Role of mGluR5 and FMRP in Mouse Primary Somatosensory Cortex

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Declaration

This work was carried out in the School of Biomedical Sciences at the University of Edinburgh. I have composed the work and analysis presented in this thesis with the exception of the following work:

1) The dendritic complexity analysis presented in figure 3.12 and the immunohistochemistry study confirming antibody specificity in figure 4.1 were performed by Dr. Sally M Till.
2) Immunoelectron microscopy studies presented in figures 3.6 and 4.9 were done in collaboration with Dr. Thomas H Gillingwater, and EM analysis for figure 4.9 was also carried out in collaboration with Dr. Sally M Till.

The work complied in this thesis has not been submitted for any other degree or professional qualification.

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Abbreviations

5HT	serotonin
AC	adenylyl cylcase
AD	adult
AKAP 79/150	A-kinase anchoring protein 79/150
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
AS	anterior snout region
ATP	adenosine triphosphate
CaMKII	calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CO	cytochorome oxidase
DAG	Diacylglycerol
Dfmr	*Drosophila* homolog of *FMR1* gene
dFMRP	*Drosophila* homolog of FMRP
Dfxr	*Drosophila* model of FXS
DG	dentate gyrus
DHPG	S-3,5-Dihydroxyphenylglycine
DmGluRA	single functional homolog of mGluRs in *drosophila*
EGFP	enhanced green fluorescent protein
EM	electron microscopy
ERK	extracellular signal-regulated kinase
FMR1	fragile X mental retardation 1
FMRP	fragile X mental retardation protein
FXS	fragile X syndrome
FXTAS	fragile X associated tremor/ataxia syndrome
$GABA_A$	gamma-aminobutyric $acid_A$
GFP	green fluorescent protein
GKAP/SAPAP/DAP	guanylate kinase-associated proteins
GluR1	subunit of AMPAR
GluR2/3	subunit of AMPAR
Gp1 mGluRs	mGluR1 and mGluR5
GRIP	glutamate-receptor-interacting protein
hnRNP	heterogeneous nuclear ribonucleoproteins

ION	infraorbital nerve
IP3	1,4,5-inositol triphosphate
IP3R	IP3 receptor
KIF17, 5, 1A	kinesin motor family
LTD	long term depression
LTP	long term potentiation
MAGUK	membrane associated guanylate kinase
MAOA	monoamine oxidase A
mGluR	metabotropic glutamate receptor
mGluR5	metabotropic glutamate receptor 5
mGluR-LTD	Gp1 mGluR dependent LTD
MPEP	2-methyl-6-(phenylethynyl)-pyridine
MR	mental retardation
MRI	magnetic resonance imaging studies
mRNA	messenger RNA
mRNPs	messenger ribonucleoprotein particles
mTOR	mammalian target of rapamycin
NMDAR	*N*-methyl-D-aspartate receptor
NR1	subunit of NMDAR
NR2B	subunit of NMDAR
nVc	trigeminal nucleus caudalis
nVi	trigeminal nucleus interpolaris
nVp	trigeminal nucleus nucleus principal
PDZ	PSD95, disc large, zona occludens 1
pERK	phosphorylated ERK
PI	phosphotidyl inositol
PI3 kinase	phophotidylinositol-3 kinase
PICK1	protein that interacts with C-kinase alpha 1
PIP2	phosphotidylinositol4,5-biphosphate
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PLCβ	phospholipase C-β
PLCβ1	phospholipase C-β1
PMBSF	posterior medial barrel subfield
PP2A	protein phosphatase 2A

PSD	postsynaptic density
PSD93	postsynaptic density protein 93 (Chapsyn 110)
PSD95	Postsynaptic density 95
RNA	ribonucleic acid
S1	mouse primary somatosensory cortex
S6K1	ribosomal protein S6 kinase 1
SAP102	synapse associated protein 102
SAP97	synapse associated protein 97
sec8	subunit of the 'exocyst' complex
SynGAP	Synaptic Ras GTPase activating protein
TARP	transmembrane AMPA receptor regulatory protein
TCAs	thalamocortical axons
TGCs	trigeminal ganglion cells
TTX	Tetrodotoxin
UTR	Untranslated region
VpM	ventroposteriormedial nucleus of thalamus

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Abstract

The accurate development of the wiring between the billions of neurons in our brain is fundamental to brain function. Development of this connectivity relies on activity-dependent modification of synapses similar to those that underlie learning and memory. Glutamate is the principal excitatory neurotransmitter in the mammalian brain and several brain disorders result from altered glutamatergic receptor signalling (Catania et al., 2007; Lau and Zukin, 2007). Genes encoding glutamate receptor associated proteins have a high incidence of mutation in cognitive disorders, especially X-linked mental retardation (MR)(Laumonnier et al., 2007). MR has long been associated with altered cortical connectivity, particularly dendritic spine dysgenesis. There is also an emerging view that aberrant local protein synthesis within dendrites and protein trafficking to dendrites underlies some forms of MR (Kelleher and Bear, 2008; Pfeiffer and Huber, 2006; Zalfa and Bagni, 2005). Most studies examining the role of glutamatergic receptors in MR have focused on adults. Little is known about how these MR genes regulate brain development despite their neurodevelopmental aetiology.

Fragile X mental retardation (FXS) is the most common form of inherited MR and results from the loss of fragile X mental retardation protein (FMRP). FMRP is a RNA binding protein and is hypothesised to have a role in protein trafficking from nucleus to sites of synapses, and regulating local protein synthesis at sites of synapses (Bagni and Greenough, 2005). A prevalent theory of FXS causation is 'metabotropic glutamate receptor (mGluR) theory of fragile X', which postulates that all functional consequences of mGluR (predominantly mGluR5)-dependent protein synthesis maybe exaggerated in FXS (Bear et al., 2004).

Primary somatosensory cortex (S1) of rodents provides an excellent model system to study the role of MR genes in development because of its stereotypic, glutamate receptor-dependent, anatomical development (Barnett et al., 2006b; Erzurumlu and Kind, 2001). Hannan et al., (2001) reported that genetic deletion of mGluR5 results in loss of 'barrels', the anatomical correlates of rodent whiskers in S1. Chapter 3 extends these findings to show

that there is expression of mGluR5 as early as P4 in S1 prior to segregation of layer 4 cells into barrels suggesting a tropic role for glutamate in barrel formation. The expression of mGluR5 is postsynaptic during barrel formation and does not regulate tangential or radial cortical development. Its effects on barrel segregation are dose dependent and are not due to a developmental delay. During late S1 development, loss of mGluR5 results in decreased spine density suggesting a role in synaptogenesis. Supporting this hypothesis in mGluR5 mutant mice there is a general decrease in expression of synaptic markers in early S1 development. Chapter 4 explores the role of FMRP in cortical development. FMRP is expressed early in S1 development with peak expression prior to synaptogenesis at P14. It is expressed postsynaptically at P7 and pre and postsynaptically at P14. FMRP does not regulate cortical arealisation during barrel formation but results in decreased barrel segregation. In the absence of FMRP, biochemical studies show altered expression of glutamatergic receptors in the neocortex P7 and P14 suggesting altered glutamatergic receptor composition at synaptic sites. During late S1 development, loss of FMRP results in increased spine density in layer 4 spiny cells. Together these data indicate a role for FMRP during early and late S1 development. Chapter 5 directly tests the mGluR theory of FXS by examining whether genetic reduction of mGluR5 levels rescues anatomical phenotypes characterised in *Fmr1*$^{-/y}$ mice. The defect in barrel formation in *Fmr1*$^{-/y}$ mice is partially rescued by reducing mGluR5 levels. However, layer 4 spine density in *Fmr1*$^{-/y}$ mice does not appear to be rescued.

Chapter 6 explores the expression patterns of three key synaptic MAGUKs (Membrane associated guanylate kinases) PSD95, SAP102 and PSD93, one of which (PSD95) is regulated by FMRP (Zalfa et al., 2007) and the others which have putative binding sites for FMRP. MAGUKs tether glutamatergic receptors to their associated signalling complexes at the postsynaptic membrane and also regulate glutamatergic receptor trafficking (Collins and Grant, 2007; Kim and Sheng, 2004). The immunohistochemical expression profiles of PSD95, SAP102 and PSD93 show dynamic regulation during S1 development that is unaffected by loss of FMRP (at P7), and biochemical data indicates that basal levels of these MAGUKs in neocortex are unaltered at P7 and P14 in *Fmr1*$^{-/y}$ mice. In *Sap102*$^{-/y}$ and *Psd95*$^{-/-}$ mice, there is altered expression of several synaptic proteins biochemically providing evidence for

differential roles of SAP102 and PSD95 in regulating expression of glutamatergic receptors at synaptic sites during early S1 development.

This thesis demonstrates that synaptic proteins associated with MR are expressed early in development and display regulatory roles in cellular processes governing S1 formation. An understanding of their role in early brain development would be critical in fully appreciating when and where they exert their regulatory effects, and this in turn would be beneficial in designing therapeutic interventions.

1

1. Introduction

The human brain is comprised of around hundred billion neurons that connect together to communicate our experiences, and thereby modify our brain and influence our behaviour. Neurons transfer information to one another through specialised cell-to-cell junctions known as synapses. The formation of synaptic connections is in part genetically programmed, but these connections are guided and modified throughout our life depending on our experiences. Although, a child's brain has a remarkable capacity to undergo changes compared to an adult's such that learning a new language or a skill is inherently easier when you are younger. During our postnatal development, there is an overproduction of synapses so that a two-year-old toddler has twice as many synapses in their cerebral cortex compared to an adult, and the excessive number of synapses are pruned over the course of early development until approximately pubescence (Huttenlocher, 1990). The process of synaptic refinement through this period is thought to be primarily mediated by activity at excitatory synapses that use glutamate as their principal neurotransmitter (reviewed in McDonald and Johnston, 1990). Glutamatergic signalling apart from regulating communication between neurons, activates intracellular cascades that regulate cellular processes such as activity dependent protein synthesis and trafficking that shape connectivity between synapses. Therefore, the establishment of normal synaptic connectivity is a precise, developmentally orchestrated process, and errors in this process are thought to be associated with neurodevelopmental disorders including mental retardation (MR).

Interplay between a host of genetic programmes and intracellular signalling cascades specify the course of our brain connections even before birth. However, during postnatal development these initially predetermined connections are validated and refined through synaptic activity (Bear et al., 2008). In the first part of the chapter, key concepts governing synapse formation and refinement during postnatal brain development will be briefly introduced (with an emphasis on the postsynaptic compartment) using findings from both *in vitro* and *in vivo* studies. The second part of the chapter will focus on an example of an inherited form of MR, fragile X syndrome

(FXS). FXS is thought to manifest from dysregulated glutamatergic signalling and protein synthesis leading to altered synaptic connectivity (Garber et al., 2008). The final part will introduce the use of mouse primary somatosensory cortex (S1) in understanding the developmental role of fragile X mental retardation protein (FMRP) that is lost in FXS, and the role of glutamatergic signalling through metabotropic glutamate receptor 5 (mGluR5), which is thought to be misregulated in FXS.

1.1 Synaptic connectivity in the postnatal brain

1.1.1 General process of excitatory synapse formation

Excitatory synapses are chemical synapses. The axon of a presynaptic neuron releases glutamate that diffuses across a narrow cleft to act on the corresponding postsynaptic site on a neuronal dendrite (Li and Sheng, 2003). Once an axon-dendrite contact is initiated, the assembly of a synapse is thought to take place rapidly such that synaptic transmission can be detected within an hour of the initial contact (Friedman et al., 2000; Okabe et al., 2001; Washbourne et al., 2002) (Figure 1.1).

Presynaptic

Several studies show the presence of pleiomorphic vesicular clusters at newly formed synapses (Ahmari et al., 2000) that are mobile in axons prior to synapse contact and are then immobilised suggesting that both pre and postsynaptic components are trafficked to the nascent synapse preassembled. One such presynaptic precursor complex about 80nm in diameter associated with dense core vesicles has been purified and found to contain active-zone components such as presynaptic scaffolding proteins Piccolo, Bassoon and RIM (Rab3-interacting molecule), SNARE (soluble *N*-ethylmaleimide-sensitive-factor-attachement-protein receptor) proteins synataxin and SNAP25 (synaptosomal-associated protein 25), and the cell adhesion molecule N-cadherin (Zhai et al., 2000; Zhai et al., 2001). Ahmari et al., (2000) using green fluorescent protein (GFP) tagged VAMP (vesicule-associated membrane protein) found GFP-VAMP to be present in axons as mobile clusters co-localised with presynaptic membrane proteins and other

synaptic vesicle proteins. The delivery of three to four of these preassembled precursors has been shown to be sufficient in forming the presynaptic active zone (Ahmari et al., 2000; Shapira et al., 2003).

Postsynaptic

Conversely, the detailed assembly of postsynaptic specialisation is less well known (Li and Sheng, 2003) and seems to be largely dependent on the interaction of PDZ (PSD95, disc large, zona occludens 1) domain containing scaffolding proteins and other synaptic proteins. The scaffolding protein, PSD95 (Postsynaptic density 95), is rapidly recruited within 20min of the axon-dendritic contact (Okabe et al., 2001) and *de novo* accumulation of PSD95 clusters at nascent synaptic sites has been shown to occur from translocation of pre-existing cytoplasmic pools of PSD95 (Bresler et al., 2001; Marrs et al., 2001). The accumulation of other postsynaptic proteins such as Homer 1, Shank 2/3 and CaMKII (Calmodulin-dependent protein kinase II) are thought to occur via gradual diffusion from the cytoplasmic pool or recruited individually (Bresler et al., 2004; Okabe et al., 2001; Petersen et al., 2003).

The synaptic delivery of ionotropic glutamatergic receptors, NMDAR (*N*-methyl-D-aspartate receptor) and AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor) is facilitated by different PDZ domain containing proteins, chaperons, endocytic adaptors and cytoskeletal elements (Waites et al., 2005). Time lapse imaging of GFP tagged NR1 (subunit of NMDAR) and GluR1 (subunit of AMPAR) show non-overlapping clusters and differential kinetics for recruitment of these receptors to nascent synapses suggesting heterogeneity in glutamatergic receptor trafficking and localisation to synapses. Moreover, while NR2B (subunit of NMDAR) is linked to the kinesin family motor KIF17 (Setou et al., 2000), GluR2/3 (subunit of AMPAR) is associated with KIF5 and KIF1A (Shin et al., 2003) through different scaffolding interactions suggesting that these glutamatergic receptors may have specific cargo routes along microtubules within dendrites. While there is evidence for trafficking of NMDAR with clusters of PSD95 (Wenthold et al., 2003), others show PSD95 to traffic to and incorporate into synapses largely independent of NMDARs (Friedman et al.,

2000; Rao et al., 1998; Washbourne et al., 2002), and that PSD95 binding is not essential for NMDAR localisation (Migaud et al., 1998; Passafaro et al., 1999; Sprengel et al., 1998). However, once recruited, NMDARs are stabilised at the synaptic site by PSD95 (Roche et al., 2001). In addition, the sec8 subunit of the 'exocyst' complex that targets secretary vesicles to the surface interacts with the PSD95 family protein SAP102 (synapse associated protein 102) and mediates trafficking of NMDARs to synaptic sites (Sans et al., 2003). Steady levels of AMPARs at the synapse is maintained by scaffolding proteins GRIP (glutamate-receptor-interacting protein), PICK1 (protein that interacts with C-kinase alpha 1) and Stargazin (Barry and Ziff, 2002; Chen et al., 2000; Scannevin and Huganir, 2000), however it is unclear whether these scaffolding proteins play a role in the morphological development of a nascent synapse (Chen et al., 2000; Li and Sheng, 2003) or unclear (Li and Sheng, 2003). Once the axon-dendritic contact is made, pre and postsynaptic sites of the synapse develop in a coordinated manner (Harris and Stevens, 1989) as discussed in subsequent sections.

1.1.2 The postsynaptic density (PSD)

The postsynaptic density (PSD) is an electron rich thickening of the excitatory postsynaptic membrane containing glutamatergic receptors and their associated signalling and cytoskeletal proteins (Collins and Grant, 2007; Collins et al., 2006; Farr et al., 2004; Husi and Grant, 2001; Husi et al., 2000; Kennedy, 1993, 1997, 2000; Kim and Sheng, 2004; Walikonis et al., 2000). The PDZ domain scaffolding proteins are crucial in assembling the PSD as they create an interface between glutamatergic receptor signalling complexes, cell adhesion molecules and actin based cytoskeletal elements (Boeckers, 2006). The members of the NMDAR associated complex at the PSD were first discovered by performing immunoblot and mass spectrometry on whole brain homogenate purified by NMDAR immunoprecipitation (Husi et al., 2000). In this proteomic characterisation, 77 molecules were identified and these comprised of receptors, adaptors, signalling molecules, cytoskeletal and novel molecules (Husi et al., 2000). Since the initial discovery, more PSD proteins have been identified by the affinity isolation of MAGUK (membrane associated guanylate kinase) bound proteins (Collins et al., 2006). Three protein complexes have been defined at the PSD on the basis of

their proteins associations: the NMDAR or MAGUK associated complex (NRC/MASC), the AMPAR complex (ARC) and the metabotropic glutamate receptor (mGluR) complex (mGC) (Collins and Grant, 2007; Collins et al., 2006; Farr et al., 2004; Husi and Grant, 2001; Husi et al., 2000; Kennedy, 2000; Walikonis et al., 2000). Although a detailed view of these complexes is beyond the scope of this introduction, below is a brief mention of how these PSD components are interlinked which is of interest in terms of signalling cascades that would be discussed in this thesis.

The PDZ protein interacting motifs of PSD95 family MAGUKs associate with NR2 subunits of NMDAR and localise these to the PSD (Husi et al., 2000; Sheng and Sala, 2001). The PDZ domains of PSD95 also interacts with stargazin, a transmembrane AMPA receptor regulatory protein (TARP) that has been shown to regulate AMPARs at the synapse (Schnell et al., 2002). Moreover, PSD95 associates with guanylate kinase-associated proteins (GKAP/SAPAP/DAP) (Kim et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997), and GKAP in turn binds to Shank (Naisbitt et al., 1997) that interacts with Homer (Sala et al., 2001), a scaffolding protein that binds to Gp1 mGluRs (Brakeman et al., 1997). Gp1 mGluRs are comprised of mGluR1 and mGluR5. GKAP-Shank-Homer interactions bring together the NRC/MASC and ARC at the PSD with the mGC complex, which is thought to be present in regions adjacent to the PSD (Boeckers, 2006; Kim and Sheng, 2004). In addition to tethering glutamatergic receptors, scaffolding proteins also facilitate interactions between receptors and their downstream signalling cascades involving various second messengers, kinases and phosphatases and other regulatory elements (Garner et al., 2000; Husi et al., 2000; Kennedy, 2000; Kim and Sheng, 2004).

1.1.3 Spines, the sites of excitatory synapses and synaptic maturation

In the late 19th century, Cajal observed by silver staining (Golgi staining) that the surface of cerebellar Purkinje cells bristled with thorn like structures, which he termed “espinas”, and proposed that these are sites of axon-dendritic connections (cited in Garcia-Lopez et al., 2007). Since then it is thought that more than 90% of excitatory synapses occur at spines, which are membranous protrusions from dendrites (reviewed in Nimchinsky et al.,

2002). Spine formation, spinogenesis, is a developmentally regulated process (reviewed in Alvarez and Sabatini, 2007). *In vivo* imaging studies of sensory cortical areas show that formation of new spines is stable throughout development, whereas during development spine elimination gradually increases to reach a peak and then rapidly drop off to a stable rate in the adult (Harms and Dunaevsky, 2007; Lippman and Dunaevsky, 2005). Along a dendrite, the density of spines characteristically increases exponentially as a function of distance from the cell body until an optimum point (about 70-100μm) and then decreases gradually in distal regions (reviewed in Nimchinsky et al., 2002).

There are three models proposed for spinogenesis. These are known as the 1) Sotelo model 2) Miller/Peters model and 3) Filopodial model (reviewed in Yuste and Bonhoeffer, 2004) (Figure 1.2). In brief these models are as follows,

Sotelo model

Sotelo model is based on evidence from studies examining formation of cerebellar Purkinje cell synapses and suggests that spinogenesis is intrinsic to a neuron and that it does not require a presynaptic axon (Sotelo, 1990). In *weaver* mutants, presynaptic granular cells that form 90% of synapses with Purkinje cells are absent. Despite Purkinje cells developing abnormal atrophic dendrites, they still have spines with postsynaptic specialisations (Hirano and Dembitzer, 1973; Rakic and Sidman, 1973; Sotelo, 1975). In addition, in *reeler* mice that have gross defects in migration of neural precursor cells in the cerebellum, there are ectopically expressed Purkinje cells with spines without any presynaptic granular cell partners (Mariani et al., 1975). Typically, cerebellar granule cells develop slightly delayed to the development of Purkinje cells, and if these granule cells are ablated by X-irradation in neonatal rats, Purkinje cells still develop spines at roughly normal densities (Sotelo, 1977). Moreover, in normal cerebellar synapse development, spinogenesis in distal dendritic braches of Purkinje cells precede their synapse formation with parallel fibres (cited in Yuste and Bonhoeffer, 2004).

Miller/Peters model

On the basis of evidence from the rat visual cortex, this model proposes that synapses induced through the effects of presynaptic terminal first occur on pyramidal cell dendritic shafts. A spine then emerges and carries the shaft synapse away from the dendritic shaft (Miller and Peters, 1981). In a Golgi study of layer 5 pyramidal cells from visual cortex at different developmental ages, Millers and Peters (1981) show that dendritic spines begin as broad protrusions that have symmetric junctions with small diameter axonal processes. These protrusions with time grow taller and mature and participate in asymmetric synapses with axonal varicosities. However, earlier dendritic shaft synapses only constitute a small proportion of adult synapses. Furthermore, based on this model one would expect afferent projection during early development to have convoluted trajectories to pull out the shaft synapse to form a spine, and such afferent projections have not been yet identified (Yuste and Bonhoeffer, 2004). Moreover, Knott et al., (2006) show that new cortical spines form synapses with boutons that already contained synapses with other spines, thus in accordance with this model, conversion of these synapses would involve synchronised elongation of one and the retraction of others which they did not observe.

Filopodial model

Cajal initially hypothesised that spines themselves are capable of moving with respect to the position of the axon, and undergo synaptic activity dependent swelling and elongation (cited in Bonhoeffer and Yuste, 2002). Vaughn, (1989) proposed the 'synaptotropic' hypothesis, in which he proposed that filopodia extend towards axonal contacts during development, and a synapse is formed on the filopodium. In favour of this model, enrichment of synapses on filopodia has been found in the hippocampus (Fiala et al., 1998), in the spinal cord (Vaughn et al., 1974) and corticorubral synapses (Saito et al., 1997). Also, confocal microscopy of hippocampal slice cultures provided the first evidence to show that long and thin dendritic protrusions known as filopodia do grow and retract during early neuronal development (Dailey and Smith, 1996). Filopodia are highly motile with changes in protrusion length about 40-50nm/min (Majewska et al., 2006) and have a rapid turn over (Zuo

et al., 2005a). The rapid motility and morphological changes in filopodia are thought to be dependent on actin dynamics (Dunaevsky et al., 1999; Fischer et al., 1998) and myosin motor activities (Fischer et al., 1998; Osterweil et al., 2005; Tada and Sheng, 2006). Whether these early filopodia persists in the adult, or whether they are precursors for new dendritic branches or spines is yet unclear. Recent two-photon imaging has characterised that the density of filopodia decreases with progressive age in various cortical regions with only 2% present in 4-5 month old mice (Grutzendler et al., 2002; Majewska and Sur, 2003; Zuo et al., 2005a). Moreover, only 0.2% of filopodia seems to transform into mature spines (Majewska et al., 2006), or in some cases they do not develop into spines (Linke et al., 1994; Mason, 1983; Wong et al., 1992).

Immature spines are long and thin in appearance and contain macular (small) PSDs (Harris et al., 1992). In contrast, mature spines are mushroom shaped with a protruding thick neck from the dendritic shaft terminating in a bulbous head (Horner, 1993) (Figure 1.2). These are enriched in F-actin (Capani et al., 2001) and are likely to contain perforated (large) PSDs (Harris and Stevens, 1989). These alterations in spine morphology are thought to be mediated via signalling through Rho and Ras family small GTPases regulating actin dynamics in the spine (Newey et al., 2005; Tada and Sheng, 2006). Furthermore, there is a positive correlation between the volume of spine head size and the size of the PSD, thereby the number of glutamatergic receptors, specifically AMPARs (Nusser et al., 1998). The spine size is also thought to be directly proportional to the number of presynaptic docked vesicles (Schikorski and Stevens, 1997, 1999).

Knott et al., (2006) in a time-lapse imaging study over a month followed by retrospective electron microscopy (EM) shows that in the adult brain, newly formed spines synapse onto existing presynaptic boutons suggesting that new spine formation may precede synapse formation. Also, new spines initially have large surface to volume ratios compared to persistent spines, and increases in volume is only evident if they become stable for few days (Knott et al., 2006) suggesting that new spines initially grow as thin, filopodia like protrusions that progressively mature by adding/increasing spine head volume.

In addition to morphological changes during development, the physiological properties of synaptic responses also alter concomitantly with changes in receptor subunit composition at the PSD. Interestingly, both *in vivo* and *in vitro* studies show a fraction of synapses in several brain regions to be 'silent' during the first postnatal week. Silent synapses lack AMPARs that can conduct current at resting membrane potentials, and only contain NMDARs, which are blocked by voltage-dependent Mg^{2+} at resting membrane potentials (Durand et al., 1996; Isaac et al., 1997; Liao et al., 1995). The fraction of silent synapses decreases with age and this is paralleled by an increase in the synaptic AMPAR expression that correlates well with the development of AMPAR mediated synaptic currents (Liao et al., 1999; Petralia et al., 1999). Furthermore, there is also a developmental switch in subunit composition of NMDARs at the synapse during the first two postnatal weeks from a predominantly NR2B enriched one to one with NR2A resulting in faster NMDAR kinetics with synapse maturation in hippocampal synapses (Petralia et al., 2005; Sans et al., 2000; van Zundert et al., 2004).

1.1.4 Synaptic activity in shaping synaptic connectivity

Recent evidence suggests that synapses form even in the absence of electrical activity or activated synaptic transmission. When there is no spontaneous or evoked release of neurotransmitter in mutants of Munc-13 and Munc-18 (mammalian homologues of *Caenorhabditis elegans* UNC-13 and UNC-18), which are presynaptic proteins important for vesicle priming and fusion, hippocampal synapses still develop normal ultrastructure suggesting that activity is not important in the initial axon-dendtritic contact development (Varoqueaux et al., 2002; Verhage et al., 2000). However, there is apoptosis of several brain regions leading to neurodegeneration in these mice and they die before spinogenesis suggesting that although synaptic connections can be formed without neurotransmitter release, it is required for maintenance of synaptic connections (Varoqueaux et al., 2002; Verhage et al., 2000). Moreover, in the presence of glutamate blockers, synaptogenesis occurs normally in hippocampal cultures (Rao and Craig, 1997). However, several lines of evidence demonstrate a role for synaptic activity in sculpting and refining synaptic connections during both early and late brain development in various brain regions (reviewed in Katz and Shatz, 1996; Waites et al., 2005).

Synaptic activity may also regulate the expression of synaptic proteins via ubiquitination, thereby regulating synapse stability. Ehlers (2003) shows that components of the PSD are co-regulated by synaptic activity and that this up or down regulation is produced by ubiquitin-mediated protein degradation.

Cajal speculated that learning would inevitably involve neuronal growth, while others such as Spencer and Tanzi argued that learning might simply alter the strength of existing synaptic connections. Hebb collating these ideas together hypothesised that "Let us assume that the persistence or repetition of reverberatory activity (or 'trace') tends to induce lasting cellular changes that adds to its stability...When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." suggesting that both changes in synaptic strength and novel synaptic growth are crucial in paradigms of learning and memory (cited in Yuste and Bonhoeffer, 2001). Mechanisms of plasticity such as synaptic strengthening (LTP-long term potentiation) and/or weakening of synapses (LTD-long term depression) are largely believed to be cellular basis of learning. There has been considerable attention given to determine whether LTP and/or LTD induction is associated with morphological changes at the synapse. Also if such morphological changes are evident, whether they contribute to enhancing the strength of the synapse or are used as future sites of plasticity (Segal, 2002; Yuste and Bonhoeffer, 2001).

Increases in spine head size and widening and shortening of spine neck in response to LTP induction paradigms has been revealed by *post hoc* EM analysis from both cortical and hippocampal tissue (Desmond and Levy, 1986, 1988; Fifkova and Anderson, 1981; Fifkova and Van Harreveld, 1977; Van Harreveld and Fifkova, 1975) as well as a concomitant increase in presynaptic docked vesicles (Schikorski and Stevens, 1999). Increase in spine size in mature spines in response to LTP is thought to result in splitting of the spine to form perforated spines (Toni et al., 1999). In contrast, recent EM analysis of hippocampal synapses after LTP induction shows that spines do not bifurcate in hippocampal neurons, but form new spines adjacent to

existing ones, which then synapse onto the same presynaptic site *en route* to forming new synapses (Fiala et al., 2002a; Harris et al., 2003). Not all such static studies find alterations in spine size or spine density in response to LTP (Chang and Greenough, 1984; Sorra and Harris, 1998). However, two photon studies elegantly show that localised or global LTP stimulation paradigms result in the emergence of new spines (Engert and Bonhoeffer, 1999; Lang et al., 2004; Matsuzaki et al., 2001; Matsuzaki et al., 2004; Murthy et al., 2001). Conversely, induction of LTD has been shown to result in decreased spine number (Zhou et al., 2004) and spine retraction (Nagerl et al., 2004; Zhou et al., 2004). Although, a recent two-photon study in cerebellar Purkinje cells by Sdrulla and Linden (2007) found no change in spine size or spine number elicited in response to either locally or globally induced LTD. They also observed no LTD induction in cells that had undergone globally evoked retraction of dendritic spines (Sdrulla and Linden, 2007). Collectively, these data may not represent discrepancies, but could result from differences in brain area and neuronal cell type.

The changes in spine number and morphology in response to either LTP or LTD are bidirectional modifications that are reversible and are accompanied by functional changes at the synapse as well (Harms and Dunaevsky, 2007). One of the prevalent forms of LTP induction is dependent on NMDAR activation leading to Ca^{2+} influx (Bliss and Collingridge, 1993; Nicoll and Malenka, 1995), and subsequent redistribution of AMPARs at the PSD (Malinow and Malenka, 2002; Song and Huganir, 2002). LTD in turn is thought to result in the removal of AMPARs from the PSD (Anwyl, 2006; Beattie et al., 2000). Matsuzaki et al., (2004) show that repetitive uncaging of glutamate results in transient changes in mature spines but persistent longer lasting enlargement of spines that previously had an immature appearance. This morphological enlargement was found to be associated with increased AMPAR mediated currents dependent on NMDAR signalling (Matsuzaki et al., 2004). It is likely that immature spines with presumably fewer AMPARs compared to mature spines may be predisposed to undergo LTP preferentially, whereas induction of LTP might be occluded in mature spines.

Therefore, the popular belief in refinement of synaptic connectivity by activity in postnatal development is that mechanisms of LTP are important in

generating new synapses and in retaining nascent synapses while conversely LTD is critical in activity-guided synapse elimination (Bear, 1998).

1.1.5 Protein synthesis in maintaining synaptic plasticity

Steward and Levy (1982) show that a high proportion of polyribosomes, clusters of ribosomes bound to mRNA (messenger RNA), are associated with spines, specifically at the base of the spine neck. This led to the hypothesis that local protein synthesis at sites of synapses may play a regulatory role in maintaining the expression of synaptic plasticity required for long-term alterations in synaptic connectivity (Schuman et al., 2006; Steward and Schuman, 2001). In agreement, tetanic stimulation of hippocampal synapses is shown to trigger translocation of polyribosomes to spines coinciding with an increase in spine size (Ostroff et al., 2002), however, whether these spines undergo LTP was not examined. The early phase of LTP (E-LTP) expression is transcription independent whereas the late phase of LTP (L-LTP) is dependent on new mRNA transcribed from the nucleus (Steward and Schuman, 2001; Sutton and Schuman, 2005). However, intermediate stages of L-LTP are maintained by post-translational modifications of existing proteins (Kang and Schuman, 1996; Kelleher et al., 2004; Otani et al., 1989). Gp1 mGluRs activation in hippocampal slices primes the induction of LTP (Cohen and Abraham, 1996) and this effect of mGluR5 on LTP requires protein synthesis (Raymond et al., 2000). Moreover, Gp1 mGluR dependent LTD (mGluR-LTD) (Huber et al., 2000; Karachot et al., 2001) requires rapid protein synthesis within minutes and NMDAR dependent form of LTD in hippocampus is underlined by mGluR activation (Oliet et al., 1997). In hippocampal dendrites severed from their cell body there is only the expression of E-LTP (Frey et al., 1988) suggesting that transport of mRNA or protein from the cell body is required to sustain LTP. However, recent studies where induction of L-LTP was examined in isolated hippocampal dendrites suggests that induction of LTP even up to 5hrs does not require trafficking of new proteins or mRNA from the cell body (Vickers et al., 2005; Vickers and Wyllie, 2007). Moreover, endocytosis of AMPAR in synaptoneurosomes seen in response to Gp1 mGluR agonist stimulation is dependent on protein synthesis (Snyder et al., 2001). A host of recent studies also demonstrate that both protein synthesis dependent LTP and mGluR-LTD activate ERK

(extracellular signal-regulated kinase) and PI3 kinase (phophotidylinositol-3 kinase) signalling cascades, which in turn regulate factors involved in protein translation initiation step. Conversely, the maintenance of L-LTP and mGluR-LTD in turn are regulated by translation initiation factors (reviewed in Pfeiffer and Huber, 2006).

Several studies characterise translocation of mRNA into dendrites and trafficking of mRNA along the dendrite is involved in the maintenance of the altered synaptic strength (Roberts et al., 1998; Steward et al., 1998; Sutton and Schuman, 2005). The process of mRNA localisation in dendrites is a complex one and is thought to require mRNA binding proteins. Collectively, mRNA binding proteins are known as hnRNP proteins (heterogeneous nuclear ribonucleoproteins) and these associate with mRNA transcripts and play a regulatory role in their function and fate (Dreyfuss et al., 2002). The mRNA translocation also involves RNA containing granules, translationally inactive messenger ribonucleoprotein particles (mRNPs), stress granules (SGs) and processing bodies (PBs). RNPs contain pre-mRNA and RNAs and their protein components are involved in pre-mRNA processing. RNP is also highly dynamic such that at a given time there are specific dissociations and associations of its protein content (Dreyfuss et al., 2002). SGs aid in reprogramming mRNA metabolism during times of oxidative or metabolic stress, and PBs are thought to mediate mRNA degradation (Bramham and Wells, 2007).

Therefore, the development of synaptic connectivity in the postnatal brain is both a spatially and temporally regulated process that is fine tuned by activity dependent protein synthesis. Dendritic spines are ultrastructural entities that have the cellular machinery and signalling molecules to locally regulate these processes involved in shaping synaptic connectivity.

1.2 Fragile X syndrome (FXS)

Fragile X syndrome (FXS) is an inherited form of MR that affects 1 in every 4000 males and 1 in every 6000-8000 females; therefore, it is the most common form of genetically inherited MR (Crawford et al., 2001; Kooy et al., 2000; Turner et al., 1996). FXS was first described as a MR transmitted in a

X-linked fashion by Martin and Bell in 1943. Lubs in 1969 described FXS as a X-linked disorder containing an unusual secondary constriction on the long arm of the X chromosome, which he termed as the 'marker X' fragile site (cited in Penagarikano et al., 2007). It was later discovered that the gene affected in FXS is the X-linked fragile X mental retardation 1 (*FMR1*) on the long arm of X chromosome at Xq27.3 (Harrison et al., 1983; Verkerk et al., 1991). *FMR1* encodes for the protein fragile X mental retardation protein (FMRP) that is lost in FXS (Ashley et al., 1993a) (Figure 1.3).

FXS in comparison to other X linked disorders has an unusual inheritance pattern referred to as the 'Sherman paradox'. Typically, a X linked recessive disorder is inherited such that female carriers would be asymptomatic while all male carriers would display symptoms of the disorder. However, in FXS, there are reports of affected females and male carriers that are unaffected. 'Sherman paradox' defines the phenomena where the risk of FXS inheritance dependent on the individual's position within the pedigree. Male carriers of FXS (see below) can transmit their alleles to nonpenetrant daughters who then go on to have affected sons while the male carrier's mother have a less of a chance of having an affected offspring suggesting that something has changed on the X chromosome over the two generations (Sherman et al., 1985). This ultimately results in increased penetrance of inheriting the fragile X mutation as the mutant gene is passed along the successive generations (Bassell and Warren, 2008). In the vast majority of cases, FXS is caused by the expansion of the trinucleotide sequence CGG in the 5' untranslated region (UTR) of *FMR1* due to meiotic instability of certain alleles of this repeat sequence (Verkerk et al., 1991). Among normal individuals, the CGG repeat is highly polymorphic in length and content and is less than 54 repeats in size, with an average of about 30 (Fu et al., 1991). FXS full mutation sets in when CGG repeat expands beyond 200 repeats, typically about 800 repeats (Oberle et al., 1991). The full mutation results in heavy methylation of *FMR1* locus with heterochromatic marks leading to transcriptional silencing of the gene possibly through histone deacetylation, although precise mechanisms of this are yet to be deciphered (Coffee et al., 1999; Sutcliffe et al., 1992).

Alleles with intermediate repeat number (55-200 repeats) carry the FXS premutation and are thought to account for the non-penetrant males described in the Sherman paradox (Bassell and Warren, 2008; Maddalena et al., 2001). The phenotype of fragile X premutation (in patients that carry 55-200 repeat numbers) is referred to as fragile X associated tremor/ataxia syndrome (FXTAS) and surprisingly, in individuals with FXTAS enhanced expression of *FMR1* transcripts has been found in blood leukocytes and CNS tissue (Tassone et al., 2007) leading to toxic RNA gain of function (Brouwer et al., 2008). FXTAS was first discovered in grandfathers of children with FXS, that presented both intention tremor and ataxia, and this phenotype is now known to progressively worsen with age and is more commonly found in male carriers (Hagerman et al., 2001; Jacquemont et al., 2004; Jacquemont et al., 2003). Other phenotypes of people with FXTAS include primary ovarian insufficiency in females and adult onset neurodegeneration that is more commonly found in males and to a lesser degree in females (Brouwer et al., 2008; Hagerman and Hagerman, 2007; Hagerman et al., 2004). As the focus of the thesis is on FXS and a detailed description of the literature on FXTAS is worthy of its own review, FXTAS will not be discussed further.

1.2.1 Symptoms of FXS

In physical appearance, people afflicted with FXS show subtle but characteristic facial features such as a long narrow face with a prominent forehead, jaw and ears (Chudley and Hagerman, 1987). Other physical features such as flat feet, hyperextensibe joints, connective tissue disorders and macroorchidism (in males) have also been observed (Penagarikano et al., 2007).

Many children with FXS display intellectual disabilities and varying behavioural deficits. Intellectual disabilities are more profound in boys with FXS with mild to severe range IQ scores observed in almost all boys as young as 3 years old (Skinner et al., 2005), whereas only 25% of girls show an IQ range comparable to that of severely affected boys. Most other female patients show sub-clinical learning deficits that could possibly be explained by the X inactivation status of females leading to mosaicism of FMRP expression thus a heterogeneous FXS phenotype (Cornish et al., 2008).

A well-characterised feature in FXS is heightened responses to sensory stimuli (reviewed in Reynolds and Lane, 2008). Males with FXS display deficits in attention such as restlessness, distractibility, and inattentiveness that worsens with progressive age (Turk, 1998) while in females this is observed to a lesser degree (Cornish et al., 2008). Clinical studies show that their attention is particularly impaired when competing stimuli are presented compared to their ability to maintain focused concentration on one particular stimulus. Moreover, their responses are delayed and they have a greater inability to inhibit task irrelevant behaviour (Munir et al., 2000).

Interestingly, about 15-30% of FXS patients are thought to present characteristics of Autism spectrum disorders (Kelleher and Bear, 2008) while 2-6% of Autistic individuals are likely to be fragile X patients (Bailey et al., 1993; Reddy et al., 2005). Hatton et al., (2006) in a longitudinal clinical study showed that misdiagnosis of fragile X children as having autism increases with age. Commonalities between FXS and autism are clearly evident at a cognitive level in skills involving social interactions and reciprocity (such as eye gaze) (Cornish et al., 2007), however, these characteristics appear to serve different functions in FXS and autism (Cornish et al., 2008). Poor skills in speech are one of the most concordant phenotypes between FXS and autism spectrum disorders, whereas poor social skills are evident in both cohorts, but there are subtle differences for its reasons (Cornish et al., 2008). For example, while patients with autism seem to actively disengage in any social interaction and show lack of social awareness, fragile X children are thought to display what is termed as the 'fragile X handshake'. Their 'handshake' is offered as a social pleasantry or an initial eye contact that is then actively followed by persistent gaze avoidance even in the case of familiarity (Cornish et al., 2008). It is thought that the gaze avoidance is primarily due to social anxiety and hyperarousal as apposed to an inherent wish to avoid social interactions (Cornish et al., 2004; Wolff et al., 1989). In FXS, the loss in the ability to understand beliefs and intentions of other people are thought to be due to deficits in processing information that would influence their working memory performance (reviewed in Cornish and Hagerman, 2008).

In addition to deficits in their sensory processing, in FXS males, hypotonia in infancy, developmental delays in motor skills and deficits in gross motor coordination has been well documented. The deficits in motor processing seem to be directly related to levels of FMRP expressed (reviewed in Kau et al., 2002). Furthermore, about 10-40% patients of FXS are also thought to suffer from epileptic seizures (Kluger et al., 1996; Musumeci et al., 1988) possibly due to increased synaptic excitability.

This is only a brief mention of some of the symptoms of FXS. Collectively, the neurological symptoms of FXS suggest perturbed normal brain processing possibly arising from altered synaptic connectivity that may lead to deficits in higher order cognitive processes.

1.2.2 Gross anatomical and ultrastructural abnormalities in the fragile X brain

Structural magnetic resonance imaging (MRI) studies of FXS patients show the gross anatomy of several brain regions to be altered. Interestingly, brain regions that are altered in FXS are regions that are thought to be the neuroanatomical substrates for the processing of correct cognitive and behavioural responses. Reiss et al., (1988) first characterised hypoplasia of the connecting tissue between the two cerebellar hemispheres known as the cerebellar vermis. The vermis is anatomically connected to limbic structures such as hippocampus and amygdala, and is thought to be involved in execution and regulation of motor behaviour (Rosenthal et al., 1988) and aspects of auditory (Huang and Burkard, 1986), visual saccadic eye movement (Hayakawa et al., 2002) and language processing (Moretti et al., 2002). Therefore, abnormalities of the cerebellar vermis may underlie some of the FXS behavioural anomalies such as hyperactivity, repetitive movements and attention deficits (Hessl et al., 2004). There is also increased volume of hippocampus, a structure known for its importance in learning and memory (Kates et al., 1997; Mazzocco et al., 1993). The amygdala, a structure involved in fear conditioning and both conscious and unconscious emotional processing is also shown to be increased in size in a study by Reiss et al., (1995). Similarly, increase in size is seen in the caudate nucleus of the basal ganglia. Caudate nucleus forms connections with the frontal lobe that

are thought to be important for shifting attention between stimuli and motor planning and execution (Reiss et al., 1995).

Ultrastructural changes in brain autopsies from FXS patients were first characterised by Rudelli et al., (1985) in an autopsy examination of a 62 years old male (Figure 1.4). He found the spines in apical dendrites of neocortex and allocortex (allocortex is regions of cerebral cortex containing fewer cellular layers than neocortex and include olfactory cortex and hippocampus) to be long and tortuous with prominent terminal heads and irregular dilations through a rapid Golgi analysis. Such morphology of spines is reminiscent of morphology of spines found in early development (Marin-Padilla, 1967). Furthermore, EM analysis of synapses indicated decreased PSD length, but postsynaptic compartments were found to form normal junctions with presynaptic terminals (Rudelli et al., 1985). In later autopsy studies this abnormal spine morphology was validated in other brain regions but no change in spine density was observed, however, by their own admission the Golgi analysis was less than optimum due incomplete dendritic impregnation (Hinton et al., 1991; Wisniewski et al., 1991). In a later study, Irwin et al., (2001) reported increased density of spines with an immature morphology similar to that characterised by Rudelli et al., (1985) in pyramidal cells from temporal and visual cortices in brain autopsies from fragile X patients. On the basis of the fact that immature spines are often associated with either development or sensory deprivation, it was hypothesised that this spine morphology in FXS reflects either a developmental delay or defects in synapse/dendritic pruning (Bagni and Greenough, 2005; Irwin et al., 2001).

1.2.3 Experimental models of FXS

The generation of experimental models of FXS has extended the characterisation of FXS and its perturbed synaptic connectivity. Most importantly, it has opened the door to both *in vivo* and *in vitro* examination of the effects of loss of FMRP. In particular the study of cellular mechanisms that are dysregulated in FXS, which in turn will elucidate the role of FMRP in regulating the development of synaptic connectivity.

