young neurons in the DG are preferentially affected by loss of Fmr1 expression, then why are Fmr1 KO mice not impaired on learning the classic hippocampal-dependent MWM? It has recently become apparent that the different subfields of the hippocampus may be associated with distinct aspects of learning (Lisman, 2003) and it appears that functional NMDA receptors in the DG are required for context discrimination learning but not normal performance on the MWM (McHugh et al., 2007). These results led us to hypothesize that Fmr1 KO mice are impaired in learning a context discrimination task.
3.2 Materials and Methods

3.2.1 Animals

C57BL/6, male Fmr1 knockout (KO) \((n = 42)\) and Fmr1 wild-type (WT) \((n = 44)\) littermate mice were used in these experiments. The animals were produced by breeding WT male C57BL/6 (Jackson Laboratories) mice with female C57BL/6 mice heterozygous for the Fmr1 gene (founded by Fmr1 KO mice provided by Dr. M. Bear). The C57BL/6 background strain was employed because they show relatively high levels of synaptic plasticity and performance on spatial learning and memory tasks (Nguyen et al., 2000a; Nguyen et al., 2000b; Holmes et al., 2002). All animals were sexed, weaned, ear-punched and tail-snipped at post-natal day 24 and group-housed with minimal enrichment (tubes and/or nesting materials). Adult male mice \((3-5\) months) were randomly assigned to behavioral, histological or electrophysiological experiments (Figure 3.1) following genotyping. Separate cohorts of mice were used as experience in behavioral tasks may alter the histological and electrophysiological outcomes measured (Eadie et al., 2005; Restivo et al., 2005). Only male mice were used in these experiments due to significant sex differences in the phenotype of FXS and the higher prevalence of FXS in males than females (Jacquemont et al., 2007). All efforts were made to minimize pain and discomfort for all animals. Experiments were carried out in accordance with international standards on animal welfare and guidelines set out by the Canadian Council on Animal Care, the University of British Columbia, and the University of Victoria (Appendix B).
3.1 Timeline of experimental procedures.

Outline of experimental procedures. Male Fmr1 knockout (KO) or wild-type (WT) littermate mice were generated, weaned and ear-punched at postnatal day 24. Mice were pseudo-randomly assigned to behavioral experiments aimed at assessing behaviors dependent on the dentate gyrus, histology experiments aimed at assessing the morphology of dendrites and dendritic spines of dentate granule neurons, or electrophysiology experiments aimed at assessing synaptic plasticity in the molecular layer of the dentate gyrus.

3.2.2 Genotyping

3.2.2.1 DNA Extraction and Purification

DNA extraction and purification was performed on ear or tail tissue, that was stored at -20°C, using PureLink Genomic DNA Purification Kit (Invitrogen). Briefly, tissue from each animal was placed in 180 µl digestion buffer and 20 µl Proteinase K in a DNAse/RNase-free 1.5 ml Eppendorf tube and incubated overnight in a thermomixer at 55°C while being agitated at 300 RPM. The sample was centrifuged at 21,000 RCF for 3 minutes and the supernatant was transferred into a new tube. 20 µl RNase A was then added, vortexed and incubated at room temperature for 2 minutes. 200 µl lysis buffer was then added to each tube and vortexed. 200 µl 100% EtOH was then added to each tube and vortexed. The lysate was transferred to a clean spin column and centrifuged at 9300 RCF for 1 minute at room temperature. The spin column was then placed in a fresh tub and washed by adding 500 µl Wash Buffer I and centrifuging at
9300 RCF for 1 min at room temperature. This process was repeated with 500 µl Wash Buffer II and centrifuged at 21,000 RCF for 3 minutes at room temperature. 100 µl Elution Buffer was added to the spin column and incubated for 1 minute followed by centrifugation at 21,000 RCF for 1 minute at room temperature. Collection tubes were either stored at -20°C or used directly in PCR.

3.2.2.2 PCR

The PCR reaction was performed by mixing 11 µl nuclease-free H₂O, 2.5 µl 10X PCR Reaction Buffer, 2.5 µl (50 mM) MgCl₂, 2.0 µl (2.5 mM) dNTP, 1.25 µl (1 µM) 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: first cycle of 5 minutes at 94°C, then 35 cycles of 60 s 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 KO allele (amplified fragments of 800 base pairs). Primers S1 = 5′GTGGTTAGCTAAAGTGAGGATGAT 3′ and S2 = 5′ CAGGTTTGTGGGGATTAACAGATC 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 10,000x SYBR-safe (1:13,333 in 1x TAE) and visualized under a BioRad Gel-Doc trans-illuminator (BioRad, Ont., Canada).

3.2.3 Contextual fear behavior

A cohort of mice (WT: n = 14, KO: n = 14) was assessed in a contextual fear discrimination task as previously described (McHugh et al., 2007). Mice were trained to discriminate between two contexts through repeated experience in both contexts. Context A consisted of four identical
conditioning chambers (30 X 25 X 25 cm; Med-Associates Inc.) placed in a sound-attenuating cubicle with a 60 dB background noise provided by a fan. An overlay of two white plastic panels created a continuous curve of side and back walls. The floor of each chamber consisted of 16 stainless steel rods of alternating diameter (0.4 and 1.0 cm) spaced 1.5 cm apart (center to center) wired to a shock generator and scrambler (Med-Associates Inc.) to deliver foot shock. Each chamber was wiped down with 70% ethanol before conditioning and between subjects. A metal pan containing a thin film of Windex was placed underneath the grid floors providing an olfactory component to context A. Context B consisted of four identical conditioning chambers (30 X 25 X 25 cm; Med-Associates Inc.) placed in a sound-attenuating cubicle in a different room from Context A with a fan providing a background noise of 60 dB. The grid floors were the same as those used in A to increase the similarity of the contexts. The side walls consisted of two black plastic panels joined at the top and sloping down to the side of the conditioning chamber to form an A-frame. A metal pan containing a thin film of Simple Green was placed underneath the grid floors providing a distinct olfactory component to context B. On each conditioning day, animals were brought to a holding room in their home cages and left undisturbed for a minimum of 30 minutes. Animals were transported from the holding room to the conditioning chambers in their home cage.

Animals received context conditioning on Days 1 through 4, during which time animals were placed in Context A (referred to as S+) and allowed to explore for 3 minutes. After the exploration period, a 2 sec 650 μA shock was presented and the animals were removed 1 minute later. On Day 5, animals were placed in Context B (referred to as S- because shock was never administered in this context) for a 3 minute generalization test. Two hours later, animals were placed in Context A for a 3 minute test (no shock was administered during this test). Day 6
followed the same procedure as day 5 but in reverse order. Days 7 through 14 consisted of discrimination training where animals were placed in both contexts on each day and Context A was again paired with shock, but Context B was not. Animals were placed in each context for a total of 4 minutes and 2 seconds. In Context A, animals received a 2 sec, 650 µA shock after 3 minutes and were left in the chamber for 1 minute following shock. In Context B, animals were placed in the chamber for an equivalent 4 minutes and 2 seconds. Time spent freezing was measured during the 3 minutes preceding shock on all days in which shock was administered and the equivalent period of time in Context B during discrimination training. The order of training followed a double alternation schedule: Day 7 S→S+, Day 8 S+→S-, Day 9 S+→S-, Day 10 S-→S+, etc. For statistical analysis and graphical presentation the data were collapsed into consecutive two-day blocks so that each block consisted of one day of S+ → S- and one day of S- → S+.

### 3.2.4 Histology

#### 3.2.4.1 Modified Golgi-Cox Protocol

A second cohort of mice (WT: n = 10, KO: n = 14) was administered a lethal injection of sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Cambridge, Ont., Canada) and perfused transcardially with 0.9% saline (60 ml). The brains were then removed, placed in vials containing 40 ml of modified Golgi-Cox solution (Gibb and Kolb, 1998), and stored in the dark for 14 days as previously described (Eadie et al., 2005). After this, the brains were switched to a 20% sucrose solution and stored in the dark for 4 days, before being blocked, sectioned (coronal) at 200 µm and mounted on 2% gelatinized slides. Sections were then processed in the following
solutions: dH2O (1 minute), ammonium hydroxide (30 minutes), dH2O (1 minute), Kodafix for film (30 minutes), dH2O (1 minute), 50% ethanol (1 minute), 70% ethanol (1 minute), 95% ethanol (1 minute), 100% ethanol (2 x 5 min), equal parts 100% ethanol/HemoDe/chloroform (10 min) and then HemoDe (2 x 15 min). After processing, all slides were cover-slipped using Permount (Fisher) and stored in a cool, dry place in the dark.

3.2.4.2 Dendritic Complexity

The dendritic complexity analysis was conducted in a manner similar as previously described (Redila and Christie, 2006). Briefly, from each brain, golgi-impregnated granule cells in the DG were selected for dendritic analyses. The location of granule cells was classified according to where the soma was located within the granule cell layer (GCL). A granule cell was classified as belonging to the subgranular zone (SGZ) if the soma was located within 30 μm of the innermost edge of the GCL (approximately the width of three granule cell bodies). The GCL itself was divided into two equal parts, the inner granule zone (IGZ) and the outer granule zone (OGZ). A cell with its soma located in the inner half of the granule cell layer was classified as belonging to the IGZ; whereas, a cell with its soma located in the outer half of the granule cell layer was classified as belonging to the OGZ. Granule cells whose soma was intersected by the midline of the GCL were not included in analyses. Only granule cells that met the following criteria were included in analyses: 1) the cells were entirely filled and there was dark and consistent impregnation throughout the extent of the dendrites; 2) cells were relatively isolated from neighboring impregnated cells, with minimal overlap of filled processes; and 3) the dendrites were completely visible, with no cut processes, and extended for the breadth of the dentate molecular layer to the hippocampal fissure while laying in the plane of the section. To quantify dendritic complexity, we performed a Sholl analysis (Sholl, 1956). For this analysis, each
neuron was traced using an upright microscope at 40x magnification with an attached drawing tube set at 10x zoom (Nikon Y-IDT). A series of concentric rings, spaced 20 μm apart, was placed over the neuron, centered on the cell body, and the number of bifurcations as a function of distance was recorded. The total dendritic length of each granule cell was also measured using a digital imaging computer program (Image-Pro Plus 5.0; MediaCybernetics, MD, USA). All analyses were conducted with the experimenter blind to the subject’s identity.

3.2.4.3 Spine Density and Type

For analyses of spine density, the number of spines per 10 μm was obtained for at least three segments per cell from primary, secondary or tertiary dendrites, and at least five cells per animal were analyzed. Each spine within this segment was categorized into filopodia (long, thin protrusion without an obvious head), thin (long, thin protrusion with a head), mushroom (relatively short protrusion with a prominent head compared to the neck), stubby (relatively short protrusion without a clear head), bifid (protrusion that branches into two distinguishable protrusions) or double (protrusion with two heads in series) types. Dendritic spines were analyzed by capturing digital images using an Olympus BX51WI suited with a 100x magnification objective and a Cool Snap-HQ CCD camera connected to a PC running ImagePro Plus 5.0 software (MediaCybernetics, MD, USA). Measurements were not made close to the terminal ends of dendrites. All analyses were conducted with the experimenter blind to the subject’s identity.
3.2.5 Electrophysiology

3.2.5.1 Slice Preparation

Individual mice from a third cohort of mice (WT: n = 18, KO: n = 16) were briefly anesthetized with isoflurane, immediately decapitated and brains removed directly into oxygenated (95% O2/5% CO2), ice-cold normal ACSF (nACSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 NaHPO4, 25 NaHCO3, 2 CaCl2, 1.3 MgCl2, and 10 dextrose, pH 7.3. Following removal of the brain, the cerebrum was longitudinally hemi-sectioned and blocked for sectioning. Transverse hippocampal slices (350 μm) were obtained in oxygenated nACSF maintained at 4°C with a cooled Vibratome 1500 (Ted Pella, Inc., Redding, CA, USA). Sections were kept in order using a custom modified 24-well plate and incubated in continuously oxygenated, nACSF at 30°C. Sections were allowed to rest for a minimum of 1 hour before recordings commenced.

3.2.5.2 Electrophysiology Recordings

Recordings were obtained in oxygenated nACSF with 5 μM bicuculline methiodide (Sigma-Aldrich, Oakville, ON, Canada) to elicit robust synaptic plasticity and isolate the excitatory contribution to synaptic plasticity. Sections were perfused at a rate of approximately 2ml / minute. Medial perforant path evoked field EPSPs (fEPSPs) were obtained using glass recording electrodes (0.5-1.5 MΩ) filled with nACSF and concentric bipolar stimulating electrodes (FHC, Bowdoin, ME, USA). The slope from a single trace was calculated from the initial slope of the fEPSP relative to the slope of the 10 ms interval immediately preceding afferent stimulation. The current magnitude (10-50 μA) was set to elicit a fEPSP approximately 30-50% of maximum for synaptic potentiation experiments and 50-70% for synaptic depression experiments. A stable
baseline (minimum 20 minutes) was obtained by delivering single pulse stimulation at 15 sec
inter-stimulus intervals.

3.2.5.3 Conditioning Stimulation Protocols

Long-term potentiation (LTP) of fEPSPs was induced using a conditioning stimulus (CS)
consisting of 4 trains of 50 pulses at 100 Hz, 30 s apart (high-frequency stimulation; HFS) and
defined as a significant increase in the absolute initial slope of the fEPSP 50-60 minutes
following the CS (“POST”) relative to the average of the absolute initial slope obtained prior to
HFS (“PRE”). Long-term depression (LTD) of fEPSPs was induced by administering 900 pulses
of low-frequency stimulation (LFS; 1 Hz) over 15 min and defined as a significant decrease in
the absolute initial slope of the fEPSP 50-60 minutes following the CS (“POST”) relative to the
average of the absolute initial slope obtained prior to LFS (“PRE”).

