NEURAL PRECURSOR CELL BIOLOGY IN THE *FMR1*-KO MOUSE

NEURAL PRECURSOR CELL BIOLOGY IN THE POSTNATAL *FMR1*-KNOCKOUT MOUSE HIPPOCAMPUS

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Lay Abstract

Fragile X syndrome is the leading inherited cause of intellectual impairment and autism spectrum disorder. The syndrome is caused by a defect in one gene. This gene has been suggested to play a role in regulating the birth of new brain cells termed neural precursor cells. The importance of neural precursor cells stems from their ability to generate neurons and glia, the main cells in the brain. In this thesis, I focus on studying neural precursor cells from the hippocampus, a brain region important for learning and memory. A mouse model was used to compare neural precursor cells from healthy and Fragile X mice during early postnatal development. I found that neural precursor cells do not divide as much as they should in the Fragile X mouse hippocampus. The results help to determine the causes for learning and memory deficits in Fragile X and potentially open avenues for intervention.

Abstract

The regulation of neural precursor cells (NPCs), which encompass neural progenitor and neural stem cells (NSCs), is fundamental for proper brain development and function. These cells are regulated by orchestrated signalling within their local environment. Aberrant aspects of cell proliferation, differentiation, survival, or integration have been linked to various neurological diseases including Fragile X syndrome (FXS)—a disorder characterized by intellectual and social changes due to the silencing of the gene encoding FMRP. The biology of hippocampal NPCs in FXS during early postnatal development has not been studied, despite high FMRP expression levels in the hippocampus at the end of the first postnatal week. In this thesis, the *Fmr1*-knockout (KO) mouse model was used to study hippocampal cell biology during early postnatal development. A tissue culture assay, used to study the effect of astrocyte-secreted factors on the proliferation of NSCs, indicated that astrocyte secreted factors from *Fmr1*-KO brains enhanced the proliferation of wild type, but not *Fmr1*-KO NSCs (Chapter 3). Next, the proliferation and cell cycle profiles of NPCs *in vitro* and *in vivo* studied with immunocytochemistry, Western blotting, and flow cytometry revealed decreased proliferation of NPCs in the *Fmr1*-KO hippocampus (Chapter 4). Finally, cells isolated from the P7 dentate gyrus and characterized by flow cytometry, showed a reduced proportion of NSCs and an increased proportion of neuroblasts—neuronal committed progenitors—in *Fmr1*-KO mice. Together, these results indicate that hippocampal NPCs show aberrant proliferation and neurogenesis during early postnatal development. This could indicate stem-cell depletion,

increased quiescence, or a developmental delay in relation to lack of FMRP and uncovers a new role for FMRP in the early postnatal hippocampus. In turn, elucidating the mechanisms that underlie FXS will aid in the development of targeted treatments.

Acknowledgments

I would like to extend my sincere gratitude to many people, without whom this thesis would not have been possible. First and foremost, I am grateful to my supervisor and mentor, Dr. Laurie Doering, for his guidance over the past five years and for giving me the freedom to explore new questions and techniques. His presence and support throughout my project enabled me to accomplish the work enclosed herein. I would also like to extend my thanks to my committee members, Dr. Ram Mishra and Dr. Jane Foster, who guided me in my scientific endeavours and provided me with constructive feedback throughout the process.

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Abbreviations

2D DIGE	two-dimensional difference in gel electrophoresis
ACM	Astrocyte conditioned media
AGG	Adenosine-guanine-guanine
ANOVA	Analysis of variance
APC	Allophycocyanin
BLBP	Brain lipid-binding protein
BMP	Bone morphogenetic protein
BV	Brilliant violet
CA	Cornu Ammonis
CD	Cluster of differentiation
CGG	Cytosine-guanine-guanine
CNS	Central nervous system
CpG	5'—C—phosphate—G—3'
CTBP1	*C-Terminal Binding Protein 1*

DAPI	4’,6-diamidino-2-phenylindole
DG	Dentate gyrus
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
E	Embryonic day
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorting
FEZ2	Fasciculation and elongation protein zeta 2
FITC	Fluorescein isothiocyanate
FMR1	*Fragile X mental retardation 1*
FGF	Fibroblast growth factor
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
G_1	Gap 1

G_2	Gap 2
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GSK3β	Glycogen synthase kinase 3β
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
KO	Knockout
M	Mitosis
MITF	Microphthalmia-associated transcription factor
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NCFC	Neural colony forming cell
NMDAR	N-methyl-D-aspartate receptor
NPCs	Neural precursor cells
NSCs	Neural stem cells

P	Postnatal day
PBS	Phosphate buffered saline
PE	Phycoerythrin
PhH3	Phospho-histone H3
PSA-NCAM	Polysialylated neuronal cell adhesion molecule
PVDF	Polyvinylidene fluoride
PTEN	Phosphatase and tensin homolog
RGCs	Radial glial cells
RIPA	Radioimmunoprecipitation assay
RBPJκ	Recombination signal binding protein for immunoglobulin kappa J
S	Synthesis
SEM	Standard error of the mean
SGZ	Subgranular zone
SHH	Sonic hedgehog
SOX2	Sex-determining region Y-box 2

SSRIs	Selective serotonin re-uptake inhibitors
SVZ	Subventricular zone
TAPs	Transit amplifying progenitor cells
TBST	Tris-buffered saline Tween-20
TN-C	Tenascin-C
TSC2	Tuberous sclerosis complex 2
VZ	Ventricular zone
Wnt	Wingless-type MMTV integration site family
WT	Wild type

Declaration of Academic Achievements

The author would like to acknowledge the following individuals for their contributions:

Dr. Hongjin Huang for 2D DIGE and mass spectrometry experiments (Chapter 3), Emily L. Taylor for help with neurosphere cultures and immunocytochemistry (Chapter 4), Krupesh Patel for help with immunocytochemistry (Chapter 4), Ashley Chen for help optimizing a flow cytometry protocol (Chapter 5), Hong Liang for technical assistance with flow cytometry (Chapters 4 & 5), Dr. Jane A. Foster for statistical advice (Chapters 3 & 4), and Dr. Angela Scott for critical reading and comments on the manuscript enclosed in Chapter 3.

All other data collection, analysis, interpretation, writing and revisions were done by the author under the guidance of Dr. Laurie C. Doering.

Chapter 1: Introduction

Fragile X Syndrome

Fragile X syndrome is the leading monogenic cause of intellectual impairment and autism spectrum disorder (Wang et al. 2012). It occurs with a frequency of 1 in 7,143 males and 1 in 11,111 females in the total population (Hunter et al. 2014). Due to its X-linked heritability, males present with more severe symptoms that include developmental delay, cognitive and memory impairments, hyperactivity, epilepsy, hypersensitivity to sensory stimuli, hand biting and flapping, and a range of physical features such as a long face, prominent ears, hyperextensible finger joints, and machroorchidism (Penagarikano et al. 2007; Hagerman et al. 2014). The majority of females with FXS have less severe cognitive problems with low-normal intelligence, but tend to have anxiety and emotional problems (Garber et al. 2008). The presentation and severity of FXS varies between individuals.

History

FXS was first characterized in 1943 when Martin and Bell constructed a pedigree of an X-linked intellectual disorder (Martin and Bell 1943). More than two decades later, Lubs (1969) discovered a constriction in the long arm of the X chromosome of family members with an X-linked inheritance pattern of intellectual impairment (Lubs 1969). The ability to visualize the constriction, or fragile site as the term was coined (Magenis et al. 1970), depended on the culture medium used (Sutherland 1977). The fragile site was

later mapped to Xq27.3 (Harrison et al. 1983). It became increasingly apparent that FXS does not follow simple Mendelian inheritance: carriers of the Fragile X chromosome are not always affected and the incidence of FXS within families increases over successive generations. This was known as the Sherman paradox (Sherman et al. 1984; Sherman et al. 1985). The Sherman paradox was resolved when the genetic basis of FXS was elucidated.

Genetic Basis

The identification of the *FMR1* gene, which was mapped to the X chromosome constriction site previously identified (Verkerk et al. 1991), started a revolutionary era in Fragile X research. Research showed that the 5`-untranslated region of *FMR1* contains a CGG trinucleotide repeat, whose size is polymorphic within the population (Fu et al. 1991). Normal alleles are stable and contain 5 – 45 CGG repeats, with a mode of 30 repeats. CGG repeat sizes of 45 – 55 trinucleotides are classified as gray zone alleles, and have a propensity to expand to premutation alleles in future generations (Fernandez-Carvajal et al. 2009). Premutations contain 56 – 200 CGG repeats, which are unstable and expand in size in subsequent generations, particularly with increasing maternal age and decreasing number of AGG interruptions in the CGG repeat locus (Yrigollen et al. 2014). Premutations have their corresponding phenotypes. Specifically, premutation carriers are at risk of developing Fragile X tremor/ataxia syndrome, which more commonly affects male carriers (Jacquemont et al. 2003), while females are also at risk of developing Fragile X-associated primary ovarian insufficiency (Sullivan et al. 2005). When CGG

repeat sizes exceed 200 trinucleotides upon maternal transmission, FXS ensues due to the hypermethylation of the CGG repeats and the upstream CpG island (Oberlé et al. 1991). Consequently, *FMR1* is epigenetically silenced leading to the loss of its encoded protein, FMRP (Pieretti et al. 1991). Incomplete methylation or a full mutation and premutation mosaicism results in less severe symptoms of FXS (Tassone et al. 1999), explaining the varied clinical presentation between individuals.

Fmr1-knockout Mouse Model

Great strides have been made in Fragile X research due to the generation of the Fragile X mouse model. The highly conserved nature of *FMR1* across species (Verkerk et al. 1991) makes FXS amenable to study in animal models. Murine FMRP shows 97% homology in amino acid sequence to its human counterpart (Ashley, Sutcliffe, et al. 1993). In addition, FMRP has similar expression patterns in terms of timing and tissue specificity in mice and humans (Hinds et al. 1993). The Fragile X mouse model was generated by inserting a neomycin cassette in exon 5 of *Fmr1* (Bakker et al. 1994). Although the Fragile X mouse model has the *Fmr1* gene knocked out while the human condition results from a CGG trinucleotide repeat expansion, both the mouse model and the human are functionally similar in that no FMRP is produced (Bakker et al. 1994). In fact, *Fmr1*-KO mice recapitulate many of the phenotypes of the human condition such as learning deficits (Dobkin et al. 2000), macro-orchidism, increased risk of seizures (Bakker et al. 1994), hyperactivity (Oostra and Hoogeveen 1997), abnormalities in synaptogenesis and synaptic structures, and an abundance of immature dendritic spines (Grossman et al.

2006). Thus, the *Fmr1*-KO mouse provides a good model to study FXS and autism spectrum disorder, with the potential to extend findings to neurodevelopmental disorders at large.

Functional Significance of FMRP

FMRP is expressed in virtually all mammalian tissues, with highest expression levels in the brain and testes (Devys et al. 1993). It is highly expressed in the hippocampus and its expression was originally believed to be restricted to neurons (Feng et al. 1997), but was later confirmed in astrocytes of the developing brain (Pacey and Doering 2007). FMRP plays an important role in the post-transcriptional regulation of several proteins by virtue of its RNA binding capacity. In fact, FMRP binds up to 4% of mRNAs in the brain (Ashley, Wilkinson, et al. 1993) and was thus far shown to have 842 mRNA targets (Darnell et al. 2011). In addition, FMRP regulates the stability (Zalfa et al. 2007; De Rubeis and Bagni 2010), transport (Feng et al. 1997), and translation of mRNAs (Darnell et al. 2011) especially those involved in synaptic plasticity (Antar et al. 2004). More recent research has indicated that FMRP is necessary for the proper biology and function of neural precursor cells (**Figure 1-1**); reviewed in (Callan and Zarnescu 2011).

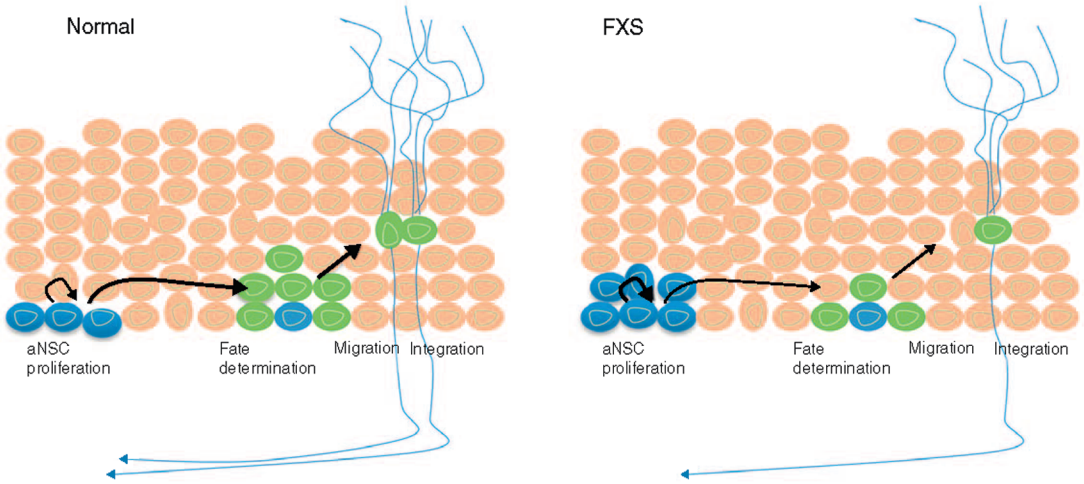

Figure 1-1. FMRP governs NPC biology. Previous reports have indicated increased proliferation of adult NSCs and decreased neurogenesis in the hippocampus of FXS disease models. Abbreviations: aNSCs (adult neural stem cells), FXS (Fragile X syndrome), NPC (neural precursor cell). Modified from (Wang et al. 2012).

FMRP and Neural Precursor Cells

NPCs show altered proliferation, differentiation, and survival in Fragile X. However, the biology of NPCs in Fragile X is not well characterized with a few research groups studying the topic. Not surprisingly, the results are not all in agreement due to the different methods, developmental stages, brain regions, and mouse strains used. Thus far, some findings indicate increased NPC proliferation in the Fragile X embryonic, early postnatal ventricular wall (Castrén et al. 2005; Tervonen et al. 2009), and DG of adult mice (Luo et al. 2010; Guo et al. 2011). Yet, others have found no differences in the proliferation of NPCs in the adult mouse DG (Eadie et al. 2009), or in human NPCs derived from fetal cortex (Bhattacharyya et al. 2008). Reports of aberrant NPC differentiation in Fragile X were also inconsistent. Findings of increased astrocytic differentiation and decreased neuronal differentiation (Luo et al. 2010; Guo et al. 2011; Telias et al. 2013) contrasted with those of increased neuronal differentiation (Eadie et al. 2009) and decreased astrocytic differentiation (Castrén et al. 2005; Eadie et al. 2009). Interestingly, the increase in the number of neurons born in the DG of *Fmr1*-KO mice is coupled with an increase in their apoptosis, resulting in no net differences in the proportions of new neurons that survive to maturity in the Fragile X brain (Eadie et al. 2009).

FMRP has also been shown to suppress the transition of radial glial cells—which act as precursor cells during cortical neurogenesis—to intermediate progenitor cells during cortical development. Consequently, lack of FMRP results in radial glial cell

depletion by an actin-dependent mechanism (Saffary and Xie 2011). Compelling evidence also indicates that *Fmr1* is enriched in neuroblasts expressing PSA-NCAM (Pennartz et al. 2004), suggesting that FMRP is necessary for neuroblast regulation. Another line of research shows that NPCs of the *Fmr1*-KO DG generate neurons with impaired functionality. Indeed, neurons show impaired NMDAR-dependent synaptic plasticity in the DG, but not in the CA1 region of the hippocampus of adult *Fmr1*-KO mice (Bostrom et al. 2015). In turn, impaired NMDAR-dependent activity can in itself lead to NPC dysregulation as NMDAR-dependent activity has been inversely correlated with NPC proliferation in the DG (Zhao et al. 2008). These findings all point to the involvement of FMRP in NPC regulation throughout life.

Deficits in hippocampus-dependent learning such as exaggerated inhibitory avoidance extinction (Dölen et al. 2007) and trace conditioning tasks (Hayashi et al. 2007) point to NPC regulation defects in the hippocampi of *Fmr1*-KO mice. NPCs contributing to lifelong neurogenesis in the DG are suggested to underlie hippocampal learning and memory (Deng et al. 2010; Snyder and Cameron 2012). More compelling evidence shows that conditional ablation of FMRP in adult NPCs results in learning deficits on the trace conditioning and delayed non-matching-to-place radial arm maze tasks, both of which require an intact hippocampus, while conditional FMRP restoration results in improved performance on these tasks (Guo et al. 2011). Studies using *Fmr1*-KO rats also corroborate results obtained with the mouse model, confirming impairments in hippocampus-dependent, but not hippocampus-independent, associative recognition

memory tasks (Till et al. 2015). Together, these findings suggest that the characteristic learning and memory impairments in FXS could at least be partially attributed to the dysfunction of hippocampal NPCs and that functional restoration of hippocampal neurogenesis may alleviate those impairments.

The dearth of NPC research in FXS is complicated by the fact that there are no studies of hippocampal NPCs during early postnatal development although the end of the first postnatal week marks the highest FMRP expression levels in the rodent hippocampus (R. Lu et al. 2004). Coincidentally, the granular layer of the rodent DG forms by the end of the first postnatal week (Bayer 1980a; Bayer 1980b) and most granule neurons are generated postnatally (Altman and Bayer 1990a). In fact, it is the NPCs that reside in the DG as it forms that are responsible for life-long neurogenesis (Mathews et al. 2010). Thus, it follows that aberrant regulation of NPCs during DG development could result in lifelong neurogenesis impairments. All these highlight the importance of studying hippocampal NPCs in relation to their microenvironment early on in postnatal development.

Neural Precursor Cells

NPCs are heterogeneous and encompass populations of NSCs and progenitor cells. NSCs differ from progenitors in that the former can self-renew, generate a large number of progeny, and are multipotent, giving rise to neurons, astrocytes, and oligodendrocytes, while the latter have limited lineage potential and self-renewal ability. Interest in NPCs has soared after the discovery of lifelong neurogenesis in the DG (Altman and Das 1965),

as well as the SVZ of the lateral ventricles (Alvarez-Buylla et al. 2002). Continuous neurogenesis challenged the central dogma of neurobiology that no new neurons are born in the adult mammalian brain (Cajal 1928) and was first met with skepticism until corroborated in the 1990s. The translational impact of adult hippocampal neurogenesis became clear with its confirmation in the human brain (Eriksson et al. 1998). While the exact role of adult neurogenesis in the DG is still debatable, it is suggested to enable the long-accepted role of the hippocampus in learning and memory; reviewed in (Deng et al. 2010; Snyder and Cameron 2012). In agreement with this, knockdown of adult neurogenesis in the rat DG is associated with impaired spatial and object recognition memory (Jessberger et al. 2009). Not surprisingly, aberrant neurogenesis is characteristic of several neurological disorders, such as epilepsy, stroke, Alzheimer's, Parkinson's, in addition to Fragile X, which underscores the importance of NPC regulation; reviewed in (Zhao et al. 2008).

Neural Stem Cells in the Embryonic Brain

Radial glial cells are the principal neural stem cells in the embryonic brain, generating both neurons and macroglia, i.e. astrocytes and oligodendrocytes; reviewed in (Kriegstein and Alvarez-Buylla 2009). Neurogenesis precedes gliogenesis. Cortical neurogenesis begins at about E9-10 in mice when neuroepithelial cells transform into radial glia. At that point, neuroepithelial cells lining the ventricles acquire glial features and express the glial markers GLAST, BLBP, and TN-C, reviewed in (Campbell and Götz 2002), and the intermediate filament proteins Nestin, Vimentin, RC1, and RC2. They also begin to

contact endothelial cells of the vasculature. Neuroepithelial cells and RGCs undergo interkinetic nuclear migration, which involves the migration of nuclei between the apical and basal surfaces of the ventricular zone depending on their cell cycle phase. Nuclei in the G1 phase are found in the mid region between the apical and basal surfaces and migrate close to the apical surface of the VZ by the S phase. Nuclei go back to the mid region in the G2 phase, and they reach the ventricular surface by the M phase. It has been proposed that interkinetic nuclear migration regulates the exposure of nuclei to proliferative or neurogenic signals by virtue of the apical-basal Notch gradient (Del Bene et al. 2008). Radial glia undergo asymmetric divisions to self-renew and generate one daughter neuron or intermediate progenitor cell (Noctor et al. 2004). In turn, intermediate progenitor cells divide symmetrically either to produce two intermediate progenitor cells or two neurons (Noctor et al. 2004). After cortical neurogenesis is complete, most RGCs terminally differentiate into astrocytes and migrate away from the VZ (Noctor et al. 2008) upon the downregulation of proneural genes such as Ngn2. Notch signalling plays a role in the switch from neurogenesis to gliogenesis. In fact, neuron committed progenitors express the Notch ligands Delta-like 1 and Jagged 1. In turn, this activates Notch signalling and promotes gliogenesis (Namihira et al. 2009). Thus, Notch signalling blocks neurogenesis and allows gliogenesis to take place instead (Itoh et al. 2003).

DG Development

The DG undergoes a prolonged period of development that starts in the embryonic period and continues postnatally (**Figure 1-2**); reviewed in (Rolando and Taylor 2014). At about

E13, hippocampal RGCs proliferate in the dentate neuroepithelium (Altman and Bayer 1990b), adjacent to the cortical hem. The importance of the cortical hem in patterning the hippocampus stems from its release of instructive signals such as Wnt (Galceran et al. 2000) and BMP (Furuta et al. 1997; Grove et al. 1998). In fact, cortical hem-derived Cajal-Retzius cells play an important role in the migration of NSCs in the developing DG through the secretion of Reelin (Förster et al. 2002; Sibbe et al. 2009). By E15.5, RGCs migrate through the dentate migratory stream toward the fimbriodentate junction, a transient neurogenic zone that maintains NPCs in an undifferentiated state (Li et al. 2009). Migrating RGCs form the secondary dentate matrix and split into two routes. The first involves RGCs and committed neuronal progenitors lining the hippocampal fissure, which leads to the formation of the subpial zone. In turn, this gives rise to the outside layer of granule cells of the suprapyramidal blade of the DG at around E17.5. The second route of RGCs and progenitors populates the future hilus, which is the main proliferative zone in the late embryonic and early postnatal periods, known as the tertiary dentate matrix (Altman and Bayer 1990a). The majority of DG granule cells are born between P0 – P4 (Bayer 1980a; Bayer 1980b) and NPCs responsible for DG adult neurogenesis populate the DG by the end of the first postnatal week (Mathews et al. 2010). The tertiary dentate matrix disappears by the end of the second postnatal week. Subsequently, DG neurogenesis is restricted to the SGZ of the DG, which is fully formed by P30. Recent research has shown that embryonic and postnatal NPCs that give rise to the DG express GFAP similar to NPCs responsible for adult neurogenesis in the DG (Seki et al. 2014).

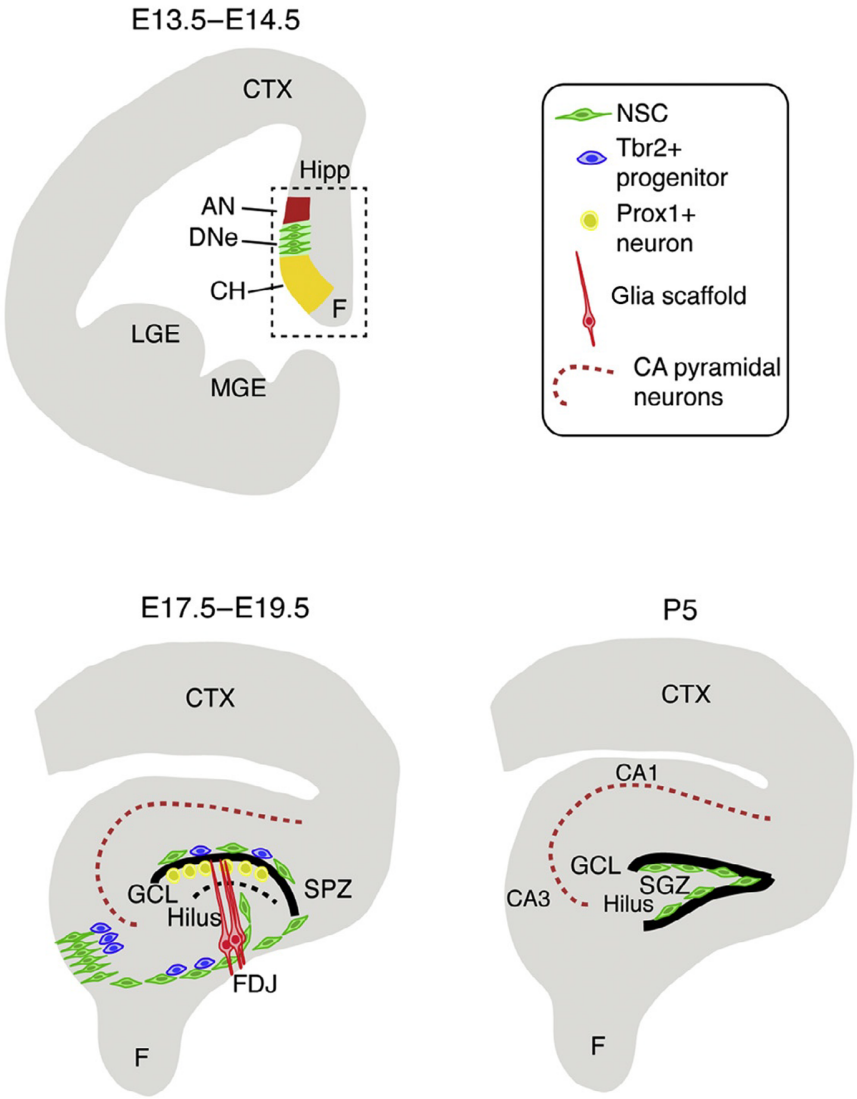

Figure 1-2. DG morphogenesis. Abbreviations: CH (cortical hem), DNe (dentate neuroepithelium), GCL (granule cell layer), FDJ (fimbriodentate junction), SGZ (subgranular zone), SPZ (subpial zone). Modified from (Rolando and Taylor 2014).

NPC Differentiation in the Adult DG

NPC maintenance, proliferation, differentiation, survival, and functional integration take place as part of a highly orchestrated process that includes a few cell divisions. Given that hippocampal neurogenesis has been better characterized than gliogenesis, it is used here as an example to explain the progression of NSCs to mature neurons (**Figure 1-3**). Quiescent NSCs (type-1) are activated and express GFAP, GLAST, and Nestin (Pastrana et al. 2009; Jungblut et al. 2012; Mich et al. 2014). NSCs divide asymmetrically to give rise to mitotic transit amplifying progenitor cells (type-2a). In turn, transit amplifying progenitor cells—still $Nestin^{+}$ and $GLAST^{+}$ (Pastrana et al. 2009; Jungblut et al. 2012)—commit to the neuronal lineage, and start expressing PSA-NCAM and Doublecortin as they divide (type-2b), generating neuroblasts (type-3). Neuroblasts express PSA-NCAM and Doublecortin, but not Nestin or GLAST. As neuroblasts exit the cell cycle, they become postmitoic and do not express the migrating cell markers, Doublecortin or PSA-NCAM. The now mature granule neurons functionally integrate in the DG neural circuit (van Praag et al. 2002); reviewed in (Kempermann et al. 2004). Newborn neuron survival is another variable regulated during neurogenesis. Under normal conditions, about 50% of newborn neurons die within 4 weeks after birth. One of the factors promoting neuronal survival is intrinsic NMDAR activity (Tashiro et al. 2006). This is intriguing given impaired NMDAR-dependent plasticity in the *Fmr1*-KO DG (Bostrom et al. 2015) along with increased newborn neuron death in the ventral hippocampus of *Fmr1*-KO adult mice (Eadie et al. 2009). Together, these findings indicate that impairments in NMDAR-

dependent plasticity may underlie the increased apoptosis of immature neurons in the *Fmr1*-KO DG.