1.2.3.1 The drosophila model of FXS

In *drosophila*, the *FMR1* gene homolog is known as *dfmr1* and the encoded protein is known as dFMRP. There is 56% overall DNA sequence similarity with the *FMR1* gene and 36% amino acid identity between the functional domains of *drosophila* and mammalian FMRP (Gao, 2002; Zarnescu et al., 2005)(refer to 1.2.4.1). There have been several loss-of-function mutations varying from weak hypomorphs to nulls generated to determine the anatomical and physiological functions of dFMRP (Dockendorff et al., 2002; Inoue et al., 2002; Lee et al., 2003; Morales et al., 2002; Zhang et al., 2001). Loss of dFMRP results in altered circadian rhythms, erratic locomotor activity that parallels behavioural patterns observed in the FXS, and also diminished courtship behaviour (Dockendorff et al., 2002), which is an inherent set of behaviours in *drosophila* that leads to their copulation. *Drosophila* null mutant neurons show exaggerated dendritic elaboration, axonal branching and abnormal synaptic formation (Pan et al., 2004), thus correlating well with the altered cortical connectivity described in FXS. This is only a brief summary of some of the findings from the *drosophila* model of FXS, and throughout the thesis where appropriate discoveries from this model will be mentioned.

1.2.3.2 The 'conventional' FXS mouse model

The most commonly studied experimental FXS model is the 'conventional' *Fmr1* mutant mouse model that is also examined in this thesis. The *FMR1* gene is highly conserved among species (Verkerk et al., 1991) and in terms of amino acid sequence homology, the murine homologue FMRP is 97% similar to its human ortholog, FMRP (Ashley et al., 1993b). The FXS mouse model that is extensively studied is known as the 'conventional' *Fmr1* mutant. It was generated by homologous recombination where *Fmr1* was selectively knocked out by interrupting exon 5 with the positive selection marker gene *neomycin* (Dutch-Belgian fragile X consortium, 1994). The conventional mouse model does not recapitulate the trinucleotide repeat expansion evident in the human disorder; nonetheless, it does cause loss of FMRP. However, the conventional mouse model of FXS is inadequate to study how the CGG repeat expansion results in FXS.

Physically, one of the consistent findings in *Fmr1*$^{-/y}$ mice is macroorchidism that is found in about 90% of FXS males (Dutch-Belgian fragile X consortium, 1994). However, no gross neuroanatomical alterations have been observed in *Fmr1*$^{-/y}$ mice (Kooy, 2003; Kooy et al., 1999).

Behavioural deficits in *Fmr1*$^{-/y}$ mice

The behavioural deficits observed in the *Fmr1*$^{-/y}$ mice are moderate in comparison to the human FXS (reviewed in Bernardet and Crusio, 2006), and the attempts of finding a robust behavioural phenotype seem to be confounded by strain variability due to the effects of modifier genes (Errijgers et al., 2008; Errijgers and Kooy, 2004). These mice typically show increased exploratory and locomotor activities (Dutch-Belgian fragile X consortium, 1994). These deficits may result from their hyperactivity or might simply reflect slowness in learning about their environment (Dutch-Belgian fragile X consortium, 1994) and is consistent with attention deficit and hyperactivity behaviour found in patients with FXS (Cornish et al., 2004). One of the most robust behavioural phenotypes in *Fmr1*$^{-/y}$ mice is reduced habituation in an open field (Kooy, 2003) that agrees well with the anxiety observed in FXS patients in novel social environments (Hagerman, 2002). The other reproducible behavioural phenotypes is increased susceptibility to audiogenic seizures (Kooy, 2003) consistent with epilepsy reported to occur in 20-25% of individuals with FXS (Hagerman, 2002; Sabaratnam et al., 2001). *Fmr1*$^{-/y}$ mice also display modest deficits in spatial leaning and some motor deficits in finding the hidden platform in Morris water maze (D'Hooge et al., 1997). In a recent study *Fmr1*$^{-/y}$ mice were shown to have exaggerated responses to inhibitory avoidance extinction. It is a test that examines retrieval of memory associated with a fear motivated learning task that is thought to involve the hippocampus and is protein synthesis dependent (Dolen et al., 2007). Altered responses in *Fmr1*$^{-/y}$ mice in inhibitory avoidance extinction (Dolen et al., 2007) and stronger effects elicited in *Fmr1*$^{-/y}$ mice to auditory stimuli (Chen and Toth, 2001) recapitulate the heightened sensory responses observed in FXS patients (Cornish et al., 2008; Hagerman, 2002).

Alteration in *Fmr1*$^{-/y}$ mice at a cellular level

At a cellular level, defects in neocortical differentiation into glutamatergic cell lineages, that are associated with increased layer 5 pyramidal cells in the cortex of postnatal *Fmr1*$^{-/y}$ mice have been shown compared to *Fmr1*$^{+/y}$ mice (Tervonen et al., 2008). Conversely, densities of paravalbumin positive inhibitory neurons in the neocortex are reduced in adult *Fmr1*$^{-/y}$ mice through all cortical layers (Selby et al., 2007). $GABA_A$ (gamma-aminobutyric $acid_A$) receptors provide the main inhibitory drive in the brain and are present along proximal dendrites of glutamatergic neurons, often near spine necks to allow inhibitory regulation of the excitatory response (Jacob et al., 2008). Several subunits of $GABA_A$ receptor are reduced in *Fmr1*$^{-/y}$ cortex compared to *Fmr1*$^{+/y}$ mice (D'Hulst et al., 2006). The number of GABAgeric synapses is also decreased in the striatum of adult *Fmr1*$^{-/y}$ mice (Centonze et al., 2008). Centonze et al., (2008) found increased frequency of both spontaneous and miniature GABA- mediated IPSCs (inhibitory postsynaptic currents) in the striatum of *Fmr1*$^{-/y}$ mice without any changes in the amplitude of responses suggesting that in *Fmr1*$^{-/y}$ mice there is increased transmitter release probability in GABAgeric neurons compared to *Fmr1*$^{+/y}$ mice (Centonze et al., 2008). Hence, loss of FMRP results in alterations in both excitatory and inhibitory cortical connectivity.

Defects in spinogenesis in *Fmr1*$^{-/y}$ mice

Ultrastructurally, in agreement with FXS autopsy findings a plethora of both *in vivo* (Comery et al., 1997; Dolen et al., 2007; Galvez and Greenough, 2005; Grossman et al., 2006b; Hayashi et al., 2007; Irwin et al., 2002; McKinney et al., 2005) and *in vitro* (de Vrij et al., 2008) studies of the *Fmr1*$^{-/y}$ mice have shown increased spine density of an immature morphology with long, thin and tortuous spines in various brain regions such as cortex and hippocampus. However, spine analysis carried out in *Fmr1*$^{-/y}$ mice in various brain regions and ages appears to suggest that FMRP may regulate spinogenesis in both region and age specific manner (refer to table 1.1).

Neocortical studies report an increased density of immature spines. Visualising neurons by using a sindbis virus to transfect neurons with

enhanced green fluorescent protein (EGFP) gene, Nimchinsky et al., (2001) found that cortical layer 5 pyramidal neurons from *Fmr1*$^{-/y}$ mice have increased density of immature spines compared to *Fmr1*$^{+/y}$ mice. This spine phenotype is also developmentally regulated with most profound spine phenotype evident during the first two postnatal weeks suggesting a developmental role for FMRP in regulating synaptogenesis. The loss of spine phenotype at the third postnatal week (the latest they examined) in *Fmr1*$^{-/y}$ mice (Nimchinsky et al., 2001) was incongruent with previous findings in the neocortex that showed increased spine density in the adult *Fmr1*$^{-/y}$ mice (Comery et al., 1997; Irwin et al., 2002). Galvez and Greenough (2005) in a Golgi study of layer 5 pyramidal cells in the same cortical area as Nimchinsky et al., (2001) show that spines in *Fmr1*$^{-/y}$ mice have an increased immature appearance at 4th postnatal week and in adult mice compared to *Fmr1*$^{+/y}$ mice suggesting that spine defects due to FMRP might be developmentally regulated and transient in their manifestation. However, technical differences in the two studies preclude any direct comparisons. In the cerebellar Purkinje cells, spines are immature in appearance with elongated necks and heads but their density is normal (Koekkoek et al., 2005). Hippocampal culture studies show decreased spine density with fewer functional synapses (Braun and Segal, 2000 but see de Vrij et al., 2008) while Golgi analysis of hippocampal CA1 pyramidal cells show normal spine density but an immature appearance (Grossman et al., 2006b).

These variations seen in the spine phenotype of *Fmr1*$^{-/y}$ mice may simply point to FMRP playing a slightly different role in different cell types, especially given the fact that different cell types have different sensitive periods and hence their activity dependent demands will differ (Yuste and Bonhoeffer, 2004). Nonetheless FMRP does play a regulatory role in spinogenesis during development, but the precise pathways in which FMRP regulates spinogenesis may differ depending on the developmental age, brain region and/or neuronal cell type. Moreover, both behavioural and neuroanatomical defects in *Fmr1*$^{-/y}$ mice indicate a regulatory role for FMRP not only in spinogenesis but also in orchestrating correct synaptic connectivity. How does FMRP mediate its effects?

1.2.4 FMRP

FMRP was first characterised as a protein containing RNA binding domains within its structure, and to also have a high affinity for its own transcripts (Ashely et al., 1993; Siomi et al., 1993). FMRP is reported to bind to 4% of human foetal mRNA and the absence of FMRP association with its RNA was hypothesised to be responsible for the 'pleiotropic phenotype' associated with FXS (Ashley et al., 1993). Since then FMRP is known as one of the hnRNP family members, thereby regulating mRNA localisation and metabolism (Bagni and Greenough, 2005).

FMRP mostly exists as a 82kDa protein, but alternative splicing of *FMR1* does result in several isoforms (Ashley et al., 1993b), and in mammals it is one of three paralogous proteins that also include FXR1 and FXR2. FMRP is expressed extensively in several tissues and is abundant in the brain (Bassell and Warren, 2008). Ultrastructural studies from adult brain show FMRP to be in neurons but not in glia. In neurons, its expression is abundant in the perikaraya and in regions enriched with ribosomes such as near or between rough endoplasmic reticulum. In the adult, FMRP expression is localised to both the neuronal neucleoplasm, within nuclear pores, along dendrites and in spines (Feng et al., 1997b).

FMRP has also been shown to be present along the dendrite in 'RNA granules' that are thought to be translationally arrested complexes of ribosomes, RNA binding proteins and RNA. FMRP associated with RNA granules are thought to be trafficked along the dendrite via microtubule interactions (Figure 1.5) (Antar et al., 2005; Kanai et al., 2004). Moreover, FMRP is shown to associate with either actively translating polyribosomes (Khandjian et al., 2004; Stefani et al., 2004) or translatingly inactive mRNPs (Brown et al., 2001; Feng et al., 1997a; Zalfa et al., 2003) or both depending on the translational state of the cell (Vasudevan and Steitz, 2007; Wang et al., 2008).

1.2.4.1 Structural domains of FMRP and mRNA target recognition

In its structure, FMRP has both a nuclear localisation (NLS) and a N terminus nuclear exporter signal (NES) (Bardoni et al., 1997; Eberhart et al., 1996;

Fridell et al., 1996; Sittler et al., 1996) suggesting that it shuttles between the nucleus and cytoplasm with its RNA cargo (Feng et al., 1997b; Tamanini et al., 1999). It also contains two KH domains and a RGG box that are characteristic RNA binding motifs among hnRNP family members (Figure 1.3) (Ashley et al., 1993a). The N terminus of FMRP also contains a loop-helix-Tudor domain that mediates its protein-protein interactions (Maurer-Stroh et al., 2003; Ramos et al., 2006). In normal individuals, nascent FMRP self-dimerises in the cytoplasm and then translocates into the nucleus where it binds to its target mRNA. FMRP with its bound mRNA cargo then exits the nucleus and is trafficked as mRNPs and/or polyribosomes to localise along the dendrite, thereby regulating local protein synthesis (Jin and Warren, 2000).

KH domains

Although the most prevalent aetiology of FXS is a trinucleotide expansion, in a rare case a patient was found to have a missense mutation (I304N) in the second KH domain (KH2) resulting in a severe FXS phenotype (De Boulle et al., 1993). This suggests that KH domains are of critical importance in protein interactions of FMRP. The mutation lies in the hydrophobic core of the KH2 domain, results in decreased stability of KH1-KH2 domains (Lewis et al., 2000; Valverde et al., 2007), and is thought to (Feng et al., 1997a; Siomi et al., 1994) alter RNA binding activity of FMRP. Adinolfi et al., (1999) in a molecular dissection of the different FMRP domains suggested that mainly the first KH domain (KH1) facilitates FMRP mRNA interactions, while KH2 may provide some contribution to the KH1-KH2 interactions. In agreement, with this prediction Brown et al., (1998) show that while I304N mutation of FMRP disrupts the structure of KH domains, it can still bind RNA. However, an *in vitro* selected RNA structure referred to as the 'kissing complex', which is a sequence specific element within a complex tertiary structure has been shown to bind to KH2 domain of FMRP with the capability of competing off FMRP from polyribosomes (Darnell et al., 2005). Interestingly, I304N mutation prevents FMRP to self-dimerise (Feng et al., 1997a), increases shuttling between the nucleus and the cytoplasm (Tamanini et al., 1999 but see Castren et al., 2001) and inhibit translation of some mRNAs and formation of the 80s initiation complex (40s and 60s ribosomal subunits

assembled together with the translation initiation machinery) (Laggerbauer et al., 2001; Li et al., 2001).

RGG Box

The RGG box is thought to strengthen RNA interactions (reviewed in Zalfa and Bagni, 2004). An intramolecular G quartet is the predominant RNA motif that is recognised by FMRP and G quartet interactions are facilitated by the RGG box (Brown et al., 2001; Darnell et al., 2001; Schaeffer et al., 2001). In a G quartet, four guanines are arranged in a square planar arrangement through hydrogen bonding with each other, and G quartet containing RNAs encode for proteins that have been shown to play a role in neuronal development, synaptic function and neuronal maturation (reviewed in Darnell et al., 2004). Interactions between G quartets of mRNA encoding FMRP, MAP1b (microtubule associated protein 1b) and Sema3F (semaphorin 3F) have been validated biophysically (Menon et al., 2008; Menon and Mihailescu, 2007; Schaeffer et al., 2001), and *Map1b* has been coimmunoprecipitated with FMRP (Lu et al., 2004; Zalfa et al., 2003) in the mouse brain as well as *App* (amyloid precursor protein mRNA) (Westmark and Malter, 2007). Furthermore, Zalfa et al., (2007) show direct binding of 3' UTR of *Psd95* that has a G quartet.

In addition to the extensively studied FMRP recognition motifs in mRNA, FMRP has been shown to bind to mRNA containing poly U stretches (Chen et al., 2003). FMRP also appears to interact with the small dendritic non-coding RNA, BC1, thereby potentially forming translation inhibitory complexes by binding to specific mRNAs targeted by BC1 (Zalfa et al., 2003). However, in another study Iacoangeli et al., (2008) observed no direct interactions between FMRP and BC1, and interactions between BC1 and FMRP target mRNA such as MAP1B and CAMKIIα to be non-specific in both *in vivo* and *in vitro* biochemical assays (Iacoangeli et al., 2008). These two differing views on FMRP and BC1 interactions are not mutually exclusive but it is possible that FMRP-BC1 association may depend on specific physiological conditions. FMRP has also been shown to interact with miRNA (microRNA) and miRNA pathway components, Dicer and AGO1 (mammalian ortholog of Argonaute 1) (Jin et al., 2004).

1.2.5 FMRP in dendritic mRNA localisation and expression

The structural binding affinity of FMRP for mRNA, its association with actively translating polyribosomes, and its expression not only in dendrites but also in dendritic spines have led to the speculation that FMRP may play a role in regulating mRNA localisation and expression (Bagni and Greenough, 2005; Bassell and Warren, 2008; Grossman et al., 2006a).

Several studies describe associations between FMRP and dendritic mRNA using various techniques. Fluorescence in situ hybridisation (FISH) *in vitro* has shown colocalisation of FMRP with *Map1b* in dendrites (Antar et al., 2005). In brain extracts, FMRP coimmunoprecipitates with mRNAs encoding both αCAMKII and the activity dependent immediate early gene Arc/Arg3.1 (activity-regulated cytoskeletal-associated protein) (Zalfa et al., 2003), and both these mRNAs are translated at synapses (reviewed in Bramham and Wells, 2007). Furthermore, PSD95 is shown to be dendritically localised (Muddashetty et al., 2007; Zalfa et al., 2007).

Consistent with evidence for associations between FMRP and its target mRNA in dendrites, a microarray study carried out in fragile X cell lines identified 113 FMRP associated mRNAs to be downregulated, but their associations with polysomes were unaltered (Brown et al., 2001). Another study identifying FMRP target mRNA using *in vitro* association assays found altered expression of some mRNA, in particular mRNA for Glucocorticoid receptor α (Miyashiro et al., 2003), and in FXS increased levels of circulating corticosteroids (Hagerman and Sobesky, 1989; Hessl et al., 2002) and abnormal expression of glucocorticoid modulating proteins (Sun et al., 2001) has also been characterised. In contrast, several studies have found that loss of FMRP does not alter basal levels of its target mRNA in dendrites (Bassell and Warren, 2008). Steward et al., (1998a) show that the expression of dendritic mRNAs for MAP2 and CAMKII as well as seizure-induced expression of Arc were all normal in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice. Quantitative PCR analysis shows no changes in basal levels of mRNA encoding PSD95 or αCAMKII, and no changes in their dendritic expression patterns in the hippocampus and cortex in the absence of FMRP (Muddashetty et al., 2007). However, Zalfa et al., (2007) demonstrate that

FMRP regulates *Psd95* stability such that in *Fmr1*$^{-/y}$ mice, total *Psd95* is decreased in the hippocampus but not in the cortex. The expression pattern of *Psd95* is also altered moderately in the molecular layers of the hippocampus (Zalfa et al., 2007). Whether FMRP regulates 1) the constitutive activity of other mRNAs and 2) subsets of mRNA in a region specific manner and 3) local mRNA expression by stabilising their expression are all open questions requiring further investigation (Bassell and Warren, 2008).

1.2.6 Interactions between Gp1 mGluRs and FMRP

Stimulation of Gp1 mGluRs has been shown to result in the synthesis of FMRP in synaptoneurosomes (Weiler et al., 1997). In hippocampal cultures, both FMRP and *Fmr1* are translocated to dendrites in a Gp1 mGluR activity-dependent manner. The Gp1 mGluR subtype, mGluR5 is thought to be particularly important in this process (Antar et al., 2004). Moreover, *in vitro* stimulation of hippocampal neurons with agonists of Gp1 mGluR results in the movement of FMRP-associated mRNPs to sites of synapses in a protein synthesis regulated manner (Ferrari et al., 2007). In turn, the chemically induced mGluR-LTD in the hippocampus (mGluR-LTD) with Gp1 mGluR agonist DHPG (S-3,5-Dihydroxyphenylglycine) requires rapid translation of pre-existing mRNA (Huber et al., 2000) and protein synthesis dependent internalisation of GluR1 (Snyder et al., 2001). In addition, hippocampal mGluR-LTD is irreversible (Oliet et al., 1997) suggesting that this form of LTD could be prelude to synapse elimination (Snyder et al., 2001). Because the synaptic expression of FMRP is enhanced with Gp1 mGluR activation and the fact that mGluR-LTD expression is dependent on protein synthesis, Huber et al., (2002) explored mGluR-LTD in *Fmr1*$^{-/y}$ mice. In contrast to their anticipated reduction in LTD expression, they found exaggerated mGluR-LTD in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Huber et al., 2002). This surprise finding taken together with a plethora of other effects of Gp1 mGluR dependent protein synthesis that appeared to coincide with several FXS phenotypes (refer to table 1.2) led Bear et al., (2004) to postulate 'The mGluR theory of fragile X mental retardation'. The theory speculates that loss of FMRP results in exaggerated effects of Gp1 mGluR dependent protein synthesis due to aberrant Gp1 mGluR signalling (Bear et al., 2004).

Moreover, FMRP can act as both a translational repressor and an activator, in so doing maintain physiologically stable levels of Gp1 mGluR dependent protein synthesis (Figure 1.6) (Bear et al., 2004).

FMRP as a translational repressor

There are several lines of evidence to suggest the role of FMRP as a translational repressor. Mammalian FMRP has been shown to inhibit mRNA translation in rabbit reticulocyte lysates (Li et al., 2001) and in microinjected *Xenopus* oocytes (Laggerbauer et al., 2001). In *drosophila*, dFMRP binds to *drosophila* homolog of MAP1B mRNA, *futsch*, and negatively regulates its expression (Zhang et al., 2001). dFMRP is also thought suppress translation through its associations with miRNA and miRNA signalling pathway (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004). Consistent with a role of FMRP as a translational repressor, there are increased levels of MAP1b, αCAMKII and Arc in synaptoneurosomes from *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice. Furthermore, the association of mRNA for these proteins with actively translating polyribosomes are increased in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Zalfa et al., 2003).

FMRP as an activator

FMRP is thought to act as a translational activator with regards to some proteins because Gp1 mGluR dependent *de novo* synthesis of PSD95 (Todd et al., 2003) and APP (Westmark and Malter, 2007) fail to occur in the absence of FMRP. A recent study by Muddashetty et al., (2007) suggests that loss of FMRP may result in excess basal translation of specific mRNAs while at the same time their *de novo* translation in response to Gp1 mGluR activation is dysregulated. In *Fmr1*$^{+/y}$ mice synaptoneurosomes, mRNA encoding PSD95 and αCAMKII immunoprecipitates with FMRP and show increased translation in response to Gp1 mGluR stimulation whereas in synaptoneurosomes from *Fmr1*$^{-/y}$ mice this Gp1 mGluR activity dependent increase is absent. Moreover, Gp1 mGluR stimulation of *Fmr1*$^{+/y}$ mice synaptoneurosomes results in the association of *Psd95* and *αCamkII* with actively translating polyribosomes while in synaptoneurosomes from *Fmr1*$^{-/y}$ mice the activity dependent recruitment of these mRNA into polyribosomes is

dysregulated. Interestingly, these mRNA were shown to be elevated in polyribosomes at basal states in *Fmr1*$^{-/y}$ mice similar to the observed increases in *Fmr1*$^{+/y}$ mice upon stimulation suggesting defects in mRNA translation (Muddashetty et al., 2007).

Phosphorylation status of FMRP is important in determining whether it acts as an activator or a repressor

The phosphorylation status of FMRP and its recruitment from translationally inactive mRNP or RNA granules to polyribosomes may determine whether it acts as a repressor or an activator (Bassell and Warren, 2008; Ronesi and Huber, 2008). Following Gp1 mGluR stimulation, PP2A (protein phsophatase 2) becomes active and rapidly dephosphorylates FMRP allowing mRNA translation to takes place. However, sustained activity of Gp1 mGluRs results in mTOR (mammalian target of rapamycin) mediated suppression of PP2A via a homer cascade leading to rephosphorylation of FMRP (Narayanan et al., 2007) allowing FMRP to act as a brake on otherwise exaggerated mRNA translation due to Gp1 mGluR activation. Concomitantly, mTOR can activate translation of mRNA independent of FMRP regulatory effects resulting in sustained maintenance of protein synthesis (Bassell and Warren, 2008). A recent study has also characterised the ribosomal protein S6 kinase (S6K1) as a potential kinase that phosphorylates FMRP in an activity dependent manner, and this phosphorylation step also appears to require signalling via the PI3K-mTOR pathway (Narayanan et al., 2008).

At synaptic sites the regulation of FMRP by Gp1 mGluR signalling appears to be complex such that in addition to regulating the translation of FMRP bound mRNA, Gp1 mGluRs also cause rapid degradation and ubiquitination of FMRP resulting in a net decrease in levels of synaptic FMRP (Hou et al., 2006). It is thought that Gp1 mGluR activity induced loss of FMRP may de-repress FMRP to allow changes in mRNA translation (Ronesi and Huber, 2008), which in turn would allow modifications of synapses in response to synaptic activity.

Altered global protein synthesis in *Fmr1*$^{-/y}$ mice

There is also an emerging view of altered basal rate of proteins expression in the absence of FMRP. Utilising a quantitative autoradiographic method, regionally selective elevations in *in vivo* basal cerebral protein synthesis has been demonstrated in adult *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Qin et al., 2005). Dolen et al., (2007) report increased basal rates of protein synthesis in *in vitro* hippocampal slices from *Fmr1*$^{-/y}$ mice relative to levels in *Fmr1*$^{+/y}$ mice. A recent quantitative proteomic analysis of protein expression in *Fmr1*$^{-/y}$ cortical cultures shows both up and down regulation of proteins involved in regulating neurotransmission, synaptic structure and dendritic mRNA transport compared to *Fmr1*$^{+/y}$ mice (Liao et al., 2008).

Defects in synaptic plasticity in *Fmr1*$^{-/y}$ mice

There is exaggerated Gp1 mGluR-LTD in *Fmr1*$^{-/y}$ mice in both hippocampus and cerebellum (Huber et al., 2000; Koekkoek et al., 2005). Moreover, this enhanced LTD persists even in the presence of protein synthesis inhibitors (Hou et al., 2006; Nosyreva and Huber, 2006) suggesting that new protein synthesis in not required probably due to the fact that basal protein synthesis is altered in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice. Decreased stability of *Psd*95 (Zalfa et al., 2007) or dysregulated translation of *Psd95* (Muddashetty et al., 2007) in *Fmr1*$^{-/y}$ mice may also results in persistent internalisation of AMPAR from the PSD as PSD95 has been shown to regulate levels of AMPARs at synaptic sites (Colledge et al., 2003; Ronesi and Huber, 2008; Xu et al., 2008).

Arc/Arg3.1 has also been shown to mediate AMPAR endocytosis via its interactions with dynamin and endophilin (Castillo et al., 2008). The *in vitro* stimulation of hippocampal neurons with Gp1 mGluR agonists results in increased synthesis of Arc, and in *Arc*$^{/-}$ mice there is decreased mGluR-LTD (Park et al., 2008). In agreement with a role for Arc in mGluR-LTD, in severed dendrites there is increased synthesis of Arc in response to Gp1 mGluR stimulation, and AMPAR internalisation during mGluR-LTD is blocked by inhibition of Arc synthesis (Waung et al., 2008). Interestingly, *Arc* is also known to associate with FMRP (Zalfa et al., 2003) and in *Fmr1*$^{-/y}$ mice rapid

induction of mGluR dependent *Arc* is impaired (Zalfa et al., 2003 *but see* Park et al., 2008). In the hippocampus, DHPG induced mGluR-LTD is impaired in *Fmr1*$^{-/y}$/*Arc*$^{-/-}$ mice compared to *Fmr1*$^{-/y}$ mice and *Fmr1*$^{+/y}$ mice suggesting that FMRP regulate Arc expression in dendrites that is required to maintain the mGluR-LTD expression (Park et al., 2008). In brief, other candidate proteins of interest that may facilitate the internalisation of surface AMPARs leading to exaggerated LTD observed in *Fmr1*$^{-/y}$ mice are the FMRP targets APP (Westmark and Malter, 2007) and MAP1B (Lu et al., 2004; Menon et al., 2008; Wei et al., 2007). The beta amyloid peptide generated by APP has been shown to mediate endocytosis of AMPARs in hippocampal cultures (Hsieh et al., 2006). In hippocampal cultures, DHPG stimulation results in increased MAP1B expression and increased associations between MAP1B and GRIP1, which is a scaffolding protein that stabilise levels of GluR2 at the synapse. Moreover, siRNA knockdown of MAP1B specifically blocks DHPG stimulated AMPAR internalisation (Davidkova and Carroll, 2007).

In addition to abnormal mGluR-LTD, mGluR5-dependent LTP expression in layer 5 of visual cortex of P13-P25 *Fmr1*$^{-/y}$ mice has been shown to be severely attenuated compared *Fmr1*$^{+/y}$ mice, and this defect seems to be primarily mediated by mGluR5 (Wilson and Cox, 2007). Levels of GluR1 are decreased in cerebral cortex homogenates and synaptic plasma membrane fractions from adult *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice expression, and there is also reduced LTP response in cortical layer 4/5 (Li et al., 2002). In addition to the predominantly reported and characterised view of FMRP associations with Gp1 mGluRs, light exposure dependent increases in FMRP in rat visual cortex have been shown to be blocked by NMDAR antagonists alluding to NMDAR dependent modulation of FMRP as well (Gabel et al., 2004). Moreover, Desai et al., (2006) show NMDAR-dependent spike time dependent plasticity (STDP), in which the temporal order of pre and post synaptic spikes determine whether a synapse is potentiated or depressed, to be impaired in the absence of FMRP. Moreover, in early postnatal (P10-P18) neocortical synapses, loss of FMRP was shown to result in robust LTD but absent LTP (Desai et al., 2006).

In summary, regulated protein synthesis is crucial in converting labile synaptic modifications into permanent changes that may be reflected by

morphological alterations at the synaptic sites (Bear et al., 2008). Loss of FMRP causes dysregulated protein synthesis and altered synaptic plasticity, in several brain regions that may underlie the phenotypes of FXS. However, the precise nature of these defects may be cell type and age specific. Albeit, FXS is a developmental disorder that is associated with aberrant protein synthesis and altered synaptic connectivity, most studies to date have examined effects of loss of FMRP in the adult brain. One could hypothesise that FMRP may play a pivotal role in regulating these cellular processes during early cortical development. Characterisation of roles of FMRP during early development would be beneficial in determining when these defects arise and whether early intervention in FXS may have greater therapeutic benefits. The mouse S1 is an excellent model to characterise the regulatory role of FMRP in the development of synaptic connections, as the anatomical and functional organisation of S1 is a tightly regulated development process as described below.

1.3 The mouse primary somatosensory cortex (S1)

Based on verbal responses to sensations experienced by electrical stimulation of the postcentral gyrus from patients who were undergoing brain surgery, Penfield and Bouldrey (1937) proposed an illustration of a cerebral cortical map where the areas receiving sensory input were mapped corresponding to the magnitude of the sensory responses elicited (cited in Schweizer et al., 2008). This sensory map of the somatosensory cortex is known as the 'sensory homunculus' and it divides the surface of our cerebral cortex proportionate to the sensory input received from our sensory modalities, hence sensory homunculus (little man) has disproportionately large lips and hands (Figure 1.7A). Moreover, the somatosensory cortical map is not static but incredibly dynamic such that it is altered by the sensory information we perceive throughout our development. For example, in string instrument players, the cortical area representing their left hand has been shown to increase in proportion to the length of time they spent practising during a period ranging from 5-20 years (Elbert et al., 1995; Johnston, 2004).

In a manner analogous to our use of fingers in somatosensory discrimination, rodents use their whiskers for explorative behaviour and spatial navigation. Their exploratory movements are designed to acquire sensory input that is essential for discriminating objects and refining their motor behaviour (Ferezou et al., 2007). Therefore, almost a third of the mouse neocortex is represented by the primary somatosensory cortex (S1), and a large proportion of this, the 'barrel cortex' is devoted to the processing of sensory information from their whiskers (Figure 1.7B). S1 of mice has a somatotopic columnar organisation (Schubert et al., 2007). Cortical columnar organisation was first characterised by Mountcastle (1957) who demonstrated that neurons in each layer in a dorsoventral column lineage have a similar property of place and similar responsiveness to a sensory stimulus (Mountcastle, 1997). In the barrel cortex, a cortical column contains about 10,000-20,000 neurons distributed along cortical layers 1-6 (Keller and Carlson, 1999; Lubke and Feldmeyer, 2007). In layer 4 of barrel cortex, the well characterised cytoarchitectonic units known as 'barrels' provide an excellent model to study cellular and molecular mechanisms of cortical organisation (Erzurumlu and Kind, 2001). Barrels replicate patterned array of whiskers and sinus hair follicles on contralateral snout in layer 4 of S1 (Figure 1.8) (Woolsey and Van der Loos, 1970) and each barrel is associated with a functional barrel column via cortical connections (Lubke and Feldmeyer, 2007). Moreover, the formation of barrels is a tightly regulated developmental process that depends on glutamatergic signalling (Barnett et al., 2006a; Erzurumlu and Kind, 2001).

1.3.1 Formation of whisker related patterns along the trigeminal pathway

1.3.1.1 The arrangement of whiskers on the rodent snout

The large mystacial vibrissae (or large whiskers) of rodent are arranged in five roughly horizontal arched rows along the whisker pad. In addition there is also an array of small whiskers positioned rostral to the main large whiskers (Snow and Wilson, 1991). The large whisker rows are labelled A, B, C, D and E in a dorsal to ventral order, with row A and B containing fewer

large whiskers (4) than the other (5-7). There are also four dorsoventrally oriented whiskers known as straddlers (guard whiskers), named alpha, beta, gamma and delta, in dorsal to ventral order proximal to the main rows (Diamond et al., 2008; Snow and Wilson, 1991). The whiskers are tactile sensors whose angular position is controlled by the follicles in the mystacial pad. The infraorbital nerve (ION) derived from the maxillary branch of the trigeminal ganglion provides sensory innervation to the whisker follicles (Zucker and Welker, 1969). Approximately 200-300 axons innervate each large mystacial vibrissa (Diamond et al., 2003), while fewer number of axons (about 50) innervate each small whisker (Li et al., 1995). Electrophysiological recordings from single trigeminal ganglion cells (TGCs) have shown that each afferent fibre innervates only a single large mystacial vibrissa (Zucker and Welker, 1969).

1.3.1.2 The whisker related pattern generated at the brainstem and the thalamus relay stations

The 'barrelette' pattern

TGCs project centrally and extend collaterals to nuclei at the caudal brainstem trigeminal complex (BSTC). TGCs respond to deflections of whiskers by firing actions potentials that cause the release of glutamate at brainstem synapses. The excitatory input is received by trigeminal nuclei nucleus principal (nVp), nuclei interpolaris (nVi) and caudalis (nVc) (Hayashi, 1980). At each nucleus, both the axons and cells aggregate to recapitulate the whisker related pattern, and is referred to as 'barrelettes' (Figure 1.8) (Bates and Killackey, 1985; Ma, 1993; Ma and Woolsey, 1984). The clustering of axons into this whisker-related pattern is evident by the end of P0 (postnatal day 0) with cytochorome oxidase (CO) staining that is utilised to reveal areas of high metabolic activity, while thionin staining reveal cellular aggregation into barrelettes by the end of P1 (Ma, 1993).

The 'barreloid' pattern

The ventroposterior medial nucleus (VpM) of the thalamus receive whisker related sensory input predominantly from the contralateral nVp and nVi that

results in both the axonal and cellular aggregation to recapture the whisker related pattern (Van der Loos, 1976). The whisker related pattern at the VpM is referred to as 'barreloids' (Figure 1.8) (Van der Loos, 1976), and CO reveals axonal segregation into barreloids at P3 (Yamakado, 1985). At P5, the dendrites of VpM orient towards the incoming trigeminal axons, thereby forming dense plexus of synapses centrally within each barreloid, but this restricted dendritic plexus becomes diffused with age and by P18, dendrites extend towards adjacent barreloids (Brown et al., 1995; Zantua et al., 1996).

1.3.1.3 The whisker related pattern generated at the cortical layer 4

The whisker pattern in layer 4 of the cortex is referred to as 'barrels' (Figure 1.8), and barrel formation is a sequential process that involves three key steps.

The clustering of thalamocortical afferents (TCAs)

The thalamocortical axons (TCAs) from VpM project to the layer 4 of cortex with weak innervation to layer 6 (Aronoff and Petersen, 2008; Petersen, 2007; Rebsam et al., 2002). TCAs enter the cortex by P0 (Agmon et al., 1993), and at P1, TCAs are uniformly distributed as a tangential plexus of fibres that overlap over more than one barrel width within a row (Rebsam et al., 2002), however, TCAs do not seem to overlap over different arcs (Killackey, 1973). The extended TCA arbours appear to be progressively retracted into whisker related patterns, and at P7 a dense plexus of TCA arbours are found almost all exclusively within a single patch corresponding to a single whisker (Killackey, 1973; Rebsam et al., 2002).

The formation of layer 4 cellular aggregates around a TCA patch

Concomitant with the clustering of TCAs, layer 4 cells also aggregate to form clusters corresponding to the whisker related pattern (Woolsey and Van der Loos, 1970). Layer 4 cells at P3 are uniformly distributed, but by P7 cells aggregate around a TCA patch forming a cell dense 'barrel wall', thus leaving a cell sparse 'barrel hollow' where layer 4 cell dendrites synapse onto TCAs

(Woolsey and Van der Loos, 1970). The segregation of layer 4 cells appears most distinct between P10-P14 (Barnett et al., 2006a).

The areal segregation of both TCAs and layer 4 cells relating to the whisker related pattern is two-fold. The large barrels corresponding to the large mystacial vibrissae form the posterior medial barrel subfield (PMBSF) while the smaller barrels anteriolateral to the PMBSF correspond to the small whiskers in anterior snout (AS) region (Welker and Van der Loos, 1986).

The layer 4 cell dendrites orient towards TCA patches

The layer 4 cell dendrites selectively orient into barrel hollows to form synapses with incoming TCAs resulting in asymmetrical dendrites, and this layer 4 cell dendritic asymmetry is thought to be maintained through to the adult. The precise timing of when this process occurs is unclear (Woolsey et al., 1975), however, selective orientation arises from pruning of dendritic branches outside a patch and elaboration of dendrites within the appropriate patch (Greenough and Chang, 1988).

1.3.2 Synaptogenesis in barrel cortex

In PMBSF region of mouse S1, both asymmetric (putative excitatory) and symmetric (putative inhibitory) synapses are present at all layers from P4 onwards (De Felipe et al., 1997). During P4-P8 corresponding to the period of anatomical barrel segregation, staining with markers for inhibitory neurons show 43% of synapses are inhibitory while 57% are excitatory (De Felipe et al., 1997). The developmental profile of asymmetric synapse density in layer 4 is a dynamic process (White et al., 1997). During P6 to P8, synapse density increases rapidly followed by a gradual increase from P9 to P12. With the onset of active whisking at P12, there is a second transient surge in synapse density between P13 and P14, with the peak of synaptogenesis at P14. The rate of synaptogenesis remains gradual until P20 and tapers off in the adult (White et al., 1997). It has also been shown that spine retraction exceeds spine formation between P16 and P25 resulting in a net loss of spines (Holtmaat et al., 2005).

In layer 4 of adult animals, 20-25% of excitatory synapses are comprised of synapses between TCAs and layer 4 spiny stellate cells (White, 1979). In the adult rodent barrel cortex, 85% of synapses are thought to be excitatory (Micheva and Beaulieu, 1996). Increasing evidence from two-photon imaging studies from the adult barrel cortex suggests that in the adult S1, synapse formation continues to stabilise (Holtmaat et al., 2005). For example, synapse formation in the adult (P34-74) S1 is more dynamic with only 50% of the population from layer 5 pyramidal cell spines persisting for at least a month (Trachtenberg et al., 2002). *In vitro* imaging of apical tufts of layer 5 pyramidal neurons and layer 2/3 pyramidal neurons in S1 from P175-225 old adult mice show 73% mature spines appear to persist for months, however, there is also transient formation of thin spines evident (Holtmaat et al., 2005). In another imaging study of spines in layer 5 and 2/3 pyramidal cells over a period of a month in 2.3-5 month old mice, transient thin spines form identifiable new synapses via EM after these spines remain persistent for 4 days (Knott et al., 2006).

1.3.3 Role of activity in the anatomical segregation of barrels

It is yet unclear what role neural activity plays or whether there is a role for either patterned or spontaneous activity in the anatomical segregation of barrels (Erzurumlu and Kind, 2001; Fox and Wong, 2005). It has been shown that blockade of all action potential or NMDAR activity at P0 does not perturb the segregation of TCAs into whisker related patterns suggesting that neither patterned or spontaneous activity is required for TCA segregation in the differentiation of S1 (Chiaia et al., 1992; Henderson et al., 1992; Schlaggar et al., 1993; Schlaggar and O'Leary, 1993). However, by P0 TCAs have already entered the cortex, therefore, blockade of activity at this time point might be too late to cause an effect. Furthermore, whisker trimming at P0 and during early development, does not disrupt the TCA segregation, despite weaker single neuron cortical responses (Fox, 1992). The temporary blockade of NMDAR and non-NMDAR dependent activity during the sensitive period of S1 (which is described below) does not alter segregation of TCAs (by CO staining) or segregation of layer 4 cells (by Nissl staining) into whisker related patterns, but disrupt the receptive field properties of TCAs (Fox et al., 1996). Collectively, these data suggest that while activity is required for refining

TCA connectivity and functional columnar organisation of S1, it does not play a role in the anatomical segregation of barrels.

Although, spontaneous release of glutamate seems to be sufficient for barrel formation as described below, it is unclear whether spontaneous activity plays a permissive role by allowing a certain threshold of activity to be reached for normal barrel development or an instructive one in guiding barrel formation by correlated patterned activity in the thalamus. However, it is clear that neural activity plays a role in synaptic plasticity and refinement of barrel cortex synapses.

1.3.4 Sensitive periods of S1

A fundamental characteristic of sensory maps of the neocortex is the capacity for plasticity in response to sensory experience or learning (Feldman and Brecht, 2005). Although plasticity is observed in the adult, during development, sensory areas exhibit heightened plasticity in response to sensory experiences, and the time frame for this developmental plasticity is specific to cortical areas and cortical layer and is known as a 'sensitive or critical period'. Below is a brief description of sensitive periods of S1,

1.3.4.1 Sensitive period for row C whisker lesion induced plasticity

The lesioning of row C follicles on the whisker pad cause the fusion of row C TCAs into a thin band while adjacent TCA rows B and D expand into row C region (Van der Loos and Woolsey, 1973). Layer 4 cells aggregate around the fused TCA row to form a 'megabarrel' (Van der Loos and Woolsey, 1973) with their dendritic arbours oriented towards the fused row C TCAs (Harris and Woolsey, 1981; Steffen and Van der Loos, 1980). This follicle lesion induced anatomical plasticity can only be induced during a very short developmental time period and fails to be manifested by P3-5 (Rebsam et al., 2005; Woolsey and Wann, 1976). Blocking firing of action potentials by TTX (tetrodotoxin) (Chiaia et al., 1992) or NMDAR-dependent activity by APV in rat S1 has been shown to diminish this neonatal row C ablation induced plasticity suggesting a role for activity in its induction (Schlaggar et al., 1993). However, Rebsam et al., (2005) in an elegant study in MAOA (monoamine oxidase A) knockout mice show that row C ablation induced plasticity can be dissociated from

anatomical segregation of barrels. In MAOA knockout mice, there is no segregation of TCAs or layer 4 cells into barrels (Cases et al., 1996) and this is shown to be due an excess build up of presynaptic serotonin (5HT) (Cases et al., 1996; Rebsam et al., 2002; Salichon et al., 2001). Early pharmacological reduction of 5HT levels in MAOA knockout mice results in rescue of segregation of TCAs and layer 4 cells within the whole barrel field (Cases et al., 1996), and reducing 5HT levels at P2 onwards until P17 in MAOA knockout mice results in rescue of barrel formation within the PMBSF area (although weaker segregation was evident when blocked between P10-P17) (Rebsam et al., 2005). Although, effects of row C whisker ablation can be rescued in MAOA knockout mice with reducing levels of 5HT, closure of critical period of row C whisker ablation induced plasticity is unchanged in these rescued animals suggesting that anatomical segregation of barrels can be dissociated from lesion induced plasticity (Rebsam et al., 2005).

1.3.4.2 Sensitive period for physiological plasticity

During S1 development, the functional synaptic plasticity of barrel cortex is dependent on glutamatergic signalling and can be induced well after the lesion induced anatomical plasticity window (Daw et al., 2007; Feldman et al., 1999).

LTP/LTD induction at TCA-layer 4 synapse

Physiologically, functional layer 4-TCA synapses are detected as early as P3 (Lu et al., 2001). LTP can be elicited at the TCA-layer 4 synapse until P7 (Crair and Malenka, 1995; Isaac et al., 1997), and expression of LTD is robust at P4-P5 but some cells has been shown elicit LTD at P9 and slightly after (Feldman et al., 1998). There is a fraction of glutamatergic synapses that are silent during development of S1 containing only NMDARs, and one LTP expression mechanism is shown to be the rapid incorporation of AMPARs at silent synapses (Isaac, 2003). The glutamatergic receptor subunit compositions at layer 4 synapses are also developmentally regulated in a time frame that somewhat parallels the synaptic plasticity sensitive period. During the first postnatal week, there is a switch from slow kainate receptor-mediated transmission to fast AMPAR-mediated synaptic transmission (Bannister et al., 2005; Daw et al., 2007; Kidd and Isaac, 1999). There is also

a change in subunit composition of NMDARs during development, such that the predominant NR1-NR2B expression during early development progressively switch to a NR1-NR2A containing one, and the increase in NR2A correlate well with the end of layer 4 sensitive period for LTP induction (Barth and Malenka, 2001).

Sensitivity periods for plasticity induced by whisker trimming

Whisker trimming instead of whisker ablation has been shown to cause changes in the physiological organisation of the barrel cortex by changing the balance of activity in the pathways from whiskers to the cortex, without affecting the anatomical organisation of the cortex (Simons and Land, 1987). In animals, where all but one whisker (D1) are trimmed at P0, and subsequently allowed to regrow prior to recording cellular responses, the responses in 'deprived' barrels surrounding the D1 barrel is enhanced to D1 whisker stimulation (Fox, 1992). This plasticity in layer 4 decreases with age and responses in deprived whiskers decrease to basal state in animals trimmed at P4 and P7. In contrast, this plasticity can be elicited in layer 2 and 3 in animals whose whiskers are trimmed at P7 suggesting that developmental sensitive period differ between cortical layers and that plasticity continues in supragranular layers well after it ends in layer 4 (Fox, 1992). This variation is thought to result from the heterogeneity in maturation of thalamocortical and intracortical synapses (O'Leary et al., 1994).

