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Convergence of Synaptic Pathophysiology in the Hippocampus of *Fmr1*$^{-/y}$ and *Syngap1*$^{+/-}$ Mice

Stephanie A. Barnes B.Sc. (Hons)

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Declaration

This work was carried out in the Centre for Integrative Physiology, School of Biomedical Sciences at the University of Edinburgh. I hereby certify that this thesis and its composition are entirely my own work, with the exception of the following:

1.) Mr Derek Thomson and Mr Mark Patrizio often carried out genotyping of mutant colonies.

2.) Initially metabolic labelling experiments were performed with the assistance of Dr. Emily Osterweil; these experiments have contributed specifically to measurements of basal protein synthesis rates in the *Syngap* heterozygous mice and *Fmr1* knockout rats.

3.) Dr. Emily Osterweil also assisted with the western blotting of hippocampal homogenates and synaptoneurosomes from *Syngap* heterozygous mice.

4.) Furthermore Mr Adam Jackson, when a summer student, contributed to some of the LTD recordings performed in hippocampal slices from *Syngap* heterozygous mice.

No part of the work contained in this thesis has been submitted for any other degree or professional qualification.

Signed …… Date……..

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Abstract

The genetic causes of intellectual disability (ID) and autism spectrum disorder (ASD) are frequently associated with mutations in genes that encode synaptic proteins. A recent screen of ID patients has revealed that approximately 4% of individuals carry spontaneous autosomal-dominant *de novo* mutations in the *SYNGAP1* gene. This gene encodes the synaptic GTPase activating protein (SYNGAP) a known regulator of Ras signalling. Investigations into the pathological consequences of *Syngap1* haploinsufficiency (*Syngap*$^{+/-}$) in mice have reported abnormalities in behaviour, synaptic plasticity and dendritic spine development. These are analogous to findings from the mouse model of fragile X syndrome (FXS; *Fmr1*$^{-/y}$), the most common inherited form of ID.

One of the prominent phenotypes reported in the mouse model of FXS is that a form of hippocampal long-term depression (LTD) mediated by the activation of Group 1 (Gp1) metabotropic glutamate (mGlu) receptors is enhanced and independent of new protein synthesis (Huber et al. 2002; Nosyreva et al. 2006). The cause of these synaptic plasticity deficits together with other cognitive abnormalities observed in FXS are thought to arise, in part, from excessive protein synthesis, the consequence of altered $mGlu_5$ receptor signalling via the Ras-ERK1/2 signalling pathway. Enhanced protein synthesis rates in *Fmr1*$^{-/y}$ mice can be corrected by either inhibiting $mGlu_5$ receptors or reducing Ras and subsequent ERK1/2 activity (Osterweil et al. 2013).

In this thesis mGluR-dependent LTD was examined at Schaffer collateral/commissural inputs to CA1 pyramidal neurones in hippocampal slices obtained from *Fmr1*$^{-/y}$, *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$*Syngap*$^{+/-}$ double mutant mice. Extracellular field recordings reveal that acute application of the Gp1 mGluR agonist dihydroxyphenylglycine (DHPG) induces a form of mGluR-dependent LTD that is enhanced and independent of new protein synthesis in CA1 of *Fmr1*$^{-/y}$ mice. In *Syngap*$^{+/-}$ mice, the magnitude of mGluR-dependent LTD is also significantly increased relative to WT littermates and insensitive to protein synthesis inhibitors.

Furthermore, in the *Fmr1*$^{-/y}$*Syngap*$^{+/-}$ double mutant, *Syngap* haploinsufficiency occludes the increase in mGluR-dependent LTD caused by the loss of FMRP.

In addition, metabolic labelling studies reveal basal protein synthesis rates to be modestly enhanced in the hippocampus of *Fmr1*$^{-/y}$ mice compared to WT mice. Importantly this phenotype translates to the rat model of FXS. In *Syngap*$^{+/-}$ hippocampal slices, basal protein synthesis rates are also significantly elevated compared to WT counterparts. Interestingly, elevated basal protein synthesis rates in *Syngap*$^{+/-}$ mice could be corrected in the hippocampus by similarly pharmacological strategies employed in *Fmr1*$^{-/y}$ mice.

The comparable neuropathophysiology we observe between *Syngap*$^{+/-}$ and *Fmr1*$^{-/y}$ mice suggests that SYNGAP and fragile X mental retardation protein (FMRP) may converge on similar biochemical pathways raising the intriguing possibility that therapeutic strategies used in the treatment of FXS may also be of benefit in treating individuals with ID caused by mutations in *SYNGAP1*.

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Chapter 1

Introduction

Intellectual disability (ID), formerly known as mental retardation, is a complex and debilitating neurodevelopmental disorder characterised by impairments in general mental ability. The prevalence of ID in developed countries is estimated at 1-3% of the general population and represents the most frequent cause of severe handicap in children (Roeleveld & Zielhuis 1997; Chelly et al. 2006). In ICD-10 (International statistical Classification of Disease and related health problems-10), the World Health Organisation (WHO) defines ID as 'arrested or incomplete development of the mind especially characterized by impairment of skills manifested during the development period, which contribute to the overall levels of intelligence such as cognition, language, motor and social abilities' (WHO 1992).

1.1.1 Diagnosis of Intellectual Disability

The diagnosis of ID is historically based on standardized intelligence testing and is considered as having an intellectual quotient (IQ) two standard deviations or more below the general population, which is equivalent to an IQ below 70. The severity of ID ranges from mild to severe and is subdivided by IQ score (Table 1.1). As all patients with ID display impaired adaptive behaviour, the American Psychiatric Association has recently revised their criteria for diagnosis of ID in DSM-5 (Diagnostic and Statistical Manual of Mental Disorders). The severity of ID is now based on adaptive functioning rather than IQ and is assessed in three domains: conceptual, social and practical skills. This revision means the severity of ID is now based on how impairments in mental abilities affect functioning needed for everyday life.

1.1.2 Symptoms of Intellectual Disability

The onset of symptoms in ID patients begins early on in childhood and can occur with or without any other mental or physical disorders. However the core symptoms of ID are rarely observed in isolation and usually coexist with other psychiatric and medical conditions, including autism spectrum disorders (ASD), language disorders, epilepsy, motor control problems, attention-deficit, hyperactivity, anxiety, sleep disorders, gastrointestinal problems and abnormal responses to sensory stimuli (Huguet et al. 2013). In fact, the prevalence of other mental disorders is at least 3-4 times greater in the ID population compared to the general population (WHO 1992). For example, the incidence of epilepsy is estimated to affect approximately 1% of the general population (van Blarikom et al. 2006), yet in the ID population the prevalence rate of epilepsy varies from ~16-50% . There also appears to be variable penetrance and expressivity, with symptoms spanning a wide spectrum of severity. This has been attributed to interactions of other genes, environmental causes and epigenetic variation in gene expression (San Martín & Pagani 2014).

Intellectual disability

Diagnosis	Intellectual Quotient
Mild	50-69
Moderate	35-49
Severe	20-34
Profound	<20

Table 1.1 Classification of intellectual disability. A diagnosis of intellectual disability (ID) is given when intellectual quotient (IQ) is < 70, as determined from standardised intelligence tests. ID is subdivided in to severity classes based on IQ, ranging from mild to profound.

1.1.3 Treatment of Intellectual Disability

Although ID is highly prevalent within the population it is very difficult to treat at present due to the lack of disease altering therapies. Current strategies for treatment of ID depend on the use of a wide range of different drugs, such as antidepressants, anxiolytics and antipsychotics. These central nervous system (CNS) active compounds treat very specific behavioural problems rather than the core symptoms of ID patients and are administrated if the patient presents with comorbidities, such as epilepsy, anxiety, attention deficit and mood disorders (Berry-Kravis 2014). Thus the development of pharmacotherapies that modify the underlying cause of the disorder will be a remarkable advance.

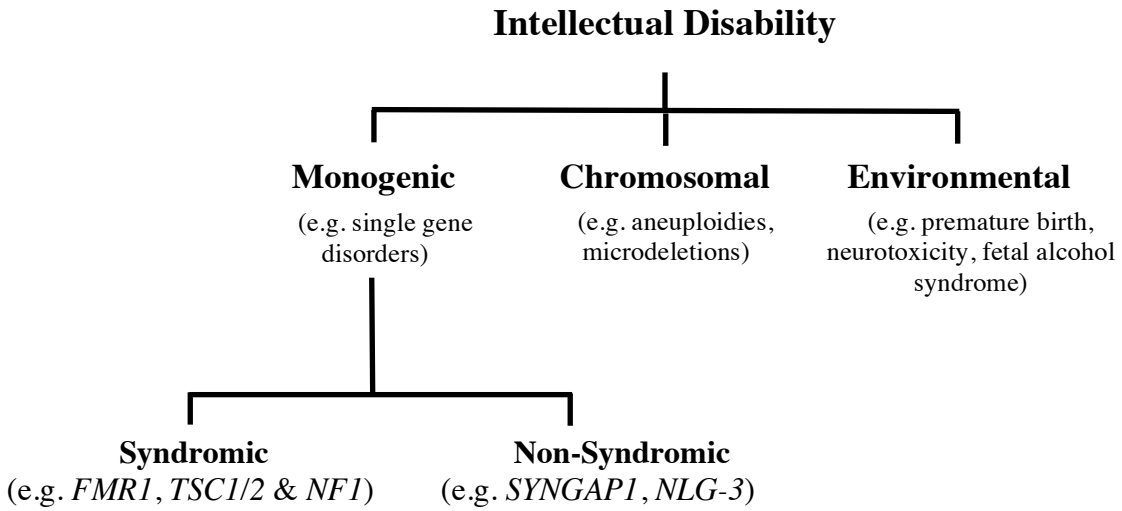


Figure 1.1 Genetic and environmental causes of intellectual disability. The causes of intellectual disability (ID) are highly heterogeneous and include environmental, chromosomal and single gene mutations. Monogenic causes of ID are further divided in to syndromic and nonsyndromic forms of ID. Syndromic causes of ID are characterised by physical, metabolic and biological features and include Tuberous Sclerosis, Fragile X syndrome and Neurofibromatosis 1, which are caused by mutations in the *TSC1/2*, *FMR1* and *NF1* genes respectively. In contrast, the only clinical manifestation associated with non-syndromic causes of ID is cognitive dysfunction, examples of which include mutations in SYNGAP1 and NLG3 that encode the synaptic GTPase activating protein (SYNGAP) and NEUROLIGIN-3 that are crucial for normal synaptic functions.

1.1.4 Causes of Intellectual Disability

The causes of ID are highly heterogeneous and include both genetic and non-genetic factors (Figure 1.1). The latter of which are caused by environmental influences that act prenatally or during early infancy leading to brain damage and include: malnutrition over pregnancy, environmental neurotoxicity, premature birth, perinatal brain ischemia, fetal alcohol syndrome, pre- or post-natal infections (Chelly & Mandel 2001). Genetic causes of ID include chromosomal abnormalities (aneuploidies, micro deletion syndrome), and single-gene mutations (Chelly et al. 2006). However an exact cause of ID has only been established in 50% of patients who present with moderate to severe ID and this will be far less in patients with milder forms of ID. Two monogenic causes of ID will be the focus of this thesis.

1.1.5 Genetics of Intellectual Disability

Over the past decade technological advances have enabled the identification of several hundred ID-related genes. For example, high-density microarrays have allowed the detection of copy number variants, whilst exome sequencing has led to the identification of single nucleotide mutations in ID patients. Inlow & Restifo (2004) estimated that there are around 282 human genes linked to ID, which since then is likely to have significantly increased. Genetic forms of ID can be subdivided in to two major categories: syndromic ID, characterised by the coexistence of clinical, radiological, metabolic and physical features (e.g. fragile X syndrome, tuberous sclerosis, Neurofibromatosis 1); and non-syndromic ID (NSID), where cognitive impairment is the only clinical manifestation of the disease (e.g. *SYNGAP1* haploinsufficiency).

Epidemiological studies of ID patients have revealed a significant gender bias, with many more males affected with monogenic forms of ID than females (Leonard & Wen 2002). This has implied that a greater number of the genes influencing cognitive function are found on the X-chromosome compared to the autosomal chromosomes, where the distribution of ID genes correlates with relative gene composition (Inlow & Restifo 2004). Further investigation by Inlow & Restifo (2004) revealed that 16% of ID genes are located on the X-chromosome, showing a four-fold overrepresentation, considering only 4% of all known genes are located here. However, this could be due to X-linked mutations being easier to map and thus identify.

The discovery of mutations in genes causing ID has led to the generation of genetically engineered animal models of the disorder. These models of ID recapitulate many of the behavioural, cognitive and physiological alterations reported in human patients. Furthermore they have allowed an in depth look at the pathophysiological mechanisms underlying the behavioural and cognitive abnormalities to this disorder in order to identify potential therapeutic targets.

1.1.6 ID genes encode proteins that belong to distinct functional networks

The generation of mouse models of ID has revealed that ID-related genes encode proteins that fall in to distinct functional subclasses, such as chromatin-remodelling, transmembrane proteins, microtubule- and actin- associated proteins, transcription and translation proteins, regulators or effectors of Ras/Rho GTPase pathways, and synaptic plasticity proteins (Chelly et al. 2006). The loss or reduced expression of these proteins leads to defects in broad range of cellular functions that underlie intellectual performance and emotional behaviour associated with ID.

The impact of altered signalling and synaptic function in the pathology of ID has been particularly emphasized by research into fragile X syndrome (FXS), the most common identifiable form of inherited ID that affects approximately 1 in 4000 males to 1 in 8000 females (Wijetunge et al. 2013). From investigations in the mouse model of FXS, it is now known that FMRP is an RNA-binding protein that functions to negatively regulate protein synthesis in the brain. It is now viewed that many symptoms associated with FXS arise from an increase in synaptic protein synthesis. Consequently this leads to abnormalities in synaptogenesis and synaptic plasticity during postnatal development, processes that underlie the cognitive impairments associated with FXS.

Furthermore, these investigations have identified key pathophysiological mechanisms generating several therapeutic hypotheses and the development of targeted pharmacotherapies. Thus, the mouse model of FXS has become one of the most advanced animal models of ID. Here this thesis will explore the behavioural, cellular and molecular abnormalities that have so far been reported in FXS and detail how these investigations have not only advanced our knowledge about FXS but also guided our own investigations in to understanding the pathophysiology of a second genetic cause of ID caused by mutations in *SYNGAP1*.

1.2 Fragile X syndrome

The term FXS was first coined by Moore et al. (1982) who identified a fragile site on the X chromosome of patients with mental retardation. Indications that the gene mutation responsible for FXS was X-linked was first described by Martin & Bell in 1943 who observed that this type of ID was transmitted in an X-linked fashion, which explained why more males than females were affected by the disorder (Martin & Bell 1943).

However it wasn't until 1991 that the FXS gene, designated the *FMR1* gene, was first identified at the fragile site at map position Xq27.3 on the long arm of the X chromosome (Verkerk et al. 1991). From Southern blot analysis it was found that the 40 kb *Fmr1* DNA fragment on the X chromosome exhibited significantly increased size variation, indicating that the FXS mutation may result from the presence of an insertion or amplification event (Verkerk et al. 1991). This coincided with the identification of an unusual number of CGG repeats, which exhibited length variation in the fragile X gene, and the presence of a hypermethylated CpG island. This led Verkerk et al. (1991) to speculate that the CGG repeat may influence local methylation and the expansion of this repeat may be responsible for the abnormal methylation status of the fragile X gene.

1.2.1 Genetics of FXS

Since these initial observations it is now known that the vast majority of FXS patients have a significant CGG expansion mutation of more than 200 units (Figure 1.2). This expansion mutation lies in the promoter region of the *FMR1* gene leading to hypermethylation of a near CpG site (Oberlé et al. 1991; Sutcliffe et al. 1992; Coffee et al. 1999). This results in little or no production of corresponding messenger RNA (mRNA) and consequently the loss or significant reduction of the fragile X mental retardation protein (FMRP), a RNA binding protein (Ashley et al. 1993). The severity of FXS appears to be negatively correlated with the level of FMRP expression (Tassone et al. 1999).

In unaffected individuals, the CGG repeat is polymorphic ranging from 5 to 54 repeats (Fu et al. 1991). However, once the number of CGG units expands into the range of 55 to 200 units, individuals are said to carry the premutation of FXS, which is linked to fragile X-associated tremor/ataxia syndrome and fragile X-associated primary ovarian insufficiency (Willemsen et al. 2011). The premutation leads to a reduction in FMRP via an unusual mechanism that targets translation rather than transcription, leading to a sparse amount of FMRP even though there is an abundance of mRNA (Tassone et al. 2000). The instability of the CGG repeat expansion means that it can increase in size from one generation to the next, increasing in penetrance, making it more likely that it will progress from a premutation to the full mutation with successive generations (Sherman et al. 1985).

Although the majority of FXS cases are the result of a mutant trinucleotide expansion of 200 units or above, there are reports of individuals carrying rare point mutations (I304N and R138Q) in the *FMR1* gene that result in a severe FXS phenotype (De Boulle et al. 1993; Collins et al. 2010). Examination of the I304N mutation revealed that there is a usual number of CGG repeats along with a normal methylation status (De Boulle et al. 1993). However the mutation is located in the mRNA binding domain, disrupting FMRPs ability to bind and regulate mRNA translation at the polyribosome (Zang et al. 2009). This indicates that the binding of FMRP to its mRNA targets is critical to its function.

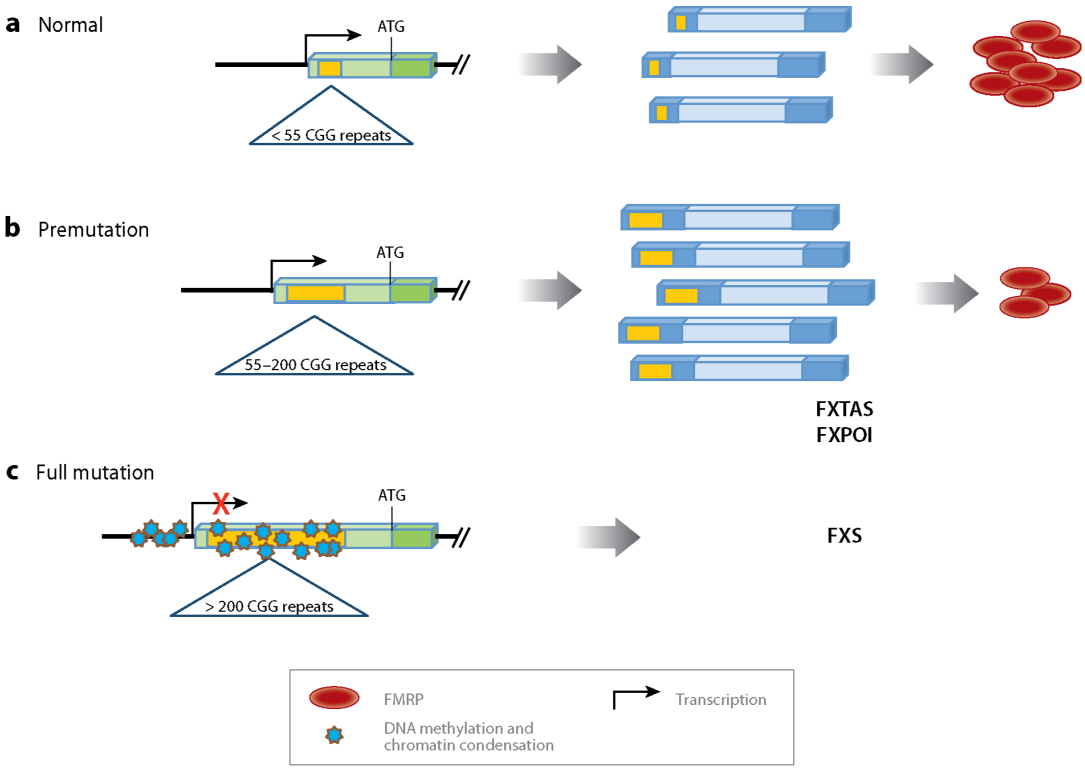


Figure 1.2 CCG expansions in the *Fmr1* gene. (A) The CGG repeat is polymorphic and can expand from 5 to 54 repeats in the *FMR1* allele leading to normal transcription and translation of FMRP. (B) Premutation alleles are caused by 55 or 200 CGG repeats that lead to excessive transcription of *FMR1* messenger RNA (mRNA) leading to fragile X associated tremor/ataxia syndrome (FXTAS) and fragile X-related primary ovarian insufficiency (FXPOI). (C) The full mutation is caused by more than 200 CGG repeats leading to transcriptional silencing and the loss of the fragile X mental retardation protein (FMRP) resulting in fragile X syndrome (FXS). Figure from Santoro et al 2012.

1.2.2 Phenotypes reported in human patients with FXS

The cognitive, behavioural and morphological symptoms of FXS are highly variable, but share a defining feature of ID. The severity of ID ranges from moderate to severe and IQ is found to decline further with age (Ashley et al. 1993; Bernardet & Crusio 2006). Affected individuals usually present with language delay and can have additional behavioural symptoms that include hyperactivity, social anxiety, impulsivity, attention deficit hyperactivity disorder and autistic-like behaviours, such as shyness, poor eye contact and hypersensitivity to sensory stimuli (Hagerman et al. 2009). The predominant physical feature observed in FXS is macroorchidism (observed in >80% of FXS males) with a variable presence of an elongated face, large and prominent ears, macrocephaly, prominent jaw and forehead, high-arched palate, and loose connective tissue leading to hyperextensible joints, flat feet and soft skin (Hagerman 2002; Hagerman et al. 2009). Additional medical problems, include childhood seizures, sleep disorders, strabismus, a susceptibility to ear and sinus infections and gastrointestinal problems (Hagerman & Hagerman 2002; Kravis & Potanos 2004). For female carriers of the full mutation, symptoms tend to be more variable and milder due the existence of one functional copy of the *Fmr1* allele on the other X chromosome. This yields some FMRP leading to a somewhat moderating effect (Mazzocco 2000).

1.2.3 FXS and Autism

FXS was one of the first genetic causes of ID to be linked to ASD, which has highly variable behavioural manifestations that differ in severity. FXS remains the most prevalent inherited cause of this disorder, however it only accounts for ~4 % of all ASD cases (Wang et al. 2010), with a further 200 genes linked to autism (Berg & Geschwind 2012). ASD is also a neurodevelopmental disorder characterised by a triad of deficits including impaired social interaction and communication as well as restrictive and repetitive behaviours, and affects approximately 1% of the general population (Zoghbi & Bear 2012; Belmonte & Bourgeron 2006). It is estimated that the prevalence of autism in FXS individuals is between 18-36% (Rogers et al. 2001; Kaufmann et al. 2004). However, a large majority of FXS males (~60-90%) display the core behaviours that are commonly observed in individuals with ASD, this

includes avoidant eye gaze, hand flapping, repetitive behaviours and abnormal speech patterns (Harris et al. 2008). Approximately one-third of male patients with FXS meet the criteria for autism, and two-thirds for ASD (Wang et al. 2010). Interestingly, several mRNA targets of FMRP encode proteins that are associated with autism, such as postsynaptic density protein 95 (PSD-95), SH3 and multiple ankyrin repeat domains 3 (SHANK3), phosphatase and tensin homolog (PTEN), mitogen activated protein kinase 1 (MAPK1), synaptic GTPase activating protein (SYNGAP), NEUROXIN1 and NEUROLIGINs. This suggests there is a great degree of molecular overlap between ID and Autism and perhaps autism risk genes converge on a common pathophysiological axis (Wang et al. 2010).

1.2.4 FXS and Epilepsy

Within the FXS population there is also high incidence of epilepsy (10-20%), which appears to greater in male FXS patients than females (Berry-Kravis 2002). Previous reports in cohorts of male FXS patients carrying the full mutation describe a variety of seizure types (Musumeci et al. 1999; Musumeci et al. 1991; Berry-Kravis 2002). The most commonly reported in FXS cases is complex partial seizures, however generalized clonic-tonic and simple partial seizures are also frequently observed (Musumeci et al. 1999; Berry-Kravis 2002). These FXS patients present with abnormal epileptiform electroencephalography (EEG) patterns resembling centrotemporal spikes that are commonly associated with benign focal epilepsy (Musumeci et al. 1991; Musumeci et al. 1999; Berry-Kravis 2002). These abnormal spiking patterns begin during childhood and are considered benign, as they tend to disappear by adolescence. However if they do persist then seizure frequency tends to be low, isolated to a specific locus within the brain, and are well controlled by anticonvulsant medication (Musumeci et al. 1999). Whether or not the epileptiform discharges observed in FXS patients exacerbate the overall behavioural and cognitive impairments remains to be determined. Interestingly, there are reports of FXS patients without a co-diagnosis of epilepsy presenting with abnormal EEG patterns indicating that they may also be at high risk of developing seizures (Berry-Kravis 2002).

1.2.5 Structure of the Fragile X Mental Retardation Protein

Shortly after the *Fmr1* gene was cloned, it was revealed that the protein product FMRP contained three RNA binding domains (Siomi et al. 1993). FMRP itself can be structurally divided into three main regions: an N-terminal region, containing two Tudor domains that function as a nuclear localization signal (NLS); a central region, which contains two K homology domains and a nuclear export signal (NES); and a C-terminal region, which contains the RGG RNA binding domains (Bagni & Oostra 2013; Figure 1.3). The NLS and NES enable FMRP to shuttle between the nucleus and cytoplasmic space whilst the KH and RGG RNA binding domains contribute to FMRPs role as a regulator of mRNA translation through binding non-coding RNA structures, which include G-quartets and kissing complexes (Brown et al. 2001; Darnell et al. 2005)..

1.2.6 FMRP in brain

FMRP is widely expressed throughout the body with protein expression levels peaking at the end of the first postnatal week before gradually declining (Wang et al. 2004). FMRP is abundantly expressed in the testes and mammalian brain where it is expressed at synapses that use the excitatory neurotransmitter glutamate (Devys et al. 1993). Functionally FMRP belongs to the family of RNA bindings proteins (RBP), which shuttle between the nucleocytoplasmic space binding and transporting mRNA (Feng et al. 1997; Eberhart et al. 1996). There are several *in vitro* studies that suggest FMRP may play a role in transporting mRNA as it has been observed in RNA granules which transport mRNA from the soma to the dendrites (De Diego Otero et al. 2002). However, FMRP does not appear imperative for the localisation of the majority of its mRNA targets (Steward et al. 1998; Dictenberg et al. 2008).

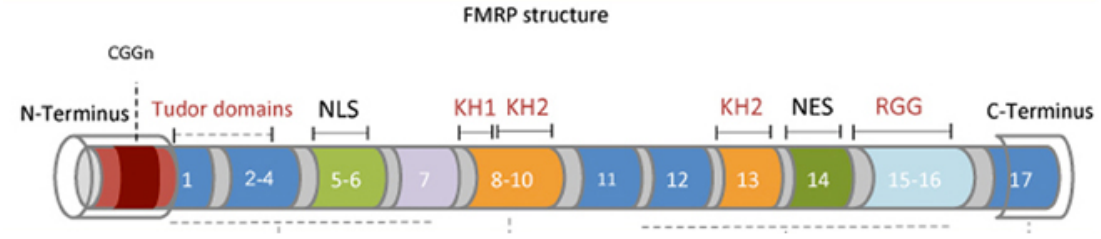


Figure 1.3 FMRP and its functional domains. The N-terminal contains two Tudor domains (TD) and a Nuclear localisation signal (NLS) whilst the central region contains two K homology domains (KH1 and KH2), and a nuclear export signal (NES) and the C-terminal containing an RGG box, which is involved in FMRP binding mRNA targets. Figure from Fernández et al. 2013.

At the synapse FMRP is found in complexes with its RNA targets on actively translating polyribosomes, suggesting it may play a role in regulating local protein synthesis at the synapse (Zalfa et al. 2007; Eberhart et al. 1996; Feng et al. 1997). Although FMRP is generally considered to negatively repress translation of its mRNA targets, there is evidence in regards to PSD-95 that FMRP modulates mRNA stability in the hippocampus (Zalfa et al. 2007). This may infer that FMRP has cell-specific dual functionality in modulating large numbers of proteins. Co-immunoprecipitation (Co-IP) studies have revealed that FMRP is associated with ~4% of all mRNA in the brain, many of which are involved in neuronal function (Darnell et al. 2011). These include three of the most prevalent proteins found at the postsynaptic density (PSD): PSD-95, an adaptor protein associated with the N-methyl-D-aspartate (NMDA) receptor; Ca^{2+}/calmodulin-protein kinase II (CaMKII), which binds Ca^{2+} and is crucial for synaptic plasticity; and SynGAP, a GTPase activating protein whose targets play key roles in neuronal survival and synaptic plasticity.

The activity of FMRP at the synapse is controlled by its phosphorylation status, which is governed by two enzymes whose activity is dependent on Group 1 (Gp1) metabotropic glutamate ($mGlu_{1/5}$) receptor stimulation. FMRP can bind to its mRNA targets once a conserved serine residue is phosphorylated by the ribosomal S6 kinase (S6K) 1 (Narayanan et al. 2007; Narayanan et al. 2008). The serine/threonine-protein phosphatase 2A (PP2A) is responsible for removing this phosphate group leading to the movement of FMRP from stalled to active polyribosomes repressing mRNA translation. The rapid dephosphorylation of FMRP in response to $mGlu_{1/5}$ receptor activation enables translation to proceed, which is shortly curbed by S6K1 phosphorylating FMRP once again. Thus, FMRP appears to form part of an intracellular signalling cascade that links $mGlu_{1/5}$ receptor activation to new protein synthesis at the synapse.