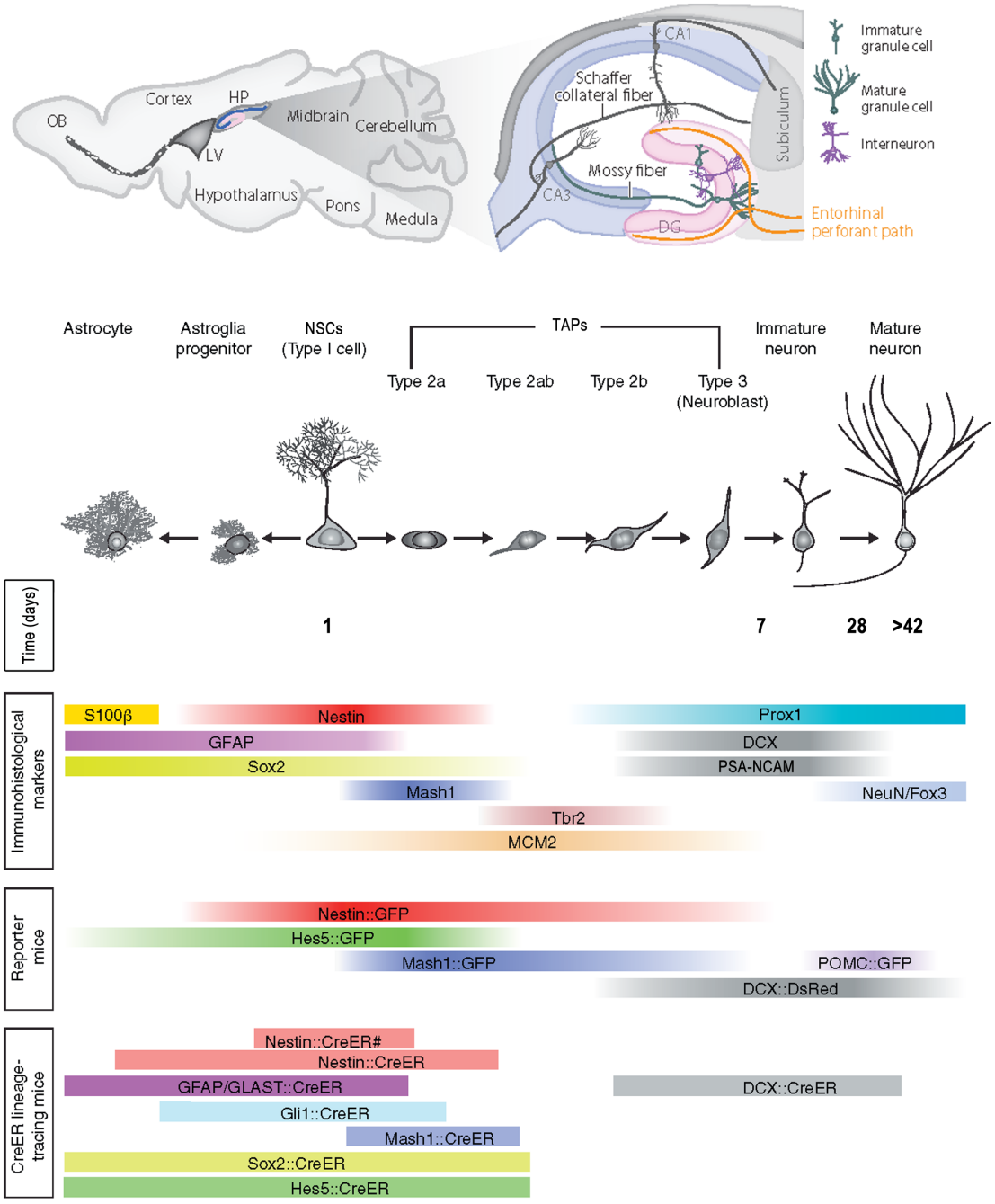

Figure 1-3. Histological expression of markers of hippocampal NSCs committing to different lineages. Abbreviations: NSCs (neural stem cells), TAPs (transit amplifying progenitor cells). Modified from (Bonaguidi et al. 2012; Christian et al. 2014).

NPC Heterogeneity

The heterogeneity of NPCs is evident from differences in their morphology, antigenicity, or their divergent responses to stimuli. For instance, slow-dividing NSCs are label retaining and are resistant to ablation by antimitotic agents, in contrast to neural progenitors and neuroblasts (Seri et al. 2004). Thus, after acute ablation of neurogenesis, NSCs are recruited to the cell cycle to repopulate the DG. Recent findings suggest that NSCs alone can have a radial or a horizontal morphology in the DG (Suh et al. 2007), and that most activated NSCs have a horizontal morphology compared to their quiescent (G_0) counterparts which have a radial morphology (Lugert et al. 2010). Despite the differences in morphology between active and quiescent NSCs, they both depend on Notch activity for their maintenance (Lugert et al. 2010). Under normal physiological conditions, only active NSCs contribute to neurogenesis. However, quiescent and active NSCs differentially respond to environmental stimuli. For instance, it has been shown that physical activity recruits quiescent, but not active, NSCs into the cell cycle, whereas seizures increase the proliferation of both active and quiescent NSCs (Lugert et al. 2010). These findings show the distinct roles of NPC populations in the postnatal brain, further highlighting the intricate balance that exists in maintaining the different populations. Notch signalling is emerging as an important pathway in mediating this fine balance.

Notch1

It has long been accepted that canonical Notch signalling maintains NPCs in an undifferentiated state. The traditional view was that differentiating neurons express Notch

ligands, which in turn activate Notch receptors on neighbouring cells. Once Notch receptors are activated in cells, they remain in an undifferentiated state in a process known as lateral inhibition (Pierfelice et al. 2011). Since then, other essential roles of Notch signalling have emerged including the regulation of self-renewal, cell cycle exit, cell proliferation, cell death, and binary fate choices of NPCs during development and in adult neurogenic regions (Yoon and Gaiano 2005; Breunig et al. 2007; Ables et al. 2010; Ehm et al. 2010). Interestingly, Notch signalling has also been shown to promote gliogenesis (Furukawa et al. 2000; Gaiano et al. 2000; Tanigaki et al. 2001), and inhibit the expression of proneural genes (Lundkvist and Lendahl 2001). Hence, the complexity of the Notch pathway explains its involvement in disease phenotypes (**Figure 1-4**). For instance, human embryonic Fragile X stem cell lines show aberrant regulation of Notch1 expression (Telias et al. 2013). Similarly, expression of the transcription co-repressor *CTBP1*—which regulates targets of Notch and Wnt signalling—is altered in human NPCs derived from the cerebral cortex of a week 14 FXS fetus (Bhattacharyya et al. 2008).

In addition to its well-established role during embryonic development, Notch signalling is essential in adult neurogenesis (Lugert et al. 2010). In keeping with this, the expression of Notch pathway components was detected in germinal zones and in neurons of the postnatal brain. Indeed, postnatal overexpression of Notch1 in $GFAP^{+}$ cells results in NPCs not exiting the cell cycle (Breunig et al. 2007). Likewise, conditional ablation of RBPJκ, a transcriptional mediator of Notch signalling, in NPCs initially led to increased neurogenesis followed by decreased neurogenesis and impaired self-renewal of NSCs in

the adult hippocampus (Ehm et al. 2010; Lugert et al. 2010). These findings show that Notch activity is indispensable for regulating NSC self-renewal. A possible mechanism underlying its role in NSC self-renewal is the resulting increase in the promoter activity of SOX2, a transcription factor expressed in undifferentiated NPCs, upon Notch activation (Ehm et al. 2010). Notch is also important for neuronal maturation as it modulates the dendritic morphology of neurons born postnatally (Breunig et al. 2007); reviewed in (Yoon and Gaiano 2005). Interestingly, Notch signalling is mediated by astrocytes. For instance, lack of GFAP and Vimentin expression in astrocytes results in reduced endocytosis of Jagged1, a Notch ligand. In turn, this causes decreased Notch signalling and increased neurogenesis (Wilhelmsson et al. 2012). Similarly, decreased Notch1 signalling in striatal astrocytes after stroke activates neurogenesis (Magnusson et al. 2014). These findings reveal the multifaceted nature of Notch signalling and the involvement of different niche components such as astrocytes in regulating NPCs and neurogenesis.

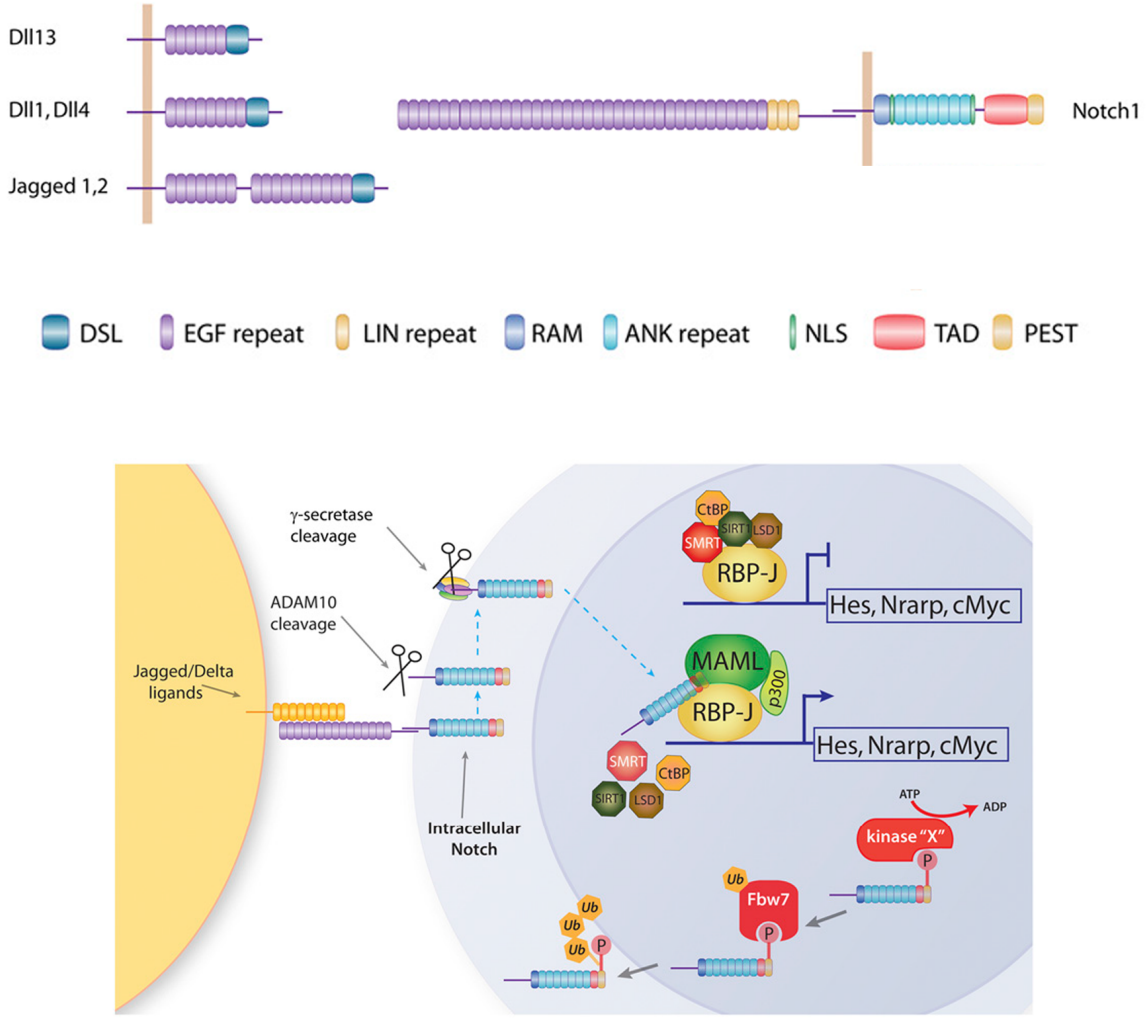

Figure 1-4. Canonical Notch signalling. Abbreviations: Hes (HES Family BHLH Transcription Factor), MAML (Mastermind-Like Transcriptional Co-activator), RBPJ (Recombination Signal Binding Protein for Immunoglobulin Kappa), Modified from (Lobry et al. 2014).

Astrocytes

Astrocytes are more dynamic than their once believed role of being support cells (Barres 2008). They produce and recycle neurotransmitters, release neurotrophic factors, maintain the blood-brain barrier, and play an important role in synapse formation; reviewed in (Hewett 2009; Clarke and Barres 2013). In fact, evidence indicates that neuron survival depends on the survival of astrocytes in co-culture systems. Cortical astrocytes lacking EGFR expression do not support the survival of WT neurons, whereas mutant midbrain astrocytes support the survival of both WT and mutant neurons (Wagner et al. 2006), highlighting both the importance of astrocytes to neuronal integrity as well as the heterogeneity of astrocytes in different brain regions. It is not surprising that astrocytes are important for the formation and maintenance of the neural circuitry given that a single astrocyte is estimated to contact between 20,000 to 120,000 synapses in rodent brains (Matyash and Kettenmann 2010). Despite their emerging importance in CNS integrity, astrocytes are still not well characterized.

Niche Astrocytes

With the exception of the SVZ and the DG, the adult brain does not provide a permissive milieu for neurogenesis. Thus, germinal regions have unique niches that allow lifelong neurogenesis in an otherwise inhibitory brain environment. Astrocytes are an important component of neurogenic niches in addition to endothelial cells, neurons, NPCs and their progeny, and morphogens; reviewed in (Ming and H. Song 2011). In fact, astrocytes are closely associated with NPCs and granule neurons in the DG (Seri et al. 2004). Research

has indicated that astrocytes support the proliferation and differentiation of co-cultured SVZ NPCs into neurons even in the absence of mitogens or serum in the culture medium (Lim and Alvarez-Buylla 1999). A similar juxtacrine effect is seen with astrocytes in contact with NPCs in the hippocampus (Ashton et al. 2012; Wilhelmsson et al. 2012). Such astrocytes express the ephrin-B2 ligand, which binds to EphB4 receptors on NPCs and neuroblasts, promoting neurogenesis (Ashton et al. 2012). Moreover, astrocytes in germinal niches have been shown to secrete factors that actively regulate NPC proliferation, neurogenesis, or both, such as thrombospondin-1 (Z. Lu and Kipnis 2010), fibroblast growth factor-2 (Kirby et al. 2013), and clusterin (Cordero-Llana et al. 2011). The differential between niche and non-niche astrocytes is evident after adult hippocampal astrocytes were shown to promote NPC proliferation and neuronal differentiation, in contrast to adult spinal cord astrocytes (S. Song et al. 2002). At a gene expression level, newborn and adult hippocampal astrocytes share more genes compared to newborn and adult spinal cord astrocytes (Barkho et al. 2006), which suggests that hippocampal astrocytes do not diverge as much throughout development in agreement with their lifelong role in regulating neurogenesis.

Astrocytes and FXS

Uncovering the extent of astrocyte involvement in neurological disease is still in its infancy especially that the functions of their highly enriched genes are not known. The fact that astrocytes express FMRP during development suggests that they play a role in critical periods of plasticity. Indeed, *Fmr1*-KO cortical astrocytes alter the dendritic

morphology and synaptic formations of WT neurons, whereas WT cortical astrocytes can correct the abnormalities of *Fmr1*-KO neurons (Jacobs and Doering 2010). This indicates that astrocytes contribute to the pathophysiology of FXS. Yet, the contribution of astrocytes to FXS is confounded by other factors such as the developmental time point, which seems to play a role. For instance, the effect of *Fmr1*-KO astrocytes on aberrant WT neuron morphology is detected after 7 DIV but not after 21 DIV, suggesting that *Fmr1*-KO astrocytes act by delaying the development of neurons compared to those in typically developing brains (Jacobs et al. 2010). Thus, it is important to study the contribution of astrocytes to disease phenotypes, such as in FXS.

Tools for Studying NPCs

The Neurosphere Assay

The neurosphere assay is one of the most common tissue culture techniques used to generate NPCs. In the absence of serum, NPCs exposed to mitogens, such as epidermal growth factor and/or fibroblast growth factor, respond by generating spherical free-floating clusters known as neurospheres (Reynolds and Weiss 1992). However, a single neurosphere culture contains heterogeneous populations of NPCs with different proliferative, self-renewal, and differentiation capacities (Reynolds and Rietze 2005). To distinguish between neural stem and progenitor cells in culture, the NCFC assay was developed, making use of the high proliferative potential of stem cells (Louis et al. 2008). Clonally derived neurosphere colonies are grown in collagen for 3 weeks before being examined based on size. Colonies larger than 2 mm in diameter contain cells with high

proliferative potential and fulfill functional criteria of neural stem cells, namely self-renewal and mutlipotency, whereas colonies smaller than 2 mm in diameter have a limited proliferative potential and are considered progenitor cells. Functional stem cell criteria were confirmed in the NCFC assay by examining the self-renewal, differentiation, and proliferative potential of neurospheres (Louis et al. 2008). Successful long term passaging of neurospheres indicates self-renewal, whereas the generation of a large number of progeny orders of magnitudes larger than the starting population confirms high proliferative potential. Finally, the withdrawal of growth factors from neurospheres and the generation of neurons, astrocytes, and oligodendrocytes in the absence of serum pointed to multipotency; reviewed in (Reynolds and Rietze 2005).

Flow Cytometry

FACS or flow cytometry is an invaluable tool in that it allows the prospective isolation of NPC populations and their progeny. By contrast, other tools only study NPC populations by retrospective analysis. One of the challenges in studying NPCs lies in their heterogeneous nature along with the lack of a single marker that uniquely defines a population subset. Hence, scientists resorted to the use of combinatorial approaches in flow cytometry, such as detecting cell surface antigens and fluorescence signals in transgenic reporter animals, to isolate cells of interest for further analysis. Additionally, the use of cell surface markers allows for a reliable flow cytometric analysis whereas intracellular marker detection poses some technical challenges. Some of the cell surface markers more commonly used in flow cytometry to date include cluster of differentiation

markers such as CD15, CD24, and CD133. While the exact function of these markers is not yet known, their expression levels have been shown to enrich for NPCs and neuroblasts (Capela and Temple 2002; Panchision et al. 2007; Walker et al. 2013; Beckervordersandforth et al. 2014). Indeed, advances in flow cytometric techniques as well as a better understanding of the functions of the available cell surface markers will be revolutionary for further characterization of NPCs.

Immunohistochemical Markers

Immunohistochemical markers label NPCs and/or their progeny, thus allowing for their identification. The combination of these markers along with cell cycle markers offers a better understanding of the biology of NPCs studied.

CD15

CD15 is a glycan expressed on the surface of cells, especially NPCs (Capela and Temple 2002), as well as glioblasts and neuroblasts (Capela and Temple 2006). Its expression is strongest in neurogenic regions (Capela and Temple 2006).

CD133

CD133 is a glycoprotein associated with membrane protrusions whose function is elusive. Its expression enriches for neurosphere-forming cells (Corti et al. 2007; Walker et al. 2013). Research has shown that $GFAP^{+}CD133^{+}$ cells isolated from the DG act as NSCs (Beckervordersandforth et al. 2014). Yet, astrocytes also express CD133 albeit weakly (Beckervordersandforth et al. 2014).

GLAST

GLAST, a glutamate/aspartate transporter, is expressed by astrocytes and radial glia, but not oligodendrocytes (Jungblut et al. 2012). It is also expressed by NSCs (Mich et al. 2014) and its expression persists in neuronal progenitors of the DG (Pastrana et al. 2009).

Ki67

Ki67 is a nuclear antigen that labels cells in the active phases of the cell cycle, starting in the late G_1 phase through the M phase (Gerdes et al. 1983). Thus, its expression indicates proliferating cells.

Nestin

Nestin is an intermediate filament protein expressed in NSCs and neural progenitors before they exit the cell cycle (Lendahl et al. 1990). Recent research has indicated that activated, but not quiescent NSCs, express Nestin (Codega et al. 2014).

PhH3

Histone H3 is specifically phosphorylated during mitosis and meiosis (Hans and Dimitrov 2001), labeling proliferating cells in the M phase.

PSA-NCAM

PSA-NCAM is a glycoprotein expressed on the surface of neuroblasts and immature neurons during development and in neurogenic regions due to its role in neurite outgrowth and cell migration (Bonfanti 2006); reviewed in (Kiss et al. 2001).

SOX2

SOX2 is a transcription factor that maintains NPCs in an undifferentiated state (Graham et al. 2003). Research has shown that it is expressed in NSCs with both horizontal and radial morphologies (Suh et al. 2007). It regulates SHH (Favaro et al. 2009) and Notch1 (Bani-Yaghoub et al. 2006) signalling.

Chapter 2: Hypothesis and Specific Aims

Previous research has implicated FMRP in regulating NPCs. Yet, no studies have examined NPCs from the early postnatal hippocampus. In this thesis, I use the *Fmr1*-KO mouse model to examine the role of FMRP in governing NPC biology from the early postnatal hippocampus. I hypothesized that *Fmr1*-KO NPCs will show increased proliferation and altered neurogenesis on the basis of previous findings. To test my hypothesis, the following specific aims were used:

1.
    a. Distinguish between the proliferation of neural progenitors from that of NSCs cultured from P1 WT and *Fmr1*-KO hippocampi using the Neural Colony Forming Cell (NCFC) Assay.
    b. Assess the effect of astrocyte-secreted factors from WT and *Fmr1*-KO brains on the proliferation of neural progenitors and NSCs.

2. Examine hippocampal expression patterns of early cell lineage and cell cycle markers *in vitro* and *in vivo* and analyze cell cycle progression of cells isolated from the hippocampus and DG.

3. Study NPCs and their progeny from the hippocampi and DG of WT and *Fmr1*-KO mice by flow cytometry.

Chapter 3: Astrocyte-Secreted Factors Selectively Alter Neural Stem and Progenitor Cell Proliferation in the Fragile X Mouse

Chapter Link

The work enclosed in this chapter has been published in *Frontiers in Cellular Neuroscience* (2016), doi: 10.3389/fncel.2016.00126.

Previous work from our laboratory has shown that FMRP is expressed in astrocytes during development. Yet, the role of astrocytes in NPC biology has not been examined in Fragile X. At the same time, the proliferation of NSCs has not been studied distinctly from that of neural progenitors during development in the context of FXS. Thus, in this study, I examined the effect of astrocyte-conditioned media on the proliferation of neural progenitors as well as NSCs from WT and *Fmr1*-KO hippocampi.

Astrocyte-secreted factors selectively alter neural stem and progenitor cell proliferation in the Fragile X mouse

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Running Head: Fragile X neural stem cells

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Highlights:

- We studied the proliferation of neural stem and progenitor cells in Fragile X.
- We examined the role of astrocyte-secreted factors in neural precursor cell biology.

- Astrocyte-secreted factors with differential expression in Fragile X identified.

Keywords: Fragile X Syndrome; neural stem cells; neurospheres; astrocyte-secreted factors; *Fmr1* knockout mice; 2D DIGE

Abstract:

An increasing body of evidence indicates that astrocytes contribute to the governance and fine tuning of stem and progenitor cell production during brain development. The effect of astrocyte function in cell production in neurodevelopment disorders is unknown. We used the Neural Colony Forming Cell assay to determine the effect of astrocyte conditioned media (ACM) on the generation of neurospheres originating from either progenitor cells or functional stem cells in the knock out (KO) Fragile X mouse model. ACM from both normal and *Fmr1*-KO mice generated higher percentages of smaller neurospheres indicative of restricted proliferation of the progenitor cell population in *Fmr1*-KO brains. Wild type neurospheres, but not KO neurospheres, showed enhanced responses to ACM from the *Fmr1*-KO mice. In particular, *Fmr1*-KO ACM increased the percentage of large neurospheres generated, representative of spheres produced from neural stem cells. We also used 2D DIGE to initiate identification of the astrocyte-secreted proteins with differential expression between *Fmr1*-KO and WT cortices and hippocampi. The results further support the critical role of astrocytes in governing neural cell production in brain development and point to significant alterations in neural cell proliferation due to astrocyte secreted factors from the Fragile X brain.

Introduction:

Fragile X Syndrome (FXS) is the most common single-gene cause of autism spectrum disorder and intellectual impairment (Wang et al., 2012), with a frequency of about 1 in 7,100 males and 1 in 11,100 females (Hunter et al., 2014). FXS is associated with impairments in learning and memory, hyperactivity, hypersensitivity to sensory stimuli, increased susceptibility to seizures, and autistic behaviours (Penagarikano et al., 2007). The majority of FXS cases result from the epigenetic transcriptional silencing of the Fragile X Mental Retardation 1 gene (*FMR1*), which subsequently prevents the expression of Fragile X Mental Retardation Protein (FMRP) (Coffee et al., 1999; 2002; Eiges et al., 2007; Pieretti et al., 1991; Verkerk et al., 1991). FMRP is responsible for the translational regulation of many mRNAs (Ascano et al., 2012; Darnell et al., 2011; Miyashiro et al., 2003) including those involved in synaptic plasticity (Bassell and Warren, 2008; Darnell et al., 2011), and has been implicated in regulating the proliferation and differentiation of embryonic and adult neural precursor cells (NPCs) (Castrén et al., 2005; Eadie et al., 2009; Guo et al., 2011; Luo et al., 2010; Tervonen et al., 2009). A loss of FMRP and the associated memory and learning impairments observed in FXS, may therefore be linked to aberrant regulation of NPCs in the hippocampus.

There is a strong correlation between learning and memory capacity and hippocampal neurogenesis. A hallmark of the dentate gyrus (DG) of the hippocampal formation is lifelong neurogenesis to which NPCs contribute (Altman and Das, 1965).

The enhanced plasticity of immature newborn neurons is suggested to enable learning and memory (Deng et al., 2010; Snyder and Cameron, 2012). In fact, many neurological diseases marked by cognitive decline such as Alzheimer's, Parkinson's, depression, and epilepsy, all show alterations in hippocampal neurogenesis (Zhao et al., 2008). Similarly, conditional knockout of FMRP in adult NPCs results in increased NPC proliferation, and the consequent impairment of hippocampus dependent learning (Guo et al., 2011). Notably, the majority of DG granule cells are born postnatally in the rodent brain (Altman and Bayer, 1990), and most adult born neurons are generated from early-born NPCs that reside in the DG as it is forming (Mathews et al., 2010). It is unknown whether early postnatal dysregulation of NPCs has ramifications on neurogenesis throughout life, or whether later interventions can correct abnormalities. Regulation of neurogenesis via FMRP is likely to impact early-born NPCs in the developing DG since expression of FMRP peaks at postnatal day 7 (Lu et al., 2004), which may underlie abnormal hippocampus-dependent memory in 3-week-old Fragile X mice (Bilousova et al., 2009).

The role of FMRP and its downstream effects have been largely limited to studies of neural populations in isolation. However, emerging evidence suggests that the glial environment and the role of FMRP in these cells are just as critical for proper brain development. Astrocytes for example, previously believed to be only support cells, secrete factors that actively regulate neurogenesis, neural function and communication (Barres, 2008). When cortical astrocytes devoid of FMRP are co-cultured with wild type (WT) neurons, the growth of the neurons is affected and normal synaptic formations

among them are limited (Jacobs and Doering, 2010a). Of particular interest, the astrocytes within the hippocampus have been shown to promote NPC proliferation and neuronal differentiation (Song et al., 2002), and we hypothesized that a lack of FMRP in astrocytes may contribute to altered neural precursor/stem cell production.

To test this hypothesis, we used the Neural Colony Forming Cell (NCFC) Assay to examine the proliferation of neural stem cells within wild type (WT) or Fragile X mice (*Fmr1*-KO) hippocampal neurospheres cultured in the presence of astrocyte conditioned media. The NCFC assay is specifically designed to distinguish between functional stem cells that form neurospheres more than 2 mm in diameter from neurospheres produced by the general precursor cell population with limited proliferative capacity (Louis et al., 2008). We found that Fragile X progenitor-derived neurospheres showed restricted proliferation in the presence of both WT and *Fmr1*-KO astrocyte conditioned media (ACM). On the other hand, the proliferation of WT neural progenitor-derived neurospheres selectively decreased with *Fmr1*-KO ACM, while that of WT stem cell-derived neurospheres was enhanced. Interestingly, one population of WT neural progenitor-derived neurospheres showed decreased proliferation in the presence of cortical *Fmr1*-KO ACM, but not hippocampal *Fmr1*-KO ACM, highlighting a regional difference in astrocyte-secreted factors. Additionally, we compared the protein expression profiles of secreted factors from cortical and hippocampal astrocytes between WT and *Fmr1*-KO brains. Of the multiple proteins with differential expression that we detected, we identified the following proteins using mass spectrometry: multidrug resistance

protein 1B, microphthalmia-associated transcription factor, haptoglobin, fasciculation and elongation protein zeta 2, antithrombin III, and serum albumin. Together, these results indicate that signals derived from *Fmr1*-KO astrocytes affect the proliferation of WT neurospheres, and that *Fmr1*-KO neurospheres have intrinsic deficits in responding to environmental cues as evident in their indiscriminate response to WT versus *Fmr1*-KO ACM.