1.3.5 Role of neural activity in maintaining and refining synaptic connectivity in barrel cortex

Recent two-photon imaging studies also demonstrate a role for sensory evoked synaptic activity in maintaining and refining correct synaptic connectivity in the barrel cortex. The sensory deprivation by trimming whiskers 1-3 days prior to imaging has been shown to decrease the spine protrusive motility by 40% during P11-P13 but not at younger (P8-P10) or older (P14-P16) ages in barrel cortex layer 2/3 pyramidal neurons. However, no changes were found in spine density, length or shape (Lendvai et al., 2000). In another study of layer 5 pyramidal neurons in barrel cortex of one-

month-old mice shows that the rate of spine elimination is decreased with either sensory deprivation by whisker trimming or by blockade of NMDARs but that rate of spine formation is unaltered. These changes appear to be reversible as both restoring sensory experience and drug withdrawal subsequently results in accelerated spine elimination (Zuo et al., 2005b).

Therefore, neural activity plays a key regulatory role in synaptic plasticity and refinement of barrel cortex synapses during S1 development. Moreover, the ongoing characterisation of components of the PSD (Husi et al., 2000) has facilitated the exploration of the role of postsynaptic glutamatergic receptors and their effector molecules in the differentiation of S1. The generation of mutant mouse models with specific deletions in PSD components have elucidated valuable information regarding the pathways downstream of glutamatergic receptor activation in barrel formation. Some of these signalling pathways that affect barrel formation have also been implicated in FXS, and these will be discussed below.

1.3.6 Role of glutamatergic receptors in barrel formation

NMDAR

The role of glutamatergic signalling in barrel formation was first suggested by a study in which the essential subunit of NMDAR, the NR1 was globally deleted. The genetic deletion of *Nr1* globally results in the loss of barrelette formation despite normal axonal projections from the mystacial whiskers, however, the postnatal lethality by P1 due to global loss of NR1 precluded the investigation of whisker related pattern formation at the thalamus and the cortex (Li et al., 1994). The ectopic expression of a transgene of NMDAR1 splice variant in $Nr1^{-/-}$ mice has been shown to rescue the phenotype of the barrelettes and normal patterning of barreloids and barrels in a transgene dose dependent manner (Iwasato et al., 1997).

Subsequently, to determine roles of cortical NMDARs, a cortex specific *Nr1* ($CxNr1^{-/-}$) knockout mouse was generated where NR1 expression was selectively deleted in cortical excitatory neurons (Iwasato et al., 2000). In $CxNr1^{-/-}$ mice, there is normal patterning of barrelettes and barreloids

(Iwasato et al., 2000). In the barrel cortex, layer 4 cells fail to form cellular aggregates and TCAs show rudimentary segregation (Iwasato et al., 2000). There is also exuberant thalamocortical arborisation in *CxNr1*$^{-/-}$ mice suggesting that postsynaptic NMDARs regulate refinement of presynaptic TCAs into whisker related patches during S1 development via a retrograde messenger(s) that regulate growth and focalisation of thalamocortical arbours (Lee et al., 2005). In addition, there is increased layer 4 neuronal dendritic arborisation with an increased spine density in *CxNr1*$^{-/-}$ mice indicating that postsynaptic NMDARs also play a regulatory role in regulating layer 4 spinogenesis and dendritic complexity (Datwani et al., 2002). Furthermore, layer 4 spiny stellate cells in *CxNr1*$^{-/-}$ mice also fail to show orientation bias compared to orientation bias observed in wildtype mice (Datwani et al., 2002). In summary, signalling through postsynaptic NMDAR receptor appears to play a regulatory role in both barrel formation and synaptogenesis.

In contrast to the effects of the pharmacological blockade of NMDAR (Schlaggar et al., 1993), *CxNr1*$^{-/-}$ mice show normal row C whisker lesioned plasticity (Iwasato et al., 2000). This discrepancy could be due to altered downstream effector signalling in *CxNr1*$^{-/-}$ mice compared to acute blockade of NMDAR. Alternatively, the presence of GABAgeric neurons in *CxNr1*$^{-/-}$ mice as the promoter Emx1 used in driving the cortex specific loss of *Nr1* is not expressed in the ganglionic eminence where GABAgeric cells originate. Irrespective of this discrepancy, NMDAR mediated glutamatergic signalling appears to play a key role in regulating barrel formation and TCA synapses.

mGluR5-PLCβ1 pathway

Recent studies suggest that loss of FMRP results in dysregulated signalling via Gp1 mGluRs leading to the pathogenesis of FXS (Bagni and Greenough, 2005; Bassell and Warren, 2008; Bear et al., 2008; Bear et al., 2004; Ronesi and Huber, 2008). Gp1 mGluRs are comprised of mGluR1 and mGluR5, and mGluR5 is the predominant subtype in the forebrain. A role for mGluR5 has been shown in the formation of whisker related patterns in mouse S1 (Hannan et al., 2001). The global loss of mGluR5 results in the partial segregation of TCAs within the PMBSF into whisker related rows, and there is

no segregation of TCAs in the AS region suggesting that mGluR5 plays a role in TCA pattern segregation during S1 development (Hannan et al., 2001). Furthermore, layer 4 cells fail to form cellular aggregates resulting in near complete loss of barrel formation in layer 4 (Hannan et al., 2001). However, it is yet unclear whether mGluR5 plays a pre or postsynaptic role in barrel formation or whether it has a dose dependent effect on barrel formation. Given the fact that the 'mGluR theory of fragile X mental retardation' predicts that the loss of FMRP results in exaggerated effects of mGluR5 signalling (Bear et al., 2004), it would be of interest to determine whether a critical level of mGluR5 is required for the anatomical segregation of barrels in S1. Another interesting question is whether mGluR5 plays a role in regulating synaptogenesis in layer 4 cells during S1 development.

Gp1 mGluRs are Gq protein coupled receptors and by definition mediate phosphotidyl inositol (PI) hydrolysis. Activation of mGluR5 activates the membrane bound phospholipase C-β (PLCβ), which then cleaves PIP2 (phosphotidylinositol4,5-biphosphate) into two second messengers, DAG (diacylglycerol) and IP3 (1,4,5-inositol triphosphate). Subsequently, DAG activates PKC (protein kinase C) and IP3 activates the IP3R (IP3 receptor) on the endoplasmic reticulum to initiate release of $Ca2^{+}$ from intracellular stores. PLCβ1 is one of four members of the PLCβ family and is the most highly expressed one in the neocortex. The expression of PLCβ1 is high in layers 2-4 of S1 during the first two postnatal weeks (Hannan et al., 2001; Hannan et al., 1998). Moreover, expression of PLCβ1 during development is largely considered to be postsynaptic (Erzurumlu and Kind, 2001). In addition, the Gp1 mGluR activated PI hydrolysis is dependent on PLCβ1 during the first postnatal week (Hannan et al., 2001). Collectively, these data suggest a role for PLCβ1 in barrel formation. In agreement with this idea, the global deletion of PLCβ1 results in impaired layer 4 cell segregation into barrels but segregation of TCAs appears to be normal (Hannan et al., 2001). The loss of layer 4 cellular segregation in *Plcβ1*$^{-/-}$ mice despite TCA segregation suggests that layer 4 cellular segregation is not completely dependent on TCA patterning nor is it driven by it. Moreover, these findings also suggest that while mGluR5 may mediate layer 4 cellular segregation via PLCβ1 activation, it may regulate TCA patterning via a different signalling pathway (Hannan et al., 2001).

Interestingly, PLCβ1 is highly expressed in intermediate-compartment-like organelle, known as botrysomes, that localise to dendrites leading to the hypothesis that PLCβ1 might be involved in protein trafficking in response to Gp1 mGluR signalling, thereby play a role in activity dependent refinement of cortical connections (Kind et al., 1997). In agreement, there are reduced symmetric/asymmetric synapses in barrel cortex in *Plcβ1*$^{-/-}$ mice at P5 compared to *Plcβ1*$^{+/+}$ mice suggesting alterations in the inhibitory and excitatory connections. There is also evidence for defects in spine maturation in layer 5 pyramidal cells (Spires et al., 2005). In layer 4 spiny cells, there is increased spine density but normal dendritic complexity in the absence of PLCβ1 (Upton et al., manuscript in preparation). Furthermore, *Plcβ1*$^{-/-}$ mice exhibit normal dendritic complexity despite loss of layer 4 cellular segregation suggesting that selective elaboration of dendrites within a TCA patch is not sufficient for barrel formation (Upton et al., manuscript in preparation).

1.3.7 cAMP-PKA pathway

cAMP

Gp1 mGluRs positively regulate the adenylyl cylcase (AC) enzymatic activity, which is an enzyme that can be activated via NMDAR signalling as well (Erzurumlu and Kind, 2001). AC catalyses the synthesis of cAMP (cyclic adenosine monophosphate) from ATP (adenosine triphosphate). In turn cAMP acts as a second messenger to intracellular effectors. In FXS, it is thought that altered cAMP metabolism may partly underlie the neuronal phenotypes observed, and this known as the 'cAMP theory of FXS' (Kelley et al., 2008; Kelley et al., 2007). In fragile X patients, there are decreased cAMP levels in platelets compared to normal controls (Berry-Kravis and Huttenlocher, 1992). Overexpression of FMRP in mouse neuronal cell lines show a positive correlation between levels of FMRP and cAMP (Berry-Kravis and Ciurlionis, 1998). Moreover, induced levels of cAMP have also shown to be downregulated in *dfmr1* fly heads, cortex from *Fmr1*$^{-/y}$ mice and human *FMR1* neural cell lines (Kelley et al., 2008; Kelley et al., 2007). Thus, Kelly et al., (2007) hypothesised that altered levels of cAMP reflect functional deficiencies in neurotransmitters and receptors signalling through FXS.

There are 10 known ACs (AC1-AC10), and both NMDAR and Gp1 mGluR signalling results in the activation of AC1 (Erzurumlu and Kind, 2001; Wang and Storm, 2003; Wong et al., 1999). A spontaneous mutation that resulted in failure of TCA patterning and layer 4 cellular segregation was first characterised as a 'barrelless' mouse (Welker et al., 1996). This spontaneous mutation was later identified as an *Adcy1* mutation that encodes AC1 (Abdel-Majid et al., 1998). In barrelless mice, the tangential extent of the TCA arborisation in layers 4 and 6 is expanded compared to the wildtype mice suggesting a presynaptic effect on barrel formation (Gheorghita et al., 2006) and consistent with this there is reduced neurotransmitter release at the TCA-layer 4 synapse (Lu et al., 2003). The presynaptic $5HT_{1B}$ receptor also negatively couples to AC activity suggesting that AC may play a presynaptic role in regulating glutamatergic signal transmission in TCAs and refining TCA segregation into whisker related patterns (reviewed in Erzurumlu and Kind, 2001).

In addition to presynaptic functional defects in barrelless mice, postsynaptically there is impaired LTP induction and AMPAR expression during development (Lu et al., 2003). To test whether AC1 mediate barrel formation pre or postsynaptically, a recent study examined the cortex specific deletion of AC1 in excitatory neurons (*CxAC1*$^{-/-}$ mice). The *CxAC1*$^{-/-}$ mice have normal TCA patterning but reduced layer 4 cellular segregation. Physiologically at TCA-layer 4 synapse maturation, *CxAC1*$^{-/-}$ mice have postsynaptic deficits such as reduced AMPA/NMDA ratios during barrel cortex development and impaired LTP. Furthermore, in *CxAC1*$^{-/-}$ there is normal neurotransmitter release presynaptically compared to wildtype mice (Iwasato et al., 2008). Collectively these data also suggest a postsynaptic role for AC1 in barrel formation. Moreover, dendrites of layer 4 spiny cells of *CxAC1*$^{-/-}$ mice show reduced dendritic asymmetry and increased dendritic span but whether loss of AC1 affects spine number or morphology is unknown (Iwasato et al., 2008).

PKA

PKA (cAMP-dependent protein kinase A) is a kinase activated by cAMP and is assembled from products of four regulatory (Rlα, Rlβ, RIIα and RIIβ) and two catalytic (Cα and Cβ) subunit genes. PKARIIβ is also localised to the NMDAR via AKAP 79/150 (A-kinase anchoring protein 79/150) (Carr et al., 1992) and PKA phosphorylation of GluR1 at S845 has been shown to mediate AMPAR trafficking at the synapse (Ehlers, 2000; Esteban et al., 2003; Lee et al., 2003). In barrelless mice, functional deficits in TCA-layer 4 have been shown to correlate with reduced PKA dependent phosphorylation and GluR1 surface expression (Lu et al., 2003) suggesting a role for PKA in barrel formation. In all viable mutants analysed where subunits of PKA were genetically deleted (Rlβ, RIIα, RlIβC, α and Cβ), only *PrkarIIβ*$^{-/-}$ mice show reduced layer 4 cellular segregation and impaired TCA patch patterning in the AS region but not in the PMBSF region (Watson et al., 2006). At P7, the expression of PKARIIβ is postsynaptic and homogenates of *PrkarIIβ*$^{-/-}$ mice show no upregulation of the other subunits of PKA, suggesting that barrel defect is due to postsynaptic loss of PKARIIβ (Watson et al., 2006). There is also reduced expression of LTP and deficits in AMPAR mediated currents during development at TCA-layer 4 synapse (Inan et al., 2006) and reduced levels of GluR1 at the PSD (Watson et al., 2006) in *PrkarIIβ*$^{-/-}$ mice compared to *PrkarIIβ*$^{+/+}$ mice. Despite these defects in AMPAR localisation, the genetic deletion of GluR1 or other subunits of AMPARs do not alter barrel formation (Watson et al., 2006). Together these data suggest that role of PKA in anatomical segregation of barrel development may occur independent of PKA mediated synaptic plasticity of barrel cortex. This idea of anatomical segregation of barrels can be dissociated from synaptic plasticity of barrel formation is consistent with the formation of a megabarrel, which forms in response to row C follicle ablation despite complete loss of peripheral receptors (Van der Loos and Woolsey, 1973). Furthermore, the sensitive period for lesion-induced plasticity and barrel development can be dissociated suggesting that these processes are regulated by different cellular mechanisms (Rebsam et al., 2005).

1.3.8 SynGAP-ERK pathway

Altered early phase ERK activation is observed in platelets from patients with FXS (Weng et al., 2008) and in cortical synaptoneurosomes from *Fmr1*$^{-/y}$ mice, ERK is dephosphorylated upon Gp1 mGluR activity whereas in *Fmr1*$^{+/y}$ mice it is phosphorylated suggesting that loss of FMRP leads to aberrant ERK signalling (Kim et al., 2008). There is an emerging view that ERK might be the converging signalling molecule for multiple divergent signalling cascades such as those mediated by NMDAR (Komiyama et al., 2002), mGluR5 (Berkeley and Levey, 2003; Choe and Wang, 2001; Gallagher et al., 2004), PKA (Cancedda et al., 2003) and PKC (Sweatt, 2004) activity.

The activation of NMDAR results in increased levels of phosphorylated ERK (pERK) (Zhu et al., 2002) and it is thought that NMDAR mediate this upregulation of pERK by inhibiting SynGAP (Synaptic RasGTPase activating protein) activity thus causing a build up of RasGTPase that leads to ERK phosphorylation (Chen et al., 1998; Kim et al., 1998). In hippocampal slices from *Syngap+/-* mice, increased levels of pERK is evident both basally and in response to NMDAR stimulation suggesting that as well as SynGAP mediated regulation of ERK phosphorylation, there may be other SynGAP independent pathways to ERK phosphorylation (Komiyama et al., 2002). SynGAP is a brain specific Ras- and Rap- GTPase activating protein with enriched expression at excitatory synapses (Kim et al., 1998). SynGAP is localised to the NMDAR complex via its C terminal interactions with the PDZ binding domains of PSD95 and SAP102 (Chen et al., 1998; Kim et al., 1998; Komiyama et al., 2002; Nonaka et al., 2006). Developing S1 expresses high levels of both *Syngap* mRNA and SynGAP protein during barrel formation and at P7 it is expressed postsynaptically (Barnett et al., 2006b). The global loss of SynGAP results in postnatal lethality at P4. The genetic deletion of *Syngap* does not affect the formation of barrelettes but results in partial segregation of barreloids (Barnett et al., 2006b). In *Syngap*$^{-/-}$ mice TCAs segregate into whisker related rows but individual patches do not form (Barnett et al., 2006b). Moreover, layer 4 cellular segregation is impaired in a SynGAP dose dependent manner with reduced layer 4 cell segregation in *Syngap*$^{+/-}$ mice and near complete loss of layer 4 cell segregation in *Syngap*$^{-/-}$ mice (Barnett et al., 2006b). SynGAP has also been shown to play a role in synaptogenesis. In

cultured hippocampal neurons of *Syngap*$^{-/-}$ mice show precocious spines with increased glutamatergic receptor and PSD95 incorporation at synaptic sites (Vazquez et al., 2004). In agreement, in adult *Syngap+/-* mice there is increased density of mature mushroom spines compared to *Syngap+/+* mice (Carlisle et al., 2008).

Defects in barrel formation due to genetic deletion of *Syngap* indicated that mutations in MAGUKs that tether SynGAP to the NMDAR might disrupt barrel formation. However, global deletion of neither PSD95 nor SAP102 resulted in altered barrel formation suggesting that there might be functional/molecular redundancy between MAGUK family members (Petrie, 2008). Consistent with this idea, *Sap102*$^{-/y}$*/Psd95*$^{+/-}$ mice show reduced layer 4 cellular segregation compared to wildtype mice (Petrie, 2008). In *Psd95*$^{-/-}$ mice, spine density in hippocampal is increased by 40% in apical dendrites of CA1 neurons and is decreased by 15% along the length of striatal neurons (Vickers et al., 2006) suggesting a role for PSD95 family MAGUKs in regulating synaptogenesis in a neuronal cell type specific manner.

1.3.9 In summary,

During the course of postnatal development, genetically programmed synaptic connections are refined and stabilised. Both synaptic activity and protein synthesis play a regulatory role in shaping this synaptic connectivity. The symptoms of FXS are underlined by altered synaptic connectivity and deficits in both synaptic activity and protein synthesis are evident in FXS. The formation of barrels in mouse S1 is glutamatergic signalling dependent and appear to involve molecules that are associated with FXS. Most importantly, regulatory roles of FMRP neuronal cell processes are extensively characterised in the literature, but despite the developmental aetiology of FXS, not many studies examine the regulatory roles of FMRP during early cortical development. Therefore, to fully understand the effects of loss of FMRP in the neuropathologies observed in FXS, it is critical to examine early regulatory functions of FMRP in neuronal cell processes. The mouse S1 provides an excellent model in examining the dysregulated cortical connectivity in FXS due to it precise developmentally regulated somatotopy.

1.3.10 Aims of the thesis

Chapter 3 aims to further characterise the role of mGluR5 in S1 organisation by buildings on the finding of Hannan et al., (2001). It tests the hypotheses that,

1) mGluR5 is expressed throughout S1 development

2) mGluR5 plays a postsynaptic role in barrel formation.

3) Effects of mGluR5 in anatomical organisation of barrel cortex is dose dependent

4) mGluR5 plays a role in synaptogenesis of layer 4 spiny cells in S1

Chapter 4 aims to explore the role of FMRP during both early and late S1 development. It tests the hypotheses that,

1) FMRP is expressed in S1 during early postnatal development

2) FMRP plays a role in barrel formation

3) FMRP regulates synaptogenesis in layer 4 spiny cells

4) Loss of FMRP affects basal expression of synaptic proteins

Chapter 5 aims to investigate the interactions between mGluR5 and FMRP by genetically manipulating levels of mGluR5 in *Fmr1*$^{-/y}$ mice. It will address the hypothesis that reducing levels of mGluR5 in *Fmr1*$^{-/y}$ mice will rescue S1 phenotypes in *Fmr1*$^{-/y}$ mice in accordance with the predictions of the 'Gp1 mGluR theory of fragile X syndrome'.

Chapter 6 aims are two fold.

Firstly, to examine the roles of PSD95 family MAGUKs (PSD95, SAP102 and PSD93) in S1 development. It tests the hypotheses that

(1) PSD95, SAP102 and PSD93 are expressed in S1 during barrel formation

(2) PSD95 and SAP102 are involved in synaptic protein trafficking during cortical development

This work was carried out as a subpart of a main project, which examined the roles of PSD95 family MAGUKs in barrel formation, which was carried out by Anne Petrie (Petrie, 2008).

Secondly, to test the hypothesis that expression of PSD95 family members PSD95, SAP102 and PSD93 are altered in *Fmr1*$^{-/y}$ mice during cortical development as all of these molecules show putative binding sites for FMRP.

The characterisation of developmental roles of mGluR5, FMRP and PSd95 family MAGUKs would further our understanding of what molecules are important in and how they regulate cellular processes that govern cortical connectivity in order to elucidate what happens in the diseased state.

2

2 Materials and Methods

2.1 Transgenic animals

There were several transgenic mutant lines used in this thesis and following is a brief description of each mutant.

2.1.1 Mglur5 mutant mice

Mglur5 mutant mice characterised in chapter 3 were generated by target deletion of exon 1 and part of intron 1 and replacing these with a *neomycin* cassette (Jia et al., 1998; Lu et al., 1997). The mice were obtained from Jackson laboratories (Bar harbor, Maine) and were bred from *Mglur5*$^{+/-}$ mice mating pairs on a mixed C57BL/6JX129 background. The presence or absence of *Mglur5* allele was determined by using the PCR primers PGK22N (5' AGG GGA GGA GTA GGA GGT GGC GCG A 3'), F (5' GCT CAC ATG CCA GGT GAC ATT ATT ATT GGA 3'), and R (5' CCA TGC TAG TTG TTG CAG AGT AAG CAA TCT GAG GT 3') (Eurofins MWG Operon, London, UK). The WT (wildtype) and KO (knockout) PCR products were run as two separate PCR reactions using the PCR program: 95^{0}C for 10min, followed by 30 cycles of amplification where each cycle consisted of 5s at 95^{0}C, 55s at 64^{0}C, and 30s at 72^{0}C. The PCR products were WT (F/R) 445bp and KO (F/PGK22K) of approximately 600bp.

2.1.2 Fmr1 mutant mice

The *Fmr1* mutant mice studied in chapter 4 is the conventional fragile X mouse model generated by interrupting exon 5 with a *neomycin* cassette (Dutch-Belgian fragile X consortium, 1994). The *Fmr1* mutant mice were obtained from two sources. All of the anatomical characterisations, apart from analysis of cortical arealisation were carried out on a C57BL/6J background in collaboration on brains shipped from Prof. M F Bear's lab. All of the biochemical analysis and measurements of cortical arealisation were carried out on mice obtained from Jackson laboratories that were backcrossed 5 generations on to a C57BL/6JOla background from the original

C57BL/6J background. The PCR genotyping program to determine the presence or absence of WT allele and *Fmr1* allele in animals used in collaboration with the Bear lab was carried out according to the methods of Dolen et al., (2007). Briefly, the PCR primers used for WT allele were S1 (5' GTG GTT AGC TAA AGT GAG GAT GAT 3') and S2 (5' CAG GTT TGT TGG GAT TAA CAG ATC 3') and the KO allele using primer M2 (5' ATC TAG TCA TGC TAT GGA TAT CAG C 3') and N2 (5' GTG GCG TCT ATG GCT TCT GAG G 3'). The WT reaction product (S1/S2) was 465bp and the KO reaction product was 800bp in size. In *Fmr1* mutant mice from the C57BL/6J0la background, the presence or absence of WT allele on the *Fmr1* locus was carried out by using PCR primers for WT allele 2009 (5' GTG GTT AGC TAA AGT GAG GAT GAT 3') and 2010 (CAG GTT TGT TGG GAT TAA CAG ATC 3'). The KO primers used were 162 (5' CCG GTT CTT TTT GTC AAG ACC G 3') and 163 (5' CGG CAG GAG CAA GGT GAG AT 3'). The WT and KO PCR products were run as two separate PCR reactions using the PCR program: 94^{0}C for 3min, followed by 35 cycles of amplification where each cycle consisted of 30s at 94^{0}C, 30s at 63^{0}C, and 1min at 72^{0}C. The PCR products were WT (2009/2010) approximately 500bp and KO (163/164) 197bp.

2.1.3 Mglur5 and Fmr1 double mutant mice

All analysis of double mutants was carried out on brains shipped from Prof. M F Bear's lab on a C57BL/6J background. The double mutants of *Mglur5* and *Fmr1* were generated by setting up breeding crosses between male *Fmr1*$^{-/y}$ mice and female *Mglur5*$^{+/-}$ mice as described in Dolen et al., (2007). The double mutants were genotyped for *Mglur5* and *Fmr1* alleles and WT alleles separately by using the PCR programs mentioned in 2.1.1 and 2.1.2.

2.1.4 Sap102 and Psd95 double mutant mice

Transgenic mice used in chapter 6 were created by setting various breedings between *Sap102* and *Psd95* mutant mice generated in Prof. S Grant's lab. Both the *Sap102* and *Psd95* lines were obtained on a C57BL/6JX129 mixed background. The *Sap102* mutant mice were created by causing a frameshift from exon 1-9 resulting in the deletion of its PDZ domains (Cuthbert et al., 2007). The *Psd95* mutant mice were created by inserting a stop codon at the

3rd PDZ domain. The resulting PSD95 protein fragment does not localise to the synaptic sites (Migaud et al., 1998).

PCR reactions were carried out on *Psd95* mutant mice by utilising primers designed to amplify a WT sequence upstream of the *PuvII* site in the PDZ3 domain. F primer (5' AAC CAA GGC TCG TGA TCC A 3') and R primer (5' TCT CTT TGG TGG GCA GTG 3') were used to identify the WT allele corresponding to a 220bp fragment. The 2kb fragment of the *Psd95* mutant allele was identified with F primer (5' CAT TCG ACC ACC AAG GGA AAG ATC 3') and R primer (5' CAG GGA GCG GGG ACG GAT GA 3'), and these primers identified a strand containing the neomycin cassette (Migaud et al., 1998).

PCR reactions were carried out on *Sap102* mutant mice by utilising the F primer (5' GGT CTC TGA AGC AGT GAT TTT T 3') and two R primers (5' TGA TGA CCC ATA GAC AGT AGG ATC A 3') and (5' CTA AAG CGC ATG CTC CAG AC 3') to yield an amplified WT PCR product of 535bp and a KO PRC product of 215bp (Cuthbert et al., 2007).

2.2 Anatomical characterisations

2.2.1 Tissue preparation for histology

All mice used were euthanised by giving a lethal dose of sodium pentobarbital (Euthanol; 200mg/kg, ip) and perfused transcardially with 0.1M phosphate buffered saline (PBS) followed by 4% (w/v) paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Brains were then removed and post-fixed for at least 04 hours at 4°C. In instances where brains were sectioned on a freezing microtome, prior to sectioning they were cryo-protected in 30% (w/v) sucrose in 0.1M PBS overnight at 4°C.

Unless otherwise mentioned, brains were sectioned either on the coronal plane or tangential to the pial surface on a freezing microtome at 48µm thickness. To obtain sections tangential to the pial surface two different techniques were used depending on which aspect of cortical development was examined. To examine developmental expression profiles of proteins of

interest, quantification of layer 4 cellular segregation and Golgi analysis in layer 4, cortices were separated and the thalamus, entorhinal cortex, hippocampus and striatum were removed leaving only the neocortical sheet. This procedure dissects all connections that could potentially otherwise distort the barrel pattern during cortical flattening. Second flattening technique was adapted where it was vital to keep the valuable landmarks intact to accurately determine the localisation of the relative position of S1 and measurement of neocortical and sensory areas. For this purpose the cortices were separated, thalamus (and part of striatum) was removed and cortex was gently flattened for at least 24 hours at 4^{o}C in between two glass slides with two glass capillary tubes utilised as spacers between the slides.

2.2.2 Immunohistochemistry

2.2.2.1 For developmental expression profiles

Brains were collected from P4, P7, P14, P21 and adult wildtype mice on a C57BL/6J0la background for mGluR5 and FMRP developmental expression profiles, and for the PSD95 family MAGUK expression profiles wildtype brains at these ages were collected from a C57BL/6JX129 background. Coronal free-floating sections were reacted overnight with primary antibodies against mGluR5 (1:10,000; Millipore, UK), FMRP (rAM1 antibody, 1:1000; gifted from Bagni C), PSD95 (1:1000; Frontier Science Co Ltd, Hokkaldo, Japan), SAP102 (1:1000; Frontier Science Co Ltd, Hokkaldo, Japan) and PSD93 (1:1000; Frontier Science Co Ltd, Hokkaldo, Japan). Tangential free-floating sections from all developmental ages mentioned above were also reacted with primary antibodies against mGluR5 and FMRP.

2.2.2.2 For cortical arealisation analysis

Tangential sections from P7 brains were utilised for neocortical and sensory area measurements and positioning of PMBSF analysis. Sections were reacted overnight with anti-serotonin transporter (5HTT) (1:2000; Calbiochem, La Jolla, CA).

All area measurements on 5HTT reacted tangential sections were made using UTHSCSA Imagetool version 3.0 software (University of Texas Health Science

Centre at San Antonio, San Antonio, TX), and measurements were carried out blind to the genotype. Areas measured are demarcated in figure 2.1. To determine whether there was any defect in cortical arealisation in the mutants analysed, areas of neocortex, primary somatosensory cortex, AS and/or PMBSF and visual cortex were measured and these areas are outlined in figure 2.1A, B and C. To analyse the positioning of PMBSF, the distances from barrel C3 to rostral (R) and caudal (C) edges of cortex were measured as shown in figure 2.1D. The ratio between rostral distance from C3 over the total length of the cortex (R/T) and the caudal distance from C3 over the total length of the cortex was then determined (C/T). All these area and positioning measurements are presented as average ± SEM.

The signal from the primary antibodies in both procedures mentioned above (section 2.2.2.1 and 2.2.2.2) was amplified utilising appropriate secondary antibodies (Dako UK Ltd, Cambridgshire, UK) and the resulting signal was visualised using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine (DAB) as the chromogen. The primary and secondary antibodies were made up in Dulbecco's Modified Eagle Medium (D-MEM: 1000mg/l D-glucose, sodium pyruvate, 25mM HEPES) (GIBCO-Invitrogen, Paisley, UK) containing 5% foetal calf serum and 0.2-0.5% Triton X-100. Immunoreacted sections were mounted on 0.5% (w/v) gelatine chrome-alum subbed slides, dehydrated in an ascending alcohol series, defattened in Xylen and coverslipped.

2.2.2.3 For layer 4 cellular segregation analysis

Thionin staining was performed on tangential sections mounted on chrome-alum gelatin coated slides to initially assess layer 4 cellular segregation qualitatively in *Mglur5* and *Fmr1* mutant mice. Thionin stain constitutes of three stock solutions: (A) 1g thionin /100ml dH_2O; (B) 0.1M glacial acetic acid; and (C) 0.1M sodium acetate. Final thionin solution contained 90mls of (B), 10ml of (C) and 2.5ml of (A). Once stained, sections were differentiated in 95% ethanol containing acetic acid (1:1000dil) appropriately, then dehydrated, defattened and coverslipped.

To quantify any layer 4 cellular segregation defects observed qualitatively, free floating tangential sections from P7 brains were reacted overnight with anti-5HTT. The signal from the 5HTT antibody was amplified with biotinylated goat anti rabbit antibody (Dako UK Ltd, Cambridgshire, UK), and visualised using an AlexaFlour 568-conjugated goat anti-rabbit secondary antibody (Invitrogen, San Diego, CA). The sections were then incubated overnight in Topro3 (Invitrogen, San Diego, CA) in 9:1-glycerol: PBS to reveal cell nuclei and were mounted on mowi oil (Poly Vinyl Alcohol, BDH) containing glycerol, ddH_2O, Tris and anti-fade reagent 1,4-Diazobicyclooctane (DABCO) (Sigma-Aldrich, Dorset, UK), and coverslipped.

All tangential sections stained with 5HTT and Topro3 were examined to determine which tangential section through layer 4 contained the optimal cellular segregation for barrel C3. Confocal images were taken from this section at X5, 10 and 20 on a Leica TCNST confocal microscope. To perform cell counts, an optical stack through barrel C3 was taken at 7μm intervals under X20. The optical slice containing the best layer 4 cellular segregation for C3 was determined qualitatively from the stack. The barrel wall to hollow cell density ratio was determined for this selected optical slice and the slice immediately before and after it as follows: In image software Adobe Photoshop version CS3, The edge of C3 TCA patch was outlined using 5HTT stained C3 TCA patch (Figure 2.2Bi), and the photoshop layer containing the TCA patch outline was then superimposed on the photoshop layer containing Topro3 labelled cell nuclei as shown in figure 2.2Bii to verify the barrel patch demarcation. Using only the layer containing the patch outline, sampling frames (32μmX52μm rectangles) were placed on the wall (~10-12 frames) and hollow (~5-8 frames) of barrel C3 as depicted in figure 2.2Biii. The placement of sample frames within barrel wall and hollow were verified by determining whether these were placed in agreement with the variation in the density of Topro3 stained nuclei between the barrel wall and hollow (Figure 2.2C). Cells within these sampling frames were counted if they overlapped two sides of the rectangle or were contained within the rectangle (Figure 2.2D). A ratio was calculated between the total cell counts in the barrel wall to that of the hollow for the three optical slices and the best ratio was taken for that particular animal. This ratio represented how well segregated barrel C3 was for each animal analysed and all steps in this

analysis procedure was carried out blind to the genotype. The values are presented as ratio of wall:hollow cell density±SEM. In addition to quantifying the layer 4 cell densities of barrel C3, size of C3 TCA patch was also measured blind to the genotype using Image J software to determine if size of TCA patch was altered. The average area of C3 TCA patch ±SEM is presented.

2.2.2.4 For barreloid and barrelette analysis

To visualise barreloids and barrelettes, coronal sections through thalamus and brainstem were taken respectively at P7. The sections were mounted and then stained in a solution containing 0.15mg/ml cytohrome C (Sigma-Aldrich, Dorset, UK), 0.5mg/ml DAB, 40mg/ml sucrose in 0.1M PB at 37^{0}C for 6-12hours as described in Wong-Riley (1979). These CO reacted slides were then dehydrated in an ascending alcohol series, defattened in xylene, and coverslipped.

2.2.3 Immuno Electron microscopy

Tissue at P7 only or both P7 and P14 was collected as previously described in 2.2.1 with the exception of adding 0.1% gluteraldehyde (TAAB Laboratories, UK) to the fixative and only post-fixing for couple of hours. Fixed tissue was sectioned coronally at 50µm thickness on a vibrotome. Coronal sections through the barrel cortex were incubated with antibodies against mGluR5 (1:500; Millipore, UK) and FMRP (rAM1 antibody, 1:250; gifted from C Bagni). DAB immunohistochemistry was performed as described in 2.2.2 but to preserve the membranous ultra structure no detergent was introduced during the immunohistochemical process. The reacted sections were further post-fixed in 1% osmium tetraoxide in 0.1M PB for 45min. All sections were embedded on glass slides in Durcupan resin and were then dehydrated in an ascending alcohol series and propylene oxide. Cortical regions containing barrels were then dissected out (~1X1mm) under a dissecting scope and were glued onto a resin block for ultra-thin (~70nm) sectioning and these were collected on formvar-coated grids (Agar Scientific, Stansted, UK). The DAB immuno product was visualised with uranyl acetate and lead citrate in LKB UltroStainer, and imaged in a Philips CM12 transmission electron-microscope.

2.2.4 Golgi

2.2.4.1 Golgi impregnation of neurons

Tissue was collected at either P21-P23 (in the case of *Mglur5* mutants) or at P30-P35 (in the case of *Fmr1* and *Fmr1/ Mglur5* mutants) as mentioned in 2.2.1. The fixed tissue was tangentially sectioned at 80μm thickness on a vibrotome and post-fixed in 1% osmium tetraoxide in 0.1M PB for 30min-1hour, and this step enables the visualisation of whisker related patterns on sections through layer 4. The sections containing barrels were the treated with 3.5% potassium dichromate overnight followed by further staining in 1.5% aqueous silver nitrate for ~6hours. After neuronal impregnation was completed, sections were mounted, dehydrated, defattened and coverslipped.

2.2.4.2 Spine density and dendritic complexity analysis

All analysis was carried out blind to the genotype. Neurons and dendrites were examined and traced utilising a Leica DMR microscope attached to a Neurolucida computer assisted tracing system (Microbrightfield Inc, USA). For all spine analysis, spines were counted along an entire length of a dendrite and in order to select an appropriate dendrite it had to fit the criteria of 1) dendrite is from a layer 4 spiny cell localised within the PMBSF, 2) a well isolated dendrite and 3) the dendrite has a normal tapered ending that is indicative of a normal ending. Dendrites were traced along its length and markers were used to denote branch points and spines under X100 oil immersion objective. Spines were binned into 10μm bins along the length. Only one dendrite was analysed per cell, and 03 independent cells were analysed per animal.

In addition to spine analysis, in *Mglur5* mutant mice dendritic complexity was also analysed. Layer 4 spiny neurons localised within the PMBSF with cell bodies located at least 20μm below the surface of the section and neuronal dendrites that could be well isolated from its neighbouring neurons were identified for tracing. Dendrites were reconstructed in three dimension under X100 oil immersion objective and dendritic parameters were calculated using NeuroExplorer software.

2.3 Biochemical characterisations

2.3.1 Tissue preparation for biochemistry

Mice used at less than P10 were sacrificed by decapitation whilst older mice underwent cervical dislocation. Brains were removed rapidly and dissections were carried out in ice-cold 320mM sucrose solution containing 1mM EDTA and 5mM Tris at pH7.4. On the basis of the biochemical assay to be employed on the tissue collected, two distinct tissue dissections were carried out.

To collect 'barrel cortex' tissue for the developmental expression profiles of mGluR5, FMRP and PSD93, firstly, the two hemispheres were separated along the midline to carry out barrel cortex dissection on each individual cerebral hemisphere (Figure 2.3). Each hemisphere was placed on its medial surface and an anterior coronal cut was made where the olfactory tubercle joins the piriform cortex followed by a posterior cut where the hippocampus extends medio-laterally just prior to its curvature along the fornix. The resulting block of tissue was then placed on its anterior surface and a third cut was made at the junction between archicortex and neocortex. Finally, a fourth cut was made at a 40^{o}C angle to the third cut that would fall approximately where the barrels would start to appear. The dissected out tissue block was then manoeuvred onto its pial surface in order to extract all subcortical tissue with particular attention given to the removal of striatum (and white matter in older tissue) to obtain a tissue block that contained primarily the neocortical sheet which lies in the barrel region. Katnelson (2002) has shown an enrichment of layer 4 barrelfield by 70-80% in this neocortical sheet.

To collect neocortical tissue for synaptosome, PSD and synaptoneurosome preparations, the two hemispheres were separated along the midline and each hemisphere was placed on its medial surface as described in the afore mentioned section. In short the thalamus, entorhinal cortex, hippocampus and striatum were removed leaving only the neocortical sheet in a manner similar to that mentioned 2.2.1.

The tissue acquired by both methodologies was then immediately snap frozen on dry ice and kept at -80^{0}C until the time of use.

2.3.1.1 Barrel cortex homogenates for developmental expression profiles

Barrel cortices from wildtype C57BL/6JOla mice at P4, P7, P14, P21 and adult were homogenised in 200µl lysis buffer containing 50mM HEPES pH7.5, 1% Triton X-100, 50mM NaCl, Protease Inhibitors (Roche) and Phosphatase Inhibitor cocktails I and II (Sigma P2850 and P5276) using a glass-glass homogeniser. A modified Lowry assay was performed on the homogenates to determine the protein concentration according to the methods in Bio-Rad DC protein assay manual.

2.3.1.2 Neocortical synaptosomes and PSDs

Synaptosomes were prepared according to the methods described in (Dunkley et al., 1986). Both neocortices from three P7 *Mglur5* mutant mice were pooled together for one sample and were homogenised in ice-cold 320mM sucrose solution containing 1mM EDTA and 5mM Tris at pH7.4 with a teflon-glass homogeniser at 700rpm. This homogenisation was sufficiently strong enough to leave a presynaptic terminal with only a PSD attached without the presence of the postsynaptic terminal. A sample of the homogenate was saved at this point and the remaining homogenate was layered on a percol gradient that consisted of a 24%, 10% and 3% and was centrifuges at 15000rpm for 12min at 4°C. The synaptosome layer formed at the interface between the 24% and 10% percol layers was then carefully aspirated out and centrifuged again at 13000rpm for 30min at 4°C in ice-cold 320mM sucrose solution containing 1mM EDTA and 5mM Tris at pH7.4 to sediment out the synaptosomes. The synaptosome pellet thus formed was given two successive washes in cold Krebs buffer containing NaCl, KCl, $MgSO_4$, glucose, $Na_2HPO_4.12H_2O$, and HEPES at 13000rpm for 10min each. The synaptosome pellet at the end was lysed in lysis buffer containing 50mM HEPES pH7.5, 1% Triton X-100, 50mM NaCl, Protease Inhibitors (Roche) and Phosphatase Inhibitor cocktails I and II (Sigma P2850 and P5276).

Half of the synaptosome volume was further centrifuged at 36,800g for 45min at 4°C to obtain the PSD pellet that is a modified step from Walikonis et al., (2000). The supernatant was saved and the pellet was lysed in a volume equivalent to that of the supernatant.

A modified Lowry assay was performed on the homogenates, synaptosomes and PSDs to determine the protein concentration according to the methods in Bio-Rad DC protein assay manual.

2.3.1.3 Neocortical synaptoneurosomes

Synaptoneurosomes from *Fmr1* mutant mice and *Sap102*/*Psd95* double mutant mice were prepared according to the methods described in Quinlan et al., (1999). Both neocortices from one animal at either P7 or P14 were pooled together for one sample. Each sample was gently homogenised (20 even strokes) in ten times the volume in homogenisation buffer containing 10mM HEPES, 2mM EDTA, 2mM EGTA, 0.5mM DTT, Protease Inhibitors (Roche) and Phosphatase Inhibitor cocktails I and II (Sigma P2850 and P5276) using a glass-glass homogeniser. A sample of the homogenate was saved at this point and the remaining homogenate was extracted through a 2ml syringe using a 18 G needle and back filled with air. The homogenate was then filtered through a double-layered 100μm nylon mesh. The filtrate obtained through this was then extracted through a syringe again and filtered once more through a 5μm pore filter. The final filtrate was centrifuged at 1000g for 10min at 4°C. The supernatant after the centrifugation step was saved, and the resulting synaptoneurosome pellet was lysed in 100μl of 1% boiling SDS. The homogenate and supernatant volumes were also resuspended in 1% SDS and all samples (homogenate, supernatant and synaptoneurosome) were boiled at 100°C for 10min.

A modified Lowry assay was performed on the homogenates, supernatants and synaptoneurosomes to determine the protein concentration according to the methods in Bio-Rad DC protein assay manual.

2.3.1.4 Immunoblotting

SDS-polyacrylamide gel electrophoresis was carried out on samples prepared by boiling in Laemmli buffer containing 5% β-mercaptoethanol (Sigma-Aldrich, Dorset, UK). Samples were loaded 10μg per lane onto SDS mini-gel (the percentage of resolving gel was determined based on the size of the protein of interest and varied between either 6, 7 or 10%) with a 4% stacking gel. Gels were run at constant current in buffer containing Tris, glycine, SDS and dH_2O and protein gels were then electroblotted onto nitrocellulose membrane (Bio-Rad Laboratories, Hertfordshire, UK) in buffer containing Tris, glycine, methanol and dH_2O at constant current. To qualitatively determine if proteins were loaded equally, after transfer, blots were stained with either 1% (w/v) amido black or 0.1% (w/v) ponceau S (Sigma-Aldrich, Dorset, UK).

Blots were then probed with primary antibodies against proteins of interest either overnight or about 4hrs at room temperature. Blots for developmental expression profiles were probed with antibodies against mGluR5 (1:4000; Neuromics, Edina, US), FMRP (clone1C3 1:2000; Millipore UK Ltd, Hertfordshire, UK) and PSD93 (1:4000; Frontier Science Co Ltd, Hokkaldo, Japan). When probing blots for the expression of different synaptic proteins, each blot was cut in two halves with top half containing the protein of interest and the bottom half containing loading controls. In probing the top half for synaptic proteins in homogenates and/or synaptoneurosome preparations, primary antibodies against NR1 (1:1000; Frontier Science Co Ltd, Hokkaldo, Japan), NR2B (1:1000 or 1:5000; BD Transduction laboratories, Oxford, UK), GluR1 (1:1000; Frontier Science Co Ltd, Hokkaldo, Japan), GluR2/3 (Upstate Biotechnology, UK), PSD95 (1:10000; Millipore UK Ltd, Hertfordshire, UK or 1:4000; Frontier Science Co Ltd, Hokkaldo, Japan), SAP102 (1:1000 or 1:5000; Santa Cruz Biotechnology, California, US), PLCβ1 (1:8000 or 1:10000 Santa Cruz Biotechnology, California, US), SynGAP (1:4000-5000; Affinity Bioreagents, Cambridge, UK) and PKARIIβ (1:20000 BD Transduction laboratories, Oxford, UK) were utilised. The bottom half of immunblots were probed with primary antibodies against β actin (1:2000 or 1:20000; Millipore UK Ltd, Hertfordshire, UK) and synaptophysin (1:5000 or 1:10000; Synaptic Systems, Goettingen, Germany) to test whether these will be appropriate

loading controls. Primary antibodies were made in either D-MEM (GIBCO-Invitrogen, Paisley, UK) containing 5% foetal calf serum or 0.2-0.5% Triton X-100 or in Odyssey blocking buffer (Li-COR Biosciences UK Ltd, Cambridge, UK) in PBS containing 0.1% Tween. If primary antibodies were made up in the latter media, membranes were blocked in Odyssey blocking buffer in PBS for 30min prior to adding the primary antibody and 0.1% Tween. Also, if primary antibodies were made up in Odyssey blocking buffer in PBS, the antibodies gave an optimum signal at low concentrations, hence why there are two concentrations of primary antibody mentioned for several antibodies. Blots were washed in either tris base saline (TBS) containing Triton X-100 (if D-MEM was used as the media for antibodies) or in PBS containing Tween (if Odyssey blocking media was used for antibodies) before incubating in appropriate secondary antibodies (1:10,000, Invitrogen, San Diego, CA). Fluorescent conjugated secondary antibodies were made up in the same media as that used for primary antibodies, and blots were incubated in the secondary antibody for 30-45min at room temperature. Blots were then thoroughly washed in appropriate buffer and imaged on an Odyssey infrared imaging system (Li-COR Biosciences UK Ltd, Cambridge, UK).