1.2.7 *Fmr1* KO mouse model

The generation of the *Fmr1* knockout (KO) mouse in 1994 has been a valuable neurobiological tool in assessing the deficits associated with the loss of FMRP (Consortium 1994). In the 'conventional' mouse model of FXS, the *Fmr1* gene is knocked out by the insertion of a neomycin cassette in to the promoter region of the *Fmr1* gene. This is opposed to the hypermethylation and transcriptional silencing of this region that is observed in FXS patients. Nevertheless in both cases FMRP, the protein product of the *Fmr1* gene, is lost. Thus, both human patients and the *Fmr1* KO mouse progress through development without any functional FMRP. There are several other animal models of FXS, including the conditional KO and a mutant carrying the point mutation at I340N (Mientjes et al. 2006; Zang et al. 2009). However the majority of studies have utilised the 'conventional' KO model, which has been shown to reproduce many of the neuropathological abnormalities observed in human patients.

1.2.8 Behavioural deficits associated with the mouse model of FXS

The generation of the mouse model of FXS has enabled the examination of a large range of behavioural traits, which have revealed correlations between the human and mouse condition validating the *Fmr1* KO mouse as model of FXS. However, it appears that many of behavioural deficits in the *Fmr1* KO mouse are more moderate than those observed in human patients and are not always consistently reported, which could be the result of strain variability from the effect of modifier genes. Also handling and housing, prior to testing may have adverse effects on the outcome of test. The availability of the *Fmr1* KO rat will now allow a more extensive set of behavioural tasks to be examined (Hamilton et al. 2014).

In terms of locomotive activity, *Fmr1* KO mice appear to have increased exploratory activity in the open field with a reduced tendency to remain close to peripheral zones (Mineur et al. 2002; Spencer et al. 2011; Qin et al. 2011). This suggests that in comparison to wild type (WT) controls, *Fmr1* KO mice are more hyperactive and have abnormal anxiety levels. There appears to be subtle impairments in the Morris water maze when the platform is hidden, with *Fmr1* KO exhibiting increased escape latencies when the position of the platform is moved after learning the initial position, as well a reduced rate of learning trial to trial (Consortium 1994; Kooy et al. 1996). This suggests that *Fmr1* KO mice may have less flexibility in learning than WT mice. In contrast, evidence from the radial maze appears to show that working memory is intact in the *Fmr1* KO mice (Mineur et al. 2002). Inhibitory avoidance (IA), a hippocampus-dependent memory, was similar between *Fmr1* KO and WT littermates. However when IA extinction (IAE) was examined, which requires new protein synthesis, they observed that IAE is exaggerated in the *Fmr1* KO mouse (Dölen et al. 2007).

The majority of sensory stimuli responses in *Fmr1* KO have been found to be impaired, with the exception of nociception in response to heat (Bernardet & Crusio 2006). It has been shown *in vivo* that *Fmr1* KO mice have increased susceptibility to both partial and generalized seizures (Osterweil et al. 2010; Chen & Toth 2001; Qiu et al. 2009). Qiu et al. (2009) investigated seizure susceptibility in the limbic system

of *Fmr1* KO mice, using amygdala kindling, which mimics epileptogenesis induced at a specific locus (i.e. partial seizure). In *Fmr1* KO mice the rate of seizure progression is accelerated compared to WT littermates (Wang et al. 2013). It is also consistently reported that *Fmr1* KO mice exhibit an increased susceptibility to audiogenic seizures (AGS), upon exposure to an >130 dB alarm sound (Osterweil et al. 2010; Yan et al. 2004; Musumeci et al. 2000). There also appear to be an increased startle response in *Fmr1* KO mice upon exposure to low frequency auditory stimuli (Nielsen et al. 2002). These findings along with altered responses to IAE (Dölen et al. 2007), highlight the similarities in hypersensory responses observed in *Fmr1* KO mice and FXS patients (Hagerman 2002).

Fmr1 KO mice also exhibit greater prepulse inhibition indicating alterations in sensorimotor processing (Frankland et al. 2004). Furthermore deficits in social interaction have been reported in the *Fmr1* KO mice exhibiting increased social anxiety in the mirror chamber test, reduced social dominance in the tube test to unfamiliar test mates (Spencer et al. 2011), and deficits in ultrasonic vocalizations in *Fmr1* KO pups that were isolated from their mothers (Roy et al. 2012).

1.2.9 Immature spine phenotype observed in FXS patients and *Fmr1* KO mice

One of the hallmark phenotypes consistently reported in the autopsy of FXS patient is the overabundance of dendritic spines with a thin, tortuous, and immature morphology (Fiala et al. 1998; Hinton et al. 1991; Wisniewski et al. 1991; Rudelli et al. 1985). This abnormal spine phenotype is observed in various cortical regions, which were qualitatively analysed by rapid Golgi staining. Only Irwin et al. (2001) observed an increase in spine density in the visual and temporal cortices of the fragile X human brain, which was isolated to the most distal dendritic segments of layer 5 pyramidal neurones (Figure 1.4). These findings indicate: firstly, that there appears to be a failure in synapse maturation during the development of the fragile X brain that persists throughout the entire lifespan; and secondly, that synaptic pruning may be aberrant leading to an increase in spine density in the *Fmr1* KO brain.

Abnormalities in spine morphology are recapitulated in the adult neocortex of *Fmr1* KO mice (Comery et al. 1997; Galvez & Greenough 2005; McKinney et al. 2005; Dölen et al. 2007; Hayashi et al. 2007) as well as in other brain regions including the hippocampus and cerebellum (Grossman et al. 2006). This suggests that FMRP may play a role in regulating spinogenesis in multiple brain regions. FMRP is localised to dendrites and spines regulating local protein synthesis upon $mGlu_{1/5}$ receptor activation, which is important for spine development (Bagni & Greenough 2005; Grossman et al. 2006). Stimulation of $mGlu_{1/5}$ receptors can induce dendritic spine elongation *in vitro*, reminiscent of the phenotype observed in the neocortex of *Fmr1* KO mice (de Vrij et al. 2008; Vanderklish & Edelman 2002). Furthermore, abnormalities in dendritic spine morphology can be corrected by either the genetic reduction of $mGlu_5$ receptor or negative allosteric modulator (NAM)s of this receptor (de Vrij et al. 2008; Yan et al. 2005). Thus, in the *Fmr1* KO brain where FMRP is absent exaggerated protein synthesis rates are thought to contribute to excessive spine growth and abnormal morphology.

In the young adult hippocampus it has been observed that *Fmr1* KO CA1 neurons possess more stubby and mushroom-like spines and fewer thin dendritic spines with no change in the overall spine density when compared to WT controls (Grossman et al. 2006). In the more mature hippocampus of *Fmr1* KO mice, Levanga et al. (2011) observed a subtle increase in the number of filopodia-like spines when compared to WT neurones. This phenotype appears to be region specific as no genotype differences were observed in the CA3 region of the hippocampus (Levenga et al. 2011).

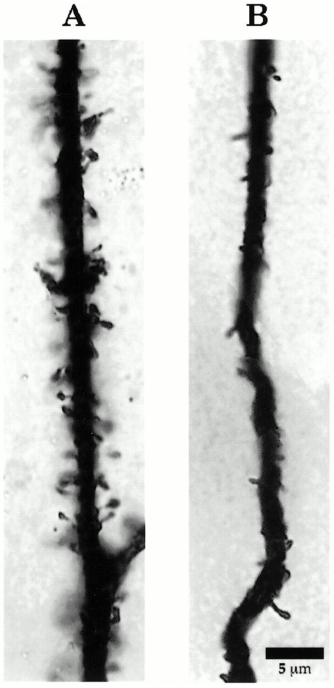


Figure 1.4 Increased spine density in the Fragile X brain. Golgi staining of distal dendrites of layer 5 pyramidal neurones reveal typical spine morphologies observed in (A) human FXS patient where an overabundance of dendritic spines are observed and (B) an unaffected individual. Figure from Irwin et al. 2000.

Unlike the neocortex where dendritic arborisations are predominantly covered in thin tortuous spines during early development which progress in to more stubby mushroom shaped spines by adulthood, there is evidence to suggest the opposite is observed in the hippocampus. Initially a high abundance of mushroom-like spines are observed in the early stages of developments, which shift towards a greater number of thinner spines in adulthood (Harris et al. 1992). Thus, in both the hippocampus and neocortex there appears to be immature dendritic spine morphology in *Fmr1* KO mice, which in the neocortex appears to persist into adulthood.

Recently dendritic spines were examined in *Fmr1* KO mice at the nanoscale using stimulated emission depletion (STED) microscopy. STED microscopy has a spatial resolution of ~50 nm, compared to 250 and 400 nm with confocal and two-photon microscopy, respectively. This has enabled a more accurate assessment of dendritic spines, such as head width, neck length and neck width, which are too small to be reliably measured by diffraction-limited microscopy techniques. This study revealed that at P14 CAl pyramidal neurones in *Fmr1* KO hippocampal slices have fewer dendritic spines with wider heads whilst at P37 there were a greater number of spines with larger heads and shorter, narrower necks compared to WT controls indicating a subtle immaturity in the hippocampus (Wijetunge et al. 2014).

1.2.10 Synaptic plasticity in the hippocampus of *Fmr1* KO mouse

The most prominent phenotype observed in FXS patients is impaired cognitive function. In the mammalian brain it is believed that activity-dependent remodelling of synaptic connections driven by plasticity mechanisms underlies key cognitive processes, such as learning and memory. Many long lasting changes in synaptic efficacy require the rapid synthesis of new proteins, suggesting that the translation of pre-existing mRNA is fundamental for the expression of certain forms of synaptic plasticity.

In response to synaptic activity, mRNAs are trafficked to dendrites where they are locally translated (Steward & Levy 1982). Interesting, one of the proteins synthesized in response to synaptic activation is FMRP which is thought to influence synaptic plasticity by functioning as a key regulator of mRNA translation (Weiler & Greenough 1993). Thus in the FXS brain where FMRP is absent, there is no longer the ability to modulate translation in response to synaptic activity, which has detrimental effects on long-lasting forms of synaptic plasticity.

Indeed, investigations into the cellular mechanisms underlying learning and memory deficits in *Fmr1* KO mice have revealed dysfunction in certain forms of synaptic plasticity. Long-term depression (LTD) mediated by Gp1 mGlu receptors (mGluR-dependent LTD) appears to be enhanced in the hippocampus of *Fmr1* KO mice (Huber et al. 2002; Zhang et al. 2009; Hou et al. 2006). Whilst investigations in to long-term plasticity (LTP) have revealed that certain forms of NMDA receptor dependent forms of LTP (NMDAR-dependent LTP) are impaired (Hu et al. 2008; Lauterborn et al. 2007; Shang et al. 2009). In contrast, there were no differences observed in NMDA receptor dependent forms of LTD (NMDAR-dependent LTD; Huber et al. 2002). NMDAR- and mGluR- dependent forms of LTD are mechanistically distinct from one another suggesting that FMRP may be specifically regulating the translation of proteins for the expression mGluR-dependent forms of synaptic plasticity.

mGluR-dependent LTD is enhanced in Hippocampus of Fmr1 KO mice

In *Fmr1* KO mice, induction of mGluR-dependent LTD leads to an exaggerated level of synaptic depression at CA1 synapses. This finding was initially reported by Huber et al. (2002) who found that upon acute application of the Gp1 agonist (*R, S*)-3,5-Dihydroxyphenlglycine (DHPG) there was a modest enhancement of mGluR-dependent LTD by ~10-15% in the hippocampus of *Fmr1* KO mice. This form of synaptic plasticity is mediated by a rapid endocytosis and persistent decrease in the surface expression of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Snyder et al. 2001; Moult et al. 2006; Zhang et al. 2008). In *Fmr1* KO mice, enhanced mGluR-dependent LTD results from

excessive AMPA receptor internalisation in response to Gp1 $mGlu_{1/5}$ receptor activation (Nakamoto et al. 2007).

Certain forms of LTP are impaired in the hippocampus the Fmr1 KO mouse

In the hippocampus investigations in to LTP mechanisms have yielded conflicting findings. Initially, examination of late phase LTP (L-LTP) by high frequency stimulation (HFS) revealed no differences in the magnitude of L-LTP between *Fmr1* KO and WT mice (Godfraind et al. 1996; Larson et al. 2005; Li et al. 2002; Auerbach & Bear 2010; Zhang et al. 2009). However, others find NMDAR-dependent LTP to be reduced in CA1 (Hu et al. 2008; Lauterborn et al. 2007; Shang et al. 2009). These discrepancies may be due to differences in stimulation protocols, which can induce distinct forms of LTP (Abraham & Williams 2003). For example, although both tetanic stimulation (Godfraind et al. 1996; Li et al. 2002) and conventional theta burst (Larson et al. 2005) produce normal LTP in *Fmr1* KO mice, changing the threshold levels of theta burst afferent stimulation (from 15 bursts of 4 pulses at 100 Hz to 5 Hz at 30 s intervals) revealed altered LTP in the *Fmr1* KO mice (Lauterborn et al. 2007).

L-LTP induced by either tetanic or conventional theta burst protocols requires both somatic transcription and translation to be sustained. In contrast, L-LTP induced by the nonconventional theta burst protocol (5 Hz, 30 s) is solely dependent on local dendritic mRNA translation (Huang & Kandel 2005). This indicates that FMRP may not regulating protein synthesis in the cell soma. Instead FMRP may only regulate local mRNA translation at synaptic sites. In addition, further examination of this nonconventional form of L-LTP revealed that the lack of LTP in *Fmr1* KO mice was accompanied by a failure in trafficking of GluA1-containing AMPA receptors and Ras-dependent activation of phosphoinositide 3-kinase (PI3K), a pathway implicated in GluA1 insertion and LTP (Zhu et al. 2002; Qin et al. 2005b). This could indicate an uncoupling of the PI3K signalling from glutamatergic synaptic activation, which prevents GluA1 receptor insertion and an increase in synaptic strength.

Stimulus-induced local protein synthesis is lost in the Fmr1 KO mouse

At individual synapses, glutamate release from the presynaptic terminus is believed to stimulate local protein synthesis. This process occurs independently of transcription and involves the translation of pre-existing dendritically localized mRNA, which is crucial for the transition from the early to late phases of certain forms of LTP and LTD (Huber et al. 2000; Huang & Kandel 2005). Evidence in support of local protein synthesis, rather than somatic protein synthesis, comes from the observation that mGluR-dependent LTD can be induced in the dendritic layer even when the somatic layer has been severed (Huber et al. 2000).

The stable expression of mGluR-dependent LTD at CA1 synapses is reliant on newly synthesized proteins to maintain the persistent removal of AMPA receptors from the cell surface (Huber et al. 2000; Snyder et al. 2001). It is hypothesized that the activation of Gp1 mGlu receptors stimulates the synthesis of new proteins that participate in the regulation of AMPA receptor endocytosis and trafficking, thus are known as "LTD proteins". If protein synthesis is blocked during mGluR-dependent LTD then AMPA receptor endocytosis will proceed as normal, but AMPA receptors will eventually recycle back to the cell surface (Snyder et al. 2001; Waung et al. 2008). In the *Fmr1* KO mouse where FMRP is absent, mGluR-dependent LTD is independent of new protein synthesis (Nosyreva & Huber 2006; Figure 1.5). This suggests that the proteins required for the maintenance of this form of LTD may already be present at the synapse in *Fmr1* KO brains.

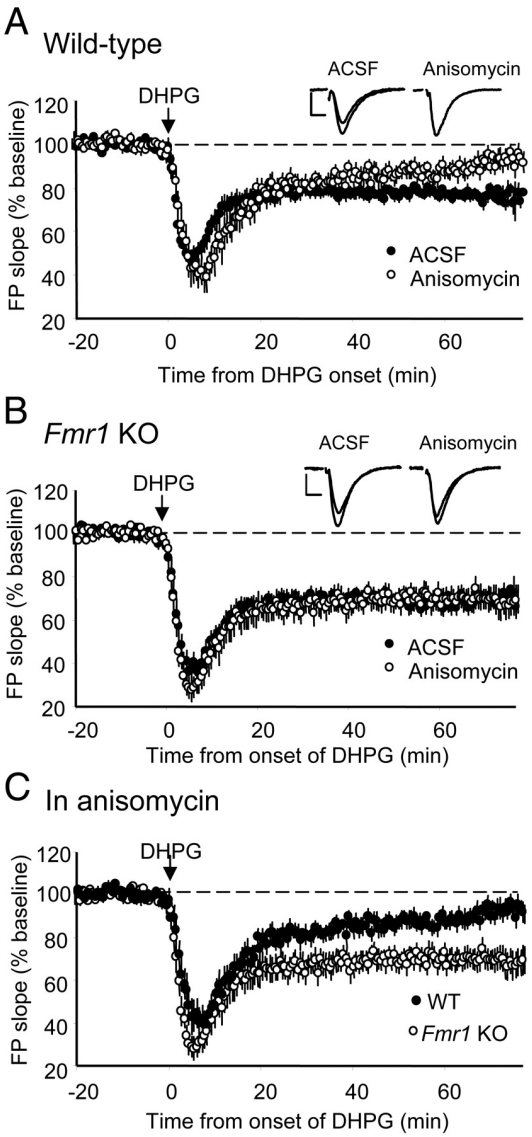


Figure 1.5 mGluR-dependent LTD is enhanced and independent of new protein synthesis in *Fmr1* KO mice. Chemical induction of metabotropic glutamate receptor-dependent long-term depression (mGluR-dependent LTD) in the presence of the protein synthesis inhibitor anisomycin in CA1 of hippocampal slices from *Fmr1* knockout (KO) and wild type (WT) controls. (A) In WT mice, anisomycin inhibits mGluR-dependent LTD induced by the Group 1 mGluR agonist R, S-dihydroxyphenylglycine (DHPG; 100 μM; 5 min). (B) In *Fmr1* KO mice mGluR-dependent LTD is insensitive to anisomycin. (C) Comparison of mGluR-dependent LTD in the presence of anisomycin in *Fmr1* KO and WT mice. Figure from Huber et al. 2002.

1.2.11 Basal protein synthesis is elevated in the *Fmr1* KO mouse

In the *Fmr1* KO brain direct measurements of basal protein synthesis both *in vitro* (Dölen et al. 2007; Osterweil et al. 2010) and *in vivo* (Qin et al. 2005a) reveal an increase in translational rates in the hippocampus and several other brain regions. Furthermore the activation of Gp1 mGlu receptors, under similar conditions that lead to elevated mGluR-dependent LTD in *Fmr1* KO mice, increased basal protein synthesis rates in WT mice but failed to further increase elevated protein synthesis rates in the *Fmr1* KO mice (Osterweil et al. 2010). This suggests that in the *Fmr1* KO hippocampus, mRNA translation is already saturated downstream of constitutive $mGlu_{1/5}$ receptor activation due to loss of FMRP translational repression.

The translation efficiency of several FMRP target mRNAs have been examined in polysomal fractions and hippocampal slice homogenates. Here PSD-95, GluA1 and GluA2 (AMPA receptor subunits) and CAMKII were found to be excessively translated in *Fmr1* KO preparations (Muddashetty et al. 2007; Osterweil et al. 2010). This indicates that FMRPs target mRNAs are abnormally translated under steady state conditions in the *Fmr1* KO brain, providing further evidence in support of FMRPs role in repressing mRNA translation. Thus it appears in the absence of FMRP there is exaggerated protein synthesis that consequently triggers excessive AMPA receptor internalisation upon activation of Gp1 mGlu receptors leading to the weakening of synaptic connections within the hippocampus.

1.2.12 The mGluR Theory of FXS

These findings that (1) Gp1 mGluR activation increases synthesis of FMRP, (2) mGluR-dependent LTD requires translation of pre-existing mRNA to be sustained, and (3) mGluR-dependent LTD is enhanced and independent of new protein synthesis led to the mGluR theory of FXS (Bear et al. 2004; Figure 1.6). This theory outlines how FMRP acts downstream of Gp1 mGlu receptors to halt the local mRNA translation of proteins required for mGluR-dependent LTD, and in the absence of FMRP, there is exaggerated protein synthesis that leads to excessive AMPA receptor

internalization and augmented LTD in response to mGluR-activation (Figure 1.6). Furthermore the model predicts that many of phenotypic features of FXS can be corrected by down regulation of Gp1 mGlu receptors. This was the first report to propose a targeted treatment to correct the core deficits that underlie the cognitive and behavioural abnormalities observed in the FXS.

In support of the mGluR theory of FXS, many of the prominent phenotypes associated with *Fmr1* KO mice can be rescued either genetically with the reduction of $mGlu_5$ receptor expression or pharmacologically by reducing $mGlu_5$ receptor signalling. The genetic reduction of $mGlu_5$ receptor expression in the *Fmr1* KO mouse corrected: increased spine morphology in L3 of the binocular visual cortex, elevated basal protein synthesis, exaggerated inhibitory avoidance extinction and an increased susceptibility AGS (Dölen et al. 2007). Whilst pharmacologically, the use of the negative allosteric modulators (NAMs) of Gp1 mGlu receptors such as 2-Methyl-6-(phenylethynyl)pyridine (MPEP), or 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine (CTEP) have been shown to rescue: increased prepulse inhibition, deficits in inhibitory avoidance and extinction, susceptibility to low-intensity auditory stimuli and AGS, hyperactivity in the open field, increased number of filopodia-like protrusions in hippocampal cultures and increased spine density in the binocular region of the visual cortex, enhanced hippocampal LTD, increased basal protein synthesis rates, and excessive internalization of AMPA receptors at basal state (Yan et al. 2005; Michalon et al. 2012; Michalon et al. 2014; de Vrij et al. 2008; Osterweil et al. 2010; Nakamoto et al. 2007). Importantly, many of these phenotypes were rescued in young adult mice indicating that therapeutics may still be beneficial to FXS patients when started in adolescence or adulthood. These studies have provided further evidence in support of the FMRP regulating mGluR-dependent protein synthesis and show that the dysregulation of this pathway underlies many of the core deficits associated with FXS.

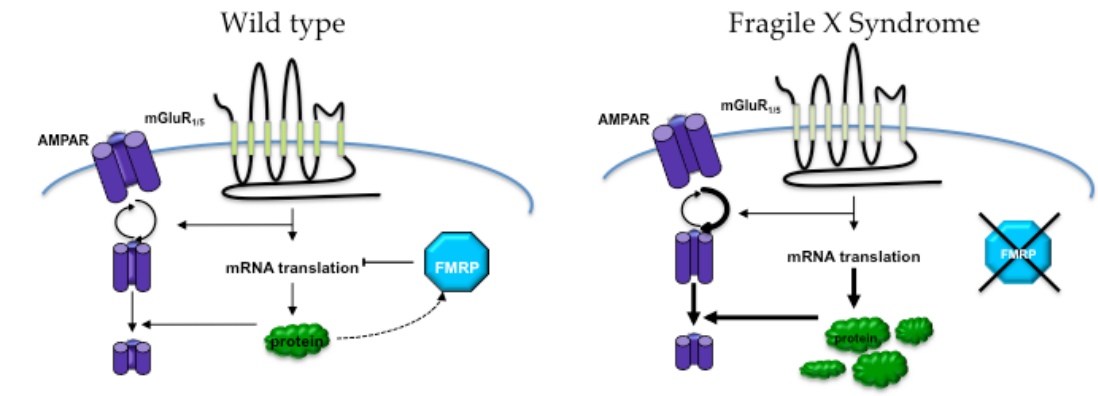


Figure 1.6 The mGluR theory of Fragile X Syndrome. (A) Model to explain exaggerated metabotropic glutamate receptor (mGluR) dependent long term depression (LTD) in CA1 of the hippocampus of *Fmr1* KO mice based on evidence that the fragile x mental retardation protein FMRP is rapidly synthesized in response to mGluR-activation and functions as a repressor of mRNA at local dendritic sites. (B) In the *Fmr1* KO mice where FMRP is absent, mRNA translational rates are increased leading to the excessive removal of AMPA receptors from the cell surface in response to an mGluR-LTD inducing stimulus.

1.2.13 $mGlu_5$ receptors couple to mRNA translational machinery

There are two key intracellular signalling pathways thought to couple $mGlu_{1/5}$ receptor activation to mRNA translation: firstly, the mammalian target of rapamycin (mTOR) pathway; and secondly, the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (Figure 1.7). Both ERK1/2 and mTOR can stimulate cap-dependent protein translation (translation of mRNA targets with 5' cap) by targeting the regulatory components of the initiation complex, primarily the eukaryotic translation initiation factor (EIF) 4E and the EIF4E binding protein (4E-BP) (Banko 2006; Ronesi & Huber 2008). The initiation step of mRNA translation begins once EIF4E recognises the 5' mRNA cap. This leads to the formation of the EIF4F complex, consisting of EIF4E, EIF4F and EIF4A, which once bound triggers the recruitment of the small ribosomal subunit (Richter & Sonenberg 2005). Furthermore both mTOR and ERK1/2 can stimulate 5'TOP mRNA translation (mRNAs containing a 5' oligopyrimidine tract) through phosphorylation of S6K1/2 and RSK, respectively, that increases the translational capacity of cell by synthesizing ribosomal subunits and translation factors (Antion et al. 2008a).

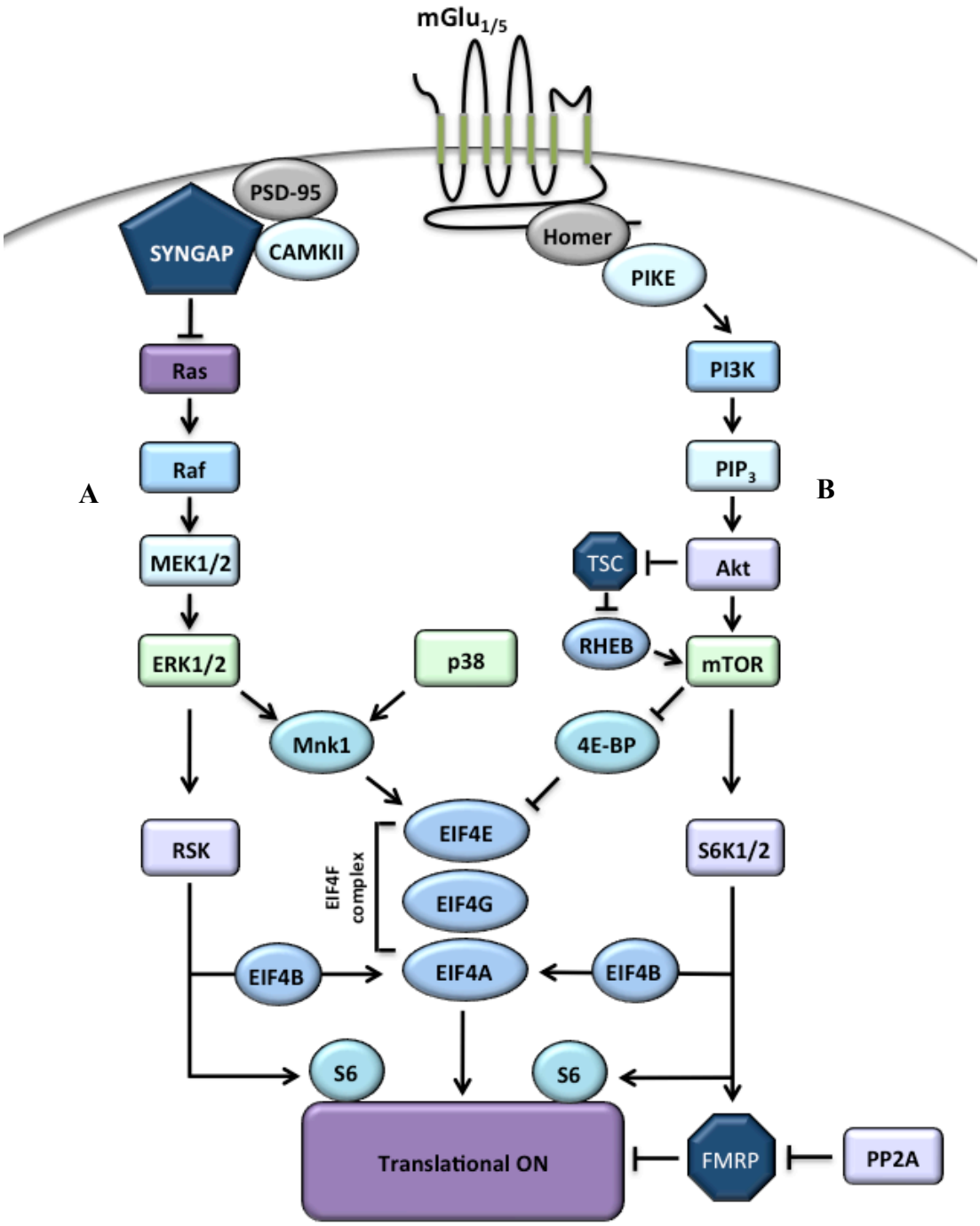


Figure 1.7 $mGlu_{1/5}$ receptor activation leads to local mRNA translation. Activation of $mGlu_{1/5}$ receptors stimulates protein synthesis via (A) the extracellular signal-regulated kinase (ERK) pathway, which in turn target multiple effectors to initiate mRNA translation and (B) the mammalian target of rapamycin (mTOR) signal pathways.