Materials and Methods:

Animals:

All animal experiments were performed in accordance with the guidelines set by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board. The transgenic mouse colony was established from breeding pairs of FVB.129P2(B6)-*Fmr1*^{tm1Cgr} mice obtained from Dr. Carl Dobkin at the New York State Institute for Basic Research in Developmental Disabilities (Staten Island, NY). The wild type (WT) and *Fmr1*-knockout (KO) mice were housed and maintained at the McMaster University Central Animal Facility.

Neural Colony Forming Cell Assay:

WT and *Fmr1*-KO postnatal day 1 (P1) mouse pups were decapitated and the brains processed for the Neural Colony Forming Cell Assay, using a modified protocol (Louis and Reynolds, 2010). Briefly, hippocampi were microdissected, minced into small pieces, and enzymatically digested for 20 minutes at 37°C (Pacey et al., 2006). The enzymes

were inactivated by incubating the cell pellet for 10 minutes at 37°C in 4 mL of 1 mg/mL solution of trypsin inhibitor (Roche, Mississauga, Canada) dissolved in NeuroCult complete NSC proliferation medium (StemCell Technologies, Vancouver, Canada). Cells were resuspended in 1 mL of NeuroCult complete NSC proliferation medium in the presence of 20 ng/mL epidermal growth factor (EGF, Sigma-Aldrich, St. Louis, USA), 10 ng/mL fibroblast growth factor-basic (FGF-2, Sigma-Aldrich), and 2 μg/mL heparin (Sigma-Aldrich). The cell suspension was passed through a 40-μm cell strainer and the cell density brought to a concentration of 650 cells/μL. For 2 replicates/culture, the following components were added in order: 1.7 mL NeuroCult NCFC Serum-Free Medium without cytokines, 330 μL NeuroCult NSC Proliferation Supplements (mouse), 6.6 μL EGF (of 10 μg/mL), 3.3 μL FGF-2 (of 10 μg/mL), 6.6 μL heparin (of 0.2%), 25 μL of cells at 650 cells/μL to a final density of 8,000 cells/35 mm culture dish, and 1.3 mL collagen solution. The neurosphere colonies were replenished with Complete NeuroCult Replenishment medium at 7 and 14 days *in* vitro (DIV) containing 0.5 μg/mL EGF, 0.25 μg/mL FGF, and 0.1 mg/mL heparin.

Astrocyte-conditioned medium:

Monolayers of cortical and hippocampal astrocytes were established (Jacobs and Doering, 2010b; Jones et al., 2012). P2 cortices of WT and *Fmr1*-KO mice were dissected and minced in Hank's Buffered Salt Solution (Life Technologies, Burlington, Canada) supplemented with HEPES (Life Technologies) and enzymatically digested in 0.25% trypsin (Life Technologies) supplemented with 1 mg/mL DNase (Roche).

Enzymatic digestion of cells was inactivated by the addition of glial medium, which is comprised of Minimal essential media (MEM) with Earl salts and L-glutamine (Life Technologies) supplemented with 10% horse serum (Life Technologies) and 0.6% glucose, and the cell suspension was passed through a 70 μm strainer. Cells were resuspended in glial medium and plated in T-75 cm^2 flasks. Hippocampal astrocyte cultures were established using a modified protocol previously published (Jones et al., 2012). P2 hippocampi of WT and *Fmr1*-KO mice were dissected and minced in Hank's Buffered Salt Solution supplemented with HEPES and mechanically digested in glial media of the same composition as that used for cortical cultures. Cells were resuspended in glial media and plated in T-75 cm^2 flasks previously coated with 0.1 mg/mL Poly-D-Lysine Hydrochloride (Sigma-Aldrich) in 0.1 M borate buffer, pH 8.5. When hippocampal and cortical astrocytes reached 50% confluence, glial medium was switched to serum-free medium containing a final concentration of 1.25 ng/mL FGF-2 and no EGF (Pacey et al., 2006).

Astrocyte-conditioned media (ACM) was collected and concentrated 10X in concentrator tubes (Sartorius Vivaspin 20 Concentrator 5000 MWCO) at 3000 x*g* at 4°C. ACM was added to the NCFC assay when applicable at 1X concentration, and the concentrations of NeuroCult NCFC Serum-Free Medium, NeuroCult NSC Proliferation Supplements (mouse), EGF (10 μg/mL), FGF-2 (10 μg/mL), and heparin (0.2%) were adjusted to 9/10X at plating when ACM was used. Neurosphere colonies were cultured in different combinations listed in **Table 3-1**.

As a control, neurosphere proliferation was tested in the presence of 1X concentrated SFM containing 1.25 ng/mL FGF-2.

Neurosphere measurements:

Neurosphere colonies were measured after 21 DIV on a 2 x 2 mm^2 grid culture dish, using the 2X objective on the EVOS XL Core Cell Imaging System (Life Technologies). Images were obtained using the 4X objective. Individual neurospheres were classified into 4 categories based on diameter: ≤0.5mm, 0.5 – 1 mm, 1 – 2 mm, ≥2 mm as previously documented (Louis and Reynolds, 2010). Analysis included WT (n=9) and *Fmr1*-KO (n=9) cultures without the addition of ACM, and with the addition of WT and *Fmr1*-KO cortical and hippocampal ACM in groups as shown in **Table 3-1**.

Table 3-1. Plating combinations of neurospheres and astrocyte conditioned media. Sample size (n) in parentheses.

Neurosphere Genotype	**Media Condition**
WT	No ACM (n=4)
	WT cortical ACM (n=4)
	WT hippocampal ACM (n=3)
	Fmr1-KO cortical ACM (n=5)
	Fmr1-KO hippocampal ACM (n=5)
Fmr1-KO	No ACM (n=5)
	WT cortical ACM (n=5)
	WT hippocampal ACM (n=5)
	Fmr1-KO cortical ACM (n=5)
	Fmr1-KO hippocampal ACM (n=5)

Proteomic analysis:

ACM from WT and *Fmr1*-KO cortices and hippocampi were analyzed for protein expression using two-dimensional difference in gel electrophoresis (2D DIGE). ACM was 2D DIGE-analyzed by *Applied Biomics* (Hayward, CA, USA). Spots with the highest differential expression between WT and *Fmr1*-KO ACM as determined by 2D DIGE were selected for identification (4 spots from cortical ACM and 4 from hippocampal ACM, **Tables 3-1, 3-S1, 3-S2**). Protein identification was based on peptide fingerprint mass mapping (using MS data) and peptide fragmentation mapping (using MS/MS data).

Statistical analyses:

Statistical analysis was performed using GraphPad Prism (version 5.0). Two-way analysis of variance followed by Bonferroni's post-hoc test was used in **Fig. 3-1**, and all other pairwise comparisons were done using a two-tailed Student's t-test (**Fig. 3-2 – 3-3**). Probabilities of $p<0.05$ were considered significant. Data are expressed as group means and error bars represent the standard error of the mean (SEM).

Results:

WT and Fmr1-KO cultures generate similar sizes of neurospheres

The NeuroCult® Neural Colony-Forming Cell (NCFC) Assay distinguishes stem from progenitor cell-derived neurospheres by generating clonally derived neurospheres in collagen that can be discriminated on the basis of size [(Louis et al., 2008); **Fig. 3-1A**, **B**, **C** and **D**]. Neurosphere colonies larger than 2 mm in diameter contain cells with high

proliferative potential and fulfill the functional criteria of stem cells (Louis et al., 2008). After 21 *DIV,* no differences were detected in the proportion of neurosphere colonies in each of the four size categories between WT and *Fmr1*-KO cultures (≤0.5mm, 0.5 – 1 mm, 1 – 2 mm, ≥2 mm diameter, **Fig. 3-1E**). However, the larger the neurosphere size category, the fewer the percentage of neurospheres in that category regardless of genotype ($F_{3,64} = 68.16$, $p<0.0001$), as expected since fewer NPCs have an increased proliferative capacity (Reynolds and Rietze, 2005). Compared to neurospheres ≤0.5mm in diameter, there were 86.9% fewer neurospheres with a diameter ≥2 mm. Together, these results indicated that while there were significantly more neurospheres with a lower proliferative potential, lack of FMRP did not affect the distribution of neurospheres in the 4 size categories after 21 *DIV* in our culture system, and thus the proportion of stem or progenitor cell-derived neurospheres was not different when comparing the two genotypes.

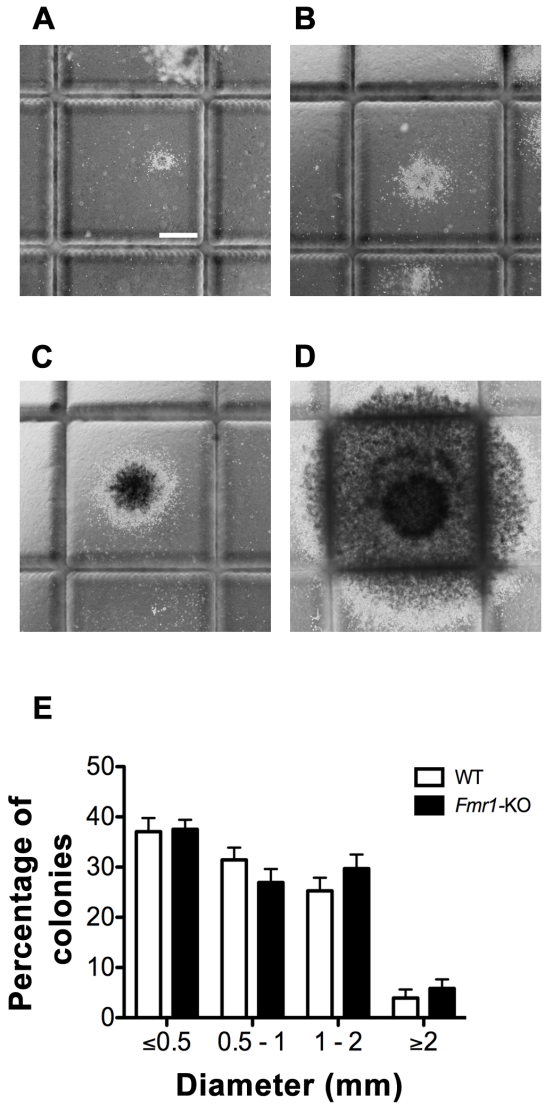

Figure 3-1. WT and *Fmr1*-KO hippocampi generated four size categories of neurosphere colonies. A. Neurospheres ≤0.5 mm in diameter. **B.** Neurospheres 0.5 – 1 mm in diameter. **C.** Neurospheres 1 – 2 mm. **D.** Neurospheres ≥2 mm. **E.** No difference in the percentage of neurosphere colonies present between WT and *Fmr1*-KO cultures in each of the four size categories. Scale bar=500μm (A – D). Abbreviations: WT: wild type, *Fmr1*-KO: *Fmr1* knockout.

ACM modulates the size of Fmr1-KO neurospheres

Our previous work has shown that signals from Fragile X astrocytes contribute to the immature neuronal morphology characteristic of Fragile X (Jacobs and Doering, 2010a; Jacobs et al., 2010), and so we examined whether Fragile X astrocyte-secreted factors could affect the proliferative capacity of neurospheres. We collected ACM from primary astrocyte cultures and added it to cultured NPCs in collagen as outlined (**Table 3-1**). When testing for variances between groups, we found a significant interaction between ACM conditions and *Fmr1*-KO neurosphere size ($F_{12, 80} = 2.69$, p=0.0042). ACM did not affect the total number of *Fmr1*-KO colonies generated (**Fig. 3-S1**).

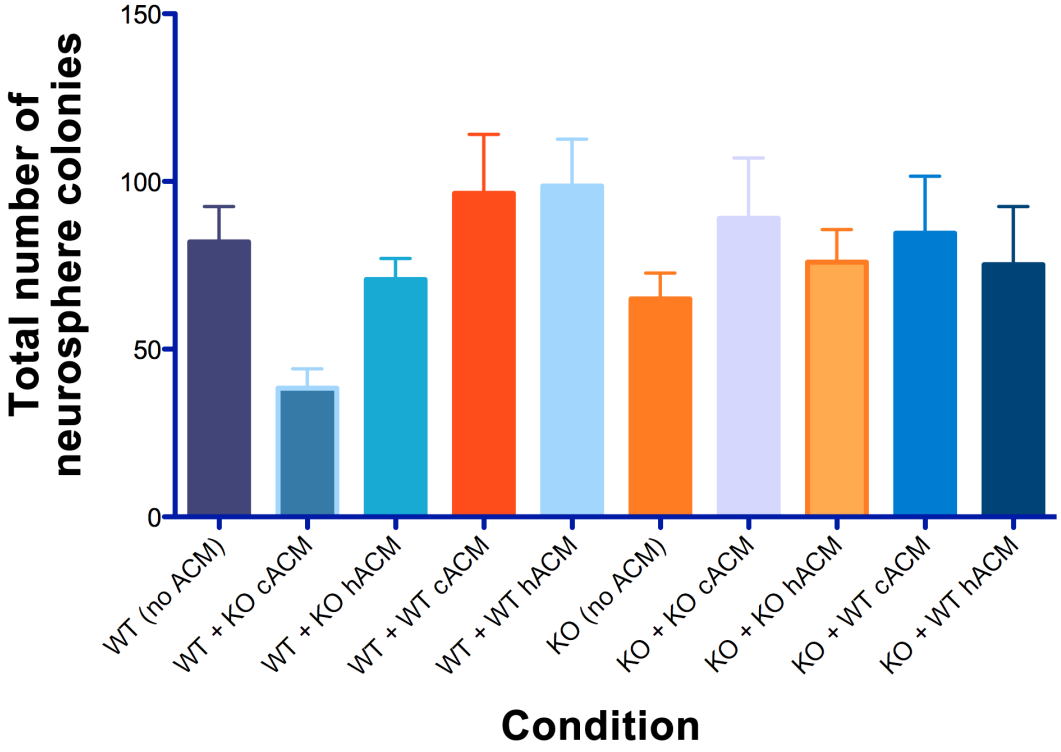

Figure 3-S1. Total number of colonies generated by the addition of ACM to WT and *Fmr1*-KO neurospheres. Abbreviations: WT: wild type, KO: *Fmr1* knockout, ACM: astrocyte conditioned media, c: cortical, h: hippocampal.

A larger proportion of *Fmr1*-KO neurospheres measured ≤0.5mm in parallel to a smaller proportion of neurospheres that were 1 – 2 mm, in the presence of WT and *Fmr1*-KO ACM. Specifically, there was an increase in the percentage of small neurospheres (≤0.5 mm) in *Fmr1*-KO cultures (34.51±2.76%) with the application of ACM from the *Fmr1*-KO cortex (48.62±3.59%, p=0.014; **Fig. 3-2A**), *Fmr1*-KO hippocampus (48.77±3.59%, p=0.014, **Fig. 3-2A**), and WT cortex (54.48±3.01%, p=0.001, **Fig. 3-2B**). At the same time, application of ACM from the *Fmr1*-KO cortex (20.73±1.35%, p=0.011, **Fig. 3-2C**), WT cortex (17.43±1.42%, p=0.0031, **Fig. 3-2D**), and WT hippocampus (21.83±3.13%, p=0.049, **Fig. 3-2D**) caused a decrease in the percentage of larger neurospheres (1 – 2 mm) relative to *Fmr1*-KO neurospheres without ACM (32.41±3.30%). Interestingly, ACM did not affect the percentages of *Fmr1*-KO neurospheres measuring 0.5 – 1 mm or ≥2 mm (**Fig. 3-S2**). These results demonstrated that astrocyte secreted factors decreased *Fmr1*-KO neurosphere size, suggesting that WT and *Fmr1*-KO ACM, from the cortex and the hippocampus, restricted the proliferation of progenitor-derived neurospheres.

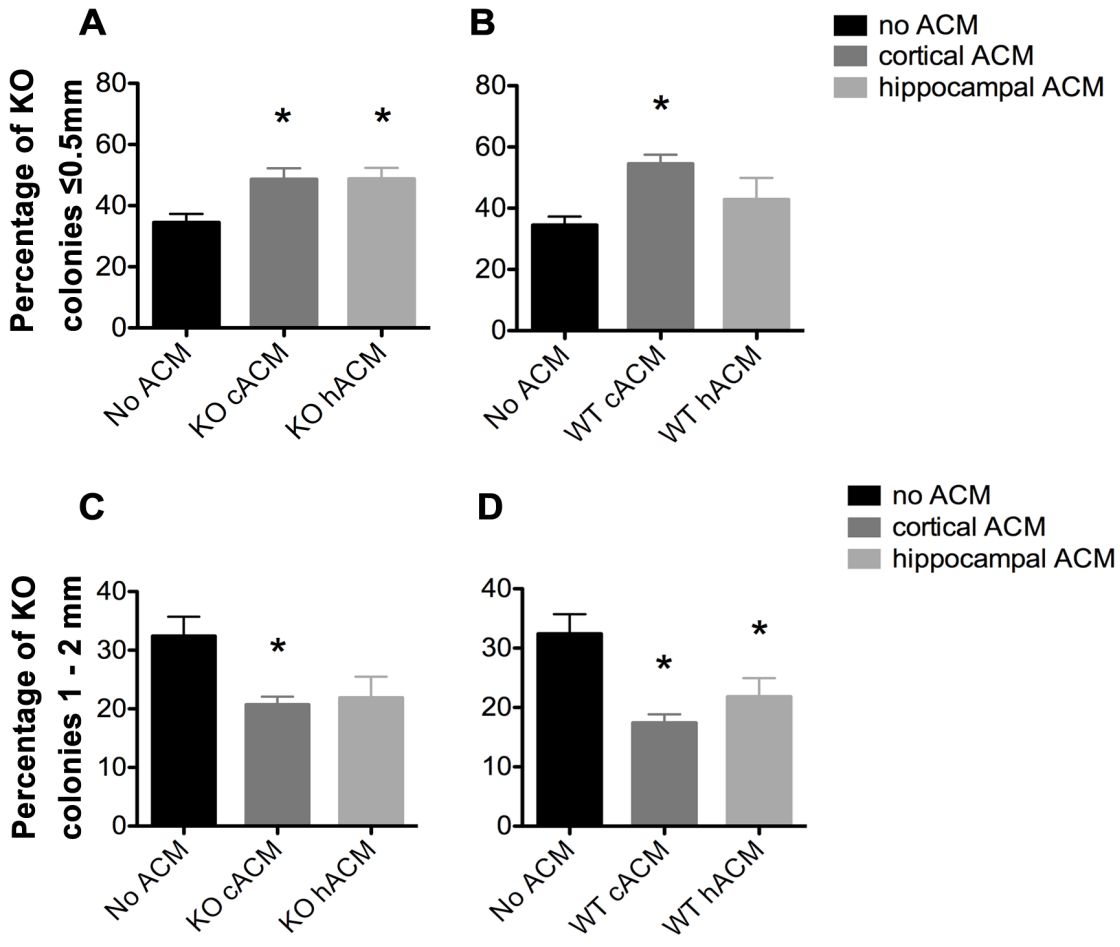

Figure 3-2. Astrocyte conditioned media (ACM) decreased the size of *Fmr1*-KO progenitor-derived neurospheres. A. Both cortical and hippocampal ACM from *Fmr1*-KO astrocytes increased the percentage of KO neurospheres ≤0.5mm relative to KO neurospheres without ACM (p=0.014; p=0.014, respectively). **B.** WT cortical ACM also increased the percentage of KO neurospheres (p=0.001) ≤0.5mm relative to KO neurospheres without ACM. **C.** Cortical *Fmr1*-KO ACM decreased the percentage of KO neurospheres 1 – 2 mm relative to KO neurospheres without ACM (p=0.011), and hippocampal KO ACM resulted in a near significant decrease (p=0.063). **D.** Cortical and

hippocampal WT ACM decreased the percentage of neurospheres 1 – 2 mm (p=0.0031, p=0.049) relative to neurospheres without ACM. * denotes a significant difference compared to neurospheres with no ACM. Abbreviations: WT: wild type, *Fmr1*-KO: *Fmr1* knockout, ACM: astrocyte conditioned media.

WT neurospheres have an enhanced sensitivity to Fmr1-KO ACM

The addition of ACM significantly affected the generation of WT neurospheres of different sizes ($F_{12, 64} = 2.69$, p=0.0013). This is evident from the reduced total number of WT colonies generated in the presence of ACM from cortical *Fmr1*-KO astrocytes (**Fig. 3-S1**). In addition, the percentage of WT neurospheres measuring ≤0.5 mm in diameter increased in the presence of both WT and *Fmr1*-KO ACM. ACM from the *Fmr1*-KO cortex (50.24±6.28%, p=0.046, **Fig. 3-3A**), *Fmr1*-KO hippocampus (45.66±1.59%, p=0.049, **Fig. 3-3A**), and WT cortex (45.04±1.59%, p=0.009; **Fig. 3-3B**) increased the percentage of WT neurospheres ≤0.5 mm relative to WT neurospheres without ACM (31.83±3.09%). Notably, ACM from WT astrocytes had no effect on WT neurospheres measuring 0.5 – 1 mm, 1 – 2 mm (**Fig. 3-3D)**, or ≥2 mm (**Fig. 3-3F**).

On the other hand, ACM from the *Fmr1*-KO cortex (20.31±2.58%, p=0.049, **Fig. 3-3C**) and *Fmr1*-KO hippocampus (19.26±3.78%, p=0.069, **Fig. 3-3C**) reduced the percentage of WT neurospheres 1 – 2 mm diameter compared to WT neurospheres without ACM (30.97±3.86%). Interestingly, only ACM from the *Fmr1*-KO cortex (18.58±3.56%, p=0.012, **Fig. 3-S2**) decreased the percentage of WT neurospheres measuring 0.5 – 1 mm compared to those without ACM (34.27±2.71%), potentially due to the genotype-region mismatch of *Fmr1*-KO cortical ACM and WT hippocampal neurospheres (data not shown).

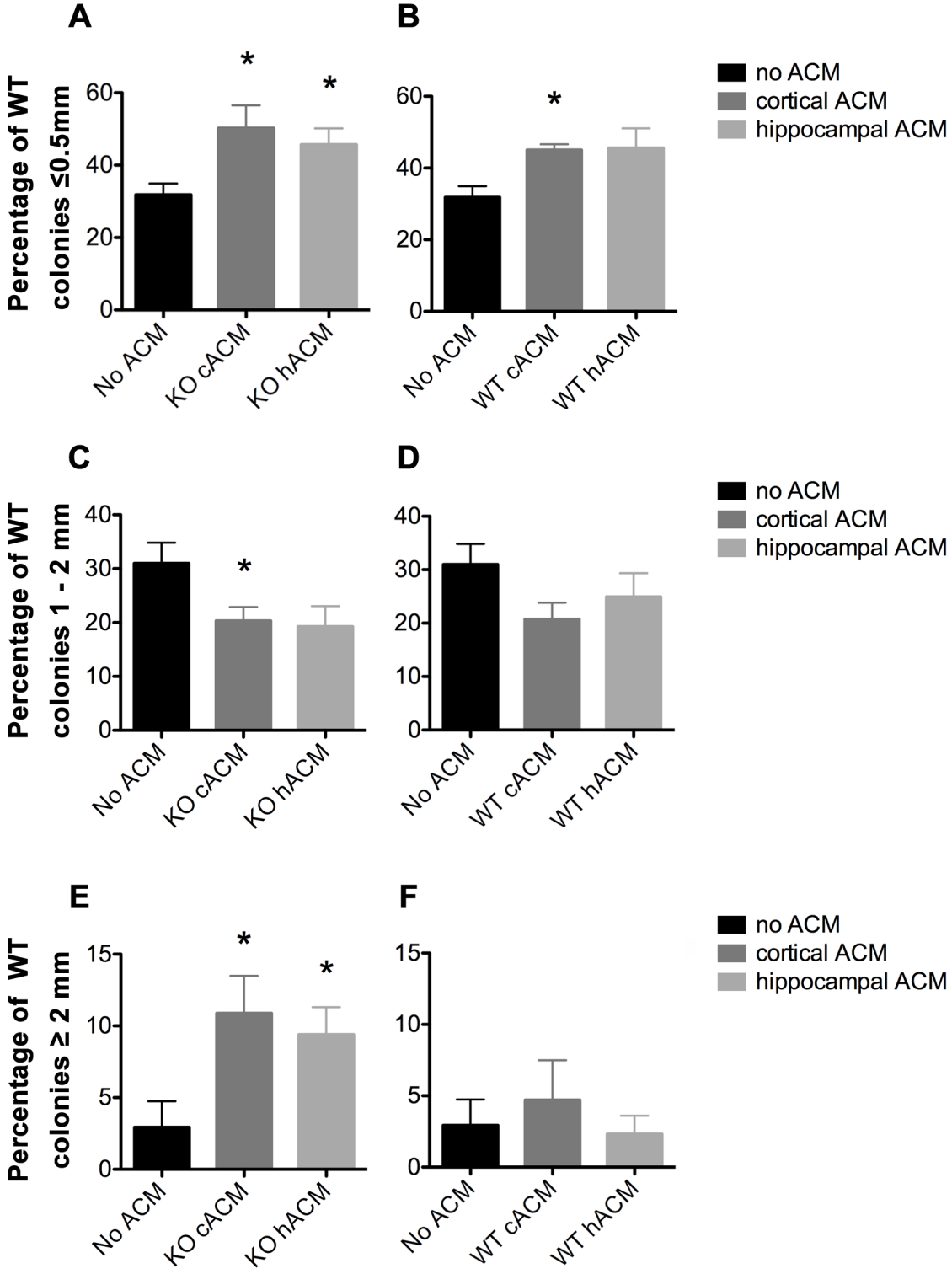
A
B
C
D
E
F
Percentage of WT colonies ≤0.5mm
Percentage of WT colonies 1 - 2 mm
Percentage of WT colonies ≥ 2 mm
no ACM
cortical ACM
hippocampal ACM
No ACM
KO cACM
KO hACM
WT cACM
WT hACM
*

Figure 3-3. WT neurospheres respond more selectively to *Fmr1*-KO astrocyte conditioned media (ACM). A. Conditioned media from cortical and hippocampal KO astrocytes increased the percentage of neurospheres ≤0.5 mm relative to WT neurospheres without ACM (p=0.046, p=0.049, respectively). **B.** WT Cortical ACM also increased the percentage of neurospheres ≤0.5 mm (p=0.009) relative to WT neurospheres without ACM. WT hippocampal ACM caused a near significant increase in the percentage of WT neurospheres (p=0.066). **C.** *Fmr1*-KO cortical and hippocampal ACM decreased the percentage of WT neurospheres 1 – 2 mm (p=0.049, p=0.069, respectively). **D.** No effect of WT ACM on WT neurospheres 1 – 2 mm in diameter. **E.** Increased percentage of WT neurospheres ≥2 mm in diameter in the presence of cortical (p=0.050) and hippocampal (p=0.047) KO ACM relative to WT neurospheres without ACM. **F**. No effect of WT ACM on WT neurospheres ≥2 mm in diameter. * denotes significant difference from WT neurospheres with no ACM (p<0.05). Abbreviations: WT: wild type, *Fmr1*-KO: *Fmr1* knockout, ACM: astrocyte conditioned media.

Fmr1-KO ACM increases stem cell production in WT neurospheres

In sharp contrast, the percentage of WT neurospheres ≥ 2 mm, originating from functional stem cells, increased in the presence of ACM from the *Fmr1*-KO cortex (10.88±2.62%, *p*=0.050; **Fig. 3-3E**) and the *Fmr1*-KO hippocampus (9.40±1.92%, *p*=0.047; **Fig. 3-3E**) in comparison to cultures without ACM (2.93±1.81%). The proportion of neurospheres ≥ 2 mm did not change in the presence of ACM from WT astrocytes from the cortex (4.71±2.78%, p=0.61; **Fig. 3-3F**) or the hippocampus (2.31±1.29%, p=0.81 **Fig. 3-3F**). Taken together, the results showed that *Fmr1*-KO ACM but not WT ACM significantly affected WT neurosphere size. This suggests that *Fmr1*-KO astrocytes provide aberrant signals in the control of NPC proliferation.

Table 3-2. Summary of results.