2.3.1.5 Performing densitometry on immunoblots

Densitometry was performed using Image J gel analyzer software. As shown in figure 2.4, a rectangular sampling frame was drawn around the first lane to select it (Analyze>Gels>Select First Lane), and the same was done for subsequent lanes (Analyze>Gels>Select Next Lane). By using the command Analyze>Gels>Plot Lanes, a plot profile was drawn for each lane which showed intensity peaks for each protein band within a lane. The area under each intensity peak corresponding to the magnitude of each protein band was measured using the wand (tracing) tool and the data was then be copied into a Microsoft excel work sheet. The expression level of each of protein of interest was first normalised to the appropriate loading control. Data for mutants were then normalised to average wildtype expression where wildtype expression was considered to be 100%. The expression levels of protein of interest in each mutant analysed is presented as % levels normalised to wildtype (WT) ±SEM.

2.4 Statistical analysis

All statistical analysis was carried out in SPSS statistical analysis software or in Microsoft excel. The significances of all anatomical measurement parameters were determined by using either a two-tailed t test in cases where there were only two independent genotype groups or a one-way ANOVA where there were more than two independent groups. Where the parameter tested between the groups was found to be significant with an ANOVA, an appropriate *posthoc* test was performed to determine which pairs were significantly different. The details of the *posthoc* test are mentioned in the appropriate results section. For all biochemical analysis, one sample t tests (using two sided p values) were performed, in which the specified control value was set to be 100%.

2.5 Methodological considerations

2.5.1 The developmental expression profiles of FMRP and MAGUKs

In classic immunohistochemical paradigms, the tissue of interest is typically fixed with 4% PFA in PB to preserve its morphology. However, several studies have characterised that fixation can potentially mask the antigen eptiopes from the antibody, most commonly those of PSD proteins. The aldehyde is thought to cross link with tissue proteins, thereby altering the structure of the epitopes, which in turn masks the epitope structure. Moreover, the cross linking of tissue proteins with classic fixation paradigms can also affect the penetration of the antibodies to the postsynaptic sites (reviewed in Gasser et al., 2006). A number of solutions have been proposed in the literature to resolve this issue. For example, 1) antigen retrieval by heat treatment (Fritschy et al., 1998) 2) protease treatment to unmask the epitopes, which is carried out subsequent to clearing out the blood with PBS during classic fixation, but prior to circulating the fixative (Fukaya and Watanabe, 2000) and 3) short-fixation techniques or using less fixative concentration to attain a compromise between labelling efficiency and morphological preservation (Geiman et al., 2002; Sassoe-Pognetto et al., 1994).

To determine expression patterns of FMRP, antigen retrieval was performed on sections for 5min at 95^{0}C prior to incubating sections in rAM1 antibody (Ferrari et al., 2007). The retrieval of antigenicity with heat treatment greatly improved the intensity of the staining, thereby enhancing the signal from the specific antigen-antibody interaction from that of the background (Figure 2.5Ai and Aii).

To improve the immunohistochemical detection of PSD95 family MAGUK expression two different protocols were utilised. One was antigen retrieval as described above and the other was perfusing brains with 2% PFA in 0.1M PB and post-fixing for only 2-3hours at 4^{0}C. For example, immunoproduct for SAP102 was more intense and clearly evident in sections from a brain that was perfused with less fixative (Figure 2.5Bii) compared to SAP102 labelled sections from a brain that had undergone classic fixation (Figure 2.5Bi). Although the pattern of SAP102 is evident with classic fixation, it is not as intense or clearly distinguishable from the background compared to the SAP102 staining achieved by a light fixation.

2.5.2 The biochemical analysis of synaptic protein expression in homogenates and in synaptic sites

Neocortical homogenates

Neocortical homogenates encompass all material in the neocortical tissue and reflect global expression of proteins of interest. For example, in an excitatory neuron, it entails overall changes in both the cell body and dendritic material. Homogenates will also contain glia and blood cells.

Synaptic sites

There are several biochemical purification protocols utilised in the literature to isolate subcellular fractions (Bagni et al., 2000; Dunkley et al., 1986; Kennedy and Greengard, 1981; Quinlan et al., 1999; Sung et al., 2004; Villasana et al., 2006). In order to determine expression of synaptic proteins in synaptic sites, three specific biochemical purification methods that have been previously characterised were utilised in this thesis. The **synaptosome**

(Figure 2.6B) involves a very strong homogenisation, with a motorised Teflon-glass homogeniser at 700rpm with 10-15 up and down strokes. The synaptosome purification by density of cellular material yields the presynaptic compartment attached to PSD. Therefore, proteins that are in the presynaptic compartment such as synaptophysin and synaptic receptors that are already inserted and/or synaptic proteins that are firmly attached to the PSD are enriched in the synaptosome preparation (Barnett et al., 2006; Dunkley et al., 1986). The lysis and centrifugation step for **PSD** (Figure 2.6B) purification from the synaptosome preparation yields the PSD, while the supernatant contains the presynaptic cellular fractions and some of the proteins that are not firmly attached to the PSD.

The **synaptoneurosome** (Figure 2.6C) utilises a weak homogenisation using a glass-glass hand held homogeniser with 20 up and down strokes. The synaptoneurosome is prepared by a method of filtration based on sizes of cellular material to yield a presynaptic compartment attached to the postsynaptic "bag". The postsynaptic bag is pinched off at the base of the spine neck (Quinlan et al., 1999). The synaptoneurosome preparation is similar to the synaptosome preparation in that it contains the presynaptic proteins. However, in contrast to the synaptosome, the synaptoneurosome contains postsynaptic proteins that are at the perisynaptic sites and within the spine as well as what is inserted at the PSD or firmly attached to the PSD. Therefore proteins found in the synaptoneurosome preparation reflects a snap shot of what is at the PSD and what is available within the spine head as well as presynaptic components.

2.5.3 Challenges of quantifying synaptic proteins

Equal loading of samples while western blotting is at the heart of accurate quantification. To load equal amounts of total protein from each sample, proteins concentrations in homogenates, synaptosome and synaptoneurosome preparations were determined using a modified Lowry assay. Proteins were loaded 10μg/lane to ensure that the total protein loaded was in equal amounts between different samples. However, using this technique a certain amount of experimental pipetting error can be introduced when loading the protein sample into each lane. Therefore, in addition to

loading equal amount of total protein, all blots were stained with amido black or Ponceau S and for a loading control that does not significantly vary between samples in addition to the protein of interest. While loading controls are the "gold standard", finding a loading control that is constant across development and is not altered by the genetic deletion is not trivial. Synaptogenesis is an incredibly dynamic process during first three postnatal weeks in S1 (Lendvai et al., 2000; White et al., 1997); therefore, slight differences in developmental age can cause large differences in protein expression of synaptic markers. In this thesis β actin, which is often used as a loading control as it is a constitutively expressed postsynaptic marker in the spine, and synaptophysin, a constitutively expressed presynaptic vesicular protein were tested as loading controls. Synaptophysin therefore is also indicative of number of synapses. The levels of β actin and synaptophysin in homogenates and synaptosomes/synaptoneurosomes were examined by equal loading of tissue and staining of blots with amido black or Ponceau S (see below). Percentage levels of loading controls for mutant mice analysed are presented normalised to mean of wildtype±SEM.

In homogenates from ***Mglur5*** **mutants**, β-actin levels did not differ significantly between the genotypes (n=6) (Figure 2.7A), however, levels of β actin were variable at P7. Synaptophysin levels were significantly reduced in *Mglur5*$^{-/-}$ mice compared to *Mglur5*$^{+/+}$ mice (63.14±7.13; $p<0.01$; one sample t test, n=5) suggesting that there might be fewer synapses present in *Mglur5*$^{-/-}$ mice compared to *Mglur5*$^{+/+}$ (Figure 2.7). Therefore, in the results presented in chapter 3, β-actin was utilised as the best possible loading control for analysing levels of synaptic protein expression in *Mglur5* mutant mice.

In *Fmr1* mutant mice, levels of β actin in both homogenates (n=3 at P7; n=5 at P14) (figure 2.8A and B) and synaptoneurosomes (n=6 at P7; n=5 at P14) (Figure 2.8A and B) were highly variable at P7 and P14. Comparatively, levels of synaptophysin varied less between genotypes in both homogenates (n=3 at P7; n=5 at P14) and synaptoneurosomes (n=6 at P7; n=5 at P14) at both ages examined (Figure 2.8A and B). Levels of synaptophysin were used as the loading control for biochemical analysis carried out in chapter 4 on *Fmr1* mutant mice.

In double mutants of *Sap102* and *Psd95*, levels of β actin were highly variable in both homogenates and synaptoneurosomes (Figure 2.9A). In comparison, levels of synaptophysin were less variable between genotypes in both homogenates and synaptoneurosomes (Figure 2.9B). In chapter 6, synaptophysin was used as the loading control in normalising expression of protein of interest.

The first and second week of postnatal development examined here corresponds to the onset of synaptogenesis and the peak of synaptogenesis (White et al., 1997). There is a rapid increase in spinogenesis between P6-P8, and P13-P14 denotes the peak of synaptogenesis (White et al., 1997). In turn, there is also a high turnover of filopodia and spines of an immature appearance during the process of forming persistent spines (Lendvai et al., 2000). Both filopodia and immature spines as well as spine necks of mature spines are enriched with actin (Figure 2.6) (Matus, 2000). Therefore, the variable levels of β actin may reflect the high turnover in actin dynamics during this developmental period.

3

Key findings:

1. Expression of mGluR5 is selectively upregulated in the cortex during the first two postnatal weeks, and mGluR5 expression in both the VpM and nVp is developmentally transient.

2. The expression of mGluR5 is postsynaptic in P7 cortical synapses.

3. Loss of mGluR5 does not affect general cortical arealisation or lamination.

4. Genetic deletion of *Mglur5* causes defects in afferent segregation in layer 4, VpM and nVp in the area subserving the AS region. Moreover, afferent segregation within the PMBSF in layer 4 of *Mglur5*$^{-/-}$ mice is less defined compared to both *Mglur5*$^{+/-}$ and *Mglur5*$^{+/+}$ mice.

5. Layer 4 cellular segregation is mGluR5 dose dependent and defects in segregation due to loss of mGluR5 do not arise from a developmental delay.

6. There is decreased spine density in layer 4 spiny neurons at P21-23 in both *Mglur5*$^{-/-}$ and *Mglur5*$^{+/-}$ mice compared to *Mglur5*$^{+/+}$ mice.

3 mGluR5 Regulates Glutamate Dependent Development of Mouse S1

3.1 Introduction

FXS is the most common form of inherited MR and its symptoms of developmental delay in motor skills and heightened sensory responses are thought to be a manifestation of altered synaptic connectivity. The 'mGluR theory of fragile X mental retardation' proposed by Bear et al., (2004) predicts that the loss of FMRP in FXS leads to exaggerated effects of Gp1 mGluR signalling on the basis that several lines of evidence suggest a correlation between exaggerated protein synthesis dependent effects of Gp1 mGluR and neurological symptoms of FXS (refer to table 1.2). Therefore, it is thought that FMRP regulates and stabilises responses to glutamatergic signalling via Gp1 mGluRs (reviewed in Bear et al., 2004; Ronesi and Huber 2008). In order to examine interactions between FMRP and mGluR5 in S1, it is important to first characterise the role of glutamatergic signalling via mGluR5 in S1 differentiation.

Glutamatergic receptors and their associated signalling cascades have been shown to play a fundamental role in activity dependent neocortical differentiation of the mouse S1 (Erzurumlu and Kind, 2001; Kind and Neumann, 2001; Barnett *et al.*, 2006; Inan and Crair, 2007; Fox, 2008). The anatomical differentiation of S1 follows three key sequential steps. The TCAs segregate into discrete 'barrel-like' bundles (Rebsam *et al.*, 2002), layer 4 cells then aggregate around the TCA-layer 4 synapse to form a cell-dense barrel wall and cell-sparse barrel hollow, and layer 4 cells prune dendrites outside a TCA patch while elaborating within the appropriate TCA patch (Woosley and Van der Loos, 1970) (refer to section 1.3.1.2). Collectively several studies have shown that the genetic deletion of the main subunit NR1 of NMDAR disrupts all three key sequential steps of anatomical differentiation of S1 suggesting that glutamatergic receptors play a crucial regulatory role in the formation and refinement of TCA-layer 4 synaptic

connections as described in detail in section 1.3.6 (Datwani et al., 2002; Iwasato et al., 2000; Lee et al., 2005).

Hannan et al., (2001) reported that loss of mGluR5 also leads to failure of layer 4 cell segregation into barrels. In *Mglur5*$^{-/-}$ mice, TCAs corresponding to the PMBSF region segregate into rows but not into discrete patches within a row and there is no segregation visible in the AS region (Hannan et al., 2001). Gp1 mGluRs coupled to Gq proteins by definition mediate PI hydrolysis via PLCβ1. The Gp1 mGluR agonist stimulated IP3 production in both rodent barrel (Bevilacqua et al., 1995) and cat visual (Dudek et al., 1989) cortices are developmentally regulated. In mouse cortical synaptoneurosomes, PI hydrolysis by Gp1 mGluR activation is PLCβ1 dependent during the first postnatal week (Hannan et al., 2001). The genetic loss of *Plcβ1* results in the failure of barrel formation despite normal TCA segregation suggesting that while mGluR5 signalling via PLCβ1 plays a regulatory role in layer 4 cellular segregation, the role of mGluR5 in segregation of TCAs may be independent of PLCβ1 signalling (Hannan et al., 2001). Welker et al., (1996) first characterised a spontaneous mutation that results in loss of both TCA and layer 4 cellular segregation referred to as the 'barrelless' mutants. Subsequently, this spontaneous mutation was identified as a mutation in *Adcy1*, the gene that produces AC1, another downstream target of Gp1 mGluRs (Abdel-Majid et al., 1998; Welker et al., 1996). Therefore, TCA segregation defects seen in *Mglur5*$^{-/-}$ mice could be mediated by mGluR5 signalling via AC1. However, recent findings by Iwasato et al., (2008) show that cortex specific deletion of *Adcy1* results in decreased layer 4 cellular segregation but normal TCA segregation into whisker related patterns. The neuroanatomical phenotypes due to the selective loss of NR1 and AC1 in the cortex and the loss of mGluR5 and PLCβ1 globally, provide evidence that clustering of TCAs does not necessarily mediate aggregation of layer 4 cells indicating that these two events can be genetically dissociated.

Despite the fact that genetic deletion of mGluR5 causes altered TCA segregation and layer 4 cellular segregation (Hannan et al., 2001), it is yet unclear whether mGluR5 regulates barrel formation pre or postsynaptically. Previously, presynaptic expression of mGluR5 has been suggested in synaptosomal preparations from P21 mice (Rodriguez-Moreno et al., 1998)

and in adult rat cortex mGluR5 expression is predominantly shown to be postsynaptic, however some immunoreactivity is also found in presynaptic axon terminals (Romano et al., 1995). A developmental immunogold EM study in rat cortex demonstrates the presence of mGluR5 confined to postsynaptic elements, including dendrites and dendritic spines at P3 and P10 in cortical layer 1 and layer 2/3 respectively (Lopez-Bendito et al., 2002). Moreover, several EM studies in the adult rat brain show localisation of mGluR5 postsynaptically in hippocampal and cerebellar synapses (Lujan et al., 1996; Nusser et al., 1994; Shigemoto et al., 1997).

To fully understand the role of mGluR5 in glutamatergic dependent early whisker related pattern formation in S1, it is important to examine the expression pattern of mGluR5 during early postnatal development when anatomical segregation of S1 takes place. *In situ* hybridisation studies in the rat brain show differential regulation of Gp1 mGluRs, mGluR1 and mGluR5 transcripts during development. *Mglur1* expression is low at birth and progressively increases during postnatal development whereas *Mglur5* is high during early postnatal developmental and declines with progressive age (Catania et al., 1994; Romano et al., 1996; Yamaguchi and Nakanishi, 1998). In rat barrel cortex, the expression pattern of mGluR5 in layer 4 is localised to 'barrel hollows' and persists through to adult but its expression is less pronounced after P14. Furthermore, mGluR5 is the most abundant Gp1 mGluR subtype in the forebrain (Blue et al., 1997; Lopez-Bendito et al., 2002). In contrast, mGluR1α is not localised in a somatotopic pattern in the barrel cortex during S1 development (Lopez-Bendito et al., 2002). Muñoz et al., (1999) in a developmental study in the mouse brain show that mGluR1α and mGluR5 are both expressed in a 'barrel-like' pattern throughout development in layer 4, which is incongruent with previous immunohistochemical studies in the rat brain. This discrepancy between Gp1 mGluR expression profiles in rat vs mouse brain could be due to cross-reactivity among Gp1 mGluR antibodies utilised or a difference in species.

In addition to the regulatory role of mGluR5 in layer 4 barrel formation characterised by Hannan et al., (2001), there is substantial evidence to suggest a regulatory role for mGluR5 in neuronal morphology. Increased density of spines with an immature appearance is associated with FXS and is

thought to be the crux of its altered synaptic connectivity (Bagni and Greenough, 2005; Galvez and Greenough, 2005; Rudelli et al., 1985). In agreement with the mGluR theory of fragile X, agonist stimulation of Gp1 mGluRs in hippocampal slice cultures and dissociated cultures has been shown to result in elongation of dendritic spines that is blocked by Ca^{2+} chelation and protein synthesis inhibition (Vanderklish and Edelman, 2002). In addition, blockade of Gp1 mGluRs both *in vivo* and *in vitro* results in reduced arborisation of cerebellar Purkinje cells (Catania et al., 2001). The Ca^{2+} release from intracellular stores in cultured hippocampal neurons affects spine morphology (Korkotian and Segal, 1999) and intracellular Ca^{2+} release is typically regulated by PLCβ1 activity. Signalling through PLCβ1 has also been suggested to play a role in development of S1 connectivity (Spires et al., 2005). In *Plcβ1*$^{-/-}$ mice, synapse formation in layers 2-4 is disrupted and there are also defects in spine maturation as marked by a decrease in the density of mushroom spines in layer 5 pyramidal cells (Spires *et al.*, 2005). Moreover, Golgi analysis of layer 4 spiny cells in *Plcβ1*$^{-/-}$ mice shows increased spine density but normal dendritic complexity (Upton, L., unpublished data).

The main focus of this chapter is to explore the role of mGluR5 in the anatomical differentiation of S1 during both early and late S1 development. Specifically, this chapter aims to examine several hypotheses. 1) mGluR5 expression in layer 4 is abundant during mouse S1 development. 2) loss of mGluR5 misregulates more fundamental processes such as early patterning of the cortex and general cortical development on the basis of the fact that Gp1 mGluRs also seem to play a more elementary role in brain development by regulating proliferation, differentiation, and survival of neural stem/progenitor cells (Brazel et al., 2005; Cappuccio et al., 2005; Di Giorgi Gerevini et al., 2004). 3) mGluR5 plays a postsynaptic role in barrel formation in a dose dependent manner and barrel defects in *Mglur5*$^{-/-}$ mice is not due to a developmental delay. 4) mGluR5 regulates whisker related patterning in the VpM and nVp. 5) mGluR5 regulates synaptogenesis in layer 4 of S1.

3.2 Results

3.2.1 Expression of mGluR5 is selectively upregulated during the first two weeks of postnatal development

As mentioned above there are discrepancies in the expression patterns for Gp1 mGluRs, mGluR1 and mGluR5 during development between rat and mouse brains (Blue et al., 1997; Lopez-Bendito et al., 2002; Munoz et al., 1999). In agreement with early defects in barrel formation (Hannan et al., 2001), this chapter tests the hypothesis that there is high expression of mGuR5 during early S1 with a mGluR5 specific antibody that showed no staining in *Mglur5*$^{-/-}$ mice compared to *Mglur5*$^{+/+}$ mice (Figure 3.1). The expression profile of mGluR5 in mouse brain was examined throughout postnatal development in both coronal and tangential sections to identify its cellular expression pattern.

The expression of mGluR5 was evident throughout development in the hippocampus (Hip), cortex (Ctx) and striatum (St) (Figure 3.2). In the striatum, mGluR5 expression appeared to be robust throughout development (Figure 3.2Ai-Ei). In both cortex and hippocampus, mGluR5 staining appeared as diffused neuropilar and no punctate staining was evident. In the hippocampus (Figure 3.2Aii-Eii), throughout development, mGluR5 staining was concentrated in the basal dendrites of pyramidal cells in striatum oriens and in striatum radiatum that contain the schaeffer collateral pathway. There was little mGluR5 staining in the cell body layer of CA1-CA3, stratum pyramidal, and cell body layer of DG (dentate gyrus), stratum granulosum. The staining in cell bodies of granule cells was more prominent at P21 and adult (AD). There was also weaker expression for mGluR5 in the polymorphic layer of DG throughout development. During early development, at P4 (Aii) and P7 (Bii) there was high mGuR5 expression in all compartments apart from the cell bodies of pyramidal and granule cells. After P7, the intensity of mGluR5 expression in strata lacunosum containing the schaeffer collateral pathway and lucidum containing the mossy fibre pathway progressively grew weaker with age. The layer specific expression of mGluR5 during development of mouse hippocampus agrees well with the mGluR5 expression characterised in the rat hippocampus (Lopez-Bendito et al., 2002).

In the cortex, diffuse neuropilar staining for mGluR5 was evident in all cortical layers throughout development with intense expression in layers 4 and 5 during the first two postnatal weeks (Figure 3.2Aiii-Eiii). At all ages, even as early as P4 there was intense albeit diffused staining in the neuropil localised to 'barrel hollows' in layer 4, which is consistent with previous findings in the rat cortex (Lopez-Bendito et al., 2002). mGluR5 staining on tangential sections through layer 4 appeared as dense neuropil staining within barrels (Figure 3.3A-E) with little if any staining in septal regions at all ages confirming the 'barrel' expression pattern observed in coronal sections. This 'barrel' expression of mGluR5 in layer 4 suggests that mGluR5 immunoreactivity is either present in dendrites of layer 4 cells or in TCAs. Qualitatively, mGluR5 cortical expression pattern was maintained throughout development with slightly less intense staining at P21 and in the adult (Figure 3.2Diii-Eiii), therefore, expression of mGluR5 within the barrel cortex was determined by examining levels of mGluR5 expression in barrel cortex homogenates during S1 development (Figure 3.2F). The developmental immunoblot showed expression of mGluR5 at all ages with levels peaking at P14 that correlates well with the peak of synaptogenesis (White *et al.*, 1997).

In contrast to both cortical and hippocampal expression of mGluR5 that is maintained throughout S1 development, its expression in the thalamic VpM was strongest at P4 and P7 (Figure 3.4A and B) and appeared less intense at P14. After P21, mGluR5 expression in VpM declined below detection (Figure 3.4D and Figure 3.2Ci-Ei). Muñoz et al., (1999) show expression of mGluR5 in trigeminal nuclei nVc and nVp at P9 and thereafter, hence, the expression of mGluR5 in the brain stem was also examined during the early time points at which formation of barrelettes take place. In trigeminal nucleus nVp, expression of mGluR5 was present at P0 and there was dense expression from P0 to P14 (Figure 3.5). From P4 onwards, staining for mGluR5 was localised in a 'barrelette-like' pattern throughout development (Figure 3.5C-F), although its expression was downregulated at P21 and in the adult (Figure 3.5F).

In summary, expression of mGluR5 was most abundant in the trigeminal system (in layer 4, VpM and nVp) during the first two weeks of postnatal

development, which is consistent with the first hypothesis that mGluR5 expression is high during the anatomical segregation of barrels (Barnett et al., 2006a). In addition to peak expression of mGluR5 coinciding with the segregation of layer 4 cells into barrels, it is also synchronised with the rapid phase of synaptogenesis during early S1 development (Lendvai et al., 2000; White et al., 1997) suggesting that mGluR5 may also play a role in synaptogenesis in addition to barrel formation.

3.2.2 Expression of mGluR5 is postsynaptic at P7

Hannan et al., (2001) hypothesised that the barrel defects seen in *Mglur5*$^{-/-}$ mice compared to *Mglur5*$^{+/+}$ is due to aberrant postsynaptic signalling in layer 4 neurons. At P7, there was diffuse neuropil staining of mGluR5 localised in a 'barrel-like' manner that may either correspond to expression of mGluR5 in layer 4 dendrites and/or expression in TCAs. Moreover, mGluR5 immunoreactivity in dense neuropil patches was present as early as P4, a time point when TCAs have already segregated in layer 4 (Barnett et al., 2006a). The expression of mGluR5 was also transient in the VpM raising the possibility that mGluR5 is transiently expressed in TCAs during development. Therefore, immuno EM was carried out to test the hypothesis whether mGluR5 acts pre and/or postsynaptically during barrel formation. The tissue processed for EM was taken by dissecting out an area containing only intensely labelled mGluR5 patches in layer 4 in coronal sections (refer to section 2.2.3). In all labelled synapses observed in this tissue, DAB immunoproduct for mGluR5 was only found postsynaptically (Figure 3.6A-C). Furthermore, mGluR5 expression was enriched in the biochemically purified synaptic preparations of synaptosomes and PSDs (Figure 3.6D). The EM data and biochemical data at P7 collectively suggests a postsynaptic locus of action for mGluR5 in barrel formation consistent with the hypothesis proposed by Hannan et al., (2001). The expression of mGluR5 at P4 in patches may reflect either glutamatergic activity dependent selective expression of mGluR5 in layer 4 dendrites contacted by TCAs or selective elaboration of dendrites within a patch already by P4. However, a presynaptic expression of mGluR5 at P4 in TCAs cannot be ruled out unless an EM analysis is carried out at P4.

3.2.3 Loss of mGluR5 does not affect general cortical development

López-Bendito et al., (2002) has shown transient expression of mGluR5 in Cajal-Retzius cells of the rat hippocampus and cerebral cortex at E18. Cajal-Retzius cells are present during early neocorticogenesis and disappear around the end of second postnatal week and they direct laminar organisation of neocortex through the release of the glycoprotein reelin (Frotscher, 1998). The transient expression of mGluR5 in Cajal-Retzius cells and its expression at P0 (Munoz et al., 1999) raises the possibility that mGluR5 may play a regulatory role in cortical patterning and cell migration.

To address the hypothesis that mGluR5 plays a role in fundamental processes such as cortical patterning and general cortical development, neocortical arealisation and cortical lamination were examined in the *Mglur5*$^{-/-}$ and *Mglur5*$^{+/-}$ mice compared to *Mglur5*$^{+/+}$ mice. Levels of mGluR5 were investigated in *Mglur5*$^{-/-}$ (n=4) and *Mglur5*$^{+/-}$ mice (n=3) to determine whether there is a dosage dependent effect of mGluR5 on cortical development. Levels of mGluR5 were decreased approximately 50% in *Mglur5*$^{+/-}$ mice (50.00±12.51; *Mglur5*$^{+/-}$ vs *Mglur5*$^{+/+}$ $p<0.05$, one sample t test) whereas no protein was detected in the *Mglur5*$^{-/-}$ mice (Figure 3.6D) relative to *Mglur5*$^{+/+}$ mice. The general body weight (Figure 3.7D) and neocortical area (Figure 3.7E) were examined in *Mglur5*$^{-/-}$ (n=9) and *Mglur5*$^{+/-}$ mice (n=9) and found to be comparable to that of *Mglur5*$^{+/+}$ mice (n=6) at P7 suggesting that genetic loss of *Mglur5* does not alter general body weight or cortical development. To examine if genetic loss of mGluR5 affected general patterning of neocortex devoted to various sensory areas, tangential sections were reacted with 5HTT to stain subregions of S1 and other sensory areas such as visual cortex (Figure 3.7). The size of whole S1 area, the area of PMBSF and AS together and PMBSF area only were all comparable between genotypes. Moreover, there were no differences between the areas of visual cortex among genotypes (Figure 3.7F). In addition, positioning of PMBSF within the neocortical sheet was unaltered in *Mglur5*$^{-/-}$ and *Mglur5*$^{+/-}$ mice compared to *Mglur5*$^{+/+}$ mice (Figure 3.7G). Collectively, these data negate the original hypothesis and suggest that loss of mGluR5 does not affect cortical patterning or general cortical development at P7. Finally, to examine whether loss of mGluR5 affected cortical lamination, coronal sections containing AS region

and PMBSF region were stained with thionin to demarcate different cortical layers and reacted with calretinin to stain the boundary between layers 4/5 (Figure 3.8). No differences were found in the thickness of layer 1-6 (Figure 3.8M) and layers 1-4/5 (Figure 3.8N) in *Mglur5*$^{-/-}$ (n=5) and *Mglur5*$^{+/-}$ mice (n=7) compared to *Mglur5*$^{+/+}$ mice (n=9) suggesting that genetic loss of mGluR5 does not alter cortical lamination.

Moreover, approximately 50% reduction in mGluR5 levels did not affect segregation of TCAs into patches in layer 4 of S1 as no differences were observed in patterning of TCAs within S1 between *Mglur5*$^{+/-}$ mice and *Mglur5*$^{+/+}$ mice. Hannan et al., (2001) reported that complete loss of mGluR5 results in deficits in TCA segregation such that only fused rows are observed within the PMBSF and that segregation is completely absent in the AS region. In contrast to these original findings, more than 20 animals analysed for this chapter showed segregation of TCAs into patches within the PMBSF in *Mglur5*$^{-/-}$ mice (Figure 3.7C), however, the TCA segregation was not as clearly defined as that in *Mglur5*$^{+/-}$ mice (Figure 3.7B) and *Mglur5*$^{+/+}$ mice (Figure 3.7A). Consistent with Hannan et al., (2001), no TCA segregation patterns were observed in the AS region in *Mglur5*$^{-/-}$ mice analysed here (Figure 3.7A). The potential reasons for the variable expressivity of TCA phenotype in *Mglur5*$^{-/-}$ mice will be discussed in section 3.3.1.

3.2.4 Deficits in layer 4 cellular segregation is mGluR5 dose dependent and is not due to a developmental delay

Given the variable expressivity of the TCA phenotype in *Mglur5*$^{-/-}$ mice, loss of mGluR5 on layer 4 cellular segregation was characterised in the existing colony (Figure 3.9). In addition to examining *Mglur5*$^{-/-}$ mice, layer 4 cellular segregation was also analysed in *Mglur5*$^{+/-}$ mice to test whether there is a dose dependent effect of mGluR5 on barrel formation. In agreement with the original findings of Hannan et al., (2001) there was near complete loss of layer 4 cellular segregation in *Mglur5*$^{-/-}$ mice at P7 (Figure 3.9C). Interestingly, thionin stained tangential sections through layer 4 in S1 from *Mglur5*$^{+/-}$ mice (Figure 3.9B) showed slightly less defined barrels compared to *Mglur5*$^{+/+}$ mice (Figure 3.9A). To determine if in fact there is a dose dependent effect of mGluR5, layer 4 cellular distribution was quantified at P7 in

tangential sections through layer 4 from *Mglur5*$^{+/-}$ mice and *Mglur5*$^{+/+}$ mice. Tangential sections were double labelled with Topro3 and 5HTT to identify cell nuclei and TCA patches respectively (refer to chapter 2). The analysis of cells in the barrel wall to hollow showed a significant decrease in the ratio of cell density in the barrel wall:hollow in *Mglur5*$^{+/-}$ mice (1.43±0.04, n=11) relative to *Mglur5*$^{+/+}$ mice (1.79±0.02, n=5) (p=0.0001, two-tailed t test) (Figure 3.9I). This confirms the hypothesis that layer 4 cellular segregation is mGluR5 dose dependent. However, the size of the TCA patch corresponding to the barrel analysed did not differ significantly between *Mglur5*$^{+/-}$ mice and *Mglur5*$^{+/+}$ mice (Figure 3.9H).

The layer 4 cellular segregation of *Mglur5*$^{-/-}$ mice at P14 and P21 was then qualitatively analysed by staining tangential sections with thionin to determine if barrel defects resulting from loss of mGluR5 at P7 represented a developmental delay in layer 4 cellular segregation (Figure 3.10). At both P14 (Figure 3.10Bi-iii) and P21 (Figure 3.10Ci-iii), although there were hints of cellular segregation, the layer 4 cellular distribution was similar to that seen at P7 (Figure 3.10Ai-iii) indicating that there was no improvement of layer 4 cellular segregation with age in *Mglur5*$^{-/-}$ mice. This suggests that barrel deficits in S1 resulting from the loss of mGluR5 are not due to a general delay in cortical development.

3.2.5 Loss of mGluR5 disrupts segregation of barrelettes and barreloids

During the first week of development, expression of mGluR5 was abundant in the VpM of the thalamus and the nVp of brainstem. In addition, no segregation of TCAs was evident in the AS region at P7 in *Mglur5*$^{-/-}$ mice. On the basis of these findings the whisker related patterns in the brainstem and thalamus at P7 were examined to determine whether the segregation of afferents into barrelettes and barreloids was affected by loss of mGluR5. In the brainstem, barrelette pattern in *Mglur5*$^{+/-}$ mice (n=9) (Figure 3.11B) was comparable to that in *Mglur5*$^{+/+}$ mice (n=2) (Figure 3.11A). However, in *Mglur5*$^{-/-}$ mice (n=8), while segregation of barrelettes was evident in the region corresponding to the PMBSF, it was hard to decipher barrelette

patches arranged in a clear row in the region corresponding to the AS (Figure 3.11C).

The barreloid pattern was variable in *Mglur5*$^{+/-}$ mice (Figure 3.11E) compared to *Mglur5*$^{+/+}$ mice (Figure 3.11D). In seven *Mglur5*$^{+/-}$ mice analysed, five had barreloid patterns comparable to *Mglur5*$^{+/+}$ mice. However, in the other two *Mglur5*$^{+/-}$ mice, segregation was clear in the region corresponding to the PMBSF region but only partial segregation was observed in AS region compared to *Mglur5*$^{+/+}$ mice. In all *Mglur5*$^{-/-}$ mice (n=5) there was clear segregation within barreloids in the area corresponding to the PMBSF, however there was near complete loss of segregation in the area corresponding to the AS region (Figure 3.11F). Therefore, the deficits in segregation of TCAs in the AS region observed in layer 4 of *Mglur5*$^{-/-}$ mice may arise from defects of patterning at relay stations in VpM and nVp of the trigeminal system and awaits analysis from a cortex specific knockout of mGluR5.

3.2.6 mGluR5 plays a role in synaptogenesis during late S1 development

Several lines of evidence suggest a role for mGluR5 in regulating neuronal morphology, thereby influencing formation of synaptic connectivity (Catania et al., 2001; Korkotian and Segal, 1999; Spires et al., 2005; Vanderklish and Edelman, 2002). The immunohistochemistry data shown here also showed high expression of mGluR5 in the barrel cortex during the first two weeks of development with levels peaking at P14 corresponding well with peak of synaptogenesis (White et al., 1997). In order to test the hypothesis that mGluR5 plays a role in synaptogenesis, Golgi analysis was carried out on tangential section from P21-P23 *Mglur5*$^{-/-}$ mice (n=7), *Mglur5*$^{+/-}$ mice (n=7) and *Mglur5*$^{+/+}$ mice (n=7). At this time point synapses have already undergone the rapid phase of synaptogenesis during the first two weeks of development and are considered less 'plastic' and spine density is relatively stable compared to early S1 development (Lendvai et al., 2000; White et al., 1997). The n for each group represents the number of animals, and from each animal three different dendrites from three different layer 4 spiny cells were analysed.

Firstly dendritic complexity of layer 4 neurons was characterised by analysing isolated Golgi filled layer 4 spiny neurons within the PMBSF from *Mglur5*$^{-/-}$ mice (Figure 3.12C and F), *Mglur5*$^{+/-}$ mice (Figure 3.12B and E) and *Mglur5*$^{+/+}$ mice (Figure 3.12A and D). No significant differences were observed in the number of dendritic crossings with increasing distance from the cell body as a function of genotypes by scholl analysis (Figure 3.12G). Similarly, analysis of total dendritic length or number of bifurcations yielded no significant differences between genotypes (Figure 3.12H and I). Hence loss of mGluR5 does not seem to regulate general dendritic elaboration or complexity of layer 4 spiny neurons.

However, spine density/10μm was significantly reduced (Figure 3.13A and C) in both *Mglur5*$^{-/-}$ mice (11.46±0.46) and *Mglur5*$^{+/-}$ mice (11.89±0.44) compared to *Mglur5*$^{+/+}$ mice (14.48±0.61) (*Mglur5*$^{+/+}$ *vs Mglur5*$^{-/-}$ $p<0.001$; *Mglur5*$^{+/+}$ *vs Mglur5*$^{+/-}$ $p<0.001$; ANOVA with a *post hoc* Bonferroni test), and this decrease in spine density was observed along the dendritic length from the cell body (Figure 3.13B). The decreased spine density in *Mglur5*$^{-/-}$ mice and *Mglur5*$^{+/-}$ mice could be due to several factors such as (1) an increase in dendritic length but no overall change in number of spines, (2) no change in dendritic length but a decrease in number of spines and/or (3) an increase in dendritic length as well as a decrease in spine number. The average length of dendrites analysed for spine density did not differ significantly between genotypes consistent with normal dendritic complexity revealed by scholl analysis. However, average number of spines per dendrite was significantly reduced in both *Mglur5*$^{-/-}$ mice (140.14±13.07) and *Mglur5*$^{+/-}$ mice (165.95±8.72) relative to *Mglur5*$^{+/+}$ mice (193.62±17.50) (*Mglur5*$^{+/+}$ *vs Mglur5*$^{-/-}$ $p<0.001$; *Mglur5*$^{+/+}$ *vs Mglur5*$^{+/-}$ $p<0.046$; ANOVA with a *post hoc* Bonferroni test) (Figure 3.13C). Therefore the decreased spine density is due to a decrease in number of spines suggesting that genetic deletion of mGluR5 results in a decreased number of synapses per dendritic length onto layer 4 cells, which agrees with the hypothesis that mGluR5 plays a role in layer 4 synaptogenesis.

During early S1 development there is a rapid phase of synaptogenesis (Lendvai et al., 2000; White et al., 1997) thus suggesting that levels of synaptic markers will be unregulated corresponding to the increased number

of synapses. To determine if levels of synaptic markers during early S1 development were altered coinciding with decreased spine density, levels of synaptic proteins were analysed in neocortical homogenates at P7. The % expression of synaptic proteins normalised to expression in *Mglur5*$^{+/+}$ mice was quantified (refer to sections 2.3.14 and 2.4.3) and statistical analysis was performed using a one sample test (refer to table in Figure 3.14). In agreement with a decreased spine density, levels of postsynaptic markers such as NR2B, SAP102 and PSD95 were significantly reduced in neocortical homogenates of *Mglur5*$^{-/-}$ mice compared to *Mglur5*$^{+/+}$ mice at P7. Moreover, levels of synaptophysin, a presynaptic vesicular protein were also significantly reduced consistent with the hypothesis that there is a decrease in number of synapses onto layer 4 neurons in both *Mglur5*$^{-/-}$ mice and *Mglur5*$^{+/-}$ mice compared to *Mglur5*$^{+/+}$ mice. Levels of SynGAP, PLCβ1 and PKARIIβ were unaltered in *Mglur5*$^{-/-}$ mice compared to *Mglur5*$^{+/+}$ mice at P7 (Figure 3.14).

Collectively, data presented here suggests a role for mGluR5 in spinogenesis/synaptogenesis but not in dendritic complexity during late S1 development.

3.3 Discussion

Previously, Hannan et al., (2001) show that mGluR5 plays a regulatory role in barrel formation. To further characterise the role of mGluR5 in the development of S1, the developmental expression profile of mGluR5 was firstly characterised to determine when and where in development its expression is high. The expression of mGluR5 was present in layer 4 localised to a 'barrel-like' pattern throughout development. Immuno EM demonstrated a postsynaptic localisation of mGluR5 in P7 cortical synapses and its expression was enriched in both the synaptosome and the PSD. Together these data characterise the presence of mGluR5 in layer 4 dendrites and spines suggesting a postsynaptic mode of action for mGluR5 in regulating barrel formation. Consistent with this idea, loss of mGluR5 resulted in deficits in layer 4 cellular segregation into barrels in a dose dependent manner whereas general cortical development, lamination and arealisation were not

affected. Furthermore, segregation of TCAs was absent in the AS region of *Mglur5*$^{-/-}$ mice and TCA segregation in PMBSF was less defined compared to *Mglur5*$^{+/+}$ mice. As well as defects in the patterning of TCAs in the area corresponding to AS region in layer 4, loss of mGluR5 disrupted formation of whisker related patterning corresponding to AS region at the nVp and VpM suggesting that mGluR5 may regulate whisker related patterning throughout the trigeminal system. In barrel cortex homogenates, expression pattern of mGluR5 correlated well with synaptogenesis during S1 development with mGluR5 gradually increasing during the first two postnatal weeks to peak at P14. During late S1 development, layer 4 spiny cells show decreased spine density in both *Mglur5*$^{-/-}$ mice and *Mglur5*$^{+/-}$ mice relative to *Mglur5*$^{+/+}$ mice but their dendritic complexity was normal. Consistent with a general decrease in number of synapses several postsynaptic markers and the presynaptic marker, synaptophysin in neocortical homogenates from *Mglur5* mutants were also downregulated compared to *Mglur5*$^{+/+}$ mice. Together these findings agree well with a putative role for mGluR5 in synapse formation. Therefore, glutamatergic signalling via mGluR5 play a regulatory role in S1 differentiation during both early and late S1 development.

3.3.1 The variable expressivity of barrel phenotype

The phenotypic expression of a trait in some instances can vary even though it is inherited in a simple Mendelian fashion (Nadeau, 2001). In these instances allelic variance and environmental factors are thought to contribute to the variable expressivity of the phenotype. The effect of modifier genes, which are genes other than the causative mutation in disease models, is a well-described phenomenon (Genin et al., 2008). For example, in cystic fibrosis mouse model, a locus in mice was identified that modified the severity of the disease (Rozmahel et al., 1996), and this gene locus was later mapped onto the human genome (Zielenski et al., 1999). Moreover, small differences among genetic composition can have obvious influences on the phenotype as evident by the wide variation of coat colours observed in a progeny of mice from a mating between two brownish 129P2/OlaHsd X C57BL/6J strains (Errijgers and Kooy, 2004).

Hannan et al., (2001), in their original characterisation of *Mglur5*$^{-/-}$ mice reported that the TCAs only segregate into fused rows but not individual patches compared to *Mglur5*$^{+/+}$ mice. However, Hannan et al., (2001) also mentioned that the precise barrel phenotype in *Mglur5*$^{-/-}$ mice is background dependent. The genetic deletion of *Mglur5* on either a mixed C57BL/6JX129 or a C57 background strain, results in defects as reported by the original findings of Hannan et al., (2001). However, on a C57BL/6J background, *Mglur5* mutation shows defects in layer 4 cellular segregation but normal TCA segregation (only within the PMBSF area) (Takasaki et al., 2008; Lu H-C personal communications). In contrast, when the *Mglur5* mutation is present on a CD-1/129 background, the segregation of layer 4 cells and TCAs appear normal (Hannan et al., 2001). The analysis carried out in this chapter on a mixed C57BL/6JX129 shows TCAs to segregate within rows, albeit less defined in *Mglur5*$^{-/-}$ mice compared to *Mglur5*$^{+/+}$ mice and near complete loss of layer 4 cellular segregation. This phenotype corresponds to the one reported on a C57BL/6J background (Takasaki et al., 2008; Lu H-C personal communications), thus suggesting that several years of inbreeding since the original findings of Hannan et al., (2001) may have inadvertently biased the colony towards one with a greater contribution from C57BL/6J.

Mutations in several other genes that result in barrel phenotypes have also been identified to be background dependent. The *PrkarIIβ* mutation on a C57BL/6J background, on which the barrel phenotype was originally characterised, the layer 4 cellular segregation defect was much severe compared to the one published on a C57BL/6JOla Hsd background (Watson et al., 2006). The mutation of *Syngap* results in poor barrel barrel formation in both *Syngap*$^{+/-}$ and *Syngap*$^{-/-}$ mice compared to *Syngap*$^{+/+}$ mice on a MF1 background (Barnett et al., 2006b), but the barrel phenotype of *Syngap*$^{+/-}$ mice is less severe on a C57BL/6X129 background (Petrie, 2008).