Mammalian target of rapamycin pathway

The $mGlu_5$ receptors couple to the mTOR signalling cascade through their association with Homer, a postsynaptic density scaffolding protein (Ronesi & Huber 2008). Homer links $mGlu_5$ receptors to PIKE, which binds and stimulates PI3K activity (Rong et al. 2003). PI3K phosphorylates PIP_2 converting it to PIP_3, which recruits Akt to the plasma membrane. PDK1 phosphorylates and activates Akt, which in turn phosphorylates and inhibits the TSC2 subunit of the tuberous sclerosis complex (TSC). TSC2 is a GAP protein, which hydrolyses active Rheb (GTP-bound) to its inactive form (GDP-bound). However in its active form, Rheb will proceed in activating mTOR, which can go on two form two complexes: mTORC1 or mTORC2.

The mTORC1 consists of mTOR in a complex with Raptor and LST8/GβL and is a target of rapamycin. The mTORC1 complex will bind and phosphorylate its target substrates, which include 4E-BPs, and p70S6 kinases (S6K1/2). Furthermore mTORC1 specifically regulates the translation of 5'TOP mRNAs by removing the TOP sequence of these mRNAs de-repressing translation of these transcripts (Antion et al. 2008a). The other mTOR complex, mTORC2 contains LST8/GβL and Rictor, which phosphorylates Akt, PKB and PKC (Costa-Mattioli et al. 2009; Figure 1.6). The PI3K-Akt-mTOR stimulates cap-dependent protein synthesis through the activation of S6K1/2, which phosphorylate ribosomal protein S6 leading to the activation of EIF4B. EIF4B potentiates EIF4A activity facilitating the formation of EIF4F (Shahbazian et al. 2006). Furthermore, in response to $mGlu_{1/5}$ activation mTOR signalling can facilitate protein synthesis by targeting 4E-BPs, increasing EIF4E availability allowing the formation the EIF4F assembly (Raught & Gingras 1999; Banko et al. 2006).

The role of mammalian target of rapamycin pathway in Fmr1 KO mice

In *Fmr1* KO mice the interaction between Homer and $mGlu_{1/5}$ is disrupted and stimulation of $mGlu_{1/5}$ receptors fail to activate mTOR signalling or initiate the translation of 5'TOP containing mRNAs (Ronesi & Huber 2008). In contrast, Sharma et al. (2010) find increased phosphorylation levels of mTOR and Akt under basal conditions in hippocampal lysates and PSD fractions from young and more mature adult *Fmr1* KO mice suggesting that PI3K-Akt-mTOR pathway is hyperactive in the absence of FMRP. Both Sharma et al. (2010) and Gross et al. (2010) also see increases in the activation state of Akt in cortical tissue from young adult mice and in cortical cultures, respectively. These discrepancies may result from differences in tissue preparation as Sharma et al. (2010) measured the protein phosphorylation levels in hippocampi that had been rapidly dissected whilst Ronesi et al. (2008) quantified protein expression levels in hippocampal slices that were left to recover for several hours in ACSF.

In the hippocampus the activation of PI3K-Akt-mTOR signalling in response to $mGlu_{1/5}$ activation is crucial for the stable expression of mGluR-dependent LTD (Hou & Klann 2004). Furthermore inhibition of mTOR signalling with rapamycin reduces basal protein synthesis in cortical synaptoneurosome preparations suggesting mTOR regulates basal levels mRNA translation (Muddashetty et al. 2007). Yet in *Fmr1* KO mice, mGluR-dependent LTD and elevated protein synthesis rates are insensitive to the mTOR inhibitor, rapamycin (Sharma et al. 2010; Osterweil et al. 2010). These findings could suggest that in the absence of FMRP this pathway is uncoupled from $mGlu_{1/5}$ receptor activation and so can no longer regulate synaptic strength (Sharma et al. 2010). Yet in WT hippocampal slices, Osterweil et al. (2010) did not observe any differences in the activation state of Akt or p70S6K (two readouts of mTOR activity), in response to $mGlu_{1/5}$ activation. This suggests that the PI3K-Akt-mTOR signalling cascade is not activated upon $mGlu_{1/5}$ receptor stimulation. These discrepancies could be due to differences in the brain region examined (cortical synaptoneurosomes *versus* hippocampal slices) and also preparation of experimental tissue.

ERK1/2-MAPK signalling pathway

In addition to the mTOR pathway, the ERK-MAPK signalling cascade is known to regulate cap-dependent protein synthesis (Banko et al. 2006). At the head of the ERK-MAPK signalling cascade is Ras, whose activity is regulated by GTPase activating proteins (GAPs), such as SynGAP and NF1. This leads to the sequential activation of proto-oncogene serine/threonine protein kinase Raf, mitogen/extracellular signal-regulated kinase (MEK1/2) and ERK1/2. Active ERK1/2 can stimulate cap-dependent translation via phosphorylation of RSK, which in turn activates S6 (Antion et al. 2008a). S6 phosphorylates EIF4b, which potentiates the activity of EIF4A increasing EIF4F complex formation. ERK1/2 can also initiate translation by activating the MAPK-interacting kinase (Mnk1), which also leads to the phosphorylation of eIF4E (Banko et al. 2006).

The role of the ERK1/2-MAPK signalling cascade in Fmr1 KO mice

In CA1 of the hippocampus, mGluR-dependent LTD is reliant on ERK-MAPK signalling (Gallagher et al. 2004). In the presence of 1,4-diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio) butadiene (U0126), an inhibitor of MEK1/2, mGluR-dependent LTD cannot be sustained (Gallagher et al. 2004). Furthermore elevated basal protein synthesis rates in the hippocampus of *Fmr1* KO mouse are selectively reduced to control levels by U0126 (Osterweil et al. 2010). In the same study it was found that ERK1/2 signalling was unaltered in *Fmr1* KO hippocampal slices and increased by a similar magnitude upon $mGlu_{1/5}$ receptor activation to WT controls. These findings suggest that the elevation in protein synthesis rates in the *Fmr1* KO hippocampus are not the result of hyperactivation of the ERK1/2 signalling but rather a hypersensitive response of the translational machinery to $mGlu_{1/5}$ receptor activation due to the loss of mRNA translation repression by FMRP (Osterweil et al. 2010). This could indicate that altered signalling in the *Fmr1* KO mouse is not the cause of augmented protein synthesis in the hippocampus but rather a consequence of the loss of FMRP. However several other studies have found steady-state levels of ERK1/2 signalling to be increased in the *Fmr1* KO brain and found mGluR-dependent LTD to be insensitive to inhibitors of this pathway (Hou et al. 2006; Kim et al. 2008).

1.2.14 Basal synaptic transmission in *Fmr1* KO mice

Examination of basal synaptic properties in *Fmr1* KO hippocampal neurones revealed smaller excitatory postsynaptic currents (EPSCs) with no significant difference in mEPSC frequency (Braun & Segal 2000; Pfeiffer & Huber 2006). Furthermore there is a reduction in AMPA/NMDA receptor ratio at P14, which disappears by 6-7 weeks of age in *Fmr1* KO mice (Pilpel et al. 2009). However, a separate study found no difference in AMPA/NMDA receptor ratios at the same time point (Hu et al. 2008). Together these findings suggest that during early development there may be delayed synapse maturation in *Fmr1* KO mice that is mediated by postsynaptic disruptions that lead to reduced excitatory drive.

1.2.15 Hyperexcitability in the hippocampus of *Fmr1* KO mice

Intracellular recordings of CA3 pyramidal neurons in *Fmr1* KO hippocampal slices have revealed epileptiform bursting in response to spontaneous synaptic activity (Osterweil et al. 2013; Zhao et al. 2004). These ictal-like responses progresses to prolonged synchronized discharges and are comparable to those observed during epileptic seizures. Typically prolonged discharges are only observed in response the activation of Gp1 mGlu receptors and are dependent on ERK1/2 activation and mRNA translation (Chuang et al. 2005; Zhao et al. 2004). Likewise inhibition of either protein synthesis or ERK1/2 activity prevents the expression of synchronized bursting in CA3 pyramidal neurones (Osterweil et al. 2013; Chuang et al. 2005; Zhao et al. 2004). Thus epileptiform bursting in CA3 pyramidal neurons of *Fmr1* KO slice resembles that observed when DHPG is applied to WT slices, indicating that in *Fmr1* KO slices there is an exaggerated response downstream of $mGlu_{1/5}$-ERK1/2 signalling leading to epileptogenesis in the FXS brain.

In agreement, inhibitors of Gp1 mGlu receptors (e.g. MPEP) or Ras-ERK1/2 signalling (e.g. lovastatin, UO126) abolish the persistence of epileptiform bursting in *Fmr1* KO slices (Osterweil et al. 2013; Zhao et al. 2004). This indicates that $mGlu_{1/5}$ receptor responses are exaggerated in the absence of FMRP due to the loss of

repression of mRNA translation. This is consistent with the finding that MPEP and lovastatin lower seizure susceptibility in *Fmr1* KO mice (Osterweil et al. 2010; Osterweil et al. 2013). Thus together these findings suggest that FMRP plays a role in regulating neuronal excitability at the synapse.

1.2.16 Passive and active membrane properties in *Fmr1* KO mice

Intrinsic properties, such as resting membrane potential ($V_{m)}$, series resistance and rheobase, have also been examined in hippocampal slices and there appears to be no change in intrinsic postsynaptic excitability in *Fmr1* KO mice (Pilpel et al. 2009). Similarly Brager et al. (2012) found no differences in V_m, input resistance (R_N), and membrane time constant (τ_m) in somatic recordings from CA1 of the hippocampus. However dendritic recordings found V_m to be significantly more depolarised, R_N was lower and τ_m was faster.

These findings were consistent with an increase in I_h (the current of h-channels), which was due to elevated proteins levels of h-channel subunits HCN1, but not HCN2, in the distal dendritic segments of CA1 field of *Fmr1* KO mice (Brager et al. 2012). Between the soma and dendrites of CA1 pyramidal neurones HCN channels are expressed in a gradient like manner with HCN expression increasing as you move away from the soma. This HCN gradient is important for regulating the propagation of signals between the soma and the dendrites. This increase in I_h in *Fmr1* KO dendrites could reduce dendritic excitation, which could be a compensatory effect due to excessive synaptic excitation within this region caused by the loss of FMRP. It will also cause an increase in membrane excitability by depolarising R_N and increasing the rebound potential that follows a hyperpolarisation, which will increase the probability of spike initiation.

1.2.17 Treatment Strategies for FXS

Collectively, these findings demonstrate how the *Fmr1* KO mouse has been a tremendously valuable tool in identifying the role of FMRP at the synapse and the mechanisms in the FXS brain that underlie the learning and behavioural abnormalities in the absence of FMRP. This has led to the identification of potential therapeutic targets and the development of pharmacotherapies that seek to correct (1) altered $mGlu_5$ receptor signalling, (2) enhanced protein synthesis rates, (3) overabundant LTD proteins, or (4) increased hyperexcitability in brain networks.

As previously discussed NAMs of the $mGlu_5$ receptor, which correct excessive protein synthesis rates, also rescue an extensive number of FXS phenotypes including abnormalities in behavioural, synaptic plasticity and spine morphology (Dölen et al. 2007; Michalon et al. 2012; Michalon et al. 2014; Osterweil et al. 2010). Two compounds which downregulate $mGlu_5$ receptor signalling, fenobam (Berry-Kravis 2014) and AFQ056 (Chelly & Mandel 2001), have now progressed into clinical trials. Similarly compounds that target downstream components of $mGlu_{1/5}$ receptor signalling have also shown therapeutic potential. In the *Fmr1* KO mouse, chronic treatment with lithium restored: exaggerated mGluR-dependent LTD (Chelly et al. 2006), increased protein synthesis rates (Leonard & Wen 2002), increased susceptibility to AGS (Min et al. 2009), and behavioural deficits including social behaviours (Mines et al. 2010).

Minocycline is an antibiotic that targets the matrix metalloproteinase 9 (MMP-9), a protein overexpressed in *Fmr1* KOs that acts downstream of $mGlu_{1/5}$ receptor and is involved in the elongation of dendritic spines (Michaluk et al. 2011). Minocycline has been shown to correct hippocampal spine abnormalities and improve behavioural outcomes of *Fmr1* KO mice in the elevated plus maze and Y-maze task (Bilousova et al. 2009) with several reports of its use in human patients (Leigh et al. 2013; Paribello et al. 2010; Utari et al. 2010). Another approach to counteract the hyperexcitability of excitatory strength in these mutants has been to enhance inhibitory function with GABA receptor agonists, such as STX209. In the *Fmr1* KO mouse, STX209 reduces the increased susceptibility to AGS (Pacey et al. 2009) and

also corrects elevated protein synthesis rates and increased spine density in the cortex (Henderson et al. 2012).

1.3 Insights Other Monogenic Causes of ID

In addition to the mouse model of FXS, investigations in several other models of ID have contributed significantly to our understanding of the underlying brain pathophysiology associated with ID/ASD. From these studies it appears that many of these ID gene mutations disrupt dendritic synapse function, which contribute significantly to the impaired cognition and behaviour associated with ID. Interestingly, there are reports that specific cell types in the mammalian brain are responsible for distinct cognitive and behavioural deficits in models of ID. Furthermore biochemical analysis of these mutants has discovered novel therapeutic interventions, which have been validated in preclinical models and in some cases found to be beneficial in human patients. Moreover, several compounds have proved successful in improving cognitive function in other monogenic causes of ID indicating that there may a common pathophysiological axis that underlies ID. Finally studies that have utilised gene reactivation or pharmacological interventions have shown considerable improvements in core symptoms even when treatment begins in adulthood raising the intriguing possibility that these neurodevelopmental disorders may not lead to a permanent and irreversibly mental disability (Zoghbi & Bear 2012).

1.3.1 The mouse model of Neurofibromatosis 1

Studies in the mouse model of Neurofibromatosis 1 (NF1) have revealed that reduced levels of neurofibromin (a Ras GAP) result in a hyperactivity of Ras-ERK1/2 signalling. *NF1* heterozygous mice exhibit memory impairments in the Morris water (Silva et al. 1997) and hippocampal LTP (Costa et al. 2002), consistent with NF1 patients who display deficits in executive functions. Interestingly, both

impairments in LTP and cognition in the *NF1* heterozygous mutant are rescued by reducing Ras activity, either genetically or pharmacologically (Costa et al. 2002). *NF1* heterozygous mice treated with lovastatin, an inhibitor of the HMG-CoA reductase enzyme that reduces Ras-ERK1/2 activity, no longer display deficits in LTP or spatial learning (Li et al. 2005). Furthermore, lovastatin has been shown to the effective in human NF1 patients (Mainberger et al. 2013).

Recently this therapeutic strategy was examined in *Fmr1* KO mice to determine whether lovastatin could correct increased translation rates downstream of $mGlu_5$-ERK1/2 signalling. Lovastatin modestly reduces ERK-MAPK activation by preventing the posttranslational farnesylation of Ras, preventing Ras translocating to the plasma membrane where it is functional. In *Fmr1* KO mice, the acute application of lovastatin to hippocampal slices rescued elevated protein synthesis rates, enhanced mGluR-dependent LTD and epileptiform bursting in CA3 pyramidal neurones (Osterweil et al. 2013). Furthermore lovastatin administered orally or by injection reduced seizure susceptibility *in vivo* (Osterweil et al. 2013).

The possibility of using lovastatin as a treatment in FXS patients and other disorders that arise from altered Ras-ERK1/2 signalling has a lot of therapeutic potential because it is already approved for treatment of hypercholesterolemia in both children and adults and has a well-known safety profile due to its widespread use (Costa et al. 2002; Osterweil et al. 2013). It would now be advantageous to see whether lovastatin can rescue additional neurocognitive phenotypes that are reported in *Fmr1* KO mouse to see whether it can be effective in the treatment of a broader spectrum of phenotypes.

1.3.2 The mouse model of Tuberous Sclerosis

Recent studies in the mouse model of tuberous sclerosis complex (TSC) have proposed a role by which mTOR signalling may also disrupt protein synthesis levels and impact synaptic strength. Like FXS and NF1, TSC is a monogenic cause of ID caused by the loss of either the *TSC1* or *TSC2* gene, which together form the TSC complex. As mentioned above, TSC represses mTOR signalling and so in its

absence, mTOR activity is excessive (Ehninger et al. 2008). Although TSC mutants share similar cognitive and behavioural abnormalities to *Fmr1* KO mice, surprisingly, examination of basal protein synthesis rates and mGluR-dependent LTD in *TSC* mutants revealed they were impaired (Auerbach et al. 2011; Chévere-Torres et al. 2012), whilst late-phase LTP was enhanced (Ehninger et al. 2008). Thus, *TSC* mutants appear to have opposing phenotypes to those observed in the *Fmr1* KO mice.

Furthermore the cognitive deficits reported in the *TSC* mutants could be corrected by positive allosteric modulators (PAM)s of $mGlu_5$ receptors, inhibitors of mTOR or by crossing *TSC* mutants with an *Fmr1* heterozygous mouse (Ehninger et al. 2008; Auerbach et al. 2011). Together this data has led to the proposal of a model by which ERK1/2 and mTOR regulate the translation of distinct pools of mRNAs that can repress one another by competing for access to the same translational machinery (Bhakar et al. 2012). Furthermore it highlights the need to for a reliable biomarker for disorder in order to identify the most effective pharmacological strategy.

1.3.3 The mouse model of Rett Syndrome

In addition to pharmacologically interventions correcting dysfunctional signalling in mouse models of ID, other groups have taken a genetic approach to see whether the reintroduction of the mutant gene can reverse cognitive deficits associated with ID and ASD. Phenotypic rescue was successively shown in a mouse model of Rett syndrome (Guy et al. 2007), a disorder predominantly caused by *de novo* mutations in the *MECP2* gene (Amir et al. 1999). The *MECP2* gene encodes the MECP2 is one of a small group of methylated DNA-binding proteins that can act as transcriptional repressors (Klose & Bird 2006). Within the brain MECP2 is ubiquitously expressed and most in abundance in mature neurones.

Like FXS, Rett syndrome is X-linked so boys with an MECP2 mutation are far more severely affected than girls, presenting with infantile encephalopathy and dying shortly after birth. Females who have two X chromosomes and therefore one

functional copy of *MECP2* express this protein in a mosaic fashion due to random X-chromosome inactivation. This means that only a proportion of cells will express the MeCP2 protein. Girls with Rett syndrome will develop symptoms between 6-18 months of age after a period of normal development but then quickly regress and show a slowed brain and head growth, ataxia, seizures, cognitive deficits and severe NSID (Ropers & Hamel 2005). In girls, Rett syndrome is the leading cause of severe mental retardation (Cobb et al. 2010). Furthermore approximately 70% of Rett syndrome patients exhibit seizures and nearly all exhibit abnormal EEG patterns (Glaze et al. 1998).

The *Mecp2* null mutants develop motor impairments, tremor, breathing abnormalities and limb stereotypies dying at 10-20 weeks of age whilst heterozygous females have a normal lifespan, displaying a delayed set of symptoms that progressively worsen in severity but eventually stabilise after several months (Guy et al. 2001). Guy et al. (2007) generated a conditional KO where endogenous *Mecp2* was silenced by the insertion of a lox stop cassette that could subsequently be deleted upon tamoxifen injections leading to the reactivation of *Mecp2*. The reintroduction of MeCP2 in *Mecp2* null male mutants had an astonishing effect on reversal of phenotypes, reducing postnatal lethality and correcting neurological symptoms. Similarly in *Mecp2* heterozygous females, activation of MeCP2 expression in adulthood reversed neurological symptoms and synaptic plasticity deficits in L-LTP. These findings indicate that the deficits caused by the loss of MeCP2 are reversible in adulthood.

The deletion of *Mecp2* in specific cells or regions can reproduce some of the phenotypes observed in Rett syndrome. For example loss of Mecp2 postnatally in excitatory neurones of the forebrain led to motor impairments, increased anxiety and impaired fear conditioning and social behaviour (Gemelli et al. 2006). Rett syndrome phenotypes have also been observed when *Mecp2* is deleted specifically in dopaminergic neurones or when lost in the amygdala (Fyffe et al. 2008; Samaco et al. 2009). In a separate study the specific restoration of gene function in only glia

cells in global Mecp2 deficient mice rescued locomotion and anxiety levels, restored breathing abnormalities and prolonged lifespan (Lioy et al. 2011). These findings highlight the role of specific cell types/regions play in certain behaviours and phenotypes and may mean subtle manipulations of specific circuits could be promising for therapeutic intervention.

1.3.4 Mutations in ID-related genes define a pathological axis

Based on these findings it is now clear that mutations in ID genes define an axis of synaptic pathophysiology. These studies have highlighted that protein synthesis rates must be tightly regulated to maintain at an optimum level of mRNA translation otherwise the repercussions are extensive. Altered protein synthesis rates in the ID brain can impact intrinsic properties of individual neurones, destabilise excitatory and inhibitory networks and causes aberrant synaptic plasticity mechanisms (Banko et al. 2006; Ronesi & Huber 2008; Auerbach et al. 2011; Osterweil et al. 2010). Deviations in protein synthesis rates, in either direction, disrupt synaptic function leading to similar cognitive impairments (Figure 1.8). Depending on which end of the spectrum the mutation lies will determine its phenotypic outcome (i.e. enhanced/impaired LTD) and treatment strategy (NAM/PAM of $mGlu_5$ receptor; inhibitors of ERK1/2 or mTOR).

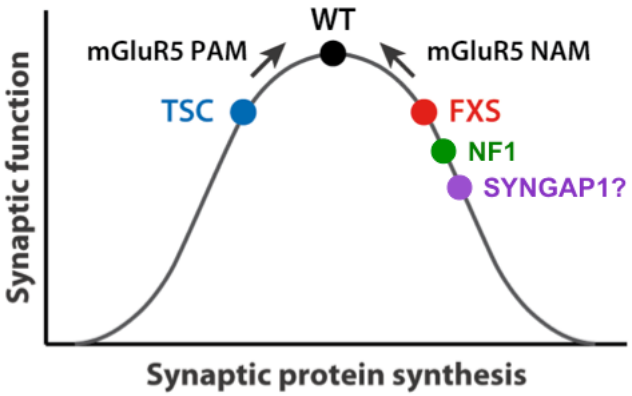


Figure 1.8 The pathophysiological axis of intellectual disability. Monogenic causes of intellectual disability (ID) appear to require an optimum level of protein synthesis for proper synaptic function. Mutations in ID-related genes that lead to increased protein synthesis rates, enhanced mGluR-dependent long-term depression (LTD) and impaired long-term potentiation (LTP), include fragile x syndrome (FXS), Neurofibromatosis 1 (NF1) and *SYNGAP1* haploinsufficiency may be amenable to negative allosteric modulators (NAM) of $mGlu_5$ receptors or inhibitors of the Ras-ERK1/2. On the opposite end of the spectrum is Tuberous Sclerosis complex (TSC) caused by a mutation in either *TSC1* or *2*, which leads to opposing phenotypes including reduced protein synthesis rates, impaired mGluR-dependent LTD and enhanced LTP that can be corrected to wild type (WT) levels with positive allosteric modulators of the $mGlu_5$ receptor. Image adapted from (Auerbach et al. 2011).

1.3.5 Convergence of proteins encoded by ID-related genes

The pathophysiological axis of ID helps explain why genetically distinct causes of ID share significant phenotypic overlap. For example there are now an accumulating number of reports that link genetic mutations in signalling molecules, which cause dysregulated Ras signalling known as 'Rasopathies' to a group of neurodevelopmental disorders with cognitive impairments, that resemble ID (Cesarini et al. 2009). These mutations are found in proteins upstream (i.e. PTPN1, SOS1, NF1, SYNGAP) and downstream of Ras signalling (i.e. Ras, BRAF, MEK1/2, FMRP). The role this pathway plays in the pathology of various genetic causes of ID have been confirmed in the mouse models of FXS and NF1, with a subset of phenotypes rescued by downregulation of the Ras-ERK1/2-MAPK signalling pathway (Osterweil et al. 2010; Li et al. 2005; Osterweil et al. 2013). In fact, the mRNA of many of the Ras related genes listed are targets of FMRP suggesting there be an indirect as well as direct interaction between these ID-related proteins (Darnell et al. 2011). Based on these findings, we have begun investigating another upstream regulator of Ras signalling – SynGAP.

1.4 Haploinsufficiency of *SYNGAP1*

Like NF1, SynGAP is a Ras GAP protein, binding members of the Ras family of G-proteins and downregulating their activity at the synapse. Here SynGAP relays glutamatergic receptor activation to intracellular signalling events that are important for cognitive processes, such as synaptic plasticity and spine morphogenesis. In recent years a number of rare *de novo* mutations in *SYNGAP1* have been identified in patients with NSID that are predicted to lead to the loss of one functional copy of *SYNGAP1*. With the availability of the mouse of model of *Syngap1* haploinsufficiency - the *Syngap* heterozygous mouse - we are now able to validate this mutant as an animal model of ID and see how closely it mimics the human condition.

Furthermore we can test whether there is phenotypic overlap with other known genetic causes of ID that result from aberrant Ras signalling. There are already encouraging findings reported in literature from investigations in the *Syngap* heterozygous mice, such as elevated ERK1/2 activity in the hippocampus and behavioural abnormalities that are consistent with ID. This evidence already hints at the suggestion there may convergence between *SYNGAP1*, *FMR1* and *NF1*. Here it will be discussed in detail the involvement of SynGAP in the physiology and pathology of learning and memory.

1.4.1 Haploinsufficiency of *SYNGAP1* is linked to intellectual disability

Mutations in the *SYNGAP1* gene have only recently been linked to a non-syndromic form of ID where the only clinical manifestation in these patients is cognitive impairment and behavioural abnormalities. Although, there are a minority of reported cases of *SYNGAP1* haploinsufficiency, small screens of NSID patients (<100 patients) have revealed that the prevalence of *SYNGAP1* mutations could be as high as 4%, which when extrapolated to the ID population could make it one of the most common causes of NSID (Hamdan et al. 2011). Furthermore, affected individuals also commonly display childhood seizures and ASD. This has fuelled

further interest in the function of this protein at the synapse, and the consequence reduced SYNGAP expression has on cellular and behaviourally processes that underlie ID.

1.4.2 Autosomal dominant *de novo* mutations in *SYNGAP1* are deleterious

Hamdan et al. (2009) was the first to identify rare *de novo* mutations in *SYNGAP1* in patients with NSID and proposed that they were likely to be pathogenic because the truncated versions of SYNGAP lacked key functional domains known to regulate synaptic plasticity mechanisms and spinogenesis. This initial study involved 94 patients with NSID (mixed ethnicity and gender) who underwent *SYNGAP1* sequence analysis, for which three point mutations (K138X, R579X and L813RFSX22) were identified. Two patients were heterozygous for nonsense mutation, K138X and R579X. The third patient was heterozygous for mutation L813RFSX22, predicted to result in a frame shift mutation that introduced a premutation stop codon. Interestingly, all mutations were absent from parental DNA samples indicating they were *de novo* mutations. All three patients presented with a similar clinical profile; a delay was observed early on in development, moderate to severe ID and language impairment. In 1 of the 3 patients a strabismus was found whilst 2 out of 3 were mildly epileptic. In both patients with a co-diagnosis of epilepsy, seizures were well controlled by valproate (reduces expression of GluA1 containing AMPA receptors at the synapse) or topiramate (inhibits AMPA receptor activity). Only one of the three mutations was found in the RasGAP domain (R579X), whilst K138X and L813RFSX22 were located in the N-terminal and SH3 domains, respectively (Figure 1.8).

Since this initial study further mutations in the *SYNGAP1* gene have been identified in patients with moderate to severe ID (Figure 1.9). These include: deletion mutations at 6p21.3 encompassing *SYNGAP1* (Writzl & Knegt 2013; Krepischi et al. 2010; Zollino et al. 2011; Berryer et al. 2012); a balanced translocation between chromosome 6 and 22, that led to the truncation of the gene (Klitten et al. 2011); frameshift mutations (Vissers et al. 2010; Hamdan et al. 2011), a single base

duplication that introduced a premature stop codon (Berryer et al. 2012); and missense mutations (Berryer et al. 2012). These mutations are predicted to result in the full truncation of the protein, leaving only one functional copy of the *SYNGAP1* gene. Where paternal and maternal testing took place, no mutations were found in *SYNGAP1* in parental DNA indicating that the majority of cases arise from heterozygous *de novo* mutations in *SYNGAP1*. The only exception was in one patient with a base duplication in *SYNGAP1*, whose father was also found to carry this mutation but in a mosaic state (Berryer et al. 2012).

Mutations in the *SYNGAP1* gene can vary in the type, location, and functional outcome: missense mutations affect stability and function; truncating mutations at the start of the protein abolish production; whilst truncating mutations in the RasGAP domain affect its activity (Berryer et al. 2012). All truncating mutations abolish the C-terminal domains that are required for SYNGAP to interact with components of the PSD complex. This indicates that pathogenic mutations of *SYNGAP1* result in a loss of function.

1.4.3 Symptoms of *SYNGAP1* haploinsufficiency

Clinical examination of these patients has unearthed a spectrum of symptoms that vary in severity. Behaviourally, it is widely reported that all patients appear to present with a psychomotor delay early on in development and language is moderately to severely impaired. One patient with a novel deletion in 6p21.3 presented with connective tissue abnormalities, similar to what is observed in FXS patients, and was classified as hyperactive with attention deficit (Krepischi et al. 2010). Zollino et al. (2011) reported a patient with severe mental retardation, poor social interaction, speech was nearly absent and seizures began which were continually difficult to manage. Autism has also been reported in several patients (Berryer et al. 2012), and autistic-like behaviours was reported in a patient with a balanced translocation mutation (Klitten et al. 2011), and another with reductions in *SYNGAP1* copy number (Pinto et al. 2010).