	Neurosphere Size	WT ACM	KO ACM
WT Neurospheres	≤0.5 mm	Δ↑	Δ↑
	0.5 – 1 mm	no Δ	Δ↓
	1 – 2 mm	no Δ	Δ↓
	≥2 mm	no Δ	Δ↑
KO Neurospheres	≤0.5 mm	Δ↑	Δ↑
	0.5 – 1 mm	no Δ	no Δ
	1 – 2 mm	Δ↓	Δ↓
	≥2 mm	no Δ	no Δ

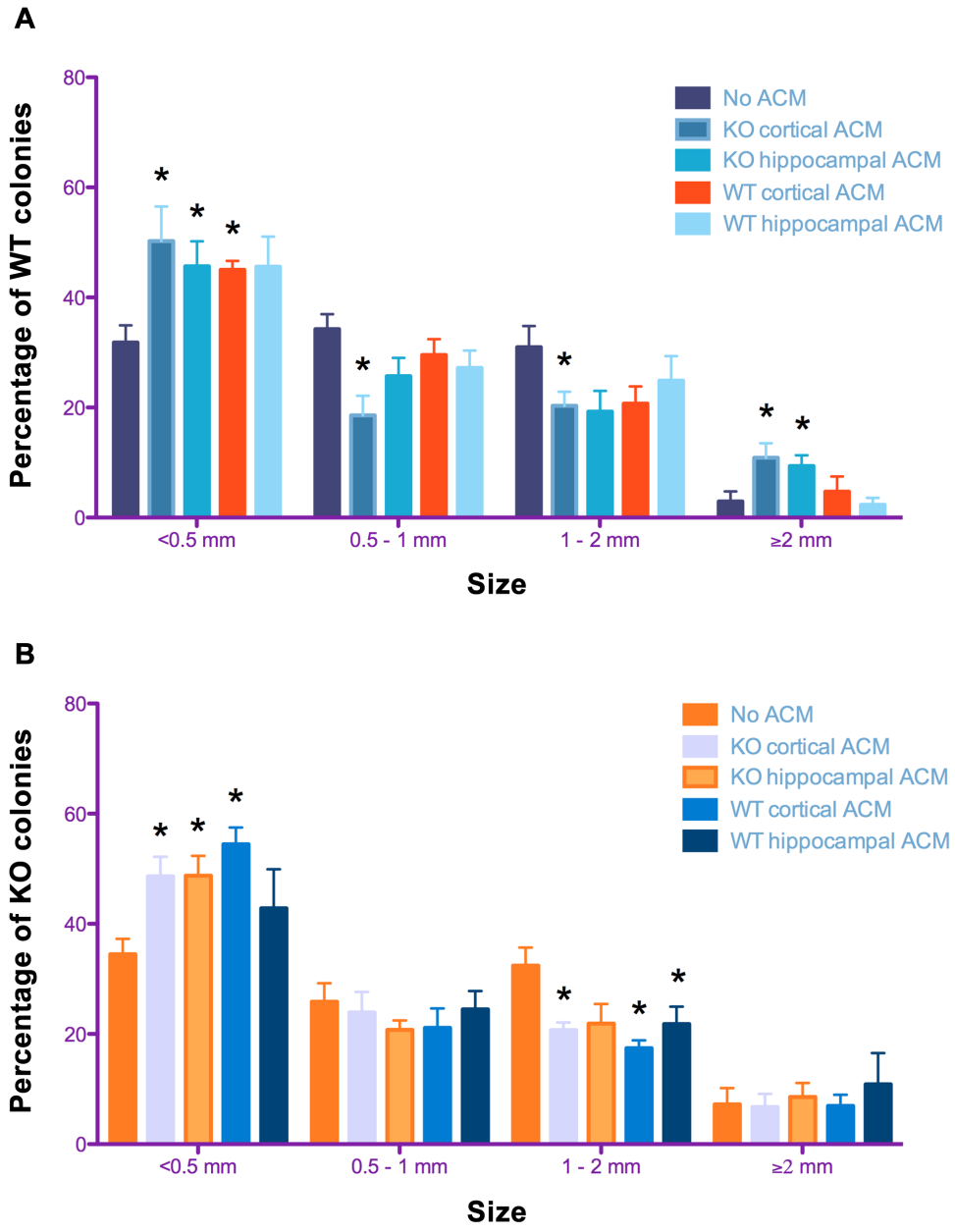

Figure 3-S2. Summary of the effect of astrocyte conditioned media (ACM) on neurosphere size. A. WT neurospheres, and **B.** *Fmr1*-KO neurospheres. * denotes significant difference from neurospheres with no ACM ($p<0.05$). Abbreviations: WT: wild type, *Fmr1*-KO: *Fmr1* knockout, ACM: astrocyte conditioned media.

Differential expression of astrocyte-secreted proteins in Fragile X

Proteomic analyses were performed on ACM to determine the identity of secreted proteins and compare between WT and *Fmr1*-KO astrocytes. 2D DIGE of spots that were at least expressed at ±1.5 fold in *Fmr1*-KO compared to WT samples revealed 37 spots in cortical ACM (**Fig. 3-4C**) and 29 spots in hippocampal ACM (**Fig. 3-4E**). Eight of the spots were identified by mass spectrometry as: multidrug resistance protein 1B, microphthalmia-associated transcription factor (MITF), haptoglobin, fasciculation and elongation protein zeta 2 (FEZ2), antithrombin III, and serum albumin (**Table 3-3**). Three spots corresponded to serum albumin, which were not immediately adjacent on the gel possibly due to post-translational modifications or disintegration. Notably, the expression of the majority of astrocyte-secreted proteins from the *Fmr1*-KO cortex was downregulated relative to WT (34 out of 37 spots, **Table 3-S1**), whereas most secreted proteins from the *Fmr1*-KO hippocampus were upregulated compared to WT (18 out of 29 spots, **Table 3-S2**). Differences between cortical and hippocampal astrocyte-secreted factors again confirm the heterogeneity of astrocytes.

Table 3-3. Identity of proteins in ACM.

Spot number	Gel	Protein	Fold difference (*Fmr1*-KO/WT)	Theoretical PI/MW	Protein Score C.I. (%)	Total Ion C.I. (%)
2	1 (cortical ACM)	Multidrug resistance protein 1B	-4.9	8.5/140,905	0	97
16		Microphthalmia-associated transcription factor (MITF)	-4.8	5.9/58,590	90	100
28		Haptoglobin	-10.6	5.9/38,728	100	100
33		Fasciculation and elongation protein zeta-2 (FEZ2)	-12.9	4.5/39,076	99	91
8	2 (hippocampal ACM)	Antithrombin-III	2.4	6.1/51,971	100	100
25		Serum albumin	-7.0	5.8/68,648	100	100
27		Serum albumin	-25.7	5.8/68,648	100	100
28		Serum albumin	-45.3	5.8/68,648	100	100

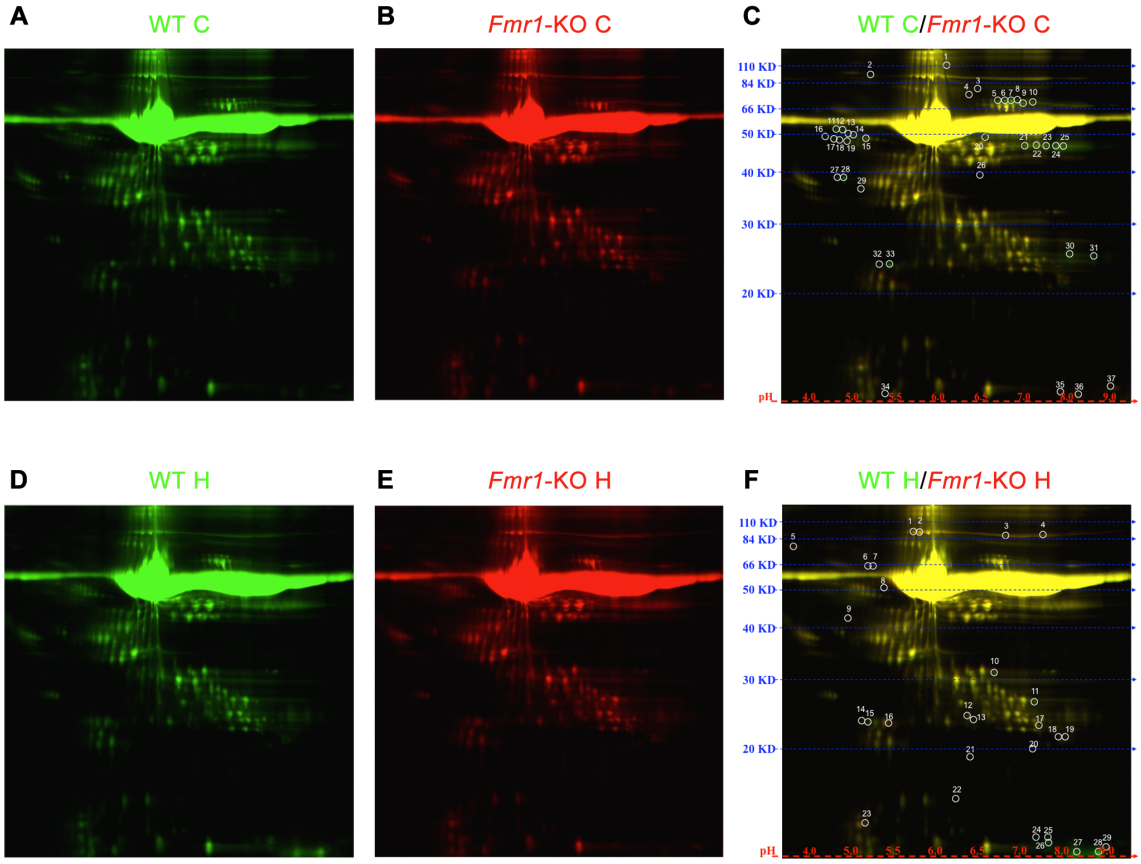

Figure 3-4. 2D DIGE of cortical and hippocampal ACM. Proteome map of ACM from **A.** WT cortical samples or **B.** *Fmr1*-KO cortical samples. **C.** 37 spots showed at least 50% expression difference in ACM from *Fmr1*-KO cortices relative to their WT counterparts. Proteome map of ACM from **D.** WT hippocampal samples or **E.** *Fmr1*-KO hippocampal samples. **F.** 29 spots showed at least 50% expression difference in ACM from *Fmr1*-KO hippocampi. Abbreviations: WT: wild type, *Fmr1*-KO: *Fmr1* knockout, ACM: astrocyte conditioned media, C: cortical, H: hippocampal, 2D DIGE: two-dimensional difference in gel electrophoresis.

Table 3-S1. Spots from cortical ACM with at least 1.5 fold difference relative to WT.

Spot # on gel	Protein expression ratio relative to WT
1	-1.6
2	-4.9
3	-2.4
4	-1.7
5	-1.9
6	-3.1
7	-3.6
8	-2.4
9	-1.7
10	-1.7
11	-2.1
12	-3.4
13	-2.1
14	-3.1
15	-1.7
16	-4.8
17	-3.6
18	-2.3
19	-1.6
20	-2.2
21	-2.1
22	-2.2
23	-3.1
24	-4.5
25	-4.2
26	-3.7
27	-5.2
28	-10.6
29	-1.9
30	-2.7
31	-3.5
32	-2.3
33	-12.9
34	-3.5
35	1.5
36	1.9
37	1.6

Table 3-S2. Spots from hippocampal ACM with at least 1.5 fold difference relative to WT.

Spot # on gel	Protein expression ratio relative to WT
1	1.6
2	1.5
3	1.6
4	1.7
5	1.7
6	2.0
7	1.8
8	2.4
9	1.8
10	-1.7
11	-1.7
12	1.6
13	1.6
14	-1.8
15	-1.7
16	1.8
17	1.8
18	1.6
19	2.1
20	1.7
21	1.8
22	-2.1
23	1.8
24	-4.9
25	-7.0
26	-8.5
27	-25.7
28	-45.3
29	-2.0

Discussion:

In this study, we examined potential differences in the proportions of neural progenitor and neural stem cells between WT and *Fmr1*-KO NPCs *in vitro* using the NCFC Assay. Our results confirmed that the NCFC Assay generates four size categories of neurospheres, in turn reflecting different proliferative potentials in the NPC population (Louis et al., 2008). We also found that *Fmr1*-KO neural progenitor cells had a non-autonomous cell deficit in correctly responding to astrocyte secreted factors. This was evident in the indiscriminate response of *Fmr1*-KO neurospheres to ACM from WT or *Fmr1*-KO astrocytes, which contrasted with the specific response of WT neurospheres to *Fmr1*-KO, but not WT, ACM. Interestingly, *Fmr1*-KO ACM increased the proliferation of WT functional neural stem cells, highlighting the abnormal signals that astrocytes send to NPCs in the absence of FMRP.

NPCs comprise populations of neural stem and progenitor cells. Neural stem cells self-renew and are multipotent, giving rise to all neural lineages, namely neurons, astrocytes, and oligodendrocytes. Neural progenitor cells, on the other hand, have limited proliferative capacity and restricted potential in differentiating to distinct lineages. Previous research has reported enhanced NPC proliferation in the embryonic SVZ (Castrén et al., 2005), the embryonic VZ and SVZ combined (Tervonen et al., 2009), and the adult DG (Guo et al., 2011; Luo et al., 2010) in association with lack of FMRP. Given this, we expected to observe differences between WT and *Fmr1*-KO cultures in the proportions of neural progenitor- or stem cell-derived neurospheres generated. Our results

alternatively show that there were in fact no inherent differences between the proliferative propensities of the neurospheres of each genotype, in line with Eadie and colleagues (2009), who reported no differences in the proliferation of NPCs in the DG of adult *Fmr1*-KO mice *in vivo* (Eadie et al., 2009). It is noteworthy to mention that our study is the first to examine hippocampal NPCs from the *Fmr1*-KO mouse brain on an FVB genetic background. While we detected no differences in neural progenitor and stem cell proliferation in our culture system between WT and *Fmr1*-KO cultures after 21 *DIV*, differences may exist at other time points especially since FMRP is developmentally regulated (Till et al., 2012). Till et al. (2012) indicate that *Fmr1*-KO mice show delays in cortical development at time points that coincide with the highest FMRP levels in normal brains. Notably, astrocytes of the developing hippocampus express FMRP (Pacey and Doering, 2007), suggesting that astrocytes contribute to the hippocampal impairments seen in Fragile X.

We identified a subset of astrocyte-secreted factors whose expression was up- or down-regulated by at least 50% in *Fmr1*-KO samples. These included multidrug resistance protein 1B, MITF, haptoglobin, and FEZ2 in cortical ACM, and antithrombin III and serum albumin in hippocampal ACM. These factors were all downregulated with the exception of antithrombin III (**Tables S1**, **S2**). Notably, the expression of multidrug resistance protein 1 has been reported in human NPCs where it promotes the proliferation of NPCs (Yamamoto et al., 2009). Thus, our finding of decreased multidrug resistance protein 1 expression in *Fmr1*-KO cortical ACM is in agreement with the decreased neural

progenitor proliferation detected in the presence of *Fmr1*-KO cortical ACM. Unlike the multidrug resistance family of proteins, the function of the FEZ family of proteins is elusive. Previous research has found that FEZ2 interacts with 59 proteins in categories that include transcription, translation, apoptosis, signal transduction, neuronal cell development, and cytoskeleton and centrosome (Alborghetti et al., 2011). The function of FEZ2 remains poorly understood at this time. The expression of albumin and antithrombin III in the developing brain under normal conditions suggests that they play an important role in brain development (Deschepper et al., 1991; Dziegielewska et al., 1984). In fact, astrocytes of the developing brain have been shown to secrete antithrombin III in culture, similar to our findings (Deschepper et al., 1991). Albumin is also necessary for astrocytes to provide energy to the developing brain (Tabernero et al., 1999) and to synthesize oleic acid (Tabernero et al., 2001). In turn, oleic acid promotes neuronal differentiation (Tabernero et al., 2001). Given that astrocytes show developmental delays in FXS (Jacobs et al., 2010), we suggest that the expression levels of albumin and antithrombin III that we detected in *Fmr1*-KO hippocampal ACM may mirror the expression levels in ACM from the WT hippocampus at earlier time points. We are currently studying the role of the astrocyte secreted factors in Fragile X in order to better understand the extent of astrocyte involvement in Fragile X and associated neurodevelopmental disorders.

Astrocyte-secreted factors, such as thrombospondin-1 (Lu and Kipnis, 2010), fibroblast growth factor-2 (Kirby et al., 2013), and clusterin (Cordero-Llana et al., 2011)

promote NPC proliferation, neurogenesis, or both. Indeed, astrocytes are an important component of the hippocampal neurogenic niche (Song et al., 2002). Our previous work has shown that signals from *Fmr1*-KO astrocytes can alter the development and growth of co-cultured WT neurons, an effect not seen with WT astrocytes (Jacobs and Doering, 2010a). Thus, we hypothesized that ACM from WT astrocytes would correct aberrant proportions of progenitor and stem cell-derived *Fmr1*-KO neurospheres, whereas *Fmr1*-KO ACM would alter the proportions of each of the WT neurosphere size categories. Our results demonstrated that ACM from *Fmr1*-KO astrocytes did alter the proliferation of WT neural progenitor and functional stem cells, restricting the proliferation of neural progenitors while increasing the proliferation of neural stem-derived neurospheres. It remains to be determined why *Fmr1*-KO ACM exerts opposite effects on neural progenitor compared to neural stem cells.

Intriguingly, the percentage of WT neurospheres measuring ≤0.5 mm increased in the presence of WT and *Fmr1*-KO ACM from the cortex and the hippocampus, similar to *Fmr1*-KO neurospheres of the same size category. These neurospheres have a limited ability for self-renewal (Louis et al., 2008). The generic response of WT neurospheres to ACM is interesting, as it was only detected in this size category, and mirrors the effect of ACM on *Fmr1*-KO neurospheres. This suggests a non-cell autonomous response of WT NPCs of limited potential as demonstrated by their generic response to astrocyte-secreted factors regardless of FMRP expression.

Lack of FMRP caused a defect in *Fmr1*-KO NPCs to respond correctly to environmental cues. *Fmr1*-KO neurospheres showed an indiscriminate response towards WT and *Fmr1*-KO ACM. We found that ACM from WT and *Fmr1*-KO astrocytes caused a global increase in the proportion of *Fmr1*-KO neurospheres ≤0.5 mm. In parallel, ACM decreased the proportion of *Fmr1*-KO neurospheres 1 – 2 mm in diameter relative to *Fmr1*-KO neurospheres of the same size without ACM. Collectively, this indicates that ACM restricted the proliferation of *Fmr1*-KO neural progenitors without affecting the proliferation of stem cells. The fact that the percentages of some *Fmr1*-KO neurosphere size categories (i.e. 0.5 – 1 mm and ≥2 mm) did not change in the presence of ACM suggests that not all NPC populations are targets of astrocyte-secreted factors in Fragile X.

In contrast to *Fmr1*-KO NPCs, only ACM from *Fmr1*-KO astrocytes decreased the percentage of WT neurospheres 1 – 2 mm, and increased that of neurospheres ≥2 mm in diameter. The selective response of WT neurospheres to *Fmr1*-KO ACM points to a non-cell autonomous defect in NPC proliferation in the absence of FMRP caused by abnormal *Fmr1*-KO astrocytic cues. This was particularly evident in the effect of ACM on neurospheres ≥2 mm in diameter, where the percentage of WT neurospheres was increased in the presence of *Fmr1*-KO ACM, while that of *Fmr1*-KO neurospheres was not. This population of neurospheres represents multipotent and proliferative functional stem cells (Louis et al., 2008). The apparent aberrant signalling of *Fmr1*-KO astrocytes

may be due to compensatory measures for the lack of responsiveness of *Fmr1*-KO neurospheres to cellular or environmental cues.

The addition of *Fmr1*-KO cortical ACM to WT neurospheres caused the most robust changes, affecting the total number of neurospheres generated (**Fig. S1**) as well as the percentages of neurospheres in all size categories relative to WT neurospheres without ACM (**Fig. S2**), which highlights the heterogeneity of astrocytes (Matyash and Kettenmann, 2010). This is likely due to the genotypic-spatial mismatch between hippocampal WT neurospheres and cortical *Fmr1*-KO ACM. Indeed, of all combinations tested, ACM from *Fmr1*-KO cortical astrocytes decreased the percentage of WT colonies measuring 0.5 – 1 mm. Thus, the proliferation of NPCs that make up colonies 0.5 – 1 mm is not solely changed by astrocyte-secreted factors from Fragile X brains. Rather, the enhanced effect of *Fmr1*-KO cortical ACM on WT hippocampal neurospheres resulted in our finding, which seems to only apply to neurospheres 0.5 – 1 mm. In agreement with the specificity of astrocytes, hippocampal astrocytes are shown to promote NPC proliferation and neurogenesis, whereas spinal cord astrocytes do not (Song et al., 2002). Similarly, ACM from the hippocampus increases the number of neurons differentiated from human NPCs, whereas cortical ACM does not (Cordero-Llana et al., 2011).

One of the functions of hippocampal neurogenesis is learning and memory (Deng et al., 2010). We thus hypothesized that impaired learning, characteristic of FXS, is attributed to aberrant NPC proliferation and neurogenesis. *Fmr1*-KO mice have demonstrated deficits in hippocampus-dependent learning such as exaggerated inhibitory

avoidance extinction (Dölen et al., 2007) and trace conditioning tasks (Hayashi et al., 2007). Interestingly, conditional ablation of FMRP from adult NPCs results in learning deficits on the trace conditioning and delayed non-matching-to-place radial arm maze tasks, both of which require an intact hippocampus, and conditional restoration of FMRP results in improved performance on these tasks (Guo et al., 2011). Therefore, functional restoration of hippocampal neurogenesis may alleviate learning and memory impairments in Fragile X.

Summary:

In this study, we examined the proliferation of hippocampal neural progenitor and stem cells from the early postnatal *Fmr1*-KO mouse brain. We found that *Fmr1*-KO progenitor-derived neurospheres showed decreased proliferation in response to WT and *Fmr1*-KO ACM, thereby demonstrating non-cell autonomous defects in responding correctly to astrocyte-secreted factors. In contrast, WT neurospheres showed a specific response to *Fmr1*-KO ACM, where *Fmr1*-KO ACM restricted the proliferation of neural progenitors, and enhanced the proliferation of stem cells. We also identified some of the astrocyte secreted factors with ±1.5 fold expression difference in *Fmr1*-KO brains. To our knowledge, this is the first time that astrocyte-secreted factors are assayed and a number of them identified with mass spectrometry in relation to Fragile X. In turn, this work opens up new possibilities in investigating the functions of these factors and uncovering the role of astrocytes in Fragile X and neurodevelopmental disorders.

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Disclosure of Potential Conflicts of Interest:

The authors indicate no potential conflicts of interest.

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Chapter 4: Aberrant Regulation of Neural Precursor Cells in the Early Postnatal Fragile X Mouse Hippocampus

Chapter Link

Previous research has implicated FMRP in governing NPCs. However, no research groups examined hippocampal NPCs at the time when FMRP normally peaks, which is P7. Thus, I studied the proliferation and cell cycle progression of hippocampal NPCs *in vitro* and *in vivo* during early postnatal development.

Aberrant regulation of neural precursor cells in the early postnatal Fragile X mouse hippocampus

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Running Head: Neural Precursor Cells in Fragile X

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Research Highlights:

- Aberrant *in vitro* regulation of *Fmr1*-KO NPCs.
- Proliferation of hippocampal NPCs is decreased in early postnatal *Fmr1*-KO brains.
- Fewer cells are detected in the G_2/M phases of the cell cycle in *Fmr1*-KO DG.

Keywords: Fragile X Syndrome; neural stem cells; neurospheres; cell cycle; *Fmr1*-knockout mice**.**

Abstract:

The regulation of neural precursor cells (NPCs) is indispensable for a properly functioning brain. Abnormalities in NPC proliferation, differentiation, survival, or integration have been linked to various neurological diseases including Fragile X syndrome. Yet, no studies have examined NPCs from the early postnatal Fragile X mouse hippocampus despite the importance of this developmental time point. In fact, the end of the first postnatal week marks the highest expression level of FMRP, the protein missing in Fragile X, in the rodent hippocampus and is when hippocampal NPCs have migrated to the dentate gyrus to give rise to lifelong neurogenesis. In this study, we examined NPCs from the early postnatal hippocampus and dentate gyrus of Fragile X mice. Using immunocytochemistry, we analyzed the expression of the precursor cell markers Nestin and SOX2, coupled with the expression of Ki67, a cell proliferation marker. Intriguingly, we found increased Nestin expression and decreased Ki67 expression, which collectively indicated reduced NPC proliferation. Western blotting on hippocampal tissue, on the other hand, showed lower expression levels of Nestin and the mitotic marker phospho-histone H3, which were in agreement with our analysis of profiles of the cell cycle phases. Together, these results showed that the absence of FMRP leads to fewer actively cycling NPCs in the early postnatal hippocampus, which is the time when the neurogenic subgranular zone of the dentate gyrus is forming.

Introduction:

Neural precursor cells (NPCs) comprise populations of multipotent, self-renewing neural stem and the more restricted neural progenitor cells. NPCs are tightly governed temporally and spatially not only to generate the correct number and subtypes of neurons and glia, but ones that also functionally integrate into neural networks in a precise manner (van Praag et al., 2002). Dysfunction of NPCs has been associated with many neurological disorders including Fragile X syndrome (FXS) (Castrén et al., 2005; Eadie et al., 2009; Guo et al., 2011; Luo et al., 2010).

FXS is the leading monogenic cause of autism spectrum disorder and inherited intellectual impairment (Wang, Bray, & Warren, 2012). Its symptoms include developmental delays, cognitive and memory impairments, repetitive behaviours, and hyperactivity (R. J. Hagerman, Portes, Gasparini, Jacquemont, & Gomez-Mancilla, 2014). FXS largely stems from the hypermethylation and silencing of the *FMR1* gene (Oberlé et al., 1991), consequently resulting in the lack of production of the Fragile X mental retardation protein (FMRP) (Pieretti et al., 1991). Mice that lack expression of FMRP, hereafter referred to as *Fmr1*-knockout (KO), recapitulate symptoms of FXS (Bakker et al., 1994). Notably, *Fmr1*-KO mice demonstrate impaired hippocampus dependent learning (Hayashi et al., 2007) that can be corrected upon the expression of FMRP in hippocampal NPCs (Guo et al., 2011). This strongly suggests that the dysfunction of hippocampal NPCs contributes to the learning and memory symptoms of FXS.

Emerging evidence indicates that NPCs primarily show altered proliferation in the absence of FMRP expression in the embryonic (Castrén et al., 2005; Tervonen et al., 2009) and adult brains (Guo et al., 2011; Luo et al., 2010). Lack of FMRP has also been associated with aberrant differentiation of NPCs into neurons and astrocytes (Castrén et al., 2005; Eadie et al., 2009; Luo et al., 2010; Telias, Segal, & Ben-Yosef, 2013). A plausible signalling pathway that may underlie aberrant NPC proliferation and differentiation is Notch1. Notch1 plays a role in regulating the self-renewal, fate determination, and cell cycle exit of NPCs during development and in adult neurogenic regions (Ables et al., 2010; Breunig, Silbereis, Vaccarino, Šestan, & Rakic, 2007; Ehm et al., 2010; Yoon & Gaiano, 2005). In fact, overexpression of Notch1 in $GFAP^+$ cells results in NPCs not exiting the cell cycle (Breunig et al., 2007). Moreover, findings from human embryonic Fragile X stem cell lines indicate aberrant regulation of Notch1 expression (Telias et al., 2013). This suggests that the cell cycle progression may be altered in *Fmr1*-KO NPCs.

We thus decided to study hippocampal NPCs from *Fmr1*-KO mice in the early postnatal period, which coincides with the highest FMRP expression in the hippocampus (Lu et al., 2004). We hypothesized that NPCs from *Fmr1*-KO hippocampi will demonstrate altered proliferation based on previous findings and that aberrant regulation of Notch1 signalling contributes to the altered proliferation. To test our hypothesis, we analyzed the *in vitro* expression of Nestin and SOX2, which label undifferentiated cells, and Ki67, which labels actively cycling cells (Gerdes, Schwab, Lemke, & Stein, 1983) in

neuropsheres. We also studied postnatal day 5 (P5) and P9 hippocampal expression of Nestin, SOX2, Notch1, and phospho-histone H3 (PhH3), which is expressed in cells in the mitotic phase of the cell cycle, using Western blotting. Our *in vitro* results showed that *Fmr1*-KO NPCs had increased expression of Nestin and decreased expression of Ki67, suggesting decreased proliferation of NPCs in culture. Intriguingly, Nestin expression was decreased *in vivo* in P9 hippocampi of *Fmr1*-KO mice along with a decrease in PhH3 expression in P9 of male mice. In line with this, our flow cytometric cell cycle analysis indicated a decrease in the relative proportion of *Fmr1*-KO cells in the G_2/M phases of the cell cycle. All together, these results showed that lack of FMRP leads to decreased proliferation of hippocampal NPCs during early postnatal development, which may suggest increased quiescence of neural stem cells, a delay in the activation of NPCs, or alternatively the depletion of NPCs in Fragile X.