3.2.5.4 Data Analyses

Evoked fEPSP responses were digitized and the initial slope of the fEPSP was analyzed as
described previously (Vasuta et al., 2007) using pClamp 10 software (Molecular Devices, Union
City, CA) (the initial slope of the EPSP was calculated relative to the baseline slope). All data
are represented as percentage change from the initial average baseline fEPSP slope, which was
defined as the average slope obtained for the 20 minutes prior to conditioning stimuli being
applied. Percentage potentiation or depression was calculated as follows: (average fEPSP 50 to
60 minutes post-CS) / (average fEPSP between -10 to 0 minutes pre-CS) x 100, or “POST” / ”PRE” x 100. A single recording was considered a single sample due to the relatively high
variability between recordings. For all groups, a minimum of 4 individuals from each genotype
contributed at least one recording.
3.2.6 Statistical methods

Differences are presented as mean ± standard error of the mean (SEM). Differences between mean values of experimental groups were compared using Student’s *t*-test or analysis of variance (ANOVA) followed by Tukey’s post-hoc tests as appropriate using Statistica 7.0 software (StatSoft, Tulsa, OK, USA) and Graph Pad Prism. The differences were considered statistically significant when *p* < 0.05.
3.3 Results

3.3.1 Acquisition of contextual fear is normal in Fmr1 KO mice

The acquisition of the context-shock association in Fmr1 KO and WT mice was measured over 4 training days (Figure 3.2A). As shown in Figure 3.2D, Fmr1 KO mice showed normal acquisition of contextual fear. Repeated measures ANOVA across training days showed no overall effect of genotype (F(1,26) = 2.87, p = 0.102) but the effect of training day (F(3,78) = 16.38, p = 0.000) and the genotype by training day interaction were not significant (F(3,78) = 2.09, p = 0.108). Overall, Fmr1 KO mice appear to show normal acquisition of contextual fear.

3.2 Contextual fear acquisition, generalization and extinction in Fmr1 KO mice.

Acquisition, generalization and extinction of contextual fear in Fmr1 KO mice. (A) Experimental design for the assessment of contextual fear acquisition. (B) Experimental design for the generalization test in shock-paired (S+) and novel context (S-) collapsed over two days of testing. (C) Outline for the assessment of extinction of conditional fear to the S+ context across two days of generalization testing and prior to shock on the first day of discrimination training. No shock was presented during these tests. (D) Percent freezing during the 3 minutes prior to shock onset across 4 days of acquisition training. (E) Percent freezing during generalization testing. (F) Percent freezing in S+ without shock prior to discrimination training. WT mice show extinction of contextual fear over this time, whereas Fmr1 KO mice do not. (* denotes P < 0.05 between Fmr1 KO and WT mice.)
3.3.2 Generalization of contextual fear is normal in *Fmr1* KO mice

Conditional fear responses will occur not only in response to the original conditional stimuli but also to stimuli which show similar or overlapping features, a process referred to as generalization. The extent to which *Fmr1* KO and WT mice generalize to a context that was similar, but distinct from, the original training context was tested over a two day period (Figure 3.2B). Freezing data from S+ and S- over the two generalization testing days were averaged together as shown in Figure 3.2E. Two-way ANOVA indicated a significant effect of context ($F_{(1,26)} = 182.4, p < 0.001$) but the effect of genotype ($F_{(1,26)} = 2.866, p = 0.102$) and the genotype by context interaction ($F_{(1,26)} = 1.298, p = 0.265$) did not reach statistical significance. This indicates that *Fmr1* KO mice show normal levels of freezing in the S+ context and equivalent generalization of contextual fear to the S- context.

3.3.3 Impaired extinction of contextual fear in *Fmr1* KO mice

No shocks were given during the generalization testing and therefore some level of extinction was expected. On the first day of discrimination training (Day 7) freezing was measured in S+ prior to shock onset. Therefore a decrease in freezing in S+ over days 5, 6 and 7 represented a measure of extinction (Figure 3.2C). As shown in Figure 3.2F, WT mice showed clear evidence of extinction across these three days, whereas *Fmr1* KO mice did not. Repeated measures ANOVA across days 6 and 7 showed significant main effects of genotype ($F_{(1,26)} = 5.79, p = 0.0235$) and day ($F_{(1,26)} = 9.60, p = 0.0046$) as well as a genotype by day interaction ($F_{(1,26)} = 5.90, p = 0.0224$). Bonferroni post-hoc comparisons indicated that *Fmr1* KO mice show significantly enhanced freezing on Day 7 ($t_{(28)} = 3.205, p < 0.01$) but not on day 6 relative to WT mice. These results suggest that *Fmr1* KO mice possess a deficit in fear extinction.
3.3.4 Impaired context discrimination in Fmr1 KO mice

The ability to of Fmr1 KO and WT mice to discriminate between the S+ context that is paired with shock and the S- context that is never paired with shock was assessed over eight days of discrimination training. Learning this discrimination is thought to require a process of “pattern separation” where the pattern of multi-modal stimuli that defines the S+ context is separated from the pattern of stimuli that define the S- context. This information can then be utilized to determine which context is “safe” and which context is “dangerous.” Results from the discrimination training phase were collapsed into two day blocks, as shown in Figure 3.3A. Repeated measures ANOVA for freezing in the S+ context showed a significant effect of Training Block (F(3,78) = 16.38, p < 0.0001) but did not show a Training Block by Genotype interaction (F(3,78) = 2.09, p = 0.108) or an overall effect of Genotype (F(1,26) = 2.87, p = 0.102). This indicates that Fmr1 KO and WT mice freeze to a similar extent in the S+ context. Repeated measures ANOVA for freezing in the S- context showed a significant effect of Training Block (F(3,78) = 28.42, p < 0.0001) and an overall effect of Genotype (F(1,26) = 10.52, p = 0.003), but did not show a Training Block by Genotype interaction (F(3,78) = 0.444, p = 0.722). This analysis indicates that Fmr1 KO mice show elevated freezing in the S- context (Figure 3.3B). A discrimination ratio was calculated for each individual animal for each training block using the following formula: (Freezing in S+) / ((Freezing in S+) + (Freezing in S-)). Discrimination ratios above 0.5 are indicative of significant discrimination. Repeated measures ANOVA on the discrimination ratios indicated a significant effect of Training Block (F(3,78) = 28.42, p < 0.0001) and a significant effect of Genotype (F(1,26) = 5.02, p = 0.034) but no Training Block by Genotype interaction (F(3,78) = .71, p = 0.549). This analysis indicates that Fmr1 KO mice show a significantly reduced discrimination ratio relative to WT mice (Figure 3.3C). Overall, the data from discrimination training indicates that Fmr1 KO mice exhibit an impairment in contextual
This deficit primarily manifests as a reduced ability to decrease freezing in the S- context and may therefore be related to the extinction deficit seen during generalization testing (see Figure 3.2F).

**3.3 Context discrimination in Fmr1 KO mice.**

(A) Experimental design for discrimination training. Data was collapsed over each consecutive two day period into Trial Blocks 1-4 for the S+ and S- contexts. (B) Percent freezing in the 3 minutes prior to shock in the S+ context and in the equivalent 3 minute period in the S- context across discrimination training. Fmr1 KO mice show a persistent elevation of freezing in the S- context relative to WT mice. (C) Discrimination ratios, calculated as (Percent freezing in S+)/((Percent freezing in S+) + (Percent freezing in S-)) across discrimination training. Fmr1 KO mice show reduced discrimination ratios relative to WT mice. (* denotes P < 0.05 between Fmr1 KO and WT mice.)
3.3.5 Young neurons in 

Analyses of dendritic morphology of dentate granule neurons revealed that neurons located in the inner granular zone (IGZ) of Fmr1 KO mice possess less complex dendritic arborization and decreased total dendritic length compared to WT controls (Figure 3.4B). Specifically, a Sholl analysis revealed that Fmr1 KO mice possess IGZ neurons with significantly less complex dendritic arbors ($F_{(1,54)} = 6.126, p = 0.0165$; Figure 3.4D). Fewer crossings between dendrites and rings 1 (WT: $1.38 \pm 0.10$, KO: $1.14 \pm 0.05$), 5 (WT: $5.91 \pm 0.34$, KO: $4.93 \pm 0.34$), 8 (WT: $5.85 \pm 0.43$, KO: $4.67 \pm 0.32$), 9 (WT: $5.41 \pm 0.42$, KO: $4.36 \pm 0.31$) and 12 (WT: $1.56 \pm 0.31$, KO: $2.38 \pm 0.26$) were observed. No differences in dendritic complexity were observed in the SGZ ($F_{(1,42)} = 0.430$, $p = 0.515$) or OGZ between genotypes ($F_{(1,82)} = 0.000$, $p = 1.000$) (Figures 3.4C & 3.4E). It appears that young neurons in the granule cell layer possess the most compromised dendritic structure. In support of this assertion, there was also a significant decrease in the total dendritic length of IGZ neurons in the Fmr1 KO mice (WT: $1031.82 \pm 54.61$, KO: $866.62 \pm 41.93$; $t_{(74)} = 3.390, p = 0.019$) (Figure 3.4G). There were no significant differences in total dendritic length in the SGZ (WT: $638.04 \pm 49.81$, KO: $586.70 \pm 46.43$; $t_{(42)} = 0.728, p = 0.471$) or OGZ (WT: $1077.43 \pm 52.28$, KO: $1069.13 \pm 35.61$; $t_{(82)} = 0.130$, $p = 0.019$) (Figures 3.4F & 3.4H). Considering that the development of neurons in the dentate gyrus is associated with a migration of cell bodies from the SGZ to the OGZ, it appears that it is the relatively young dentate granule neurons (cell bodies in the IGZ) in the DG of Fmr1 KO mice that are significantly less complex compared to controls.
3.4 Dendritic morphology of granule neurons in the DG of Fmr1 KO mice.

The complexity and total dendritic length of dendrites on young dentate granule neurons are decreased in tissue obtained from Fmr1 KO. (A) Example image of a golgi-impregnated neuron in the dentate gyrus (DG) under (20x magnification; scale bar = 20 μm; large, dashed black line demarcates fissure and small, dashed red lines demarcate the granule cell layer). New neurons are born in the SGZ of the DG and migrate into the IGZ of the granule cell layer and into the OGZ as they mature. Note the occasional overlapping dendritic branch from a separate neuron. (B) Examples of traced golgi-impregnated neurons from the IGZ are shown representing the decreased dendritic complexity observed in Fmr1 KO mice compared to controls. (C-E) Sholl analysis for SGZ, IGZ, and OGZ neurons from WT and Fmr1 KO mice showing significant reductions in dendritic complexity in IGZ neurons in tissue obtained from Fmr1 KO mice. (F-H) Total dendritic length was obtained for neurons in the SGZ, IGZ and OGZ. Total dendritic length was reduced specifically on neurons with cell bodies located in the IGZ. (* denotes p < 0.05)
3.3.6 Young neurons in *Fmr1* KO mice show decreased dendritic spine density

There was a significant decrease in the density of dendritic spines in young neurons in the *Fmr1* KO animals. This occurred specifically on dentate granule neurons with their cell bodies located in the IGZ (WT: 8.32 ±1.13, KO: 5.66 ±0.23; t(12) = 3.315, p = 0.006; Figure 3.5A). The density of spines was not significantly different on more mature DG neurons with their cell bodies located in the OGZ (WT: 12.05 ±2.98, KO: 8.97 ±0.35; t(12) = 1.559, p = 0.145; Figure 3.5B). It is noteworthy, however, that within the IGZ of WT and *Fmr1* KO mice, there was not a significant difference in the types of spines we observed. Both filopodia/thin spines (WT: 20.85 ±6.21, KO: 17.06 ±3.7; t(12) = 0.601, p = 0.559) and mushroom/stubby spines (WT: 78.70 ±6.05, KO: 82.36 ±3.70; t(12) = 0.593, p = 0.564) were equivalently represented in the IGZ of KO and WT mice (Figure 3.5C). Similarly, there were no differences in the number of filopodia/thin spines (WT: 18.28 ±6.45, KO: 17.94 ±2.81; t(12) = 0.062, p = 0.952) and mushroom/stubby spines (WT: 81.23 ±6.96, KO: 80.46 ±3.04; t(12) = 0.126, p = 0.900) in the OGZ of KO and WT mice (Figure 3.5D).

Based on previous research emphasizing an increase in long, thin dendritic spines and filopodia in *Fmr1* KO mice, we hypothesized that there may be an increase in the proportion of filopodia spines on neurons with cell bodies located in the OGZ of *Fmr1* KO mice attempting to re-populate the dendrites with new sites for synaptic transmission. Indeed, there was an approximately 3-fold increase in the percentage of dendritic spines with a filopodia morphology in tissue obtained from *Fmr1* KO mice (WT: 1.47 ±0.77, KO: 6.82 ±1.65; t(12) = 2.451, p = 0.031). In short, it appears that the main spine abnormality in the dentate gyrus of *Fmr1* KO mice is a decreased spine density that primarily affects younger neurons located in the IGZ. This
may be compensated for by an increase in filopodia-like dendritic spines in older neurons with their cell bodies in the OGZ.

3.5 Dendritic spines on granule neurons in the DG of Fmr1 KO mice.

The density of dendritic spines on young dentate granule neurons is decreased in tissue obtained from Fmr1 KO mice. (A & B) Spine density, as measured by counting spines per 10 μm segment of dendrite, revealed a significant reduction in tissue obtained from Fmr1 KO mice on neurons with their cell bodies located in the IGZ, but not on neurons with their cell bodies located in the OGZ. (D&E) No significant differences in spine types were apparent. (* denotes p < 0.05)

3.3.7 Long-term potentiation is impaired in the DG of Fmr1 KO mice

The majority of fast synaptic glutamatergic transmission in the brain occurs at dendritic spines on principal neurons. The decrease in the complexity of dendrites and the density and
type of dendritic spines suggested abnormalities in the efficacy of synaptic transmission. Long-term potentiation (LTP) was assessed in the middle molecular layer of the DG by comparing the slope of the field excitatory postsynaptic potential (fEPSP) before (“PRE”) and after (“POST”) applying high-frequency stimulation (HFS) to the medial perforant path input (Figures 3.6A and 3.6B). WT mice showed significant LTP as indicated by an increase in the average absolute slope of the fEPSP pre-HFS to post-HFS ($t_{(28)}$, $p < 0.000$). LTP was significantly attenuated in the $Fmrl$ KO mice (WT: 73.01 ±8.64, KO: 44.27 ±7.51; $t_{(28)} = 7.564$, $p = 0.034$). This LTP was NMDA receptor dependent in both genotypes, as inclusion of the NMDA-receptor antagonist APV (50 µM) severely attenuated LTP in both WT and $Fmrl$ KO slices (WT: 8.17 ±3.75; KO: 3.79 ±6.98). There was no significant difference in LTP between genotypes with the application of APV ($t_{(22)} = 0.500$, $p = 0.622$) (Figure 3.6C).
3.6 NMDA-dependent LTP in the DG of Fmr1 KO mice.