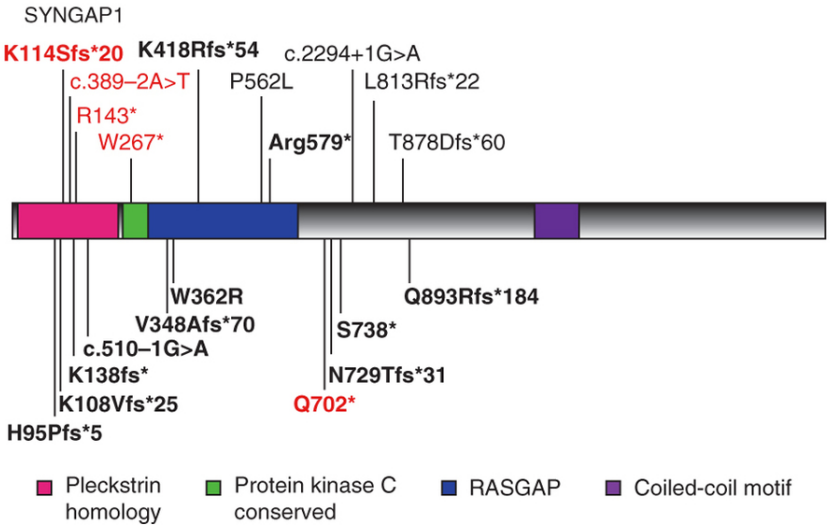


Figure 1.9 Mutations in SYNGAP1 are linked to non-syndromic intellectual disability. The *SYNGAP1* gene encodes the synaptic GTPase activating protein (SYNGAP). SYNGAP contains several functional domains including: pleckstin homology domain, C2 domain, RASGAP domain and a coiled-coil motif. Above depicts localisation of *de novo SYNGAP1* mutations identified in patients with nonsyndromic intellectual disability. Figure from (Carvill et al. 2013).

1.4.4 *SYNGAP1* Haploinsufficiency and Epilepsy

The recent identification of ID patients with mutations in the *SYNGAP1* gene has also revealed that epilepsy is a frequent comorbidity (Hamdan et al. 2009; Pinto et al. 2010; Writzl & Knegt 2013; Zollino et al. 2011; Berryer et al. 2012; Klitten et al. 2011). Furthermore *SYNGAP1* mutations have also been linked to a form of epileptic encephalopathy, an extremely debilitating group of epilepsies characterized by refractory seizures and cognitive arrest that carry a poor prognosis (Carvill et al. 2013).

Epileptic seizures in *SYNGAP1* haploinsufficiency occur early on during childhood with the age of onset varying from 3 months to 4 years of age, affecting both males and females. All *SYNGAP1* haploinsufficiency patients present with generalized seizures either clonic-tonic, absence or myoclonic seizure, with one report of febrile seizures (Hamdan et al. 2011). The frequency of seizures can be high, with one patient reported to have up to 100 seizures per day prior to treatment (Writzl & Knegt 2013). To assess the incidence of epilepsy in patients with *SYNGAP1* haploinsufficiency patients, greater cohorts of *SYNGAP1* haploinsufficiency patients are required. However, together these findings suggest that *SYNGAP1* may play a role in the pathogenesis of generalized epilepsy.

Abnormal EEG patterns are observed in all patients with *SYNGAP1* haploinsufficiency, which are predominantly characterized by generalized spike waves or bioccipital spikes. It would also be interesting to see if abnormal EEG patterns extend to *SYNGAP1* haploinsufficiency patients that do not present with seizures to see whether it may be a biomarker for the disorder. Following treatment with anticonvulsants, such as topiramate and valproic acid, the majority of patients become seizure free or are well managed (Hamdan et al. 2009; Vissers et al. 2010; Hamdan et al. 2011; Writzl & Knegt 2013; Berryer et al. 2012; Zollino et al. 2011). There were three cases where seizures were not well controlled, with a relapse of seizures (Klitten et al. 2011; Berryer et al. 2012) indicating that the degree of severity varies considerably amongst patients. Thus it appears that both the *FMR1* and *SYNGAP1* gene can influence seizure susceptibility, with the greatest risk presenting during childhood.

1.4.5 The *SYNGAP1* gene

Prior to any disease association, SynGAP was originally discovered in 1998 by two independent laboratories through the screening and sequencing PSD-95 binding proteins (Kim et al. 1998; Chen et al. 1998). Both groups cloned *Syngap* cDNA, later identified as *Syngap α1* and began characterising its properties. The human gene that encodes SYNGAP, *SYNGAP1*, is located on chromosome 6 (6p21.3) and encodes a protein consisting of 1343 amino acids with a molecular weight of ~140kDa. Western blot analysis, using an antibody against the C-terminal tail, revealed a doublet/triplet band, which indicates that multiple SynGAP isoforms may arise from alternative splicing of the 5' end (Kim et al. 1998). In fact, both the N- and C-terminal domains can both differ amongst SynGAP isoforms. Four distinct N-terminal isoforms of SynGAP have been identified, denoted as A, B, C and E, which arise from alternative transcription start sites (Li et al. 2001). The C-terminus of SynGAP can also vary as a result of alternative splicing of *Syngap* mRNA, giving rise to SynGAP isoforms α1, α2, β1, β2, β3, β4, and γ.

1.4.6 SynGAP protein structure

Analysis of the amino acid structure revealed that SynGAP contains several functional domains (Figure 1.1). Each isoform contains a highly conserved central GAP domain that activates the intrinsic GTPase activity of Ras (Chen et al. 1998), and is crucial for regulation of synaptic transmission (Rumbaugh et al. 2006). Similarly, a calcium/lipid binding (C2) domain are common to all isoforms and serves as Ca^{2+} dependent membrane binding domain (Chen et al. 1998). SynGAP A, B and E (but not SynGAP C) contain unique peptide sequences with a complete pleckstin homology (PH) domain for phospholipid binding, which may be important for membrane trafficking and protein-protein interactions (Li et al. 2001). Furthermore the presence of the C2 and PH domains suggests that SynGAP may respond to changes in intracellular Ca^{2+} and phospholipid second messengers, such as PIP_2 and PIP_3.

Alternative splicing of *Syngap* mRNA leads to multiple the C-terminal isoforms for which there are at least seven variants (α1, α2, β1, β2, β3, β4, and γ) encoding five different protein isoforms all of which have unique C-terminal tails (Li et al. 2001). The C-terminus contains several phosphorylation sites, which are targets of CAMKII and protein tyrosine kinases (H. Chen et al. 1998). There appears to be 10 sequential histidine residues that may serve as binding sites for divalent metal ions, such as Zn^{2+} and Cu^{2+}. Furthermore there is a proline-rich region between positions 770 and 800 that is highlighted in the Uniprot database as being a potential SH3 binding domain, which would likely serve as a protein-protein interaction domain.

SynGAP α1 contains a PDZ binding domain (QTRV) in its C-terminal tail enabling it to bind to scaffolding proteins, such as PSD-95, SAP102 (synapse associated protein 102) and MUPP1 (Chen et al. 1998; Kim et al. 1998; Li et al. 2001; Krapivinsky et al. 2004). Deletion of the C-terminus of SynGAP, specifically the QTRV motif, prevents binding of SynGAP to the PDZ domains of PSD-95 and SAP102 (Kim et al. 1998). In contrast, SynGAP α2 lacks a PDZ binding domain and instead has a unique 48 amino acid sequence at the C-terminus (McMahon et al. 2012).

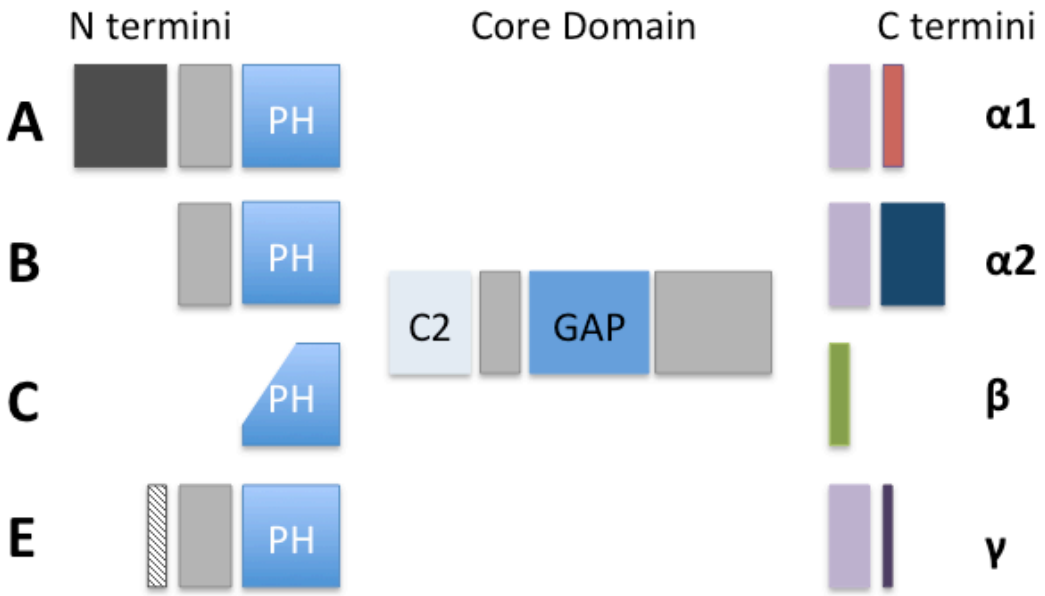


Figure 1.10 The protein domains of SynGAP isoforms. The central domain consists of pleckstin homology (PH), a calcium/lipid binding (C2) domain and a conserved GTPase activating protein (GAP) domain. N-terminal isoforms include A, B, C and E, which arise from different transcription sites, whilst different C-terminal isoforms arise from alternative splicing of *Syngap* mRNA.

1.4.7 SynGAP localisation

All SynGAP isoforms are specifically expressed in the brain (Li et al. 2001), predominantly in the cortex, hippocampus and olfactory bulb with lower expression detected in the cerebellum, brainstem, thalamus and brain stem (Kim et al. 1998). SynGAP expression shows dynamic changes throughout development with mRNA levels continually increasing in the first postnatal weeks, peaking at P14 before declining into adulthood (McMahon et al. 2012; Clement et al. 2012). Immunoblots of rat cortical homogenates and cytosolic and membrane fractions revealed that SynGAP α1 is membrane associated and found solely in excitatory neurones where it displays a punctate-like expression (Kim et al. 1998). In contrast, SynGAP β is detected in both excitatory and inhibitory neurones (Moon et al. 2008). In the hippocampus, the highest level of immunoreactivity for both SynGAP α1 and β is detected in the dendritic fields of CA1-3 and in the molecular layer of the dentate gyrus (Moon et al. 2008). Western blot analysis of subcellular fractions from rat forebrains revealed that SynGAP is highly enriched in the PSD, where it co-localises with GluN2B subunit of the NMDA receptor and scaffolding proteins PSD-95 and SAP102 supporting the notion that SynGAP is located within the NMDA receptor complex (Chen et al. 1998).

The expression of γ variant is markedly reduced compared to the α and β forms. SynGAP β expression is restricted to the PSD, whilst SynGAP α1 is detected in both the PSD and other subcellular locations (Li et al. 2001). This is surprising considering only SynGAP α1 possesses the QTRV motif required for binding to PSD-95, which is thought to anchor SynGAP α1 within the PSD. Yet SynGAP β, which lacks a PDZ binding domain, is isolated to the PSD fraction suggesting it may have a binding partner that localises it to the PSD. Interestingly, Vazquez et al. (2004) demonstrated that mutation of the QTRV motif (ΔSXV) in the C-terminus of SynGAP did not alter the targeting of recombinant SynGAP to the synapse suggesting PSD-95 binding is not crucial for targeting SynGAP to spines.

There is evidence to suggest that SynGAP localisation at the PSD is regulated in an activity-dependent manner. In hippocampal neuronal cultures, depolarisation of cells with either high extracellular K^+ or NMDA, led to the redistribution of SynGAP away from the postsynaptic membrane to the cytosolic side of the PSD, coincidently in the same general location where CAMKII is concentrated (Yang et al. 2011). Yang et al. (2011) proposes that upon depolarisation, the movement of SynGAP away from the PSD may free up the PDZ domain of PSD-95 to other proteins, for example AMPA receptors that bind to PSD-95 indirectly via transmembrane AMPA receptor regulatory protein (TARP)s.

1.4.8 SynGAP interactions

SynGAP α1 interacts with scaffolding proteins at the PSD

SynGAP α1 co-immunoprecipitates with PSD-95 and SAP102, two PSD scaffolding proteins that are highly abundant at the synapse (Kim et al. 1998). Furthermore, the carboxyl terminus containing the QTRV motif strongly interacts with PSD-95 in a yeast two-hybrid assay (Chen et al. 1998). Deletion analysis of the C-terminus of SynGAP α1 revealed that the QTRV sequence is crucial for SynGAP binding to the PDZ domain of PSD-95 and SAP102 (Kim et al. 1998). At the PSD it is likely that SynGAP α1 is in a complex with PSD-95 and SAP102 so is ideally positioned to regulate Ras dependent signalling upon synaptic stimulation.

SynGAP interacts with MUPP1

SynGAP has been shown to interact with MUPP1 a large scaffolding protein, which has multiple PDZ domains. MUPP1 forms part of the NMDA receptor signalling complex at excitatory synapses and allows CAMKII to indirectly interact with SynGAP α1 (Krapivinsky et al. 2004). The interaction between MUPP1 and SynGAP can be partially disrupted by truncation of the PDZ recognition motif. However, disruption of MUPP1 interaction with SynGAP leads to the dephosphorylation of SynGAP, inactivation of p38 MAP kinase and an increase the

frequency and amplitude of AMPA receptor mediated mEPSCs (Krapivinsky et al. 2004; Rama et al. 2008).

SynGAP β interacts with CAMKII

SynGAP β, which lacks the PDZ binding domain directly binds to the α subunit of CAMKII, which is thought to localise SynGAP β to the PSD fraction (Li et al. 2001). The interaction between SynGAP β and CAMKII is lost upon autophosphorylation of the α subunit of CAMKII. This could indicate that the interaction between these two proteins is regulated in an activity-dependent manner.

SynGAP interacts with the GluN2B subunit of NMDA receptors

SynGAP associates with the GluN2B subunit of NMDA receptors through its association with PSD-95 (Kim et al. 1998). In turn PSD-95 interacts with the PDZ-binding motif (ESDV) located on the C-terminal tail of the GluN2B subunit. Stimulation of cultured neurones with NMDA results in an increase in SynGAP phosphorylation at specific serine residues indicating that SynGAP function is regulated in an activity-dependent manner. Similar to *Syngap* KD neurones, GluN2B mutants show enhanced AMPA receptor-mediated synaptic transmission caused by an increase in AMPA receptor insertion that is dependent on protein synthesis (Wang et al. 2013). This indicates that both GluN2B and SynGAP negatively regulate excitatory synapse strength. Furthermore, genetic knockout of GluN2B leads to increased protein synthesis rates, which can be corrected, along with increased mEPSC amplitude, with the MEK1/2/ERK1/2 inhibitor U0126 (Wang et al. 2013). If *Syngap* was knocked down in GluN2B neurones, this did not further increase mEPSC amplitude, whilst overexpression of SynGAP corrected enhanced mEPSC amplitude. Thus SynGAP acts downstream of GluN2B and they both regulate the same intracellular pathways. This evidence suggests that positive modulation of the GluN2B-containing NMDA receptors could be a potential therapeutic target for downregulating hyperactive pathways that are present in *Syngap* heterozygous mice.

SynGAP interacts with Unc51.1 and 51.2

SynGAP has been shown to play a role in the early stages of cerebellar granule cell axon formation. Unc51.1 and Unc51.2 are serine/threonine kinases that are localized to axonal shafts and growth cones where they play a role in axon elongation. A yeast two-hybrid screen, prepared from P6 mouse cerebellum revealed that the C terminal tail of Unc51.1 and 51.2 interact with both SynGAP α1 and α2, showing higher affinity for α2 isoform (Tomoda et al. 2004). SynGAP protein levels were detected from as early as E16 and levels continued to increase in the cerebellum peaking at P20. In granule cell cultures, SynGAP α2 was localized to extending axons whilst α1 was found only at the cell soma. Overexpression of SynGAP in granule cell cultures led to axon truncation, which could be either fully or partially restored with co-expression of Ras or Unc51.1 respectively.

Unc51.1 suppresses the GAP activity of SynGAP, upregulating both Ras and Rab5 activity, through which it exerts control of axon formation (Tomoda et al. 2004). Rab5 is a member of the Ras-like small GTPases, localized to synaptic vesicles, which is essential for endocytic membrane fission and trafficking necessary for axon formation. Overexpression of SynGAP leads to abnormal vesicular structures within extending axons of granule cells. Thus SynGAP may be regulating axon formation through two pathways: Rab5 for organisation of membrane vesicles and Ras for long-term maintenance of gene expression.

1.4.9 SynGAP regulates Ras family of G-proteins

SynGAP is highly enriched at the PSD, forming part of the NMDA receptor complex, where it couples NMDA receptor activation to downstream Ras mediated signalling cascades (Chen et al. 1998). SynGAP contains 29 consensus sites (RXXS/T) for phosphorylation by CAMKII and it has been demonstrated *in vitro* that CAMKII regulates the activity of SynGAP (Oh et al. 2004). Initially, it was observed that when endogenous CAMKII activity is increased by pre-phosphorylation in isolated PSDs, GAP activity was inhibited by 80-93%. This turned out to be an artefact from residual adenosine triphosphate (ATP) and the

presence of pyrophosphatase in the Ras GAP assay (Chen et al. 1998). In fact, Oh et al. (2004) went on to show that when CAMKII phosphorylates SynGAP, its GAP activity increases by 70-95%. Phosphorylation of SynGAP by CAMKII is reversible and occurs at multiple sites.

In WT cortical neurones, transfection of constitutively active CAMKII (T286D) leads to a reduction in AMPA receptor-mediated mEPSC amplitude. In the absence of SynGAP, mEPSC amplitude is elevated and cannot be corrected by transfection of constitutively active CAMKII (T286D) indicating that CAMKII lies upstream of SynGAP and can only regulate AMPA receptor mediated synaptic transmission when SynGAP is present (Wang et al. 2013). CAMKII in turn is activated by either Ca^{2+} entry in to the synapse predominantly via NMDA receptor activation or through a Ca^{2+}-independent mechanism that results from the autophosphorylation of CAMKII at T286 (Coultrap & Bayer 2012).

SynGAP in its activated form specifically binds and inactivates members of the Ras family of small G-proteins, which include Ras, Rap1, Rap2 and Rab5 (Ye & Carew 2010; Tomoda et al. 2004; Figure 1.11). Although, SynGAP specifically activates both Ras and Rap, an *in vitro* study found that SynGAP preferentially activates Rap GTPases, stimulating Rap GTPase activity more potently than Ras GTPase activity (Krapivinsky et al. 2004). Ras G-proteins converge on to the multiple downstream signalling cascades including the ERK1/2-MAPK, PI3K and p38-MAPK pathways, which mediate the phosphorylation of synaptic plasticity proteins as well as their local transcription, translation and trafficking at dendritic spines (Stornetta & Zhu 2011). Ras mediated pathways play a crucial role in the expression of certain forms of synaptic plasticity by mediating the trafficking of AMPA receptors that lead to changes in synaptic strength (Stornetta & Zhu 2011). Overexpression of SynGAP in neuronal cultures decreases the number of AMPA receptors at the synapse (Rumbaugh et al. 2006), whilst *Syngap* heterozygote and KO cortical cultures show an increase in the number of AMPA receptor clusters at the synapse and fewer morphological silent synapses than their WT counterparts (Kim et al. 2003).

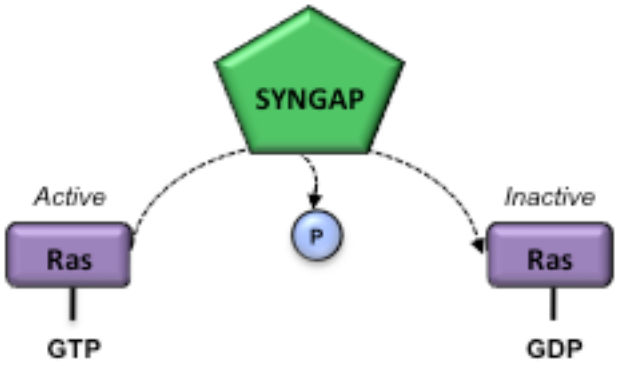


Figure 1.11 SynGAP binding Ras family of G proteins. The synaptic GTPase activating protein (SynGAP) bind GTP-bound Ras (active) family members (Ras, Rap1 and Rap2) and convert these small G proteins to their GDP-bound (inactive) form.

1.4.10 SynGAP isoforms: Expression and function

SynGAP isoform expression is regulated in activity-dependent, which in turn has differential effects on synaptic strength depending on which combinations of N- and C-terminal isoforms are expressed (Figure 1.12). Stimulation of cortical mouse cultures changes the relative abundance of N- terminal SynGAP isoforms without changing total mRNA levels (McMahon et al. 2012). Both *Syngap* B and C mRNA are upregulated, whilst *Syngap* A is down regulated. These activity-dependent changes in *Syngap* N-terminal isoform mRNA levels are abolished by inhibition of network activity with TTX. In contrast, increased network activity has no effect on protein levels of C-terminal isoforms α1 and α2 (McMahon et al. 2012). However it is still unknown what combinations of SynGAP isoforms exist in the mammalian brain and how activity-dependent changes in SynGAP N- terminal isoforms arise.

The functional consequences of the differential expression of SynGAP isoforms were examined in forebrain neurones transfected with plasmids containing combinations of N- and C- terminal isoforms. Examination of mEPSCs in transfected forebrain cultures revealed that expression of N-terminal isoforms SynGAP A, B or C with the α1 C-terminal tail led an increase in the number of silent synapses, whilst expression of SynGAP A or B isoforms with α2 had no effect on the proportion of silent synapses (McMahon et al. 2012). The functional properties of mEPSCs were assessed in terms of mean mEPSC amplitude and frequency. In SynGAPAα1 the mean mEPSC amplitude, indicative of postsynaptic receptor density, is decreased whilst being elevated in Bα2 and Cα2 neurones, with no change detected in SynGAPAα2 expressing neurones. The mEPSC event frequency, that reflects presynaptic release probability, is also influenced by SynGAP isoform expression. In neurones transfected with SynGAPAα1, event frequency was drastically reduced compared to WT neurones, whilst SynGAPBα2 expression cells displayed an increase in mEPSC frequency. Neurones expressing SynGAPAα2 had comparable mEPSC values to control cells. Thus, it appears that the distinct combinations of N- and C- terminal domains determine the functional effects of SynGAP at excitatory synapses. Whether these different isoform combinations preferentially bind to different Ras family members requires further investigation but could provide further

insight in to how SynGAP is able to regulate synaptic strength in opposing directions.

1.4.11 Mouse model of *Syngap1* haploinsufficiency

The *Syngap* heterozygous mouse was first generated by Kim et al. (2003) by insertion of a neomycin cassette into the site of exon 7 and 8 of the *Syngap1* gene that resulted in the deletion of the region encoding the C2 domain of SynGAP protein. This insertion led to the generation of transcripts with premature stop codons. Since then, several groups have generated their own versions via the insertion of a neomycin cassette in to the *Syngap1* allele. Vazquez et al. (2004) generated a *Syngap* heterozygous mouse by inserting a neomycin cassette flanked by two lox P sites into intron 3 of the *Syngap1* gene with an additional lox P site at intron 9. This meant that upon exposure to cre recombinase exons 4-9 were deleted, which encoded the PH and C2 domain of the SynGAP protein. Komiyama et al. (2002) used a similar approach except the neomycin cassette was inserted in to a region that resulted in the deletion of the region encoding C2 and GAP domain. All transgenic lines generated mutants that had only one functional *Syngap1* allele. Thus *Syngap* heterozygous neurons displayed approximately half as much SynGAP protein expression, whilst full knockouts produce no detectable levels of SynGAP protein (Vazquez et al. 2004). Clement et al. (2012) developed a conditional KO SynGAP transgenic mouse where SynGAP expression could be lost in temporal manner. In addition this mouse line was engineered so that SynGAP could be conditionally rescued at a later developmental time point, correcting reduced SynGAP expression to WT levels.

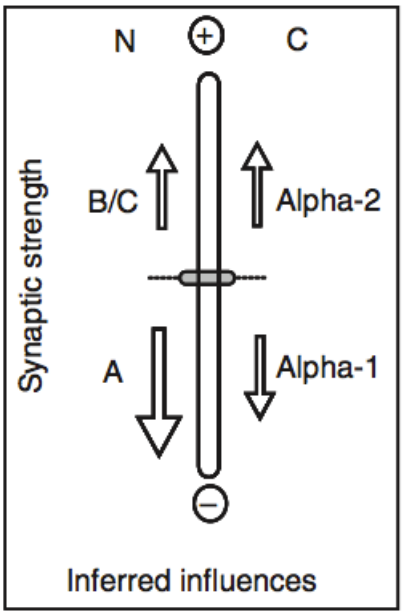


Figure 1.12 SynGAP N- and C-termini isoforms determine synaptic strength. Schematic illustrating the functional consequences of SynGAP isoform expression on synaptic strength based on recorded miniature excitatory postsynaptic currents (mEPSC) in forebrain cultures. Overexpression of N-terminal isoform Syngap A and C-terminal isoform α1 reduce synaptic strength, with Syngap A having greater influence on synaptic strength than any other isoform. In contrast expression of N-terminal isoform B and C and C-terminal isoform α2 increase synaptic strength. Figure from McMahon et al. 2012.

1.4.12 Behavioural phenotypes in *Syngap* mutant mice

Initial observations of *Syngap* KO mice reported no gross anatomical abnormalities and pups appeared to feed and breathe normally. However, the majority of pups died between P2-7, indicating that SynGAP expression is crucial for postnatal viability (Komiyama et al. 2002). Although it remains unclear why *Syngap* KO mice die, there is evidence that suggests *Syngap* null mice have enhanced cortical apoptosis (Knuesel et al. 2005). In stark contrast *Syngap* heterozygote mice, which have a 50% reduction in SynGAP protein expression levels, appear to develop normally and are viable and fertile. They display no sign of seizures, tremor, ataxia or other neurological abnormalities (Komiyama et al. 2002). There also appears to be normal development of tissues and organs, similar to WT mice. This might not be surprising considering SynGAP expression is isolated to the brain, however no gross anatomical abnormalities were detected in the brain either at P5 (Kim et al. 2003).

Behavioural studies of *Syngap* heterozygous mice have reported a multitude of deficits indicating that SynGAP expression is crucial for the normal development of human cognition. One of the most robust behavioural phenotypes to be observed in *Syngap* heterozygous mice is increased locomotive activity in the open field at both juvenile and adult ages (Guo et al. 2009; Clement et al. 2012; Muhia et al. 2010). This is accompanied by increased exploratory behaviour of the central zone compared to WT counterparts that remain close to the periphery during exploration. This suggests *Syngap* heterozygous mice are more hyperactive than WT counterparts and may have abnormal anxiety levels. In the elevated plus maze, spontaneous anxiety-like behaviour was assessed by the amount of time spent in the open arm relative to the closed arm. *Syngap* heterozygote mice spend more time in the open arm, indicating a reduced anxiety level compared to WT mice (Muhia et al. 2010). Although contextual fear conditioning appeared normal in *Syngap* heterozygote mice, cued fear conditioning was impaired with *Syngap* heterozygotes displaying significantly less freezing upon receiving an audio cue, which had been previously paired a with a foot shock (Muhia et al. 2010).

In the Morris water maze *Syngap* heterozygous mice display a slower rate of learning, initially swimming much longer path lengths to reach a submerged platform relative to their WT counterparts (Muhia et al. 2010; Komiyama et al. 2002). They also display mild deficits in spatial memory when the platform is moved from its pre-learnt location. Working and reference memory were also examined in the radial arm maze and found to be significantly impaired in *Syngap* heterozygote mice (Muhia et al. 2010).

With the exception of thermal nociception, *Syngap* heterozygous mice appear to have enhanced responses to sensory stimuli *in vivo*. *Syngap* heterozygote mice display a reduced seizure threshold upon exposure to audiogenic and fluorothyl-induced seizures, enhanced acoustic startle and reduced prepulse inhibition (Clement et al. 2012). Electroencephalography (EEG) recordings in *Syngap* mutants revealed frequent and widespread cortical epileptiform discharges that were absent in WT littermates (Ozkan et al. 2014). Furthermore it is observed *in vitro*, using laser photolysis of caged glutamate paired with fast-voltage-sensitive dye imaging, that signal propagation through the hippocampus and cortex is dramatically amplified in brain slices prepared from neonatal and adult *Syngap* heterozygous mice, respectively (Clement et al. 2012) (Ozkan et al. 2014). Muhia et al. (2010) found a significant reduction in calbindin-positive inhibitory interneurons in the hippocampus that may contribute to network instability in a brain region that is most vulnerable to epileptic activity. This data suggests that there is disrupted processing of auditory information leading to hypersensitive responses to auditory stimuli as well as alterations in sensory-motor gating in the *Syngap* heterozygote brain and could explain the increased incidence of epilepsy in patients with only one functional copy of *SYNGAP1*.

In terms of sociability, when *Syngap* heterozygote mice were presented with an unfamiliar mouse there sociality scored normally. Yet if *Syngap* heterozygotes had to discriminate between a familiar or novel mouse they did not show a preference, unlike WT mice. This indicates that *Syngap* heterozygote mice are unable to discriminate a conspecific in a social setting that is required for an organized social

hierarchy amongst a group of animals. Furthermore, if they were given the choice between socialising with another mouse or isolation, *Syngap* heterozygotes showed a preference for isolation compared to WT siblings suggesting reduced social interaction (Guo et al. 2009).