Materials and Methods:

Animals:

All animal experiments were performed in accordance with the guidelines set out by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board. The *Fmr1*-KO mouse colony was established from breeding pairs of FVB.129P2(B6)-*Fmr1*^{tm1Cgr} mice. The *Fmr1*-KO mouse was originally engineered by inserting a neomycin cassette in exon 5 of the *Fmr1* gene (Bakker et al., 1994). The WT and *Fmr1*-KO mice were maintained as individual strains, and were housed and bred at the McMaster University Central Animal Facility.

Neurosphere Assay:

WT and *Fmr1*-KO P1 mouse pups were euthanized and the brains processed for the neurosphere assay (Pacey, Stead, Gleave, Tomczyk, & Doering, 2006). Both male and female pups were used for 4 DIV experiments, whereas only male pups were used in 5 and 8 DIV experiments since symptoms of FXS are more pronounced in males (Jacquemont, Hagerman, Hagerman, & Leehey, 2007). Briefly, hippocampi were microdissected, minced into small pieces, and enzymatically digested for 15 minutes at 37°C as described in Pacey et al. (Pacey et al., 2006). Trypsin was inactivated with a 1 mg/mL solution of trypsin inhibitor (Roche, Mississauga, Canada) at 37°C for 10 minutes. Cells were resuspended in 2 mL of serum free medium in the presence of 5 ng/mL of fibroblast growth factor-basic (FGF-2, Sigma-Aldrich, St. Louis, USA) and 20 ng/mL of epidermal growth factor (EGF, Sigma-Aldrich), and were plated at 90 cells/μL for 4 DIV, or 10 cells/μL for 5 and 8 DIV.

Immunocytochemistry:

Neurospheres were plated on poly-L-lysine (1 mg/ml, Sigma-Aldrich)- and laminin (0.1 mg/ml, Life Technologies, Burlington, Canada)-coated coverslips in serum free medium for 1 hour at 37°C before being fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cell membranes were permeabilized in 0.1% v/v Triton-X 100 in phosphate-buffered saline (PBS) and non-specific binding sites were blocked in 1% w/v bovine serum albumin (Sigma Aldrich) in PBS. Neurospheres were incubated with primary antibodies overnight at 4°C. After washing 4 times, secondary antibodies were applied

for 3 h at room temperature. Coverslips were mounted with Vectashield fluorescent mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies) counterstain. The following antibodies, diluted in PBS, were used: mouse monoclonal anti-Nestin (Abcam, Eugene, USA, 1:300), rabbit polyclonal anti-SOX2 (Abcam, 1:750), and rabbit polyclonal anti-Ki67 (Novocastra, Concord, Canada, 1:1000), anti-mouse AlexaFluor 594 (Life technologies, 1:1500), and anti-rabbit FITC (Jackson, Westgrove, USA, 1:100).

Epifluorescence Microscopy:

Fixed neurospheres were examined using the Zeiss Axioskop 2 MOT Plus fluorescent microscope. The number of cells positive for Nestin, SOX2, and Ki67 per neurosphere were counted live and converted to percentage positive cells/neurosphere based on the number of DAPI counterstained nuclei.

Western Blotting:

P5 and P9 hippocampi from both male and female (N=6 per genotype, sex, and time point; a total of 48 samples) mice were used to detect if there are sex differences in relation to hippocampal marker expression in FXS during development. Hippocampi were microdissected and homogenized in 1X RIPA buffer supplemented with protease and phosphatase inhibitor tablets (Roche). Protein concentrations were determined using the DC protein assay (Bio-Rad, Mississauga, Canada). Proteins (50 μg protein/lane) were resolved on Stain-Free 4 – 15% gradient polyacrylamide electrophoresis precast gels

(TGX Precast Gels, Bio-Rad) in reducing, denaturing conditions. The trihalo compound in gels was activated by 1-minute UV exposure (302 nm). Proteins were transferred to PVDF membranes (Bio-Rad) using the Transblot Turbo for 5, 7, or 10 minutes (Bio-Rad) and the total protein was imaged on the ChemiDoc MP imager (Bio-Rad). After blocking in 5% nonfat milk in Tris-buffered saline Tween-20 (TBST), primary antibodies against rabbit monoclonal to SOX2 (Abcam, 1:1500), rabbit monoclonal to Notch1 (Abcam, 1:2000), or rabbit monoclonal to phospho-histone H3 (PhH3, Abcam, 1:2500) in 5% nonfat milk in TBST, or mouse monoclonal to Nestin (Abcam, 1:500) in 5% BSA were incubated overnight at 4°C. Washes in TBST were followed by secondary donkey anti-mouse HRP antibody (GE Healthcare Life Sciences, Mississauga, Canada, 1:5000) or donkey anti-rabbit HRP antibody (GE Healthcare Life Sciences, 1:5000) incubation for 1 h. Western blots were detected using Clarity ECL substrate (Bio-Rad), followed by analysis on Image Lab (Bio-Rad). Protein expression was normalized to total protein. Relative protein expression was calculated in reference to the same WT control sample per time point. Data from two technical replicates were averaged prior to statistical analysis.

Cell Cycle Analysis:

P4 hippocampi (N=5) or P7 dentate gyri (DG, N=6) mice were microdissected and enzymatically dissociated in TrypLE Express (Life Technologies) supplemented with 200 units/mL DNase I (Roche) and 1 mM $MgCl_2$ for 45 minutes, modified from a previously published protocol (Panchision et al., 2007). Cells were resuspended in flow cytometry

buffer, comprising 1X HBSS Ca^{2+}/Mg^{2+} free, pH 7.2 (Life Technologies), containing 1.55 g/L glucose and 0.5% bovine serum albumin, fraction V (Sigma-Aldrich). Cells were subsequently washed in 1X PBS Ca^{2+}/Mg^{2+} free (Life Technologies), fixed by drop wise addition of ice cold ethanol to a final concentration of 70% and incubated at -20°C for a minimum of 24 hours before flow cytometric analysis. Prior to analysis, cells were washed, incubated in 100 μg/mL RNase A (Sigma-Aldrich) for 40 minutes at room temperature, and stained with 50 μg/mL propidium iodide (Sigma-Aldrich) in 0.1% Triton-X (Sigma-Aldrich). Samples were analyzed on Becton Dickinson LSRII (BD, Mississauga, Canada) and FlowJo data analysis software (version X.0.8, Tree Star, Ashland, USA).

Statistics:

Statistical analysis was performed using GraphPad Prism (version 5.0). Two-way analysis of variance was used followed by Bonferroni's post-hoc test (**Fig. 4-5; Table 4-1)** or by two-tailed t-tests with Welch's correction when appropriate (**Fig. 4-1 – 4-2, 4-4**). Spearman correlation analysis was used in **Fig. 4-2C – F**. Two-tailed t-tests were used in **Fig. 4-3**. Probabilities of $p<0.05$ were considered significant. Data are expressed as means and error bars represent the standard error of the mean (SEM).

Results:

SOX2 expression increases in Fmr1-KO neurospheres after 4 DIV

To study the proliferation of early postnatal NPCs in Fragile X, we first examined the expression of the early cell lineage markers, Nestin and SOX2, after 4 days *in vitro* (DIV) in neurospheres cultured from P1 hippocampi. Percentages of $SOX2^+$ or $Nestin^+$ cells per neurosphere were calculated based on the number of DAPI counterstained nuclei. We found that the majority of cells were positive for Nestin and SOX2, and that larger neurospheres had a smaller proportion of cells labeled with SOX2 or Nestin (**Fig. 4-1**). Lack of FMRP expression differentially affected SOX2 and Nestin labeling ($F_{1, 3704} = 8.37$, p=0.0038). Specifically, *Fmr1*-KO neurospheres had a significantly higher percentage of $SOX2^+$ cells compared to wild type (WT) neurospheres (p<0.0001, WT: 86.53 ± 0.50%, *Fmr1*-KO: 89.49 ± 0.29%, **Fig. 4-1A)**.

We further examined the expression of SOX2 and Nestin in relation to neurosphere size, separately analyzing neurospheres with ≤50 cells and 51 – 100 cells. *Fmr1*-KO neurospheres ≤50 cells had a larger percentage of $SOX2^+$ cells compared to their WT counterparts (p=0.0003, WT: 87.74 ± 0.51%, *Fmr1*-KO: 89.87 ± 0.29%, **Fig. 4-1B**). Interestingly, *Fmr1*-KO neurospheres 51 – 100 cells had a significantly higher percentage of $Nestin^+$ cells (p=0.041, WT: 78.36 ± 1.35%; *Fmr1*-KO: 81.50 ± 0.70%, **Fig. 4-1C**) as well as $SOX2^+$ cells compared to WT neurospheres (p<0.0001, WT: 77.70 ± 1.45%, *Fmr1*-KO: 86.91 ± 0.60%**)**. Together, these results showed that, while there was an increased percentage of $SOX2^+$ cells in all *Fmr1*-KO neurosphere sizes after 4 DIV,

the increased percentage of Nestin^{+} cells in *Fmr1*-KO neurospheres was only evident in larger neurospheres (51 – 100 cells). This prompted further experiments to assess the relation of SOX2 and Nestin expression to FMRP at different time points during development.

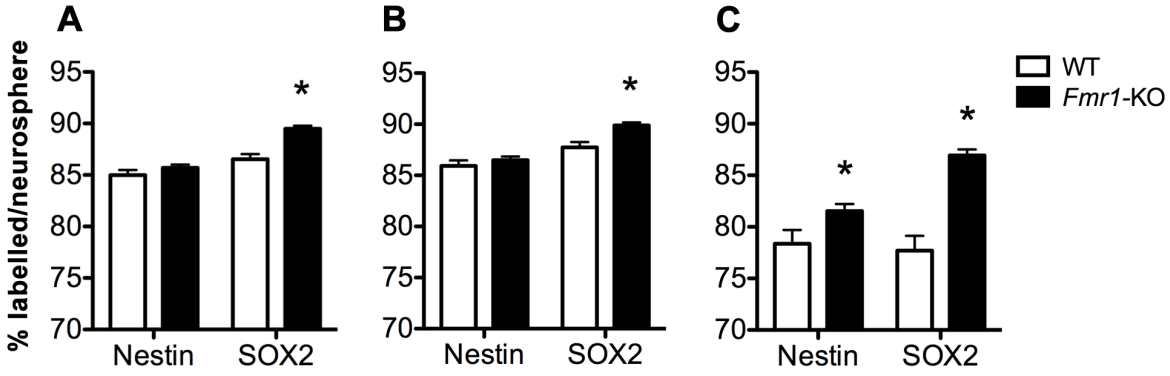

Figure 4-1. *Fmr1*-KO neurospheres express more SOX2 and Nestin after 4 DIV. A. Increased percentage of $SOX2^+$ cells in all *Fmr1*-KO neurosphere sizes combined (WT: 86.53 ± 0.50%, *Fmr1*-KO: 89.49 ± 0.29%; $p<0.0001$; $n_{WT}= 655$ neurospheres, $n_{KO}= 1199$ neurospheres; $N_{WT}=4$, $N_{KO}=3$ cultures). **B.** Increased percentage of $SOX2^+$ cells in *Fmr1*-KO neurospheres ≤50 cells (WT: 87.74 ± 0.51%, *Fmr1*-KO: 89.87 ± 0.29%; $p=0.0003$; $n_{WT}= 573$ neurospheres, $n_{KO}= 1017$ neurospheres). **C.** Increased percentage of $SOX2^+$ cells and $Nestin^+$ cells in *Fmr1*-KO neurospheres 51 – 100 cells (WT: 77.70 ± 1.45%, *Fmr1*-KO: 86.91 ± 0.60%; $p<0.0001$; WT: 78.36 ± 1.35%; *Fmr1*-KO: 81.50 ± 0.70%, $p=0.041$, respectively; $n_{WT}= 79$ neurospheres, $n_{KO}= 160$ neurospheres). Abbreviations: DIV: days *in vitro*, WT: wild type, *Fmr1*-KO: *Fmr1*-knockout.

A trend towards decreased Nestin expression in intermediate-sized Fmr1-KO neurospheres after 5 DIV

We next performed immunocytochemistry on neurospheres cultured for 5 or 8 DIV from the P1 hippocampus of male mice. After 5 DIV, $SOX2^+$ cells exceeded $Nestin^+$ cells regardless of genotype ($F_{1, 1644} = 71.77$, $p<0.0001$), which was true of the different neurosphere sizes examined. It is worth noting that neurospheres at this time point were small (<100 cells). Intriguingly, *Fmr1*-KO neurospheres 51 – 100 cells had a smaller proportion of cells labeled with Nestin or SOX2 ($F_{1, 72} = 6.21$, $p=0.015$) and there was a trend towards a decreased proportion of $Nestin^+$ cells compared to WT ($p=0.060$).

Nestin expression increases in large Fmr1-KO neurospheres after 8 DIV

After 8 DIV, we found that *Fmr1*-KO neurospheres had an increased percentage of $Nestin^+$ cells, but not $SOX2^+$ cells compared to their WT counterparts ($p=0.0110$, WT: 88.24 ± 0.68%; *Fmr1*-KO: 90.35 ± 0.47%, **Fig. 4-2A**). This was particularly evident in *Fmr1*-KO neurospheres ≥101 cells, which showed a significantly higher percentage of $Nestin^+$ cells ($p=0.0019$, WT: 70.41 ± 2.72%; *Fmr1*-KO: 80.40 ± 1.55%, **Fig. 4-2B**). Neurospheres ≤50 cells or 51 – 100 cells did not show genotypic differences in SOX2 or Nestin expression. Thus, the fact that *Fmr1*-KO neurospheres had more cells expressing Nestin primarily in larger neurospheres indicated dysregulation in the more proliferative NPC populations through enhanced Nestin but not SOX2 expression.

Examining the correlation between Nestin and SOX2 expression after 8 DIV corroborated our findings of dysregulated proliferation of neurospheres from *Fmr1*-KO

hippocampi. We found that there was a stronger correlation between the percentage of $Nestin^+$ and $SOX2^+$ cells in WT compared to *Fmr1*-KO neurospheres. Intriguingly, the correlation between the percentage of $Nestin^+$ to $SOX2^+$ cells became stronger with increasing WT neurosphere size (51 – 100 cells: r_S=0.5486, p<0.0001, **Fig. 4-2C**; ≥101 cells: r_S=0.7920, p<0.0001, **Fig. 4-2E**). This contrasted with *Fmr1*-KO neurospheres in which the correlation between Nestin and SOX2 labelling was lower than in WT neurospheres and remained similar across the two neurosphere size categories (51 – 100 cells: r_S=0.3685, p=0.0020, **Fig. 4-2D**; ≥101 cells: r_S=0.3724, p=0.0018; **Fig. 4-2F**). The Nestin-SOX2 correlation differences between WT and *Fmr1*-KO neurospheres stemmed from the increased expression of Nestin, but not SOX2, in larger *Fmr1*-KO neurospheres and suggested the dysregulation of $Nestin^+$ NPCs.

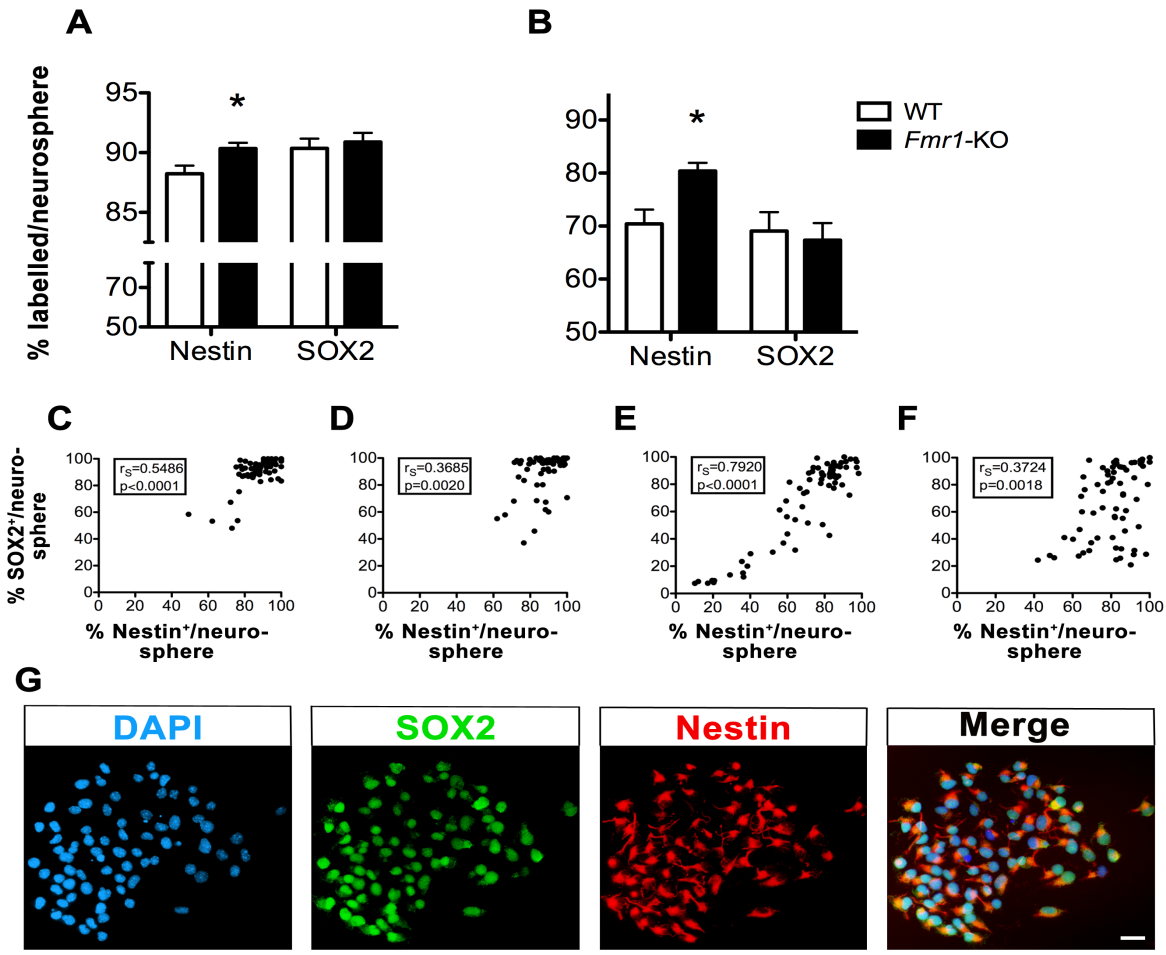

Figure 4-2. *Fmr1*-KO neurospheres contain more Nestin$^+$ cells in large neurospheres after 8 DIV. A. The percentage of Nestin$^+$ cells was increased in all *Fmr1*-KO neurosphere sizes combined (WT: 88.24 ± 0.68%; *Fmr1*-KO: 90.35 ± 0.47%; p=0.0110, n_{WT} =439, n_{KO}=459 neurospheres, N_{WT}=5, N_{KO}=3 cultures). **B.** The percentage of Nestin$^+$ cells was especially increased in *Fmr1*-KO neurospheres ≥101 cells (WT: 70.41 ± 2.72%; *Fmr1*-KO: 80.40 ± 1.55%; p=0.0019; n_{WT}=71, n_{KO}=68 neurospheres). **C-F.** The correlation between Nestin and SOX2 percent-labelled cells in neurospheres. **C.** WT neurospheres 51 – 100 cells. **D.** *Fmr1*-KO neurospheres 51 – 100 cells. **E.** WT neurospheres ≥101 cells. **F.** *Fmr1*-

KO neurospheres ≥101 cells. **G.** Sample photomicrographs of neurospheres after 8 DIV. Scale bar, 20 μm. Abbreviations: DIV: days *in vitro*, WT: wild type, *Fmr1*-KO: *Fmr1*-knockout.

Table 4-1. Summary of ANOVA main effects.

Percentage of	Figure	DIV/ neurosphere size	Effect	ANOVA	P-value
Cells labeled with SOX2$^+$ or Nestin$^+$	4-1A	4 / all sizes	Label x genotype	$F_{1, 3704} = 8.37$	p=0.0038
SOX2$^+$ cells exceeded Nestin$^+$ cells	-	5 / all sizes	Label	$F_{1, 1644} = 71.77$	p<0.0001
↓ SOX2$^+$ or Nestin$^+$ cells	-	5/ 50 – 100 cells	Genotype	$F_{1, 72} = 6.21$	p=0.015
SOX2$^+$ cells exceeded Nestin$^+$ cells	4-2A	8 / all sizes	Label	$F_{1, 1792} = 3.63$	p=0.057
↑ SOX2$^+$ or Nestin$^+$ cells	4-2A	8 / all sizes	Genotype	$F_{1, 1792} = 3.59$	p=0.058
SOX2$^+$ cells exceeded Nestin$^+$ cells	-	8 / ≤50 cells	Label	$F_{1, 1246} = 44.15$	p<0.0001

Fmr1-KO neurospheres show decreased proliferation after 5 and 8 DIV

We next assessed the proliferation of *Fmr1*-KO NPCs. We performed immunocytochemistry with an antibody directed against Ki67 on neurospheres cultured from P1 male hippocampi after 5 and 8 DIV. Overall, we found that the larger the neurospheres, the smaller the proportion of $Ki67^+$ cells, indicating that larger neurospheres had fewer cells that were actively cycling compared to smaller neurospheres. After 5 DIV, *Fmr1*-KO neurospheres had a significantly smaller proportion of $Ki67^+$ cells in all neurosphere sizes combined ($p<0.0001$, WT: 87.50 ± 0.50%, *Fmr1*-KO: 82.01 ± 0.70%) and in neurospheres ≤50 cells ($p<0.0001$, WT: 88.29 ± 0.56%, *Fmr1*-KO: 82.78 ± 0.73%). No significant differences were observed in larger neurospheres at this time point.

Fmr1-KO neurospheres grown for 8 DIV also showed a significant reduction in the proportion of $Ki67^+$ cells compared to WT ($p<0.0001$, WT: 77.42 ± 0.72%, *Fmr1*-KO: 64.83 ± 1.24%, **Fig. 4-3A**). The reduction in Ki67 labelling was evident in neurospheres ≤50 cells ($p<0.0001$, WT: 82.59 ± 0.76%, *Fmr1*-KO: 74.16 ± 1.22%, **Fig. 4-3B**), 51 – 100 cells ($p<0.0001$, WT: 76.49 ± 1.47%, *Fmr1*-KO: 62.81 ± 1.89%, **Fig. 4-3C**), and neurospheres ≥101 cells ($p<0.0001$, WT: 63.04 ± 1.79%; *Fmr1*-KO: 40.60 ± 2.74, **Fig. 4-3D**). The decreased Ki67 expression indicated that cells in *Fmr1*-KO neurospheres were less actively cycling compared to their WT counterparts. Interestingly, when comparing the proportion of $Nestin^+$ cells to that of $Ki67^+$ cells in WT neurospheres after 8 DIV, we found that there was about a 10% difference between the proportion of

Ki67^{+} and Nestin^{+} cells in each size category (**Table 4-2**), suggesting that 10% of NPCs were not actively cycling in WT neurospheres. However, the difference in the proportion of Nestin^{+} and Ki67^{+} cells was enhanced in *Fmr1*-KO neurospheres to 20% in neurospheres ≤100 cells and to 40% in neurospheres ≥101 cells, possibly suggesting increased quiescence. Furthermore, the pronounced reduction in Ki67 expression in all *Fmr1*-KO neurosphere sizes suggested that signalling pathways besides Nestin were affected in Fragile X. This led us to study Notch1 cytoplasmic expression and validate our findings *in vivo.*

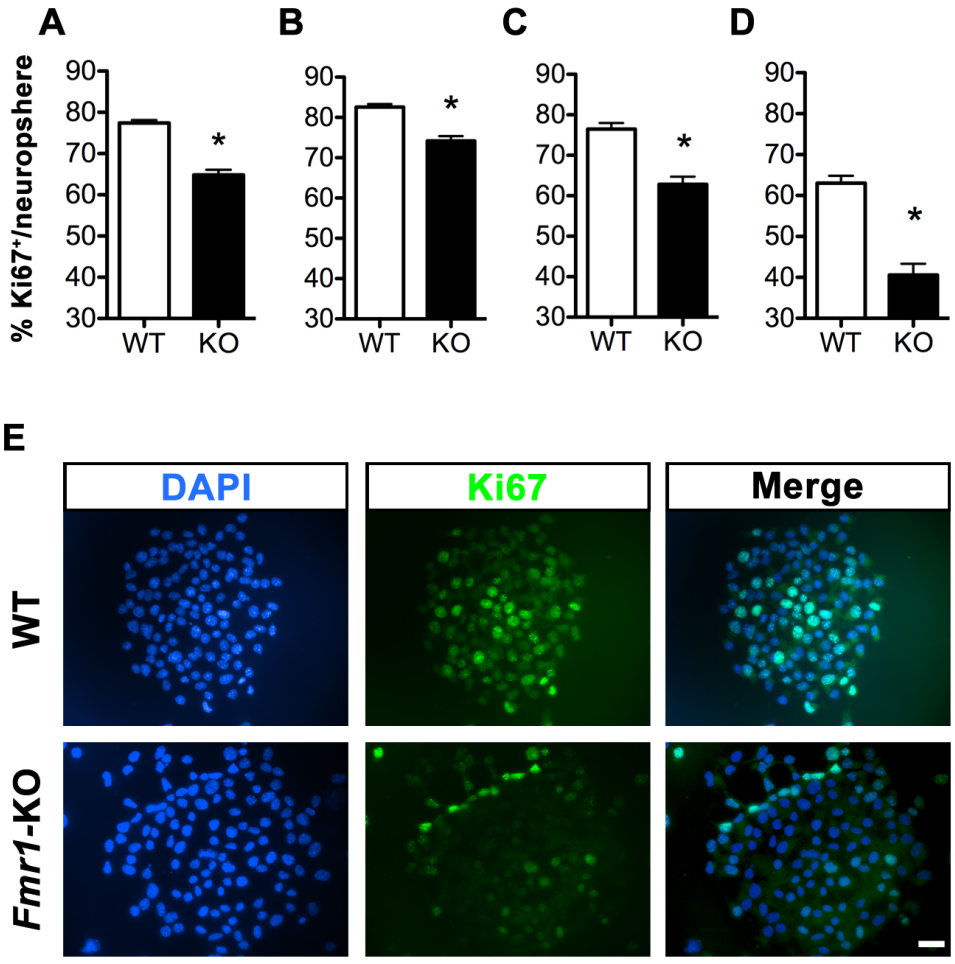

Figure 4-3. *Fmr1*-KO neurospheres express less Ki67 after 8 DIV. A – D. The percentage of $Ki67^+$ cells was decreased in *Fmr1*-KO neurospheres. **A.** All neurosphere sizes combined (WT: 77.42 ± 0.7226%, *Fmr1*-KO: 64.83 ± 1.236%, $p<0.0001$, $n_{WT}=542$, $n_{KO}=283$ neurospheres, $N_{WT}=4$, $N_{KO}=4$ cultures). **B.** Neurospheres ≤50 cells (WT: 82.59 ± 0.7555%, *Fmr1*-KO: 74.16 ± 1.218%, $p<0.0001$, $n_{WT}=314$, $n_{KO}=156$ neurospheres). **C.** Neurospheres 51 – 100 cells (WT: 76.49 ± 1.466%, *Fmr1*-KO: 62.81 ± 1.894%, $p<0.0001$; $n_{WT}=123$, $n_{KO}=73$ neurospheres). **D.** Neurospheres ≥101 cells (WT: 63.04 ± 1.79%; *Fmr1*-KO: 40.60 ± 2.74; $p<0.0001$; $n_{WT}=105$, $n_{KO}=54$ neurospheres). **E.**

Representative photomicrographs of WT and *Fmr1*-KO neurospheres labeled with Ki67. Scale bar, 20 μm. Abbreviations: DIV: days *in vitro*, WT: wild type, KO: *Fmr1*-knockout.