The existence of inbred strains of mice allows the investigation of a mutation of a certain gene on a non-homogeneous background, which is more reflective of what happens in humans, mimicking the multiple genetic interactions in human disorders. However, a single mutation resulting in a variable phenotype depending on the background precludes direct comparisons among different effects of the mutation studied. Developing congenic strains

by backcrossing mice with a particular genetic mutation onto a specific background strain such as C57BL/6J over a successive number of generations enables the investigation of this mutation of interest on a homogeneous background. For example, backcrossing a mutation such as *Mglur5* that was originally on a C57BL/6X129 background for ten generations, onto a C67BL/6J background, results in the only similarity between the new *Mglur5* mutant on a C67BL/6J strain and the original C57BL/6X129 strain being the single chromosome segment containing the *Mglur5* locus. This segment is estimated to be 20cM in length and contains 300-1000 genes (Montagutelli, 2000). In practise, backcrossing over 5-6 generations results in a colony where there is 96-97% strain homogeneity. The maintenance of mutants on congenic strains is a powerful approach that minimises the effects of modifier genes and provides the opportunity to examine interactions between two/three genes of interest by creating double/triple mutant lines.

3.3.2 Pattern development in the trigeminal system is regulated by heterogeneous glutamate signalling

Glutamatergic signalling has been shown to play a pivotal role in pattern formation throughout the trigeminal system. Several mutants with deletions in various PSD components have been studied to date in order to molecularly dissect the role of glutamate and glutamate receptor associated signalling complexes in whisker related pattern formation in the trigeminal system. An emerging view from these studies is that there is heterogeneity in glutamatergic receptors and their associated signalling complexes in regulating whisker related pattern formation throughout the trigeminal system.

The immunohistochemical expression pattern of mGluR5 presented here together with previously characterised expression patterns of mGluR5 (Lopez-Bendito et al., 2002; Munoz et al., 1999) and *Mglur5* (Yamaguchi and Nakanishi, 1998) indicates high expression of mGluR5 throughout the trigeminal system during barrel formation. In agreement, data presented in this chapter suggest that glutamatergic signalling through mGluR5 is important for pattern formation in areas subserving AS whiskers throughout

the trigeminal system such that genetic deletion of mGluR5 results in defects of segregation of afferents at barrelettes, barreloids and barrels in the AS region. However, within the PMBSF area, afferent segregation into mystacial vibrissae patterns is moderately affected in the *Mglur5*$^{-/-}$ but segregation of afferents into barreloids and barrelettes is normal. It is unclear why afferent pattern segregation is affected differentially between regions receiving input from AS whiskers and mystacial vibrissae. Analysis of region specific mGluR5 mutants would aid in the understanding of whether loss of TCA patterning in AS region in *Mglur5*$^{-/-}$ mice is due to misregulated pattern formation throughout the trigeminal system or whether mGluR5 is involved in regulating the final relay of the trigeminal system at VpM to layer 4 synapses.

Another important finding in this chapter is that genetic deletion of mGluR5 also affects layer 4 cellular segregation in a dose dependent manner. In *Plcβ1*$^{-/-}$ mice, segregation of layer 4 cells into barrels is also defective (Hannan et al., 2001) suggesting that glutamatergic signalling via mGluR5 may regulate barrel formation through PLCβ1. In contrast to *Mglur5* expression, *Plcβ1* is not expressed in the thalamus (Watanabe et al., 1998) during development and furthermore, pattern formation at nVp and VpM is normal in *Plcβ1*$^{-/-}$ mice (Kind, PC, unpublished data). Despite the fact that layer 4 cells in *Plcβ1*$^{-/-}$ mice fail to form barrels, their TCAs segregate normally (Hannan et al., 2001). Therefore, while mGluR5 may regulate layer 4 cellular segregation via PLCβ1, it may recruit different downstream effectors in regulating TCA segregation.

AC1 is a potential downstream candidate for mGluR5 signalling, and AC1 is present throughout the trigeminal system during early postnatal development (Matsuoka et al., 1997; Nicol et al., 2005). The genetic deletion of AC1 globally results in the loss of TCA and layer 4 cellular segregation (Abdel-Majid et al., 1998) as first demonstrated in barrelless mice (Welker et al., 1996). Recently, Iwasato et al., (2001) show that cortex specific deletion of AC1 results in decreased layer 4 cellular segregation but TCA clustering is normal, suggesting that activity dependent postsynaptic signalling mediated via AC1 is not required for TCA patterning while layer 4 cellular segregation is largely independent of postsynaptic AC1 signalling (Iwasato et al., 2008).

However, in barrelless mice, segregation of barreloids is mildly affected whereas barrelettes segregate normally (Welker et al., 1996). Therefore, it appears that glutamatergic signalling via AC1 plays a regulatory role in the anatomical segregation of whisker related barrels and to a lesser degree in barreloid formation. Other adenylyl cylases such as AC4 (Defer et al., 2000) and AC9 (Antoni et al., 1998) that are expressed in cortical neurons and are also regulated by the principal G-protein Gq, which is associated with mGluR5 may mediate downstream signalling of mGluR5 in TCA segregation but to date a role for these in S1 pattern formation has not been characterised.

Glutamatergic signalling via NMDARs also regulate pattern formation throughout the trigeminal system. Loss of NMDARs results in deficits in pattern formation in the brainstem (Li et al., 1994), thalamus (Iwasato et al., 2000) and cortex (Iwasato et al., 2000). However, NMDAR associated signalling molecules appear to regulate different aspects of whisker related pattern formation in the trigeminal system. The loss of the regulatory subunit RIIβ of PKA that is tethered to the NMDAR complex via AKAP79/150 results in decreased layer 4 cellular segregation. The genetic deletion of *PrkarIIβ* selectively disrupts TCA segregation in the AS region but segregation in the PMBSF region is normal (Inan et al., 2006; Watson et al., 2006). It is also interesting to note that glutamatergic signalling via mGluR5 can also utilise PKARIIβ as a downstream effector via the AC1-cAMP pathway, and genetic mutation of *Mglur5* affects whisker related pattern formation in the AS region as shown in this chapter. However, formation of barreloids and barrelettes are normal in *PrkarIIβ*$^{-/-}$ mice (Watson et al., 2006) suggesting that PKARIIβ may only regulate whisker related patterns at layer 4.

The genetic ablation of another signalling molecule, SynGAP that is found in a complex with NMDAR, affects layer 4 cellular segregation in a dose dependent manner similar to that of *Mglur5* mutants (Barnett et al., 2006b). The loss of SynGAP affects layer 4 cellular segregation in a dose dependent manner with complete loss of layer 4 cellular segregation in *Syngap*$^{-/-}$ mice and decreased segregation in *Syngap*$^{+/-}$ mice compared to *Syngap*$^{+/+}$ mice (Barnett et al., 2006b). Moreover, their TCAs in the PMBSF region only segregate into rows while there is no TCA segregation evident in the AS region (Barnett et al., 2006b) which corresponds to the original findings of

Hannan et al., (2001) in terms of TCA patterning in *Mglur5*$^{-/-}$ mice. It is a possibility that SynGAP may regulate barrel formation via NMDAR or mGluR5 mediated glutamatergic signalling. However, no interactions between mGluR5 and SynGAP have been characterised in the literature. The genetic deletion of *Syngap* affects whisker related barreloid formation but not barrelette formation (Barnett et al., 2006b) suggesting that while SynGAP may also regulate barreloid formation it is not crucial for segregation of barrelettes.

Hence, glutamate receptors may mediate their regulatory actions on cellular processes of whisker related pattern formation in trigeminal system by differentially recruiting specific pathways that may overlap but are also distinct during development.

3.3.3 Role for mGluR5 in regulating spines

There is a plethora of evidence for a role for glutamatergic receptors in regulating spine density, shape and plasticity (Bagni and Greenough, 2005; Genoux and Montgomery, 2007; Nikonenko et al., 2002). The 'mGluR theory of fragile X mental retardation' postulates that exaggerated Gp1 mGluR signalling underlie many of the symptoms of FXS (Bear et al., 2004). In the mouse model of FXS, there is an increase in spine density with increased number of immature spines (Comery et al., 1997). The mRNA binding FMRP translocates to dendrites and dendritic spines by Gp1 mGluR activation (Antar et al., 2004; Ferrari et al., 2007) and consistent with the idea that dysregulated mGluR5 signalling may manifest defects in spine density in FXS, agonist activation of Gp1 mGluRs in cultured hippocampal neurones results in increased frequency of elongated spines (Vanderklish and Edelman, 2002). Moreover, translocation in liposarcoma (TLS), which is another mRNA binding protein is also translocated to dendritic spines by mGluR5 activation. The Gp1 mGluR agonist induced increase in TLS localisation to postsynaptic spines observed in control hippocampal cultures is abolished in neuronal cultures from *Mglur5*$^{-/-}$ mice (Fujii et al., 2005). TLS associates with the actin stabilising protein Ndl-L (Fujii and Takumi, 2005), and in *TLS*$^{-/-}$ mice there is decreased spine density with an increase in the number of filopodia-like protrusions (Fujii et al., 2005). The interaction between TLS

and mGluR5 thus also support a role for mGluR5 in regulating spine shape and formation.

The Golgi analysis of layer 4 spiny cells in S1 presented here shows decreased spine density in both *Mglur5*$^{-/-}$ mice and *Mglur5*$^{+/-}$ mice compared to *Mglur5*$^{+/+}$ mice. This is consistent with the idea of an interaction between FMRP and mGluR5 in regulating spinogenesis. However, Dolen et al., (2007) found no alterations in spine density in layer 3 neurons in primary visual cortex from *Mglur5*$^{+/-}$ mice. This discrepancy in the effect of loss of mGluR5 on spine density may simply reflect differences in the neuronal populations examined. However, Dolen et al., (2007) did find deficits in ocular dominance plasticity in layer 4 of *Mglur5*$^{+/-}$ mice compared to *Mglur5*$^{+/+}$ mice. In normal mice, contralateral eye-lid closure during monocular deprivation paradigm results in depression of the deprived eye in 3 days. In 7 days there are significant changes in both deprived and non-deprived ipsilateral eye. The deprived eye de-depresses compared to day 3 but is still significantly depotentiated compared to day 0, whereas the non-deprived ipsilateral eye potentiates after 7 days (Frenkel and Bear, 2004). In *Mglur5*$^{+/-}$ mice, there was no depression of the deprived eye response in 3 days (Dolen et al., 2007). This could be explained by the fact that synapses of *Mglur5*$^{+/-}$ mice are less likely to undergo depression as they have reduced number of spines and their LTD expression may already be saturated.

The role of mGluR5 in regulating spine density may also depend on the neuronal cell type specific manner within a layer as well in addition to layer specific differences. HC Lu and colleagues (personal communications) found increased spine density in layer 4 pyramidal cells from analysis of spine density on coronal sections. The Golgi analysis presented in this chapter was carried out in tangential sections thereby making it difficult to accurately identify which excitatory neuronal cell type was analysed. However, in rat cortical layer 4 of S1, the majority of excitatory neuronal population is formed by spiny stellate and atypical star pyramidal neurons with pyramidal neurons making a small contribution to the overall layer 4 excitatory neuronal population (Feldmeyer and Sakmann, 2000; Staiger et al., 2004). In the future it would be beneficial to characterise the spine density as well as

spine morphology in a neuronal cell type specific manner to fully understand the regulatory role of mGluR5 in spinogenesis.

3.3.4 Role of synaptic plasticity in barrel formation

It is evident that patterns of activity are necessary for strengthening or weakening of TCA-layer 4 synapses (Crair and Malenka, 1995), setting up the developmental functional columnar organisation and plasticity of S1 (Fox, 2002) and spine maturation (Lendvai et al., 2000). Although studies so far show clear supportive evidence for a role for glutamate receptors in the anatomical segregation of barrels in layer 4 of S1, it is unclear what type of neural activity is required for barrel formation and whether it plays a permissive or an instructive role in barrel formation.

Studies with pharmacological blockade of action potentials by TTX (Chiaia et al., 1992) or blockade of NMDAR by APV or MK108 (Fox et al., 1996; Henderson et al., 1992; Schlaggar et al., 1993) show that segregation of TCAs are unaltered suggesting that patterned or spontaneous activity may not be required for segregation of TCAs. Several lines of evidence suggest that spontaneous release of glutamate from TCAs is sufficient to allow TCA segregation and layer 4 cellular segregation, perhaps eluding to a tropic role for glutamate. For example, in spite of complete loss of peripheral receptors due to row C follicle ablation, layer 4 cells still aggregate around fused TCAs (Van der Loos and Woolsey, 1973).

Moreover, there is also an emerging view that synaptic plasticity of layer 4 synapses does not underlie the anatomical segregation of barrels. Loss of PKARIIβ results in decreased layer 4 cellular segregation (Inan et al., 2006; Watson et al., 2006). PKA phosphorylates GluR1 at S845, thereby mediating AMPAR trafficking at the synapse (Ehlers, 2000; Esteban et al., 2003; Lee et al., 2003). In addition to barrel defects, *PrkarIIβ*$^{-/-}$ mice show deficits in LTP at TCA-layer 4 synapses (Inan et al., 2006) and decreased insertion of GluR1 subunit of AMPAR into the PSD in S1 (Watson et al., 2006), but genetic deletion of AMPAR subunits does not affect barrel formation (Watson et al., 2006). Furthermore, while loss of NR1(Iwasato et al., 2000), PKARIIβ (Inan et al., 2006) and AC1 (Iwasato et al., 2008) cause defects in barrel formation,

lesion-induced plasticity of TCA fusion due to row C whisker ablation is unaltered in these mutants (but see Takasaki et al., 2008). The sensitive period for lesion-induced plasticity can be dissociated from anatomical barrel development (Rebsam et al., 2005; Woolsey and Wann, 1976), thereby suggesting that these two processes are regulated by different cellular mechanisms. The formation of barrels in addition to various postsynaptic signalling pathways mentioned in this thesis, is also regulated by presynaptic monoaminergic afferents such as serotonin (Cases et al., 1996). In monoamine oxidase A (MAOA) mutant mice, there is excess amounts of serotonin present and the excess amount of serotonin during barrel formation results in lack of whisker related patterning in both TCAs and layer 4 cells. However, in MAOA mutant, normal barreloid and barrelette segregation takes place (Cases et al., 1996). A recent elegant study by Rebsam et al., (2005) show that while barrel formation can be reinstated until P11 with pharmacological restoration of levels of serotonin to normal levels with parachlorophenylalanine (PCPA) infusion in MAOA mutant mice, row C whisker lesion plasticity still ends at P3. The results of this study suggest that while restoration of presynaptic activity resulted in barrel formation, it is insufficient for extending row C whisker lesion plasticity that normally closes at P3/P4 (Woolsey and Wann, 1976), thus effectively dissociating the two processes.

Therefore, while it is clear that glutamate receptors such as mGluR5 play a role in the anatomical segregation of barrel formation, it is not clear precisely how they mediate barrel formation. The early expression of mGluR5 at P4 subsequent to TCA segregation but prior to layer 4 cellular segregation suggests that spontaneous release of glutamate from TCAs may act as a neurotropic agent during early neuronal development relaying positional information from TCAs to layer 4 dendrites in order to set up cortical whisker related patterns. It is also apparent that during late S1 development, mGluR5 plays a role in synaptogenesis but not in dendritic complexity. Nonetheless, glutamate signalling appears to play a critical role in the establishment of cortical connectivity and plasticity, and various forms of cognitive disorders appear to be caused by either misregulated glutamatergic signalling through glutamate receptors or mutations in glutamate receptors and associated signalling proteins at the PSD (Bear et al., 2004; Grant et al., 2005). In FXS,

where glutamate signalling via mGluR5 is thought to be dysregulated (Bear et al., 2004), one could hypothesis that the effects of mGluR5 on barrel formation would be exaggerated/altered in *Fmr1*$^{-/y}$ mice. More importantly, if FMRP plays a role in regulating signalling effects of mGluR5, given the fact that mGluR5 regulates whisker related pattern formation in S1, it could be hypothesised that FMRP may also play a regulatory role in barrel formation.

4

Key findings:

1. The cortical expression of FMRP peaks during the first two postnatal weeks, and its expression in the VpM is developmentally transient.

2. FMRP is postsynaptic in P7 cortical synapses, whereas at P14 it is expressed heterogeneously in both pre and postsynaptic compartments.

3. Loss FMRP does not affect general growth, cortical arealisation or afferent segregation in the trigeminal system at P7.

4. Genetic deletion of *Fmr1* results in decreased layer 4 cellular segregation at P7.

5. In P30-P35 *Fmr1*$^{-/y}$ mice, layer 4 spiny cells exhibit increased spine density.

6. During early S1 development loss of FMRP alters levels of synaptic proteins suggesting a role for it in trafficking and localising these proteins at developing synapses.

4 FMRP Regulates the Development of Mouse S1

4.1 Introduction

FXS is the most common form of inherited MR and unlike most forms of MR, FXS aetiology is a single gene, *FMR1* (Harrison et al., 1983; Verkerk et al., 1991). It is also a disorder of trinucleotide repeat instability, as FXS genotype is typically an abnormal expansion of CGG repeats in the 5' end of *FMR1* that leads to its transcriptional silencing (Coffee et al., 1999; Sutcliffe et al., 1992). The resulting phenotype is manifested by the loss of FMRP that is encoded by *FMR1* (Ashley et al., 1993). FMRP is a member of the RNA binding hnRNP family, and is involved in mRNA trafficking from nucleus to cytoplasm including its own mRNA. At synapses, FMRP is thought to regulate Gp1 mGluR dependent local protein synthesis (reviewed in Bagni and Greenough, 2005). Immunogold studies by Feng et al., (1997) show localisation of FMRP expression in the nucleoplasm, within nuclear pores, along dendrites, dendritic branch points and spines in the adult brain providing first anatomical evidence for the putative role of FMRP as a regulatory protein that shuttles between nucleus and cytoplasm of a neurone.

There are several lines of evidence to suggest a role for FMRP in the cellular processes governing S1 differentiation during cortical development. Firstly, stimulation of rodent whiskers is shown to result in elevated FMRP levels in adult barrel cortex synaptosomes in a Gp1 mGluR dependent manner (Todd and Mack, 2000; Todd et al., 2003). Secondly, FMRP is also shown to play a regulatory role in spinogenesis of layer 5 pyramidal cells in S1 (Nimchinsky et al., 2001). Layer 5 pyramidal cells of S1 in *Fmr1*$^{-/y}$ mice transfected with EGFP show increased spine length by 28% and spine density by 33% at the end of the first postnatal week. The increase in layer 5 dendritic spine length evident at P7 lessens with progressive age. In contrast the increase in spine density was absent by the second postnatal week (Nimchinsky et al., 2001) suggesting that in layer 5 pyramidal cells, FMRP plays a developmentally transient role in regulating spine density and morphology. A subsequent study of Golgi impregnated layer 5 dendrites in S1 from juvenile (1 month

old) and adult (4 months old) *Fmr1*$^{-/y}$ mice documented an increase in spine density at these older ages compared to *Fmr1*$^{+/y}$ mice (Galvez and Greenough, 2005). At these adult ages, there is increased density of immature spines whereas the density of spines with a mature morphology is decreased (Galvez and Greenough, 2005). Despite the differences in techniques, collectively these two studies suggest that FMRP plays a regulatory role in spinogenesis both during early S1 development and in the adult S1. Galvez et al., (2003) in another Golgi study show that layer 4 spiny neurons of juvenile *Fmr1*$^{-/y}$ mice have increased dendritic matter in the inter-barrel regions (sepate) relative to the *Fmr1*$^{+/y}$ mice suggesting that FMRP may also play a role in refining dendritic elaborations within a barrel patch in the final step of differentiation of S1 (section 1.3.1.3). However, it is not known whether FMRP 1) is expressed in the barrel cortex during development, 2) regulate barrel formation and 3) regulate synaptogenesis in layer 4 spiny cells.

There is also evidence for alterations in synaptic strength of S1 circuitry in *Fmr1*$^{-/y}$ mice. Bureau et al., (2008) found transient deficits in the excitatory synaptic projection connecting cortical layer 4 to 3 in *Fmr1*$^{-/y}$ mice. In *Fmr1*$^{-/y}$ mice, the strength of layer 4 to 3 projections within a barrel column is selectively weakened and their layer 4 axonal arbours are spatially diffused in layers 2/3 compared to *Fmr1*$^{+/y}$ mice in the second postnatal week. The weakening of this synaptic projection to whisker trimming normally seen in *Fmr1*$^{+/y}$ mice is also abolished in *Fmr1*$^{-/y}$ mice (Bureau et al., 2008). However, by three weeks of age these deficits are ameliorated suggesting that FMRP plays a developmentally transient role in refining synaptic connectivity between layer 4 to 2/3 in S1. As well as anatomical and functional synaptic changes observed in layer 2/3, dramatic alterations at the TCA synapse onto layer 4 in *Fmr1*$^{-/y}$ mice have also been characterised by Harlow et al., (2007) during S1 development. In *Fmr1*$^{-/y}$ mice, there is an elevation in the NMDA/AMPA ratio towards the end of the first postnatal week in contrast to *Fmr1*$^{+/y}$ mice that normally show decreased NMDA/AMPA ratios at the end of the first postnatal week due to a rise in AMPAR transmission (refer to section 1.3.4.2). Induction of LTP is also absent at TCA-layer 4 synapse during the first postnatal week in *Fmr1*$^{-/y}$ mice but robust LTP is found at P7, an age at which there is little or no LTP induced in *Fmr1*$^{+/y}$ mice.

A parsimonious view of these studies indicates a role for FMRP in the development of S1 organisation. However, while a role for FMRP in synaptic function has been explored recently in layer 4 during early S1 development, anatomical studies to date have been primarily focused on investigating the role of FMRP in juvenile or adult S1. Anatomical organisation of barrels in S1 occurs during the first postnatal week, although layer 4 cellular segregation is refined through to the second postnatal week as well (refer to section 1.3.1.3). Therefore, it is important to investigate the role of FMRP during this anatomical sensitive period to fully characterise its involvement in S1 organisation. Most notably, FXS is a disorder of development, so an understanding of the role of FMRP during development is of crucial benefit in developing therapeutic interventions. This is highlighted in the study by Gatto and Broadie (2008), where in the drosophila model of FXS, a near complete reversal of *dFmr1* mutant phenotypes were achieved by the reintroduction of dFMRP (the *Drosophila* homologue of FMRP) during early development, whereas late intervention only resulted in a slight improvement.

The main focus of this chapter is to examine the role of FMRP in S1 cortical organisation during development. Specifically, it aims to explore several hypotheses, 1) FMRP is expressed in S1 during development 2) FMRP plays a role in TCA segregation into whisker related patterns 3) Loss of FMRP affects layer 4 cellular segregation postsynaptically 4) FMRP regulates whisker related patterns at VpM and nVp 5) FMRP plays a role in layer 4 synaptogenesis and 6) FMRP is involved in trafficking and localisation of synaptic proteins during early S1 development.

4.2 Results

4.2.1 Expression of FMRP peaks during the S1 sensitive period

Previous studies carried out in S1 indicate that FMRP might be expressed early in S1 development. For example, FMRP plays a developmental role in synaptogenesis in layer 5 pyramidal cells from S1 in *Fmr1*$^{-/y}$ mice (Nimchinsky et al., 2001) and it appears to be involved in dendritic pruning of layer 4 spiny cells (Galvez et al., 2003). However, early FMRP expression

has not been examined in S1, therefore, to determine whether FMRP is expressed in layer 4 of S1 during barrel formation, its expression pattern was examined during S1 development in both coronal and tangential section using an antibody specific to FMRP known as rAM1. The rAM1 antibody is raised against the C-terminus of human FMRP to a peptide spanning the amino acid residues 516-632. Because the interactions between FMRP and its related proteins FXR1P and FXR2P are believed to be carried out through N terminus of FMRP, this antibody was only expected to stain for FMRP immunoreactivity (Ferrari et al., 2007). Consistent with this idea, there was no staining observed in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice confirming the specificity of rAM1 antibody (Figure 4.1).

Immunostaining for FMRP on coronal sections showed FMRP expression in the cortex and hippocampus throughout development. Its expression in the thalamus and striatum was developmentally regulated such that the intensity of the staining in these regions was weaker after P14 (Figure 4.2Ai-Ei). The FMRP immunostaining in the hippocampus was high throughout development in the cellular layers, strata pyramidal containing cell bodies of pyramidal cells and strata granulosum containing cell bodies of granule cells in DG (Figure 4.2Aii-Eii). There was early cytoplasmic expression for FMRP predominantly in the polymorphic layer of DG from P4 to P14 although it was evident in strata oriens, radiatum, lacunosum and lucidum (only expressed from P4-P7 in these compartments), but this staining only appeared to sparsely label a subpopulation of cells (Figure Aii-Cii). Furthermore, it was largely absent at P21 and in the adult suggesting that FMRP could be transiently expressed in a subpopulation, perhaps in an inhibitory cell population.

FMRP expression was present in all cortical layers throughout development (Figure 4.2Aiii-Eiii). Its expression was dense in layers 2/3 and 4 during P4-P14 (Figure 4.2Aiii-Ciii). In P21 and adult, FMRP staining appeared less intense in all cortical layers compared to early ages (Figure Ciii-Eiii). FMRP expression was localised to a 'barrel-like' pattern in layer 4 throughout development (Figure 4.3A-E), and this was further evident in tangential section through layer 4, in which a clear 'barrel-like' pattern was observed at all developmental ages (Figure 4.4Ai-Ei and Aii-Eii). In tangential sections,

FMRP staining at P4 appeared diffused (Figure 4.4F) at higher magnifications compared to the predominantly cytoplasmic expression in the adult (Figure 4.4G). In coronal sections, FMRP expression was found in the cytoplasm as well as in the dendritic processes at P4 (Figure 4.5Ai-Aii), whereas in the adult the expression was dense in the cytoplasm with some staining at the origin of dendritic process (Figure 4.5Bi-Bii). This expression agrees well with previously characterised predominantly cytoplasmic FMRP expression in the layer 5 pyramidal cells of frontal cortex in adult rats (Feng et al., 1997) and in the human cerebral cortical cells (Devys et al., 1993). There was also punctate expression of FMRP evident in the hippocampus at P14, and these puncta were localised to the cell body layers where FMRP expression was high (Figure 4.6A). This punctate expression of FMRP was found in both hippocampus (Figure 4.6B) and cortex at P21 (Figure 4.7A and B). In the cortex, puncta was apparent throughout all cortical layers and appeared to be localised to either the cell body or areas immediately adjacent to it (Figure 4.7B). The punctate FMRP expression appeared to be developmentally transient, and was largely absent in the adult. To qualitatively determine whether levels of FMRP are developmentally regulated, levels of FMRP in barrel cortex homogenates were examined throughout development (Figure 4.2F). Immunoblot for FMRP during development showed a progressive increase in FMRP during P4-P14, with expression low at P4, peaking around P7 to P14. After P14, expression of FMRP gradually declined, and comparatively low levels were observed at P21 and in the adult. The density of cells expressing FMRP appeared to be downregulated between P14 and P21 in cortex (Figure 4.2Aiii-Eiii and 4.3). It would be interesting to determine whether this downregulation reflects a general developmental decrease in the density of cells during cortical development (ie. would you see it with thionin staining?) or whether FMRP expression is lost in a subset of cells during cortical development (for example, in an inhibitory/interneuron population).

In the VpM nucleus of the thalamus, immunohistochemical staining for FMRP showed high expression at P4 and P7 (Figure 4.8A and B) but the intensity of this expression was greatly reduced at and after P14 (Figure 4.2Ai-Ei and 4.7).

In summary, in agreement with the first hypothesis, expression of FMRP was observed throughout development, and high levels of expression were observed in all brain regions examined during the first two postnatal weeks. High expression of FMRP during first two weeks in layer 4 of S1 is also consistent with the hypotheses that FMRP plays a role in barrel formation and in synaptogenesis of layer 4 spiny cells.

4.2.2 FMRP localisation is heterogeneous during S1 development

An immunogold EM study of FMRP in adult rat brain shows a somatodendritic expression of FMRP with expression localised to the cell body, dendrites and dendritic spines (Feng et al., 1997). In addition to this postsynaptic localisation of FMRP, it is also expressed in axonal terminals at a relatively low number of synapses indicating a presynaptic role as well (Feng et al., 1997). The transient expression of FMRP in VpM during S1 development characterised in 4.2.2 also raised the possibility that FMRP might be presynaptically expressed during barrel formation. To determine whether FMRP is expressed pre or postsynaptically during barrel formation, immuno EM analysis was carried out at P7 and P14.

The tissue sections processed for EM were selected from layer 4 where barrel patches were visible, and particular care was taken to dissect out only tissue within a barrel patch. At P7 FMRP expression was ubiquitous in the neuronal cytoplasm and along the dendrite (Figure 4.9A). At P7, labelling was found only in the postsynaptic compartment at the labelled synapses (Figure 4.9B-D) suggesting that during barrel formation FMRP is expressed postsynaptically. In contrast to FMRP localisation at P7, FMRP localisation at P14 was found to be more heterogeneous. At synaptic sites, DAB immuno product was found in either post (Figure 4.9E), pre (Figure 4.9G) or in both pre and postsynaptic compartments (Figure 4.9F) indicative of both a pre and postsynaptic loci of action. At both ages unlabelled synapses were also observed suggesting that FMRP is only in a subset of synapses during S1 development.

In the synaptoneurosome preparations, expression of FMRP was found at both P7 and P14 (Figure 4.8H) showing synaptic localisation of FMRP. Some

light staining was observed in the homogenates from *Fmr1*$^{-/y}$ mice with the FMRP antibody (clone 1C3, Millipore UK Ltd, Hertfordshire, UK) used for biochemical analysis. This light staining has been reported previously (Feng et al., 1997; Primerano et al., 2002) and is thought to be due to this antibody cross-reacting with FMRP related proteins, FXR1 and FXR2 in addition to FMRP.

4.2.3 General growth and cortical arealisation is normal in Fmr1$^{-/y}$ mice

Both early expression of *Fmr1* mRNA (Hinds et al., 1993) and FMRP expression (Till, S, personal communications) have been characterised in the embryonic brain suggesting that FMRP may play a role during early cortical pattern formation and arealisation. Therefore, cortical arealisation was analysed to determine whether FMRP plays a regulatory role during development in specifying the delineation of neocortex to different areas receiving varying sensory input. In addition, accelerated prepubescent growth has been reported in children with FXS (Loesch et al., 1995), therefore bodyweight was also measured at P7 in *Fmr1*$^{-/y}$ mice to determine whether loss of FMRP affects general body growth.

There were no differences found in either the body weight (figure 4.10C) or the size of neocortex (Figure 4.10D) at P7 between *Fmr1*$^{-/y}$ (n=10) Figure 4.10B) and *Fmr1*$^{+/y}$ (n=11) (Figure 4.10A) mice suggesting normal gross development in *Fmr1*$^{-/y}$ mice at this developmental stage. TCA segregation into whisker related rows and patches along a row was normal in *Fmr1*$^{-/y}$ mice (Figure 4.10B) suggesting that FMRP does not regulate TCA segregation during S1 differentiation. There were also no measurable differences between genotypes in the size of S1, AS and PMBSF together, PMBSF and visual cortex (Figure 4.10E) denoting that cortical arealisation occurred normally in *Fmr1*$^{-/y}$ mice. Finally, the positioning of PMBSF within neorcortex was also normal in these mutants (Figure 4.10F). Collectively, these data suggest that loss of FMRP does not affect general patterning of sensory areas or general body growth at P7.

4.2.4 Loss of FMRP affects barrel segregation

Given the fact that FMRP expression is high postsynaptically at P7, it is plausible that FMRP may play a role in layer 4 cellular segregation. To examine this hypothesis, analysis of layer 4 cellular segregation was carried out. To quantitatively assess if there is a defect in cellular segregation in *Fmr1*$^{-/y}$ mice, tangential sections were double labelled with 5HTT and Topro3 to identify TCA patch and cell nuclei respectively (Figure 4.11 A and B) to determine the ratio of cell nuclei between the barrel wall and hollow. The ratio of cell density in wall: hollow was significantly reduced in *Fmr1*$^{-/y}$ mice (1.45±0.03, n=6) compared to *Fmr1*$^{+/y}$ mice (1.64±0.02,n=9; $p<0.001$, two tailed t test) (Figure 4.11C). Some *Fmr1*$^{-/y}$ mice exhibited this defect more than the others suggesting a variable phenotype possibly due to variable penetrance of *Fmr1*. The TCA patch size of the barrel analysed was not significantly different between genotypes (Figure 4.11D). This agrees with the hypothesis that FMRP regulates layer 4 cellular segregation during barrel formation but negates the hypothesis that it is involved in regulating TCA segregation at P7.

4.2.5 Normal segregation of barrelettes and barreloids in *Fmr1*$^{-/y}$ mice

To determine whether FMRP plays a role in segregation at the intermediate relay stations of the trigeminal system leading to barrel formation, barreloid and barrelette segregation was examined by using CO staining at P7. In *Fmr1*$^{-/y}$ mice (n=3), a clear barrelette pattern was visible in areas subserving the AS and PMBSF regions in nVp nucleus (Figure 4.12) comparable to *Fmr1*$^{+/y}$ mice (n=3). Also, no differences in segregation of barreloid pattern were observed between *Fmr1*$^{-/y}$ mice (n=9) (Figure 4.13B, D, F and H) and *Fmr1*$^{+/y}$ mice (n=5) (Figure 4.13A, C, E and G) in the thalamic VpM nucleus. Collectively, these data suggest that FMRP does not play a regulatory role in whisker related pattern formation in the subcortical relay stations of the trigeminal system during development.

4.2.6 FMRP plays a role in spinogenesis during late S1 development

In layer 5 pyramidal cells of S1, the increased spine density with an immature morphology observed in *Fmr1*$^{-/y}$ mice is thought to be developmentally regulated (Galvez and Greenough, 2005; Nimchinsky et al., 2001). Nimchinsky et al., (2001) show the magnitude of increased spine density in layer 5 pyramidal cells to decrease with age and to be absent by P21. Nonetheless, this alleviation of spine dysgenesis in *Fmr1*$^{-/y}$ mice seems to be transient as it is observed again in the adult (Galvez and Greenough, 2005). Therefore, as a starting point to characterise whether FMRP plays a regulatory role in layer 4 dendritic spine formation, Golgi analysis was carried out on layer 4 spiny cells at P30-P35 on tangential sections (Figure 4.14A-E). For spine analysis, spines were counted on three independent dendrites selected from three independent cells within the PMBSF, and the n stated here refers to the number of animals. Layer 4 spiny cells of *Fmr1*$^{-/y}$ mice (n=4) (Figure 4.15A) showed increased spine density relative to *Fmr1*$^{+/y}$ mice (n=3) (Figure 4.15B) along its dendritic length (Figure 4.15C) and spine density/10μm was significantly increased by 31% in *Fmr1*$^{-/y}$ mice (10.60±0.51) compared to *Fmr1*$^{+/y}$ mice (8.06±0.31; p=0.013, two tailed t test) (Figure 4.14D). There was a trend towards an increase in average number of spines per dendrite (Figure 4.15F) in *Fmr1*$^{-/y}$ mice (150.75±12.01) compared to *Fmr1*$^{+/y}$ mice (114.78±21.27;) while dendritic length (Figure 4.15E) did not differ significantly between the two genotypes suggesting that number of spines per dendritic length was increased rather than an increase in both spine number and length of dendrite. This defect in spine density due to loss of FMRP in layer 4 spiny neurons is consistent with the hypothesis that FMRP plays a role in synaptogenesis during S1 development.

4.2.7 Altered levels of synaptic proteins during S1 development in *Fmr1*$^{-/y}$ mice

In adult *Fmr1*$^{-/y}$ mice, reduced cortical LTP expression is associated with reduced levels of GluR1 in cortical homogenates (Li et al., 2002). Moreover, Harlow et al., (2007) found an increased NMDA/AMPA ratio at the end of the critical period for LTP induction in *Fmr1*$^{-/y}$ mice, which is aberrant to the decreased NMDA/AMPA ratio normally seen in *Fmr1*$^{+/y}$ mice at this time

point. However, they found no detectable changes in GluR1 levels in cortical homogenates at these ages in *Fmr1*$^{-/y}$ mice relative to *Fmr1*$^{+/y}$ mice. Therefore, to determine whether levels of glutamatergic receptors are altered at synaptic sites during S1 development, glutamatergic receptor subunit levels were examined in P7 and P14 neocortical homogenates and synaptoneurosomes from *Fmr1*$^{-/y}$ and *Fmr1*$^{+/y}$ mice (refer to section 2.3.1.4 and 2.4.2). Please refer to table 4.1 for all values and statistical significances.

In *Fmr1*$^{-/y}$ mice there were no detectable changes in levels of GluR1, GluR2/3, NR2B and pGluR1 (GluR1 phosphorylated at S485) in P7 homogenates compared to *Fmr1*$^{+/y}$ mice at P7 (Figure 4.16A). However, in P7 synaptoneurosomes, levels of both GluR1 and GluR2/3 were significantly reduced while levels of NR2B and pGluR1 were unchanged in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Figure 4.16B). This suggests that while there are no general alterations in glutamatergic subunit levels, levels of AMPAR subunits are decreased at synaptic sites in *Fmr1*$^{-/y}$ mice at P7. At P14, in comparison to *Fmr1*$^{+/y}$ mice, levels of GluR2/3 and NR2B were significantly reduced in homogenates from *Fmr1*$^{-/y}$ mice (Figure 4.17A). No changes in GluR1 or pGluR1 levels were detected in *Fmr1*$^{-/y}$ mice homogenates relative to *Fmr1*$^{+/y}$ mice at P14. In the synaptoneurosomes from P14 *Fmr1*$^{-/y}$ mice, GluR1 levels were significantly reduced and pGluR1 showed a trend towards a decrease, while GluR2/3 and NR2B did not differ significantly compared to *Fmr1*$^{+/y}$ mice (Figure 4.17B). Collectively these data suggest that at P14, levels of GluR1 is reduced, which is consistent with increased internalisation of GluR1 subunits characterised in synaptic sites of adult *Fmr1*$^{-/y}$ mice (Nakamoto et al., 2007). In contrast, while levels of NR2B and GluR2/3 expression were generally downregulated in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice, levels of these subunits at synaptic sites were unaltered at P14.

FMRP is a mRNA binding protein, and one way in which it is thought to recognise its target mRNA is by recognising a binding motif known as the G quartet (Brown et al., 2001; Darnell et al., 2001; Schaeffer et al., 2001). Sequence analysis of the mRNA for synaptic proteins, PLCβ1 and SynGAP mRNA show that these proteins have putative G quartet motifs in their coding region and 3'UTR region respectively (Stoney, P, personal communications). To determine whether basal levels of these synaptic

proteins are altered their expression was also determined in P7 and P14 neocortical homogenates and synaptoneurosomes (results described here are summarised in table 4.1).

In P7 homogenates from *Fmr1*$^{-/y}$ mice, there were no changes in levels of SynGAP and PLCβ1 compared to *Fmr1*$^{+/y}$ mice (Figure 4.16A). However, in P7 synaptoneurosomes from *Fmr1*$^{-/y}$ mice, both PLCβ1 and SynGAP levels were decreased compared to *Fmr1*$^{+/y}$ mice (Figure 4.16B). No changes in levels of PLCβ1 and SynGAP were detected in either P14 homogenates or synaptoneurosomes from *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice. This suggests that trafficking and localisation of these synaptic proteins is impaired in *Fmr1*$^{-/y}$ mice at P7 but not at P14 relative to *Fmr1*$^{+/y}$ mice.

4.3 Discussion

Previous work has shown a role for FMRP in regulating synaptic connectivity in layer 5 (Nimchinsky et al., 2001) and layer 2/3 (Bureau et al., 2008) during S1 development. Moreover, in barrel cortex of adult *Fmr1*$^{-/y}$ mice, there is increased layer 4 dendritic material in the inter-barrel region suggesting that FMRP plays a role in S1 layer 4 synaptic connectivity. The focus of this chapter was to determine whether FMRP plays a role in S1 differentiation during development. Consistent with a role for it in barrel formation and synaptogenesis, FMRP expression in the barrel cortex was highest during P7-P14, a time frame that corresponds well with when the anatomical segregation of barrels are refined (Barnett et al., 2006a) and the peak of synaptogenesis in layer 4 (White et al., 1997). At P7, FMRP expression was postsynaptic, however, at P14 it was found in pre, post or both compartments. *Fmr1*$^{-/y}$ mice displayed normal body weight and cortical arealisation at P7 suggesting that general growth and early cortical patterning is not regulated by FMRP. In agreement with the hypothesis that FMRP regulates S1 differentiation, loss of FMRP at P7 resulted in decreased layer 4 cellular segregation, but TCA patterning into whisker related patterns was unaffected suggesting that FMRP does not regulate afferent patterning. Moreover, whisker related afferent pattern formation at VpM and nVp were also normal in *Fmr1*$^{-/y}$ mice at P7 suggesting that FMRP does not regulate

pattern formation at barreloids and barrelettes. In addition to its role in anatomical segregation of barrels during early S1 development, loss of FMRP also resulted in increased spine density in layer 4 spiny cells in P30-P35 mice consistent with the hypothesis that FMRP regulates synaptogenesis in layer 4 during S1 development. Basal levels of synaptic proteins were also found to be altered in *Fmr1*$^{-/y}$ mice at P7 and P14, especially at synaptic sites in P7 *Fmr1*$^{-/y}$ mice suggesting that FMRP plays an early regulatory role in localisation of synaptic proteins. An early expression and role for FMRP in S1 cortical organisation is evident in collating the data presented in this chapter indicating that FMRP plays a critical role in governing synaptic connectivity during early brain development in addition to its proposed regulatory role in the adult brain.

4.3.1 Cellular basis of FMRP dependent barrel formation

Whisker stimulation results in a Gp1 mGluR dependent enhancement of FMRP expression in barrel cortex synaptosomes and polysome fractions (Todd and Mack, 2000; Todd et al., 2003) suggesting that its expression in the barrel cortex is dependent on glutamatergic signalling mediated via Gp1 mGluRs. Genetic mutation of molecules involved in postsynaptic signalling cascades downstream of Gp1 mGluRs all seem to result in largely overlapping defects in layer 4 barrel formation. Therefore, it is possible that these are postsynaptic signalling cascades that FMRP could interact with, in regulating layer 4 barrel formation during S1 development. It is also plausible that FMRP may regulate or be regulated by presynaptic messengers in its role in layer 4 of S1.

Chapter 3 in this thesis together with previous finding by Hannan et al., (2001) show that mGluR5 plays a postsynaptic role in layer 4 cellular segregation in a dose dependent manner with a near complete loss of layer 4 cellular segregation in *Mglur5*$^{-/-}$ mice at P7, consistent with the idea that barrel defects seen in *Fmr1*$^{-/y}$ mice could due to effects of Gp1 mGluR signalling. In mouse cortical synaptoneurosomes, Gp1 mGluR activated PI hydrolysis is PLCβ1 dependent (Hannan et al., 2001). This chapter shows that in P7 synaptoneurosomes from *Fmr1*$^{-/y}$ mice, levels of PLCβ1 are significantly reduced compared to *Fmr1*$^{+/y}$ mice suggesting that FMRP and

PLCβ1 may interact in regulating barrel formation. Loss of PLCβ1 results in near complete loss or greatly reduced layer 4 cellular segregation (Hannan et al., 2001) while their TCA segregation is normal. The similarities in barrel defects between *Fmr1*$^{-/y}$ and *Plcβ1*$^{-/-}$ are consistent with the idea that FMRP might be involved in the Gp1 mGluR-PLCβ1 signalling pathway in regulating layer 4 cellular segregation.

Biochemical data presented in this chapter show significantly reduced GluR1 levels at P7 and P14, and there is a trend towards decreased phosphorylation of GluR1 at S845 in synaptoneurosomes from P14 *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice. PKA is a downstream target of both NMDAR and Gp1 mGluR signalling (Barnett et al., 2006a; Erzurumlu and Kind, 2001). NMDAR-mediated GluR1 phosphorylation at S845 is mediated by PKA, and phosphorylation of GluR1 at S845 is thought to prime AMPARs for membrane insertion (Ehlers, 2000; Esteban et al., 2003; Lee et al., 2003). During barrel development, NMDAR is thought to regulate PAKRIIβ through Ca^{2+}/calmodulin dependent AC1 or AC8 (Nicol et al., 2005). In *PrkarIIβ*$^{-/-}$ mice, despite defects in layer 4 cellular segregation, induction of LTP at TCA-layer 4 synapse, and reduced levels of GluR1 at the PSD (Inan et al., 2006; Watson et al., 2006), mutants of either GluR1 or other subunits of AMPAR display normal barrel segregation (Watson et al., 2006). Furthermore, *Adcy8*$^{-/-}$ mice show normal barrel formation (Abdel-Majid et al., 1998), while *CxAC1*$^{-/-}$ (Iwasato et al., 2008) show reduced layer 4 cell segregation into barrels but normal TCA patterning suggesting a postsynaptic role of AC1 in layer 4 barrel segregation, and the subtle barrel defects in *CxAC1*$^{-/-}$ mice closely resemble the barrel phenotype reported in this chapter for *Fmr1*$^{-/y}$ mice. In addition to a postsynaptic role of AC1, AC1 might also regulate barrel formation presynaptically via its interactions with 5-HT_{1B} receptor. These data collectively, suggest that although PAKRIIβ may mediate synaptic functions of layer 4 via NMDARs, it may also act via an alternative route in regulating anatomical segregation of barrels such as through Gp 1 mGluRs mediated catalytic activity of AC1 (Erzurumlu and Kind, 2001), and FMRP could be associated with this pathway.

cAMP mediates the effects of mGluR signalling and is produced by the catalytic activity of AC. It is also regulated by effectors of mGluR cascade

such as Ca^{2+}, PKA and PKC (Chern, 2000). The cAMP theory of fragile X states that altered cAMP metabolism may underlie some of the neurobehavioural phenotypes of FXS (Kelley et al., 2008; Kelley et al., 2007). In both *Fmr1* and *dfmr1* mutant, induction of cAMP is reduced and treatment with lithium, an activator of cAMP cascade alleviates aberrant courtship behaviour in *drosophila* model of FXS (McBride et al., 2005) and susceptibility to seizure in *Fmr1*$^{-/y}$ mice (Min et al., 2009). Lithium also affects PI metabolism (Berridge et al., 1989), therefore its effects on FXS could be regulated by PLCβ1- mediated pathways as well. Moreover, Gp1 mGluR activation of PLCβ1 results in PKC activation that in turn can regulate cAMP metabolism. Nonetheless, FMRP could potentially mediate its effects on barrel formation by associating with cAMP-PKA pathways in addition to its potential interactions with PLCβ1.