NMDA-dependent long-term potentiation (LTP) is significantly attenuated in the dentate gyrus of Fmr1 KO mice. (A & B) Field excitatory postsynaptic potentials (fEPSPs) were elicited every 15 seconds for 20 minutes in WT and Fmr1 KO mice prior to high-frequency stimulation (HFS). Vertical scale bar is 0.5 mV and the horizontal scale bar is 5 ms. fEPSPs were obtained for 60 minutes following HFS. Traces are averages from 10 minutes immediately prior to HFS (“PRE”) or 50-60 minutes after HFS (“POST”). (C) The absolute slope of the EPSP 50-60 minutes after the HFS was significantly increased relative to the 10 minutes immediately preceding HFS in both genotypes demonstrating LTP. However, the magnitude of the increase was significantly smaller in tissue obtained from Fmr1 KO mice. HFS induced a form of synaptic plasticity that appears to be dependent on the NMDA-receptor in both genotypes, because the NMDA antagonist, APV, blocked LTP in tissue obtained from WT and Fmr1 KO mice.
3.3.8 Long-term depression is impaired in the DG of Fmr1 KO mice

The inverse of LTP is long-term depression (LTD), a persistent reduction in the absolute slope of the EPSP following low-frequency stimulation (LFS). LTD has received considerable attention in the mouse model of FXS due to the discovery an *increase* in LTD dependent on metabotropic glutamate receptors, but not NMDA receptors, in other brain regions. In the DG, LTD was assessed in the middle molecular layer by comparing the slope of the evoked field excitatory postsynaptic potential (fEPSP) before (“PRE”) and after (“POST”) applying LFS to the medial perforant path (Figures 3.7A & 3.7B). WT mice showed significant LTD as indicated by a decrease in the average absolute slope of the fEPSP pre-LFS to post-LFS ($t_{(26)} = 5.582, p < 0.000$). LTD was not observed in tissue obtained from Fmr1 KO mice (WT: -21.63 ± 4.03, KO: -1.05 ± 3.57; $t_{(21)} = 3.700, p = 0.001$). Application of the NMDA antagonist APV (50 µM) during administration of the LFS led to a near complete block of LTD in WT mice, and LTD was again not observed in Fmr1 KO mice (WT: -2.59 ± 4.84, KO: -5.63 ± 5.41; $t_{(12)} = 1.222, p = 0.245$) (Figure 3.7C). Similar to the LTP results described in Figure 3.6, it appears that NMDA-dependent LTD is impaired in the DG of Fmr1 KO mice.
NMDA-dependent LTD in the DG of Fmr1 KO mice.

NMDA-dependent long-term depression (LTD) is significantly attenuated in the dentate gyrus of Fmr1 KO mice. (A & B) Field excitatory postsynaptic potentials (fEPSPs) were elicited every 15 seconds for 20 minutes in WT and Fmr1 KO mice prior to low-frequency stimulation (LFS). Vertical scale bar is 0.5 mV and horizontal scale bar is 5 ms. fEPSPs were obtained for 60 minutes following LFS. Traces are averages from 10 minutes immediately prior to LFS (“PRE”) or 50-60 minutes after LFS (“POST”). (C) The absolute slope of the fEPSP was significantly decreased 50-60 minutes following the LFS compared to the 10 minutes immediately preceding the LFS in WT tissue demonstrating LTD. In contrast, tissue obtained from Fmr1 KO mice did not show LTD. LFS induced a form of synaptic plasticity that, similar to HFS, appears to be dependent on the NMDA-receptor in both genotypes because LTD was not observed in tissue obtained from WT and Fmr1 KO mice when the NMDA antagonist, APV, was included.
3.4 Discussion

Elucidation of learning impairment in the mouse model of FXS (Fmr1 knockout (KO) mouse) has been surprisingly difficult since its generation more than 15 years ago (1994). The present results demonstrate a novel learning impairment in the mouse model of the most common form of inherited mental retardation. The employed task, context discrimination, is dependent upon the dentate gyrus (DG) subfield of the hippocampus (McHugh et al., 2007). The impetus for this assessment stemmed from our recent study showing aberrant adult neurogenesis in the DG of Fmr1 KO mice (Eadie et al., 2009). Structural analyses of young neurons in the DG of Fmr1 KO mice revealed decreased dendritic complexity and dendritic spine density. This was associated with strikingly impaired NMDA-dependent forms of synaptic plasticity (i.e., long-term potentiation and long-term depression). A picture emerges suggesting that young neurons are more severely affected by loss of expression of Fmr1, which manifests as learning impairments associated with the DG, a region of the brain unique in its disproportionate number of young neurons across the lifespan.

Assessment of Fmr1 KO mice on acquisition of the classic Morris water maze (MWM), commonly used to assess hippocampal-dependent learning, has not consistently revealed a robust learning impairment (D'Hooge et al., 1997; Eadie et al., 2009; Paradee et al., 1999; Peier et al., 2000; Van Dam et al., 2000). Normally, production of FMRP is amongst the highest in the hippocampus of the mammalian brain, making it especially perplexing that loss of FMRP expression does not impair learning in the classic MWM (Hinds et al., 1993).

An emerging notion is that the various subfields of the hippocampus are associated with different components or types of learning (Lisman, 2003; Nakazawa et al., 2004). Using genetic techniques, Tonegawa and colleagues have deleted the obligatory subunit specifically in each
subfield of the hippocampus and assessed the consequence on learning ability. Their data suggest that normal performance on the classic MWM requires functional NMDA receptors in the CA1 subfield but not in the DG (McHugh et al., 2007; Nakazawa et al., 2004; Nakazawa et al., 2002; Rondi-Reig et al., 2001). Instead, functional NMDA receptors in the DG are required for pattern separation. Akin to DG-specific NR1 KO mice, we found that Fmr1 KO mice are impaired on the DG-dependent contextual fear discrimination task.

Contextual fear relies upon proper information processing and inhibitory control, two defining features of cognitive impairment in FXS (Cornish et al., 2004). Impairments in information processing lead to difficulties in the formation of associations (e.g., context-shock) while impairments in inhibitory control lead to difficulties in the separation of the associations. Interestingly, Fmr1 KO mice are unable to discriminate between a novel and familiar object effectively, a finding suggested to involve impairments in inhibitory control (Frankland et al., 2004). Fmr1 KO mice also exhibit abnormalities in both anxiety and stress that potentially contribute to an ability to discriminate between overlapping associations involving fear (Eadie et al., 2009; Markham et al., 2006). Arguing against this interpretation is the observation of normal learning on the MWM, a task that presumably involves a degree of stress.

The DG is a unique region of the mammalian brain due to its ability to continually add new neurons, and thus possessing a disproportionate number of young neurons at any given point (Altman, 1963; Altman and Das, 1965; Altman and Das, 1966; Christie and Cameron, 2006; Gage, 2002). Emerging evidence suggests that young neurons in the DG may disproportionately affect the behavioral function of the DG (Ge et al., 2008; Ge et al., 2007; Kee et al., 2007). Neurogenesis in the adult DG appears to recapitulate primary neurodevelopment in a number of ways including the birth of new neurons from progenitor cells located in the subgranular zone.
(SGZ) and migration of their cell body through the inner and outer granular zones (IGZ and OGZ, respectively) of the granule cell layer (Kempermann et al., 2004). Based on our recent study showing abnormalities in the early stages of neurogenesis in *Fmr1* KO mice (Eadie et al., 2009), we hypothesized that young neurons in the DG are structurally and functionally abnormal. Our results indicate that young neurons are structurally abnormal, possessing a decrease in both dendritic complexity and total dendritic length in neurons located in the inner granule zone of the DG. This same population of neurons also exhibited a decrease in the density of dendritic spines, the primary sites of fast, excitatory synaptic transmission. Most studies into neuronal structure in FMRP-deficient neurons have focused on cortical neurons in adult tissue, reporting increases in dendritic spine density and length (Comery et al., 1997; Hinton et al., 1991; Irwin et al., 2002; Irwin et al., 2001). Although superficially at odds with our results, brain region and neuronal age may account for these differences. In fact, dendritic spine analyses conducted in the CA1 subfield of the hippocampus reported small, but significant differences between *Fmr1* KO and WT mice in the CA1 subfield of the hippocampus that are essentially opposite to that previously observed in neocortex of *Fmr1* KO mice (Grossman et al., 2006). Neurons cultured from the hippocampus also show more substantial morphological abnormalities that are also superficially at odds with that reported on the adult neocortical neurons. For example, analyses of 7 and 21-day old cultured hippocampal neurons revealed shorter dendrites, fewer spines and no significant differences in spine length (Braun and Segal, 2000). Similarly, Castren and colleagues (2005) reported less neurites and decreased dendritic length on cells generated from mouse and human FMRP-deficient neurospheres (Castren et al., 2005). It appears that the morphology of mammalian FMRP-deficient neurons depends on both brain region and neuronal age.
Emerging evidence indicates that young neurons in the DG disproportionately contribute to both synaptic plasticity and the overall function of the DG (Snyder et al., 2001; van Praag et al., 2002; Schmidt-Hieber et al., 2004; Ge et al., 2007; Kee et al., 2007; Ge et al., 2008), perhaps due to a more immature dendritic spine profile (Zhao et al., 2006). Based on the aforementioned result that Fmr1 KO mice possess decreased density of dendritic spines on young neurons and impaired DG-dependent learning, we hypothesized that synaptic plasticity in the DG is impaired in Fmr1 KO mice. Data presented here appear to support this notion. We observed a decrease in the ability of dentate granule neurons to persistently increase (long-term potentiation; LTP) or decrease (long-term depression; LTD) synaptic efficacy following high or low frequency stimulation. Both forms of synaptic plasticity appear to be NMDA-dependent.

Although the majority of research into the receptor-dependence of aberrant synaptic plasticity in the mouse model of FXS has focused on the metabotropic glutamate receptor, emerging evidence implicates abnormalities in the basic glutamate neurotransmission (AMPA receptor) and the classic glutamate receptor involved in synaptic plasticity (NMDA receptor) during neuronal development in the Fmr1 KO mouse. Seeburg’s group has recently reported that early postnatal Fmr1 KO mice exhibit a significant decrease in AMPA-mediated EPSCs relative to controls in the hippocampal CA1 subfield, and that this difference is abolished when assessed in adulthood (Pilpel et al., 2009). Similarly in layer V of neocortex, P10-18 Fmr1 KO mice exhibit impaired spike-timing LTP (Desai et al., 2006).

The data presented here are superficially appear at odds with the current model that neuronal loss of FMRP induces an increase in long, thin dendritic spines and enhanced metabotropic glutamate receptor (mGluR)-dependent LTD. However, when neuronal age is taken into account across studies that have investigated both structural and synaptic plasticity, another model
emerges suggesting that loss of FMRP decreases synaptogenesis and impairs major forms of synaptic plasticity. For example, it was recently reported that FMRP is normally associated with mRNAs of key synaptic scaffolding proteins such as PSD-95, SAP-97 and SAPAP1, 2 and 3, as well as key postsynaptic glutamate receptors such as NR1, NR2B and GluR1 in 7-day old cultured hippocampal and neocortical neurons (Schutt et al., 2009). Another related recent finding is impaired LTP in the CA1 subfield of the hippocampus and neocortex of 2-week old Fmr1 KO mice associated with impaired synaptic delivery of GluR1-containing AMPA receptors (Hu et al., 2008). The abnormalities in plasticity described during neuronal development may predispose mature FMRP-deficient neurons to long, thin dendritic spines and enhanced metabotropic glutamate receptor (mGluR)-dependent LTD.

Investigation of the effects of loss of Fmr1 expression on the DG has revealed impairments in the production of new neurons and the morphology of young neurons. The predilection for young neurons appears to have significant consequences on the functioning of this brain region due to its relatively unique ability to exhibit adult neurogenesis. Indeed, assessment of Fmr1 KO mice on a DG-dependent learning task revealed a novel learning impairment. The DG should be considered an important target for investigation into other forms of neurodevelopmental disability and an important target for novel therapeutics aimed at improving cognitive ability in the most common form of inherited mental retardation.
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4. General Discussion

The research described in this thesis sought to test the hypotheses that lack of expression of the \textit{Fmr1} gene deleteriously alters structural and functional plasticity in the mammalian dentate gyrus (DG), and impairs aspects of learning and emotion associated with this brain region. A central design of this research was to compare plasticity associated with the DG, at various levels of analysis, using an array of techniques, between \textit{Fmr1} KO and WT littermate mice. This thesis is novel because we investigated plasticity in the DG in the mouse model of FXS for the first time, and revealed impairments in structure, function and behavior related to this brain region.

4.1 Brief Summary of Findings

4.1.1 Behavior

We had two main hypotheses regarding the behavior of \textit{Fmr1} KO mice. First, we hypothesized that \textit{Fmr1} KO mice exhibit behaviors reflective of decreased anxiety, such as spending more time in exposed regions of behavioral testing appariati. Second, we hypothesized that \textit{Fmr1} KO mice are impaired in the learning of particular tasks, such as the reversal phase of the Morris water maze and context discrimination. We found that \textit{Fmr1} KO mice do exhibit behaviors reflective of decreased anxiety such as (1) increased time spent in the centre of an open field, (2) increased entries to open arms of an elevated plus maze during initial period of testing, (3) decreased defecations in the open field, and (4) blunted stress-induced corticosterone response. Regarding learning ability, we were unable to replicate an impairment in \textit{Fmr1} KO mice in any phase, of any form, of the water maze. In contrast, we discovered an impairment in the ability of \textit{Fmr1} KO mice to learn context discrimination, a task associated with NMDA receptors specifically in the DG.
4.1.2 Neurogenesis

We had three main hypotheses regarding adult hippocampal neurogenesis in Fmr1 KO mice. First, we hypothesized that Fmr1 KO mice would exhibit decreased cell proliferation. Second, we hypothesized that Fmr1 KO mice would exhibit decreased cell survival. Third, we hypothesized that Fmr1 KO mice would exhibit decreased neuronal proliferation. We found that Fmr1 KO mice exhibit no difference in cell proliferation using three different experimental approaches. We found that Fmr1 KO mice exhibit a decrease in cell survival; however, this was limited to the ventral subregion of the DG. Third, we found an increase in neuronal differentiation. This too was limited to the ventral subregion of the DG. It appears that abnormalities exist in the process of neurogenesis specifically in the ventral subregion of the DG.