Table 4-2. Neurosphere expression of Nestin and Ki67 after 8 DIV.

Percent labeled in WT neurospheres			
Marker	**≤50 cells**	**51 – 100 cells**	**≥101 cells**
Nestin	92.59%	87.5%	70.41%
Ki67	82.59%	76.49%	63.04%
Percent labeled in *Fmr1*-KO neurospheres			
Marker	**≤50 cells**	**51 – 100 cells**	**≥101 cells**
Nestin	92.71%	84.94%	80.40%
Ki67	74.16%	62.81%	40.60%

In Vivo expression of PhH3 increases in Fmr1-KO hippocampi of P5 female mice

To confirm the results that we obtained *in vitro,* we performed Western blotting on P5 and P9 hippocampal tissue samples from male and female WT and *Fmr1*-KO mice. We used antibodies directed against Nestin, Notch1, SOX2, and PhH3. Histone H3 is specifically phosphorylated during mitosis and meiosis (Hans & Dimitrov, 2001), allowing for the detection of cells in the M phase of the cell cycle. We detected increased PhH3 expression in *Fmr1*-KO samples regardless of sex ($F_{1, 20}$=4.78, p=0.041, **Fig. 4-4A**) indicative of increased mitosis. Further analysis showed that this genotypic difference was mainly driven by a 38% increase in PhH3 expression among *Fmr1*-KO females (p=0.029) and was not detected when analyzing male samples alone.

Additionally, there was a trend towards increased SOX2 expression levels in female hippocampi compared to males, regardless of genotype ($F_{1, 20}$=4.03, p=0.059, **Fig. 4-4B**). However, there were no genotypic or sex differences in Notch1 or Nestin expression in P5 hippocampi.

Expression of PhH3 and Nestin decrease in Fmr1-KO hippocampi of P9 mice

We further assessed the expression patterns of PhH3, Nestin, Notch1, and SOX2 in hippocampal tissue at P9 and detected differences in the expression of PhH3 and Nestin. Expression levels of PhH3 decreased by 39% in *Fmr1*-KO male hippocampi relative to WT males (p=0.0412, **Fig. 4-4D**). *Fmr1*-KO hippocampi also showed a 35% decrease in Nestin expression compared to their WT counterparts regardless of sex ($F_{1, 20}$=5.74, p=0.027, **Fig. 4-4E**). There were no genotypic or sex differences in the expression of

Notch1 or SOX2 in P9 hippocampi. The parallel decrease in Nestin and PhH3 expression, particularly in P9 *Fmr1*-KO samples, indicated that there were fewer Nestin^{+} NPCs and fewer cells dividing. These results prompted us to study the cell cycle progression in Fragile X.

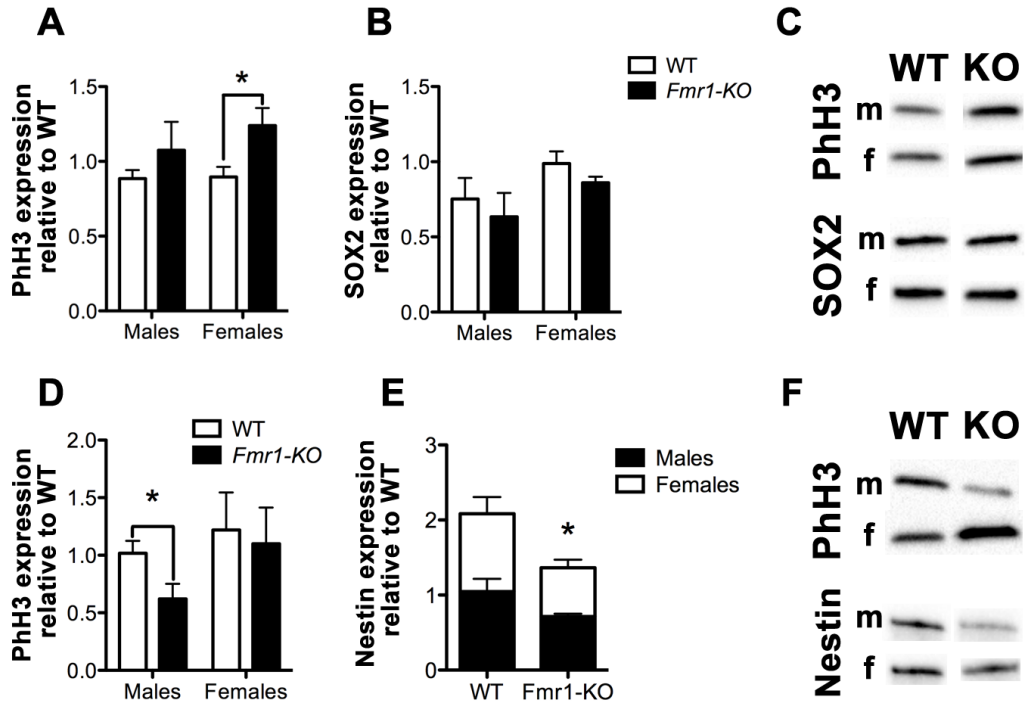

Figure 4-4. Decreased proliferation in *Fmr1*-KO hippocampi of P9 male mice. A – C. Western blot analysis of hippocampi from P5 mice. **A.** Increased relative PhH3 expression in *Fmr1*-KO hippocampi of female mice (WT_f: 0.90 ± 0.07, *Fmr1*-KO_f: 1.24 ± 0.14; p=0.029). **B.** A trend towards higher relative SOX2 expression in the hippocampi of female mice regardless of genotype ($F_{1, 20}$=4.03; p=0.059). **C.** Representative Western blot analysis. **D – F.** Western blot analysis of hippocampi from P9 mice. **D.** Decreased relative PhH3 expression in *Fmr1*-KO hippocampi of male mice (WT_m: 1.02 ± 0.11, *Fmr1*-KO_m: 0.62 ± 0.13; p=0.041). **E.** Decreased relative Nestin expression in *Fmr1*-KO hippocampi ($F_{1, 20}$=5.74, p=0.027). **F.** Representative Western blot analysis. Abbreviations: WT: wild type, KO: *Fmr1*-knockout, PhH3: phospho-histone H3, m: male, f: female. Band sizes of PhH3, SOX2, and Nestin are 17 kDa, 35 kDa, and 200 kDa, respectively.

The proportion of Fmr1-KO cells in the G_2/M phases of the cell cycle decreases in Fmr1-KO DG

Our next question was whether the cell cycle progression was altered in *Fmr1*-KO hippocampal NPCs. We microdissected hippocampi and labeled fixed cells with propidium iodide, which stoichiometrically binds to DNA and thus allows for its quantification. We used the Watson Pragmatic algorithm on FlowJo to analyze DNA peaks (Watson, Chambers, & Smith, 1987). We detected no differences between WT and *Fmr1*-KO P4 hippocampal samples in the proportion of cells in the G_1, S, or G_2/M phases. This was likely due to the NPC population being diluted with hippocampal cells. Thus, we next examined the cell cycle profile of cells from the DG of WT and *Fmr1*-KO mice at P7 (**Fig. 4-5B)**. We converted the absolute percentage of cells in each phase (G_1, S, or G_2/M) relative to WT. The relative proportions of cells in each phase varied with FMRP expression ($F_{2, 29} = 3.49$, p=0.044). Particularly, there was a 22% decrease in the proportion of *Fmr1*-KO cells in the G_2/M phases compared to WT ($p<0.05$, **Fig. 4-5C**). These results confirmed our Western blot and immunocytochemistry findings of decreased proliferation of hippocampal NPCs in *Fmr1*-KO mice in the early postnatal brain. All together, the decreased proliferation of hippocampal NPCs in the early postnatal *Fmr1*-KO brains suggests increased stem cell quiescence, depletion, or a developmental delay relative to WT NPCs.

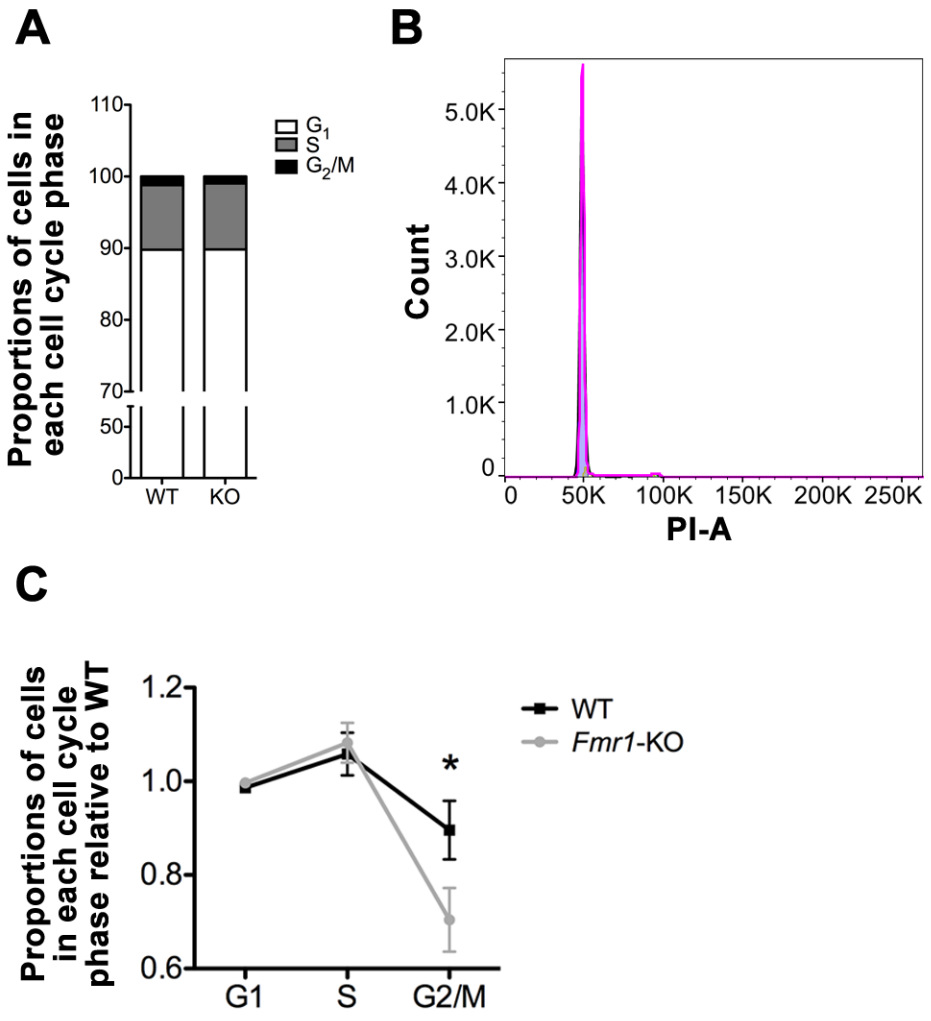

Figure 4-5. Fewer cells in the G_2/M phases of the cell cycle in the DG of P7 *Fmr1*-KO mice. A. Distribution of cells in the G_1, S, and G_2/M phases of the cell cycle. **B.** Representative cell cycle analysis with the first peak at 50K representing cells in G_1 and the second peak at 100K representing cells in the G_2/M phases. **C.** Decreased relative proportion of *Fmr1*-KO cells in the G_2/M phases (WT: 0.90 ± 0.062, *Fmr1*-KO: 0.70 ± 0.068, $p<0.05$). Abbreviations: WT: wild type, KO: *Fmr1*-knockout, P7: postnatal day 7, DG: dentate gyrus.

Discussion:

We show decreased NPC proliferation in the hippocampi of *Fmr1*-KO mice. We detected increased Nestin and decreased Ki67 immunopositive cells *in vitro*, decreased PhH3 and Nestin expression in P9 hippocampi, and a decrease in the proportion of cells in the G_2/M phases of the cell cycle in *Fmr1*-KO DG. Our findings were surprising in light of previous research, which primarily showed increased NPC proliferation in the hippocampi of adult mice (Guo et al., 2011; Luo et al., 2010) and in the ventricular zone of embryonic mice (Castrén et al., 2005; Tervonen et al., 2009) in the absence of FMRP expression. However, to our knowledge, this is the first study to examine hippocampal NPCs early in development in the context of Fragile X.

Reduced proliferation of Fmr1-KO NPCs

The proliferation of hippocampal NPCs peaks in the first postnatal week in rodents (Georg Kuhn & Blomgren, 2011; Ortega-Martínez & Trejo, 2015), which is also the time of highest FMRP expression in the hippocampus (Lu et al., 2004). In fact, the subgranular zone of the DG where neurogenesis persists into adulthood is not fully formed until P7 (Li, Kataoka, Coughlin, & Pleasure, 2009). Thus, it can be deduced that lack of FMRP will cause the most pronounced changes in the proliferation of hippocampal NPCs within this time frame; sensitive time windows is reviewed in (Meredith, Dawitz, & Kramvis, 2012). In line with this, our results largely showed that NPC proliferation was reduced in Fragile X hippocampi in the early postnatal period. We found lower expression levels of PhH3 in the hippocampi of P9 male mice, which indicated decreased mitosis. In parallel,

we detected reduced Nestin expression levels in the hippocampi of P9 *Fmr1*-KO mice. Nestin is expressed in undifferentiated NPCs (Lendahl, Zimmerman, & McKay, 1990), and its expression decreases upon the cell's progression through the G_2 phase of the cell cycle (Sunabori et al., 2008). The mirrored decrease in Nestin and PhH3 expression indicated that fewer NPCs were actively proliferating. These findings were in agreement with our flow cytometric cell cycle experiments, which showed decreased relative proportions of cells in the G_2/M phases from the DG of *Fmr1*-KO mice at P7. It remains to be determined if neural stem cells in Fragile X are more quiescent and thus are not labeled with Nestin, Ki67, or PhH3; if the pool of NPCs is depleted all together; or if Fragile X NPCs exhibit a developmental delay in reaching milestones in a timely manner compared to WT NPCs. Additionally, determining the functional consequences of decreased NPC proliferation is incumbent upon identifying the NPC populations that are affected in Fragile X given that different NPC populations have different roles in the DG (Decarolis et al., 2013; Lugert et al., 2010).

Aberrant activation of Fmr1-KO neural stem cells in vitro

Quiescent neural stem cells do not express Nestin until they are activated (Codega et al., 2014). However, research shows that quiescent and activated neural stem cells interconvert *in vitro* (Codega et al., 2014). Our results indicated that there were 63% $Ki67^+$ and 70% $Nestin^+$ cells in WT neurospheres ≥101 cells compared with 40% $Ki67^+$ and 80% $Nestin^+$ cells in *Fmr1*-KO neurospheres in the same size category after 8 DIV (**Table 4-2**). Our finding of increased Nestin expression in larger *Fmr1*-KO neurospheres

despite the pronounced decrease in Ki67 expression in neurospheres of all sizes examined suggests that *Fmr1*-KO neural stem cells, represented by the larger neurospheres, may be aberrantly activated *in vitro*. Moreover, the decrease in Ki67 indicated that fewer NPCs were actively cycling. Thus, we propose that the increased proportion of $Nestin^+$ cells arose from quiescent stem cells that were abnormally activated in *Fmr1*-KO cultures rather than an increase in the proliferation or differentiation of NPCs. It would be interesting to examine the expression of Ki67 in larger neurospheres at later time points following the increased Nestin expression to assess whether the activated $Nestin^+$ stem cells enter the cell cycle and divide. We were not able to test this in our culture system due to the continued proliferation and merging of neurospheres.

Abnormal stem cell maintenance

It is now well established that abnormal activation of stem cells leads to stem cell depletion. Under homeostatic conditions, the majority of stem cells remain quiescent until activated to enter the cell cycle and divide (Baserga, 1968). Some of the factors shown to regulate neural stem cell maintenance are PTEN (Amiri et al., 2012; Bonaguidi et al., 2011), c-myc and N-myc (Wey & Knoepfler, 2010; Zinin et al., 2014), and Notch (Chapouton et al., 2010); reviewed in (Cheung & Rando, 2013; Orford & Scadden, 2008). Although we did not detect differences in the expression level of cytoplasmic Notch1 at P5 or P9, we cannot rule out that Notch activity is indeed altered. For instance, there could be an imbalance between the maintenance and differentiation of NPCs mediated by Notch (Lugert et al., 2010). Additionally, other effectors of Notch may be

dysregulated in Fragile X such as Hes1, Hes5, or at other time points not studied. In agreement with abnormal stem cell regulation, recent findings indicate decreased cancer incidence among Fragile X patients as well as decreased c-myc expression (Rosales-Reynoso et al., 2010). Lower expression of c-myc could imply the depletion of NPCs (Wey & Knoepfler, 2010), which future studies can confirm.

Dynamic changes in postnatal brain development: sensitive time-windows

Postnatal development is marked by dynamic changes in the brain. Atypical changes that happen during time-sensitive windows when the brain is particularly vulnerable inevitably lead to impairments; reviewed in (Meredith, 2015; Meredith et al., 2012). We detected transient genotypic and sex differences early in development. Indeed, PhH3 levels were higher in P5 *Fmr1*-KO females compared to their WT counterparts. This starkly contrasted with the lower expression levels of PhH3 in the P9 hippocampi of male mice and with our immunocytochemistry and cell cycle results of decreased proliferation, suggesting a transient genotypic difference restricted to female mice. The sex difference in PhH3 expression among *Fmr1*-KO mice is interesting and poses the question of whether it has functional consequences such as symptom presentation. Male and female FXS patients present with different symptoms: female patients tend to have anxiety symptoms rather than prominent cognitive impairments, whereas male patients have prominent intellectual and learning impairments (Garber, Visootsak, & Warren, 2008; R. J. Hagerman et al., 2014). Another protein that showed a temporary differential sex expression was SOX2, whose expression levels were higher in P5 females compared to

males, regardless of genotype. Interestingly, the sex difference in SOX2 expression was diminished by P9. Thus, our finding of increased proportions of $SOX2^+$ cells in *Fmr1*-KO neurospheres after 4 DIV was likely confounded by sex differences in SOX2 expression early on in the hippocampus. This is particularly true since we did not detect genotypic differences in SOX2 expression in neurospheres cultured from male mice after 5 or 8 DIV or by Western blotting, confirming our conclusion that SOX2 expression was not in fact altered in *Fmr1*-KO hippocampi. Together, these transient changes underscore the ever-changing developing brain and the importance of mapping out findings to different stages of brain development in relation to sex in order to design optimal treatment interventions. It remains to be determined if the decreased proliferation of NPCs in the DG of *Fmr1*-KO is restricted to early postnatal development, if it persists into adulthood, or if it is overcompensated later in life and increases to levels higher than in DG of WT mice as may be suggested by previous studies in the adult hippocampus.

Conclusion:

In conclusion, we found decreased NPC proliferation in the early postnatal hippocampus of Fragile X mice and propose that the underlying cause could be increased NPC quiescence, depletion, or alternatively a developmental delay in reaching milestones in a timely manner in comparison with WT NPCs. Our results also suggest that quiescent neural stem cells are aberrantly activated in *Fmr1*-KO cultures. This is the first study to examine hippocampal NPC biology during early postnatal development, a time that is crucial for the developing DG and likely has long lasting consequences on lifelong

neurogenesis (Mathews et al., 2010). In turn, impairments in hippocampal neurogenesis contribute to the learning and memory impairments characteristic of FXS.

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Chapter 5: Abnormal Hippocampal Neurogenesis in the Early Postnatal Fragile X Mouse

Chapter Link

Following up with work from Chapter 4, I aimed to identify the population that is specifically altered in the early postnatal *Fmr1*-KO hippocampus and DG. I compared neural populations in the WT and *Fmr1*-KO P4 hippocampus and P7 DG: NSCs, neural progenitors, neuroblasts, immature neurons, neurons, and astrocytes. This study allowed me to examine hippocampal neurogenesis and the generation of NPC progeny in *Fmr1*-KO brains relative to WT.

Abnormal hippocampal neurogenesis in the early postnatal Fragile X mouse

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Keywords: Fragile X Syndrome; neural stem cells; neuroblasts; *Fmr1* knockout mice; hippocampal neurogenesis; flow cytometry

Abstract:

Several reports show abnormal neural stem and progenitor cell (collectively termed as neural precursor cells or NPCs) proliferation, differentiation, and survival in relation to Fragile X syndrome. However, these reports have been inconsistent in terms of how the proliferation and differentiation of NPCs are altered. In this study, we examined populations of NPCs and their progeny in the early postnatal hippocampus and dentate gyrus of the Fragile X knockout (KO) mouse model, which has not been studied before. We used flow cytometry and the expression of the cell surface markers CD15, CD24, CD133, GLAST, and PSA-NCAM for this purpose. We found a decreased proportion of neural stem cells ($GLAST^{+}CD15^{+}CD133^{+}$) and an increased proportion of neuroblasts ($PSA\text{-}NCAM^{+}CD15^{+}$) in the dentate gyrus of P7 *Fmr1*-KO mice, indicating aberrant neurogenesis in the early postnatal Fragile X hippocampus. Additionally, we found that CD15 and CD133 expression varied by brain region and that their expression was not interchangeable in the hippocampus.

Introduction:

Fragile X syndrome (FXS) is the leading monogenic cause of autism spectrum disorders (ASD) and inherited intellectual impairment (T. Wang, Bray, & Warren, 2012). The majority of FXS cases arise from the CGG trinucleotide repeat expansion in the 5`-untranslated region of the *FMR1* gene (Fu et al., 1991), resulting in the hypermethylation and epigenetic transcriptional silencing of *FMR1* (Oberlé et al., 1991). Consequently, the protein product, the Fragile X mental retardation protein (FMRP), is produced in limited amounts (Pieretti et al., 1991). Symptoms of FXS include cognitive and memory difficulty, epilepsy, anxiety, autistic behaviours, flexible joints, and machro-orchidism (Hagerman, Portes, Gasparini, Jacquemont, & Gomez-Mancilla, 2014). Mice that do not express FMRP, *Fmr1*-knockout (KO) mice, recapitulate many of the phenotypes of FXS (Bakker et al., 1994). Previous studies have indicated abnormal neural stem (NSC) and progenitor cell biology, collectively referred to as neural precursor cells (NPCs) from hereon, in Fragile X; reviewed in (Callan & Zarnescu, 2011). Yet, there has been some discrepancy in how NPCs are altered in FXS likely due to the different brain regions and developmental time points studied. Additionally, no studies have examined NPCs in the early postnatal rodent hippocampus, despite the importance of this developmental time point in the formation of the dentate gyrus (DG) of the hippocampal formation.

Hippocampal NPCs give rise to lifelong neurogenesis in the DG, first reported in rodents (Altman & Das, 1965) and later confirmed in humans (Eriksson et al., 1998). DG development begins on embryonic day (E)13 (Rolando & Taylor, 2014) and continues

postnatally in rodents. Yet, peak generation of DG granule cells occurs between postnatal days (P)0 – 4, and the majority of granule neurons are generated by the third postnatal week (Bayer, 1980a; 1980b). Notably, hippocampal NPCs, which give rise to lifelong neurogenesis, populate the DG by the end of the first postnatal week (Mathews et al., 2010), which is also the time of peak hippocampal FMRP expression (Lu et al., 2004), suggesting that FMRP plays an important role in the hippocampus within the first postnatal week. In turn, early postnatal hippocampal NPC biology has important functional consequences during development and later on in adult neurogenesis. This is especially true given that newborn neurons in the DG are suggested to play an important role in learning and memory (Kempermann, Wiskott, & Gage, 2004b) by virtue of the DG being the first processing station of cortical sensory information relayed to the hippocampus; reviewed in (Amaral, Scharfman, & Lavenex, 2007). Moreover, the overlap between the cognitive and psychological symptoms of FXS and the role of hippocampal neurogenesis in memory and plasticity strongly suggests that the dysfunction of hippocampal NPCs contributes to the symptoms of FXS.

Studying homogenous populations of NPCs has been challenging due to the lack of markers that uniquely define a single population. However, great strides have been made in the analysis and prospective isolation of NPCs and their progeny using fluorescence activated cell sorting. To this end, transgenic reporter mice and cell surface antigens have been utilized, including SOX1-GFP (Barraud, Thompson, Kirik, Björklund, & Parmar, 2005), Nestin-GFP (Kawaguchi et al., 2001), CD15 (Lewis X or stage-specific embryonic antigen 1) (Capela & Temple, 2002), CD133 (prominin-1)

(Corti et al., 2007), PSA-NCAM (polysialylated neuronal cell adhesion molecule) (Pennartz et al., 2004), and GLAST (glutamate aspartate transporter) (Jungblut et al., 2012).

Given that no single marker can isolate a homogenous neural population, the use of a combination of markers is inevitable. In this study, we analyzed the expression of cell surface antigens to study populations of hippocampal NPCs and their progeny in the first postnatal week of *Fmr1*-KO mouse brains. Specifically, we examined the expression of CD15, CD133, and CD24 (heat stable antigen) as previously reported (Panchision et al., 2007) in cells isolated from the entire hippocampus of P4 mice. CD15 and CD133 expression accompanied with low expression levels of CD24 enrich for NPCs, whereas high expression levels of CD24 labels neuroblasts (Panchision et al., 2007). Next, we studied neural populations in the DG at P7, using PSA-NCAM, GLAST, CD15 and CD133. GLAST is expressed in neural stem cells and astrocytes (Jungblut et al., 2012), PSA-NCAM is expressed in neuroblasts and immature neurons (Kempermann, Jessberger, Steiner, & Kronenberg, 2004a; Pennartz et al., 2004), whereas CD15 and CD133 are expressed in NPCs (Capela & Temple, 2002; Walker et al., 2013). We did not detect any differences in the expression of CD15, CD133, and CD24 or their combinations between wild type (WT) and *Fmr1*-KO mice at P4. This could be due to the use of entire hippocampi, which diluted NPCs and masked genetic differences. We did, however, find a decrease in the proportion of neural stem cells ($GLAST^{+}CD15^{+}CD133^{+}$), coupled with an increase in neuroblasts ($PSA\text{-}NCAM^{+}CD15^{+}$) in *Fmr1*-KO DG at P7. To

our knowledge, this is the first study to use flow cytometric analysis of NPCs and their progeny in the context of Fragile X.

Materials and Methods:

Animals:

All animal experiments were performed in accordance with the guidelines set out by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board. The *Fmr1*-KO mouse colony was established from breeding pairs of FVB.129P2(B6)-*Fmr1*^{tm1Cgr} mice. The wild-type (WT) and *Fmr1*-KO mice were maintained as individual strains, and were housed and bred at the McMaster University Central Animal Facility. Both male and female animals were used.

Flow Cytometry:

Postnatal day 4 (P4) hippocampi (N=6 samples in 5 independent runs) or P7 – 8 dentate gyri (N=5 samples in 3 independent runs) were microdissected and enzymatically dissociated in TrypLE Express (Life Technologies, Burlington, Canada) supplemented with 200 units/mL DNase I (Roche, Mississauga, Canada) and 1 mM $MgCl_2$ for 45 minutes, modified from a previously published protocol (Panchision et al., 2007). Cells were resuspended in flow cytometry buffer, comprising 1x HBSS Ca^{2+}/Mg^{2+} free, pH 7.2 (Life Technologies), containing 1.55 g/L glucose and 0.5% bovine serum albumin, fraction V (Sigma-Aldrich, St. Louis, USA). Cells were blocked on ice in flow cytometry buffer containing FC receptor block (eBioscience, San Diego, USA) for 20 minutes and were subsequently incubated for 30 minutes on ice in the dark using fluourophore-

conjugated primary antibodies. Cells were analyzed on Becton Dickinson LSRII or Fortessa Cell Analyzer (BD, Mississauga, Canada) and FlowJo data analysis software (version X.0.8, Tree Star, Ashland, USA). A minimum of 30,000 events was collected per sample. Dead cells, debris, and aggregates were excluded from analysis by gating the main population on the basis of 7AAD viability dye staining and forward and side scatter (**Fig. 5-S1**). Negative gates were set against unstained cells and matched fluorescence-minus-one controls. The following antibodies were used: CD15 (Alexa fluor 488-conjugated mouse IgM, eBioscience), CD24 (allophycocyanin-conjugated rat IgG2b, eBioscience), CD133 (phycoerythrin-conjugated rat IgG2a, λ, BioLegend, San Diego, USA), CD133 (brilliant violet 421-conjugated rat IgG2a, λ, BioLegend), PSA-NCAM (phycoerythrin-conjugated mouse IgM, Miltenyi Biotec, San Diego, USA), and GLAST (allophycocyanin-conjugated mouse IgG2a, Miltenyi Biotec).