Thus, it appears that *Syngap* heterozygote mice have deficits in working and reference memory, along with increased hyperactivity, abnormal anxiety levels and impairments in social recognition. This validates the *Syngap* heterozygous mouse as a robust model of *SYNGAP1* haploinsufficiency recapitulating the neuropathophysiology associated with the human condition. Many of the behavioural phenotypes described above overlap with those reported in the mouse model of FXS. This suggests that the consequence of either loss of FMRP or reduced SynGAP expression levels result in disruptions of similar neural circuits, most notably in the amygdala and hippocampus. Furthermore, many of these abnormal behaviours, including increased susceptibility to AGS, spatial learning, response inhibition and temporal discrimination, are linked to altered glutamatergic transmission, which may make them amenable to therapeutic interventions.

A recent study investigated which distinct cellular populations may contribute to behavioural abnormalities observed in the *Syngap* heterozygous mice (Ozkan et al. 2014). Here they crossed a conditional *Syngap* KO line with a mouse driver line that would specifically induce cre-mediated recombination of *Syngap1* gene in forebrain glutamatergic neurones and glia within regions that included the frontal cortex and hippocampus. Disruption of *Syngap1* in forebrain pyramidal cells was sufficient to induce similar behavioural deficits to those reported in the conventional *Syngap* heterozygous mice, which included increased time spent in the open arm of the elevated plus maze, hyperactivity in the open field, impairments in spontaneous alternation test, reduced freezing during contextual fear conditioning and an increased susceptibility to flurothyl-induced seizures (Ozkan et al. 2014). These findings suggest that *Syngap1* haploinsufficiency in forebrain glutamatergic neurons and glia are specifically responsible for certain cognitive deficits observed in the *Syngap* heterozygous mouse.

Efforts have been taken to see whether behavioural and cognitive impairments in *Syngap* heterozygous mice can be rescued by the conditional reversal of *Syngap1* haploinsufficiency. Initially a similar approach was taken to that reported by Guy et al. (2010) who successively reversed the deficits reported in Rett Syndrome. Here a mouse line was engineered with a lox P sites on exon 5/6 and 7/8 of the *Syngap1* gene, which was crossed with a hemizygous inducible and ubiquitously-expression Cre-ERt2 driver line that was effective in rescuing gene expression in adult mice (Guy et al., 2007). Prior to genetic reversal animals were put through a battery of behavioural tasks at 8 weeks, administered tamoxifen for 5 consecutive days and then retested 1 month later in the behavioural battery following genetic reversal. However, both *Syngap* heterozygous mice with and without genetic reversal displayed increased time in the open arm of the elevated plus maze, increased activity in the open field, and a lack of spontaneous alternation in the T-maze (Clement et al., 2012). Thus reintroduction of *Syngap* expression in adulthood did not rescue basic working memory in *Syngap* heterozygous mice, like it did in the mouse model of Rett syndrome, suggesting that *Syngap1* haploinsufficiency is disorder of development.

This suggests that loss of SynGAP is particularly damaging to the developmental brain and may mean there is a more limited time window for therapeutic intervention. Unlike Mecp2 whose expression is preferentially abundant in mature neurones (Shahbazian et al. 2002), *Syngap* mRNA and protein expression peaks early on in postnatal development (P14) (Clement et al. 2012). Thus perhaps the reversal of *Syngap1* haploinsufficiency at over 8 weeks of age was too late when the majority of behavioural and synaptic plasticity deficits have manifested and led to secondary consequences.

The region-specific deletion of *Syngap1* using a conditional KO crossed with an EMX1-ire-Cre driver mice, which induces Cre-mediated recombination in forebrain glutamatergic neurones, lead to a reduction of SynGAP expression in the prefrontal cortex and hippocampus, without a detectable difference in SynGAP expression in the striatum. These mice displayed similar behavioural deficits to those reported in

the conventional *Syngap* heterozygous mice, which included increased time spent in the open arm of the elevated plus maze, hyperactivity in the open field, impairments in spontaneous alternation test, reduced percentage freezing during contextual fear conditioning and an increased susceptibility to audiogenic seizures (Ozkan et al. 2014). Interestingly, when the mutation was specifically reversed in the glutamatergic neurones and glia in the forebrain all behavioural deficits were prevented with the exception of hyperactive in the open field and increased susceptibility to flurothyl-induced seizures indicating that brain regions outwith of the forebrain may be contributing to these abnormalities (Ozkan et al. 2014).

1.4.13 Spine phenotypes in *Syngap* heterozygous mice

SynGAP expression levels increase during the first two weeks of postnatal life peaking at P14, which coincides with synaptogenesis (Clement et al. 2012). Examination of spine development in hippocampal cultures at DIV 10 revealed that *Syngap* KO pyramidal neurones had a subtle increase in the number of protrusions, which was absent in more mature cultures (DIV 21) (Vazquez et al. 2004). Unlike WT neurones that displayed thinner elongated spines, in *Syngap* KO neurones the head width and area of protrusions were markedly increased. This increase in spine head size was also observed when more mature *Syngap* KO neurones were examined (DIV 21), indicating that abnormalities in spine morphology persist in to adulthood. Similarly, Carlisle et al. (2008) found that adult hippocampal neurones from *Syngap* heterozygous mice had more mushroom-shaped spines with a larger head volume and length relative to WT neurones. The abnormal spine morphology Vazquez et al. (2004) reported in *Syngap* KO neurones at DIV 10 could be reversed towards that of WT neurones by the transient expression of SynGAP.

In *Syngap* KO neurones, the increased dendritic spine size was accompanied with larger clusters of synaptic proteins including PSD-95, GluA1 and an GluN1 (Vazquez et al. 2004). Although the number of clusters was similar between genotypes, the proximity of these clusters to the head of the spine was abnormal in *Syngap* KO neurones. In WT neurones, protein clusters were smaller and localised to the dendritic shafts whilst in *Syngap* KO neurones they were localised in the tips of spines. The composition of these clusters was also altered, with more *Syngap* KO protrusions containing AMPA receptors and NMDA receptors, as opposed to just NMDA receptors, indicating that the proportion of silent synapses in *Syngap* KO neurones are far fewer at DIV 10 than in WT neurones.

Clement et al. (2012) investigated dendritic spine size, density and dynamics in granule cells of the dentate gyrus. At P9, *Syngap* heterozygote granule cells had similar spine head widths, however by P14 head diameter was significantly larger and this phenotype persisted in to adulthood. This, in turn, shifted the morphological classification towards more mushroom-like spines with fewer stubby spines by P14. However, overall spine density was unaltered in *Syngap* mutants *versus* WT mice. Examination of dendritic branches revealed that dendritic arborisations were unaltered in *Syngap* heterozygote mice but overall spatial volume occupied by these arborisations was decreased relative to WT counterparts (Clement et al. 2012). At P14, spine motility was reduced to an adult-like rate in *Syngap* mutants relative to WT mice. The replication of spine phenotype in slice provides further support for the SynGAP playing a key role in the maturation rate of dendritic spines in multiple brain regions perturbing the enlargement of spines during early development.

Since spine structure is regulated by actin dynamics that is in turn is regulated by cofilin signalling (Gu et al. 2010). Basal levels of phosphorylated cofilin were examined in both hippocampal and forebrain homogenates from young and adult *Syngap* heterozygous mice, respectively (Carlisle et al. 2008; Clement et al. 2012). Both groups reported increased phosphorylation of cofilin at steady-state levels. This is thought to be mediated by aberrant Ras signalling, which consequently effects Rac downstream signalling (Carlisle et al. 2008). Rac phosphorylates and inactivates cofilin leading to an increase in actin polymerization thought to underlie exaggerated spine growth.

In the hippocampus, the loss of FMRP or reduced expression of SynGAP appears to impact spine development. In both mutants, spine morphology was abnormal in CA1 pyramidal neurones at both young and adult ages. Data from the *Syngap* heterozygous mutants at P14 Clement et al. (2012) agrees with those findings reported by Grossman et al. (2010) in *Fmr1* KOs at a similar time point. However, Levanga et al. (2011) reported that the increase in spine head width in *Fmr1* KO mice did not persist in to adulthood. They observed a shift towards a greater number of elongated protrusions in the hippocampus of adult *Fmr1* KO mice. However, these findings are not consistently reported in *Fmr1* KO mice making it difficult to draw comparisons.

1.4.14 *Syngap* heterozygous mice display incomplete cellular segregation in the somatosensory cortex

The role of SynGAP in the development and lamination of the cortex was assessed by Barnett et al. (2006). Here it was found that there is complete loss of cellular segregation into barrels in layer 4 of the primary somatosensory cortex in *Syngap* KO mice. Examination of these afferents in *Syngap* KO brains revealed that they terminate and segregate in to rows in layer 4 correctly but do not segregate into a barrel-like pattern. This incomplete segregation of TCAs was also observed in the barreloids of thalamus but not in the barrelettes of the brainstem. In *Syngap* heterozygotes, normal segregation of TCAs into whisker-related patches is observed, yet there was significant reduction in barrel segregation, with a reduction in the ratio of cells in the barrel wall relative to barrel hollow.

1.4.15 Synaptic transmission in the hippocampus of *Syngap* heterozygous mice

During development SynGAP plays a significant role in regulating glutamatergic signalling at the synapse. A recent study by Clement et al. (2012) investigated basal synaptic transmission in the medial perforant pathway and Schaffer collateral pathway of the hippocampus at various time points to elucidate whether basal synaptic activity of AMPA receptors in *Syngap* heterozygous mice is altered. Based on the I/O relationship they found synaptic transmission to be normal at very early ages (P9) but increased in *Syngap* heterozygous mice by P14. This increase in I/O function was accompanied by an increase in the ratio of AMPA/NMDA receptor currents in DGNs, the result of increased incorporation of AMPA receptors. By P21 synaptic transmission in WT reached the same level observed *Syngap* heterozygous slices. This is in agreement with other reports that find basal synaptic transmission in the hippocampus of adult heterozygous mice is intact (Komiyama et al. 2002; Kim et al. 2003). Thus, SynGAP might regulate a ‘critical period’ of synapse maturation and the reduction of SynGAP results in a premature acceleration of synaptic strength during early development.

In contrast, the I/O relationship in layer2/3 of the medial prefrontal cortex was absent at young ages but increased in adult *Syngap* heterozygous acute slices, where mEPSC frequency and amplitude were also elevated (Ozkan et al. 2014). In the same study a reduction in miniature inhibitory postsynaptic currents (mIPSCs) and a reduced firing rate in parvalbumin positive inhibitory neurones at 6 weeks of age, was observed in medial prefrontal slices from *Syngap* heterozygous mice. Genetic reversal of *Syngap* haploinsufficiency failed to restore elevated synaptic strength in L2/3 of the medial prefrontal cortex in *Syngap* heterozygous mutants (Ozkan et al. 2014). The observed imbalance in excitation to inhibition (E/I) ratio in adult *Syngap* heterozygous mice is also reported in the mouse model of NF1 (Cui et al. 2008).

These findings suggest that during development, reduced SynGAP expression increases E/I ratio in different cell types and time points, which underlies the hyperexcitability phenotypes reported in the hippocampus and prefrontal cortex at the same developmental time points in *Syngap* heterozygous brain (Clement et al. 2012; Ozkan et al. 2014). This increase in excitatory drive from granule cells and pyramidal neurons of CA1 and layer2/3 of the medial prefrontal cortex will likely disrupt local synaptic circuits, which is supported by amplified signal propagation that is observed in these regions (Clement et al. 2012; Ozkan et al. 2014).

The relationship between loss/reduced levels of SynGAP and synaptic activity of AMPA receptor has also been examined in neuronal cultures. Analysis of mEPSCs revealed that AMPA receptor mediated synaptic transmission was potentiated in *Syngap* KO and KD (KD of *Syngap* α) hippocampal neurones relative to WT counterparts (Wang et al. 2013; Rumbaugh et al. 2006). The potentiation of mEPSCs resulted from an increase in the frequency of events with no change in the amplitude (Rumbaugh et al. 2006), the latter partially contradicting other studies which have shown an increase in mEPSC amplitude as well (Vazquez et al. 2004; Clement et al. 2012). In contrast, Wang et al. (2013) observed an increase in mEPSC amplitude not in frequency in cortical neurones with KD levels of SynGAP. The same group went on to show that the increase in mEPSC amplitude was the result of enhanced incorporation of GluA2-containing AMPA receptors indicating that under basal

conditions SynGAP represses the insertion of Ca^{2+} permeable AMPA receptors. The potentiation of mEPSCs amplitudes could be corrected by either the expression of WT SynGAP in KO/KD neurones, or by the inhibition of protein synthesis, mTOR or ERK1/2 (Wang et al. 2013).

1.4.16 LTP is impaired in the hippocampus of *Syngap* heterozygous mice

Electrophysiological recordings in hippocampal CA1 have revealed that LTP, induced by a variety of stimulation paradigms, is consistently impaired in *Syngap* heterozygous mice (Komiyama et al. 2002; Kim et al. 2003; Ozkan et al. 2014). There was significantly less potentiation observed in *Syngap* mutants relative to WT slices, upon application of HFS (2 × trains of 100 Hz and 6 × trains of 100 Hz) or theta-burst stimulation to the Schaffer collateral pathway. This LTP deficit occurred in the absence of any alterations in basal synaptic transmission, paired pulse ratios or NMDA receptor mediated synaptic currents and could be restored to WT levels by the genetic reversal of *Syngap* haploinsufficiency (Komiyama et al. 2002; Ozkan et al. 2014). This finding suggests that basal synaptic activity of AMPA receptors in *Syngap* mutants is normal, however their recruitment to the membrane surface upon LTP-inducing stimuli is defective.

Examination of downstream signalling pathways involved in LTP expression following theta burst stimulation revealed that both Ras-GAP and ERK1/2 activity levels were dramatically increased in WT slices. In contrast, no significant increases in the activation state of Ras-GAP and ERK1/2 were observed in *Syngap* mutant hippocampal slices (Ozkan et al. 2014), however basal activity levels of Ras-GAP and ERK1/2 were already significantly elevated suggesting that this pathway is saturated in the hippocampus of *Syngap* heterozygous mice. Genetic reversal of *Syngap* haploinsufficiency normalised increased levels of basal Ras and ERK1/2 activity (Ozkan et al. 2014).

1.4.17 NMDAR-dependent LTD in the hippocampus of *Syngap* heterozygous mice

The role of SynGAP in NMDAR-dependent forms of LTD has been examined in hippocampal CA1 and appears specific to certain LTD stimulation protocols. LTD induced by paired pulse low frequency stimulation (PP-LFS) appears intact whilst LTD induced by the acute application of NMDA (5 min in presence of high Ca^{2+}) is significantly impaired (Carlisle et al. 2008; Kim et al. 2003). Although PP-LFS is normally considered a Gp1 mGluR-dependent form of LTD, experimental conditions were manipulated so that only NMDA receptors would be activated (ACSF contained the AMPA receptor inhibitor NBQX and no Mg^{2+}).

1.4.18 Basal protein synthesis Rates are elevated in the cortex of *Syngap* heterozygous mice

In *Syngap* KD cortical cultures, basal levels of protein synthesis were assessed using fluorescent non-canonical amino acid tagging (FUNCAT) (Wang et al. 2013). This technique involves replacing endogenous methionine with azidohomoalanine (AHA), which is incorporated into newly translated proteins in methionine's place. In *Syngap* KD neurones there was an increased AHA signal in dendrites suggesting that during basal conditions SynGAP suppresses mRNA translation at local synaptic sites. The introduction of WT *Syngap* in KD neurones, rescued enhanced protein synthesis rates, however overexpression of *Syngap* in WT neurones did not suppress protein synthesis rates further. Similarly, enhanced protein synthesis rates in *Syngap* KD neurones could be corrected by either mTOR or ERK1/2 inhibition suggesting that increased translational rates lie downstream of ERK1/2 and mTOR signalling.

1.4.19 Downstream signalling cascades regulated by SynGAP

SynGAP negatively regulates the activity of Ras family members, which include Ras, Rap1 and Rap2. These small G-proteins cycle between an active (GTP-bound) and inactive (GDP-bound) state regulated by guanine exchange factors (GEF) and GAP proteins. GAP proteins, such as SynGAP, convert active Ras in to its inactive form by enhancing the rate of GTP hydrolysis. This prevents further activation of downstream targets of Ras signalling, which include the ERK1/2-MAPK, PI3K-Akt-mTOR and P38-MAPK pathways. Ras-mediated signalling plays a crucial role in regulating protein synthesis rates and the expression of certain forms of synaptic plasticity (Osterweil et al. 2010; Hou & Klann 2004; Gallagher et al. 2004; Sharma et al. 2010). Thus not surprisingly, in the *Syngap* heterozygous brain Ras activity is significantly elevated, which is likely to have detrimental effects on a wide array of cellular processes considering the number of effectors that lie downstream of Ras signalling (Carlisle et al. 2008).

mTOR signalling cascade

At present the activity of mTOR and components of this pathway have not been assessed when SynGAP expression is either lost or reduced. However, inhibition of mTOR signalling by rapamycin corrected enhanced mEPSC amplitude and elevated protein synthesis rates in *Syngap* KD cortical cultures (Wang et al. 2013). Likewise overexpression of mTOR increased mEPSC amplitude in WT cultures to a level that was observed in *Syngap* KD neurones. These findings suggest that in the absence of SynGAP the mTOR signalling pathway is hyperactive and under basal conditions SynGAP normally downregulates mTOR activity, inhibiting AMPA receptor insertion via a protein synthesis dependent mechanism.

ERK1/2-MAPK signalling cascade

Basal levels of MEK1/2 and ERK1/2 activity are enhanced in the hippocampus of *Syngap* heterozygote mice in the absence of any changes in total protein expression (Komiyama et al. 2002; Ozkan et al. 2014), which is reproduced in *Syngap* KO neuronal cultures (Rumbaugh et al. 2006) but not consistently (Krapivinsky et al. 2004). Likewise overexpression of SynGAP reduces ERK1/2 activity in neuronal cultures whilst inhibition of the Ras-ERK1/2 pathway with U0126 corrects the

increase in mEPSC amplitude observed in *Syngap* KD cortical neurones (Wang et al. 2013). Together this data suggests that ERK1/2 is a downstream target of SynGAP and under basal conditions SynGAP suppresses the activity of ERK1/2 signalling limiting the insertion of AMPA receptors at the synapse. Thus, when SynGAP expression levels are reduced or absent, ERK1/2-MAPK signalling cascade is hyperactive.

p38 signalling cascade

In *Syngap* KO cortical neurones, it was reported that p38 activity was markedly reduced compared to WT neurones (Rumbaugh et al. 2006). Yet Krapivinsky et al. (2004) finds p38 activity to be increased in *Syngap* KO hippocampal neurones. These discrepancies could be due to differences in neuronal cultures (cortical neurones vs. hippocampal neurone). Also, Krapivinsky et al. (2004) uses hippocampal cultures from mice that only lack the α isoform of SynGAP.

1.4.20 Convergence of FMRP and SynGAP

The mouse model of *Syngap1* haploinsufficiency shares many of the cognitive disturbances reported in *Fmr1* KO mice, including learning and memory deficits, impaired social interactions and an increased susceptibility to epileptic seizures. Furthermore there appears to be further phenotypic between *Syngap* heterozygous and *Fmr1* KO mice at the cellular and molecular level. Two key pathways implicated in the pathology of FXS, ERK1/2 and mTOR, appear to be hyperactive and dysregulated in *Syngap* heterozygous brain. Furthermore, protein synthesis rates are elevated in *Syngap* KD cortical cultures and accompanied by increases in AMPA receptor insertion at the postsynapse (Wang et al. 2013). Likewise, NMDAR-dependent forms of LTP are impaired in the hippocampus of *Syngap* heterozygous mice consistent with *Fmr1* KO mice (Komiyama et al. 2002; Kim et al. 2003; Ozkan et al. 2014).

1.5 Aims of this thesis

Based on pathophysiological axis of ID, one would predict that mutations in *Fmr1* and *Syngap1* reside on the same part of the spectrum, perhaps even share the same pathological axis (Figure 1.7). This is founded on the evidence that: (1) Ras-ERK1/2 activity is elevated in the hippocampus of *Syngap* heterozygous mice, (2) LTP is impaired, and (3) protein synthesis rates are increased in *Syngap* KD cultures. If true, then one would suspect that $mGlu_{1/5}$ dependent synaptic plasticity and protein synthesis to be exaggerated in the hippocampus of *Syngap* heterozygous mice, the consequence of hyperactivation of Ras-mediated signalling. Thus, the focus of this thesis is to determine whether these two genetically distinct causes of ID share similar a hippocampal pathophysiology.

Chapter 3: Characterise mGluR-dependent synaptic plasticity and basal protein synthesis rates in the hippocampus of *Fmr1* KO and *Syngap* heterozygous rodents to determine whether:

1.) mGluR-dependent LTD is enhanced and independent of new protein in our mouse model of FXS.
2.) mRNA translational rates are elevated in *Fmr1* KO mice and if these phenotype translates to the rat model of FXS.
3.) *Syngap* heterozygous mice share a similar hippocampal pathophysiology to *Fmr1* KO mice.
4.) mutations in *Fmr1* and *Syngap1* converge on the same pathophysiological axis.

Chapter 4: Analyse the activation states of key components of the ERK1/2 and mTOR signalling cascade and measure expression levels of synaptic proteins to determine whether:

1.) *Syngap1* haploinsufficiency results from a hyperactivation of ERK1/2 and/or mTOR signalling.
2.) protein synthesis rates are saturated downstream of $mGlu_{1/5}$ receptor activation.
3.) "LTD proteins" are overabundant in the *Syngap* heterozygous brain.

Chapter 5: Pharmacological rescue of the hippocampal pathophysiology in *Syngap* heterozygous mice to determine whether:

1.) elevated protein rates and mGluR-dependent LTD lie downstream of ERK1/2 or mTOR signalling.
2.) therapeutic interventions utilised in the *Fmr1* KO can restore normal synaptic function in *Syngap* heterozygous mice
3.) alterations in $mGlu_5$ receptor signalling can be restored in adult mice following phenotypic onset.

Chapter 2

Materials & Methods

2.1 Common Methods

There are several rodent transgenic lines used in this thesis and below gives a brief description of each mutant line. There are some particulars that are common to all and are mentioned here.

2.1.1 Housing and Breeding

All animals were group housed and maintained in a facility that was kept on a 12:12 h light: dark cycle and treated in accordance with UK Home Office regulations. All experimental animals were male, and mutants were generated with wild-type (WT) littermate controls. Experiments were performed blind to genotype, unless otherwise stated.

2.1.2 Generation of the *Fmr1* Knockout Mouse

Fmr1 knockout (KO) mice were originally purchased from Jackson laboratories and were generated by interrupting exon 5 with the positive selection marker gene, neomycin Figure 2.1 A. Mice were backcrossed by at least 10 generations on to the C57Black/6JOla background (Harlan), from the original C57Black6J (Jackson), by crossing an *Fmr1* heterozygous females mouse WT males selected from previous litters.

2.1.3 Generation of the *Syngap* Heterozygous Mouse

The *Syngap* mutant mouse was generated by Komiyama et al. (2002) at the Sanger Institute. Deletion of the *Syngap1* gene involved the insertion of a targeting vector that consisted of a coding sequence for hemagglutinin epitope tag followed by stop codons and an internal ribosomal entry site (IRES)-lacZpolyA_MC1neo-polyA cassette, which resulted in the deletion of exons encoding the C2 domain and part of the GAP domain of the *Syngap1* gene (Figure 2.1 B). Immunoblotting of hippocampal extracts revealed a 50% reduction in SynGAP expression (Komiyama et al 2002).

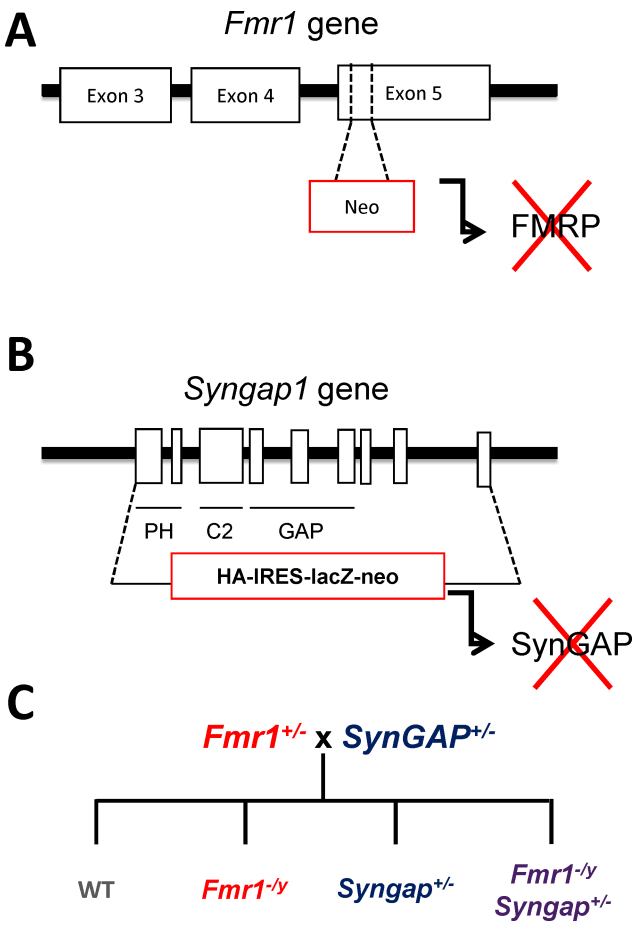


Figure 2.1 Generation of *Fmr1* KO, *Syngap* Heterozygous & Double Mutant Lines. (A) The *Fmr1* knockout (KO) mouse was generated by the insertion of a neomycin cassette into exon 5 of the *Fmr1* gene. (B) Exonic structure of the *Syngap1* gene with the targeting vector that contains: HA; hemaglutinin sequence; IRES, internal ribosome entry site, LacZ, ß-galactosidase gene; neo, neomycin resistant gene. The resultant vector deletes the region encoding the C2 and GAP domains of the *Syngap1* gene. (C) Mating of an *Fmr1* KO female with *Syngap* heterozygous male generates WT, *Fmr1* KO and *Syngap* heterozygous single mutants, and the *Fmr1* KO *Syngap* heterozygous double mutant.

Animals were initially bred on an MF1 genetic background (Sanger Institute) before being partially backcrossed onto a C57Black6JOla line. *Syngap* heterozygous mutants were generated by mating *Syngap* heterozygous females with WT C57black6JOla males.

2.1.4 Generation of a *Syngap* Heterozygote and *Fmr1* Knockout Double Mutant

Syngap heterozygous and *Fmr1* KO double mutant mice were generated by crossing a *Syngap* heterozygote males with female *Fmr1* heterozygotes, which were partially and fully backcrossed and bred on a C57Black6JOla background, respectively. From the cross there were four possible outcomes: WT, *Fmr1* KO single mutant, *Fmr1* KO and *Syngap* heterozygote double mutant and *Syngap* heterozygote single mutant (Figure 2.1 C). Only first generations of litters were used for experimental purposes.

2.1.5 Generation of the *Fmr1* KO Rat

The rat model of FXS was purchased from Sigma SAGE laboratories and bred on a Sprague Dawley background. *Fmr1* KO rats were generated using a zinc-nuclease methodology that involves these DNA-binding proteins creating a double strand break at the *Fmr1* locus, deleting the gene and resulting in the loss of FMRP (Hamilton et al. 2014).

2.1.6 Genotyping

DNA Digest

Tissue in the form of ear and tail clips was collected in 1.5 ml eppendorfs from mice at P14 or during hippocampal slice preparation. DNA was extracted using the Wizard® SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions with a minor modification to the DNA elution step.

For DNA digestion, 20 mg of tissue sample was digested in 275 µl of digestion solution master mix consisting of 200 µl nuclei lysis solution, 50 µl EDTA (0.5 M,

pH 8.0), 20 µl proteinase K (20 mg/ml) and 5 µl RNase A solution (4 mg/ml). Samples were mixed and incubated overnight (16-18 hours) in a water bath at 55 °C.

DNA Purification

For DNA extraction and purification, 250 µl of Wizard® SV Lysis Buffer was added to each sample and mixed. Sample lysate was transferred to a separate Wizard® SV mini-column assembly and spun at 13000 × g for 3 minutes. The mini-column was removed and the liquid was discarded. Next, 650 µl of Wizard® SV wash solution (with 95% ethanol) was added to each assembly and centrifuged at 13,000 × g for 1 minute. This step was repeated for a total of 4 washes discarding liquid waste between washes. After the final wash the mini-column was spun at 13000 × g for 2 minutes to dry the binding matrix. The mini-column was transferred to fresh 1.5 ml eppendorf tubes and 250 µl of nuclease-free water was added and left to incubate at room temperature for 4 minutes. The mini-column plus elution tube was then centrifuged at 13000 × *g* for 3 minutes. The mini-column was removed and the DNA stored in the elution tube at -20 °C.