4.1.3 Neuromorphology

We had three specific hypotheses relating to dendritic morphology in Fmr1 KO mice. First, we hypothesized that granule neurons in the DG of Fmr1 KO mice would show an overall decrease in length and complexity. Second, we hypothesized that we would observe an increase in the proportion of dendritic spines that appear long and thin. Third, we hypothesized that we would not observe a difference in the density of dendritic spines on dentate granule neurons from Fmr1 KO mice. We found that granule neurons in the DG of Fmr1 KO mice do indeed show an overall decrease in length and complexity. Analyses of dendritic spines revealed unexpected results. We observed decreased dendritic spine density in Fmr1 KO mice compared to controls, but no differences in the morphology of dendritic spines between genotypes. These effects were only observed in neurons in the inner granular zone, the location of relatively young neurons in
the DG. It appears that the dendritic morphology of young neurons in the DG is less elaborate within the context of loss of transcription of the Fmr1 gene.

4.1.4 Synaptic plasticity

We had two specific hypotheses relating to synaptic plasticity in Fmr1 KO mice. First, we hypothesized a decrease in long-term potentiation (LTP). Second, we hypothesized a decrease in long-term depression (LTD). We observed an attenuation of LTP in the DG of Fmr1 KO mice. This form of LTP appeared to be NMDA-dependent. In addition, we observed a decrease in LTD in the DG of Fmr1 KO mice when using a conditioning stimulus protocol that induces an NMDA-dependent LTD in WT controls. In short, bidirectional, NMDA-dependent synaptic plasticity is impaired in the DG of the mouse model of FXS.

4.1.5 Glutamate receptors

We had two specific hypotheses relating to glutamate receptors in the DG of Fmr1 KO mice. First, we hypothesized that AMPA-mediated synaptic transmission would be decreased in Fmr1 KO mice. Second, we hypothesized that NMDA-mediated synaptic transmission would be unaltered. We observed a decrease in the magnitude of the NMDA-mediated currents, rather than the AMPA-mediated currents in neurons in the DG of Fmr1 KO mice (Appendix D.1). We suggest that the decreased NMDA currents may underlie impairments in NMDA-dependent LTP and LTD.
4.2 Behavior of Fmr1 KO Mice

An understanding of the effects of a lack of FMRP on the whole mammalian nervous system is arguably best gleaned from an assessment of the behavior of individuals with a lack of FMRP. In the introduction, we thoroughly reviewed the range of cognitive and behavioral abnormalities of humans with FXS. The key symptom of patients with complete loss of expression of the Fmr1 gene appears to be intellectual disability (ID); however, alterations in anxiety and stress responsiveness are also significant (Jacquemont et al., 2007). Mimicry of these key symptoms of FXS in the Fmr1 KO mouse model has been challenging for many researchers. Our research began with an assessment of Fmr1 KO mice on classic tests of learning/memory and anxiety. Following our observation of altered NMDA-dependent synaptic plasticity in the DG of Fmr1 KO mice, we returned to behavioral experiments to test learning in Fmr1 KO mice using a task that is dependent upon functional NMDA receptors in the DG. Our hope was to discover a robust learning impairment in the mouse model of the most common form of inherited ID.

4.2.1 Modified SHIRPA

Individuals with FXS can present with a variety of symptoms. It was thus critical to conduct a general assessment, akin to a general physical exam, of our Fmr1 KO mice. This information is critical for proper interpretation of our behavioral results. A modified SHIRPA behavioral assessment of Fmr1 KO mice did not reveal any obvious differences from WT mice (Appendix C.1).
4.2.2 Anxiety, stress, and fear

Our initial behavioral experiments included tests of anxiety, stress, and fear, because these tests can be confounded by previous behavioral testing. Specifically, we chose to assess Fmr1 KO mice on the open field, elevated plus maze, corticosterone-response and fear conditioning. We chose the open field primarily because it has been used by many other researchers, yielding differences between Fmr1 KO and WT mice. It was important for us to ensure that we observed similar behaviors. In contrast, very few studies had tested Fmr1 KO mice on the elevated plus maze, a separate test of anxiety. This would provide novel data and insight into behaviors of Fmr1 KO mice. A few studies had suggested that lack of Fmr1 expression leads to a protracted corticosterone response to stressors. As such, we felt that it would be important to assess stress responsiveness in Fmr1 KO mice in our laboratory. Similarly, investigations of fear conditioning in Fmr1 KO mice has yielded conflicting results from other laboratories, spurring us to investigate this behavior. Convergent data from these experiments generated a relatively clear picture of the behavioral phenotype of the Fmr1 KO mice in our colony.

4.2.2.1 Behavior of Fmr1 KO mice in the open field

Anxiety levels in mice can be inferred from their behavior in relatively simple testing apparati. One of the first behavioral experiments we conducted was simply to observe where Fmr1 KO mice spend their time in an open arena. Rodents that spend more time near the walls of the open field, away from the more brightly illuminated and exposed central region, are inferred to be more anxious (Crawley, 1985; Crawley, 1989). Surprisingly, Fmr1 KO mice showed exactly the opposite; they spent more time in the central region of the maze. Our tentative conclusion was that loss of FMRP in mice causes them to be less anxious. Another dependent variable used in this behavioral experiment was the number of defecations because
this can be reflective of an imbalance in autonomic nervous system function. \textit{Fmr1} KO mice exhibited significantly less defecations than WT mice corroborating the conclusion that loss of FMRP leads to decreased anxiety in mice. Decreased defecations could also simply be a function of loss of FMRP expression in the gastrointestinal system. Quantification of home cage defecations did not reveal a significant difference arguing against this interpretation and supporting the conclusion that an imbalance in autonomic nervous system activity exists in \textit{Fmr1} KO mice.

The conclusion that \textit{Fmr1} KO mice appear less anxious is not novel. In fact, seven of eight studies that have investigated thigmotaxis in the open field report that \textit{Fmr1} KO mice exhibit an increase in the time or distance in the centre of the open field compared to WT mice (Hayashi et al., 2007; Peier et al., 2000; Qin et al., 2002; Qin et al., 2005; Restivo et al., 2005; Spencer et al., 2005; Spencer et al., 2006; Yan et al., 2005). The study that did not observe this difference actually reported that \textit{Fmr1} KO mice spend more time near the walls of the open field (Restivo et al., 2005). These authors suggest that background strain may account for this effect. This appears unlikely considering that other studies have used the same background strain (C57BL/6) reporting the opposite result (Spencer et al., 2005; Spencer et al., 2006; Yan et al., 2005).

Despite the interesting sex difference in anxiety in individuals with FXS, with females being more commonly and severely affected, only a single study has investigated sex differences in the open field of \textit{Fmr1} KO mice. Qin and colleagues (2005) noted that female mice with the \textit{Fmr1} gene deletion do \textit{not} exhibit an increase in time spent in the centre of the open field. More studies should investigate the basis for these interesting sex differences.
Several authors have also noted an increase in locomotion in the open field, consistent with the prevalence of hyperactivity in males with FXS. However, even within the same laboratory this result appears to be inconsistent (Mineur et al., 2002; Spencer et al., 2005; Spencer et al., 2006). The reasons why we have not observed an increase in locomotion of Fmr1 KO mice in the open field is unclear. However, it is of note that in our laboratory, we did not observe changes in locomotion on several independent tasks including swim speed in the water maze. It is unclear why we did not observed increased locomotion in our experiments. As anxiety may also be related to locomotion, it is possible that a decrease in anxiety may mask hyperactivity in our mice. Many unidentified factors can be responsible for significant inter-laboratory differences in the behavior of mice, even when methodological variables are rigorously controlled (Crabbe et al., 1999). This appears to be particularly true for the assessment of genetic manipulations on behavioral assays of anxiety and locomotion (Wahlsten et al., 2003).

In short, our observations in the open field suggestive of a decrease in anxiety in male Fmr1 KO mice are consistent with the majority of other researchers. One important inconsistency is our inability to observe an increase in locomotion in Fmr1 KO mice (Mineur et al., 2002; Spencer et al., 2005; Spencer et al., 2006). Although the former observation of decreased anxiety is less consistent with what is observed in clinical populations, it may be a more robust behavioral abnormality in Fmr1 KO mice.

4.2.2.2 Behavior of Fmr1 KO mice on the elevated plus maze

The second behavioral apparatus employed to assess anxiety in Fmr1 KO mice was the elevated-plus maze (EPM). This task is commonly used to assess anxiety, particularly the efficacy of anxiolytics, in rodents (Pellow et al., 1985). The EPM consists of a four-armed platform elevated approximately 1 m above the ground. Two opposing arms have walls while
the two other arms do not. Mice that spend more time in the closed arms and do not venture into the open arms are inferred to be more anxious. As described in Chapter 2, we found that Fmr1 KO mice enter the open arms more than WT mice in the first minute of a 5 minute testing session. This result corroborates our conclusion from observations of Fmr1 KO mice in the open field that Fmr1 KO mice exhibit decreased anxiety.

Our study is only the third study to assess the behavior of Fmr1 KO mice on the elevated plus maze (Eadie et al., 2009). In 2002, Nielsen et al. reported no difference in behavior on the elevated plus maze between Fmr1 KO mice and controls (Nielsen et al., 2002). These authors reported on multiple genetic backgrounds. This group, in contrast to several other studies cited above, did not report any significant differences following assessment in the open field. It is unclear why these authors’ results differ from ours and those of others. Mineur et al. (2002) also studied the behavior of Fmr1 KO mice on the EPM (Mineur et al., 2002). These authors reported an increase in total arm entries and no difference in open arm entries. One difference between these two studies is the order of testing. In our study, we examined behavior in the open field prior to the EPM whereas Mineur et al. employed the EPM prior to the open field. Results from these tasks may be particularly susceptible to the degree of handling prior to assessment (Hatcher et al., 2001). In addition, this latter study may not have observed significant differences in open/closed arm entries because they did not analyze within-session data. Clearly more studies are needed to replicate our finding of increased open arm entries within the first minute of testing of Fmr1 KO mice in the EPM.

The overall finding that Fmr1 KO mice exhibit decreased anxiety whereas humans with FXS exhibit increased anxiety at first appears difficult to reconcile. Several reasons have been put forth including species differences, inadequate construct validity of tasks employed and
inadequacies with the mouse model such as molecular leakiness (Yan et al., 2004). For example, future models could attempt to use alternative techniques to knockout expression of the *Fmr1* gene, such as siRNA (small interfering RNA). Other reasons for this discrepancy may include a lack of environmental factors, such as social stressors, required to precipitate the effect observed in humans.

### 4.2.2.3 Acute stress-induced corticosterone response in *Fmr1* KO mice

Anxiety and stress are closely related phenomena. For example, Barlow has defined anxiety as a mood-state associated with preparation for a possible negative future experience (Barlow, 2002). Similarly, stress is often defined as a change in homeostasis in response to a stressor (Kopin, 1995). Testing anxiety in mice using tasks such as the open field and elevated plus maze do in fact involve a stressor: exposure to an open environment. In light of this, we chose to assess the stress response of *Fmr1* KO mice directly. Based on results from the open field and elevated plus maze, we hypothesized that *Fmr1* KO mice would exhibit an attenuated corticosterone response to acute restraint stress. This was in fact our observation. These data suggest that loss of FMRP in mice blunts the stress response in mice, manifesting as an apparent decrease in anxiety.

Very few experiments have carefully assessed corticosterone/cortisol levels in *Fmr1* KO mice or patients with FXS. Normally, in both mice and humans, corticosterone and cortisol spike just prior to the active phase (i.e., the dark phase for rodents and the light phase for humans). Studies that have investigated corticosterone and cortisol in *Fmr1* KO mice and patients with FXS have yielded significant, albeit complicated, results. Reiss and colleagues obtained salivary cortisol samples from children with FXS four times per day for 3 days (Wisbeck et al., 2000) with a social stressor occurring on day 1. All days revealed significantly
higher cortisol levels immediately before lunch and bedtime, suggesting abnormal diurnal variations in cortisol in individual’s with FXS, and perhaps persistently elevated cortisol in response to social stress. Subsequently, this same laboratory conducted a larger, better controlled study (Hessl et al., 2002). Salivary cortisol levels were obtained from 109 children with FXS with non-affected siblings used as controls. Again, the children with FXS exhibited higher salivary cortisol levels on routine days but only immediately before bedtime. On the evaluation day, children with FXS had higher cortisol levels during an IQ evaluation that occurred at approximately 11AM and the increased cortisol persisted until approximately 3PM, suggesting that evaluation induced an elevation in cortisol in patients with FXS relative to their unaffected siblings. Greenough and colleagues attempted a similar study in Fmr1 KO mice (Markham et al., 2006). Male and female Fmr1 KO and WT mice were subjected to 30 minutes of restraint stress in a capped, ventilated 50 mL Faulkner tubes and sacrificed at various time intervals after removal from the restrainer. Similar to our study, a competitive ELISA for corticosterone was used to assess genotype and sex differences. The male Fmr1 KO mice revealed a blunted corticosterone response immediately after removal from the restraint tube, consistent with our study performed in the same manner with the exception that we employed a 3 hour restraint session rather than a 30 minute restraint session. Interestingly, Greenough and colleagues found that the genotype effect was lost when the mice were sacrificed 15 minutes after removal from the restraint tube and inversed when the mice were sacrificed 1 hour after removal from the restraint tube. These data suggest that environmental stressors can increase or decrease the level of corticosterone in Fmr1 KO mice depending on the time delay between a stressor and testing. It is unclear how these results in mice relate to the aforementioned findings in humans with FXS. In addition, female Fmr1 KO mice may have a different pattern of
corticosterone response to an acute stressor. Female Fmr1 KO mice showed a significant decrease in corticosterone following a 15 minute delay between removal from the restraint tube and the time of sacrifice. Similarly, we did not observe a significant difference in corticosterone response in Fmr1 KO or heterozygous female mice. In contrast to these studies, Qin and Smith (2008) recently reported no significant differences between male Fmr1 KO and WT mice before or after an acute stressor. One major difference between the study from Qin and Smith and both the studies from Greenough’s group and ours is the restraint tube itself. The restraint tube used by Qin and Smith was a manufactured Mouse Restrainer designed to prevent all movement; whereas, the restrainer used by our group and Greenough’s was a ventilated 50 mL Faulkner tube that did permit some movement. More recently, Nielsen et al. (2009) reported lower corticosterone levels 120 minutes following removal from the restraint tube in male Fmr1 KO mice. The data did not reveal any other obvious differences despite assessments at multiple time points and following multiple types of stressors (Nielsen et al., 2009). In short, we have observed a blunted corticosterone response in male Fmr1 KO mice which is consistent with at least one other study using a similar method (Markham et al., 2006). Other studies suggest that the relationship between a stressor and the physiological cortisol/corticosterone response is likely context and sex dependent (Hessl et al., 2002; Markham et al., 2006). Further studies are warranted to more carefully assess the cortisol/corticosterone response in humans with FXS and the mouse model of FXS.