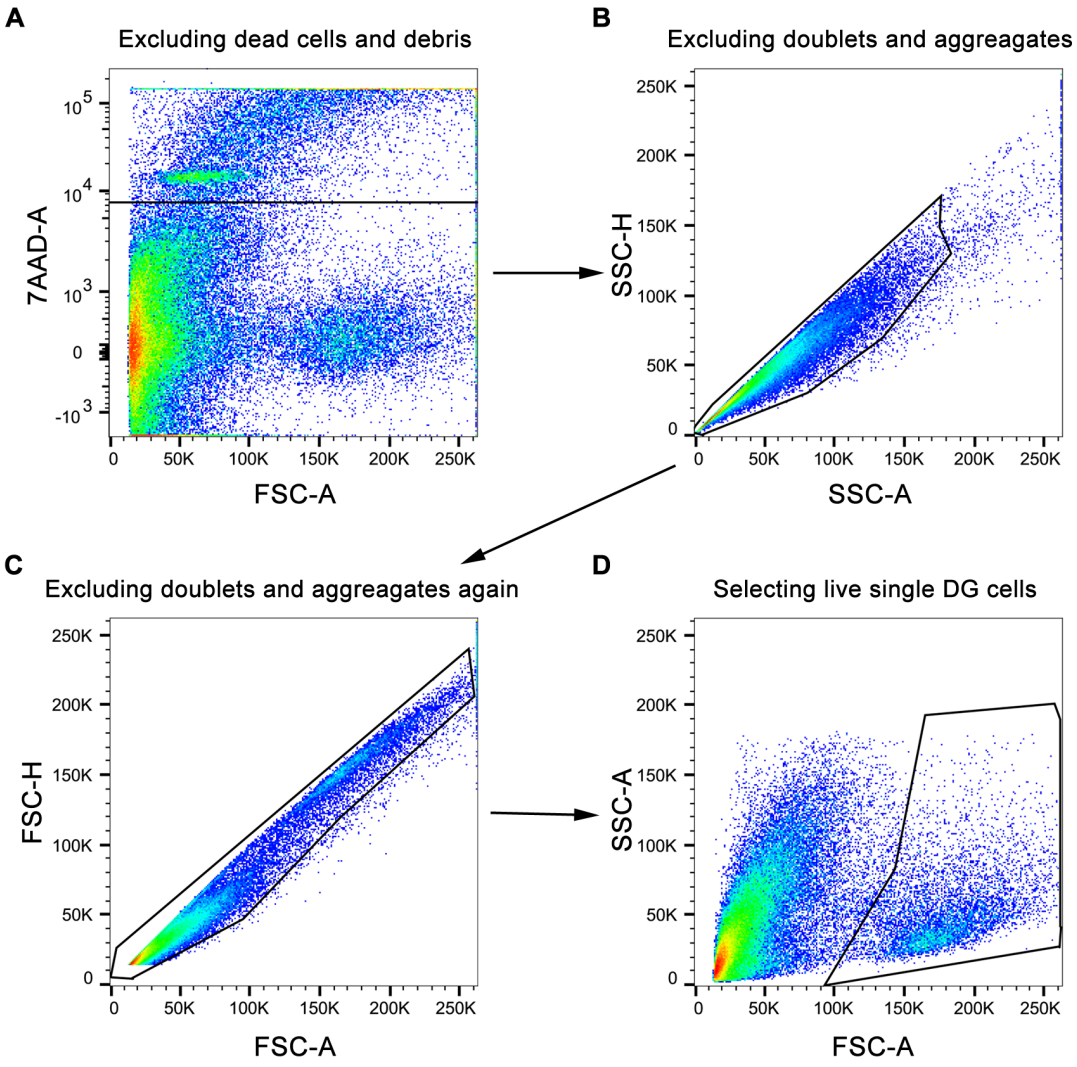

Figure 5-S1. Gating strategy to isolate single live DG cells. A. Dead cells and debris are eliminated based on 7AAD staining. **B – C.** Aggregates and doublets are eliminated on the basis of **B.** the pulse area and height of side scatter and **C.** on the area and height of forward scatter. **D.** DG cells are selected on the basis of forward and side scatter.

Statistical Analysis:

Statistical analysis was performed on GraphPad Prism (version 5.0). Two-way analysis of variance followed by pairwise two-tailed Student's t-tests was used in **Figure 5-1**, **5-2**, and **5-4**, and two-tailed Student's t-test was used in **Figure 5-3**. Probabilities of $p<0.05$ were considered significant. Data are expressed as means and error bars represent the standard error of the mean (SEM).

Results:

Similar populations of NPCs in WT and Fmr1-KO P4 hippocampi

To characterize hippocampal NPC populations, we compared the expression of the cell surface markers CD133, CD15, and CD24 in single cells dissociated from WT and *Fmr1*-KO P4 hippocampi using flow cytometry. The majority of cells (85%) were positive for CD24, followed by CD133 (37%) and CD15 (11%). The proportions of cells labelled with each of the 3 antigens were different ($F_{2, 30} = 628.43$, $p<0.0001$, **Figure 5-1**). We found no differences in the expression of any of these markers between WT and *Fmr1*-KO samples.

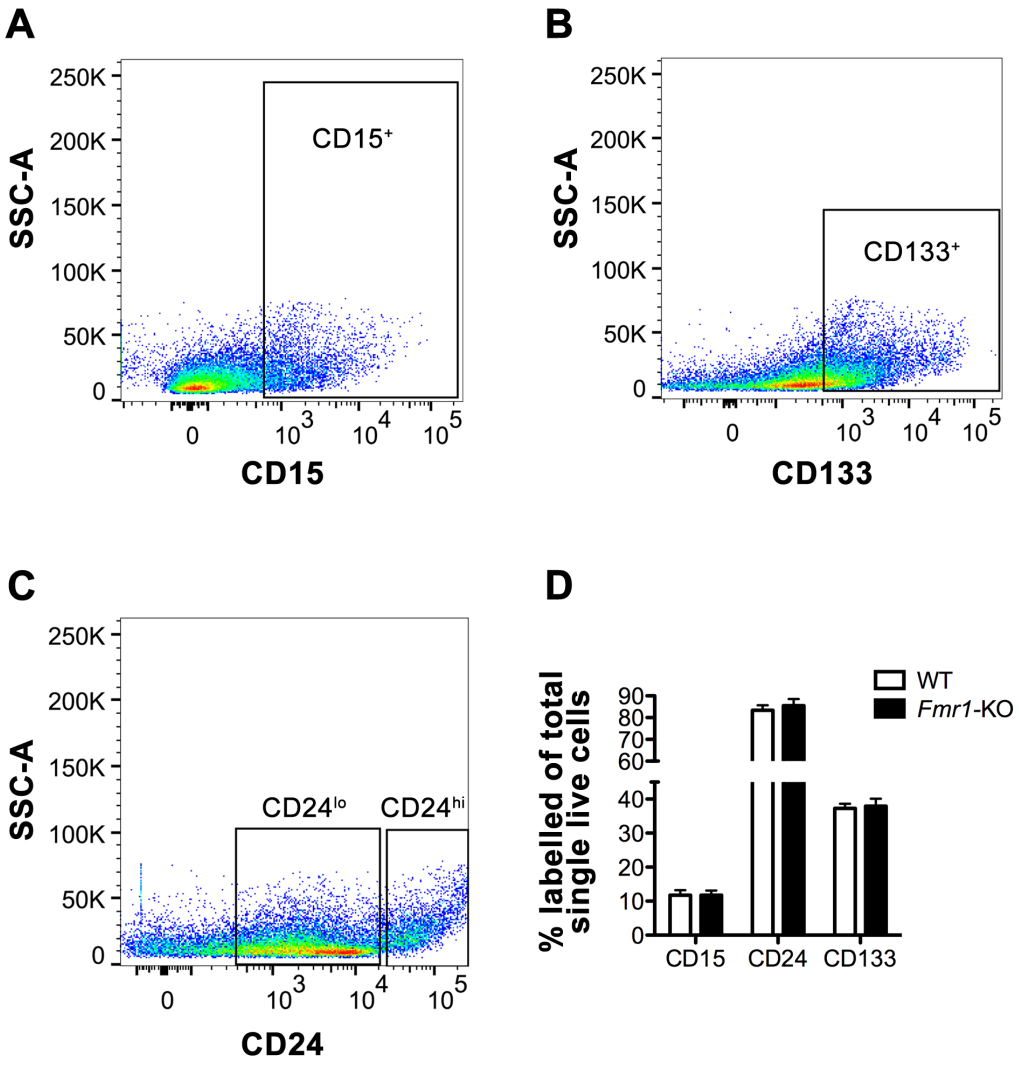

Figure 5-1. Expression of CD15, CD24, and CD133 in P4 hippocampi. A – C. Representative pseudocolour plots of gating against **A.** CD15, **B.** CD133, **C.** CD24. **D.** No differences in the expression of CD15, CD133, or CD24 between cells from WT and *Fmr1*-KO hippocampi at P4. Abbreviations: WT: wild type, KO: *Fmr1*-knockout, P4: postnatal day 4.

Given that the expression of CD133 can enrich for NPCs (Corti et al., 2007; Panchision et al., 2007; Walker et al., 2013; J. Wang, O'Bara, Pol, & Sim, 2013), and that only low and intermediate CD133 expression levels give rise to neurospheres in culture (Walker et al., 2013), we studied cells based on their expression levels of CD133, namely low, intermediate, and high levels of CD133. The majority of $CD133^{+}$ cells expressed it at a low level, followed by intermediate and high expression levels (22%, 11%, and 3% respectively of total single live cells, **Figure 5-2A – B**). There were no differences in the proportion of cells expressing low, intermediate, or high levels of CD133 between WT and *Fmr1*-KO samples.

To further characterize $CD133^{+}$ populations, we analyzed their co-expression of CD15 and CD24. $CD133^{+}CD24^{lo}$ have been shown to give rise to neurons, astrocytes, and oligodendrocytes in culture (Panchision et al., 2007). This, in addition to the fact that only cells with low or intermediate levels of CD133 generate neurospheres (Walker et al., 2013), suggests that NPCs would be $CD133^{lo}CD24^{lo}$ or $CD133^{med}CD24^{lo}$. In line with this, we found that most $CD133^{lo}$ and $CD133^{med}$ cells were $CD24^{lo}CD15^{-}$ (77% and 57% of $CD133^{lo}$ and $CD133^{med}$, respectively, which was equivalent to 16% and 6% of the total single live cells from the P4 hippocampus; **Figure 5-2D – E, 5-2G – H**). Given that CD15 enriches for neurospheres (Capela & Temple, 2002), we expect NPCs to co-express CD15. Indeed, further analysis of the $CD133^{lo/med}CD24^{lo}$ populations revealed a prominent $CD133^{med}CD15^{+}CD24^{lo}$ population, which we suggest is the population that contributes to the generation of neurospheres rather than the $CD133^{med}CD15^{-}CD24^{lo}$ population. Interestingly, ~40% of $CD133^{hi}$ cells expressed high levels of CD24, which

represented ~1.1% of total single live hippocampal cells (**Figure 5-2C**). These cells represent post-mitotic neuroblasts (Panchision et al., 2007; Pruszak, Ludwig, Blak, Alavian, & Isacson, 2009; Walker et al., 2013), particularly that $CD133^{+}CD24^{hi}$ do not generate neurospheres (Corti et al., 2007). There were no differences in the proportions of any of the aforementioned populations between WT and *Fmr1*-KO samples from the hippocampi of P4 mice (**Figure 5-2**).

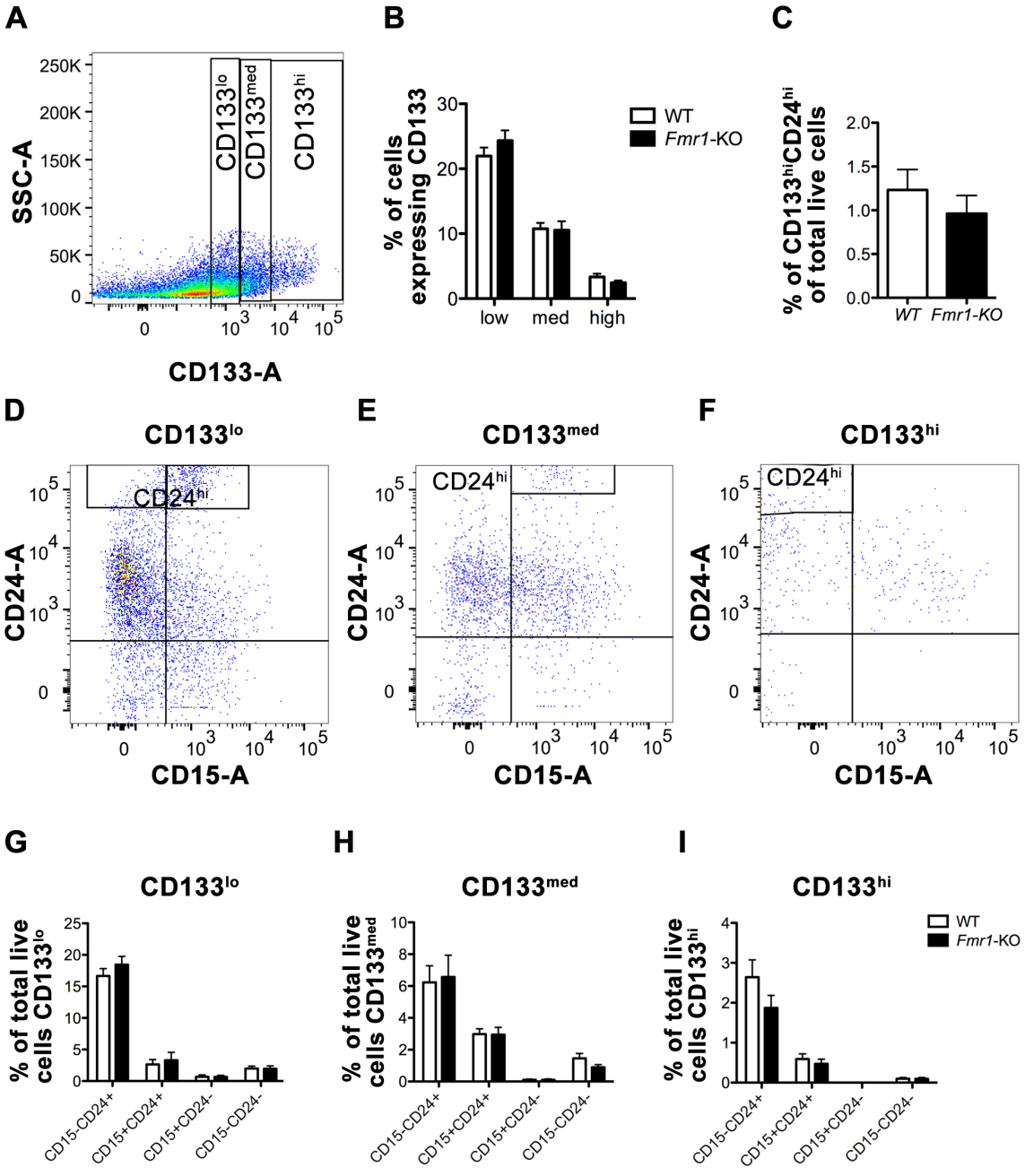

Figure 5-2. CD15 and CD24 expression in relation to CD133 expression levels in P4 hippocampi. A. Representative pseudocolour plot of CD133 expression levels. **B.** No difference in the expression levels of CD133 between WT and *Fmr1*-KO mice (expressed as % of total single live cells). **C.** No difference in the proportion of the $CD133^{hi}CD24^{hi}$

populations between WT and *Fmr1*-KO samples. **D – F** Representative pseudocolour plots of CD15-CD24 expression in **D.** $CD133^{lo}$, **E.** $CD133^{med}$, and **F.** $CD133^{hi}$ cells. **G – I** No difference in the proportions of $CD15^{-}CD24^{+}$, $CD15^{+}CD24^{+}$, $CD15^{+}CD24^{-}$, or $CD15^{-}CD24^{-}$ between WT and *Fmr1*-KO in the **G.** $CD133^{lo}$, **H.** $CD133^{med}$, or **F.** $CD133^{hi}$ populations. Abbreviations: WT: wild type, KO: *Fmr1*-knockout, P4: postnatal day 4, CD15: Lewis X or stage-specific embryonic antigen 1, CD24: heat stable antigen, CD133: Prominin-1.

Reduced neural stem cells and excess neuroblasts in the Fmr1-KO DG at P7

To study NPCs and their progeny, we microdissected DG from P7 – 8 brains and labelled dissociated cells with PSA-NCAM, GLAST, CD133, and CD15. There was no difference in the proportions of cells labelled with each of PSA-NCAM, GLAST, CD133, and CD15 between WT and *Fmr1*-KO samples (**Figure 5-3**).

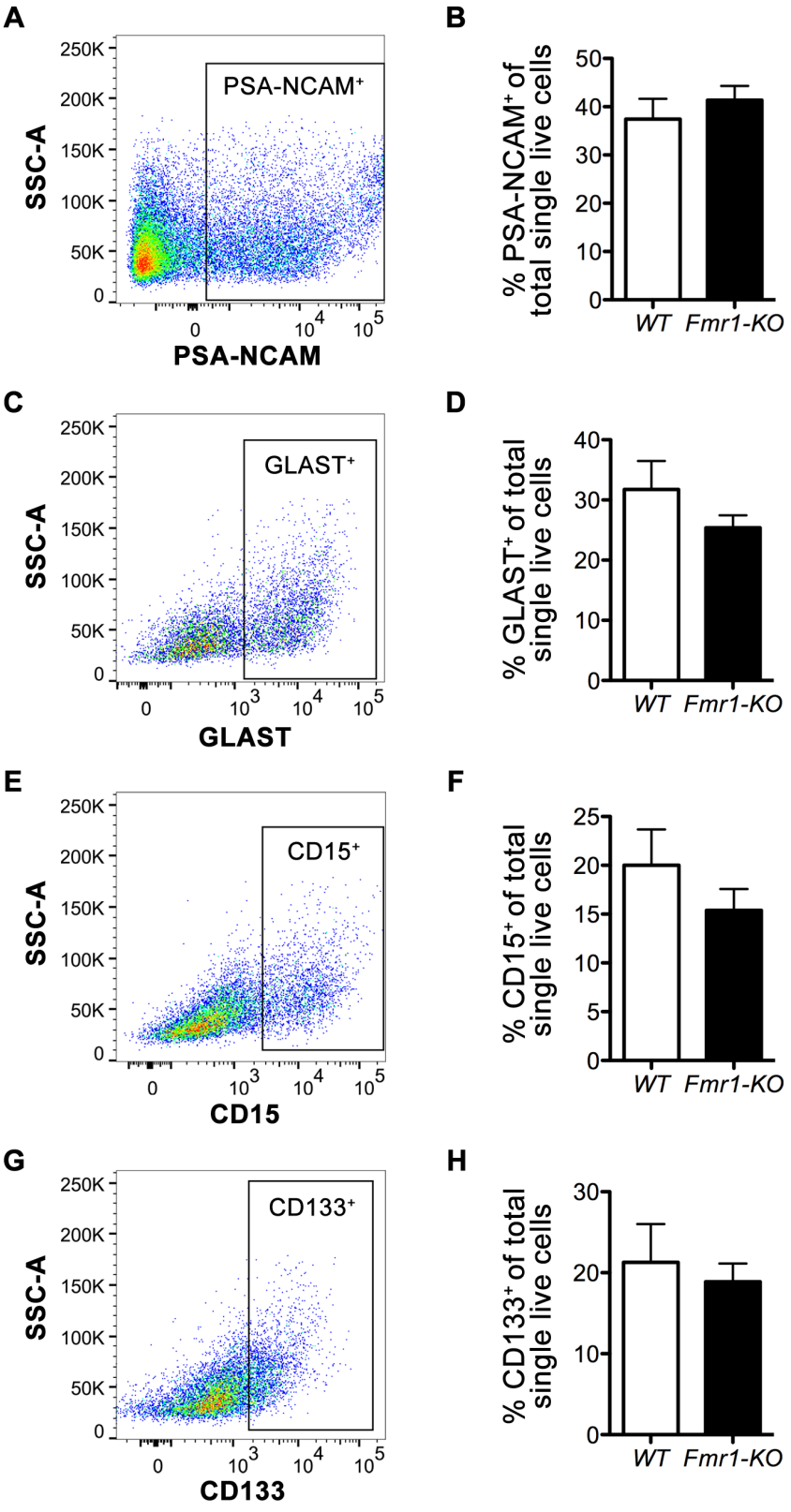
A
SSC-A
PSA-NCAM
PSA-NCAM+
B
% PSA-NCAM+ of total single live cells
WT
Fmr1-KO
C
GLAST
GLAST+
D
% GLAST+ of total single live cells
E
CD15
CD15+
F
% CD15+ of total single live cells
G
CD133
CD133+
H
% CD133+ of total single live cells

Figure 5-3. Expression of PSA-NCAM, GLAST, CD15, and CD133 in the P7 DG. A, C, E, G. Representative pseudocolour plots. **B, D, F, H.** No differences between WT and *Fmr1*-KO in the expression of **B.** PSA-NCAM, **D.** GLAST, **F.** CD15, or **H.** CD133. Abbreviations: WT: wild type, KO: *Fmr1*-knockout, P7: postnatal day 7, DG: dentate gyrus, PSA-NCAM: polysialylated neuronal cell adhesion molecule, GLAST: glutamate aspartate transporter, CD15: Lewis X or stage-specific embryonic antigen 1, CD133: Prominin-1.

We next gated single live DG cells against PSA-NCAM and GLAST expression and consistently found 4 prominent populations (**Figure 5-4A**). There was a significant interaction between the proportions of cells labelled with combinations of GLAST/PSA-NCAM and genotype ($F_{3, 32} = 3.21$, p=0.036, **Figure 5-4A – B**). Further analysis revealed a significant decrease in the proportion of *Fmr1*-KO GLAST$^+$PSA-NCAM$^-$ cells (p=0.043, WT: 16.68 ± 1.69%, *Fmr1*-KO: 11.54 ± 1.31%), which contains astrocytes and NSCs, mirrored with an increase in the proportion of GLAST$^-$PSA-NCAM$^+$ cells, which contains neuroblasts and immature neurons, compared to WT (p=0.026, WT: 20.90 ± 2.60%, *Fmr1*-KO: 27.38 ± 0.76%).

To better characterize the GLAST$^+$PSA-NCAM$^-$ and GLAST$^-$PSA-NCAM$^+$ populations, we analyzed their expression of CD15 and CD133. Previous research has shown that GLAST labels NSCs, in addition to astrocytes and radial glia (Jungblut et al., 2012) in agreement with findings reporting similar GLAST expression to GFAP expression in the GFAP-GFP$^+$EGFR$^+$ putative stem cell population (Pastrana, Cheng, & Doetsch, 2009). In line with NSC expression of GLAST, Mich and colleagues have shown that quiescent stem cells express high levels of GLAST and are GLAST^{high}PSA-NCAM$^-$ (Mich et al., 2014). Thus, GLAST allows for identifying NSCs without the use of transgenic reporter mice, when combined with other markers. Given that stem cells of the DG are GFAP-GFP$^+$CD133$^+$ (Beckervordersandforth et al., 2014) and that the CD15$^+$ population also contains stem cells (Capela & Temple, 2002; Daynac et al., 2013), we concluded that GLAST$^+$CD15$^+$CD133$^+$ cells contained the neural stem cell population. GLAST expression also persists in the neural progenitor stage (Pastrana et al., 2009),

during which it is co-expressed with PSA-NCAM in the DG (Jungblut et al., 2012). PSA-NCAM is expressed in other migrating immature cells such as neuroblasts and immature neurons besides its expression in neural progenitor cells, owing to the fact that PSA-NCAM$^+$ cells show enriched expression of chemokines, Nestin, Notch1, and Doublecortin (Pennartz et al., 2004). Gating the GLAST and PSA-NCAM populations against CD15 and CD133 yielded a total of 16 populations. Out of the 16 populations, the following 4 were the most prominent: GLAST$^-$ PSA-NCAM$^-$CD133$^-$CD15$^-$ (~40% of total cells), GLAST$^-$PSA-NCAM$^+$CD133$^-$CD15$^-$ (~15%), GLAST$^+$PSA-NCAM$^+$CD133$^+$CD15$^+$ (~10%), and GLAST$^+$PSA-NCAM$^-$CD133$^+$CD15$^+$ (~10%), representing mature neurons and oligodendrocytes, immature neurons, transit amplifying progenitor cells, and stem cells, respectively.

We found that the proportion of *Fmr1*-KO cells in the GLAST$^+$PSA-NCAM$^-$ population was significantly decreased ($F_{1, 32} = 9.34$, p=0.0045, **Figure 5-4C – D**) in addition to significant differences in CD15/CD133 labelling combinations in the GLAST$^+$PSA-NCAM$^-$ population regardless of genotype ($F_{3, 32} = 43.30$, p<0.0001, **Figure 5-4D**). The decrease in the proportion of *Fmr1*-KO cells in the GLAST$^+$PSA-NCAM$^-$ population was primarily driven by a decreased proportion of GLAST$^+$PSA-NCAM$^-$CD133$^+$CD15$^+$ cells compared to WT (p=0.037, WT: 9.15 ± 1.20%, *Fmr1*-KO: 5.99 ± 0.41%, **Figure 5-4D**). This population represents neural stem cells, as explained above. In parallel, there was an increased proportion of *Fmr1*-KO cells in the GLAST$^-$ PSA-NCAM$^+$ population ($F_{1, 32} = 10.03$, p=0.0034, **Figure 5-4G – H**) besides the population's different antigenicity to combinations of CD15 and CD133 regardless of

genotype ($F_{3, 32} = 188.30$, $p<0.0001$, **Figure 5-4H**). The genotype difference among GLAST$^-$PSA-NCAM$^+$ cells resulted from an increased proportion of GLAST$^-$PSA-NCAM$^+$CD133$^-$CD15$^+$ cells in *Fmr1*-KO (p=0.002, WT: 2.34 ± 0.34%, *Fmr1*-KO: 4.42 ± 0.31%, **Figure 5-4H**) at P7, representing a neuroblast population. Interestingly, there were no differences between WT and *Fmr1*-KO GLAST$^+$PSA-NCAM$^+$ or GLAST$^-$PSA-NCAM$^-$ populations or their subpopulations (**Figure 5-4B, F, J**).