Finally, in P7 synaptoneurosomes from *Fmr1*$^{-/y}$ mice, basal levels of synGAP at the synaptic sites are also decreased compared to *Fmr1*$^{+/y}$ mice. SynGAP negatively regulates the ERK signalling pathway in a NMDAR activity dependent manner (Komiyama et al., 2002), and there is evidence to suggest a role for FMRP in regulating NMDAR dependent signalling effects (Desai et al., 2006; Gabel et al., 2004; Muddashetty et al., 2007). Early induction ERK is reported to be deficient in both *Fmr1*$^{-/y}$ mice (Kim et al., 2008) and in platelets from patients with FXS (Weng et al., 2008). In cortical synaptoneurosomes from *Fmr1*$^{-/y}$ mice, activation of Gp1 mGluRs leads to dephosphorylation of ERK in contrast to activity dependent ERK phosphorylation seen in the *Fmr1*$^{+/y}$ mice (Kim et al., 2008). Gp1 mGluRs has also been shown to activate phosphorylation of ERK in the hippocampus (Gallagher et al., 2004). Although, it has not been shown that SynGAP is a downstream effector of Gp1 mGluR signalling, chapter 3 shows that levels of SynGAP are decreased in synaptosomes from *Mglur5*$^{-/-}$ mice. Furthermore, loss of SynGAP affects layer 4 cell segregation in a dose dependent manner with near complete loss of segregation in *Syngap*$^{-/-}$ mice (Barnett et al., 2006b). *Syngap*$^{-/-}$ mice in addition to defects in layer 4 cell segregation, show altered afferent patterning. Their TCAs only segregate into rows and pattern formation in the region subserving AS is also disrupted in *Syngap*$^{-/-}$ mice (Barnett et al., 2006b). These defects in the trigeminal pathway of *Syngap*$^{-/-}$ mice parallels the phenotype of *Mglur5* mutant mice characterised in chapter

3. Therefore, it is possible that the molecular mechanisms by which FMRP and SynGAP mediate barrel formation could converge to mediate their effects.

In summary, FMRP may mediate its effects on barrel formation through its interactions with signalling cascades downstream of Gp1 mGluRs in a manner consistent with both the mGluR theory of fragile X (ie.exaggerated effects of Gp1 mGluRs in FXS) (Bear et al., 2004) and the cAMP theory of FXS (ie. altered cAMP metabolism in FXS) (Kelley et al., 2008; Kelley et al., 2007), bearing in mind that these two theories are not mutually exclusive. Although, only postsynaptic mechanisms are discussed here, a presynaptic role for FMRP in regulating barrel development cannot be ruled out, and in such a scenario AC1 could be a potential candidate for regulating FMRP mediated effects on barrel development, due to its presynaptic effects in S1 differentiation in addition to its postsynaptic role (Abdel-Majid et al., 1998; Erzurumlu and Kind, 2001; Iwasato et al., 2008).

4.3.2 FMRP locus of action in layer 4 of S1: pre vs post synaptic

FMRP is thought to be activated in a Gp1 mGluR dependent manner in mediating its regulatory effects in cellular processes (Bagni and Greenough, 2005; Bassell and Warren, 2008; Bear et al., 2008; Bear et al., 2004; Ronesi and Huber, 2008). In determining roles of FMRP in cellular processes regulating synaptic connectivity during cortical development, it is important to determine whether its effects are presynaptic or postsynaptic. Especially, to find potential therapeutic targets for FXS, it is crucial to understand where FMRP exerts its effects and this would also be important in elucidating signalling pathways regulated by or regulating FMRP.

The data presented in this chapter show FMRP localisation postsynaptically at P7, while at P14 it has a heterogeneous expression with FMRP expressed both pre and postsynaptically. EM analysis presented here was performed on cortical tissue that was contained within a barrel patch to ensure that synapses examined were layer 4 synapses. However, these may not necessarily be TCA-layer 4 cell synapses, or even synapses onto layer 4 neurons. Therefore, the presynaptic expression at P14 could reflect

presynaptic expression in either TCA-layer 4 synapses and/or cortico-cortical synapses.

Data presented in this chapter show that at P7 in *Fmr1*$^{-/y}$ mice, there is decreased layer 4 cellular segregation but normal TCA patterning and segregation. In addition, afferent segregation at VpM and nVp is also normal at P7 in *Fmr1*$^{-/y}$ mice suggesting that FMRP does not regulate patterning of whisker related afferents along the trigeminal system. The data together with the postsynaptic localisation of FMRP suggests that FMRP regulate layer 4 cell segregation postsynaptically. Chapter 3 in this thesis show that mGluR5 regulates barrel formation postsynaptically at P7. Therefore, a postsynaptic mode of action for FMRP during barrel formation is consistent with the idea that FMRP mediates it effects in a Gp1 mGluR dependent manner, and suggests that it acts downstream to mGluR5 signalling. However, a presynaptic role for FMRP in regulating other cellular processes during S1 development cannot be ruled out as there are several lines of evidence to support a role for FMRP presynaptically as well as postsynaptically in developing and refining synaptic connections.

Evidence for a postsynaptic role

In agreement with a postsynaptic role, Harlow et al., (2007) found no alteration in the paired pulse depression of TCA-layer 4 synapse at P4 and P7 in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice denoting that FMRP does not regulate presynaptic release of glutamate at this age. In hippocampal cultures of *Fmr1*$^{-/y}$ mice, acute expression of FMRP does not regulate presynaptic release probability, strength of functional synapses or their maturation suggesting a postsynaptic role for FMRP (Pfeiffer and Huber, 2007). Moreover, an immuno EM study by Weiler et al., (1997) show FMRP expression localised only to the postsynaptic site in the adult cerebral cortex and hippocampus.

Evidence for a presynaptic role

A presynaptic but not postsynaptic genotype of *Fmr1* has also been shown to result in aberrant synaptic connectivity in organotypic slices from P5/6 old

mosaic *Fmr1*$^{+/-}$ mice (Hanson and Madison, 2007). In an organtypic hippocampal culture study from mosaic *Fmr1*$^{+/-}$ mice where FMRP expressing cells (WT cells) were visualised by mating females with a mouse line expressing the GFP transgene to visualise FMRP expressing cells (express GFP), Hanson and Madison (2007) show that there is a greater incidence of WT presynaptic neurons forming synaptic connections than KO presynaptic neurons (cells that lack FMRP). In addition, postsynaptic WT neurons did not determine the probability that a neuron will receive a connection. Together, these data suggest that presynaptic expression of FMRP is important in establishing functional synaptic connections (Hanson and Madison, 2007). Consistent with this idea, Bureau et al., (2008) also found transient alterations in layer 4 to 2/3 connectivity of S1 in young *Fmr1*$^{-/y}$ mice suggesting a presynaptic role for FMRP during S1 development.

Evidence for both a pre and postsynaptic role

EM studies of immunogold labelled FMRP in adult rat cerebral cortex have shown expression of FMRP predominantly localised to the postsynaptic compartment with sparse labelling in presynaptic terminals (Feng et al., 1997). Antar et al., (2006) demonstrated *in vitro* that in developing hippocampal cultures, FMRP is localised to axons and growth cones, and as synapses mature FMRP is present in both axons and dendrites. In the *drosophila* model of FXS, dfxr loss of dFMRP results in a presynaptic phenotype of enlarged presynaptic terminals, altered neural function and increased axonal growth and complexity (Pan et al., 2004; Zhang et al., 2001). However, early induction of dFMRP in *dfmr1* null mutants reversed the anatomical phenotype of *dfxr* but not the functional neurotransmission defects alluding to a further role for dFMRP postsynaptically (Gatto and Broadie, 2008).

In light of these findings, FMRP may play a role presynaptically in S1 differentiation during development, the generation of cortex and thalamic specific mutants of FMRP will be important is answering this question.

4.3.3 Role of FMRP in regulating spines

Autopsy studies first described the long, thin spine morphology and increase in spine density observed in FXS (Hinton et al., 1991; Irwin et al., 2001; Rudelli et al., 1985). Since then spine dysgenesis due to loss of FMRP has been recapitulated in both *in vivo* and *in vitro* studies (refer to table 1.1) (Comery et al., 1997; de Vrij et al., 2008; Galvez and Greenough, 2005; Irwin et al., 2002; Nimchinsky et al., 2001; Pan et al., 2004). It is increasingly becoming evident that the specific spine phenotype associated with loss of FMRP is dependent on the neuronal population examined and also the developmental age studied (refer to section 1.2.2.3 and table 1.1) (Beckel-Mitchener and Greenough, 2004).

This chapter shows that in layer 4 spiny cells, spine density is increased by 31% in P30-P35 *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice. The volume of spine head is proportional to its synaptic area, number of postsynaptic receptors and number of presynaptic docked vesicles (Harris and Stevens, 1989; Nusser et al., 1998; Schikorski and Stevens, 1999). Interestingly, biochemical data presented in this chapter show that basal levels of AMPAR subunits are decreased at synaptic sites at both P7 and P14 in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice suggesting that spine head size is decreased at these ages. In synaptoneurosomes from *Fmr1*$^{-/y}$ mice, levels of GluR1 and GluR2/3 are decreased at P7 compared to *Fmr1*$^{+/y}$ mice. In P14 synaptoneurosomes from *Fmr1*$^{-/y}$ mice, levels of GluR1 and pGluR1 are decreased compared to *Fmr1*$^{+/y}$ mice. Although biochemical data is obtained from neocortical synaptoneurosomes, and reflect changes overall in neocortical synapses, these data indicate that spine morphology is altered in *Fmr1*$^{-/y}$ mice at these early developmental stages of S1. Nimchinksy et al., (2001) reports that in *Fmr1*$^{-/y}$ mice, spines have an immature appearance and are longer and thinner in layer 5 pyramidal cells in S1 during the first two postnatal weeks compared to *Fmr1*$^{+/y}$ mice. Although, they do not document the spine head width, it is likely that these spines are longer and thinner with smaller spine heads. In layer 4, P7 denotes the onset of synaptogenesis, but spine density is still sparse compared to the adult, whereas, P14 is the peak of synaptogenesis (White et al., 1997). Therefore, spine dynamics are rapid during these time points and there is a high

turnover of immature spines or fiolopodia seeking out synaptic connection (refer to sections 1.1.3, 1.3.2, and 2.4.3). Therefore, it is important to examine the spine morphology and density during these early time points to determine whether there is an early developmental role for FMRP in layer 4 spiny cell spinogenesis.

Anther popular theory on FXS dendritic spine dysgenesis is either a defect in spine maturation or synapse elimination/pruning may underlie the prevalence of immature spines seen in the adult brain (Bagni and Greenough, 2005). Using an organotypic slice hippocampal culture system, Pfeiffer and Huber (2007) demonstrated that acute postsynaptic expression of FMRP in *Fmr1*$^{-/y}$ neurones was sufficient to revert the increased in spine density to that of *Fmr1*$^{+/y}$ neurones. In addition to altered synapse formation, Galvez et al., (2003) also described an increase in the amount of layer 4 spiny cell dendritic material orientated towards barrel septae in adult *Fmr1*$^{-/y}$ mice relative to *Fmr1*$^{+/y}$ mice suggesting a defect in dendritic pruning. However, using the Golgi-Cox method, the analysis in this study was carried out by examining the orientation of spiny cell dendrites with respect to layer 4 cell segregation and only in the adult brain (Galvez et al., 2003). Therefore, it is important to study the elaboration and retraction of layer 4 spiny cell dendrites with respect to a TCA patch as dendrites of layer 4 spiny cells orient towards the TCA patch (Woolsey et al., 1975). In the initial experiments in which Golgi staining was carried out on tangential sections, the osmium stain was found to label barrel patches serendipitously (Upton, L, personal communications) (refer to Figure 4.14), thus enabling the characterisation of dendritic orientation with respect to a barrel patch. In addition to examining dendritic orientation, analysis of dendritic complexity of layer 4 spiny cells would provide valuable information on the role of FMRP in regulating layer 4 spiny cell complexity, growth and pruning.

4.3.4 Role of FMRP in synaptic plasticity

This chapter shows that in synaptoneurosomes from *Fmr1*$^{-/y}$ mice there are altered levels synaptic markers, specifically at P7 proposing that during S1 sensitive period, trafficking of key synaptic proteins might be impaired. Such

impairments may adversely affect synaptic connectivity and consolidation of synapses during this early window of S1 plasticity.

Harlow et al., (2007) found increased NMDA/AMPA ratio at the first postnatal week of *Fmr1*$^{-/y}$ mice at TCA-layer 4 synapse and deficits in induction of LTP during the sensitive period for eliciting LTP at the TCA-layer 4 synapse. Surprisingly, they found LTP at P7 in *Fmr1*$^{-/y}$ mice, an age which typically denotes the closure of LTP induction in TCA-layer 4 synapse in wildtype animals (Crair and Malenka, 1995; Isaac et al., 1997). Although, the exact mechanisms involved in ending the closure of this developmental plasticity period is unclear, it is marked by an increase in AMPAR-mediated synaptic transmission and a developmental NMDAR subunit switch from NR2B to NR2A (Daw et al., 2007). In *Fmr1*$^{-/y}$ mice, no changes were found in the AMPAR quantal events, the developmental switch of NMDAR subunit composition or levels of GluR1 in homogenates compared to *Fmr1*$^{+/y}$ mice (Harlow et al., 2007). Date from neocortical synaptoneurosomes from *Fmr1*$^{-/y}$ mice at P7 presented here demonstrates reduced levels of GluR1 and GluR2/3 and no changes in NR2B compared to *Fmr1*$^{+/y}$ mice. This is consistent with an increased NMDA/AMPA ratio, and robust LTP suggests that synapses with low levels of AMPARs are primed to undergo LTP. At P14 in *Fmr1*$^{-/y}$ mice, levels of GluR2/3 and NR2B were significantly reduced in homogenates (GluR1 decreased but not significant), while in synaptoneurosomes GluR1 and pGluR1 were significantly decreased (GluR2/3 and NR2B were decreased but not significant) relative to *Fmr1*$^{+/y}$ mice suggesting that at P14, in addition to altered levels of AMPARs, there may be global changes in NMDARs as well. This agrees with the gradual decrease in NMDA/AMPA ratio observed after P7 by Harlow et al., (2007) in *Fmr1*$^{-/y}$ mice. It is possible that levels of NR2A may also be altered in *Fmr1*$^{-/y}$ mice during S1 development.

A recent study by Pilpel et al., (2008) show similar functional defects during development in the hippocampus in another *Fmr1* null mutant, referred to as Fmr1 KO2 (Mientjes et al., 2006). In hippocampal CA1, compared to wildtype animals a decreased AMPA/NMDA ratio is observed in Fmr1 KO2, and this appeared to be caused by a downregulation in AMPA with a concomitant upregulation of NMDA component at P14 but not at 6-7 weeks (Pilpel et al., 2008). These changes are also accompanied by an increased NMDAR-

dependent LTP induction at this developmental age but this altered LTP is not evident in the adult (Pilpel et al., 2008). Moreover, in *Fmr1*$^{-/y}$ mice there are deficits in synaptic delivery and trafficking of GluR1 receptors at P14 compared to *Fmr1*$^{+/y}$ mice due to aberrant Ras signalling at hippocampal CA1 and cortical layer 2/3 (Hu et al., 2008). Collectively, the data from the barrel cortex and hippocampus suggest a developmental delay in maturation of synapses in *Fmr1*$^{-/y}$ mice. It would be interesting to determine whether the there are any changes in the kainate receptor component that is present early in TCA-layer 4 synapse in *Fmr1*$^{-/y}$ mice relative to *Fmr1*$^{+/y}$ mice.

Persistent alterations in synaptic plasticity in neocortex have also been described in the neocortex of *Fmr1*$^{-/y}$ mice (Desai et al., 2006; Li et al., 2002; Pilpel et al., 2008; Wilson and Cox, 2007), and impaired GluR1 trafficking seems to one of the factors that underlie these synaptic alterations (Li et al., 2002). Therefore, it would be interesting to characterise levels of glutamatergic receptors in synaptoneurosomes from adult *Fmr1*$^{-/y}$ mice to determine whether these early defects in trafficking and localisation of synaptic proteins persist into the adult. Recently, Gibson et al., (2008) show changes at layer 4 local synaptic connectivity that may result in severe impairments in local feed back loop to layer 4 of S1 leading to hyperexcitability of layer 4 neuronal circuitry.

FXS is a neurodevelopmental disorder and it is clear that expression of FMRP is abundant in brain regions examined during early development suggesting that it plays a critical role in regulating cellular processes that lead to formation and establishment of correct synaptic connectivity. Consistent with this idea loss of FMRP leads to defects in layer 4 cellular segregation, spinogenesis in S1 and altered synaptic trafficking and localisation in the neocortex during early postnatal development. Hence, in treating patients with FXS, an early intervention and diagnosis would be of more benefit.

5

Key findings:

1. The basal expression of mGluR5 is largely unaffected during the first two postnatal weeks in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice.

2. Similarly, loss of FMRP does not appear to affect basal cellular expression patterns of mGluR5 at P7.

3. Decreasing levels of mGluR5 signalling in *Fmr1*$^{-/y}$ mice results in the rescue of their decreased layer 4 cellular segregation to that of *Mglur5*$^{+/-}$ mice but not wildtype mice at P7.

4. Preliminary data suggests that decreasing levels of mGluR5 in *Fmr1*$^{-/y}$ mice does not ameliorate the increased layer 4 spinogenesis during late S1 development.

5 Interactions between mGluR5 and FMRP during S1 development

5.1 Introduction

'The mGluR theory of fragile X mental retardation' postulates that "The psychiatric and neurological aspects of FXS are a consequence of exaggerated responses to mGluR1/5 activation" (Bear et al., 2004). This theory was originally proposed on the premise of several coincidental findings as follows (Bear et al., 2004): Stimulation of Gp1 mGluRs result in synaptic protein synthesis (Weiler and Greenough, 1993) that also include synthesis of FMRP (Weiler et al., 1997). Many lasting functional and structural synaptic modifications upon Gp1 mGluR stimulation such as LTD expression in the hippocampus and internalisation of glutamatergic receptors in *in vitro* hippocampal cultures depend on the translation of new proteins (Huber et al., 2000; Karachot et al., 2001; Merlin et al., 1998; Naie and Manahan-Vaughan, 2005; Raymond et al., 2000; Vanderklish and Edelman, 2002; Zho et al., 2002). Interestingly, in *Fmr1*$^{-/y}$ mice, one of the Gp1 mGluR activated protein synthesis dependent processes, mGluR-LTD was found to be exaggerated by 20% (Huber et al., 2002). Moreover, several studies ensuing this initial finding suggested that effects of exaggerated Gp1 mGluR signalling correlated well with several symptoms of FXS (reviewed in Bear et al., 2004; refer to table 1.2). Concomitantly, several lines of evidence suggested a role for FMRP as a regulator of protein synthesis, both as a translational repressor (Brown et al., 2001; Li et al., 2001) and as a translational activator (Todd et al., 2003).

Since its proposal the mGluR theory of fragile X has gained supportive evidence, and is considered as one of the prevalent hypothesis for the manifestation of FXS. More studies have identified effects of dysregulated Gp1 mGluR signalling in FXS experimental models that agree well with the clinical phenotypes of FXS. For example, prolonged epileptiform discharges in hippocampal slices, which correlate well with increased seizure susceptibility in patients with FXS (Chuang et al., 2005) and elongated spines and enhanced LTD in cerebellar Purkinje cell-parallel fibre synapses, which is

consistent with the developmental delay in motor skills evident in patients with FXS (Koekkoek et al., 2005). Moreover, evidence show that in *Fmr1*$^{-/y}$ mice there is altered basal protein synthesis (Liao et al., 2008; Qin et al., 2005) whilst Gp1 mGluR stimulation dependent translation is altered and/or absent (Bassell and Warren, 2008; Grossman et al., 2006a; Hou et al., 2006; Muddashetty et al., 2007; Ronesi and Huber, 2008; Todd et al., 2003).

In essence, the mGluR theory of fragile X suggests that mGluR5 and FMRP potentially act in opposition to regulate processes that require Gp1 mGluR activity, and is a simplistic model to test, which predicts that neuropathological symptoms of FXS can be ameliorated by reducing signalling via Gp1 mGluR (Bear et al., 2004; Dolen and Bear, 2008). Several studies in recent times have tested this theory and lend supporting evidence to it. Consistent with the predictions of the mGluR theory of fragile X, the pharmacological application of mGluR5 specific antagonist MPEP (2-methyl-6-(phenylethynyl)-pyridine has been shown to rescue several FXS morphological, physiological and behavioural phenotypes (de Vrij et al., 2008; McBride et al., 2005; Michel et al., 2004; Nakamoto et al., 2007; Pan et al., 2008; Tucker et al., 2006; Yan et al., 2005). The acute application of MPEP, corrects the increased filopodial protrusions in *Fmr1*$^{-/y}$ hippocampal neurons to that of wildtype neurons *in vitro* (de Vrij et al., 2008). MPEP also corrects the excessive internalisation of synaptic AMPARs observed at basal levels in *Fmr1*$^{-/y}$ neurons (Nakamoto et al., 2007). The mushroom bodies in *drosophila* are thought to be important in learning and memory (Pascual and Preat, 2001), and defects in mushroom bodies caused by the fusion of mushroom body β lobes are found in *dfmr1* mutant flies (Michel et al., 2004). Administration of MPEP rescues these mushroom body defects and deficits in experience dependent courtship behaviour in *dfmr1* mutants (McBride et al., 2005), as well as the increased presynaptic architectural complexity in *dfmr1* mutants (Pan et al., 2008). The correction of behavioural deficits in FXS experimental models by MPEP treatments is also reported in *Fmr1*$^{-/y}$ mice (Yan et al., 2005). The acute administration of MPEP in *Fmr1*$^{-/y}$ mice suppresses their seizure phenotype and their preferential tendency to spend more time in the open field (Yan et al., 2005). However, at high concentrations, MPEP has been shown to have off target effects (Heidbreder

et al., 2003) such as blocking NMDARs (Lea and Faden, 2006; Spooren et al., 2001).

The recent findings of Dolen et al., (2007) add further support to the mGluR theory of fragile X. Using a genetic strategy whereby levels of mGluR5 in *Fmr1*$^{-/y}$ mice are reduced, they show correction of multiple FXS phenotypes that included behavioural deficits such as enhanced inhibitory avoidance extinction and increased susceptibility to audiogenic seizures, physiological defects such as exaggerated hippocampal LTD and ocular dominance plasticity, and morphological deficits such as increased layer 3 spine density in *Fmr1*$^{-/y}$*/Mglur5*$^{+/-}$ mice to that of wildtype mice (Dolen et al., 2007). In addition, they show increased basal protein synthesis in *Fmr1*$^{-/y}$ mice, which can also be corrected to levels comparable to wildtype mice by reducing mGluR5 signalling by 50%. However, macroorchidism in *Fmr1*$^{-/y}$ mice is not alleviated in *Fmr1*$^{-/y}$*/Mglur5*$^{+/-}$ mice (Dolen et al., 2007). Therefore, not only acute blockade of mGluR5 but genetic (chronic) reduction of mGluR5 also provides support for the mGluR theory of fragile X, and mGluR5 appears to be the key Gp1 mGluR required (Dolen et al., 2007).

Given the success of blockade of mGluR5 signalling in ameliorating phenotypes of FXS in experimental models, several attempts are underway to trial mGluR5 specific antagonists in phase I clinical trials (Berry-Kravis et al., 2009). Most encouragingly, the recent pilot open-labelled single dose trial of fenobam, a highly potent and highly selective mGluR5 antagonist comparable to MPEP, in a small cohort of adult fragile X patients suggests that fenobam is safe to use in humans. A single dose of fenobam has been shown have safe pharmacokinetics in metabolism (Berry-Kravis et al., 2009) and not result in side effects of metabolic intolerance that have been previously described in patients with anxiety disorders (Friedmann et al., 1980; Pecknold et al., 1982). Moreover, the study reports of rapid improvements in prepulse inhibition, a measure of sensorimotor gating and inhibitory control with the intake of a single dose in fragile X patients (Berry-Kravis et al., 2009).

Despite the success in improving FXS symptoms with reducing signalling via mGluR5, almost all studies to date utilising either genetic reduction or pharmacological blockade of mGluR5 have only examined the effects of these

strategies in the adult. Therefore, interactions between mGluR5 and FMRP during early stages of cortical development are yet widely unexplored. In addition, their interactions at a cellular level and the mechanisms responsible for the amelioration observed with reduced Gp1 mGluR signalling in FXS are all open questions.

This chapter first examines whether cellular expression pattern of and levels of mGluR5 are altered in *Fmr1*$^{-/y}$ mice during early S1 development at basal levels to determine whether FMRP regulates mGluR5 protein expression. Secondly, it aims to examine the interactions between FMRP and mGluR5 during S1 development; specifically, to test the hypothesis that the anatomical defects observed in the S1 of *Fmr1*$^{-/y}$ mice will be rescued by genetically reducing levels of mGluR5. The defects of barrel formation during early S1 and defects in spine density of layer 4 spiny cells during late S1 development in *Fmr1*$^{-/y}$ mice will be investigated in the double mutant *Fmr1*$^{-/y}$/*Mglur5*$^{+/-}$ mice, and there are several possible outcomes. These S1 phenotypes in *Fmr1*$^{-/y}$ mice could be ameliorated, exacerbated or remain persistent in *Fmr1*$^{-/y}$/*Mglur5*$^{+/-}$ mice. Levels of mGluR5 in *Mglur5*$^{+/-}$ mice are reduced by 50% (chapter 3 and Dolen et al., 2007), and the *Fmr1*$^{-/y}$/*Mglur5*$^{+/-}$ mice with reduced levels of mGluR5 were generated by crossing female *Fmr1*$^{+/-}$ mice with male *Mglur5*$^{+/-}$ mice in collaboration with the Bear lab (Dolen et al., 2007).

5.2 Results

5.2.1 The basal expression of mGluR5 is largely unaltered in the absence of FMRP

To answer the question whether FMRP regulates mGluR5 protein synthesis, the regional and cellular expression of mGluR5 was examined at P7 in *Fmr1*$^{-/y}$ mice to determine whether the absence of FMRP affects the basal cellular localisation and levels of mGluR5 expression (Figure 5.1). The regional expression of mGluR5 in areas of cortex, hippocampus, thalamus (in particular in VpM) and striatum appeared not to differ in P7 *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Figure 5.1A-D). The barreloid pattern evident by

staining of mGluR5 in VpM of the thalamus was also evident in *Fmr1*$^{-/y}$ mice (Figure 5.1C and D). In the hippocampus of both *Fmr1*$^{-/y}$ and *Fmr1*$^{+/y}$ mice, expression of mGluR5 was concentrated in basal dendrites of pyramidal cells in stratum oriens, but expression was absent in the stratum pyramidal, which contains their cell bodies. In both genotypes, mGluR5 was also expressed in other CA1-CA3 layers, strata radiatum, lacunosum and moleculare, with highest expression in stratum lacunosum, which contains the schaeffer collateral and perforant pathways. The mGluR5 expression in CA3 stratum lucidem that contains mossy fibres from DG was also unaltered in *Fmr1*$^{-/y}$ mice. There was also little if any mGluR5 expression in the cell body layer stratum granulosum of DG, whereas the polymorphic layer of DG had some mGluR5 staining in both genotypes (Figure 5.1E and F). The mGluR5 expression in the cortex also did not differ between the two genotypes at P7 (Figure 5.1G and H). mGluR5 was expressed throughout all cortical layers but expression was most intense in cortical layers 4 and 5 in both genotypes. In both the hippocampus and cortex, mGluR5 staining appeared to be diffused and no punctuate staining was observed at P7 in either *Fmr1*$^{-/y}$ or *Fmr1*$^{+/y}$ mice. This diffuse neuropil staining was further evident in tangential section across layer 4 from both genotypes at P7, where mGlur5 expression was localised to barrel patches in both AS and PMBSF regions. Furthermore, there was little if any expression of mGluR5 observed in the inter-barrel septal regions (Figure 5.2A-D).

To determine whether there were any changes quantitatively despite normal cellular expression of mGluR5 in *Fmr1*$^{-/y}$ mice, levels of mGluR5 was examined in both neocortical homogenates and synaptoneurosomes isolated from P7 and P14 in *Fmr1*$^{-/y}$ and *Fmr1*$^{+/y}$ mice (Figure 5.3). The genetic deletion of FMRP did not alter global levels of mGluR5 in neocortical homogenates of *Fmr1*$^{-/y}$ mice at both P7 (n=6) and P14 (n=4) relative to *Fmr1*$^{+/y}$ mice (Figure 5.3A). This is consistent with findings of Dolen et al (2007) where they found no changes in levels of mGluR5 in visual cortex homogenates from P30 *Fmr1*$^{-/y}$ mice. In synaptoneurosomes, there were no significant changes in levels of mGluR5 expression at both ages in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (n=3 at P7; n=5 at P14) (Figure 5.3B) suggesting that loss of FMRP does not alter levels of mGluR5 at the synapse. Collectively, these data indicate that while FMRP is thought to constrain

effects of Gp1 mGluR dependent signalling, it may not directly regulate expression of mGluR5. Alternatively, FMRP may regulate expression of mGluR5 in an activity dependent manner, and examining the expression profile of mGluR5 at basal levels would not reveal this. For example, will whisker stimulation in *Fmr1*$^{-/y}$ mice lead to altered levels of mGluR5 expression in neocortical synaptoneurosomes from these mice?

5.2.2 The basal cellular expression of FMRP is largely unaltered in the absence of mGluR5

FMRP expression is translocated to dendrites *in vitro* in response to Gp1 mGluR activation (Antar et al., 2004; Ferrari et al., 2007). Therefore, genetic deletion of mGluR5 may lead to mislocalisation of basal FMRP expression. To determine whether the loss of mGluR5 results in impaired cellular expression of FMRP, immunohistochemical studies were carried out at P7 to determine expression of FMRP in *Mglur5*$^{-/-}$, *Mglur5*$^{+/-}$ and *Mglur5*$^{+/+}$ mice (Figure 5.4). In coronal sections from these mice, no changes in the regional expression of FMRP in cortex, hippocampus, VpM of thalamus (Figure 5.4J-L) or striatum (Figure 5.4A-C) was observed among the genotypes. In the hippocampus, the dense FMRP staining in the cell body layer of CA1-CA3, stratum pyramidal and the DG cell body layer, stratum granulosum evident in *Mglur5*$^{+/+}$ mice (Figure 5.4D) was also present in both *Mglur5*$^{+/-}$ and *Mglur5*$^{-/-}$ mice (Figure 5.4E and F). In all genotypes, there was little if any staining in the dendrites of hippocampal pyramidal cells.

In the cortex, FMRP expression was present throughout all cortical layers irrespective of the genotype (Figure 5.4G-I). FMRP expression was abundant in the cytoplasm of cortical neurons but staining was also evident in proximal dendrites. The abundant cytoplasmic expression of FMRP was also observed in tangential sections though layer 4 where a clear pattern of layer 4 cells segregating into barrels was apparent in *Mglur5*$^{+/+}$ mice (Figure 5.4M), whereas in *Mglur5*$^{-/-}$ mice (Figure 5.4N), such a barrel pattern was less obvious. At P7, thionin staining of layer 4 cells show that layer 4 cells of *Mglur5*$^{-/-}$ mice fail to aggregate into barrels while TCAs segregate into rough patches within a row compared to *Mglur5*$^{+/+}$ mice (chapter 3.2.4 and 3.2.3). At P7, FMRP is postsynaptically localised (chapter 4.3.2) suggesting that its

expression is localised to layer 4 cell cytoplasm and dendrites. Interestingly, in tangential sections from *Mglur5*$^{-/-}$ mice, the cytoplasmic FMRP staining revealed layer 4 cells aggregating into a rough pattern of rows relative to *Mglur5*$^{+/+}$ mice. This is in contrast to the roughly uniform layer 4 cellular distribution observed in *Mglur5*$^{-/-}$ mice revealed by thionin staining. Thionin staining reveals all cells by staining nissl substance found in granular endoplasmic reticulum and ribosomes occurring in cell bodies and dendrites. Therefore, the layer 4 cellular pattern revealed with the cytoplasmic FMRP suggests that FMRP may only be expressed in a subset of cortical cell types. In summary, the basal cellular localisation of FMRP was largely unaffected by the loss of mGluR5, however, its activity dependent translocation to synaptic sites (Antar et al., 2004; Ferrari et al., 2007; Zalfa et al., 2007) might be affected in *Mglur5*$^{+/-}$ and *Mglur5*$^{-/-}$ mice and needs to be examined.

5.2.3 Reducing levels of mGluR5 results in the partial rescue of defects in barrel formation in *Fmr1*$^{-/y}$ mice

Altered cortical connectivity is often associated with FXS, and chapter 4 shows that loss of FMRP results in decreased layer 4 cellular segregation into barrels. In this chapter, the question is whether reducing levels of mGluR5 will rescue these barrel defects in *Fmr1*$^{-/y}$ mice. However, the caveat in this rescue attempt is that mGluR5 itself affects barrel formation in a dose dependent manner. The ratio of layer 4 cells in barrel wall to hollow was analysed in wildtype, *Mglur5*$^{+/-}$, *Fmr1*$^{-/y}$ and *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice at P7 on a C57BL/6J background to determine whether the decreased barrel wall: hollow in *Fmr1*$^{-/y}$ can be rescued by reducing mGluR5 levels. Statistical analysis was performed using ANOVA with *post hoc* Fisher's LSD test. *Fmr1*$^{-/y}$ mice (Figure 5.5B and 5.6A) showed significantly reduced cellular segregation of layer 4 cells as reported in chapter 4 (1.45±0.03;n=6) compared to wildtype mice (1.64±0.02; n=9; $p<0.0001$) (Figure 5.5A and 5.6A). Consistent with the phenotype found on a C57BL/6X129 background due to genetic deletion of *Mglur5*, *Mglur5*$^{+/-}$ mice (Figure 5.5C and 5.6A) on this background also showed significantly reduced layer 4 cellular segregation compared to wildtype mice (1.53±0.02, n=8; p=0.002) on C57BL/6J background, and their barrel segregation was significantly better than *Fmr1*$^{-/y}$ mice (p=0.039). The barrel segregation in *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$

mice (1.54±0.03, n=6) was significantly improved compared to *Fmr1*$^{-/y}$ mice (p=0.037), and was comparable to that of *Mglur5*$^{+/-}$ mice. However, segregation in *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ was significantly different from the wildtype mice (p=0.007). This suggests that reducing levels of mGluR5 in *Fmr1*$^{-/y}$ mice rescues the early defects in barrel segregation to that of *Mglur5*$^{+/-}$ mice but not wildtype mice consistent with the idea that levels of mGluR5 are also a critical factor in normal barrel segregation. While there are deficits in layer 4 cellular segregation, the TCA patch size corresponding to the barrel analysed did not differ significantly between genotypes (Figure 5.5A-D; 5.6B).

5.2.4 Reducing levels of mGluR5 does not appear to rescue defects in spine density in Fmr1$^{-/y}$ mice

Spine dysgenesis is a feature of FXS in humans and is the most well characterised neuropathalogical finding in both *in vivo* and *in vitro* experimental models of FXS (reviewed in Beckel-Mitchener and Greenough, 2004). In agreement with spine analysis studies of fragile X in other cortical areas, layer 4 spiny cells also have increased spine density in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (chapter 4.3.4). Also, the genetic deletion of mGluR5 affects spinogenesis in layer 4 spiny cells such that *Mglur5*$^{+/-}$ mice show decreased spine density on a C57BL/6JX129 background and is found to be as severe as the spine phenotype in *Mglur5*$^{-/-}$ mice (chapter 32.5). Here, spine analysis of layer 4 spiny cells in PMBSF was carried out in wildtype, *Mglur5*$^{+/-}$, *Fmr1*$^{-/y}$ and *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice at P30-35 on a C57BL/6J background to determine whether reduced levels of mGluR5 in *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice will rescue the spine phenotype in *Fmr1*$^{-/y}$ mice. The availability of mice from Picower Centre prevented a complete detailed study for this thesis, however, some clear findings are suggested from the limited number of mice [(wildtype (n=3), *Fmr1*$^{-/y}$ (n=4) and *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ (n=2) mice)] examined. From each animal, three dendrites with normal tapered endings were analysed from three independent cells (refer to section 2.2.4.2) and statistical analysis was performed using an ANOVA with *post hoc* Fisher's LSD test.

In *Fmr1*$^{-/y}$ mice, spine density was increased throughout the dendritic length compared to wildtype mice (Figure 5.7A-C and D). The spine density/10μm

was significantly increased in *Fmr1*$^{-/y}$ mice (10.60±0.79; n=4) relative to wildtype mice (8.06±0.31; n=3; p=0.019) (Figure 5.7E). The spine density in *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice (11.01±0.47; n=2) was also significantly increased compared to wildtype mice (p=0.021) (Figure 5.7E). The average dendritic length of layer 4 spiny cells did not differ between genotypes (Figure 5.7F). However, there was a trend towards an increase in the average spine count along the dendritic length in both *Fmr1*$^{-/y}$ (150.75±12.01) and *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ (171.17±1.5) mice compared to wildtype mice (114.78±21.27) (Figure 5.7G). Collectively, these preliminary data suggests that decreasing levels of mGluR5 in *Fmr1*$^{-/y}$ mice does not appear to rescue their spine density phenotype in layer 4 spiny cells. Further analysis is required in both *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ and *Mglur5*$^{+/-}$ mice to validate findings presented here. However, although it is only a sample of 2 animals that was analysed for *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice, the robustness of the spine phenotype observed in this small cohort makes it unlikely that more animals will alter these preliminary findings.

5.3 Discussion

The focus of this chapter was to investigate the interactions between mGluR5 and FMRP during S1 development. To first address the question whether there are regulatory interactions between the two at basal levels, expression profiles of mGluR5 and FMRP were characterised in *Fmr1*$^{-/y}$ and *Mglur5* mutants respectively. The immunohistochemical studies presented suggest that during early S1 development (P7), the genetic loss of FMRP does not alter the basal expression profile of mGluR5. Despite unaltered basal expression of mGluR5 observed in *Fmr1*$^{-/y}$ mice as presented here and in Dolen et al., (2007), several lines of evidence suggest that protein synthesis dependent Gp1 mGluR mediated functional consequences such as hippocampal LTD, prolonged epileptiform bursts and AMPAR internalisation are exaggerated in *Fmr1*$^{-/y}$ mice (Bear et al., 2004; Chuang et al., 2005; Hou et al., 2006; Koekkoek et al., 2005). It is likely that while the loss of FMRP does not alter basal expression of mGluR5, the functions of downstream effectors of Gp1 mGluR mediated signalling cascades regulated by FMRP are dysregulated resulting in perturbed cellular processes. The loss of mGluR5

also largely did not affect the predominantly cytoplasmic expression pattern of FMRP at P7. However, FMRP has been shown to be translocate to sites of active synapses in a Gp1 mGluR dependent manner (Antar et al., 2004; Ferrari et al., 2007) suggesting that loss of mGluR5 might dysregulate synaptic activity dependent localisation of FMRP leading to aberrant targeting of FMRP and its cargo mRNA. Therefore, one could hypothesise that the interactions between mGluR5 and FMRP might be activity dependent and may not be evident when examining at basal levels.

Secondly, this chapter examines the interactions between FMRP and mGluR5 by exploring whether the anatomical phenotypes in S1 of *Fmr1*$^{-/y}$ mice will ameliorate, exacerbate or persist with the reduction in levels of mGluR5 (ie. in *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice). In *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice, barrel segregation was better than *Fmr1*$^{-/y}$ mice and is comparable to segregation in *Mglur5*$^{+/-}$ mice. However, defects in spine density evident in *Fmr1*$^{-/y}$ mice persisted in *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice. Chapter 3 showed that defects in layer 4 spine density in *Mglur5*$^{+/-}$ mice were comparable to those in *Mglur5*$^{-/-}$ mice (section 3.2.6) suggesting that levels of mGluR5 are critical in spinogenesis. A parsimonious view of these data is that levels of mGluR5 are critical in the regulation of different cellular processes. Furthermore, signalling via mGluR5 may also have differential roles in regulating different cellular phenotypes.

5.3.1 Levels of mGluR5 are of critical importance in normal cortical development

Data presented in this thesis suggest levels of mGluR5 are critical in regulating S1 differentiation and that the levels required may vary depending on the cellular process. For example, when the contralateral eye is closed during paradigms of monocular depravation, layer 4 cells in visual cortex of *Mglur5*$^{+/-}$ mice show lack of deprived eye depression compared to wildtype mice (Dolen et al., 2007). This agrees well with decreased spine density in layer 4 cell dendritic spines suggesting that these cells are less likely to undergo depression resulting in a 'hypoplastic' response (Dolen et al., 2007). It could be that depression in these synapses is already saturated but homeostatic mechanisms have returned activity levels to normal, as these

mice do not exhibit any alterations in visually evoked action potentials to sensory stimuli (Dolen et al., 2007).

The critical level of mGluR5 required in regulating cellular processes may also depend on the neuronal cell type involved. The reduced signalling via mGluR5 does not alter spine density in layer 3 pyramidal cells in *Mglur5*$^{+/-}$ mice compared to *Mglur5*$^{+/+}$ mice (Dolen et al., 2007). However, data presented in chapter 3 shows that reducing mGluR5 levels by 50% results in decreased spine density in *Mglur5*$^{+/-}$ mice that is comparable to *Mglur5*$^{-/-}$ mice. Conversely, loss of mGluR5 has been shown to result in increased spine density in layer 4 pyramidal cells (Hui-Chen, personal communications).

Finally, glutamatergic signalling via mGluR5 is also involved in regulating normal cognitive behaviour. *Mglur5*$^{-/-}$ mice show deficits in spatial learning, reward based learning, reference and working memory performance and contextual fear conditioning (Chiamulera et al., 2001; Lu et al., 1997; Naie and Manahan-Vaughan, 2004). However, these studies did not examine whether these behavioural deficits due to loss of mGluR5 is also dose dependent. Therefore, it is important to decipher the regulatory role of mGluR5 in different cortical processes with particular attention given to neuronal cell type in question and the developmental ages in question. Furthermore, it is critical to determine the levels of mGluR5 required to maintain processes regulated via mGluR5 signalling.

5.3.2 The complexities of the interaction between mGluR5 and FMRP

Despite the caveat of the importance of glutamatergic signalling through mGluR5 in normal cortical processes, decreasing levels of mGluR5 in *Fmr1*$^{-/y}$ mice has been shown to rescue several FXS phenotypes (Dolen et al., 2007). As mentioned above both mGluR5 and FMRP are important in cognitive processes (Bernardet and Crusio, 2006). However, the precise nature of the interactions may be cell type dependent, age dependent and also may vary depending on the cellular process being regulated. For example, protein synthesis appears to be critical in processes of learning and memory (Costa-Mattioli et al., 2009; Hernandez and Abel, 2008; Klann and Sweatt, 2008) and complete genetic deletion of both mGluR5 (Naie and Manahan-Vaughan, 2005) and FMRP (Bolduc et al., 2008) result in dysregulated protein

synthesis and impaired learning and memory. However, reducing levels of mGluR5 in *Fmr1*$^{-/y}$ mice rescued the excessive elevated levels of basal protein synthesis as well as exaggerated inhibitory avoidance learning behaviour, which is a task that is protein synthesis dependent (Dolen et al., 2007). A key thing to note is that in both of these cases, both *Mglur5*$^{+/-}$ and *Fmr1*$^{-/y}$/ *Mglur5*$^{+/-}$ mice were comparable to wildtype mice and had no defects in either protein synthesis or inhibitory avoidance learning (Dolen et al., 2007).