Fmr1 PCR reaction

For each DNA sample, one reaction was performed with two sets of primer pairs: 2009/2010 (Sigma-Aldrich: 2009, 5' – GTG GTT AGC TAA AGT GAG GAT GAT -3'; 2010 5'- CAG GTT TGT TGG GAT TAA CAG ATC –3'), amplifies the 500 bp product of the WT allele; and 162/163 (Sigma-Aldrich: 162, 5' –CCG GTT CTT TTT GTC AAG ACC G -3'; 163, 5'- CGG CAG GAG CAA GGT GAG AT -3'), detects the neomycin cassette of the KO allele. The PCR reaction mix for each reaction, consisted of 4.25 µl of double distilled water (ddH_2O), 6.25 µl of Promega GoTaq Green Master Mix and 0.5µl of 2009 (20 µM), 0.5 µl of 2010 (20 µM), or 0.5 µl of 162 (20 µM) and 0.5 µl of 163 (20 µM). From the PCR reaction mix 11.5 µl was transferred to PCR tubes (Scientific Laboratory Supplies Ltd) and 1 µl of the DNA sample was added. The thermocycling conditions of the PCR reaction for primer pairs 2009/2010 and 162/163 are summarised in Table 2.1. The PCR products consisted of a 197 bp band, which represents the neomycin cassette of the *Fmr1* KO allele, and a 500bp band, which indicates the presence of the *Fmr1* gene. These PCR

products were run on a 0.75 % agarose (Sigma-Aldrich) gel at 50 mV for 35 minutes with a 100 bp DNA ladder (New England BioLabs).

Syngap PCR reaction

To detect the presence of the transgenic cassette in the *Syngap* allele two sets of primers were used: Syn12R (Sigma-Aldrich; 5' – CAT ACA AGA ATT GCT GCA TAG AAC -3'), a common reverse primer; in conjunction with either Fcass1A (Sigma-Aldrich; 5' – CTT CCT CGT GCT TTA CGG TAT C -3'), a forward primer complementary to the transgenic cassette or Syn11R (Sigma-Aldrich; 5' – TTC ATG GAG CGG GAA CAC CTC ATA T -3'), a forward primer complementary to the WT sequence. The PCR reaction mix consisted of 4.25 µl of ddH_2O, 6.25 µl of Promega GoTaq Green Master Mix, 0.50 µl of Syn12R (20 µM) and 0.50 µl of Fcass1A (20 µM) or 0.50 µl of Syn11R (20 µM). 1 µl of purified DNA was added to the reaction mix and spun. Thermocycling conditions are summarised in Table 2.1. The PCR product of the reaction is approximately 1 kb for the KO allele and 2.5 kb for the WT reaction. These are detected by running the PCR products on a 0.6% agarose gel at 50 mV for 35 minutes with a 1 kb DNA ladder (New England BioLabs).

Table 2.1 Thermocycling conditions for *Syngap* and *Fmr1* genotyping.

	Genotyping Thermocycling Conditions			
	Syngap1		***Fmr1***	
Step	**Temperature (ºC)**	**Duration**	**Temperature (ºC)**	**Duration**
1	**95**	**15 min**	**94**	**15 min**
2	**95**	**30 sec**	**94**	**30 sec**
3	**94**	**10 sec**	**63**	**10 sec**
4	**55**	**30 sec**	**Go to Step 3 34X**	**30 sec**
5	**68**	**3 min**	**72**	**1 min**
6	**Go to Step 3 10X**		**72**	**2 min**
7	**94**	**10 sec**	**4**	**1 hr**
8	**55**	**30 sec**		
9	**68**	**3 mins**		
10	**Add 20 secs/cycle**			
11	**Go to Step 7 28X**			
12	**68**	**7 min**		
13	**4**	**1hr**		

2.2 Methods for Electrophysiology

2.2.1 Preparation of Horizontal Hippocampal Slices for Extracellular & Intracellular Recordings

Horizontal hippocampal slices from male mice were prepared from postnatal day 20-32 (P20-32). For experiments involving a comparison between WT and mutant mice, animals were chosen at random, and all experiments and data analyses were performed blind to the genotype of the animal. After slice preparation on each experimental day, the ear and tail were preserved for use in genotyping. All chemicals for ACSF were purchased from Sigma-Aldrich.

Briefly, animals were deeply anaesthetized by inhalation of isofluorane released as fumes from liquid stock, and killed by decapitation. The skin covering the skull was cut along the midline and peeled back. Using forceps the skull was then cut along the midline and towards both ear bones. Brains were removed using a spatula and 400 μm thick slices were cut using a Leica VT1200S in pre-oxygenated ice-cold dissection buffer containing (in mM): NaCl, 86; NaH_2PO_4, 1.2; KCl, 25; $NaHCO_3$, 25; glucose, 20; sucrose, 75; $CaCl_2$, 0.5; $MgCl_2$, 7; saturated with 95% O_2, 5% CO_2.

Slices were hemisected and recovered for 30 minutes at 35 ^oC in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; NaH_2PO_4, 1.2; KCl, 25; $NaHCO_3$, 25; glucose, 20; $CaCl_2$, 2; $MgCl_2$; 1, saturated with 95% O_2, 5% CO_2. The hippocampus was sectioned from the rest of the slice and an incision made through CA1/CA3 boundary. Slices were left to recover for a further 30 minutes at room temperature prior to any experimentation.

2.2.2 Extracellular Recordings in CA1 of the Hippocampus

For electrophysiological recordings, slices were placed in a submersion chamber heated to either room temperature or 30 $^{\circ}C$ (Fine Science Tools) and perfused with pre-oxygenated ACSF containing picrotoxin (50 μM; Abcam), at a rate of 4 ml/min, kept constant using a peristaltic pump (Watson Marlow). For field recordings at 30 °C, ACSF was heated and oxygenated on a Stuart Scientific hotplate.

Field excitatory postsynaptic potentials (fEPSPs) were recorded with extracellular recording glass microelectrodes (1-4 MΩ; Harvard Apparatus) filled with ACSF and placed in the *stratum radiatum* of CA1 (Figure 2.2). Synaptic responses were evoked by applying a current pulse (Stimulator; Digitimer Ltd) every 30 seconds to the Schaffer collateral axons with a concentric bipolar nickel/ chromium (80%/20%;) stimulating electrode.

2.2.3 Stimulation Paradigms

Stable baseline responses were recorded every 30 seconds for 30 minutes before the induction of any stimulation paradigm. For chemical inductions the following compounds were used. (*R*, *S*)-3, 5-Dihydroxyphenylglycine (DHPG), D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), anisomycin, picrotoxin and rapamycin were purchased from Abcam. CTEP (RO4956371) and U0126-EtOH (U0126) were purchased from SelleckChem and lovastatin was purchased from Merck. DHPG and D-AP5 were dissolved in ddH_2O, whilst anisomycin, rapamycin, CTEP and U0126 were dissolved in Dimethylsulphoxide (DMSO). To maintain the DMSO concentration constant (0.0005%) between experiments, picrotoxin was dissolved in either DMSO or ddH_2O depending on whether additional inhibitors (e.g. CTEP, U0126, rapamycin) were to be added to the ACSF.

Input-Output Response

Basal synaptic transmission (input-output relationship) was examined in the CA1 by increasing the duration of the current pulse (input) in increments of 20 μs from 20-200 μs and recording their respective output values by measuring the slope of the fEPSP waveform relative to the amplitude of the presynaptic volley.

From the input-output curve the stimulation intensity that yielded 40% of the maximum response (prior to contamination by a population- or P- spike) was estimated and used to determine paired-pulse ratios and establish a new baseline prior the induction of a long-term depression (LTD) paradigm.

Paired-Pulse Facilitation

Paired-pulse facilitation, a form of presynaptic facilitation induced by two stimuli presented in rapid succession, was measured by examining the ratio of the fEPSP slope of the second pulse relative to the first. Each pulse lasted 0.1 ms delivered at an inter-pulse interval ranging from 20-100 ms.

Induction of Metabotropic Glutamate Receptor Dependent LTD

mGluR-dependent LTD was induced in CA1 by applying either: 900 paired pulses at 1 Hz to the Schaffer collateral pathway (PP-LFS), lasting 15 minutes; or the Gp1 mGlu receptor agonist DHPG (30 μM, 50 μM or 100 μM) in the presence of the NMDA receptor antagonist (D-AP5; 50 μM), perfusing the slice for 5 minutes before the slice was re-perfused in ACSF containing picrotoxin (50 μM;).

For mGluR-dependent LTD experiments in the presence of anisomycin (20 μM), rapamycin (20 nM) or U0126 (5 μM or 20 μM), hippocampal slices were preincubated in inhibitors or combinations of inhibitors for at 20-30 minutes prior to LTD induction and remained in the bathing solution throughout the remainder of the recording.

2.2.4 Field Analysis

Waveform data was collected using WinLTP 1999-2009 (WinLTP Ltd., University of Bristol), amplified 1000 times (npi electronics), filtered at 1.3 kHz and digitized (National Instruments) at 20 kHz. The data was exported to Microsoft Excel and statistical analysis performed in Origin. After genotyping, normalised data were averaged across experiments and expressed as a mean ± SEM. Statistics were performed using each animal as an '*n*'. Significant differences between two groups) were determined using a Student's *t*-test ($p < 0.05$). For experiments involving more than two genotypes a one-way analysis of variance (ANOVA) with a Bonferroni *post-hoc* test was used to determine significance ($p < 0.05$).

2.3 Methods for Biochemistry

2.3.1 Preparation of Transverse Hippocampal Slices

Protein levels were measured following the protocol outlined in Osterweil et al. (2010). On each day 4 animals from both mutant and WT groups were prepared from young adult rodent males (P25-32) in an interleaved manner. Animals were given an overdose of isoflurane released as fumes from liquid stock, and killed by decapitation. The brains were collected using a spatula and the hippocampus was dissected in pre-oxygenated ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; NaH_2PO_4, 1.25; KCl, 3; $NaHCO_3$, 26; glucose, 10; $CaCl_2$, 2; $MgCl_2$, 1, saturated in 95% O_2 and 5% CO_2. Slices were cut (500 μm) using a Stoelting tissue slicer. Four dorsal hippocampal slices were taken and left to recover in netwells (Corning, Sigma-Aldrich) for at least 4 hours at 30 ^oC in preoxygenated ACSF to allow protein synthesis rates to recover.

2.3.2 Metabolic Labelling

Slices were transferred to a second recovery chamber containing preoxygenated ACSF containing actinomycin D (ActD, 25μM; Tocris), heated to 30 ^oC and left to incubate for 30 minutes to inhibit transcription. For DHPG (100 μM) experiments, slices were incubated in ~0.37 MBq/ml of ^{35}S-Met/Cys express protein labelling mix (Perkin Elmer) -/+ drug for 5 mins and then transferred to fresh ACSF with approximately ~0.37 Mbq/ml ^{35}S–Met/Cys for 40 minutes to measure protein synthesis. For metabolic labelling experiments in the presence of CTEP (10 μM & 20 μM), U0126 (5 μM), lovastatin (50 μM & 100 μM) or rapamycin (20 nM), slices were incubated +/- drug during ActD exposure (30 min) and transferred to fresh ACSF containing ~0.37 Mbq/ml of ^{35}S-Met/Cys -/+ drug for 45 minutes. Slices were rapidly frozen on dry ice and stored at -80 ^oC.

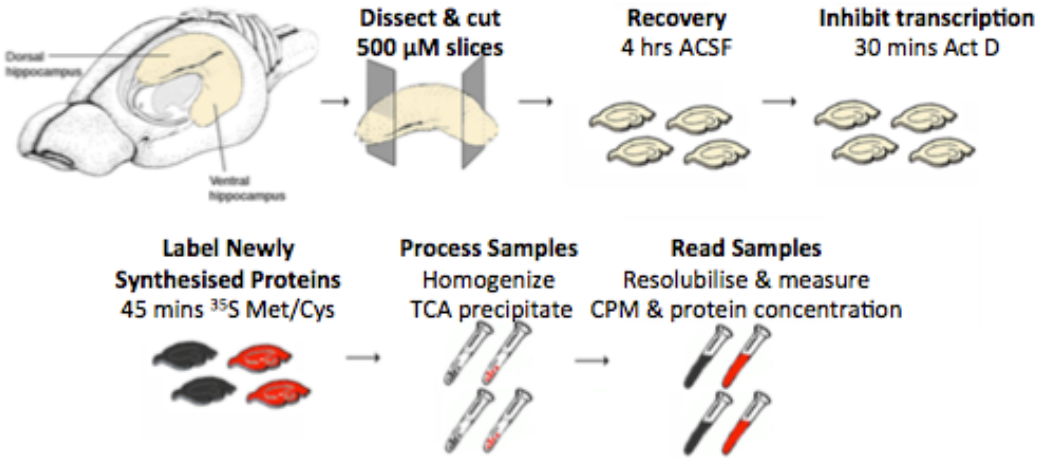


Figure 2.2 Measuring basal protein synthesis in dorsal hippocampal slices. Hippocampi were rapidly dissected from rodents between P25-32 and four dorsal transverse hippocampal slices were prepared. Slices were left to recover in artificial cerebrospinal fluid (ACSF) for 4 hrs. before transcription was blocked with Actinomycin D (ActD; 25 µM) ± vehicle or inhibitor for 30 mins. Newly synthesized proteins were metabolically labelled with ^{35}S Methionine-Cysteine (Met/Cys) protein labelling mix ± vehicle or inhibitor for 45 minutes. Samples were homogenized and proteins precipitated using trichloroacetic acid (TCA) and resolubilised. Counts were measured and normalised to overall protein concentration. Image adapted from www.biomedcentral.com/content/figures/1471-2202-6-36-3-1.jpg.

2.3.3 Processing

To measure protein levels, slices were homogenized in 150 µl of homogenising buffer containing 10 mM HEPES pH 7.4, 2 mM EDTA, 2 mM EGTA, 1% triton X-100 (Sigma-Aldrich), protease and phosphatase inhibitors. From the homogenate, 75 µl was removed and taken for western blotting (see section 2.4.3) by adding 50 µl of x4 Laemmli samples buffer containing 10% β-mercaptoethanol (Sigma-Aldrich) and frozen at -20 ^{o}C. To the remaining homogenate, proteins were precipitated using trichloroacetic acid (TCA, 12.5%; Sigma-Aldrich), vortexed and incubated on ice for 10 minutes before being spun at 13000 × g in a microfuge for 10 minutes at 4 ^{o}C. The supernatant was removed and 500 µl of ice-cold ddH_2O was added to the homogenate, inverted and spun at 13000 × g for 5 minutes at 4 ^{o}C. Once again the supernatant was removed and replaced with 150 µl of NaOH (1 M) and left to incubate at 37 ^{o}C to dissolve the pellet. The pH was readjusted by adding 50 µl of HCl (0.33 M) and 50 µl of sample was added to scintillation vials (Perkin Elmer) containing 10 ml of scintillation cocktail (Promega) in triplicates. Sample vials were loaded into a scintillation counter, set to read ^{35}S at 2 minutes per sample, which gave the number of counts per minute (CPM; Figure 2.3).

2.3.4 BSA Protein Assay for Metabolically Labelled Hippocampal Slices

The protein concentration of each sample was determined using a Bio-Rad DC kit. Initially BSA standards were made using a 10mg/ml BSA stock (New England Bio Lab), which was diluted with ddH_2O to make a 2mg/ml solution. This 2mg/ml stock was then used to make serial dilutions in ddH_2O, which ranged from 2mg/ml to 0.0625 mg/ml. For the protein assay, 5 µl of either BSA standard or protein sample were added to a 96-well Nunc plates (Scientific Laboratory Supplies Ltd) in triplicate. Next, 25 µl of reagent A containing reagent S' (20 µl of reagent S for every ml of reagent A) was added, followed by 200 µl of reagent B. Plated samples were t left to incubate at room temperature for 15 minutes before being read on a FluoStar Optima plate reader (BMG Labtech) at an absorbance of 740 nm. Proteins concentrations for each sample were calculated based on the BSA standard curve.

2.3.5 Analysis of Protein Samples

The scintillation counts for each sample were divided by the protein concentration, as calculated from the BSA standard curve to find cpm/µg. This value was normalised to all other samples by dividing cpm/µg by the cpm for the ACSF control sample. To find the number of cpm/µg/µl the previous value was divided by volume of sample added to the scintillation (50 µl).

$$\text{CPM/µg/µl} = \frac{100\ \text{(cpm/µg)/ (CPM for ACSF)}}{\text{Volume of sample added to scintillant}}$$

Once the average CPM/µg/µl was calculated for each triplicate. The mean and S.E. were calculated for each hippocampal slice. The average % change in protein synthesis for each set of hippocampal slices were calculated and expressed as a percentage of the WT control.

$$\text{\% Change in basal protein synthesis} = \frac{100\ \text{(CPM/µg/µl for Mutant}}{\text{CPM/µg/µl for WT pair}}$$

Statistics were performed using each animal as an '*n*'. Significant differences between genotypes (WT *versus* mutant) were determined by a paired Student's *t*-test ($p < 0.05$). However for comparisons of more than two experimental factors (genotype & treatment) significance was determined using a two-way ANOVA with Bonferroni *post-hoc* test ($p < 0.05$).

2.3.6 Western Blotting of Metabolically Labelled Hippocampal Slices

Loading, Running & Transfer of a Polyacrylamide Gel

SDS-polyacrylamide gel electrophoresis was performed on metabolically labelled protein samples by boiling samples containing 4X Laemmli buffer and 10% β-mercaptoethanol. For every sample, 10 µl was loaded per lane in to Bio-Rad Mini-protean precast gels (percentage of polyacrylamide gel varied from 7.5% to 12% depending on the protein of interest). Gels were run at constant current in buffer containing (in mM): Tris, 25; glycine, 190; SDS, 0.1%; ddH_2O. Protein gels were electroblotted on to nitrocellulose membrane (Bio-Rad) in buffer containing (in mM): Tris, 25; glycine, 190; methanol, 20%; ddH_2O.

2.3.7 Western blotting of Hippocampal Homogenates and Synaptoneurosome Preparations

Animals were given an overdose of isoflurane released as fumes from liquid stock, and killed by decapitation. The brains were collected using a spatula in pre-oxygenated ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; NaH_2PO_4, 1.25; KCl, 3; $NaHCO_3$, 26; glucose, 10; $CaCl_2$, 2; $MgCl_2$, 1, saturated in 95% O_2 and 5% CO_2. Hippocampi were dissected and frozen on dry and stored at -80 ^oC. Samples were shipped on dry ice to M.I.T (Boston, USA) for homogenization and western blotting.

Homogenization of Hippocampi

Hippocampi were homogenized in 2 ml glass homogenizers in ice cold homogenizing buffer containing (in mM): HEPES, 10; NaCl, 150; EDTA, 2; EGTA, 2; protease inhibitor cocktail III. From the homogenate 108 µl was removed and added to 12 µl of Triton X-100. From this sample 20 µl was removed for a protein assay. To the remaining volume, 100 µl of 4X Laemmeli buffer and 10% β-mercaptoethanol was added and boiled.

Preparation of Synaptoneurosomes from Hippocampal Homogenates

Hippocampal homogenate was filtrated through a 105 µm filter holder using a 3 ml syringe (18G needle). The filter was removed and liquid re-filtrated through a 5 µm filter holder. Filtrate was spun for 10 minutes at 1000 × g at 4 ^{o}C to collect the synaptoneurosome pellet, which was then resuspended in 150 µl homogenization buffer and Triton X-100. From the synaptoneurosome homogenate 100 µl was added to 100 µl 4X Laemmeli buffer and 10% β-mercaptoethanol and boiled.

BSA Protein Assay for Hippocampal Homogenate and Synaptoneurosome Preparations

The protein concentration of hippocampal and synaptoneurosome homogenates were measured using a Bio-Rad DC kit as outlined in section 2.4.4. However, once proteins concentrations were calculated using the BSA standard curve, all protein concentrations were adjusted to 1 mg/ml.

Making Gels for Western blotting

Polyacrylamide gels (10%) were made from two gels: a separating and stacking gel. The separating gel contained in ml: ddH_2O, 61.5; 30% acrylamide, 49.5; separating buffer, 37.5; 10% SDS, 1.5; 10% ammonium persulfate (APS), 1.5; Temed, 75 µl. The stacking gel was composed of (in ml): ddH_2O, 45.75; 30% acrylamide, 9.75; stacking buffer, 18.75; 10% SDS, 0.75; 10% APS, 0.75; Temed, 75 µl; few crystals of bromophenol blue. The separation gel was poured into a gel multicaster and a 0.5-1ml of butanol was poured on to the top of each gel and left for 20-30 minutes to polymerise. Once gels were set the butanol was poured off and the tops of the gels washed with ddH_2O.

Loading, Running & Transfer of a Polyacrylamide Gel

For loading, hippocampal and synaptoneurosomes samples were boiled and 10 µl/ per lane was loaded in to gels. Gels were run in a dodeca cell at constant voltage 150 V in x1 Tris/Glycine/SDS buffer (TGS) diluted in ddH_2O from 10X stock (Bio-Rad), which contained (in mM): Tris 25mM, glycine 192, 0.1% SDS and ddH_2O. Protein gels were electroblotted on to nitrocellulose membrane (Bio-Rad) in transfer buffer containing 1x TG buffer (Bio-Rad), methanol 20% and ddH_2O.

MemCode stain of Membrane

To visualise all proteins on membranes for quantification purposes a Thermo Scientific MemCode Reversible Protein Stain was used according to manufacturer's instructions. Briefly, membranes were incubated in MemCode stain at room temperature for 1 minute and replaced with destain shaking vigorously. Destain was left to incubate for 5 minutes with membranes being continually shaken. Membranes were washed repeatedly with ddH_2O and scanned on greyscale.

For LICOR Imaging of Proteins of Interest

Western blot membranes were cut at specific molecular weights (50, 75 or/and 100 kDA) so that multiple proteins could be examined on the same gel. They were blocked with Odyssey blocking buffer (Li-COR Bioscience) made up in 1 x phosphate buffered saline (PBS; Sigma-Aldrich) in a 1:1 ratio for 30 minutes. Block was poured off and antibodies made up in a 1:1 ratio of Odyssey blocking buffer and PBS with 0.1% tween (Boston Bio Products) and membranes were then probed with primary antibody (Table 2.2) and incubated at room temperature for a minimum of 4 hrs. or overnight at 4 ^oC. Membranes were repeatedly washed with PBS and 0.1% Tween. The respective fluorescent conjugated secondary, either anti-rabbit or anti-mouse (Li-COR Biosciences), were diluted 1 in 10000 in blocking buffer in a 1:1 ratio with 1 x PBS and Tween 0.1% and left to incubate for 45 minutes on a rocker. The secondary antibody was then discarded and blots washed with 1x PBS repeatedly. Membranes were imaged on an Odyssey infrared imaging system (Li-COR Bioscience). Blots were stripped with 1 x stripping buffer (Millipore) for 30

minutes and then repeatedly washed with 1 X PBS before being reprobed in primary antibody.

For ECL Imaging of Proteins of Interest

For enhanced chemiluminesence (ECL), membranes were cut at specific molecular weights (50, 75, 100 or/and 150 kDA) so that multiple proteins could be examined on the same gel. They were then blocked with 5% BSA in Tris buffered saline with Tween (TBS-T, Bio-Rad) containing (in mM): Tris pH 7.5, 20; NaCl, 150; Tween-20 0.1%. The block was poured off and membranes were probed with primary antibodies made up in 5% BSA and TBS-T and left overnight at 4 ^{o}C (Table 2.2). Primary antibodies were poured off and membranes were washed in TBS-T. Blots were incubated in secondary antibodies, either anti-rabbit HRP or anti-mouse HRP secondary antibodies (GE Healthcare), diluted 1 in 5000 in 3% BSA in TBS-T for 30 minutes at room temperature then membranes were washed repeatedly with TBS-T. Using the ECL Plus Kit, reagent A and B were mixed in a 40:1 ratio and poured over the blots and imaged. An exposure series was performed, which ranged from 1 second to 5 minutes and films were scanned on gray scale. If blots were to be reprobed they were washed in TBS-T on a shaker. TBS-T was poured off and replaced with 100 ml stripping buffer and 700 µl of beta-mercaptoethanol (Sigma) and blots left to incubate to 55 ^{o}C for 45 minutes. Blots were washed with several times with ddH_2O, which was replaced with TBS-T and left to incubate at room temperature for 1 hr. Membrane were then left to incubate in 5% BSA block (described above) and left to incubate for 1 hr. at room temperature before incubated once again being incubated in their appropriate primary antibody overnight at 4 ^{o}C.

Protein Quantification

Imaged gels were uploaded in to Image J and densitometry was performed using the analyser software (for full blots of each anti-protein antibody see appendix). Protein bands were analysed and included in figures if the band was sufficiently quantifiable without the interference of contaminants, such as air bubbles. The expression levels of proteins examined via licor methodology were normalised to the loading control β-actin (Abcam) whilst those obtained via ECL were normalised to total protein stain

obtained by MemCode stain. Protein expression levels were also expressed as a ratio between the phosphorylated and unphosphorylated form of the protein. To identify and quantify $mGlu_5$ receptor expression in hippocampal homogenates and synaptoneurosome preparations, an $mGlu_5$ KO sample was run with all other samples. This is because there is no distinctive band produced, rather a smear, which can be determined from the known molecular weight of $mGlu_5$ and using the $mGlu_5$ KO sample as a comparison to determine the boundaries for quantification. For GluA2 and PTEN, several bands were observed on the blot, so the one selected was based on the predicted molecular weight of each protein. In hindsight, to be confident that the right band was selected for GluA2 or PTEN, homogenates of GluA2 or PTEN KOs would need to be run alongside samples. For anti phospho/total ERK1/2 a double band was produced that was analysed individua

When comparing WT and mutant values, the WT protein levels were considered to be 100% and mutant values were expressed as a percentage ± S.E.M. of the WT value. When both genotypes and drugs treatments were being compared, all were normalised to the WT vehicle value and expressed as a percentage ± S.E.M. Significant differences between two groups (e.g. WT versus mutants) paired Student's *t*-test ($p < 0.05$). However for more than two groups (genotype x treatment) significance was determined using a two-way ANOVA and Bonferroni *post-hoc* test ($p < 0.05$). It should be noted that western blots performed via ECL methodology were only technically replicated once, whilst western blottings performed via licor were technically replicates 3-4 times.

Table 2.2 Primary antibodies for western blotting

Antigen	Host	Company	Dilution	Apparent MW (KDa)
Actin	Rabbit	Abcam	1:20,000	42
Akt	Rabbit	CST	1:1000	60
CAMKII alpha	Mouse	Zymed	1:10000	50
ERK1/2 (p44/42 MAP Kinase)	Rabbit	CST	1:1000	44+42
GluA1	Rabbit	Upstate 06-306	1:4000	100
GluA2	Mouse	Chemicon	1:1000	102
$mGlu_5$	Rabbit	Neuromics	1:500	132
mTOR	Rabbit	CST	1:1000	289
GluN1	Goat	Sc-1467	1:4000	120
GluN2A	Rabbit	MoPro A6473	1:500	180
GluN2B	Goat	Sc-1469	1:500	178
p38-MAPK	Rabbit	CST	1:200	38
p70 S6 Kinase	Rabbit	CST	1:1000	70
Phospho-Akt (Ser473)	Rabbit	CST	1:1000	60
Phospho-Akt (Thr308)	Rabbit	CST	1:1000	60
Phospho-ERK1/2 (p44/42 MAP Kinase [Thr202/Tyr204])	Rabbit	CST	1:1000	44+42
Phospho-mTOR (Ser2448)	Rabbit	CST	1:1000	289
Phospho-p38 (Thr180/Tyr182)	Rabbit	CST	1:200	38
Phospho-p70 S6 Kinase (Thr389)	Rabbit	CST	1:1000	70+85
Phospho-PTEN (Ser380/Thr382/383)	Rabbit	CST	1:1000	54
Phospho-S6 Ribosomal Protein (Ser235/236)	Rabbit	CST	1:500	32
Phospho-S6 Ribosomal Protein (Ser240/241)	Rabbit	CST	1:500	32
PTEN	Rabbit	CST	1:500	54
S6 Ribosomal Protein	Rabbit	CST	1:500	32

Chapter 3

Characterisation of Hippocampal Pathophysiology in *Fmr1*$^{-/y}$ & *Syngap*$^{+/-}$ Mutant Mice

Chapter 3.1 Key Findings

1. In CA1, chemically induced mGluR-dependent LTD induces a robust and long-lasting form of synaptic depression that involves both presynaptic and postsynaptic mechanisms in young adult hippocampal slices.

2. Consistent with previous reports, mGluR-dependent LTD and is enhanced and independent of new protein synthesis and is accompanied by increased basal protein synthesis rates in the hippocampus of *Fmr1* KO mice

3. Importantly, the electrophysiological and biochemical phenotypes we observe in the hippocampus of *Fmr1* KO mice translate to the rat model of FXS.

4. Similarly to *Fmr1* KO mice, mGluR-dependent LTD is increased and independent of new protein synthesis in the hippocampus of *Syngap* heterozygous mice, which also show exaggerated levels of basal protein synthesis.

5. In the *Fmr1* KO x *Syngap* heterozygous double mutants, *Syngap1* haploinsufficiency occludes the increase in mGluR-dependent LTD caused by the loss of FMRP.

3.2 Introduction

FXS and *SYNGAP1* haploinsufficiency are two highly pathogenic causes of ID and ASD that have dramatic consequences on the developing brain. FXS is caused by a CGG expansion mutation in the *FMR1* gene triggering partial or complete loss of the RNA-binding protein FMRP whilst *SYNGAP1* haploinsufficiency arises from autosomal-dominant *de novo* mutations in the *SYNGAP1* gene leading to the truncation of the full-length protein. Extensive investigations in the *Fmr1* KO mouse have revealed abnormalities in long-lasting forms of synaptic plasticity and protein synthesis rates downstream of the $mGlu_5$-ERK1/2 signalling (Huber et al. 2002; Osterweil et al. 2010). This has implicated $mGlu_5$-ERK1/2 signalling in the pathogenesis of ID and ASD and led to the development of novel therapeutics that specifically downregulate this signalling cascade.