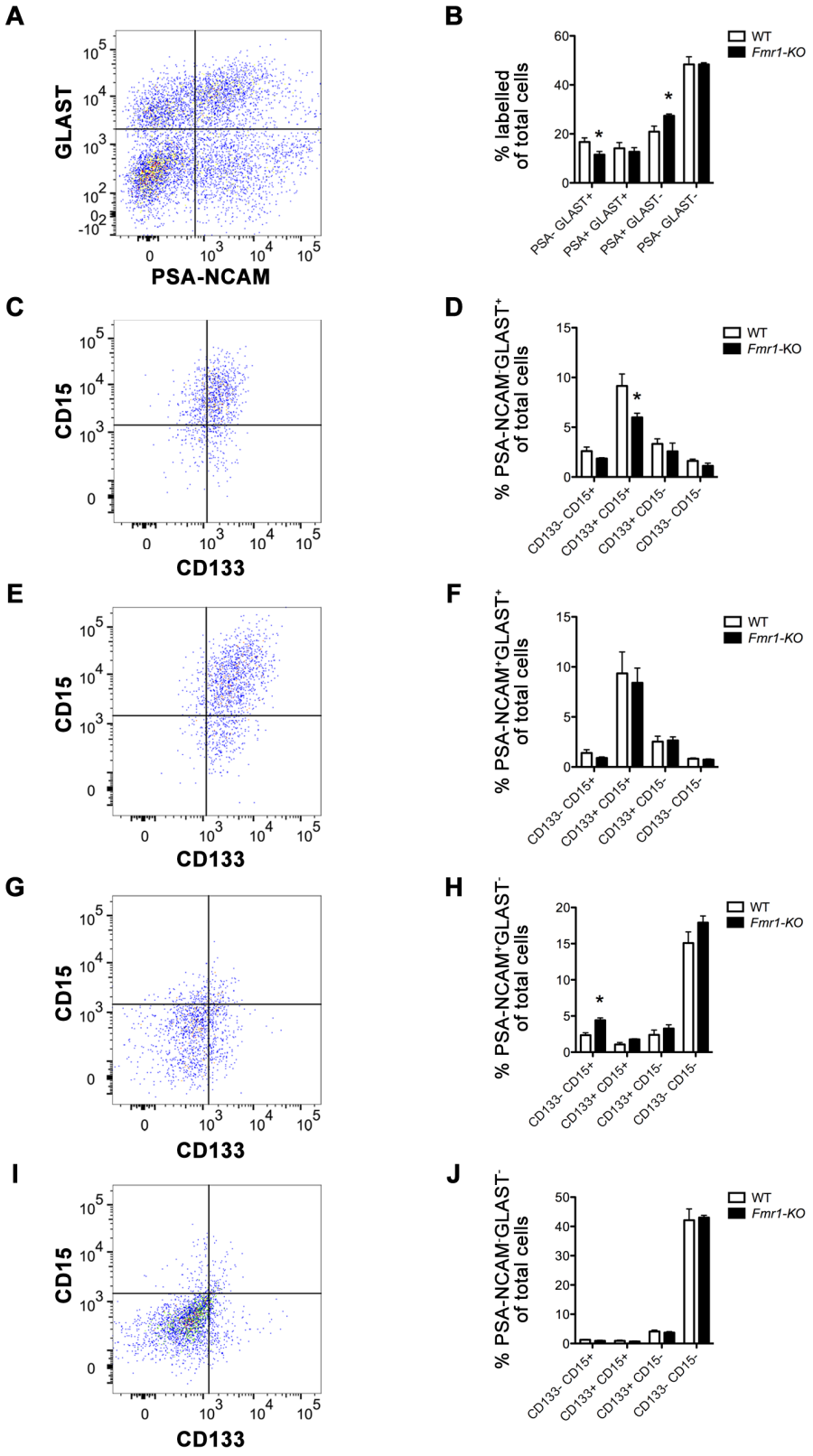
A
GLAST
PSA-NCAM
B
% labelled of total cells
WT
Fmr1-KO
PSA- GLAST+
PSA+ GLAST+
PSA+ GLAST-
PSA- GLAST-
C
CD15
CD133
D
% PSA-NCAM-GLAST+ of total cells
CD133- CD15+
CD133+ CD15+
CD133+ CD15-
CD133- CD15-
E
F
% PSA-NCAM+GLAST+ of total cells
G
H
% PSA-NCAM+GLAST- of total cells
I
J
% PSA-NCAM-GLAST- of total cells

Figure 5-4. Reduced neural stem cell and increased neuroblast proportions in the *Fmr1*-KO P7 DG. A. Representative pseudocolour plot of gating single live cells against PSA-NCAM and GLAST showed **B.** Decreased proportion of PSA-NCAM$^-$GLAST$^+$ cells (p=0.043, WT: 16.68 ± 1.69%, *Fmr1*-KO: 11.54 ± 1.31%) and increased proportion of PSA-NCAM$^+$GLAST$^-$ cells (p=0.026, WT: 20.90 ± 2.60%, *Fmr1*-KO: 27.38 ± 0.76%) in *Fmr1*-KO cells; expressed as percentage of total single live cells. **C.** Gating the PSA-NCAM$^-$GLAST$^+$ population against CD15 and CD133 indicated **D.** Decreased proportion of PSA-NCAM$^-$GLAST$^+$CD133$^+$CD15$^+$ in *Fmr1*-KO DG (p=0.037, WT: 9.15 ± 1.20%, *Fmr1*-KO: 5.99 ± 0.41%) compared to WT; expressed as percentage of total single live cells. **E.** Gating the PSA-NCAM$^+$GLAST$^+$ population against CD15 and CD133 showed **F.** No differences between WT and *Fmr1*-KO. **G.** Gating the PSA-NCAM$^+$GLAST$^-$ population against CD15 and CD133 showed **H.** Increased proportion of PSA-NCAM$^+$GLAST$^-$CD133$^-$CD15$^+$ in *Fmr1*-KO DG (p=0.002, WT: 2.34 ± 0.34%, *Fmr1*-KO: 4.42 ± 0.31%) compared to WT; expressed as percentage of total single live cells. **I.** Gating the PSA-NCAM$^-$GLAST$^-$ population against CD15 and CD133 showed **J.** No differences between WT and *Fmr1*-KO. Abbreviations: WT: wild type, KO: *Fmr1*-knockout, P7: postnatal day 7, DG: dentate gyrus, PSA-NCAM: polysialylated neuronal cell adhesion molecule, GLAST: glutamate aspartate transporter, CD15: Lewis X or stage-specific embryonic antigen 1, CD133: Prominin-1.

Expression of CD15 and CD133 overlaps but is not equivalent in the early postnatal hippocampus and DG

While both CD15 and CD133 enrich for NPCs (Capela & Temple, 2002; Walker et al., 2013), our findings indicated that their expression overlapped but was not equivalent in the hippocampus, unlike in the forebrain (Panchision et al., 2007). For instance, CD133 labelled 3 times as many cells as CD15 in the P4 hippocampus (**Figure 5-1D**). Most $CD15^+$ cells were also $CD133^+$, but not vice versa. Additionally, virtually no $CD15^+$ cells expressed high levels of CD24, in agreement with previous findings (Pruszak et al., 2009). In contrast, many of the cells that expressed high levels of CD133 also expressed high levels of CD24.

Comparing the expression pattern of CD15 and CD133 between cells from the P4 hippocampus and the P7 DG again showed a difference in expression between the two brain regions. The proportion of $CD15^+$ cells was higher in the P7 DG compared to the P4 hippocampus (21% vs 11%), while that of $CD133^+$ was lower in the P7 DG (22% compared to 37% in the P4 hippocampus; **Figure 5-1, 5-3**). The fact that CD15 was enhanced in the DG as opposed to the whole hippocampal formation suggested that it might be a more specific NPC marker owing to the DG being a germinal region. Alternatively, the difference in CD15 and CD133 expression might be linked to the different time points examined (P4 vs. P7), rather than a difference in expression between the hippocampal formation vs the DG.

Discussion:

In this study, we analyzed cell surface marker expression to study NPCs and their progeny using flow cytometry. To our knowledge, this is the first study to use flow cytometric analysis to study populations of hippocampal NPCs and their progeny in Fragile X. We showed a decrease in the proportion of neural stem cells paralleled with an increase in the proportion of neuroblasts in the DG of *Fmr1*-KO mice at P7, as demonstrated by the percentages of $GLAST^{+}CD133^{+}CD15^{+}$ cells and PSA-$NCAM^{+}CD15^{+}$ cells, respectively. We also showed that microdissection of the DG rather than the whole hippocampus is important to detect differences in NPC proportions.

NPC populations and their progeny in FXS

Flow cytometry provides a powerful tool to study NPCs and their progeny. As NPCs divide to generate mature cells, their expression profile of different markers changes. We made use of the differential expression of cell surface markers on NPCs to study their lineage progression in Fragile X. First, neural stem cells of the DG, which are positive for GFAP, GLAST, and Nestin (Jungblut et al., 2012; Mich et al., 2014; Pastrana et al., 2009), give rise to neural progenitor cells. In turn, progenitor cells retain Nestin and GLAST expression (Jungblut et al., 2012; Pastrana et al., 2009), and start expressing PSA-NCAM and Doublecortin as they divide. Progenitor cells then give rise to neuroblasts, which lose Nestin and GLAST expression, but remain PSA-$NCAM^{+}Doublecortin^{+}$. Neuroblasts exit the cell cycle and generate postmitotic neurons, which do not express the migrating cell markers, Doublecortin or PSA-NCAM, as they

mature, reviewed in (Kempermann et al., 2004a). Identifying the NPC population that is affected in Fragile X will provide a better understanding of the function of FMRP in governing NPCs. In turn, this will help optimize targeted treatments for FXS particularly that NPC populations show differential responses to environmental stimuli (Decarolis et al., 2013; Lugert et al., 2010).

NPC biology in FXS

Previous work has shown that FMRP plays a role in regulating the proliferation and differentiation of NPCs. However, the results on how NPC biology is affected in Fragile X have not been consistent. On the one hand, some studies found increased NPC proliferation in the Fragile X embryonic, early postnatal ventricular wall (Castrén et al., 2005; Tervonen et al., 2009), or adult DG (Guo et al., 2011; Luo et al., 2010). On the other, Eadie et al (2009) found no differences in the proliferation of NPCs in the adult DG (Eadie et al., 2009). NPC differentiation into astrocytes and neurons is also altered in Fragile X with reports of increased astrocytic differentiation and decreased neuronal differentiation (Guo et al., 2011; Luo et al., 2010; Telias, Segal, & Ben-Yosef, 2013), increased neuronal differentiation and decreased astrocytic differentiation (Castrén et al., 2005), or no changes in the proportions of neurons and astrocytes that survive to maturity (Eadie et al., 2009) in Fragile X. These inconsistencies could arise from the use of different mouse strains, methods, developmental stages, or brain regions. Notably, differences in mouse strains result in pleiotropic effects on neurogenesis (Pozniak & Pleasure, 2006). Additionally, some learning impairments characteristic of Fragile X,

such as in the hippocampus-dependent cross-shaped water maze task (Dobkin, Rabe, Dumas, & Idrissi, 2000), are detected in the FVB-129 mouse strain, but not the C57BL/6. Thus, the experimental design could in fact affect the results obtained and suggests the importance of extending the results to clinical populations.

The significance of the early postnatal period in hippocampal NPC biology in FXS

Much of the rodent DG develops postnatally. Hippocampal NPCs, which give rise to lifelong neurogenesis, populate the DG by the end of the first postnatal week (Mathews et al., 2010), corresponding to peak FMRP expression in the hippocampi of normal mouse brains (Lu et al., 2004). This highlights the importance of the early postnatal period in the development of the DG, particularly in the context of FXS. Surprisingly, no research has examined NPCs from the early postnatal hippocampus or DG. Our findings of decreased proportions of $GLAST^{+}CD133^{+}CD15^{+}$ cells, representing NSCs, coupled with increased proportions of $PSA\text{-}NCAM^{+}GLAST^{-}CD15^{+}CD133^{-}$ cells, which represented neuroblasts, in the *Fmr1*-KO DG at P7 was surprising in light of previous research, which mainly showed increased NPC proliferation. However, our previous experiments have consistently detected decreased rather than increased proliferation of NPCs. This supports the notion that NSCs may be depleted in the *Fmr1*-KO DG, which arises due to precocious cell proliferation, followed by later reductions in NPC proliferation. Indeed, we detected an already smaller pool of NSCs generating an increased proportion of neuroblasts. Interestingly, many of the reported targets of FMRP regulate the cell cycle such as phosphatase and tensin homolog (PTEN), mammalian target of rapamycin

(mTOR), tuberous sclerosis complex 2 (TSC2), glycogen synthase kinase 3β (GSK3β), and β-catenin (Darnell et al., 2011). This could explain the finding that FXS patients have a lower risk of developing some cancers (Lucá et al., 2013; Rosales-Reynoso et al., 2010), by virtue of FMRP governing the translation of cell cycle regulators. It remains to be determined if our findings of decreased NSCs and increased neuroblasts are restricted to the early postnatal DG or if they persist into adulthood, as would be suggested by NSC depletion and the decreased cancer risk among FXS patients.

The increased proportion of neuroblasts detected in our study is in agreement with previous reports, which showed an initial increase in neuronal differentiation coupled with increased cell death, resulting in a comparable number of mature neurons in WT and *Fmr1*-KO DG (Eadie et al., 2009). We did not detect a difference in the proportions of immature or mature neurons from the P7 DG between WT or *Fmr1*-KO brains. This suggests increased apoptosis of *Fmr1*-KO neuroblasts. Alternatively, it is possible that the increase in neuroblasts in the P7 *Fmr1*-KO DG will yield more neurons at later time points. The importance of FMRP expression to neuroblast biology is underscored by the fact that *Fmr1* is upregulated in PSA-NCAM$^+$ cells, which encompass neuroblasts and immature neurons (Pennartz et al., 2004). Together, our results showed that aberrant hippocampal neurogenesis takes place early in development in the Fragile X brain.

Conclusion:

We report aberrant neurogenesis in the P7 DG of *Fmr1*-KO mice using a combination of cell surface markers and flow cytometry. We specify the NPC population that is affected

at this time point, namely GLAST^{+}CD15^{+}CD133^{+}, which we suggest are neural stem cells. We also show increased neuroblasts, PSA-NCAM^{+}CD15^{+}. These results could indicate that NSCs are depleted in the *Fmr1*-KO DG, which future studies can confirm.

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Chapter 6: Discussion

In these experiments, I sought to answer how NPC biology is dysregulated in the hippocampi of *Fmr1*-KO mice during the early postnatal period of development. I found decreased NPC proliferation, aberrant neurogenesis, and abnormal concentrations of astrocyte-secreted factors in *Fmr1*-KO brains relative to WT. These findings are novel given that no other groups studied hippocampal NPCs during postnatal development despite the importance of this time period in the development of the DG. In turn, altered biology of NPCs during the early postnatal period likely has consequences on lifelong neurogenesis, with functional impairments in learning and memory, as is the case in FXS. Intriguingly, previous studies largely found increased proliferation of NPCs and altered neurogenesis in FXS.

Astrocyte Involvement in FXS (Chapter 3)

The first study aimed to examine populations of NSCs and neural progenitor cells *in vitro*, before assessing their response to astrocyte-secreted factors. It was previously unknown whether FMRP governs NSCs and neural progenitor cells distinctly from each other and how astrocyte-secreted factors from *Fmr1*-KO brains might affect the proliferation of different NPC populations. Although we found similar proportions of NSCs and neural progenitors in *Fmr1*-KO and WT cultures after 21 DIV, we cannot preclude that genotypic differences in the proliferation of NPC populations might exist at other time points or under a different experimental design. This is particularly true given that FMRP is developmentally regulated (Till 2010). Additionally, we have not assessed

the differentiation or cell survival rates in the neurosphere colonies, which if different between genotypes, will have an effect on the colony sizes.

Interestingly, ACM from *Fmr1*-KO brains increased the proportion of WT but not *Fmr1*-KO NSC colonies. The significance of this is twofold. It indicates that *Fmr1*-KO astrocytes aberrantly secrete factors that affect the proliferation of WT NSCs; yet, *Fmr1*-KO NSCs are not responsive to such signals pointing to a deficit or resistance in NSC response to environmental cues. In other words, there are cell-autonomous and non-cell-autonomous defects in the proliferation of NPCs from the *Fmr1*-KO hippocampus. It would be interesting to examine the effect of juxtacrine astrocyte signalling in the proliferation of *Fmr1*-KO NPCs. Astrocytes are known to signal through direct contact with cells (Ashton et al. 2012; Wilhelmsson et al. 2012). In fact, comparison between the effects of signalling through astrocyte contact and astrocyte secreted factors shows that NPCs co-cultured on an astrocyte monolayer were more effective in differentiating into neurons than those cultured with ACM (S. Song et al. 2002). This suggests that contact with *Fmr1*-KO astrocytes may have more potent effects on NPC regulation. It remains to be established whether this effect is specific to NPC differentiation, or if it extends to other aspects of NPC biology.

We also compared protein expression in ACM from *Fmr1*-KO cortices and hippocampi to that of WT. Interestingly, when examining proteins that varied in their expression from WT by at least 50%, we found that the majority of secreted factors from cortical astrocytes (34 out of 37) were down-regulated in contrast to secreted factors from

the hippocampus where the majority was up-regulated (18 out of 29) in *Fmr1*-KO. This indicates that FMRP may regulate astrocyte-secreted factors in a context dependent manner. It is worth noting that at P2, when we cultured astrocytes, the hippocampal formation is far less developed relative to the cortex (Georg Kuhn and Blomgren 2011), which may explain these differences. Next, we identified some of the astrocyte-secreted factors with differential expression in *Fmr1*-KO ACM. These included multidrug resistance protein 1B, MITF, haptoglobin, FEZ2, antithrombin III, and serum albumin. The identification of these proteins opens new possibilities in understanding astrocyte function in general, and the role of astrocyte secreted factors in FXS in particular. Given that the function of some of these proteins, such as FEZ2 (Fujita et al. 2004; Alborghetti et al. 2011), is still elusive, current experiments in the lab are underway to better characterize these proteins and uncover their role in FXS.

Decreased NPC Proliferation (Chapter 4)

The purpose of the second study was to examine the proliferation of hippocampal NPCs. I analyzed the expression of markers of uncommitted precursors (Nestin and SOX2), and cell cycle phases (Ki67) *in vitro*; and Nestin, SOX2, the M phase marker PhH3, and Notch 1 *in vivo*. I found a decreased proportion of cells expressing Ki67 in *Fmr1*-KO neurospheres after 5 and 8 DIV relative to WT. This finding was true of all neurosphere sizes examined and was not due to increased differentiation or cell death given that there were no differences in the expression of Nestin or SOX2 between WT and *Fmr1*-KO smaller neurospheres (≤100 cells). Interestingly, the proportion of $Nestin^{+}$ cells was

higher specifically in *Fmr1*-KO neurospheres ≥101 cells after 8 DIV, indicating that *Fmr1*-KO NSCs might be aberrantly activated in our culture system (Codega et al. 2014). This is similar to findings from the first study and shows that specifically NSCs from *Fmr1*-KO hippocampi show abnormal responses to environmental cues represented by our culture system.

Next, I looked at the *in vivo* expression of Nestin, SOX2, PhH3, and Notch1 in the hippocampi of P5 and P9 male and female mice and analyzed the cell cycle progression of cells from the entire hippocampal formation at P4 or from the P7 DG. I found increased expression levels of PhH3 in P5 hippocampi, especially in female mice, which contrasts with the decreased expression levels of PhH3 in the hippocampi of P9 male mice. The shift in PhH3 expression levels underscores the dynamic changes that happen during brain development and may underlie the differences between our findings and those of other research groups. The decrease in PhH3 expression in male hippocampi at P9 falls in line with our *in vitro* findings and suggests reduced cell proliferation in the hippocampus. In agreement with this, I found a lower proportion of cells in the G_2/M phases of the cell cycle from the DG of P7 *Fmr1*-KO mice, but not in the hippocampi of P4 mice. Together, the reduced expression levels of PhH3 and Nestin in the P9 hippocampus of *Fmr1*-KO mice indicate that hippocampal NPCs show decreased proliferation in *Fmr1*-KO brains.

The decreased Nestin expression in the P9 *Fmr1*-KO hippocampus regardless of sex was intriguing and starkly contrasted with our *in vitro* findings of increased Nestin

expression in *Fmr1*-KO cultures. This apparent discrepancy can be explained by the different experimental designs: Nestin is expressed in activated NSCs as well as in neural progenitors (Kempermann et al. 2004; Codega et al. 2014). Thus, it is conceivable that *Fmr1*-KO NSCs may have been abnormally activated in our culture system, and that this activation does not occur *in vivo* unless the mice are exposed to specific environmental stimuli. Alternatively, given that the decreased *in vivo* expression of Nestin represents a global decrease in the different NPC populations that express Nestin, including activated NSCs and neural progenitors, I cannot pinpoint which NPC populations were affected *in vivo*. Thus, it remains to be determined if NSCs are indeed more quiescent in *Fmr1*-KO hippocampi and thus show reductions in Ki67 and PhH3 labelling; if the cell cycle length is altered in *Fmr1*-KO brains and thus affects the proportion of cells in the M phase; if the pool of NPCs is depleted all together; or if *Fmr1*-KO NPCs exhibit a developmental delay in reaching milestones in a timely manner during postnatal hippocampus development relative to their WT counterparts. It is crucial to identify the specific NPC populations that are dysregulated in FXS since NPC populations have different roles in the DG (Lugert et al. 2010; Decarolis et al. 2013). For instance, exercise enhances neurogenesis by recruiting quiescent, but not active, NSCs to the cell cycle (Lugert et al. 2010). By contrast, seizures increase the proliferation of both quiescent and active NSCs (Lugert et al. 2010). These findings show that environmental stimuli have differential effects on NPC populations and highlight the importance of identifying the NPC populations altered in FXS. In turn, this provides a better understanding of the role of FMRP in governing NPCs, consequently allowing for the optimization of treatment

interventions targeting the appropriate NPC populations.

Decreased NSCs and Increased Neuroblasts in the P7 DG of *Fmr1*-KO Mice (Chapter 5)

The third study aimed to compare NPC populations and their progeny between WT and *Fmr1*-KO mice using FACS. I found similar proportions of NPCs, neuroblasts, and neurons in the P4 hippocampi of *Fmr1*-KO relative to WT, as determined by the combinatorial expression of CD15, CD24, and CD133. Interestingly, analyzing cells from the P7 DG of *Fmr1*-KO mice revealed a lower proportion of NSCs ($GLAST^+CD15^+CD133^+$) accompanied by a larger proportion of neuroblasts (PSA-$NCAM^+CD15^+$) relative to WT. The lower proportion of NSCs in the *Fmr1*-KO DG likely underlies our earlier findings of decreased NPC proliferation (**Chapter 4**). The increase in the proportion of neuroblasts, however, is intriguing especially that there is a lower percentage of NSCs. In turn, this could indicate stem cell depletion, which could be accounted for by the increased differentiation of NSCs to neuroblasts at the expense of NSC maintenance. In agreement with this, lack of FMRP has been shown to result in precocious differentiation of RGCs to intermediate progenitors during embryonic cortical neurogenesis, ultimately resulting in RGC depletion (Saffary and Xie 2011). Given that we did not detect a difference in the proportion of neurons (PSA-$NCAM^-GLAST^-CD15^-CD133^-$), we suggest that a larger proportion of immature neurons from the *Fmr1*-KO DG undergo apoptosis relative to WT, as previously reported in the adult DG (Eadie et al. 2009). This could be attributed to impaired NMDAR plasticity in the *Fmr1*-KO mouse

DG (Bostrom et al. 2015), the activity of which regulates the survival of newborn neurons (Tashiro et al. 2006).

Significance

Altered NPC biology has been associated with several functional impairments, such as emotional dysregulation, seizures, and difficulties in learning and memory; reviewed in (Zhao et al. 2008), which are all symptoms of FXS. Decreased NPC proliferation in the DG of *Fmr1*-KO mice (**Chapter 4**) likely arises from NSC depletion (**Chapter 5**). In turn, NSC depletion might be attributed to similar mechanisms as those that result in decreased cancer incidence among FXS patients (Rosales-Reynoso et al. 2010; Lucá et al. 2013). Indeed, many cell cycle regulators and tumor suppressor genes are targets of FMRP such as mTOR, PTEN, GSK3β, β-catenin, and TSC2 (Darnell et al. 2011). Interestingly, mutations in the tumour suppressor gene, PTEN, have been found in children with ASD and are associated with macrocephaly (Pramparo et al. 2015); reviewed in (Zhou and Parada 2012). PTEN plays an important role in NSC maintenance. In fact, knockout of PTEN in adult mouse NPCs first results in their increased proliferation, followed by NSC depletion (Amiri et al. 2012). There have also been reports of decreased Wnt signalling (Luo et al. 2010) and increased mTOR signalling (Sharma et al. 2010) in relation to Fragile X. Enhancing mTOR signalling in radial glia by knocking out *Tsc2* increases the proliferation of NPCs at the expense of neuronal differentiation (Way et al. 2009). The additive effect of these cell cycle regulators and their contribution to reduced NPC proliferation in the early postnatal *Fmr1*-KO

hippocampus remains to be determined, particularly that mTOR, PTEN, GSK3β, and TSC2 are all players in the PI3K/AKT signalling pathway with some antagonistic effects; reviewed in (Chalhoub and Baker 2009). In addition to decreased NPC proliferation (**Chapter 4**), I also found aberrant *Fmr1*-KO NSC responses to environmental stimuli as was evident in their lack of response to ACM (**Chapter 3**) and their enhanced activation in culture (**Chapter 4**).

Abnormalities in hippocampal NPC biology could underlie some of the phenotypes exhibited by *Fmr1*-KO mice, which show deficits in hippocampus-dependent learning such as exaggerated inhibitory avoidance extinction (Dölen et al. 2007) and trace conditioning tasks (Hayashi et al. 2007). Interestingly, conditional restoration of FMRP in NPCs results in improved performance on hippocampus-dependent tasks (Guo et al. 2011). Therefore, functional restoration of hippocampal neurogenesis can alleviate cognitive impairments associated with Fragile X. In agreement with this, SSRIs are often prescribed to FXS patients, which mechanistically work in part by increasing hippocampal neurogenesis; reviewed in (Sahay and Hen 2007). Additionally, behavioural interventions can rescue learning deficits by correcting neurogenesis even in the absence of pharmacological treatments. In fact, physical exercise has been shown to rescue neurogenesis after NSCs and progenitors were depleted, without restoring NPCs (Ables et al. 2010). This suggests that modulating hippocampal neurogenesis alleviates some of the symptoms of FXS.

Limitations of the Research

We report decreased NPC proliferation in the early postnatal *Fmr1*-KO hippocampus and DG, which contrasts with previous findings of increased NPC proliferation in the embryonic and early postnatal ventricular zone (Castrén et al. 2005; Tervonen et al. 2009) and the adult DG of FXS mice (Luo et al. 2010; Guo et al. 2011), or with no reported differences in NPC proliferation in the adult DG of *Fmr1*-KO mice (Eadie et al. 2009) and in human NPCs derived from fetal cortex (Bhattacharyya et al. 2008). These apparent discrepancies could arise from the use of different disease models, human NPCs compared to mouse models, the use of *Fmr1*-KO mice on the FVB or C57BL/6 genetic backgrounds, different genetic manipulations such as inducible *Fmr1*-KO and knockin mice (Guo et al. 2011), methods, developmental time points, or brain regions examined. Notably, differences in mouse strains are known to have pleiotropic effects on neurogenesis, with reports of highest NPC proliferation in WT C57BL/6 mice compared to other background strains (Pozniak and Pleasure 2006). Additionally, some learning impairments characteristic of Fragile X, such as in the hippocampus-dependent cross-shaped water maze task (Dobkin et al. 2000), are detected in the FVB-129 mouse strain, but not the C57BL/6. Thus, differences in findings of altered NPC biology in FXS can be, at least partially, attributed to the experimental design. It remains to be determined how decreased NPC proliferation in the early postnatal *Fmr1*-KO mouse hippocampus on the FVB genetic background compares to its adult counterpart, and how these results translate to clinical populations.

Clinical Considerations

There is currently no targeted treatment for FXS. Current pharmacological treatments are prescribed on an individual basis to alleviate symptoms of anxiety, ADHD, and aggression. These include SSRIs, stimulants, and antipsychotics (Hagerman et al. 2014). Early behavioural interventions combined with off-label pharmacological treatments were shown to be beneficial to the point where two cases demonstrated normal IQ after the combined treatment (Winarni et al. 2012). Additionally, toddlers with ASD receiving early comprehensive interventions outperform those who receive traditional community interventions on adaptive behaviour measures after two years from starting the interventions (Dawson et al. 2010). Similar to the clinical population, non-pharmacological interventions can correct symptoms of FXS in animal models indicating the translational validity of the behavioural results obtained in animal studies. For instance, environmental enrichment rescued anxiety symptoms in adult *Fmr1*-KO mice (Restivo et al. 2005). Additionally, spaced rather than continuous training corrected learning impairments in *Fmr1*-KO mice (Seese et al. 2014), stressing the importance of optimizing targeted interventions since modulations in behavioural treatment paradigms can change the outcome.

Conclusions

In this thesis, I used various methods to examine NPCs from the early postnatal *Fmr1*-KO hippocampus, a time point that marks the formation of the DG blades (Bayer 1980a; Bayer 1980b) and is when FMRP expression is highest in the rodent hippocampus (R. Lu

et al. 2004). I found:

- Astrocyte-secreted factors from *Fmr1*-KO cortices and hippocampi send aberrant signals affecting the proliferation of NPCs.
- *Fmr1*-KO NSCs show intrinsic deficits in responding to astrocytic cues.
- Decreased proliferation along with decreased expression of the NPC marker, Nestin, in *Fmr1*-KO hippocampi.
- Decreased proportions of NSCs coupled with increased proportions of neuroblasts.

While these results contrasted with most previous findings that studied *Fmr1*-KO NPCs in other brain regions or developmental time points, this study is the first to examine NPCs from the developing hippocampus and DG. This raises the question of whether our findings are specific to the time point in question or whether they hold true throughout development and adulthood and are a result of our experimental design and mouse strain. It is important to answer these questions in order to understand the role of FMRP in the developmental trajectory of DG NPCs and translate the findings to clinical populations. In turn, identifying the cellular and molecular underpinnings of FXS will allow for optimizing pharmacological and behavioural interventions.

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