In cellular processes that appear to require critical levels of mGluR5 signalling such as barrel formation and layer 4 spinogenesis, the interactions between mGluR5 and FMRP are more complex. For example, genetically reducing levels of mGluR5 rescues the barrel formation in *Fmr1*$^{-/y}$ mice to that of *Mglur5*$^{+/-}$ mice. However, it does not appear to rescue the spine phenotype in layer 4 spiny cells, a cellular process, in which reducing mGluR5 levels by 50% had a similar effect to complete loss of mGluR5. Moreover, although the layer 4 spiny cell spine phenotypes in *Fmr1*$^{-/y}$ and *Mglur5*$^{+/-}$ mice are in opposition, the double mutant display a spine phenotype that is as severe as *Fmr1*$^{-/y}$ mice. This outcome suggests that while both mGluR5 and FMRP are involved in regulating spinogenesis in layer 4 spiny cells, their regulatory effects in this process may not be mediated via a simple direct 'upstream downstream' pathway. It could be that glutamatergic signalling via mGluR5 and FMRP act on two independent pathways that converge at multiple levels or may converge at the end to regulate spinogenesis in layer 4 spiny cells.

Moreover, in addition to the complex nature of mGluR5 in regulating spine density as discussed, role of FMRP in spinogenesis is also developmentally regulated (Nimchinsky et al., 2001). For example, in layer 5 pyramidal cells of S1 the increased spine length and density is only evident during the first two weeks of postnatal life, and is absent during the third week of postnatal development (Nimchinsky et al., 2001). However, another study show that layer 5 pyramidal cells from S1 at P25 as well as in the adult to have increased spine density (Galvez and Greenough, 2005). There is also evidence to suggest that role of FMRP in spinogenesis may be region specific, (Braun and Segal, 2000 but see de Vrij et al., 2008) such that in contrast to the increased spine length observed in the cortex (Beckel-Mitchener and

Greenough, 2004) and the cerebellum (Koekkoek et al., 2005), an *in vitro* hippocampal culture study show neurons from *Fmr1*$^{-/y}$ mice (21 days *in vitro*) to have decreased spine length and density (Braun and Segal, 2000). However, another hippocampal culture study (20 days *in vitro*) (de Vrij et al., 2008) and a Golgi study in adult hippocampus CA1 region find the spine length to be increased (Grossman et al., 2006b). It is possible that these conflicting data on the spine phenotype of hippocampal neurons from *Fmr1*$^{-/y}$ mice may reflect differences in techniques (refer to table 1.1) and ages rather than a region specific effect. Therefore, to fully appreciate the interactions between mGluR5 and FMRP it is important to investigate their interactions in the context of neuronal cell type, brain region and developmental stage. It may also be fruitful to see whether the outcome would be different if acute reduction of mGluR5 were achieved in *Fmr1*$^{-/y}$ mice either by mating these to floxed *Mglur5* mutants or by pharmacological blockade. The chronic loss of mGluR5 may have multiple effects that could be detrimental to cellular processes; therefore, examining acute localised reduction of mGluR5 will eliminate this possibility.

Hence, while treatment of FXS with antagonists of mGluR5 and genetic manipulation of mGluR5 has been shown to be successful in a number of *in vitro* and *in vivo* studies, it is critical to understand the developmentally regulated role of FMRP and mGluR5 and their interactions to advocate better therapeutic interventions. The mouse S1 provides an excellent model in addition to other existing ones to characterise these roles because formation of S1 is a developmentally regulated sequential process that regulate cortical connectivity. Studies are underway to determine the role of FMRP in S1 formation throughout development to determine whether defects identified here persist through to adult or whether these are due to a developmental delay. Moreover, in addition to critical ages where FMRP plays a role in barrel formation its role in synaptogenesis needs to be further characterised. The increased spine density with an immature appearance is a hallmark of FXS (Beckel-Mitchener and Greenough, 2004), therefore, it is important to investigate whether there are any morphological changes in layer 4 spiny cells in addition to changes in spine density in *Fmr1*$^{-/y}$ mice. Once these cellular processes are characterised in *Fmr1*$^{-/y}$ mice, the genetic manipulation of mGluR5 levels in these mice will reveal whether a) decreasing levels of

mGluR5 differentially affect the S1 phenotype in *Fmr1*$^{-/y}$ mice and b) whether the postnatal age of intervention is critical.

6

Key findings:

1. The spatiotemporal expression profiles of PSD95, SAP102 and PSD93 are developmentally regulated.

2. Preliminary data suggests that there is differential compensation among PSD95 family MAGUKs.

3. Loss of PSD95 results in altered trafficking and localisation of NMDAR subunits NR2B and NR1, AMPAR subunit GluR1 as well as SynGAP at P7.

4. Despite the fact that PSD95 family MAGUKs, PSD95, SAP102 and PSD93 have putative binding domains for FMRP targeting, loss of FMRP does not alter their basal expression profiles during early S1 development.

6 Role of MAGUKs in synaptic protein trafficking and localisation during early S1 development-implications for FXS

6.1 Introduction

MAGUKs are scaffolding proteins that play a critical role in the assembly of the postsynaptic compartment of excitatory synapses by recruiting glutamatergic receptors and specific cytoskeletal and signalling proteins to the PSD. Through their domain specific interactions, MAGUKs form microsignalling domains within the synaptic spine, thereby regulating the size and strength of either basal or evoked synaptic responses (Collins and Grant, 2007; Kim and Sheng, 2004). The best-characterised MAGUK is PSD95 (also known as synapse associated protein 90, SAP90). The PSD95 family of MAGUKs includes PSD93/Chapsyn110, SAP102 and SAP97 (Bredt and Nicoll, 2003; Kim and Sheng, 2004). These proteins are characterised by three PDZ (PSD95, Disc large, Zona occludens) domains, a SH3 (Src Homology 3) domain and a GK (Guanylate Kinase) domain that facilitate a plethora of protein interactions (Kim and Sheng, 2004). It could be hypothesised that loss of these genes may preclude normal synaptic connectivity in the brain and perturb processes involved in learning and memory (Gardoni et al., 2009; Lau and Zukin, 2007; Laumonnier et al., 2007). Mutations in *SAP102* have been shown to cause non-syndromic X-linked MR (Tarpey et al., 2004). Moreover, deregulated PSD95 and SAP97 localisation has been shown in mouse models of Parkinson disease (Nash et al., 2005; Picconi et al., 2004).

Todd et al., (2003) also proposed that expression of PSD95 is dysregulated in FXS. By sequence analysis they found *Psd95* to have a putative FMRP binding G quartet in its 3' UTR (Todd et al., 2003). Furthermore, Gp1 mGluR agonist stimulation dependent increases in levels of PSD95 seen in cortical cultures is absent in cultures from *Fmr1*$^{-/y}$ mice suggesting that FMRP is required for its translation (Todd et al., 2003). The Gp1 mGluR dependent increases in

PSD95 and Gp1 mGluR dependent incorporation of *Psd95* mRNA into actively translating polyribosomes evident in brain synaptoneurosomes from adult *Fmr1*$^{+/y}$ mice is also absent in *Fmr1*$^{-/y}$ mice (Muddashetty et al., 2007). In a recent study, Zalfa et al., (2007) show that the C terminus of FMRP directly interacts with the 3' UTR of *Psd95* mRNA *in vivo*, and that *Psd95* is dendritically localised with FMRP. However, *Psd95* localisation in dendrites is unaltered in cortical cultures from *Fmr1*$^{-/y}$ mice at basal levels, but levels of total *Psd95* mRNA is decreased in brain homogenates (Zalfa et al., 2007). PSD95 is also decreased in the hippocampus in adult *Fmr1*$^{-/y}$ mice, but not in the cerebellum or cortex compared to *Fmr1*$^{+/y}$ mice (Zalfa et al., 2007). In hippocampus, stabilisation of *Psd95* transcripts in response to Gp1 mGluR stimulation found in *Fmr1*$^{+/y}$ mice is also lost in *Fmr1*$^{-/y}$ mice suggesting that *Psd95* mRNA stability is affected with loss of FMRP (Zalfa et al., 2007). Moreover, consistent with a role for NMDAR mediated regulation of FMRP (Desai et al., 2006; Gabel et al., 2004), deficits in activity dependent *Psd95* translation in *Fmr1*$^{-/y}$ mice is also in part dependent on activation of NMDARs (Muddashetty et al., 2007).

Biochemical analysis show that PSD95 is enriched in the PSD fraction from rat brain (Cho et al., 1992) and immunogold labelling for PSD95 in both lysed and intact forebrain synaptosomes only label PSDs confirming a postsynaptic localisation for PSD95 (Hunt et al., 1996). A presynaptic localisation of PSD95 is also reported via EM studies in rat cerebellar (Kistner et al., 1993) and visual cortical (Aoki et al., 2001) synapses. PSD93 has a somatodendritic expression similar to PSD95 and is purified in the detergent insoluble PSD fraction (Kim et al., 1996). In hippocampus, SAP102 is enriched in the synaptosome preparations and EM studies show its localisation in dendritic shafts and spines (Muller et al., 1996). SAP97 is found presynaptically in hippocampal axons and axon terminals (Muller et al., 1996), as well as postsynaptically at PSDs of asymmetric synapses in rat cerebral cortex synapses (Valtschanoff et al., 2000). The MAGUK expression colocalises at postsynaptic sites, and is developmentally regulated. SAP102 gradually increases with age while PSD95 undergoes a dramatic increase around the onset of synaptogenesis (Petralia et al., 2005; Sans et al., 2000; Watson et al., 2006). Sans et al., (2000) in an immunogold study labelling synapses in rat CA1 stratum radiatum at different developmental ages show that

colocalisation of SAP102 and PSD95 at synapses increases with age. They found no evidence for synapses containing one or the other MAGUK (Sans et al., 2000). PSD93 expression at synapses also increased with age, and in the adult, PSD93 shows a preference to colocalise with PSD95 as 33% of synapses contained both PSD95-93, while 16% contained SAP102-PSD93 (Sans et al., 2000).

Over the years considerable attention has been given to decipher the association of ionotropic glutamatergic receptors, NMDAR and AMPAR with PSD95 family MAGUKs. The C termini of NR2 subunits of NMDAR bind to the first two PDZ domains of PSD95, PSD93 and SAP102 (Sheng and Sala, 2001). The GluR1 subunit of AMPAR binds to SAP97 directly (Leonard et al., 1998), and has been shown to colocalise with SAP97 via EM studies (Valtschanoff et al., 2000). GluR1 also indirectly associate with PSD95 via its interactions with stargazin that regulates AMPARs at the synapse (Schnell et al., 2002). The expression of glutamatergic receptors are dynamically regulated during development, in particular that of NR2 subunits (Barnett et al., 2006b; Monyer et al., 1994; Petralia et al., 2005; Sans et al., 2000). In both hippocampus and cortex, NR2B is expressed early and gradually declines on or after peak of synaptogenesis at P14, while NR2A is predominant in mature synapses (Barnett et al., 2006b; Sans et al., 2000; van Zundert et al., 2004). Sans et al., (2000) postulated that during neuronal synapse maturation, PSD95-NR2A complex might replace immature SAP102-NR2B complexes based on a coimmunoprecipitation study in adult hippocampus, which showed preferential association between NR2B-SAP102 and NR2A-PSD95. However, this maybe an over simplified view of the complex nature of NR2-MAGUK interactions at synaptic sites. For example, such a parallel developmental expression profile is observed between NR2 subunits and SAP102/PSD95 MAGUKs in the visual cortex (Shi et al., 1997; Yoshii et al., 2003) and the barrel cortex (Barnett et al., 2006b). However, at these synapses, the expression of SAP102 is maintained up to adulthood and PSD95 expression is evident even at young ages (<P10) negating the idea of a presence of only one or other MAGUK as synapses mature. Moreover, Al-Hallaq et al., (2007) show no discrimination between NR1/NR2A, NR1/NR2B and PSD95, SAP102 and PSD93 in coimmunoprecipitation studies carried out in P42 hippocampus.

There is also an emerging view of MAGUKs in trafficking synaptic proteins to sites of synapses. Interactions between PSD95 family MAGUKs and dendritic microtubules and/or kinesin motor family members have been characterised consistent with a role for PSD95 family MAGUKs in protein trafficking (Kim and Sheng, 2004). GK domains of PSD95 and SAP97 directly interact with KIFIBα (Kinesin family member 1Bα) (Mok et al., 2002). Furthermore, PSD95 family MAGUKs indirectly bind to myosin V through their GK domain interactions with GKAP (Naisbitt et al., 2000), whereas PSD93 associate with MAP1A (Microtubule associate protein 1A) (Brenman et al., 1998). EM and immunohistochemical studies in cultured cortical neurons show transport of NMDARs associated with SAP102 along microtubules on large tubulovesicular organelles during nascent synapse formation (Washbourne et al., 2004). Sans et al., (2003) also found an interaction between PDZ domains of SAP102 and Sec8, a subunit of 'exocyst' complex that was important in the delivery of NMDARs to the surface in *in vitro* heterologous cells and hippocampal cultures.

Despite evidence for direct associations between NR2 subunits and PSD95 family MAGUKs, deletion of MAGUKs does not result in alterations in glutamate receptor basal transmission *in vivo* suggesting that there maybe molecular redundancy among MAGUKs leading to functional compensation. In *Psd95*$^{-/-}$, *Sap102*$^{-/y}$ and *Psd93*$^{-/-}$ mice, basal NMDAR synaptic transmission is unaltered (Carlisle et al., 2008; Cuthbert et al., 2007; Migaud et al., 1998). However, *Psd95*$^{-/-}$ mice (Beique et al., 2006, Carlisle et al., 2008 but see Migaud et al., 1998) exhibit deficits in basal AMPAR transmission but AMPAR EPSCs are unaltered in *Sap102*$^{-/y}$ mice (Cuthbert et al., 2007) and *Psd93*$^{-/-}$ mice (Carlisle et al., 2008) at hippocampal synapses. While genetic deletion of SynGAP (Barnett et al., 2006b), which interacts with the NMDAR via the PDZ domains of both PSD95 and SAP102 (Chen et al., 1998; Kim et al., 1998; Komiyama et al., 2002; Nonaka et al., 2006) and cortex specific deletion of NR1 (Iwasato et al., 2000) results in barrel deficits in layer 4 of S1, *Psd95*$^{-/-}$ and *Sap102*$^{-/y}$ mice display normal barrel segregation. Moreover, in *Psd95*$^{-/-}$ mice, SynGAP still associates with the PSD (Barnett et al., 2006b) suggesting functional redundancy among MAGUKs. In support, Cuthbert et al., (2007) show increased levels of SAP102 in adult hippocampal homogenates from *Psd95*$^{-/-}$ mice.

This chapter has two distinct focuses. Firstly, to examine PSD95 family MAGUKs during S1 development and secondly to examine whether basal expression of PSD95 family MAGUKs are altered in *Fmr1*$^{-/y}$ mice during development. More specifically, the first part of the chapter examines 1) the developmental cellular expression patterns of PSD95, SAP102 and PSD93 to determine whether they are developmentally regulated, 2) whether their cellular expression patterns overlap during development and 3) whether PSD95 and SAP102 are involved in the trafficking and localisation of glutamatergic receptors and SynGAP during development. The second half of the chapter examines whether basal expression of PSD95, SAP102 and PSD93 are altered in *Fmr1*$^{-/y}$ mice during early S1 development to determine whether their expression is regulated by FMRP.

6.2 Results

6.2.1 The spatiotemporal expression profiles of PSD95, SAP102 and PSD93 are developmentally regulated

Previous biochemical studies show expression levels of MAGUKs, PSD95 and SAP102 to be developmentally regulated in both hippocampus and cortex (Petralia et al., 2005; Sans et al., 2000; Watson et al., 2006), In addition, expression patterns of *Psd95*, *Sap102* and *Psd93* mRNA are developmentally regulated in a number of brain regions (Fukaya et al., 1999). However, little is known about their protein expression pattern during development. Therefore, immunohistochemical analysis on coronal sections was carried out throughout development (refer to 2.4.1) to answer the specific questions 1) are PSD95, SAP102 and PSD93 developmentally regulated? 2) do their cellular expression patterns overlap or are they distinct during development? The developmental expression profiles will give an indication as to whether they exert their effects throughout development in cohort or in an age, layer and cell specific manner.

In general, PSD95 was expressed throughout development in the cortex. Its expression in the hippocampus was concentrated in dendritic fields throughout development, and there was some immunoreactivity present in

the cell body layers at later ages (Figure 6.1Ai-Av). In the striatum, PSD95 expression became more intense with age (Figure 6.1Ai-Av), and its expression in the thalamus was maintained throughout development (Figure 6.1Ai-Av and Figure 6.3Ai-Aii). SAP102 consistent with PSD95 expression was also present throughout the cortex during development, and its expression in hippocampus was dynamically regulated, with SAP102 expression predominant in specific dendritic fields of CA1-CA3 (Figure 6.1Ai-Av). In both the striatum and thalamus, SAP102 expression in the adult (Figure 6.3Av) was less intense compared to developmental ages. Cortical expression of PSD93 was also present throughout development, and its expression was regulated in a region specific manner in the hippocampus (Figure Ci-Cv). There was little if any PSD93 immunoreactivity in the adult thalamus (Cv). In contrast to SAP102 but consistent with PSD95 expression, PSD93 staining in the striatum appeared more intense with age (Figure 6.1Ci-Cv). The expression patterns of PSD95, SAP102 and PSD93 characterised here agree well with their mRNA profiles reported by Fukaya et al., (1999). Consistent with protein expression patterns for striatum documented here, *Psd95 and Psd93* mRNA expression in the striatum increases with age, while in *Sap102* mRNA expression in the adult striatum is markedly reduced (Fukaya et al., 1999).

6.2.1.1 In the cortex

PSD95 was expressed in neuropil throughout all cortical layers during development (Figure 6.2Ai-Av), with prominent expression in layers 2, 3 and 4 after P14 through to adult. PSD95 expression was concentrated in layer 4 at all ages and staining appeared diffused in a 'barrel-like' pattern at all ages. This is consistent with *Psd95* mRNA profile in the cortex characterised by Fukaya et al., (1999), which shows abundant *Psd95* expression in all cortical layers after P7. Diffused neuropilar staining for SAP102 was also found in the cortex at all ages, but the staining pattern was dynamically regulated during development in a layer specific manner. At P4 (Figure 6.2Bi) SAP102 was dense in layers 4-6 but was less so in the supragranular layers. From P7 to P14 (Figure 6.2Bii-Biii) its expression was abundant in all cortical layers. In P21 and adult cortex (Figure 6.2Biv-v), SAP102 expression persisted in layers 1-4, but was reduced in the infragranular layers. SAP102 staining was

dense in a 'barrel' like pattern in layer 4 similar to that of PSD95 at all ages. In agreement, *Sap102* mRNA expression at P7 is relatively high in layers 1-4, but after P7 appears in all layers but there is a general overall decrease in its expression in the adult cortex (Fukaya et al., 1999). PSD93 was also expressed throughout all cortical layers during development. However, in contrast to PSD95 and SAP102 staining, PSD93 was localised predominantly to layer 5 pyramidal cells at all ages with intense staining in their cell soma and dendritic processes (Figure 6.2Ci-Cv). From P7 onwards, there were hints of PSD93 expression in a 'barrel' like pattern in layer 4, but this expression seemed to be masked by its intense somatodendritic labelling of layer 5 cells (Figure 6.2Cii-v). The examination of PSD93 expression on tangential sections through layer 4 will confirm whether it is expressed at layer 4. Fukaya et al., (1999) show abundant expression of *Psd93* mRNA in all cortical layers throughout development with the intensity of expression slightly decreased in the adult.

In barrel cortex homogenates, Watson et al., (2006) show that levels of SAP102 increased gradually through development, whereas PSD95 increases rapidly during the time of synaptogenesis at P14. To determine whether PSD93 expression was also developmentally regulated a developmental immunoblot was carried out using barrel cortex homogenates. Levels of PSD93 increased gradually over development, and its expression was highest at P14. While levels of SAP102 and PSD95 are maintained through to adulthood (Watson et al., 2006), PSD93 levels in contrast gradually declined with age after P14 (Figure 6.2D).

6.2.1.2 In the thalamus

In the VpM nucleus of thalamus, PSD95 staining was diffused throughout development and was localised in a 'barreloid' like pattern (Figure 6.3Ai-Aii). PSD95 staining was also evident in the VpL (ventroposterior lateral) nucleus throughout development as well. SAP102 expression and (Figure 6.3Bi) PSD93 (6.3Ci) expression was also localised in a 'barreloid' like pattern in the VpM during early development, especially at P7. In the adult VpM, SAP102 staining appeared to be downregulated, while there was near complete loss of PSD93 expression (Figure 6.3Bii and Cii). In a similar manner, expression of

SAP102 and PSD93 was high in the VpL during early development, but was markedly reduced in the adult. Consistent with PSD95 expression in the thalamus, *Psd95* mRNA is expressed throughout development (Fukaya et al., 1999). In contrast to SAP102 expression, *Sap102* mRNA is low in the thalamus throughout development (Fukaya et al., 1999). In addition, although there is early expression of PSD93 in the thalamus, *Psd93* mRNA is not detected in the thalamus at all ages (Fukaya et al., 1999).

6.2.1.3 In hippocampus

All three MAGUKs, PSD95, SAP102 and PSD93 were expressed throughout hippocampus during its development but there were subtle differences in where each of these were predominantly expressed at different ages. At P4, all three were expressed diffusely in all hippocampal compartments (Figure 6.4Ai-Ci). PSD95 staining at P4 was largely absent from the cell body layers of CA1-CA3 (stratum pyramidal) and DG (stratum granulosum), whereas both SAP102 and PSD93 expression was found in the cell bodies of pyramidal and granular cells at P4. PSD93 staining at P4 was concentrated in stratum molecular, which contains the distal apical dendrites of pyramidal cells, and there was also weak expression of SAP102 in these apical dendrites (Figure 6.4Ci). Between P4 and P7, PSD95 expression was largely unaltered in the hippocampus with some staining evident in the apical dendrites of pyramidal cells and in strata molecular and lacunosum containing the perforant pathway (Figure 6.4Aii). SAP102 (Figure 6.4Bii) and PSD93 (Figure 6.4Cii) immunostaining was concentrated in apical dendrites of pyramidal cells stratum moleculare. In addition, PSD93 staining was also high in CA3 stratum lucidum, which contains the mossy fibres from DG granule cells at P7. At P14, PSD95 expression was apparent in cell bodies of CA1-CA3 pyramidal cells but not DG granule cells. In addition PSD95 expression at P14 was found in stratum lacunosum containing the schaeffer collateral and perforant pathways as well as in apical dendrites of pyramidal cells in stratum moleculare (Figure 6.4Aiii). By P14, SAP102 expression was concentrated in apical dendrites of pyramidal cells and there was also staining in stratum lacunosum (Figure 6.4Biii). PSD93 staining at P14 was highest in strata molecular and lucidum, especially in the region containing the mossy fibre pathway (Figure 6.4Ciii). By P21, diffuse staining for PSD95

increased throughout all compartments but there was very weak if any staining in the cell bodies of DG (Figure 6.4Aiv). SAP102 staining at P21 was most intense in the apical dendrites of pyramidal cells in stratum moleculare and staining was also present in strata lacunosum and radiatum containing the perforant and schaeffer collateral pathways (Figure 6.4Biv). At P21, PSD93 was highest in apical dendrites of pyramidal cells and in the region containing the mossy fibre pathway. PSD93 immunoreactivity was also concentrated in the schaeffer collateral and perforant pathway similar to SAP102 (Figure 6.4Civ). In the adult hippocampus, staining patterns for PSD95 and PSD93 were similar, albeit PSD93 immunoreactivity was more intense. In contrast to PSD95, PSD93 appeared to have high expression in the cell bodies of both pyramidal and granular cells (Figure 6.4Cv), whereas there was little staining for PSD95 in the cell bodies of granule cells (Figure 6.4 Av). The SAP102 expression in the hippocampus was strongest in the apical dendrites of pyramidal cells and in the perforant and schaeffer collateral pathways in the adult. In contrast to both PSD95 and PSD93, there was very weak if any staining for SAP102 in the mossy fibre pathway (Bv). In a similar manner to PSD93 and PSD95 immunoreactivity, SAP102 expression was also observed in cell bodies of pyramidal cells in the adult. The immunoreactivity of PSD95 and PSD93 progressively increased in the basal dendrites of pyramidal cells in the stratum oriens with age, while expression of SAP102 in basal dendrites was maintained at a steady intensity throughout development.

In summary, expression of PSD95, SAP102 and PSD93 were dynamically regulated throughout development in the hippocampus. Although Fukaya et al., (1999) show their mRNA expression to be present throughout development in the hippocampus, it is difficult to determine whether the mRNA expression profiles are also regulated in a layer specific manner.

6.2.2 Differential compensation among PSD95 family MAGUKs at the level of protein expression

In layer 4 of S1, PSD95 and SAP102 expression was predominant throughout S1 development suggesting that these MAGUKs may play a regulatory role in barrel formation, especially as these tether SynGAP to the NMDAR (Datwani

et al., 2002; Iwasato et al., 2000; Iwasato et al., 1997) and loss of NR1 and SynGAP (Barnett et al., 2006b) both result in barrel deficits. However, deletion of either *Psd95* or *Sap102* does not disrupt barrel formation (Petrie, 2008). Moreover, PSD95$^{-/-}$ or SAP102$^{-/y}$ mice exhibit normal brain morphology and gross anatomy (Cuthbert et al., 2007; Migaud et al., 1998) suggesting that there is molecular compensation between PSD95 family MAGUKs. In support of this hypothesis, in *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice layer 4 cells have reduced cellular segregation and their TCA patch size is decreased compared to wildtype mice (Petrie, 2008). To determine whether levels of PSD95, SAP102 and PSD93 were differentially regulated, their levels were quantified in neocortical homogenates and synaptoneurosomes from P7 *Psd95*$^{-/-}$ (n=3), *Sap102*$^{-/y}$ (n=3), and *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ (n=2) mice compared to wildtype mice (n=3). Table 6.1 presents the level of protein expression normalised to WT (wildtype mice)±SEM (refer to 2.4.2) for levels of SAP102, PSD95 and PSD93 found in each genotype. Data were analysed using a one sample t test.

In homogenates and synaptoneurosomes, there was no expression of SAP102 in *Sap102*$^{-/y}$ mice and *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice suggesting that *Sap102*$^{-/y}$ mice make no functional SAP102 (Figure 6.5A and B). Also, there was little if any expression of PSD95 in homogenates and synaptoneurosomes from *Psd95*$^{-/-}$ mice showing that *Psd95*$^{-/-}$ mice do not express any functional PSD95 either (Figure 6.5A and B). Moreover, this verified the specificity of SAP102 and PSD95 antibodies utilised. Levels of PSD95 were also significantly reduced in *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice relative to wildtype mice in homogenates by ~60% ($p<0.05$) (Figure 6.5A) while in synaptoneurosomes there was a trend towards a decrease by ~65% (Figure 6.5B). Level of PSD95 were down regulated slightly more than by 50% as expected in a *Psd95*$^{+/-}$ but this is possibly due to the fact that there were only two samples of *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice. There was a trend towards an increase in SAP102 expression in both homogenates and synaptoneurosomes from *Psd95*$^{-/-}$ mice compared to wildtype mice (Figure 6.5A and B) suggesting that SAP102 may compensate for PSD95 at levels of protein expression consistent with previous data, which show upregulation of SAP102 in adult hippocampus from *Psd95*$^{-/-}$ mice (Cuthbert et al., 2007). Conversely, Levels of PSD95 were unaltered in homogenates and synaptoneurosomes from *Sap102*$^{-/y}$ mice compared to

wildtype mice (Figure 6.5A and B). There was a trend towards a decrease in levels of PSD93 in homogenates and synaptoneurosomes from *Psd95*$^{-/-}$ mice relative to wildtype mice (Figure 6.5A and B). Levels of PSD93 were unaltered in either homogenates or synaptoneurosomes from *Sap102*$^{-/y}$ mice suggesting that loss of SAP102 does not affect levels of PSD93. In homogenates and synaptoneurosomes from *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice, levels of PSD93 were variable but were not significantly altered in either the homogenates or synaptoneurosomes.

6.2.3 Loss of SAP102 and PSD95 affects levels of glutamatergic receptor expression

NR2 subunits of NMDAR have been shown to interact with PSD95, SAP102 and PSD93 (Sheng and Sala, 2001) while certain splice variants of NR1 have been shown to interact with PSD95 (Kornau et al., 1995). GluR1 also associate with PSD95 indirectly via its interaction with TARPs (Bredt and Nicoll, 2003). It could be hypothesised that loss of either PSD95 or SAP102 or both will lead to dysregulated receptor localisation during development. Therefore, levels of glutamatergic receptor subunits were examined in P7 homogenates and synaptoneurosomes to determine if loss of SAP102 and/or PSD95 alters levels of receptor subunit expression globally or their localisation at sites of synapses respectively. Table 6.2 presents the level of protein expression normalised to WT (wildtype mice)±SEM (refer to 2.4.2) for receptor subunit expression found in each genotype. Statistical analysis was carried out using one sample t test.

In homogenates, NR2B levels were comparable between genotypes (Figure 6.6A), whereas levels of NR1 and GluR1 were both significantly reduced in homogenates from *Psd95*$^{-/-}$ (for NR1 $p<0.05$; for GluR1 $p<0.05$) and there was a trend towards a decrease in both NR1 and GluR1 in *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice compared to wildtype mice. Levels of NR1 and GluR1 were unaltered in homogenates from *Sap102*$^{-/y}$ mice (Figure 6.6A). In summary, data from homogenates suggest that loss of PSD95 but not SAP102 affect levels of NR1 and GluR1 globally, while levels of NR2B are unaffected. In synaptoneurosomes, levels of NR2B were significantly decreased in *Psd95*$^{-/-}$ ($p<0.02$) while there was a trend towards a decrease in both *Sap102*$^{-/y}$ and

Sap102$^{-/y}$/*Psd95*$^{+/-}$ mice in comparison to wildtype mice (Figure 6.6B) suggesting that NR2B localisation at synaptic sites is impaired in the absence of PSD95. Levels of GluR1 were also significantly decreased in *Psd95*$^{-/-}$ ($p<0.05$) and a trend towards a decrease in *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice relative to wildtype mice, but GluR1 levels were unaltered in *Sap102*$^{-/y}$ mice (Figure 6.6B) suggesting that GluR1 localisation at synaptic sites is predominantly affected by loss of PSD95. Conversely, levels of GluR2/3 were unaltered in any of the genotypes in synaptoneurosomes (Figure 6.6B). Levels of NR1 in synaptoneurosomes from *Sap102*$^{-/y}$ were significantly decreased ($p<0.02$), while there was a trend towards a decrease in *Psd95*$^{-/-}$ and *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice compared to wildtype mice (Figure 6.6B). Collectively, these data suggests that synaptic localisation of glutamatergic receptors are mainly altered by loss of PSD95.

6.2.4 Loss of SAP102 and PSD95 alters synaptic localisation of SynGAP

On the basis of the fact that SynGAP is a synaptic signalling protein found in a complex with NMDAR, SAP102 and PSD95 (Komiyama et al., 2002), it could be hypothesised that loss of PSD95 and/or SAP102 would affect localisation of SynGAP. To examine this hypothesis, levels of SynGAP were quantified in homogenates and synaptoneurosomes from P7 *Psd95*$^{-/-}$ (n=4), *Sap102*$^{-/y}$ (n=4), and *Sap102*$^{-/y}$*/Psd95*$^{+/-}$(n=2) mice compared to the wildtype mice (n=4). Table 6.2 presents the level of SynGAP expression normalised to WT (wildtype mice)±SEM (refer to 2.4.2) found in each genotype. Statistical analysis was carried out using one sample t test.

In homogenates, SynGAP levels were unaltered in all genotypes (Figure 6.7A) but levels were significantly reduced in synaptoneurosomes from *Psd95*$^{-/-}$ mice ($p<0.02$), while there was a trend towards a decrease in *Sap102*$^{-/y}$ and *Sap102*$^{-/y}$*/Psd95*$^{+/-}$ mice (Figure 6.7B). This suggests that although levels of SynGAP are globally unaffected, SynGAP localisation at synaptic sites is impaired by loss of PSD95.

6.2.5 The basal expression profiles of PSD95, SAP102 and PSD93 are unaffected by loss of FMRP at P7

Previous studies show that *Psd95* mRNA has a G quartet, and that direct interactions between *Psd95* and FMRP regulate the stability of *Psd95* mRNA in a region specific manner (Todd et al., 2003; Zalfa et al., 2007). Sequence analysis of *Sap102* mRNA shows a putative G quartet in its 3' UTR (Stoney, P, personal communications) similar to PSD95 (Todd et al., 2003), and *Psd93* mRNA show a structural motif that corresponds to a kissing complex (Till, S, personal communications). To determine whether loss of FMRP affects basal expression profiles of PSD95, SAP102 and PSD93 during early development their expression profiles were examined in *Fmr1*$^{-/y}$ mice at P7 by qualitative immunohistochemistry and quantitative immunoblotting at P7 and P14.

6.2.5.1 Basal cellular expression patterns of PSD95, SAP102 and PSD93 are unaltered in Fmr1$^{-/y}$ mice at P7

In the cortex, PSD95 expression was evident in all cortical layers at P7 with predominant expression in layer 4 in a 'barrel-like' pattern in *Fmr1*$^{+/y}$ mice and this diffused neuropilar staining for PSD95 was also observed in *Fmr1*$^{-/y}$ mice (Figure 6.8A, B, G and H). In the VpM nucleus of thalamus, the expression of PSD95 is localised to a 'barreloid' pattern as seen in *Fmr1*$^{+/y}$ mice and this pattern was clearly evident in both genotypes (Figure 6.8A, B, C and D). The expression of PSD95 in the striatum was also unaffected by loss of FMRP (Figure 6.8A and B). In the hippocampus PSD95 immunoreactivity was diffused in all layers with relatively high expression in strata lacunosum and molecular containing the perforant pathway and apical dendrites of pyramidal cells in both genotypes (Figure 6.8 A, B, E and F).

Similarly, SAP102 staining appeared diffuse throughout all cortical layers with its expression in layer 4 concentrated in 'barrel-like' neuropil patches in both *Fmr1*$^{-/y}$ and *Fmr1*$^{+/y}$ mice (Figure 6.9A, B, G and H). SAP102 expression in the striatum, and its localisation in the VpM in a 'barreloid-like' pattern did not differ between genotypes (Figure 6.9A and B). In the hippocampus of both genotypes, SAP102 staining was diffused in all compartments with most pronounced staining in strata oriens containing basal dendrites and

molecular containing apical dendrites of pyramidal cells. In both genotypes, there was also weak immunoreactivity for stratum lacunosum containing schaeffer collateral and perforant pathways (Figure 6.9A, B, C and D).

The PSD93 expression in the cell soma and dendrites of layer 5 pyramidal cells of *Fmr1*$^{+/y}$ mice was also observed in the cortex of *Fmr1*$^{-/y}$ mice. In both genotypes, PSD93 was expressed through all cortical layers and there was hint of a 'barrel-like' pattern in layer 4 that appeared to be masked by the intense PSD93 staining in layer 5 pyramidal dendrites (Figure 6.10A, B, G and H). PSD93 expression in the striatum and its staining in a 'barreloid' like pattern in the VpM were present in both genotypes (Figure 6.10A and B). In the hippocampus of both *Fmr1*$^{-/y}$ and *Fmr1*$^{+/y}$ mice, there was diffused neuropil staining in all layers and similar to SAP102 immunoreactivity, PSD93 staining was highest in the strata oriens and molecular (Figure 6.10A, B, E and F) with weak staining in the stratum lacunosum.

In summary, there were no obvious alterations in the basal cellular expression patterns of PSD95, SAP102 and PSD93 in various brain regions between *Fmr1*$^{-/y}$ and *Fmr1*$^{+/y}$ mice at P7, which is consistent with previous in situ hybridisation studies that show no alterations in basal *Psd95* mRNA cellular expression in hippocampus and cortex in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice suggesting that FMRP may not regulate localisation of *Psd95* mRNA and proteins at basal levels (Muddashetty et al., 2007; Zalfa et al., 2007). However, it is unknown whether *Sap102* and *Psd93* mRNA expression is affected by loss of FMRP.

When comparing the cellular expression patterns of PSD95, SAP102 and PSD93 in P7 wildtype mice on a C57BL/6X129 presented in section 6.1.2.3 to that presented here on a C57BL/6J0la, it was apparent that although profiles of these MAGUKs largely overlapped between the two wildtype backgrounds, the relative intensities of their staining varied, specifically in the hippocampus. It could be due to strain differences which has been shown to influence expression of these MAGUKs (Pollak et al., 2006; Pollak et al., 2005) or because the two characterisation studies were carried out on two separate occasions. However, the comparisons between *Fmr1*$^{-/y}$ mice and

Fmr1$^{+/y}$ mice at P7 were made on tissue taken from littermates on the C57BL/6JOla background and immunoreacted under the same conditions.

6.2.5.2 Basal levels of PSD95, SAP102 and PSD93 are unaltered in neocortex from *Fmr1*$^{-/y}$ mice during early S1 development

Although loss of FMRP does not alter cellular expression patterns of PSD95, SAP102 and PSD93, it is possible that their basal levels of expression might be altered due to loss of FMRP. For example, although mRNA expression patterns are unaffected, levels of *Psd95* are selectively decreased in the adult hippocampus (Zalfa et al., 2007). Therefore, levels of PSD95, SAP102 and PSD93 were examined in neocortical homogenates and synaptoneurosomes from *Fmr1*$^{-/y}$ and *Fmr1*$^{+/y}$ mice at P7 (n=3-6/genotype) and P14 (n=4-5/genotype). Levels of protein expression in *Fmr1*$^{-/y}$ mice were analysed normalised to WT (*Fmr1*$^{+/y}$ mice)±SEM (refer to 2.4.2) and statistical analysis performed using one sample t test.

Basal levels of PSD95, SAP102 and PSD93 in homogenates or synaptoneurosomes from *Fmr1*$^{-/y}$ mice were unaltered compared to *Fmr1*$^{+/y}$ mice at P7 (Figure 6.11A and B). Similarly, no changes were detected in basal levels of PSD95, PSD93 and SAP102 expression in homogenates from *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice at P14 (Figure 6.12A). No changes were detected in PSD95, SAP102 or PSD93 levels at P14 in synaptoneurosomes between the two genotypes (Figure 6.12B) suggesting that their synaptic localisation and expression is unaffected by loss of FMRP. In summary, basal levels of PSD95, SAP102 and PSD93 were unaltered globally or at synaptic sites in *Fmr1*$^{-/y}$ mice at P7, moreover, even at P14 their basal expression levels were largely unchanged apart from SAP102 that was elevated globally. The unaltered levels of PSD95 expression quantified here from *Fmr1*$^{-/y}$ mice at P7 and P14 are consistent with previous quantification studies where no changes in PSD95 were seen in adult cortical homogenates (Zalfa et al., 2007) and in P18-P21 cortical synaptoneurosomes (Muddashetty et al., 2007) from *Fmr1*$^{-/y}$ mice.

6.3 Discussion

The first part of this chapter demonstrated that expression profiles of PSD95, SAP102 and PSD93 were dynamic during development and alluded to a developmental role for these scaffolding proteins in the assembly and localisation of synaptic proteins during early development. The cellular expression patterns of PSD95, SAP102 and PSD93 were dynamically regulated throughout development in various brain regions. During development, in addition to overlapping expression patterns, there were distinct differences in their expression in a layer specific manner in areas such as the hippocampus and cortex. For example, in the cortex both PSD95 and SAP102 were strongly expressed in layer 4 with diffuse neuropil staining localised in a 'barrel' like pattern. In contrast, while PSD93 appeared to be expressed in layer 4, it was predominantly expressed somatodendritically in layer 5 pyramidal cells. Moreover, expression of all PSD95, SAP102 and PSD93 was evident in VpM in a 'barreloid' like pattern during early development but there was near complete loss of PSD93 expression in the adult VpM, while SAP102 expression was less intense with age. Thus, the transient expression of SAP102 and PSD93 in VpM suggests that the expressions of these MAGUKs are regulated in different brain regions in an age specific manner. In support of the idea that their early expression may indicate a significant developmental role, loss of SAP102 and PSD95 resulted in deficits in synaptic protein expression at P7, in particular PSD95 in trafficking and localisation of synaptic proteins. Despite the fact that NR1 and SynGAP both play a role in barrel formation, loss of PSD95 or SAP102 does not cause defects in whisker related barrel patterns (Barnett et al., 2006b) suggesting functional compensation among MAGUKs. In support, the data presented here show a trend towards an increase in basal levels of SAP102 globally and at synaptic sites in *Psd95*$^{-/y}$ mice, but levels of PSD95 were unchanged in *Sap102*$^{-/y}$ mice compared to wildtype mice. Collectively, these data suggests that compensation among MAGUKs is heterogeneous.

Second part of this chapter examined whether basal expression profiles of PSD95, SAP102 and PSD93 were affected in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice during early development. Consistent with previous findings on both PSD95 and *Psd95* mRNA in the juvenile *Fmr1*$^{-/y}$ mice (Muddashetty et

al., 2007; Zalfa et al., 2007), the basal expression profile of PSD95 during early development characterised in this chapter was also unaltered in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice at P7. SAP102 and PSD93 also have putative FMRP binding sites, however, overall basal expression profiles of these two MAGUKs were unaltered in *Fmr1*$^{-/y}$ mice during early neocortical development. However, it is possible that either the stability of *Sap102* and *Pas93* mRNA or their basal translation is compromised in *Fmr1*$^{-/y}$ mice in a similar manner to *Psd95* mRNA and this needs to be examined in the future.

6.3.1 Expression of MAGUKs are developmentally regulated

Previous studies have extensively characterised the developmental expression profiles of MAGUKs, PSD95, SAP102 and PSD93 biochemically in different brain regions. In rat hippocampal homogenates, SAP102 levels are observed early during development while PSD95 and PSD93 are present by P10 and then undergo a dramatic increase between P10 and P35. Conversely, levels of SAP102 increase gradually over time with the maximum increase between P2-P10 (Sans et al., 2000). A similar gradual increase in SAP102 levels is also observed in homogenates of superior colliculus (Shi et al., 1997) and visual cortex (Yoshii et al., 2003), with rapid increases in levels of PSD95 between P12 and P16. This developmental regulation is mimicked in homogenates from barrel cortex as well (Barnett et al., 2006b). Interestingly, at a first glance, PSD95 levels in barrel cortex homogenates seem to follow a similar pattern to that described in other brain regions. PSD95 is present after P7 and then undergoes a rapid increase corresponding to the period of synaptogenesis. However, PSD95 expression is found at P0 in barrel cortex homogenates, and then shown to gradually increase at P4 and P7 followed by abundant expression of PSD95 after P14 at high concentrations of PSD95 antibody (Barnett et al., 2006b). It is a possibility that studies in other brain regions may have utilised PSD95 antibody for immunoblotting at a concentration yielding an optimal signal during late development, thereby effectively masking any presence of PSD95 earlier on in development. However, it is clear that PSD95 goes through a large upregulation at later ages compared to SAP102. From data presented here, there are low levels of PSD93 expression in barrel cortex homogenates during early development, and PSD93 expression peaks at P14 to the peak of synaptogenesis (White et

al., 1997). In contrast to PSD95 and SAP102 expression patterns that maintain increased expression through to adulthood (Barnett et al., 2006b), PSD93 levels seem to gradually decline with age after P14.

The developmental cellular expression patterns of PSD95, SAP102 and PSD93 shown here agree well with their mRNA expression profiles during development (Fukaya et al., 1999). However, the transient early expression of PSD93 in thalamic nuclei contrasts with its mRNA as there is near complete absence of *Psd93* mRNA in the thalamus during development (Fukaya et al., 1999). Ultrastructural studies of hippocampal CA1 synapses in stratum radiatum by Sans et al., (2000) show that while PSD95 is developmentally upregulated, SAP102, PSD95 and PSD93 overlap in their localisation at synaptic sites. The immunohistochemical patterns for these proteins presented here agree with this finding as these MAGUKs do overlap in their cellular expression profiles. The cellular expression patterns of PSD95 and PSD93 in the adult hippocampus are very similar, which agrees with Sans et al., (2000) immuno EM studies that show preferential association between PSD95 and PSD93 in adult hippocampal synapses.

However, the abundance of the expression of these three MAGUKs appears to vary depending on the cellular layer/cell type and age. For example, PSD95, SAP102 and PSD93 are expressed throughout the cortex during development. However, both PSD95 and SAP102 dense neuropil staining in layer 4 is patchy corresponding to a 'barrel' like pattern, whereas PSD93 is predominantly expressed somatodendritically in layer 5 pyramidal cells. There is some evidence of PSD93 expression in layer 4 as well but it appears to be effectively mask by its expression in layer 5, therefore PSD93 staining in tangential sections would determine whether it is in fact expressed in layer 4. Moreover, in hippocampal regions containing the mossy fibre pathway, intense PSD93 expression is present after P14, and PSD95 expression after P14/P21 but there is very little expression of SAP102 in these regions throughout development. The overlapping yet distinct expression patterns of PSD95, SAP102 and PSD93 suggests that while these MAGUKs have overlapping roles, these may also have specific regulatory effects on the cellular processes they govern in a cell type specific and age specific manner. For example, while both *Psd95*$^{-/-}$ (Migaud et al., 1998) and *Sap102*$^{/y}$

(Cuthbert et al., 2007) mice exhibit learning deficits in the Morris water maze task, their deficits are distinct. The *Psd95*$^{-/-}$ mice show inability to learn the position of the hidden platform (Migaud et al., 1998), while *Sap102*$^{-/y}$ mice exhibit impaired and less efficient strategies in finding the hidden platform (Cuthbert et al., 2007).