Currently, FXS is the most common inherited form of ID and the leading genetic cause of ASD (Wang et al. 2010). However recently, the genetic screening of small cohorts of ID patients has revealed that mutations in *SYNGAP1* are also highly prevalent in the ID population with a high comorbidity of epilepsy and ASD (Hamdan et al. 2009; Hamdan et al. 2011). Already, investigations in the mouse model of this disorder have begun to elucidate the pathophysiological deficits associated with the reduction of SynGAP expression in the mammalian brain. Intriguingly, *Syngap* heterozygous mice share similar behavioural deficits to the *Fmr1* KO mouse, including subtle learning and memory deficits in the Morris water maze, hyperactivity in the open field, abnormal anxiety levels, an increased susceptibility to sensory stimuli and impairments in social interaction. Furthermore *Syngap* heterozygous mice exhibit dendritic spine abnormalities that suggest *Syngap1* haploinsufficiency may arise from altered synaptic maturation and plasticity.

Indeed investigations into synaptic plasticity mechanisms in *Syngap* heterozygous mice have revealed that LTP and NMDA receptor mediated forms of LTD are impaired in the hippocampus (Carlisle et al. 2008; Komiyama et al. 2002; Kim et al. 2003) and are accompanied by alterations in Ras-ERK1/2 activity. At the synapse, SynGAP is an upstream regulator of the Ras family of G-proteins. Ras family members converge on a diverse range of cellular processes including those that regulate synaptic plasticity and protein synthesis (Ye & Carew 2010; Stornetta & Zhu 2011). Through investigations in the mouse model of NF1, which expresses reduced levels of the Ras GAP neurofibromin, the role of Ras signalling in the neuropathology of ID has been firmly established. Similarly to *Syngap* heterozygous mice, in the *NF1* heterozygous mouse the activity levels of Ras and ERK1/2 are elevated, and this is accompanied by impairments in LTP and deficits in spatial memory (Silva et al. 1997; Costa et al. 2002; Li et al. 2005). Importantly, these hippocampal-dependent phenotypes can both be rescued by disrupting Ras signalling either genetically or pharmacologically (Guilding et al. 2007; Li et al. 2005).

Furthermore it has recently been reported that the pharmacological reduction of Ras-ERK1/2 signalling corrects elevated protein synthesis rates, r enhanced mGluR-dependent and epileptogenesis in the mouse model of FXS (Osterweil et al. 2013). However in the *Fmr1* KO brain, Ras-mediated signalling appears intact even though translational rates are saturated downstream of $mGlu_5$ receptor activation (Osterweil et al. 2010). This suggests that the translational apparatus may be hypersensitive to constitutive $mGlu_5$ signalling due to the loss of functional repression by FMRP.

In contrast, Ras-ERK1/2 activity is increased in the hippocampus of *Syngap* heterozygous mice (Rumbaugh et al. 2006; Komiyama et al. 2002; Ozkan et al. 2014), suggesting that the pathophysiology associated with this disorder may too result from a hyperactivity, as opposed to a hypersensitivity, of the Ras-ERK1/2 signalling pathway. Thus it was hypothesized that this could consequently lead to similar alterations in $mGlu_5$ dependent synaptic plasticity and basal protein synthesis to those reported in *Fmr1* KO mice. In support of this hypothesis, *Syngap* KD cortical cultures show increased protein synthesis rates (Wang et al. 2013). This

suggests that SynGAP may lie within a signalling pathway that couples cell surface receptors to the mRNA translational machinery.

Thus in this chapter, Gp1 mGluR-dependent LTD and basal protein synthesis rates are examined in the hippocampus of *Syngap* heterozygous mice, alongside *Fmr1* KO mice. Firstly, we want to establish whether the key electrophysiological and biochemical findings reported in the *Fmr1* KO can be reproduced in our mouse model of FXS. Secondly, whether *Syngap* heterozygous mice share similar abnormalities in mGluR-dependent synaptic plasticity and protein synthesis, and thirdly, whether the *Fmr1* KO and *Syngap* heterozygous share a common pathophysiological axis.

3.3 mGluR-dependent LTD at CA1 synapses

Extracellular recordings were performed in the CA1 field of horizontal hippocampal slices prepared from WT males between P20-30 by placing a stimulating electrode in the Schaffer collateral pathway and recording from the apical dendrites of CA1 pyramidal neurones, which project in to *stratum radiatum* (Figure 3.1 A). In CA1, the application of a single current pulse every 30 seconds to the Schaffer Collateral pathway, generated a fEPSP that is the summation postsynaptic potentials from a population of CA1 apical dendrites. The evoked fEPSP consisted of a short negative potential from which the slope and amplitude are used as a measure of synaptic strength (Figure 3.1 B). By plotting the slope of fEPSP (mV/ms) against time (mins), changes in synaptic strength can be observed by an increase or decrease in the slope of the fEPSP. Once synaptic responses stabilise and produce a baseline of 20 minutes or more the mean of baseline response is calculated and all responses from that point are normalised to this value and expressed as a percentage of the baseline response. The magnitude of LTD is based on the last 20 minutes of a recording and is expressed as a percentage of the predrug baseline. In the absence of an LTD or LTP inducing stimulus, fEPSPs recordings will remain stable for at least 80 minutes (Figure 3.1 C).

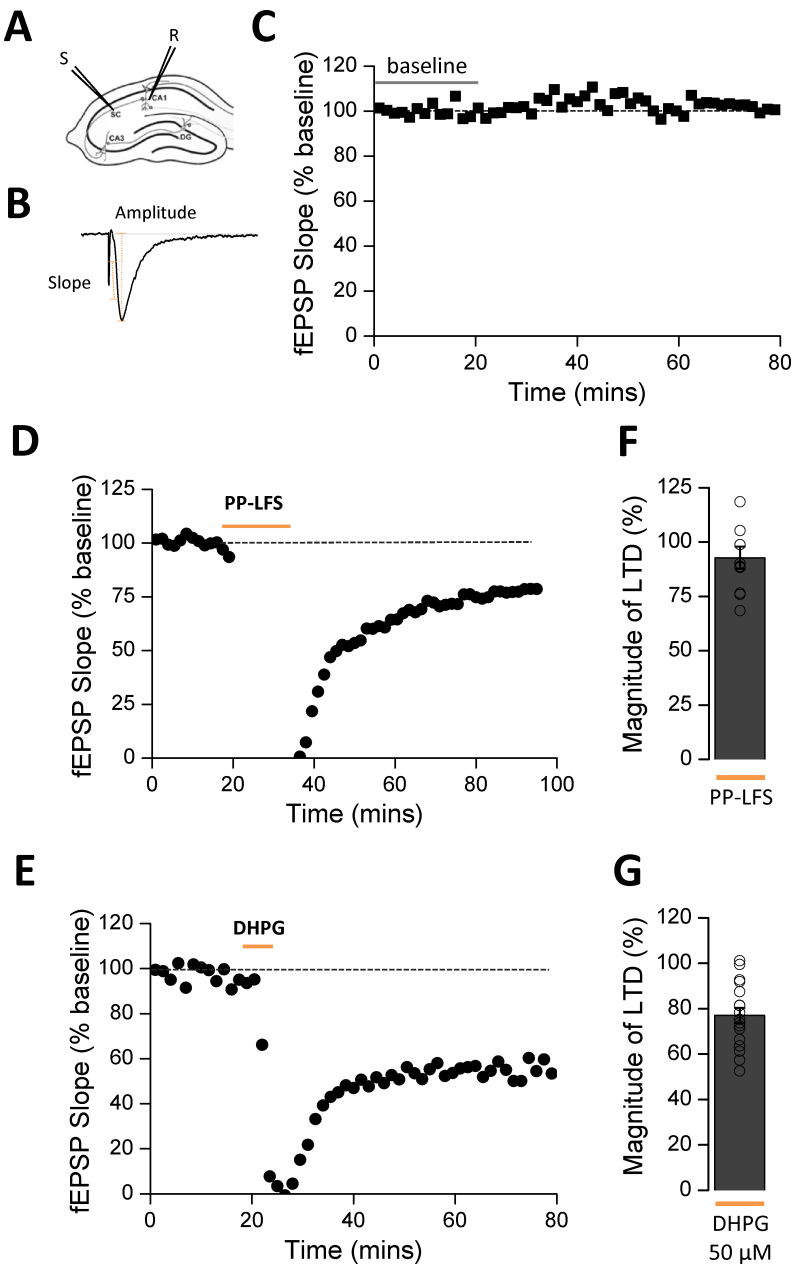


Figure 3.1 mGluR-dependent LTD in CA1 of the hippocampus. (A) Schematic of extracellular recordings in the hippocampus showing placement of stimulating (S) electrode in the Schaffer collateral pathway and recording (R) electrode in *stratum radiatum* of CA1. Image adapted from Biel et al. (2009). (B) Application of single current pulse generates a fEPSP from which the slope (mV/ms) expressed as a % of the mean baseline fEPSP and plotted against time. (C) Stable baseline fEPSPs were recorded for 80 minutes in CA1 region of hippocampal slices. Example recordings of mGluR-dependent LTD induced by either: (D) applying 900-paired pulses at 1 Hz to the Schaffer collateral pathway or (E) the acute application of the Gp1 mGlu receptor agonist (DHPG, 50 µM). (F) PP-LFS leads to high LTD failure rate compared to (G) DHPG (50 µM) induced mGluR-dependent LTD in hippocampal slices from WT mice.

3.3.1 Chemically-induced mGluR-dependent LTD evokes a robust form of synaptic depression in CA1

At Schaffer collateral/CA1 synapses mGluR-dependent LTD can be induced either: pharmacologically, by the application of orthosteric agonists of $mGlu_{1/5}$ receptors such as DHPG (Palmer et al. 1997); or synaptically, by repeatedly applying paired-pulses at 1 Hz for 15 minutes to the Schaffer collateral pathway (Huber et al. 2000; Kemp & Bashir 1999; Figure 3.1 C and D). Preliminary investigations of mGluR-dependent LTD in hippocampal slices from WT mice revealed that on average the chemical induction of mGluR-dependent LTD was far more reliable at inducing a depression of synaptic responses compared to PP-LFS (Figure 3.1 E & F). It was calculated that PP-LFS led to a depression of synaptic responses (of > 10%) in 4 out of 10 slices, and of those that depressed the mean LTD was 21 ± 4%. In contrast, only 4 out of 18 slices failed to depress, more than 10%, following chemical induction of LTD (50 μM DHPG), which had a mean LTD of 28 ± 3% (n = 14).

To improve the consistency of PP-LFS induced LTD it was recommended that the stimulation intensity be increased to at least 70% of the maximum response prior to LTD induction. Alternatively the number of paired-pulses could be increased to 1200 pulses, however we were advised that the former was more reliable (Personal communication, B. Auerbach, M.I.T.). Thus, the stimulus intensity was increased to 70% of the maximal response, but LTD was still only observed in 4 out of 8 slices with a mean LTD of 18 ± 3% (n = 4). Furthermore, it was a concern that high stimulation intensities for long periods of time could jeopardise slice health. Recently we were advised to increase the frequency at which pulses were delivered to the Schaffer collateral pathway by applying 900 paired pulses at 3 Hz rather than at 1 Hz (Personal communication, Professor A. Randall, University of Exeter). However, in this thesis investigations preceded using DHPG-induced mGluR-dependent LTD.

3.3.2 Chemical mGluR-dependent LTD induces a long-lasting form of synaptic depression

Further investigations in to chemically induced mGluR-dependent LTD revealed that upon application of DHPG (50-100 μM) there was a transient loss of synaptic responses within minutes, which only partially recovered over the 60-minute recording. Depressed fEPSPs stabilised and remained depressed for several hours (Mean LTD: 80 mins 53 ± 2%; 140 mins 53 ± 4%; n = 3; Figure 3.2 A). Furthermore a single application of DHPG (100 μM) did not induce a saturating level of LTD in hippocampal slices from young adult mice as further depression of synaptic responses was observed following a second application of DHPG (1^{st} 81 ± 3%; 2^{nd} 76 ± 6%; n = 8, t-test $*p$ = 0.03; Figure 3.2 B).

3.3.3 mGluR-dependent LTD reduces presynaptic function

Previous studies in young rats (P4-18) revealed that the expression of mGluR-dependent LTD involves a reduction in presynaptic function (Fitzjohn et al. 2001; Bolshakov & Siegelbaum 1994). Here the paired pulse ratio (%) of fEPSPs was examined during baseline recordings and 60 minutes after DHPG application at Schaffer Collateral/CA1 synapses in hippocampal slices from young adult mice (P25-30). The application of DHPG (100 μM) led to a significant increase in the PPR (PPR: before DHPG 1.39 ± 0.08%; after DHPG 1.67 ± 0.08%; n = 10; t-test $*p$ = 0.03; Figure 3.2 C), suggesting that mGluR-dependent LTD leads to a reduction in the release probability of glutamate from the presynaptic terminus.

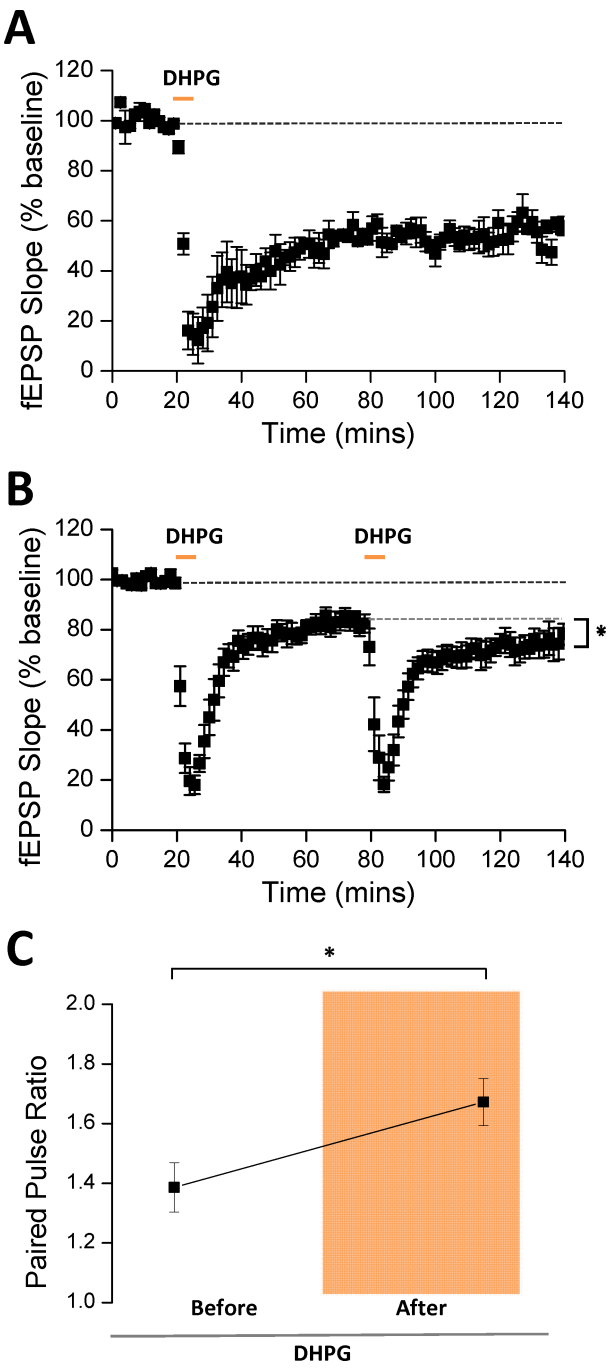


Figure 3.2 DHPG-induced mGluR-dependent LTD in CA1 of the hippocampus. Acute application of Gp1 mGlu receptor agonist DHPG (100 µM, 5 mins) induces a rapid depression of fEPSPs that only partially recovered in WT hippocampal slices. (A) DHPG-induced a long lasting depression of fEPSPs (80 mins 53 ± 2%; 140 mins 53 ± 4%; n = 3). (B) A second application of DHPG leads to further depression of synaptic responses (1st 81 ± 3; 2nd 72 ± 5; n = 8, *p = 0.03). (C) Paired pulse ratios (PPR)s were measured during baseline and 60 minutes after DHPG stimulation and reveal an increase in the PPR following DHPG application (PPR: baseline 1.39 ± 0.08%; DHPG 1.67 ± 0.08%; n = 10; t-test *p = 0.03).

3.3.4 GluR-dependent LTD is dependent on new protein synthesis

In CA1 of the hippocampus mGluR-dependent LTD is dependent on new protein synthesis (Huber et al. 2000). To investigate the protein synthesis dependency of mGluR-dependent LTD in CA1, hippocampal slices were incubated in the translational inhibitor anisomycin (20 μM) at various time points. Anisomycin had no effect on basal synaptic transmission and stable fEPSPs were obtained for at least 80 minutes in the presence of the drug alone (Figure 3.3A). Preincubation of hippocampal slices in anisomycin (20 μM) for at least 20 minutes prior to the induction of LTD and throughout the remainder of the recording strongly abolishes mGluR-dependent LTD in CA1. The acute application of anisomycin (5 mins) during DHPG wash-on failed to inhibit mGluR-dependent LTD (Figure 3.3B). Preincubation of hippocampal slices for 20 mins only, prior to mGluR-dependent LTD, caused a gradual return of fEPSP back towards baseline values (Figure 3.3C). However, if anisomycin was applied during LTD induction, but not before, then fEPSP rapidly returned to baseline level following DHPG washout (Figure 3.3D).

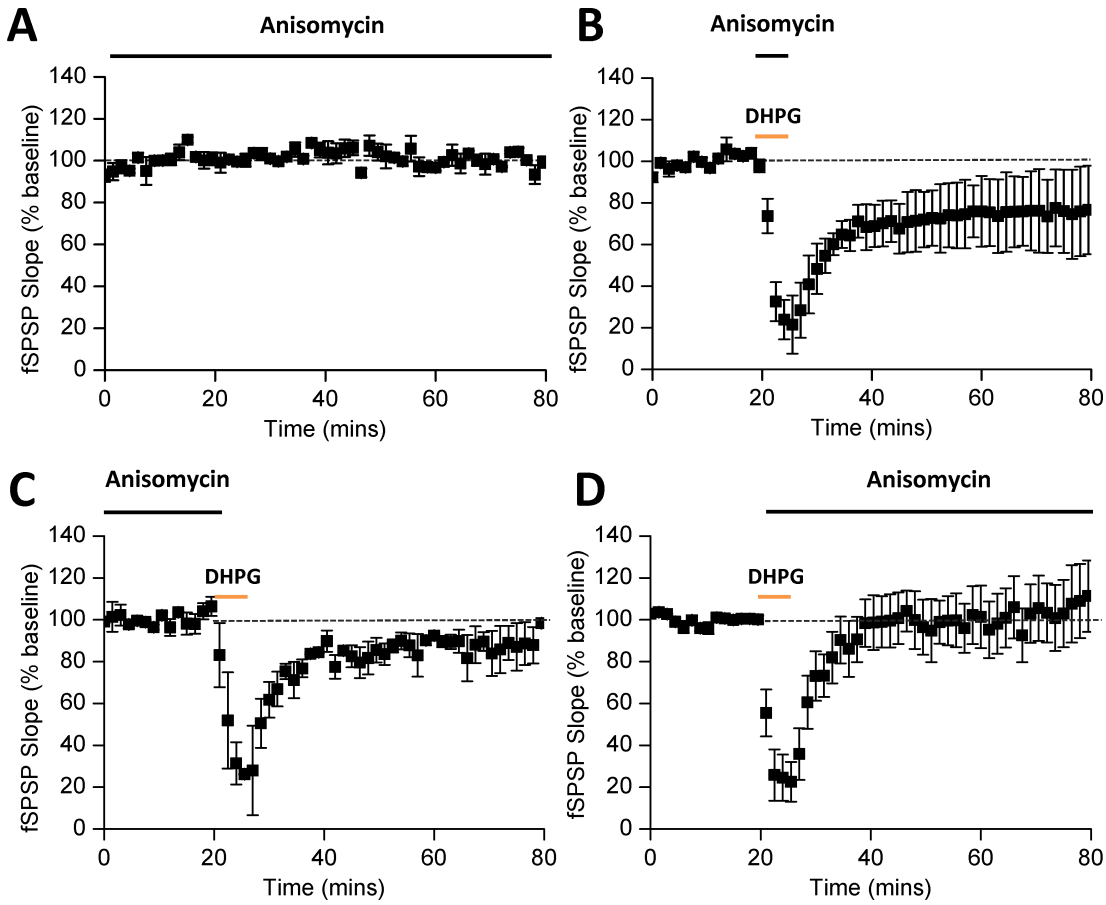


Figure 3.3 The expression of mGluR-dependent LTD requires new protein synthesis. (A) In the presence of anisomycin basal synaptic responses remain stable during an 80 minute recording in WT hippocampal slices (n = 2). (B) The acute application of anisomycin (5 mins, 20 µM) failed to inhibit DHPG-induced mGluR-dependent (5 mins, 100 µM) in the WT hippocampus (WT 76 ± 19%, n = 3). (C) Preincubation of WT hippocampal slices in anisomycin (20 min, 20µM) prevented the full expression of DHPG-induced mGluR-dependent LTD in CA1 of WT mice (88 ± 7%, n = 3). (D) mGluR-dependent LTD was fully inhibited when anisomycin was added (60 mins, 20 µM) to the ASCF during and after DHPG washout (WT 103 ± 15%, n = 3).

3.4 Results in the *Fmr1* KO Mouse

3.4.1 mGluR-dependent LTD is enhanced in *Fmr1* KO mice

One of the key findings reported in the *Fmr1* KO mouse, between P21-30, is that mGluR-dependent LTD is enhanced in CA1 of the hippocampus (Huber et al. 2002). In contrast NMDAR-dependent LTD, whose expression and maintenance does not require new protein synthesis, is intact (Huber et al. 2002). Here, mGluR-dependent LTD was examined in hippocampal slices from young adult *Fmr1* KO and WT mice (P20-30) with the acute application DHPG. Consistent with previous findings the acute application of 30 μM (Figure 3.4 A) and 100 μM DHPG (Figure 3.4 B) induced a depression of synaptic responses that was significantly greater in *Fmr1* KO slices compared to WT controls (30 μM: WT: 99 ± 9%, n = 9; *Fmr1* KO: 79 ± 9%, n = 11, 100 μM: WT: 77 ± 5%, n = 17; *Fmr1* KO: 63 ± 4%, n = 19), showing a modest effect size of ~ 20% and ~14% between genotypes respectively (Figure 3.4 C). The difference in the magnitude of LTD between *Fmr1* KO and WT mice is in close agreement with the originally findings reported by Huber et al. (2002).

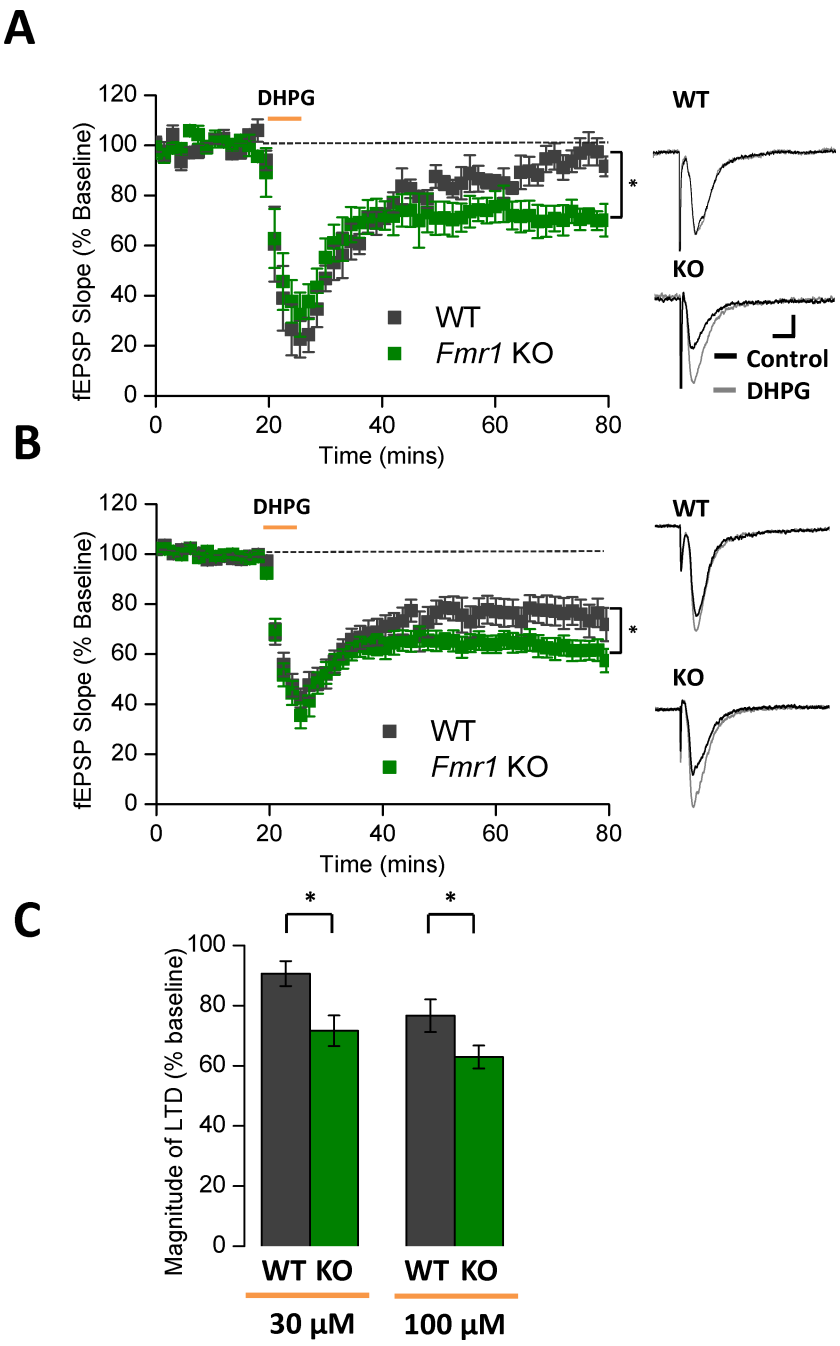


Figure 3.4 mGluR-dependent LTD is enhanced in CA1 of the hippocampus in *Fmr1* KO Mice. Extracellular recordings were performed in CA1 region of hippocampal slices from *Fmr1* KO and WT littermates. (A) The application the Gp1 mGluR agonist DHPG (5 mins, 30 µM) induced a significantly greater depression in the slope of fEPSP in *Fmr1* KO slices compared to WT littermates (WT 91 ± 4%, n = 6; KO 72 ± 5%, n = 7; t-test *p = 0.02). (B) Similarly, application of DHPG (5 mins, 100 µM) induced a significantly greater LTD in *Fmr1* KO and WT littermates (WT 77 ± 5%, n = 17; KO 63 ± 4%, n = 17; t-test *p = 0.04). (C) Summary of LTD magnitudes in *Fmr1* KO and WT littermates induced by the application 30 µM and 100 µM DHPG. Bar chart represents the mean (± S.E.) depression of fEPSP relative to pre-drug baseline. Scale bar 200 µV, 10 ms.

3.4.2 mGluR-dependent LTD is independent of new protein synthesis in *Fmr1* KO mice

In the hippocampus of *Fmr1* KO mice it is reported that mGluR-dependent LTD is insensitive to translational inhibitors, such as anisomycin and cycloheximide (Nosyreva & Huber 2006). This indicates that elevated mGluR-dependent LTD is no longer dependent on new protein synthesis, which supports the role of FMRP as an RNA-binding protein that suppresses translation of its RNA targets at the synapse. Here the dependency of mGluR-dependent LTD was examined in hippocampal slices from *Fmr1* KO and WT littermates (Figure 3.5A). Hippocampal slices were incubated in the translational inhibitor anisomycin (20 μM) for at least 20 minutes prior to the induction of mGluR-dependent LTD, which was present throughout the remainder of the experiment. Anisomycin had no effect on basal synaptic transmission; however immediately following DHPG washout depressed fEPSPs were strongly inhibited in WT slices (WT Anisomycin: 91 ± 5%, $n = 8$). In contrast, anisomycin had no effect on the establishment of LTD in *Fmr1* KO slices (KO anisomycin: 65 ± 7%, $n = 10$) making the differences in LTD magnitude between *Fmr1* KO and WT mice far more evident ($^*p = 0.01$; Figure 3.5B). Thus in agreement with existing findings, DHPG-induced mGluR-dependent LTD is enhanced and independent of new protein synthesis in our mouse model of FXS (Huber et al. 2002; Nosyreva & Huber 2006).