4.2.2.4 Fear response in Fmr1 KO mice

Fear is often defined as an alarm response to an immediate or imminent danger. The assessment of fear conditioning in rodents, compared to the assessment of stress, is often a matter of degree regarding the intensity of the stimulus employed to induce the emotion
Typically fear is induced in rodents by delivering an electric foot shock. Fear memory is typically measured by the amount of freezing behavior the animal exhibits in the context in which the shock (unconditioned stimulus) was previously administered. The shock can be paired to a conditioned stimulus such as a tone (auditory conditioning) or some other aspect of the environment (context conditioning) in which it receives the unconditioned stimulus (e.g., the electric foot shock).

Experiments designed to assess fear conditioning in the mouse model of FXS have yielded inconsistent results. Despite this, some evidence appears to indicate that at least some aspects of the fear response is abnormal in Fmr1 KO mice. Paradee et al. (1999) were the first to assess fear conditioning in Fmr1 KO mice (Paradee et al., 1999). In their experiment, all mice were allowed to explore a chamber for 2 minutes at which point a 30 second, non-aversive tone was delivered. A 2 second foot shock immediately followed the tone and the mice were left in the chamber for a further 30 seconds before being returned to their home cage. On the following day, the mice were returned to the chamber and freezing behavior was assessed. Ninety minutes later, the mice were placed in a different context where freezing behavior was assessed in response to the 30 second, non-aversive tone. Although there was no significant difference in freezing behavior prior to the tone in this latter situation (suggesting no difference in basal freezing behavior), Fmr1 KO mice froze less in response to exposure to both the context and tone that were paired with the foot shock 1 day prior. These authors attributed this finding to amygdala dysfunction due to a lack of impairment in LTP observed in the CA1 subfield of the hippocampus of Fmr1 KO mice (Godfraind et al., 1996). In contrast, Peier et al. (2000), using a similar paradigm as that described by Paradee et al., did not observe any significant differences in context or auditory fear conditioning in Fmr1 KO mice compared to controls (Peier et al.,
2000). Similarly, assessment of *Fmr1* KO mice crossed with YAC mice over-expressing *Fmr1* did not yield significant differences in auditory or contextual fear conditioning. In a separate study using a similar paradigm, Van Dam et al. (2000) reported normal basal freezing in *Fmr1* KO mice and a trend towards decreased contextual fear (Van Dam et al., 2000). The discrepancy between the above results does not appear to be related to variables such as age and genetic background. One notable difference is the apparatus used for fear conditioning. The study that reported an impairment in fear conditioning in *Fmr1* KO mice (Paradee et al.) employed a 7-inch wide chamber from Coulbourne Instruments; whereas, the methods of the two other studies (Peier et al. & Van Dam et al.) were based on a study by Paylor et al. (1994) that used a larger chamber (Paylor et al., 1994). Future studies investigating fear conditioning in *Fmr1* KO mice should compare chamber size and be mindful of other variables related to the testing apparatus.

As part of our experiments, we also assessed the ability of *Fmr1* KO and WT mice to acquire context-conditioned fear (described in chapter 3). Mice were placed into a particular context daily, where they were left to explore for 3 minutes, at which time a foot shock was delivered. The mice were left in the context for a further 2 minutes. This occurred over 3 days with the percentage freezing behavior obtained for the first 3 minutes on each day. Both groups increased their freezing behavior equally across the first 3 days. Two more days of exposure to the same context, and a different context, yielded equivalent freezing behavior in *Fmr1* KO and WT mice. On subsequent days, *Fmr1* KO and WT mice were trained to freeze more in the context that they were previously shocked compared to a context that they never received a shock (context discrimination training). During this phase of testing, *Fmr1* KO mice began to show differences compared to WT mice (to be discussed further in the section Learning below). In short, our
observation of a lack of significant difference in context fear conditioning is consistent with findings from the majority of other laboratories.

4.2.3 Spatial and context learning

The most difficult, and arguably the most important, phenotype of Fragile-X syndrome (FXS) to model in mice has been intellectual disability (ID). This difficulty is surprising in light of the severity of ID in males with FXS. The importance of modeling ID in Fmr1 KO mice is obvious. How can changes in neurobiology be linked to ID in Fmr1 KO mice if a behavioral assay of ID does not exist? How can those involved with the development of therapeutics to treat ID in FXS be tested if a behavioral assay of ID does not exist? Based on results presented throughout this thesis showing impaired plasticity specifically in the hippocampal dentate gyrus (DG), we hypothesized that a novel learning impairment would manifest if Fmr1 KO mice were tested on a task dependent on functional NMDA receptors specifically in the DG.

In the study reporting on the generation of the mouse model of FXS in 1994, the authors reported on learning behavior as assessed using the Morris water maze (MWM) (1994). In this task, mice are placed into a pool of opaque water that contains a platform just below the surface of the water in one quadrant. Using extra-maze cues, the mice learn to locate the hidden platform, and do so, better and better with each trial and day tested. In other words, rodents normally exhibit spatial learning and memory on the MWM. To the surprise of the researchers, Fmr1 KO mice performed as well as their WT controls on this task. We have replicated this null result.
Acquisition of the classic MWM appears to be associated with the CA1 subfield of the hippocampus. Moser and colleagues have shown that lesions to the hippocampus proper (CA subfields) are sufficient to induce impairments in rats on acquisition of the MWM (Moser et al., 1993). Further, Morris and colleagues have shown that infusion of the NMDA-antagonist to the hippocampus impairs the ability of rats to learn the Morris water maze (Morris et al., 1986).

Similarly, Tonegawa and colleagues (2001) have shown that deletion of the obligatory subunit of the NMDA receptor, NR1, specifically in the CA1 subfield is sufficient to impair the ability of mice to learn the MWM (Rondi-Reig et al., 2001). More recently, this same group has deleted the NR1 subunit of the NMDA receptor specifically in the DG subfield and found that mice learn the classic MWM equally well as controls.

\textit{Fmr1} KO mice show learning impairments similar to the DG-specific NR1 KO mice (McHugh et al., 2007). For example, \textit{Fmr1} KO mice are not impaired on the classic MWM. This task appears to be dependent upon NMDA-receptors in the CA1 subfield of the hippocampus. Indeed, NMDA-dependent synaptic plasticity appears normal in the CA1 subfield of the hippocampus of \textit{Fmr1} KO mice (Godfraind et al., 1996; Huber et al., 2002). It is clear that \textit{Fmr1} KO mice are not impaired on acquisition of the MWM arguing that neurobiological abnormalities observed in the hippocampal CA1 are not of paramount importance in the pathophysiology of ID in FXS.

Several authors have suggested that \textit{Fmr1} KO mice are impaired on \textit{reversal} learning. For example, the Dutch-Belgian Consortium (1994) reported that \textit{Fmr1} KO mice do not learn to locate the hidden platform as well as controls when the platform is moved to the opposite quadrant following acquisition of the memory for the initial location. A few years later these authors replicated these data, but described the impairment as “mild” (D’Hooge et al., 1997).
Paradee et al. (1999) subsequently reported their findings on acquisition and reversal phases of the MWM concluding that “Contrary to earlier reports, near-normal performance was observed” (Paradee et al., 1999). Paradee et al. suggest that the reason for their inability to replicate the effect may be a function of the greater number of backcrossings of the Fmr1 gene deletion from the 129 to the C57BL/6 genetic background in their study. Van Dam et al. (2000) performed a similar study, but used a plus-shaped water maze instead of the typical circular MWM (Van Dam et al., 2000). These authors reported that Fmr1 KO mice could learn to find a hidden platform in a single arm equally well as controls; however, Fmr1 KO mice made less entries into the correct arm in a reversal phase of the task where the hidden platform was moved to a different arm. The mice used in this study had been backcrossed onto the C57BL/6 genetic background for at least 10 generations. It is thus unclear how the Paradee study differs from these others in this regard. Overall it appears that loss of FMRP may impair reversal learning in the water maze. Despite these initial findings nearly a decade ago, not a single study designed to test the ability of various manipulations to rescue the phenotype of Fmr1 KO mice has used this paradigm. This is surprising in light of the dearth of behaviors reflective of ID in the mouse model of FXS.

In our laboratory, we were unable to replicate the putative impairment of Fmr1 KO mice in reversal learning in either the classic MWM or the Plus-shaped water maze. Consistent with other studies, we also did not observe significant differences between genotypes in the acquisition phase of these behavioral tasks. The background strain that we employed was the C57BL/6 strain. It is possible that, like the null results from Paradee et al. (1999), a greater number of backcrossings onto this background strain may account for these null findings. It is also possible that the prior exposure that the Fmr1 KO mice received in our study on the open field and elevated plus maze primed the Fmr1 KO mice for better performance on the classic
MWM. Further studies are warranted into understanding a putative difference in reversal learning.

It is important to note that reversal learning in the water maze has *not* been shown to be dependent on the hippocampus. Assuming that an impairment in reversal learning, but not acquisition, of the MWM truly exists in the *Fmr1* KO mice, then one would predict that the prefrontal cortex (PFC), not the CA1 subfield of the hippocampus, would show abnormalities (de Bruin et al., 1994). More work is clearly warranted into elucidating reversal learning impairments in *Fmr1* KO mice, and relating these findings to altered plasticity in the PFC (Meredith et al., 2007).

Our inability to replicate the subtle impairment on the reversal phase of the water maze, the unclear relationship between this putative learning impairment and the DG, and the importance of describing a robust learning impairment in the mouse model of FXS provided the impetus to seek out a learning behavior that is dependent on the DG. As previously mentioned, Tonegawa and colleagues had reported that loss of the obligatory NMDA receptor subunit NR1 *specifically in the DG subfield of the hippocampus* did *not* cause impaired learning on the classic MWM, but instead revealed an impairment in context discrimination. In collaboration with Dr. Fanselow’s laboratory at UCLA, we elucidated a novel learning/memory impairment in *Fmr1* KO mice. In this experiment, mice were placed each day into both a context where they received a foot shock (S+) and a different context where they did not receive a foot shock (S-). Within each 5 minute session, mice in the foot shock context (S+) received a foot shock at the 3 minute time point. Freezing behavior was scored for the first 3 minutes in both the S+ and S- context. Over several days, freezing behavior normally increases in the S+ context and decreases in the S- context. Since DG-specific *NR1* KO mice do not learn to discriminate between the S+ and S- contexts as
well as controls and Fmr1 KO mice show impaired NMDA-dependent synaptic plasticity in the DG, we hypothesized that Fmr1 KO mice would be impaired in their ability to discriminate between contexts, similar to the DG-specific NRI KO mice.

The assessment of Fmr1 KO mice on the context discrimination task proved fruitful. The Fmr1 KO mice did not discriminate as well as controls across 8 days of testing. Interestingly, this appeared to be largely a function of persistently elevated freezing in the S- context. This appears to be strikingly similar to the finding from McHugh et al. (2007) showing that DG-specific NRI KO mice freeze more in the S- context (McHugh et al., 2007). Prior to the context discrimination testing, mice are assessed for basal freezing, context fear-conditioning and generalization of fear. These measures did not reveal significant differences between the genotypes suggesting that testing across several days is required to observe impaired in context discrimination. These studies illuminate a learning/memory impairment in the mouse model of FXS. We suggest that this learning impairment is related to impaired NMDA-dependent plasticity specifically in the DG.

It would be interesting to determine if the fear component is necessary to observe an impairment in discrimination learning in Fmr1 KO mice in the future. Aberrancies in stress responsiveness and anxiety observed in Fmr1 KO mice would support this hypothesis. However, the observations that Fmr1 KO mice are not impaired on the Morris water maze (MWM) and the fact that the MWM may be considered a stressful learning task argues against this possibility. Further research is warranted to carefully investigate whether a fear component is required to resolve an impairment in learning/memory involving pattern separation. It would also be of interest to determine if there other aspects of the contexts such as novelty or social components that influence this effect.
4.2.4 Extinction in *Fmr1* KO mice

Bear and colleagues have recently suggested that the learning impairment in *Fmr1* KO mice is increased fear extinction. This is based on results following an inhibitory avoidance (IA) task. In contrast, we observed a decrease in extinction following contextual fear conditioning. In the IA task, rodents are placed into an illuminated chamber of a two-chamber apparatus, and administered a foot shock upon entrance into the dark chamber (Van Laethem and Vandamme, 2006). The latency of the rodents to enter the dark chamber upon a subsequent trial is measured. The assumption is that if the rodent has formed a memory of the pairing trial, then they will exhibit an increased latency to re-enter the dark chamber. This assumption is confounded by the observations that rodents seek out a dark environment when confronted with an anxiety-invoking situation (even if they have been previously shocked in that context) and the consistent observation that *Fmr1* KO mice have a tendency to spend more time in the exposed, well lit regions of behavioral apparati such as the open field (Elliott et al., 2004). In addition, a consistent lack of difference across trials in an IA task may reflect an inability of mice to maintain the ability to discriminate between two similar contexts. Thus our data provide some insight into the potential cause of increased extinction of IA reported by Bear and colleagues. Further studies into the effects of loss of FMRP on extinction are warranted.
4.3 Neurogenesis

One of our initial hypotheses was that adult neurogenesis is decreased in the dentate gyrus (DG) of Fmr1 KO mice. Chapter 2 describes our assessment of multiple phases integral to the process of adult hippocampal neurogenesis in male Fmr1 KO mice. Our data did not support the hypothesis that the quantity of new neurons is decreased within the context of the DG of adult, male Fmr1 KO mice. However, we did observe significant differences in two main phases of adult neurogenesis specific to the ventral DG: decreased cell survival and increased neuronal differentiation. Although these two abnormalities nullified an overall change in the quantity of new neurons produced, it highlighted the fact that new neurons may be produced in an abnormal fashion in the DG of the mouse model of FXS. Overall, we did not observe robust differences in the amount of cell proliferation using three different techniques. It is possible that the increased neuronal differentiation and decreased cell survival may be linked in a negative feedback manner in the ventral dentate gyrus; however, it is also possible that these two effects are independent.