6.3.2 A role for MAGUKs in synaptic protein trafficking and localisation

6.3.2.1 Regulation of NMDAR expression at synaptic sites

In biochemical data presented here, global levels of NR2B are unaltered in the homogenates from *Psd95*$^{-/-}$ mice at P7, however, levels of NR2B in synaptoneurosomes from these mice were decreased suggesting that PSD95 is involved in localising NR2B at synaptic sites. In agreement, there are several lines of evidence to support a role for PSD95 in regulating trafficking and localisation of NMDARs at synaptic sites. The C-terminal T/SXV (X represents any amino acid) motifs of NR2A and NR2B subunits of NMDAR directly interact with PDZ domains of PSD95, SAP102 and PSD93 (Kim et al., 1996; Kornau et al., 1995; Lau and Zukin, 2007; Muller et al., 1996; Niethammer et al., 1996). In nascent synapses, NMDARs are transported to dendrites along microtubules, and PSD95 has been shown to be the link between NR2B and adaptor proteins such as mLin10 that couple NMDAR containing vesicles to kinesin motors (Guillaud et al., 2003; Setou et al., 2000). In young (*in vitro* day 3/4) visual cortical cultures, NMDAR transport packets have been shown to move along microtubules at about 4μm/min and to be recruited to sites of axon-dendritic contacts within minutes either concurrent or independent of the presence of PSD95 (Washbourne et al., 2002).

A role for PSD95 in stabilising levels of NMDARs is also evident from the literature. In *in vitro* heterologous cells and cortical cultures, PSD95 inhibits NR2B-mediated internalisation and enhances clustering of NMDARs at the surface, whereas the deletion of PDZ domain of NR2B results in increased NMDAR internalisation suggesting that PSD95 may play a regulatory role in stabilising NMDARs at synaptic sites (Roche et al., 2001). In *in vitro* heterologous expression systems, it has also been shown that NR2A and

NR2B subunits have different affinities with PSD95, which may determine their stability and mobility at the PSD (Cousins et al., 2009). The increase in PSD95 mediated surface expression of NR1/NR2A is shown to dependent on palmitoylation of PSD95 (Cousins et al., 2008). The palmitoylation of PSD95 at residues C3 and C5 is necessary for the synaptic targeting of PSD95. Conversely, there is also some evidence to suggest that PSD95 is not necessary for localisation of NMDARs such as *in vitro* disruption of PSD95 clustering had no effect on synaptic localisation of NMDAR (Pasaro et al., 1999). Furthermore, *Psd95*$^{-/-}$ mice were originally characterised as having normal basal synaptic NMAR-mediated synaptic transmission (Migaud et al., 1998), but a recent study show that these *Psd95*$^{-/-}$ mice do exhibit defects in NMDAR-mediated basal synaptic transmission (Carlisle et al., 2008).

In both *Sap102*$^{-/y}$ and *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice, there is a trend towards a decrease in levels of NR2B at P7 suggesting that SAP102 may also be involved in trafficking and localisation of NR2B in addition to PSD95. In support, disruption of SAP102 interaction with sec8 exocyst complex decreases delivery of synaptic NMDARs and cell surface delivery of NMDAR *in vitro* (Sans et al., 2003). The mammalian homologue of *drosophila melanogaster* partner of inscuteable (mPins) interacts with both SAP102 and PSD95 and assists the formation of NMDAR-MAGUK complex. The dominant negative forms of mPins or siRNA knockdown of mPins have also been shown to decrease dendritic expression of SAP102 and alter surface expression of NMDARs (Sans et al., 2005).

In addition to changes in NR2B expression, levels of NR1 are also significantly downregulated globally in *Psd95*$^{-/-}$ mice at P7. In synaptoneurosomes, levels of NR1 are decreased in *Sap102*$^{-/y}$ mice and there was a trend towards a decrease in *Psd95*$^{-/-}$ and *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice compared to wildtype mice. These data suggest that levels of NR1 are generally decreased in the absence of PSD95, while its localisation at synaptic sites may also be affected by loss of both SAP102. The C1 cassette at the C terminus of NR1 splice variant contains an endoplasmic reticulum (ER) retention signal, and exit of NR1 from ER requires suppression of C1 by the PDZ interacting domain of C2' cassette (Standley et al., 2000; Xia et al., 2001). Standley et al., (2000) show that all PSD95 family MAGUKs interact

with specific amino acid sequences in C2', and that SAP102 and NR1 coimmunoprecipitate in microsomal fractions suggesting that they associate early in the secretory pathway of NR1. Therefore, PSD95 and/or SAP102 may interact with NR1 early in its biosynthetic pathway and mediate exit of NR1 from the ER, thereby loss of these MAGUKs will alter expression of NR1 generally or at synaptic sites. In summary, PSD95 family MAGUKs appear to play a key role in localisation of NMDARs to synaptic sites during development.

6.3.2.2 Regulation of AMPAR expression at synaptic sites

In neocortical homogenates and synaptoneurosomes from *Psd95*$^{-/-}$ mice, levels of GluR1 subunit of AMPAR are reduced compared to wildtype mice at P7 suggesting that PSD95 also plays a key role in regulating general expression of AMPAR receptors as well as synaptic localisation of AMPARs. Several lines of evidence suggest that PSD95 may indirectly regulate AMPAR expression. The first two PDZ domains of PSD95 (Schnell et al., 2002) directly bind the C terminus of stargazin that has been shown to regulate AMPARs at the synapse. *In vitro* overexpression of PSD95 in dissociated neurons and organotypic slice cultures show enhanced amplitude of AMPAR-mediated excitatory postsynaptic currents (EPSCs) (Beique and Andrade, 2003; El-Husseini et al., 2000; Schnell et al., 2002). An *in vitro* study by Xu et al., (2008) using a lentivirus-mediated replacement strategy that allow simultaneous knockdown of endogenous PSD95 by shRNA and expression of recombinant forms of PSD95 within the same cell, demonstrates that the full length of PSD95 is required for its regulatory effects on AMPAR. Furthermore, C terminal SH3-GK domains are necessary for PSD95 localisation at the synapse and N terminal domain mediated dimerisation of PSD95 is important for regulating AMPAR basal transmission (Xu et al., 2008).

Elias et al., (2006) show that SAP102 play a dominant role in AMPAR synaptic clustering whereas PSD95 and PSD93 play little role in immature hippocampal synapses (<P8). However, data presented in this chapter show that levels of GluR1 are unaltered in neocortical homogenates and synaptoneurosomes from *Sap102*$^{-/y}$ mice at P7 compared to wildtype mice

suggesting that at these synapses SAP102 may not play a dominant regulatory role in AMPAR trafficking.

Finally, the expression levels of GluR2/3 subunits of AMPAR were unaltered in synaptoneurosomes from *Sap102*$^{-/y}$, *Psd95*$^{-/-}$ and *Sap102*$^{-/y}$*/Psd95*$^{+/-}$ mice relative to wildtype mice indicating that at least at P7 loss of neither SAP102 nor PSD95 affects trafficking or localisation of GluR2/3 at synaptic sites. Consistent with this idea, other PDZ domain containing scaffolding proteins have been shown to regulate the stability of Glu2/3 expression at synapses. The PDZ containing glutamate receptor interacting protein/AMPAR binding protein (GRIP/ABP) and protein interacting with C kinase 1 (PICK1) have been shown to regulate trafficking and targeting of GluR2/3 to synapses (Bredt and Nicoll, 2003; Malinow and Malenka, 2002).

6.3.2.3 Regulation of SynGAP expression at synaptic sites

SynGAP interacts with PSD95 and SAP102 both *in vitro* and *in vivo* (Kim et al., 1998), and negatively regulates Ras-ERK pathway that regulate synaptic plasticity (Barnett et al., 2006a). In *in vitro* hippocampal and cortical cultures of *Syngap*$^{-/-}$ mice (Rumbaugh et al., 2006; Vazquez et al., 2004) and in siRNA knockdown of SynGAP, increased basal AMPAR transmission and synaptic AMPAR clustering is observed (Rumbaugh et al., 2006). Conversely, overexpression of SynGAP results in depressed AMPAR EPSCs, synaptic AMPAR clustering and insertion (Rumbaugh et al., 2006). Despite, interactions between SynGAP and PSD95 that is thought to be important in its synaptic localisation (Kim et al., 1998), SynGAP is found in the PSD from barrel cortex in *Psd95*$^{-/-}$ mice (Barnett et al., 2006b) suggesting that levels of SynGAP attached to the PSD is not depend on PSD95 or that SAP102 compensates for loss of PSD95. The data presented in this chapter show that levels of SynGAP are normal globally in *Sap102*$^{-/y}$, *Psd95*$^{-/-}$ and *Sap102*$^{-/y}$*/Psd95*$^{+/-}$ mice relative to wildtype mice suggesting that loss of PSD95 and/or SAP102 does not affect general levels of SynGAP in the neocortex. However, SynGAP levels are significantly reduced in synaptoneurosomes from *Psd95*$^{-/-}$ mice, while there is a trend towards a decrease in Sap102$^{-/y}$ and *Sap102*$^{-/y}$*/Psd95*$^{+/-}$ mice relative to wildtype mice at P7 suggesting that there is decreased levels of SynGAP localised to synaptic sites. Therefore, the fact that

SynGAP is still present in the PSD from *Psd95*$^{-/-}$ mice (Barnett et al., 2006b) suggest that although levels of SynGAP are decreased in the postsynaptic compartment, its interactions at the PSD are unaltered. Also, SAP102 mice may compensate for loss of PSD95 in *Psd95*$^{-/-}$ mice, thereby regulating levels of SynGAP at the synapse.

PSD95 family MAGUKs are largely thought to have a postsynaptic localisation (Kim and Sheng, 2004). Albeit, a presynaptic localisation has also been reported for PSD95 in adult rat visual cortex but not SAP102 (Aoki et al., 2001 *but see* Fukaya and Watanabe 2000). This raises a fundamental question as to whether these observed changes in NMDAR and AMPAR subunits in response to loss of PSD95 are dependent on pre or postsynaptic effects of PSD95. Therefore, it is important to investigate the localisation of SAP102 and PSD95 by EM analysis at P7 to determine their synaptic localisation. Moreover, levels of PSD95 rapidly increase between P7 and P14 in barrel cortex homogenates (Barnett et al., 2006b), therefore, taking tissue at P7 may introduce high variability in expression of proteins regulated by PSD95. In the future, it is important to characterise the levels of glutamatergic receptors and associated signalling proteins in synaptoneurosomes from adult *Sap102*$^{-/y}$, *Psd95*$^{-/-}$ and *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice when synapses are mature. This will elucidate whether altered protein expressions characterised during early development in this chapter persists in the adult or whether there is developmentally dependent differential regulation by PSD95 family MAGUKs. Additionally, examining preparation of synaptosomes and PSDs from these mice will isolate direct changes at the PSD such as receptor insertion and/or endocytosis due to loss of SAP102 and PSD95. Alternatively, changes in glutamatergic receptor insertion or endocytosis can also be examined by performing immunolabelling studies *in vitro* studies in cultures from *Sap102*$^{-/y}$, *Psd95*$^{-/-}$ and *Sap102*$^{-/y}$/*Psd95*$^{+/-}$ mice.

6.3.3 Functional redundancy among MAGUKs

In mutants of PSD95 family MAGUKs despite altered expression of synaptic plasticity, basal synaptic transmission via NMDAR and AMPAR is relatively unchanged (Carlisle et al., 2008; Cuthbert et al., 2007; McGee et al., 2001; Migaud et al., 1998) leading to the hypothesis that there is molecular

redundancy among MAGUKs leading to functional compensation. Elias et al., (2006) in an elegant study in hippocampal slices where endogenous MAGUK levels were manipulated by shRNA mediated knockdown in *Psd95*$^{-/-}$*/Psd93*$^{-/-}$ mice show that in synapses in adult *Psd95*$^{-/-}$*/Psd93*$^{-/-}$ mice, SAP102 levels are elevated by 20% and functionally compensate for PSD95 and PSD93 in NMDAR and AMPAR trafficking (Elias et al., 2006). In support, the double mutants of SAP102 and PSD95 die postnatally by P3 with only 4% of pups being double knockouts compared to the expected 12.5% by Mendelian inheritance while individual null mutants survive and breed well (Cuthbert et al., 2007). Moreover, levels of SAP102 are increased in adult hippocampal homogenates from *Psd95*$^{-/y}$ mice (Cuthbert et al., 2007). The *Sap102*$^{-/y}$*/Psd95*$^{+/-}$ mice show reduced barrel segregation suggesting that there maybe differential molecular redundancy between PSD95 family MAGUKs in regulating S1 differentiation (Petrie, 2008). The data presented in this chapter shows a trend towards a decrease in SAP102 in P7 neocortical homogenates and synaptoneurosomes from *Psd95*$^{-/-}$ mice in support of functional compensation.

However, while PSD95 family MAGUKs may overlap in regulating cellular processes, these may have non-overlapping functions as well. For example, *Psd95*$^{-/-}$ mice display enhanced LTP and no induction of LTD, whereas *Psd93*$^{-/-}$ mice conversely show deficits in LTP and normal LTD at hippocampal CA1-CA3 synapse (Carlisle et al., 2008). These results indicate that there maybe heterogeneity with respect to expression of PSD95 and PSD93 in hippocampal synapses. The cellular expression patterns described in this chapter for PSD95 and PSD93 in the hippocampus show that the expression profiles of these proteins are dynamically regulated during early development in a layer specific manner.

Collectively, it can be hypothesised that PSD95 family MAGUKs exhibit heterogeneity in a neuronal cell type specific manner, which is developmentally regulated, thereby dynamically regulating cellular processes involved in formation and maintenance of synaptic connections throughout development.

6.3.4 Implication for FXS

The mGluR theory of fragile X mental retardation postulates that many of the symptoms of FXS are manifested by exaggerated effects of Gp1 mGluRs (Bear et al., 2004). Synaptic plasticity mediated via Gp1 mGluR signalling is thought to depend on *de novo* synthesis of proteins and FMRP is thought act as a repressor or activator of Gp1 mGluR signalling dependent protein synthesis, thereby regulating its downstream effects (Bear et al., 2008; Bear et al., 2004). The majority of FMRP bound cargo mRNAs are thought to travel along microtubules in dendrites as polyribosome-free-mRNPs (Wang et al., 2008), and stimulation with Gp1 mGluR agonists results in the translocation of these translationally arrested FMRP-mRNPs into the postsynaptic compartment to be recruited to polyribosomes that are translationally active (Wang et al., 2008; Zalfa et al., 2007). EM studies in the visual cortex of P15 show that there are fewer dendritic spine synapses containing polyribosomes in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice suggesting that translation of mRNA is impaired in *Fmr1*$^{-/y}$ mice (Weiler et al., 2004). In agreement with this, there is evidence for altered mRNA targeting and elevated and/or decreased levels of mRNA translation at basal levels in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Dolen et al., 2007; Hou et al., 2006; Liao et al., 2008; Miyashiro et al., 2003; Qin et al., 2005; Zalfa et al., 2003).

Psd95 mRNA has been characterised as one of the FMRP target mRNAs that is dysregulated in *Fmr1*$^{-/y}$ mice (Muddashetty et al., 2007; Todd et al., 2003; Zalfa et al., 2007). Zalfa et al., (2007) show that *Psd95* polysomal profile of whole brain and hippocampi homogenates are unaltered in adult *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice. However, there are selective decreases in levels of *Psd95* mRNA and PSD95 in the hippocampus, and *Psd95* mRNA is selectively unstable in the hippocampus from *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Zalfa et al., 2007). Conversely, Muddashetty et al., (2007) show altered polysome profiles in P18-21 cortical synaptoneurosomes from *Fmr1*$^{-/y}$ mice such that there is exaggerated incorporation of *PSd95* mRNA into polyribosomes compared to *Fmr1*$^{+/y}$ mice resulting in basally elevated PSD95 synthesis (Muddashetty et al., 2007). However, in *Fmr1*$^{-/y}$ mice, Gp1 mGluR (Muddashetty et al., 2007; Todd et al., 2003) and NMDAR (Muddashetty et al., 2007) mediated increases in *Psd95* translation evident in *Fmr1*$^{+/y}$ mice is

absent suggesting that activity induced PSD95 synthesis is impaired. Collectively, these data indicate that PSD95 synthesis is impaired in FXS in a region specific manner, and that how these deficits in PSD95 synthesis arise may also depend on the brain region.

In addition to examining the cortical expression profile of PSD95 in *Fmr1*$^{-/y}$, this chapter also examines the expression profiles of other FMRP putative MAGUK targets SAP102 and PSD93 and show that basal expression profiles of these MAGUKs during development is unaltered in *Fmr1*$^{-/y}$ mice relative to *Fmr1*$^{+/y}$ mice. This is consistent with previous studies that show no alterations in the cellular distribution of *Psd95* mRNA or levels of PSD95 in the adult cortex at basal levels (Muddashetty et al., 2007; Zalfa et al., 2007). Both *Sap102* and *Psd93* mRNA show putative FMRP binding motifs; therefore, it is possible that *Sap102* and *Psd93* mRNA may also be misregulated at the level of translation or mRNA stability in *Fmr1*$^{-/y}$ mice similar to *Psd95* mRNA and this aspect needs to be further explored. The first part of this chapter also shows that both PSD95 and SAP102, especially PSD95 are involved in trafficking and localisation of glutamatergic receptors and other synaptic proteins such as SynGAP during early neocortical development. Therefore, impaired translation of these scaffolding proteins could have deleterious effects in early synapse formation and maturation.

In experimental models of FXS, it is becoming increasingly evident that there are developmental delays in synaptic maturation in cortex and hippocampus (Harlow et al., 2007; Pilpel et al., 2008) (refer to 4.3.4). For example, chapter 4 shows that basal levels of GluR1 are decreased at cortical synaptoneurosomes in P7 and P14, and this could in part be due to misregulated trafficking and localisation of GluR1 mediated by impaired PSD95 functioning. In agreement, coimmunoprecipitation studies show association of *Psd95* and *Glur1/2* mRNA with FMRP in P18-P21 cortical synaptoneurosomes (Muddashetty et al., 2007; Zalfa et al., 2007). However, it is unclear whether FMRP directly interacts with GluR1/2 or whether its interactions are mediated by PSD95. Moreover, in *Fmr1*$^{-/y}$ mice, activity induced synthesis of synaptic proteins such as PSD95 is deficient (Muddashetty et al., 2007; Todd et al., 2003) while there is increased activity dependent internalisation of GluR1 (Nakamoto et al., 2007) compared to

Fmr1$^{+/y}$ mice. In light of this evidence, altered synaptic connectivity in FXS is thought to be manifested by dysregulated basal proteins synthesis and impaired activity induced synthesis (Bear et al., 2008; Pfeiffer and Huber, 2006; Ronesi and Huber, 2008). There is also evidence to suggest that the stimulus induced kinesin motor dependent transportation and localisation of some FMRP bound target mRNAs are also impaired in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice (Dictenberg et al., 2008) suggesting that impaired delivery of mRNA to sites of active synapses is also defective in FXS (Bagni and Greenough, 2005; Kelleher and Bear, 2008). Therefore, the translation of PSD95 family MAGUK mRNA and their activity dependent synthesis in *Fmr1*$^{-/y}$ mice require further examination during early development to fully appreciate their regulatory roles in processes of synaptic connectivity that are dysregulated in FXS.

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7 In conclusion

During our early postnatal life, sensory experiences refine and validate our synapses in order to establish proper synaptic connectivity. In FXS, the most common form of inherited MR, altered synaptic connectivity is thought to be the neuroanatomical basis for its symptoms (Hessl et al., 2004). The 'mGluR theory of fragile X' hypothesises that the altered synaptic connectivity evident in FXS arises as a result of exaggerated Gp1 mGluR signalling (Bear et al., 2004). Despite the fact that FXS is a developmental form of MR, little is known about the role of FMRP developmentally or the interactions between mGluR5 and FMRP. The focus of this thesis was to examine the role of mGluR5 and FMRP during early postnatal development and their interactions, for which the mouse S1 was used as an experimental model. The S1 is an excellent model for this purpose because of its well-characterised somatotopical organisation at both the anatomical and physiological level. The differentiation of S1 is a developmentally regulated process, and its synaptic connections are refined and stabilised postnatally in a glutamatergic activity dependent manner (Barnett et al., 2006; Erzurumlu and Kind, 2001; Fox and Wong, 2005). Moreover, several proteins that regulate the formation of S1 have also been implicated in FXS (Barnett et al., 2006; Inan et al., 2006; Iwasato et al., 2008; Watson et al., 2006).

Findings presented in this thesis show that not only both FMRP and mGluR5 are expressed in S1 but that they also play a regulatory role in its anatomical organisation during both early and late S1 development. FMRP and mGluR5 are expressed abundantly at various brain regions during early development compared to the adult indicating a critical role during early brain development. At a cellular level, mGluR5 expression is neuropilar while FMRP is predominantly cytoplasmic with little expression in dendrites at basal levels. Consistent with this expression pattern, in *drosophila*, dFMRP is primarily localised to cell bodies and DmGluRA, which is the single functional homolog of mGluRs in *drosophila*, is localised to synaptic neuropil suggesting that their expression patterns are evolutionarily conserved (Pan et al., 2008). Ultrastructurally, both mGluR5 and FMRP are expressed postsynaptically at P7 in layer 4 synapses; whereas FMRP is present both pre

and postsyaptically in synapses at P14. In agreement with a postsynaptic role for mGluR5 and FMRP during the anatomical segregation of barrels, genetic mutations of both *Mglur5* and *Fmr1* result in decreased aggregation of layer 4 cells into barrels. The effects of mGluR5 in barrel formation are dose dependent, which is predicted by the mGluR theory of fragile X and important for therapeutic interventions. Also consistent with the theory decreasing levels of mGluR5 in *Fmr1*$^{-/y}$ mice rescues defects in barrel formation to that of *Mglur5*$^{+/-}$ mice but not wildtype mice. FMRP and mGluR5 also plays a role in synaptogenesis of layer 4 spiny neurons during late S1 development. They appear to have antagonistic regulatory roles in synaptogenesis, in which genetic deletion of *Mglur5* results in decreased spine density whereas genetic deletion of *Fmr1* results in increased spine density. In regulating synaptogenesis, effect of loss of mGluR5 by approximately 50% has a similar effect to that following complete loss of mGluR5. The genetic reduction of *Mglur5* in Fmr1$^{-/y}$ mice does not appear to rescue the increased layer 4 spine phenotype suggesting that during early spinogenesis mGluR5 either signals mainly via FMRP rather than two antagonistic pathways or that mGluR5 and FMRP regulate two independent pathways that converge on regulating spinognesis.

Therapeutic interventions based on the mGluR theory of fragile X suggests that rescue of FXS phenotypes are possible by attenuating signalling through Gp1 mGluR receptors, of which mGluR5 is the predominant forebrain subtype. Several lines of evidence support this theory and indicate that Gp1 mGluRs are a good candidate for therapeutic strategies (de Vrij et al., 2008; Dolen et al., 2007; McBride et al., 2005; Pan et al., 2008; Yan et al., 2005). However, collating data from this thesis together with previous findings suggest that interactions between mGluR5 and FMRP may differ depending on the 1) age, 2) brain region and neuronal cell type and 3) cellular processes they govern and these differences need to be taken into consideration when designing therapeutic interventions for FXS.

7.1 *The factors that may influence interactions between mGluR5 and FMRP*

7.1.1 Age

In layer 5 pyramidal cells of S1 transfected with EGFP, loss of FMRP results in increased spine length and density but it is a developmentally transient phenotype, with the maximum effect observed during the first two weeks of postnatal development and no spine abnormalities observed by P27 (Nimchinsky et al., 2001). Glavez and Greenhough (2001) in a Golgi analysis of layer 5 pyramidal cells show increased spine length and overall density concurrent with a decrease in density of mature spines at P25 and P73-76. The caveat in comparing these two studies is that the spine analysis was carried out using two different techniques. It is thought that the thin and tortuous immature spine phenotype of FXS arise from a development delay (Beckel-Mitchener and Greenough, 2004). The biochemical data presented in chapter 4 from *Fmr1*$^{-/y}$ mice, at both P7 and P14 show that there are reduced levels of glutamatergic receptor subunits, in particular GluR1 at synaptic sites in the neocortex. The size of the spine head is directly proportional to the postsynaptic number of receptors and their associated signalling complexes (Nusser et al., 1998). Therefore, it can be hypothesised that layer 4 spiny cells of S1 may also have an immature spine morphology during S1 development. The spine analysis of layer 4 spiny cells in S1 presented in chapters 4 and 5 show increased spine density at P30-P35, therefore, it is important to characterise spine density at early postnatal developmental ages such as P7 and P14, and during late ages such as P21, P30-P35 and adult. It may not be possible to successively carry out Golgi analysis during early time points because the young tissue may not survive the strong chemical treatments in Golgi, in which case intracellular filling of layer 4 spiny cells at various developmental stages would be a preferable option.

One of the key aims of characterising expression of FMRP during development and effects of loss of FMRP in the anatomical organisation of S1 is to establish when and where FMRP plays a regulatory role during early cortical development. This would be insightful in determining whether

therapeutic strategies need to be focused on development and/or whether early diagnosis is beneficial in subsequent treatment. Dolen et al., (2007) successfully show that several anatomical, physiological and behavioural abnormalities in adult *Fmr1*$^{-/y}$ mice can be rescued by reducing levels of mGluR5, thus showing that reducing mGluR5 levels in early development is beneficial in the adult. However, a key question that needs to be addressed is whether reducing levels of mGluR5 at conception in *Fmr1*$^{-/y}$ mice rescues their phenotypes at earlier time points. For example, the primary cause of Rett syndrome, a neurodevelopmental and a neurodegenerative MR that afflicts 1:10000 females is mutations in the X-linked *MECP2*. In a mouse model in which *Mecp2* is endogenously silenced, the conditional reactivation of MeCP2 expression results in the phenotypical rescue in both immature and mature adult mice (Guy et al., 2007). In *drosophila* model of FXS, targeted neuronal induction of dFMRP during early development rescues exuberant synaptic connectivity defects at the NMJ of *dfmr1* mutant flies. Although, acute expression of dFMRP partially alleviate NMJ architectural defects in the adult *dfmr1* fly, it was not to the same degree as the early intervention (Gatto and Broadie, 2008). On the basis of this study, Gatto and Broadie (2008) proposed that FMRP plays an early role in setting up normal structural architecture during synaptogenesis. Consistent with this idea, there is high expression of FMRP during early S1 development, and loss of FMRP leads to early defects in barrel cortex organisation as shown in chapter 4. Moreover, decreasing levels of mGluR5 can rescue the decreased barrel formation in Fmr1$^{-/y}$ mice at P7 suggesting an early role for FMRP and mGluR5-FMRP interactions in S1 cortical organisation.

7.1.2 Brain region or neuronal cell type

There are subtle differences in the spine abnormalities reported in the literature due to loss of FMRP and mGluR5 depending on the brain region/cell type. For example, Golgi analyses in *Fmr1*$^{-/y}$ mice compared to *Fmr1*$^{+/y}$ mice show increased spine length and density in layer 5 cells of visual cortex (adult) (Comery et al., 1997; McKinney et al., 2005), increased spine length but not density in hippocampal CA1 pyramidal cells (adult) (Grossman et al., 2006) and increased spine density in layer 3 pyramidal cells of visual cortex (P30) (Dolen et al., 2007). In *in vitro* hippocampal culture studies from *Fmr1*-

$^{/y}$ mice, 21 days *in vitro* neurons labelled with DiI exhibit decreased spine length and density (Braun and Segal, 2000), whereas 20 days *in vitro* neurons transfected with mCherry show increased density of filopodial extensions and no change in spine density (de Vrij et al., 2008). These inconsistencies, specifically in terms of characterisation of the hippocampal spine abnormalities in *Fmr1*$^{-/y}$ mice may reflect technical differences or different parameters investigated. Moreover, neuronal cell type specific effects are also observed in mutants of *Mglur5* as well. Golgi analysis show that loss of mGluR5 by approximately 50% does not affect spine density of layer 3 pyramidal neurons in visual cortex at P30 (Dolen et al., 2007) but affects layer 4 excitatory neuronal populations. The data presented in chapter 3 show that decreased spine density of layer 4 spiny cells in both *Mglur5*$^{+/-}$ and *Mglur5*$^{-/-}$ mice at P21-P23 compared to *Mglur5*$^{+/+}$ mice. Conversely, layer 4 pyramidal cells exhibit increased spine density at P45 (Lu H-C, personal communications) suggesting that loss of mGluR5 differentially affect spine density in different neuronal cell types even within the same cortical layer (although an explanation based on age cannot be ruled out).

The characterisation of spine densities of Golgi impregnated cells in different neuronal cell types such as 1) layer 2/3 cells from S1, 2) layer 3 pyramidal cells of visual cortex and 3) layer 4 spiny cells of S1 in P30-P35 tangential sections from *Fmr1*$^{-/y}$, *Mglur5*$^{+/-}$ and *Fmr1*$^{-/y}$/*Mglur5*$^{+/-}$ mice will provide data to test the hypothesis whether interaction between mGluR5 and FMRP vary depending on the brain region or neuronal cell type. In layer 3 of visual cortex, one would expect to see outcomes corresponding to Dolen et al., (2007), which are increased spine density in *Fmr1*$^{-/y}$ mice, no change in *Mglur5*$^{+/-}$ mice and rescue of spine phenotype in *Fmr1*$^{-/y}$/*Mglur5*$^{+/-}$ mice compared to willdtype mice. It would be interesting to determine whether FMRP and mGluR5 interact to regulate spinogenesis in layer 2/3 of S1 in a similar manner to layer 3 of visual cortex or whether the outcomes would be different.

7.1.3 Neuronal cellular process

The interactions between FMRP and mGluR5 may also depend on the cellular process that they govern. In support, chapter 5 shows that while reducing

levels of mGluR5 rescues barrel defects in *Fmr1*$^{-/y}$ mice, defects in layer 4 spine density are not. For example, FMRP and mGluR5 may associate with two independent pathways both of which regulate spine formation or alternatively the two could converge on a signalling pathway that would ultimately regulate spine formation. Pan and Broadie (2007) show that at the *drosophila* NMJ, dFMRP and DmGluRA perform overlapping and independent mechanisms in regulating expression of postsynaptic AMPAR like glutamate receptors (GluRs) at synaptic sites. In *drosophila* NMJ synapse there are five subunits of GluRs (GluRIIA-E) with three consistent subunits and two variable (GluRIIA and B) subunits that determine their receptor kinetics and channel open probabilities (Featherstone et al., 2005; Marrus et al., 2004; Qin et al., 2005). At *dfmr1* mutant synaptic sites, individual GluRIIA increases while GluRIIB is decreased, but overall levels of total GluRs do not change. Conversely, in *dmGluRA* mutant, both GluRIIA and B increase resulting in increased total levels of GluRs. In *dfmr1*/*dmGluRA* double mutant, there is an additive increase in levels of GluRIIA at the synapse. GluRIIB levels also increases additively in *dfmr1*/*dmGluRA* double mutant with levels significantly decreased compared to wildtypes but increased compared to *dfmr1* mutant (Pan and Broadie, 2007). They hypothesise that while dFMRP and DmGluRA converge on the same pathway to produce additive effects on insertion of GluRs at synaptic sites, the ratio of GluRIIA:B at the synaptic site is regulated by independent mechanisms (Pan and Broadie, 2007). Similarly, while decreasing levels of mGluR5 alleviate spine phenotype in layer 3 of visual cortex in *Fmr1*$^{-/y}$ mice, it does not correct the spine phenotype in layer 4 of S1 suggesting that in layer 4 there are other independent mechanisms regulating spine phenotype. Alternatively, mGluR5 and FMRP could be in the same pathway but work synergistically to regulate spinogenesis in layer 4.

Another cellular process in S1 that FMRP is thought to regulate is layer 4 dendritic pruning during S1 differentiation (Galvez et al., 2003). In adult *Fmr1*$^{-/y}$ mice, there is increased dendritic matter in inter-barrel septae compared to *Fmr1*$^{+/y}$ mice (Galvez et al., 2003). The aberrant dendritic pruning of layer 4 cells in a patch would result in layer 4 dendrites synapsing onto TCAs from adjacent patches, which would in turn lead to altered S1 connectivity in *Fmr1*$^{-/y}$ mice. Examining whether genetic deletion of *Fmr1* affects selective elaboration and/or retraction of layer 4 spiny cell dendrites

within a barrel patch during development will elucidate whether this is a developmentally regulated process. This is another assay of altered S1 cortical connectivity that can be explored to determine whether FMRP and mGluR5 interact in regulating dendritic pruning of layer 4 spiny cells during development.

7.1.4 Therapeutic implications from differential interactions between FMRP and mGluR5

The age, neuronal cell type and cell process specific interactions of FMRP and mGluR5 have important implications in treating both males and females affected with fragile X. In females, the process of X inactivation during embryonic development results in silencing of one of the X chromosomes. Once X inactivation takes place in a neuroprogenitor cell, all daughter cells will maintain the inactivation of the same chromosome. As a result, females that are heterozygous for the mutant *FMR1* allele are mosaics, with some cells expressing normal levels of FMRP ($FMRP^{+ve}$), while others lack FMRP ($FMRP^{-ve}$) expression. On average in female heterozygous, X inactivation leads to 50% of their cells expressing genes located on one X chromosome and 50% from the other X chromosome (Hadjantonakis et al., 2001). Heterozygotic FXS females, in whom cells are significantly skewed towards their normal X chromosome, will display milder phenotypes or even be asymptomatic carriers. Alternatively, if a higher proportion of the wildtype X chromosome is inactivated they will show severe phenotypes comparable to those observed in male FXS males (Heine-Suner et al., 2003; Marco and Skuse, 2006; Martinez et al., 2005; Oostra and Willemsen, 2002). In most FXS females patients that have been diagnosed their phenotypes are reported to be highly variable (Cornish et al., 2008; Oostra and Willemsen, 2002). Only 25% appear to exhibit significant cognitive impairments comparable to fragile X males (Cornish et al., 2008) whereas others show subclinical learning disabilities (Bennetto and Pennington, 2002; Cornish et al., 2008). One explanation for the majority of FXS female patients showing a milder manifestation of the MR is that there is a high propensity towards skewed X inactivation of the X chromosome carrying the *FMR1* allele in these patients (Oostra and Willemsen, 2002). In turn, these data suggest that in females presented with severe FXS, there is only a small percent of normally

functioning FMRP^{+ve} cells. Decreasing the dosage of mGluR5 affects cellular processes such as spinogenesis in a neuronal cell type specific manner, in which case decreasing levels of mGluR5 in these female patients that are **severely** affected with FXS will misregulate mGluR5 signalling dependent effects in their normal FMRP^{+ve} cell population. Moreover, it might be better not to therapeutically intervene in females with **milder** symptoms (see section 7.4).

The differential interactions between mGluR5 and FMRP in regulating different neuronal cell processes have serious implications for how neuronal networks function and compensate. One of the fundamental questions is how does mGluR5 and FMRP mediate its regulatory effects at specific neuronal cell populations to produce the final overall functional output in the cortical circuitry. In the sensory cortical circuit, the majority of afferent fibres from the appropriate thalamic relay nuclei terminate in layer 4 by forming synapses with excitatory spiny cells (Hubel and Wiesel, 1962; McCormick, 1992; McGuire et al., 1984), of which 80% is consisted of spiny stellate cells while a small fraction is star pyramidal (Ahmed et al., 1994; Feldmeyer et al., 1999; Hirsch, 1995). Both of these classes of layer 4 spiny cells relay excitatory drive to pyramidal cells in layer 2/3, and layer 2/3 distribute this excitation laterally and vertically to other cortical connections (Feldmeyer and Sakmann, 2000). Layer 5 relays the excitation received from layer 2/3 to subcortical brain regions (Armstrong-James et al., 1992; Feldmeyer and Sakmann, 2000). Dolen et al., (2007) show rescue of neuropathological phenotypes of cortical layer 2/3 in *Fmr1*$^{-/y}$ mice by reducing their levels of mGluR5 by 50%, and layer 2/3 is the cortical layer that distribute the excitatory drive within the cortex received from cortical layer 4. Therefore, the correction of cortical circuitry at this level in *Fmr1*$^{-/y}$ mice to produce a rescue of behavioural phenotypes is an encouraging step towards finding treatment for FXS. It is also in support of examining the mGluR theory of fragile X in various experimental paradigms to better understand interactions between Gp1 mGluRs and FMRP.

7.2 Other therapeutic targets for the treatment of FXS

The prevailing view of FMRP is that it is a mRNA binding protein, which regulates both mRNA trafficking along dendrites and local protein synthesis at synaptic sites in an activity dependent manner (Bagni and Greenough, 2005; Bassell and Warren, 2008; Bear et al., 2008; Ronesi and Huber, 2008). In deciphering roles of FMRP in regulating cellular processes, important questions are 1) what are the molecular mechanisms of FMRP mediated repression and activation of its target mRNA? and 2) what are the signalling pathways associated with mGluR5 dependent protein synthesis that are regulated by FMRP? (Bassell and Warren, 2008; Ronesi and Huber, 2008).

7.2.1 The molecular regulators of mTOR/ERK pathway leading to mRNA translation

The phosphorylation status of FMRP is thought to be a critical factor, which determines whether FMRP acts as a translational repressor or as an activator (Ronesi and Huber, 2008). Signalling via Gp1 mGluRs activates both the MEK/ERK pathway and PI3K/mTOR pathway, and these regulate translation initiation (Klann and Dever, 2004). The characterisation of phosphatase and/or kinases that directly regulate the phosphorylation status of FMRP in an activity dependent manner, thereby regulate ERK and mTOR mediated synaptic changes would provide putative candidates for drug treatments in FXS. Recent *in vivo* and *in vitro* evidence suggest that Gp1 mGluR stimulation leads to rapid activation of PP2A, which dephosphorylates FMRP allowing mRNA translation to take place (Narayanan et al., 2007). The persistent stimulation of Gp1 mGluRs activates S6K1 via mTOR pathway resulting in the subsequent phophorylation of FMRP and mRNA translation inhibition (Narayanan et al., 2008). Kim et al., (2008) show deficits in early phase ERK activation such that Gp1 mGluR leads to rapid dephosphorylation of ERK as apposed to ERK phophorylation that normally takes place, and it appears to be mediated via elevated levels of PP2A and tyrosine phosphotase in response to mGluR1 and GluR5 activation respectively (Kim et al., 2008). Treatment with phosphatase blockers such as okadaic acid has been shown to successfully restore the aberrant ERK signalling in synaptoneurosomes from *Fmr1*$^{-/y}$ mice in response to Gp1 mGluRs.

7.2.2 Lithium treatment

There is anecdotal evidence to suggest that lithium may have beneficial effects on mood stabilisation and aggression in FXS patients (Hagerman, 2002). Treatment with lithium has also been shown to correct susceptibility to seizures (Min et al., 2009) in *Fmr1*$^{-/y}$ mice and courtship behaviour in drosophila model of FXS (McBride et al., 2005). Lithium effects has been described in terms of both GSK3β (glycogen synthase kinase β) (Min et al., 2009) and PI metabolism (Berridge et al., 1989; McBride et al., 2005). In several brain regions, GSK3β activity has been shown to be elevated in *Fmr1*$^{-/y}$ mice, and inhibition of mGluR5 signalling lowers elevated levels of GSK3β in *Fmr1*$^{-/y}$ mice suggesting an interaction between mGluR5 and GSK3β (Min et al., 2009).

Chapter 4 shows that basal levels of PLCβ1 at synaptic sites in *Fmr1*$^{-/y}$ mice are downregulated at P7. Interestingly, several parallels can be drawn between the phenotypes of PLCβ1$^{-/-}$ and *Fmr1*$^{-/y}$ mice. PLCβ1$^{-/-}$ mice have a high incidence of seizures (Kind, 2008) and have impairments in implantation of embryos (Filis, P personal communications). Patients with FXS suffer from increased seizure susceptibility (Bernardet and Crusio, 2006), and premature ovarian failure is reported in female permutation carriers of fragile X (De Caro et al., 2008; Martin and Arici, 2008). Moreover, *PLCβ1*$^{-/-}$ mice show increased spine density of layer 4 neurons (Upton, L, personal communications), and show near complete loss of layer 4 cellular segregation into barrels (Hannan et al., 2001). The examination of barrel formation and layer 4 spinogenesis in double mutants of PLCβ1$^{-/-}$ and *Fmr1*$^{-/y}$ mice would be useful in gaining an insight into whether there are interactions between FMRP and PLCβ1 during cortical organisation of S1.

7.2.3 The cAMP theory of fragile X

The cAMP theory of FXS hypothesises that neuronal phenotypes of FXS are partly due to altered cAMP metabolism (Kelley et al., 2008; Kelley et al., 2007). For example, decreased cAMP levels in platelets are reported in human fragile X patients compared to normal controls (Berry-Kravis and Huttenlocher, 1992). Kelly et al., (2007) hypothesises that altered levels of

cAMP reflect functional deficiencies in neurotransmitters and receptors signalling through FXS. Gp1 mGluRs signalling activates AC1 that catalyses production of cAMP from ATP. Cortex specific deletion of AC1 results in reduced segregation of barrels but normal TCA patterning (Iwasato et al., 2008), and this phenotype in S1 is similar to that of *Fmr1*$^{-/y}$ mice characterised in this thesis. Furthermore, loss of PKA that is activated by cAMP results in deceased layer 4 cellular segregation but there are no defects in TCA patterning (Inan et al., 2006; Watson et al., 2006). Therefore, interactions between FMRP and AC1/PKA pathway would be of interest in understanding molecular pathways that FMRP regulate and/or is regulated by.

7.3 *Altered protein synthesis in FXS*

In FXS, altered synaptic connectivity may underlie or result from altered synaptic plasticity and protein synthesis. Long-term alterations in synaptic activity (ie.LTP and LTD paradigms) are largely believed to be the mechanism that governs memory formation and consolidation. In the 'synaptic tagging' model of Frey and Morris (1997), it is thought that synthesis of new proteins needs to be specifically targeted to active synapses for strengthening these synapses to consolidate synaptic changes and memory of a particular experience. Recently, Kelleher and Bear (2008) put forward the intriguing possibility that in forms of MR such as FXS, altered protein synthesis in response to activity may result in aberrant synaptic tagging and capture of proteins leading to deficits in synaptic connectivity and synapse consolidations that may underlie the cognitive impairments observed in FXS. The biochemical analysis carried out in this thesis, only examined levels of synaptic proteins in *Fmr1*$^{-/y}$ mice at basal levels. It is important to characterise their expression in response to Gp1 mGluR activation, such as in agonist stimulated synaptoneurosomes from *Fmr1*$^{-/y}$ mice or in *in vitro* cultures from *Fmr1*$^{-/y}$ mice. Moreover, there is evidence for FMRP regulating stability of mRNA expression profiles such as in PSD95 mRNA (Zalfa et al., 2007), therefore it is important to investigate the mRNA profiles of putative FMRP targets suggested here in *Fmr1*$^{-/y}$ mice and to determine whether there

are direct interactions between these proteins and FMRP as predicted by their putative binding sites.

7.4 *Treating female patients with FXS*

Despite only 25% of females affected to the same degree as male patients with FXS, there is no firm evidence to suggest that the severity of the phenotype correlates with levels of FMRP in these patient (Lightbody et al., 2006). Some studies suggest of a connection between FMRP and cognitive deficits (Cornish et al., 1998, 2001), while others do not report of a definitive relationship (Bennetto et al., 2001). For example, a study in females with the full mutation, only 24% of the variability observed in full scale IQ score was explained by levels of FMRP (Tassone et al., 1999), while another demonstrated that approximately 28% of the variance in IQ correlated with FMRP levels in females with full and permutation of FXS while in males it accounted for about 75% of the variability seen in IQ scores (Loesch et al., 2004). These variabilities could arise from the fact that levels of FMRP in the brain cannot be measured directly, and measurements are taken indirectly such as from patients' plasma.

Another explanation for these discrepancies is that in heterozygote females mosaic for FMRP expression, some cellular processes might be regulated in a cell non-autonomous manner while other are regulated cell-autonomously. In cell non-autonomous events, a factor such as a neurotrophic agent secreted by $FMRP^{+ve}$ cells could potentially rescue defects in $FMRP^{-ve}$ cells caused by loss of FMRP. In *Fmr1*$^{+/-}$ mice, the $FMRP^{-ve}$ cells can be marked by mating these mice with male mice carrying marker genes such as EGFP, thereby effectively visualising only this subset of cells. It is then possible to examine whether these cells display cellular defects characterised in *Fmr1*$^{-/y}$ mice such as defects in barrel formation. If these $FMRP^{-ve}$ cells display normal barrel segregation, this suggest that FMRP regulates barrel formation cell non-autonomously and molecular dissection of such an event may provide valuable information of factors secreted by $FMRP^{+ve}$ cells that allow normal cellular processing to occur in $FMRP^{-ve}$ cells. Findings from these studies would not only benefit female patients with FXS but also affected males as well.

Prof. JD Watson described the discovery of fragile X gene, *FMR1* in 1991 as the “first major human triumph of the Human Genome Project”. Since then elucidating roles of the protein encoded by *FMR1*, FMRP has been of interest for several reasons. FMRP is associated with cellular processes of synaptic plasticity, protein synthesis and synaptic connectivity and evidence to date suggests that it plays a pivotal role in stabilising these processes. Loss of FMRP is responsible for FXS, and FXS is the most common form of inherited MR and the most common monogenic cause of autism. Hence, understanding the developmental role of FMRP in cellular processes is of benefit in designing therapeutic strategies for both FXS and autism.

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Appendices