Although these are the first experiments examining adult hippocampal neurogenesis in Fmr1 KO mice, these data can be interpreted within the context of studies examining the effects of Fmr1 deletion on primary neurogenesis (i.e., during development) in the mouse or aborted fetus, and within the context of the genesis of other cell types lacking Fmr1 expression in other systems.

Castren et al. (2005) assessed proliferation and neuronal differentiation of neurospheres generated from both Fmr1 KO mice and a fetus carrying the FXS mutation (Castren et al., 2005). Although differences in proliferation were not observed, cells from the Fmr1 KO mice neurospheres showed a 3-5 fold increase in neuronal differentiation. This is similar to our observation of increased neuronal differentiation in the adult DG of Fmr1 KO mice. The
generated neurons also showed shorter neurites suggesting that, although more cells acquired a neuronal phenotype, their morphology is aberrant. These authors also investigated cell proliferation in the early post-natal period of Fmr1 KO mice and did not observe a significant difference in cortex, also consistent with our finding of no difference in cell proliferation in the adult hippocampal DG of Fmr1 KO mice. These authors did however observe a 4.5 fold increase in cell proliferation in the subventricular zone (SVZ) of early post-natal Fmr1 KO mice. An interesting future direction may be to investigate adult neurogenesis in the SVZ of Fmr1 KO mice. Despite these interesting results, more research is clearly required into the study of neurogenesis in humans in light of a recent report showing that cells isolated from the cortex of one FXS fetus and analyzed in vitro did not show alterations in neurogenesis (Bhattacharyya et al., 2008).

One of the hallmarks of FXS in males is macroorchidism, or enlarged testes (Hagerman, 2002). Although analyses of testicular biopsies indicate that this effect appears to be primarily related to increased interstitial edema and/or alterations in tubule diameter and length, alterations in the genesis of testicular cells have also been reported. For example, increased cell proliferation of Sertoli cells has been reported, based on analyses of E12-15 Fmr1 KO mice (Slegtenhorst-Eegdeman et al., 1998). Also, Bachner et al. (1993) analyzed levels of Fmr1 mRNA in spermatogonia of normal mice and found a spike in the immature testes that declined in early adult life (Bachner et al., 1993). Similarly, it was recently shown that female Drosophila lacking the Fmr1 gene (dFmr1) have egg chambers with both abnormally decreased and increased number of germ cells. Alterations in cell cycle progression have been implicated as some germ cells showed increases in proteins such as cyclin E and PhosphoHistone H3. Although these findings are informative in terms of understanding spermatogenesis and
oogenesis, the significance of these findings to reproduction in patients with FXS is questionable in light of the fact that humans with FXS do not appear to show decreased fecundity (Hagerman, 2002). In light of the data presented in this thesis, the data do beg some interesting questions such as: Could proliferation of neuroprogenitor cells in the DG of female mice be abnormal? And, could this relate to alterations in cell cycle progression? These leaps would of course be based on the weak assumption that the factors contributing to cytogenesis is consistent across systems.

In short, FMRP appears to play an important role in cytogenesis in a variety of systems including the adult hippocampus. It is likely that the factors that FMRP interact with to influence the various phases of cytogenesis will depend on cellular age, cellular environment, species and sex. A key future direction for this research is to characterize neurogenesis in the adult hippocampus of female Fmr1 KO mice. Preliminary data in fact suggest a decrease in cell proliferation, although more data is required for confirmation including assessment of the phase of the estrus cycle that the mice are in upon sacrifice. Another potentially important future direction is the assessment of neurogenesis in human brain tissue. Although difficult to obtain, brain banks do exist which include hippocampal tissue from humans with FXS (e.g., The Brain and Tissue Bank for Developmental Disorders, NICHD). Histological analyses of endogeneous proteins associated with distinct phases of neurogenesis could be used in the study of human tissue (Eriksson et al., 1998).

A few potential pitfalls in the study of neurogenesis are worthy of note as every method used to study neurogenesis carries advantages and disadvantages (Christie and Cameron, 2006; Taupin, 2007). As such, it is our belief that the use of multiple techniques providing convergent evidence is the most appropriate approach (Eadie et al., 2005). The classic approach to study
adult neurogenesis in rodents is to administer (intraperitoneal or i.p.) a thymidine analogue (exogenous marker) that can incorporate itself into replicating DNA (thus marking mitosis) and then sacrifice the animal at various time points following the injection. This effectively marks the birth date of a cohort of newborn cells. Depending on the time lag between injection and sacrifice, the subsequently labeled BrdU-immunoreactive cells will be fixed at the correlated cellular age. Typical time lags between injection and sacrifice for analyses of cell proliferation range from 2-24 hours when a single injection is employed; whereas, typical time points for analyses of cell survival range from 3 to 8 weeks (Wojtowicz and Kee, 2006). It appears that there is considerable attrition of BrdU-positive cells between these early and late time points suggesting that a considerable amount of cell death occurs in the neurogenic process (Olson et al., 2006). In fact, due to practical difficulties using various markers of cell death, the proportional decrease in BrdU-positive cells between early and late time points is often used as a proxy indicator of cell death. Some major issues are associated with the use of BrdU to study adult neurogenesis. Various independent variables, such as exercise or genetic manipulations such as Fmr1 deletion, may indirectly influence the amount and length of time that the hippocampus is exposed to BrdU. For example, voluntary exercise has been suggested to increase the permeability of the blood-brain-barrier increasing the amount of BrdU available to the hippocampus, artificially indicating that exercise increases cell proliferation. Our previous research showed that saturating doses of BrdU and the use of an endogenous marker, Ki67, indicated that exercise does increase cell proliferation independent of this potential confound (Christie et al., 2006). A similar approach was applied to our assessment of adult neurogenesis in Fmr1 KO mice. Using BrdU, we found no difference between genotypes in all subregions of the DG analyzed when a 3-hour lag between injection and sacrifice was employed. When a 4-
week time lag was employed, significantly less BrdU-positive cells were observed in the ventral subregion of the hippocampal DG of Fmr1 KO mice. Two endogenous protein markers of cell proliferation were also stained for: Ki67 and PCNA. The results from the Ki67 staining corroborated our conclusion based on the 3-hour BrdU analysis that there is no difference in cell proliferation between the genotypes. However, we observed a significant decrease in the expression of PCNA in the dorsal DG and a trend for a decrease in the ventral DG. This inconsistent result may be either a function of type-I error or an effect of loss of FMRP on the expression of PCNA independent of its role in cell division. A potential relationship between these two proteins may be an interesting future direction. The most parsimonious conclusion is that Fmr1 deletion decreases cell survival in the ventral DG and does not affect cell proliferation.

An assessment of cell differentiation requires the use of both the exogenous marker BrdU and endogenous markers of glial (e.g., S100β) or neuronal fate (e.g., NeuN). In addition, these molecules must be tagged with fluorescence molecules with non-overlapping excitation and emission spectra, and subsequently analyzed using confocal microscopy to assess, in 3-dimensions, the co-labeling of BrdU with the glial or neuronal marker (Kempermann, 2006). Surprisingly, neuronal differentiation appeared to be increased in the ventral DG, a second abnormal finding associated with neurogenesis in this subregion. When the decreased cell survival and increased neuronal differentiation in the ventral DG of Fmr1 KO mice are both taken into consideration, an estimated overall difference in the number of new neurons produced is not significantly different. This conclusion was corroborated by staining for the immature neuronal marker doublecortin (DCX) which did not show significant differences in either the dorsal or ventral DG between genotypes. Future studies should consider staining for other
endogenous markers of neurogenesis (e.g., PSA-NCAM, NeuroD) to provide further convergent evidence regarding this conclusion.

An intriguing finding from our studies of adult neurogenesis in Fmr1 KO mice was that alterations were largely restricted to the ventral subregion of the DG. As reviewed in the introduction, the ventral subregion of the hippocampus is more significantly linked to subcortical regions of the brain and may be particularly associated with emotions such as anxiety and depression (Sahay et al., 2007; Sahay and Hen, 2007). This parallels our observations of decreased anxiety and impairments in learning and memory involving a fear component.
4.4 Neuromorphology

New neurons proliferate in the subgranular zone (SGZ) of the DG, and extend their axons to the CA3 subfield of the hippocampus and dendrites towards the hippocampal fissure. Across several weeks, the cell bodies of the neurons migrate through the granule cell layer (GCL) (Frotscher et al., 2007). In contrast to neocortical development, all neurons migrate in this direction and generally do not migrate past cohorts of neurons generated earlier. This means that neurons in the inner GCL are typically younger than neurons in the outer GCL. This well-documented observation permitted the testing of the hypothesis that adult neurogenesis in the DG produces young neurons with an abnormal morphology in *Fmr1* KO mice by defining young neurons as neurons with cell bodies located close to the hilus (inner granular zone; IGZ) and older neurons as neurons with cell bodies located close to the molecular layer (outer granular zone; IGZ). The granule cell layer was arbitrarily divided into the OGZ and IGZ at the midpoint between the superficial and deep edges. We found that loss of FMRP causes aberrancies in the dendritic morphology of dentate granule neurons with cell bodies in the IGZ, but not the OGZ (results presented in Chapter 3). These putatively younger neurons appear to possess a decrease in dendritic complexity and total dendritic length, as well as a decrease in the density of dendritic spines.

The literature on dendritic spine morphology aberrancies resulting from loss of FMRP is far more extensive than that regarding the effects of loss of FMRP on neurogenesis (Bagni and Greenough, 2005; Beckel-Mitchener and Greenough, 2004; Irwin et al., 2000). In fact, the similarities in cortical dendritic spine morphology has been heralded as a triumph for the validity of the *Fmr1* KO mouse as a model of FXS. Similar to neurogenesis, it will become apparent that much more work is required to resolve the whole picture of how *Fmr1* deletion affects cell
morphology. The context in which these morphological analyses are conducted and the techniques employed appear critically important (Irwin et al., 2002).

Rudelli et al. (1985) were the first to implicate dendritic spines in an autopsy of a single patient with FXS reporting “Dendritic spine abnormalities of the type observed in trisomic chromosomal disorders” (Rudelli et al., 1985). In a subsequent report, Rudelli et al (1991) employed a rapid-Golgi histological analysis on a larger sample size and reported “very long, thin tortuous dendritic spines with prominent terminal heads and irregular dilatations” in cortical tissue obtained from individuals with FXS (Hinton et al., 1991). These authors also did not report differences in neuronal densities in the cortex between tissue obtained from patients with FXS and control tissue.

The generation of Fmr1 KO mice in 1994 permitted further analyses of the effects of loss of Fmr1 expression on the morphology of neurons in the mammalian brain in vivo (1994). In 1997, Greenough and colleagues reported that Fmr1 KO mice possess “long, thin, tortuous postsynaptic spines” in the transgenic mice relative to controls (Comery et al., 1997). In addition, these authors reported an increase in the density of dendritic spines. The neurons that these authors chose to focus on were layer V pyramidal neurons in occipital cortex. In 2001, this same group reported similar dendritic spine characteristics and density in multiple regions of the cerebral cortex in tissue obtained from patient’s with FXS (Irwin et al., 2001). The approach, again, employed a modified Golgi histological technique on tissue obtained upon autopsy. The conclusion has been drawn in several reviews that FXS is a disorder characterized by a high-density of long, thin, tortuous dendritic spines in multiple brain regions. Unfortunately, this conclusion has been called into question in several studies employing different techniques and studying different brain regions.
Svoboda’s group injected a viral vector containing EGFP into the barrel cortex of Fmr1 KO mice and controls in postnatal week 1, 2 and 4 and subsequently analyzed >16,000 dendritic spines using two-photon laser-scanning microscopy in fixed tissue sections (Nimchinsky et al., 2001). Significant increases in spine density and length were apparent at postnatal week 1 but reached levels that were non-significant by postnatal week 4. Interestingly, this effect appeared to be dependent on activity as analyses of organotypic slice cultures in a similar fashion did not reveal any significant differences between genotypes. A clear discrepancy exists between these findings and the previous studies: spine abnormalities were not apparent in the cortex of 4-week postnatal Fmr1 KO mice whereas they were evident in the cortex of 16-week postnatal Fmr1 KO mice. These authors suggest several explanations for the discrepancy. First, the authors suggest that the somatosensory cortex may develop differently from the temporal and occipital cortices, although they also suggest that this is an unlikely explanation as the time course of dendritic spine development is comparable between these regions. Second, the efficiency of transfection of the viral vector decreases with age and may selectively label cells lacking morphological abnormalities, although no changes in cell-cell variability argue against this interpretation. Third, dendritic spine abnormalities may decrease within the first month of postnatal life, and then increase with further development. Fourth, small dendritic spines may not have been detected in the 4-week postnatal group as a function of limitations of the resolution of the two-photon laser scanning microscope. Regardless of the true reason for this discrepancy, it is evident that immature neurons may be more severely affected by loss of FMRP.

Clearly, the majority of studies into dendritic spines have focused on the cerebral cortex. However, emerging data suggest fundamentally different conclusions from data on dendritic spine abnormalities in the hippocampus. Perhaps the earliest suggestion for this difference can
be found in a report from 2000 showing that 7 and 21-day old cultured hippocampal neurons possess shorter dendrites, fewer spines and no significant differences in spine length (Braun and Segal, 2000). More recently, Greenough’s group studied the CA1 subfield of the young, adult hippocampus of Fmr1 KO mice and reported results essentially opposite to that previously observed in neocortex of Fmr1 KO mice (Grossman et al., 2006). It appears that the morphological development of FMRP-deficient neurons may exhibit different abnormalities relative to the cerebral cortex. The DG is arguably even more distinct from the cerebral cortex in that the principal neurons are of the granule, rather than pyramidal, type.

The above discussion suggests that the cellular age and cellular environment influence the neuronal morphology observed in response to Fmr1 deletion. It appears that young neurons may be preferentially affected, supporting our observation of significant impairments in the morphology of young dentate granule neurons located in the inner granular zone of Fmr1 KO mice. It also appears that the morphological abnormalities observed in the DG may be fundamentally different from that observed in the neocortex, and this is consistent with previous in vitro and in vivo observations. In short, a blanket statement regarding how loss of FMRP affects the morphology of neurons may not be possible; conclusions regarding the morphology of FMRP-deficient neurons must be qualified by at least cellular age and cellular environment.

Future experiments should continue to employ multiple techniques to study the morphology of neurons in FXS and Fmr1 KO mice. In addition, these studies should employ multiple developmental ages including adults in an attempt to better understand alterations across the development of a population of neurons. It would also be possible to attempt to replicate our findings in the DG of humans with FXS to assess if this is a result unique to mice. A more accurate picture will undoubtedly emerge if such studies are conducted.
4.5 Synaptic Plasticity

The maturation process of new neurons in the DG is related to the physiology of the entire structure. From the time point immediately post-proliferation to mature, fully functional neuron, dentate granule cells proceed through electrophysiological phases characterized by differing compositions of functional ion channels and receptors (Duan et al., 2008). The effect appears to be greater synaptic plasticity for young dentate granule neurons (Ge et al., 2008; Ge et al., 2007). Thus, our previous observations of aberrant neuronal structure in young neurons led to the specific hypothesis that synaptic plasticity is impaired in the DG of Fmr1 KO mice. Our results (presented in Chapter 3) indicate that both long-term potentiation (LTP) and long-term depression (LTD) are impaired in the adult DG of Fmr1 KO mice. This decreased bidirectional synaptic plasticity was found to be largely NMDA-dependent, a finding that diverges from most previous research into the effects of loss of FMRP on synaptic plasticity in other brain regions.

The “mGluR theory of fragile X mental retardation” has been extremely influential in the study of the effects of loss of FMRP on synaptic plasticity. In fact, the third most cited paper from Trends in Neuroscience is titled “The mGluR theory of fragile X mental retardation” (Bear et al., 2004). Since the publication of this article in 2004 by Dr. Mark Bear and colleagues, the metabotropic glutamate receptor has been the focus of most research in models of FXS. In fact, Dr. Bear is now involved in the start-up of a pharmaceutical company geared towards the development and testing of mGluR antagonists for the treatment of patients with FXS. The results from Dr. Bear’s group and others regarding NMDA-dependent synaptic plasticity in the CA1 subfield is in stark contrast to the results we present here for the DG subfield of the hippocampus. Dr. Bear and colleagues report that NMDA-dependent LTD is normal in the CA1 subfield of Fmr1 KO mice. Previous research has shown that NMDA-dependent LTP is also
normal in the CA1 subfield of Fmr1 KO mice. Important future directions for our work in the DG will be to assess mGluR-dependent LTD and the role that mGluRs play in mitigating the effect of loss of FMRP on impaired NMDA-dependent, bidirectional synaptic plasticity. The question naturally follows: How does increased mGluR-dependent LTD lead to intellectual disability? The first obvious step would be to assess Fmr1 KO mice on a learning and memory task associated with the CA1 subfield of the hippocampus. Fmr1 KO mice have consistently shown to learn tasks classically associated with this brain region (e.g., the Morris water maze) as well as controls. It has been suggested that impaired extinction of inhibitory avoidance may be associated with enhanced mGluR-LTD in the CA1 subfield; however, this relationship is tenuous.

More recently, Dr. Lynch’s group has reported other subtle impairments in LTP in the CA1 subfield of the hippocampus of Fmr1 KO mice (Lauterborn et al., 2007). Administration of a weak, but not strong, theta-burst conditioning stimulus to the Schaffer’s collaterals of CA1 revealed reduced LTP in Fmr1 KO mice. Administration of BDNF was found to rescue this effect despite no differences in basal BDNF levels between genotypes. Oddly, Dr. Larson, a former student of Dr. Lynch had previously conducted the same experiment and did not observe the effect (Larson et al., 2005). Personal correspondence with both authors did not yield possible reasons for this discrepancy. Other non-classical forms of synaptic plasticity may also be altered in the CA1 subfield of Fmr1 KO mice. For example, Dr. Zhou from the University of Toronto has recently reported impaired glycine-induced LTP in the CA1 subfield of Fmr1 KO mice (Shang et al., 2009). This form of LTP is dependent upon both NMDA and mGluR receptors.

The evaluation of synaptic plasticity in the CA1 subfield of young Fmr1 KO mice has yielded clear impairments. Two recent papers have been published showing robust differences in
synaptic plasticity in the CA1 subfield of young Fmr1 KO mice. Dr. Seeburg’s group from the Max Planck Institute in Heidelberg has recently (2009) reported increased LTP using a low-frequency stimulation pairing protocol in Fmr1 KO mice at 2 weeks postnatal, but not at 6-7 weeks postnatal (Pilpel et al., 2009). This was associated with a decreased AMPA current and increased NMDA current only at 2 weeks postnatal. Both effects normalized with development. This report is similar to a previous study demonstrating absent LTP in the CA1 subfield of early postnatal Fmr1 KO mice (Desai et al., 2006). Another recent paper evaluating LTP in the CA1 subfield of young (2-week old) Fmr1 KO mice reported impaired LTP that is dependent upon synaptic delivery of GluR1 (Hu et al., 2008). The authors suggest that abnormalities in the RAS-signaling pathway underlie this effect. These data suggest that, at least in the CA1 subfield of the hippocampus, loss of FMRP disproportionately impairs synaptic plasticity in young mice.

Synaptic plasticity in brain regions other than the hippocampus has also been investigated. Dr. Peter Carlen from the University of Toronto was the first to show that LTP is reduced in the somatosensory cortex of 16-week old mice (Li et al., 2002). Western blot analyses suggested that this was due to a decrease in the expression of the AMPA receptor subunit GluR1. These abnormalities were not observed in the CA1 subfield of the hippocampus. Other abnormalities in LTP have been noted in prefrontal cortex. Meredith et al. (2007) found that the pairing of a single presynaptic stimulation and a single postsynaptic action potential (AP) 5 ms later induced LTP in WT but not Fmr1 KO mice in prefrontal cortex (Meredith et al., 2007). This effect was lost when 5 postsynaptic APs were used, suggesting that the threshold for LTP induction is shifted by loss of FMRP. It is unclear whether the threshold for LTP induction has shifted in the hippocampal DG. We conducted one experiment attempting to further saturate LTP in the DG by administering the typical HFS three times and extending the post-CS duration out to 90
minutes from the typical 60 minutes. No difference between genotypes was observed 90 minutes post-CS; however, the Fmr1 KO group appeared to be continuing to decline towards baseline. Further experiments may be warranted to more fully address this issue. Impairments in cortical LTP may be more striking if young Fmr1 KO mice are used in these experiments, similar to the pattern emerging in the CA1 subfield of the hippocampus. This would be more consistent with Dr. Svoboda’s observation that the morphology of neurons is significantly impaired in the neocortex of young Fmr1 KO mice, tapering off over the first weeks of postnatal development (Nimchinsky et al., 2001). This interpretation has important consequences for the results presented in this thesis. First, if loss of FMRP disproportionately affects young neurons, then the DG, with a continually renewing pool of young neurons, may be strikingly abnormal across the lifespan in FXS. Second, learning and memory ability dependent upon the hippocampal DG may be particularly affected by loss of Fmr1 expression. An important future direction of this research would be to assess synaptic plasticity using whole-cell electrophysiological recordings for inner and outer granular zone neurons. This research is currently underway, and preliminary results show that the CS protocol typically used (3 publications) to induce LTP in whole-cell configuration in the DG in fact induces LTD when recording from IGZ neurons. More basic research is required to understand synaptic plasticity in young neurons in the DG before this experimental paradigm can be applied to an abnormal condition. Also, the fact that we report that NMDA-dependent LTP and LTD are impaired in the DG of Fmr1 KO mice may warrant further investigation into alterations in this important receptor in models of FXS, particularly in the context of the immature brain. In short, we hypothesize that our observations of robust impairments in classic forms of synaptic plasticity (i.e., NMDA-dependent LTP and LTD) in the DG decrease the flexibility of long-term synaptic communication that underlies intellectual
disability in FXS. We suggest that this is associated with the unique ability of this brain region to produce young, principal neurons across the lifespan.

An important caveat to the observation of impaired LTP and LTD in the DG of *Fmr1* KO mice is that the *Fmr1* KO mice do not appear to be impaired on acquisition of the MWM, despite the fact that there are many examples in the literature of a correlation between these two outcomes. It is possible that this specific dysfunction in the DG is not associated with impairment on this task. Support for this notion comes from a recent study showing that mice lacking functional NMDA receptors specifically in the DG, and thus NMDA-dependent LTP, are *not* impaired on the MWM. Further studies should confirm these observations.

A second important caveat is the observation that both LTP and LTD are decreased, as under certain experimental conditions a decrease in one is associated with an increase in the other (Bienenstock et al., 1982). However, it is of note that this is not always the case and that under certain circumstances, such as impaired function of the NMDA receptor, one might expect both NMDA-dependent LTP and LTD to be decreased (Fox et al., 2006; Liu et al., 2004; Malenka, 1991).
4.6 Glutamate Receptors

The plethora of neuronal abnormalities observed in Fmr1 KO mice all are related to the loss of production of the protein FMRP. An understanding of the normal role of FMRP in the neuron is therefore informative. As reviewed in the introduction, FMRP has the capacity to bind a number of different mRNA, many of which are associated with dendritic and synaptic structure and function (Brown et al., 2001; Darnell et al., 2001). Recent data suggest that the transcripts for the NMDA receptor subunits NR1 and NR2B normally associate with FMRP, suggesting that loss of FMRP may lead to reductions in functional NMDA receptors at the synapse (Schutt et al., 2009). It is likely that translation of these transcripts normally occur following dephosphorylation of serine residue 499 of the FMRP (Bassell and Warren, 2008). In contrast, the majority of physiological studies conducted on Fmr1 KO mice have suggested that AMPA-mediated synaptic transmission may be decreased, in specific regions of the brain and under specific conditions, in Fmr1 KO mice (Hu et al., 2008; Li et al., 2002; Pilpel et al., 2009). These results spurred us to investigate AMPA and NMDA-mediated excitatory postsynaptic currents using whole-cell electrophysiology in the DG of Fmr1 KO mice. We found a significant decrease in NMDA-mediated synaptic transmission in Fmr1 KO mice compared to WT littermates (Appendix D.1). We hypothesize that loss of FMRP decreases NMDA-mediated synaptic transmission and that this underlies impairments in bidirectional, NMDA-dependent synaptic plasticity.

To our knowledge, only two other published studies exist investigating AMPA and NMDA-mediated synaptic transmission in Fmr1 KO mice. First, Dr. Seeburg’s group has recently shown that the AMPA/NMDA (A/N) ratio is decreased in the CA1 subfield of Fmr1 KO mice (Pilpel et al., 2009). This effect was only observed in 2-week old mice, and not 6-7 week old
mice. The decreased A/N ratio appears to be primarily a function of decreased AMPA-mediated synaptic transmission. Similar to morphological data, it appears that loss of FMRP has more drastic effects on the developing nervous system. An alteration in A/N ratio in developing neurons is consistent with our observations in adult Fmr1 KO mice because the DG is continually producing young, developing neurons. In contrast to our findings of an increased A/N ratio due to decreased NMDA-mediated synaptic transmission, the direction of change of the A/N ratio was in the opposite direction than that reported from Seeburg’s group. This difference may relate to the fact that we have investigated granule neurons in the adult dentate gyrus, whereas Dr. Seeburg’s group investigated pyramidal neurons in the CA1 subfield of young mice.

A second article on this topic was published very recently (February 2010) in the journal Neuron showing that a critical period for impaired synaptic plasticity exists in the somatosensory cortex of Fmr1 KO mice (Harlow et al., 2010). These authors report that the A/N ratio increases between postnatal days 4 and 7 in WT mice, but decreases in Fmr1 KO mice. Alterations in both AMPA and NMDA currents appear to be responsible for this effect. It is unclear how these finding relate to that from Dr. Seeburg’s group due to potential differences in the stage of neuronal development within the respective subregions at the time of assessment.

We are aware of one other group with unpublished results that are similar to our results regarding AMPA and NMDA-mediated currents in the adult DG of Fmr1 KO mice. Yun, Park and Trommer presented an abstract at the Society for Neuroscience meeting in 2009 reporting decreased NMDA-mediated synaptic transmission in the DG of Fmr1 KO mice. This is consistent with our observation of an increase in the A/N ratio due to a decrease in the NMDA-mediated component.
In short, it appears that neurons developing without FMRP exhibit alterations in the ratio of AMPA to NMDA-mediated synaptic transmission. Discrepancies in the few emerging findings on this topic suggest that loss of FMRP may alter the direction and magnitude of the A/N ratio depending on brain region, cell type and neuronal age. The fact that the DG is unique in its ability to exhibit neurogenesis across the lifespan may confer a disproportionate susceptibility to the effects of loss of FMRP on synaptic transmission. Future studies should investigate how alterations in the A/N ratio during neuronal development are compensated for in mature neurons. Future studies should also investigate if alterations in the A/N ratio during neuronal development predispose to enhanced mGluR-LTD in mature neurons.
4.7 Conclusion

Fragile-X syndrome (FXS) is caused by loss of expression of the *Fmr1* gene to the Fragile-X Mental Retardation Protein (FMRP) (Pieretti et al., 1991; Verkerk et al., 1991). Key symptoms in FXS include severe intellectual disability and emotional lability (Hagerman, 2002). The *Fmr1* gene has a high degree of homology between mouse and human permitting the generation of a mouse model of FXS (*Fmr1* KO mice) (Kirkpatrick et al., 2001). Since the generation of the mouse model of FXS, robust impairments in synaptic plasticity associated with a clear learning impairment have been elusive (1994; Eadie et al., 2009). We noted that studies investigating the normal expression of *Fmr1* indicate that high levels of expression occur in cells in developing systems as well as granule neurons in the hippocampus (*i.e.*, the dentate gyrus; DG) (Hinds et al., 1993). Indeed, the DG can be considered to be in a constant state of development due to the established observation of its relatively unique ability to exhibit neurogenesis across the lifespan (Altman, 1963; Altman and Das, 1965; Christie and Cameron, 2006; Gage, 2002). The DG had been previously ignored in the study of *Fmr1* KO mice, and we hypothesized that an investigation of plasticity in the DG would reveal robust alterations in plasticity associated with impaired learning and memory.

We began with an attempt to replicate reports of subtle learning and memory impairments in *Fmr1* KO mice (1994; Eadie et al., 2009). We were unable to replicate putative learning and memory impairments. Instead, we noted decreased anxiety in *Fmr1* KO mice – a finding that seemed inconsistent with the human literature. Encouragingly, we noted that many researchers studying *Fmr1* KO mice had drawn the same conclusion from their experiments (Hayashi et al., 2007; Peier et al., 2000; Qin et al., 2002; Qin et al., 2005; Restivo et al., 2005; Spencer et al., 2005). Next, we studied the process of adult neurogenesis in the dentate gyrus (DG) *Fmr1* KO
mice. Although we did not observe substantial differences in the number of new neurons produced, we did observe some abnormalities in particular phases of neurogenesis. This was apparent in the ventral subregion of the hippocampus, a subregion that is suggested to be more closely associated with emotion compared to the dorsal hippocampus (Moser and Moser, 1998). This appeared to parallel our initial behavioral findings. We then hypothesized that the new neurons produced in the DG may be structurally abnormal. We found decreased dendritic length and complexity as well as decreased dendritic spine density on young dentate granule neurons. In other words, although the quantity of neurogenesis appeared unaffected by loss of FMRP, the quality of the new neurons appears to be markedly affected by this genetic manipulation.

Our initial studies of the physiology of the DG included an assessment of long-term potentiation (LTP) using field electrophysiology techniques. We found a significant attenuation (approximately 60%) of LTP providing the first functional data associated with the effects of loss of FMRP on the DG. We then showed that this form of synaptic plasticity is NMDA-dependent. We also found that LTD was abolished in the DG of Fmr1 KO mice. Similar to LTP, this form of synaptic plasticity was found to be NMDA-dependent in the DG.

We have very recently investigated the mechanism underlying impaired NMDA-dependent bidirectional synaptic plasticity. We have shown that NMDA-mediated synaptic transmission is significantly decreased in the DG of Fmr1 KO mice; whereas, AMPA-mediated synaptic transmission appears largely normal. We suggest that decreased NMDA-mediated synaptic transmission underlies impairments in NMDA-dependent bidirectional synaptic plasticity in the DG of Fmr1 KO mice.
We chose to assess *Fmr1* KO mice on a task dependent on functional NMDA receptors in the DG called context discrimination. Mice with a specific genetic deletion of NR1 (the obligatory subunit of the NMDA receptor) perform poorly on this task (McHugh et al., 2007). We found that *Fmr1* KO mice perform poorly on this task, similar to the DG-specific NR1 KO mice, revealing a learning impairment in the mouse model of FXS. We hypothesize that decreased NMDA-mediated synaptic transmission and plasticity in the DG underlie this finding. Interestingly, assessment of DG-specific NR1 KO mice on tests of anxiety has revealed that they are less anxious than controls (Barkus et al., 2010; Niewoehner et al., 2007). (Behavioral and electrophysiological similarities between *Fmr1* KO and DG-NR1 KO mice are shown in Appendix E.1 and E.2) Again, this is similar to our behavioral observations in *Fmr1* KO mice. Data is accumulating to suggest that impaired NMDA receptor functioning in the DG subfield of the hippocampus may be at the root of learning and emotional impairments in the mouse model of Fragile X syndrome. (A working model of effects of transcriptional repression of *Fmr1* on neuronal abnormalities in dentate granule neurons is shown in Appendix F.1)

In conclusion, this thesis demonstrates that loss of *Fmr1* expression markedly impairs structural and functional plasticity specifically in the DG subfield of the adult mammalian brain. Convergent data suggests that this may be related to the unique property of the DG to continually produce new neurons across the lifespan. Decreased NMDA-mediated synaptic transmission and plasticity appear to be the most markedly affected by loss of FMRP. Emerging evidence suggests that NMDA receptor hypofunction in the DG may be sufficient to account for the behavioral impairments in *Fmr1* KO mice. The most parsimonious theory of the pathophysiology of FXS appears to be NMDA receptor hypofunction in the DG subfield of the hippocampus.
4.8 References


Eadie BD, Zhang WN, Boehme F, Gil-Mohapel J, Kainer L, Simpson JM, Christie BR. 2009. Fmr1 knockout mice show reduced anxiety and alterations in neurogenesis that are specific to the ventral dentate gyrus. Neurobiol Dis.


Appendices

Appendix A - List of Publications


Parkinson PF, Kannangara TS, Eadie BD, Burgess BL, Wellington CL, Christie BR. Cognition, learning behaviour and hippocampal synaptic plasticity are not disrupted in mice over-expressing the cholesterol transporter ABCG1. Lipids Health Dis. 2009 Feb 24;8:5. PubMed PMID: 19239689; PubMed Central PMCID: PMC2654451.


Zhao X, Ueba T, Christie BR, Barkho B, McConnell MJ, Nakashima K, Lein ES,

12634277. (* denotes co-first author.)
Appendix B - Ethics Approval

The University of British Columbia

ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A04-1011
Investigator or Course Director: Brian R. Christie
Department: Psychology
Animals: Mice 280
Approval Date: 2005-2-4

Funding Sources:

<table>
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<tr>
<th>Funding Agency</th>
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<tr>
<td>Canadian Institutes of Health Research</td>
<td>Role of Kainate Receptors in synaptic physiology in the hippocampal dentate gyrus.</td>
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<tr>
<td>BC Ministry of Children and Family Development</td>
<td>Ultrastructural Analysis of Neuronal and Synaptic Development in Fragile-X Syndrome</td>
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Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

https://rise.ubc.ca/rise/Doc/0/761T814T181KD8A3OM71M9U18F/fromString.html 15/02/2010
REQUEST FOR CONTINUATION OR AMENDMENT OF AN APPROVED PROJECT

Please submit 14 copies (signed original plus 13 photocopies) of the request containing appropriate pages from the Application to Use Animals for Research/Teaching and all attachments to the Animal Care Committee, Office of Research Services. Include only those pages that indicate changes from the original protocol. Compliance with the original protocol is mandatory, except for amendments indicated in this application.

Current ACC Protocol Number: 2007-006
Application for: Amendment to the original application.
Continuation with NO changes.
Continuation with personnel changes only.

Short Title of Project: Effects of Exercise on structural and functional plasticity in the hippocampus
Principal Investigator/Instructor: Brian Christie
Academic Rank: Associate Professor
Uvic Faculty/Department: Division of Medical Sciences and Dept. of Biology

Office Address:
Medical Sciences Building, Room 218
University of Victoria
Victoria BC V8W 2Y2

Telephone Number (Office): 250-472-4244
Fax Number: 250-472-5505

E-Mail Address: declaration: I, the undersigned, assure that all animals used in this proposal will be cared for in accordance with the principles promulgated by the Canadian Council on Animal Care and the University of Victoria.

Name: Brian Christie
Date: 20-May-2009

For Office of Research Services Use Only

Start Date: 26 May 2009
End Date: 01 May 2010

Protocol Description (Lay Summary: Suitable for Press Release)
In lay terms provide a brief description of the research and the procedures to be used.

Protocol Changes

Changes to protocol using the Application to Use Animals For Teaching Form (Check appropriate boxes where changes occur.)

Justification:
Addition of personnel only: Jairus Streight, Namat Majiaess, Larissa Szalvik, Lindsay Dodd, Crystal Bostrom (see section 15 of new animal care application form: attached)

Revised: July 15, 2000
Application to Use Animals for Research

Please note, you must use Adobe Acrobat 7 or higher (not Reader) in order to save and submit this form. Click on blue text to display additional information.

1. Project

Title (including major species, e.g. rat, mouse)
Mouse breeding protocol

STATUS
☐ New Application
☒ Renewal of Protocol #: 2008-016(1)
☐ Pilot Project
☐ Amendment of Protocol #: 2008-016(1)

2. Contact Information

PRINCIPAL INVESTIGATOR
Surname: Christie
First Name: Brian
Initial: R.
Rank / Position (please indicate): Associate Professor
Department: Division of Medical Sciences and Dept of Biology
Business Telephone: 250-472-4244
Laboratory Telephone: 250-721-8798
Residence Telephone: 260-477-4973
Emergency Telephone: Pager / Cell Telephone: 250-544-0569 (res)
E-mail Address: brain64@uvic.ca

Laboratory Address
Medical Sciences Building, Room 250

NAME OF DESIGNATED ALTERNATE FOR EMERGENCIES
A. (mandatory)
Surname: Wiebe
First Name: Evelyn
Emergency Telephone: 250-544-0569 (res)

B. (optional)
Surname
First Name
Emergency Telephone

3. Declaration

The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of University of Victoria. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be renewed on an annual basis.

Principal Investigator: Brian Christie
Date: May 2009

UVic Department Chair (Required for all Applications): Signature
Date: Nov 2009

Oscar Casiro
When the Department Chair is the Principal Investigator, the signature of the Dean is required

4. Approvals

University Veterinarian Signature
Chairman, University Animal Care Committee Signature
# Appendix C - SHIRPA

Table C.1 General assessment of the behavior of *Fmr1* KO mice.
Significant differences were not observed.

## SHIRPA Summary

![Table C.1](image-url)

## Startle

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## LeftRef

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<tr>
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Appendix D - AMPA and NMDA-mediated EPSCs in the DG of Fmr1 KO Mice

Figure D.1 AMPA and NMDA-mediated currents

Recorded neurons were filled with 0.2% biocytin and labeled with a fluorescent probe after recordings to confirm the recording from granule neurons and assess the gross morphology of neurons. Qualitative assessment of the morphology of dentate granule neurons from Fmr1 KO and WT mice did not reveal obvious differences. (a) Input-Output (IO) function of AMPA-mediated excitatory postsynaptic currents (EPSCs). The amplitude of the EPSC increased equivalently in both genotypes with increasing afferent stimulation (increasing pulse width from 0 to 300 µS). Sample AMPA-mediated EPSCs from both Fmr1 KO and WT mice at approximately 50% of maximum amplitude are shown. (b) Input-Output (IO) function of NMDA-mediated excitatory postsynaptic currents (EPSCs). The amplitude of the EPSC increased equivalently in both genotypes with increasing afferent stimulation (increasing pulse width from 0 to 300 µS). Sample NMDA-mediated EPSCs from both Fmr1 KO and WT mice at approximately 50% of maximum amplitude are shown. Vertical scale bar = 50 pA. Horizontal scale bar = 50 ms. (c) Histogram showing decreased NMDA-mediated EPSCs in Fmr1 KO mice compared to controls, and no differences in AMPA-mediated EPSCs. (d) The average AMPA/NMDA ratio from neurons recorded from Fmr1 KO mice is significantly higher compared to WT mice.
Appendix E - Behavioral and Electrophysiological Similarities Between *Fmr1* and DG-NR1 KO Mice

Table E.1 Behavioral similarities between *Fmr1* KO mice and DG-NR1 KO mice.

<table>
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<th><em>Fmr1</em> KO mice</th>
<th>DG-NR1-KO mice</th>
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<tr>
<td>Learning Ability</td>
<td>• No impairment on acquisition of the Morris water maze</td>
<td>• No impairment on acquisition of the Morris water maze</td>
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<tr>
<td></td>
<td>• No impairment in the acquisition of contextual fear conditioning</td>
<td>• No impairment in the acquisition of contextual fear conditioning</td>
</tr>
<tr>
<td></td>
<td>• <em>Impairment in context discrimination learning</em></td>
<td>• <em>Impairment in context discrimination learning</em></td>
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<tr>
<td>Anxiety</td>
<td>• Apparent decrease in anxiety on the open field</td>
<td>• Apparent decrease in anxiety on the open field</td>
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</table>

Table E.2 Electrophysiological similarities between *Fmr1* KO mice and DG-NR1 KO mice.

<table>
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<th></th>
<th><em>Fmr1</em> KO mice</th>
<th>DG-NR1-KO mice</th>
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</thead>
<tbody>
<tr>
<td>LTP &amp; LTD</td>
<td>• No impairment in NMDA-dependent LTP in the CA1 subfield of the hippocampus</td>
<td>• No impairment in LTP in the CA1 subfield of the hippocampus</td>
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<tr>
<td></td>
<td>• Attenuated NMDA-dependent LTP in the DG subfield of the hippocampus</td>
<td>• Abolished LTP in the DG subfield of the hippocampus</td>
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<tr>
<td></td>
<td>• Attenuated NMDA-dependent LTD in the DG subfield of the hippocampus</td>
<td>• LTD?</td>
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<tr>
<td>Paired-pulse plasticity</td>
<td>• No change in paired pulse plasticity</td>
<td>• No change in paired pulse plasticity</td>
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<tr>
<td>NMDA &amp; AMPA-mediated EPSCs</td>
<td>• Decreased NMDA-mediated EPSCs</td>
<td>• ?</td>
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Appendix F - Working Model

F.1 Working model of effects of loss of FMRP on dentate granule neurons.

At the neural level, we hypothesize that key symptoms in Fragile-X syndrome are caused by decreased expression of key synaptic molecules in dentate granule neurons.

At the genetic level, Fragile-X syndrome is caused by transcriptional repression of the Fmr1 gene.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>blue</td>
<td>Protein</td>
</tr>
<tr>
<td>*</td>
<td>mRNA and protein for synaptic molecules (e.g., NR1, NR2B, PSD-95).</td>
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