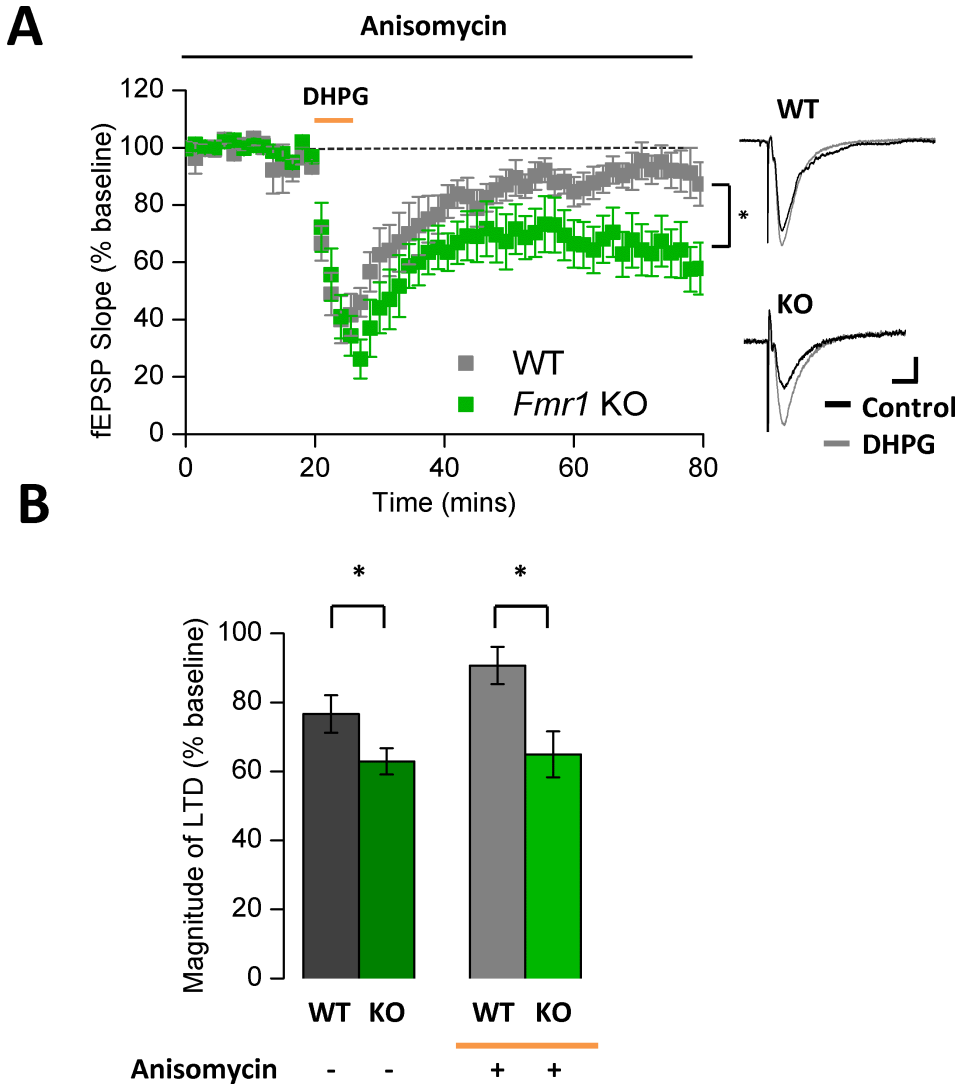


Figure 3.5 mGluR-dependent LTD is Independent of protein synthesis in the hippocampus of *Fmr1* KO mice. (A) In WT slices the protein synthesis inhibitor anisomycin (20 µM) prevented DHPG-induced mGluR-dependent LTD (5 min, 100 µM), whilst the magnitude of LTD in *Fmr1*$^{-/y}$ mutants remained intact (WT 91 ± 6%, n = 8; KO 65 ± 7%, n = 9, t-test *p = 0.01). (B) Summary of LTD magnitude with and without anisomycin in *Fmr1* KO and WT mice. Bar chart represents the mean (± S.E.) depression of fEPSP relative to pre-drug baseline. Scale bar 200 µV, 10 ms.

3.4.3 Basal synaptic transmission is intact *Fmr1* KO mice

Basal synaptic properties, such as input-output function and PPRs, have been previously examined in *Fmr1* KO mice and reported to be intact (Huber et al. 2002; Pilpel et al. 2009). Here we assessed I/O function in *Fmr1* KO mice and WT littermates by increasing the duration of the current pulse applied to the Schaffer collateral pathway from 20-200 μs in 20 μs intervals, plotting the slope of fEPSPs against the amplitude of the presynaptic volley (Figure 3.6A). In agreement with previous reports, basal synaptic transmission is intact in CA1 of young adult *Fmr1* KO mice (Huber et al. 2002). This indicates that the elevation in mGluR-dependent LTD in *Fmr1* KO mouse is not the consequence of general synaptic dysfunction.

PPRs were also examined in *Fmr1* KO and WT littermates by applying two concurrent pulses to the Schaffer collateral pathway at three different pulse intervals, ranging from 20-100 ms (Figure 3.6 B). No significant differences were observed in PPRs between *Fmr1* KO and WT mice at any inter-pulse interval (20 ms: WT 1.65 ± 0.05%, $n = 4$; KO 1.28 ± 0.14%, $n = 5$; 50 ms: WT 1.81 ± 0.07%, $n = 4$; KO 1.57 ± 0.11%, $n = 5$; 100: WT 1.42 ± 0.06%, $n = 4$; KO 1.42 ± 0.07%, $n = 5$; ANOVA, $p > 0.05$), indicating that there does not appear to be a presynaptic change in the absence of FMRP.

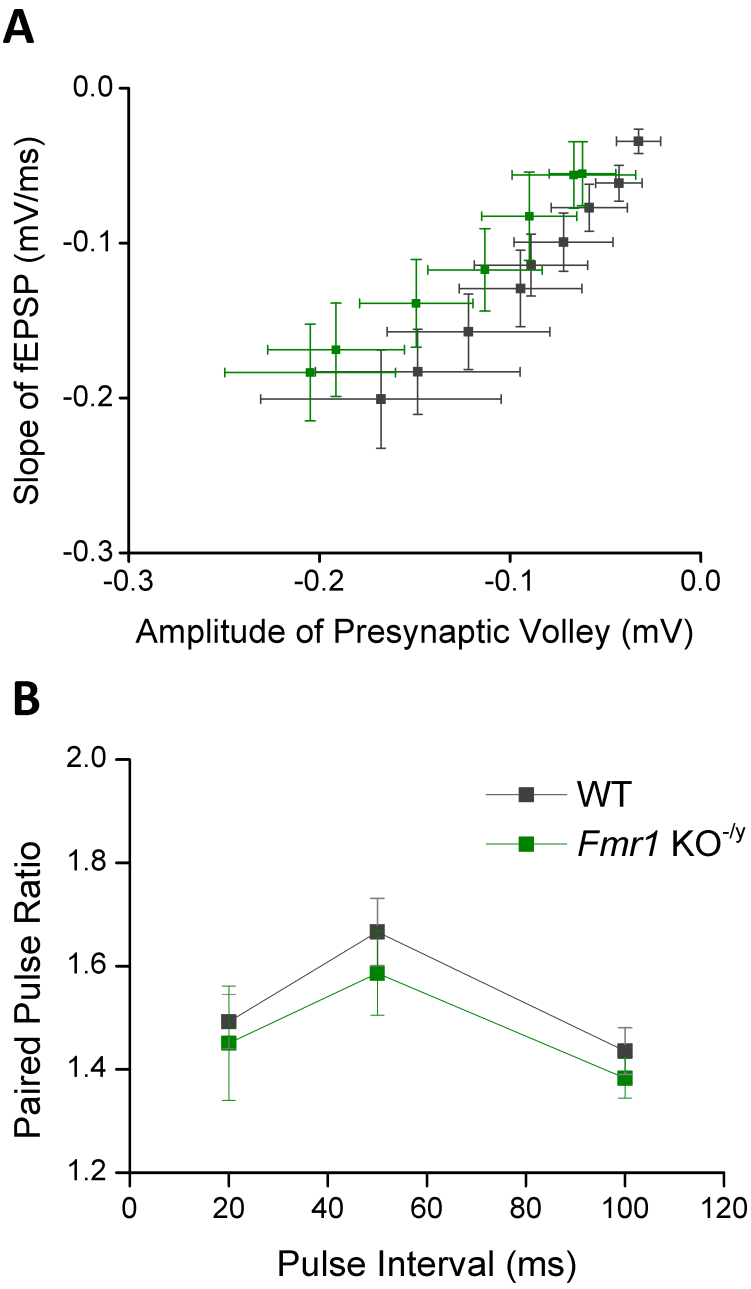


Figure 3.6 Basal Synaptic Transmission is intact in the hippocampus of *Fmr1* KO Mice. Extracellular recordings were performed in CA1 of hippocampal slices from WT and *Fmr1* knockout (KO) mice. (A) Input-output (IO) function was assessed in *Fmr1* KO mice and WT littermates by increasing the duration of the current pulse from 20 to 200 µs with increments of 20 µs. I/O function was intact in the hippocampus of *Fmr1* KO mice compared to WT litters (*t*-test of slopes following linear regression, $p > 0.05$). (B) Application of two concurrent pulses, delivered at 3 different pulse intervals (20, 50 and 100 ms) to the Schaffer collateral pathway leads to facilitation of the second fEPSP slope. Paired-pulse ratios, calculated from the slope of the second pulse to the first, are similar between WT and *Fmr1* KO mice at all three inter-pulse intervals (ANOVA $p > 0.05$).

3.4.4 Basal Protein Synthesis Rates are elevated in *Fmr1* KO Mice

FMRP binds and regulates the translation of several hundred mRNAs (Darnell et al. 2011). Thus in FXS where FMRP is absent, it is hypothesized that mRNA translation is derepressed leading to excessive translation of FMRPs mRNA transcripts (Bear et al. 2004). Basal protein synthesis rates can be measured directly using a radioactive amino acid substitute that can either be administered *in vivo* by injection (Qin et al. 2005a), or *in vitro* by incubating brains slices in ACSF containing a protein synthesis labeling mix, such as ^{35}S-Met/Cys (Dölen et al. 2007; Osterweil et al. 2010). As mRNA is translated the radioactive isotope is incorporated into newly synthesized proteins and can then be quantitatively measured. Using this techniques, both *in vitro* and *in vivo* studies have revealed that protein synthesis rates are elevated in the hippocampus and several other brain structures in the Fmr1 KO mouse, which may explain why mGluR-dependent LTD is no longer dependent on new protein synthesis in this mutant (Qin et al. 2005a; Dölen et al. 2007; Osterweil et al. 2010).

As we were able to reproduce the electrophysiological phenotypes in our mouse model of FXS, we decided to examine protein synthesis rates following the same experimental strategy implored by Osterweil et al. (2010). Basal protein synthesis rates were assessed in dorsal hippocampal slices prepared from *Fmr1* KO and WT littermates at P25-30 by measuring the incorporation of ^{35}S-Met/Cys in to newly synthesized proteins. Figure 3.7A outlines the experiment timeline in which hippocampal slices were prepared from *Fmr1* KO and WT mice. Hippocampal slices were left to recover for 4 hours in ACSF, before transcription was blocked with actinomycin D (25 μM) and protein synthesis measured by incubating slices in ^{35}S Met/Cys protein labelling mix for 45 minutes. In *Fmr1* KO mice ^{35}S incorporation was significantly enhanced in comparison to WT littermates (*Fmr1* KO 121 ± 7%; WT 100 ± 2%; $n = 6$; *t*-test $*p = 0.03$; Figure 3.7 B). The magnitude of this elevated protein synthesis rate in *Fmr1* KOs is in agreement with other *in vitro* measurements in the same region (Osterweil et al. 2010) and those reported in the ventral hippocampus (Dölen et al. 2007), both of which were obtained from *Fmr1* KO on a different strain background.

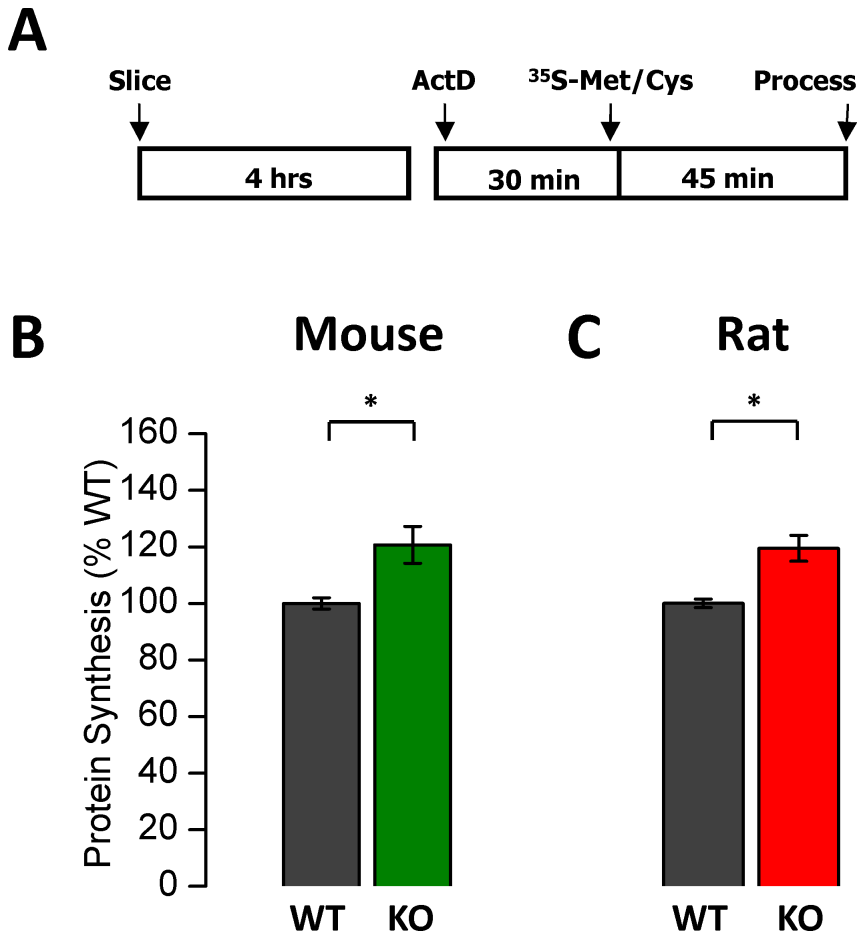


Figure 3.7 Basal protein synthesis rates are elevated in the hippocampus of *Fmr1* KO mice and rats. (A) Schematic of experimental timeline for ^{35}[S]-Met/Cys metabolic labeling: slices were recovered in ACSF for 4 hours, incubated in actinomycin D (Act-D), then protein synthesis was measured with ^{35}[S]-Met/Cys for 45 mins. (B) Basal protein synthesis levels were significantly elevated in dorsal hippocampal slices from *Fmr1* KO *versus* WT mice (*Fmr1*$^{-/y}$: 121 ± 7%; WT: 100 ± 2%; n = 6; t-test *p = 0.03). (C) Increased protein synthesis rates were also observed in hippocampal slices prepared from *Fmr1* KO *versus* WT rats (KO: 119 ± 5%; WT: 100 ± 2%; t-test *p = 0.022; n = 8).

3.5. Results from the *Fmr1* KO Rat

3.5.1 *Fmr1* KO Rat recapitulates core deficits reported in the mouse model of FXS

Recently SAGE laboratories generated the rat model of FXS using a zinc-finger nuclease methodology (Cui et al. 2011; Hamilton et al. 2014). Here we investigated whether the core physiological and biochemical phenotypes reported in the *Fmr1* KO mouse translate to the rat model of FXS. mGluR-dependent LTD and its dependence on new protein synthesis were assessed in the hippocampus of *Fmr1* KO rats. Consistent with the *Fmr1* KO mouse, mGluR-dependent LTD (50 μM) in *Fmr1* KO rats was enhanced and insensitive to the translational inhibitor cycloheximide (100 μM) at CA1 synapses (Personal communication, A. Jackson). Furthermore basal protein synthesis levels in dorsal hippocampal slices from young adult *Fmr1* KO rats (P25-32) were exaggerated compared to WT littermates (*Fmr1* KO 119 ± 5%; WT 100 ± 2%; $n = 11$; *t*-test $*p = 0.002$; Figure 3.7 C). This indicates that the core hippocampal phenotypes reported in in the mouse model of FXS translate to other mammalian species. Thus, it would be interesting to see whether pharmacological strategies employed in correcting deficits in the *Fmr1* KO mouse are efficacious in the *Fmr1* KO rats.

3.6. Results in *Syngap* Heterozygote Mouse

3.6.1 mGluR-dependent LTD is enhanced in *Syngap* heterozygote mice

Electrophysiological recordings in the hippocampus of *Syngap* heterozygous mice have revealed impairments in NMDAR-dependent forms of LTP and LTD (Komiyama et al. 2002; Kim et al. 2003; Carlisle et al. 2008). At present, mGluR-dependent forms of synaptic plasticity have not been investigated in *Syngap* heterozygous mice even though signaling downstream of $mGlu_5$ receptors is hyperactivity in the hippocampus of *Syngap* heterozygous mice.

To examine mGluR-dependent LTD at Schaffer collateral synapses in CA1, extracellular recordings were performed in horizontal hippocampal slices from *Syngap* heterozygotes and WT littermates. Here it was observed that the application of DHPG (30 μM) induced a depression of synaptic responses that was significantly increased in *Syngap* heterozygous mice compared to WT littermates (30 μM: WT 90.9 ± 2.88%, $n = 10$ *vs.* Het 75 ± 6%, $n = 12$, *t*-test $*p = 0.02$). Similarly, induction of mGluR-dependent LTD with 100 μM DHPG elicited a significantly greater LTD in *Syngap* heterozygous mice compared to WT littermates (100 μM: 74 ± 6%, $n = 12$ *vs.* Het 56 ± 5%, $n = 14$, *t*-test $*p = 0.03$). Thus consistent with *Fmr1* KO mice reduced SynGAP expression results in enhanced depression of synaptic responses upon activation of the Gp1 mGlu receptors. Furthermore the magnitude of LTD observed in both WT and *Syngap* heterozygous mice shows a DHPG dose-dependent relationship.

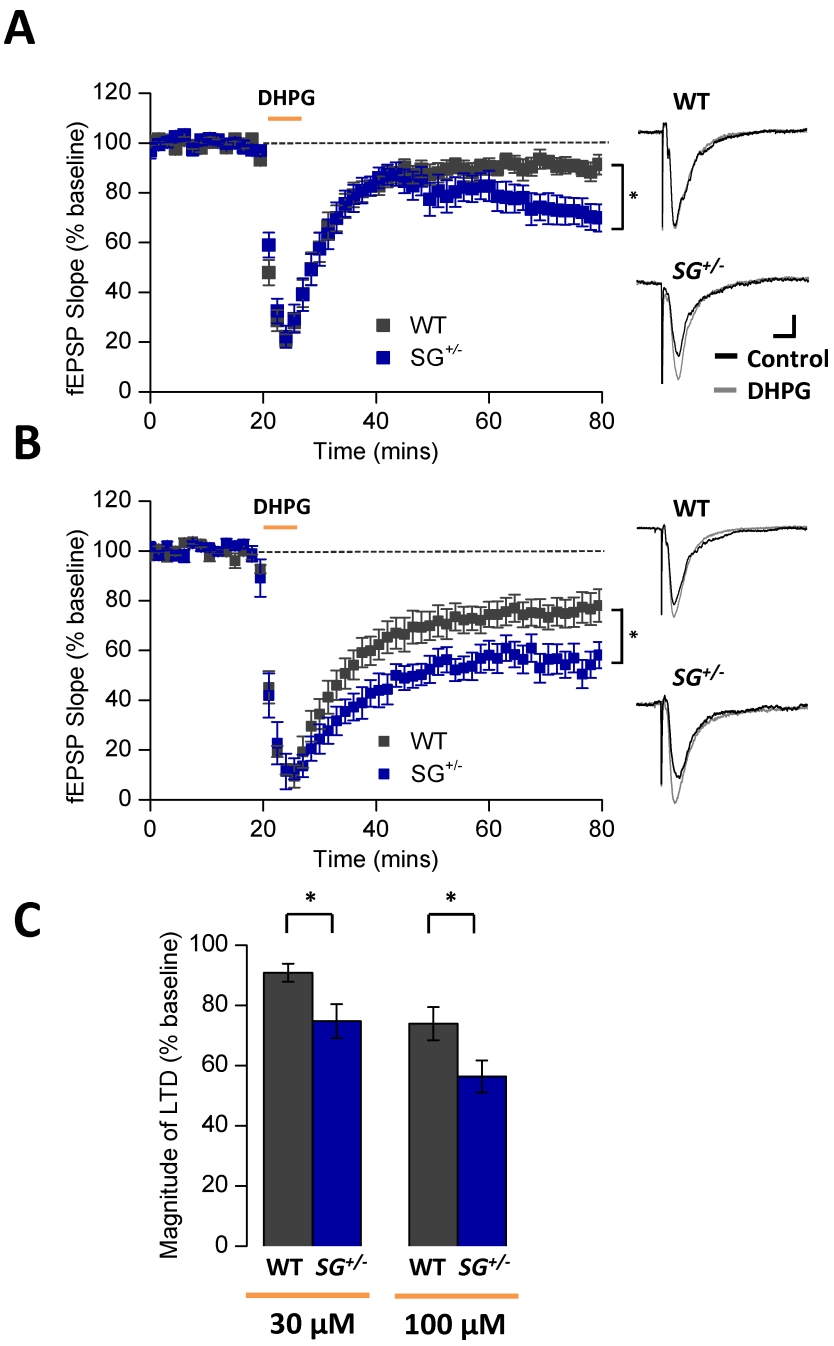


Figure 3.8 DHPG-induced mGluR-dependent LTD is enhanced in the hippocampus of *Syngap* heterozygous mice. Extracellular recordings were performed in CA1 region of hippocampal slices from *Syngap* heterozygous ($SG^{+/-}$, Het) and WT littermates. (A) The application of DHPG (5 mins, 30 µM) induced LTD that was significantly increased in *Syngap* het mice *versus* WT littermates (WT 91 ± 3%, $n = 10$; $SG^{+/-}$ 75 ± 6%, $n = 12$; *t*-test $*p = 0.02$) (B) Similarly, the application of DHPG (5 mins, 100 µM) induced a significantly greater LTD in *Syngap* het hippocampal slices compared to WT littermates (WT 74 ± 6%, $n = 12$; $SG^{+/-}$ 56 ± 5%, $n = 14$; *t*-test $*p = 0.03$). (C) Summary of LTD magnitude induced by the application of 30 µM and 100 µM DHPG in *Syngap* heterozygous and WT mice. Bar chart represents the mean (± S.E.) depression of fEPSP relative to pre-drug baseline. Scale bar 200 µV, 10 ms.

3.6.2 mGluR-dependent LTD is independent of new Protein synthesis in *Syngap* heterozygous mice

At the synapse, SynGAP down regulates Ras activated ERK1/2 and mTOR signalling, both of which initiate cap-dependent mRNA translation that is required to maintain long-lasting forms of synaptic plasticity. In *Syngap* heterozygotes where SynGAP expression is reduced, the activity of Ras and ERK1/2 are increased (Komiyama et al. 2002; Carlisle et al. 2008), which could lead to the over-activation of the translational machinery governing basal protein synthesis rates. Together with the finding that mGluR-dependent LTD is enhanced in CA1 of the hippocampus, one would predict that activity-dependent protein synthesis at local dendritic sites may be lost in the *Syngap* heterozygous mice.

To examine the consequence of *Syngap1* haploinsufficiency on the protein synthesis-dependent component of mGluR-dependent LTD, slices were preincubated in the translational inhibitor anisomycin (20 μM) for at least 20 minutes prior to LTD induction. In WT mice anisomycin inhibited mGluR-dependent LTD induced by the acute application of DHPG (50 μM), whilst vehicle treated slices showed a typical level of depression (WT veh 77.0 ± 3.3%, n = 18 *vs.* WT aniso 90 ± 4%, n = 12, t-test *p = 0.01; Figure 3.9 A). In contrast, inhibition of translation in hippocampal slices from *Syngap* mutants had no significant effect on the magnitude of LTD and synaptic responses remained depressed to a similar level as vehicle treated slices (Het veh 62 ± 5 %, n = 15 *vs.* Het aniso 64 ± 4 %, n = 14, t-test p = 0.728, Figure 3.9 B). Thus in the presence of anisomycin the differences in LTD magnitude between genotypes was far more pronounced (*p = 0.000096; Figure 3.9 C). This indicates that mGluR-dependent LTD is independent of new protein synthesis in the hippocampus of *Syngap* heterozygous mice. This situation could arise, if the proteins required for the maintenance of LTD are already present and overabundant at CA1 synapses expressing reduced levels of SynGAP.

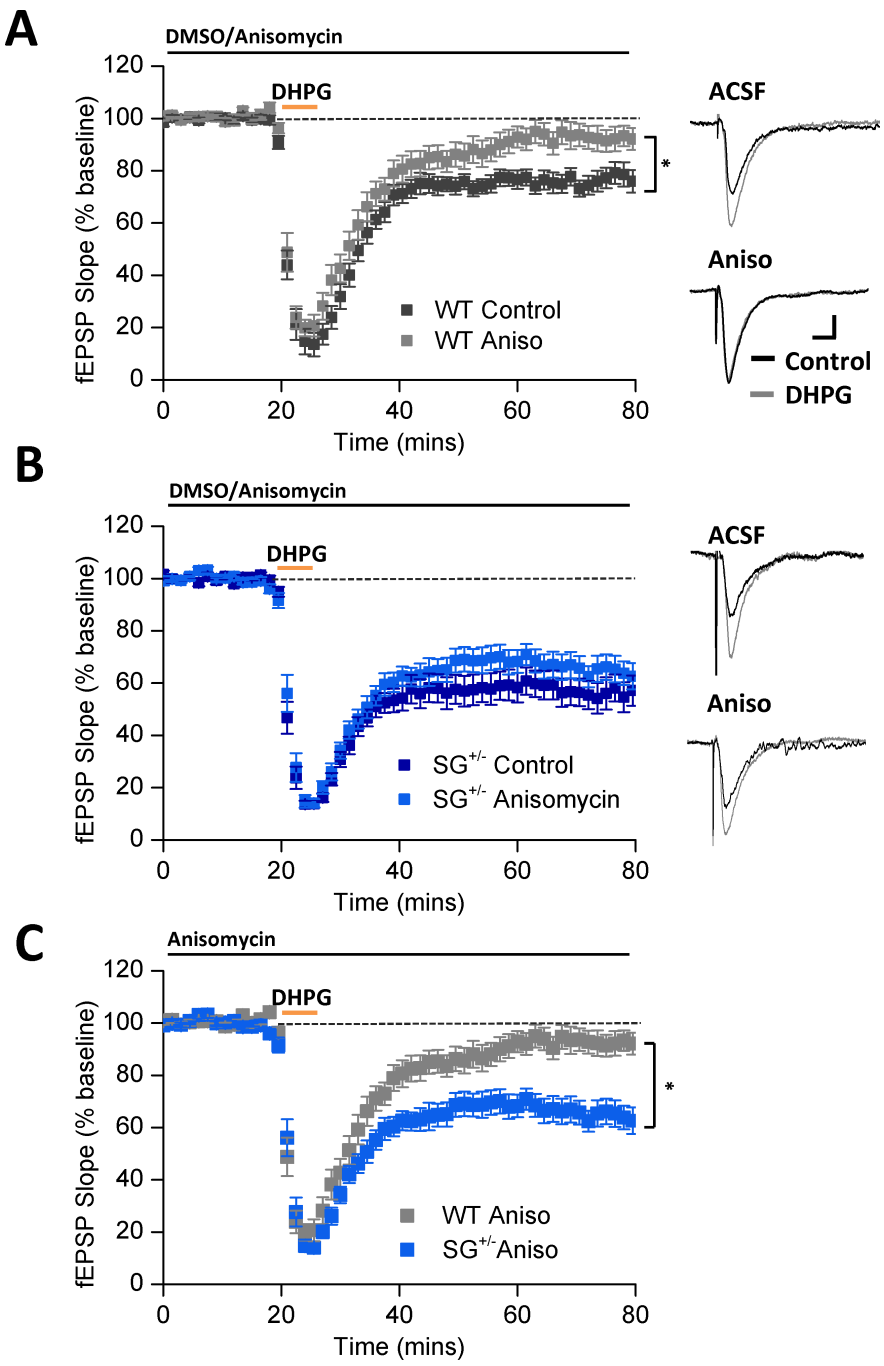


Figure 3.9 mGluR-dependent LTD is independent of protein synthesis in the hippocampus of *Syngap* heterozygous mice. (A) In WT slices the protein synthesis inhibitor anisomycin (aniso, 20 µM) prevented the expression of DHPG-induced mGluR-dependent LTD (5mins, 50 µM) in CA1 of the hippocampus (WT veh 77.0 ± 3 %, n = 18 *vs.* WT aniso 90 ± 4 %, n = 12, t-test *p = 0.01). In contrast, DHPG-induced mGluR-dependent LTD was intact in the presence of anisomycin in CA1 of *Syngap* heterozygous ($SG^{+/-}$, Het) slices ($SG^{+/-}$ veh 62 ± 5 %, n = 15 *vs.* $SG^{+/-}$ aniso 64 ± 4 %, n = 14, t-test p = 0.728). (C) Comparison of DHPG induced mGluR-dependent LTD in the presence of anisomycin in WT and het hippocampal slices (t-test *p = 0.000096). Scale bar 200 µV, 10 ms.

3.6.3 Basal synaptic transmission is intact in *Syngap* heterozygous mice

In *Syngap* heterozygous mice there is dramatic increase in AMPA receptor mediated synaptic transmission during the early stages of development (Clement et al. 2012). This abnormal increase in synaptic strength is observed at multiple synapses in the hippocampus and arose from an increase in AMPA receptor expression at excitatory synapses. This was initially detected as a difference in I/O function in hippocampal slices from *Syngap* heterozygous and WT mice. However by P21, basal synaptic transmission in *Syngap* heterozygous mice is indistinguishable from WT mice, as AMPA receptor expression increases at WT synapses. This indicates that *Syngap1* haploinsufficiency results in an early maturation of synapses.

Here I/O function was examined at Schaffer collateral synapses in CA1 of hippocampal slices from young adult *Syngap* heterozygous and WT mice (P25-32). Consistent with previous reports there was no detectable difference in basal synaptic transmission between *Syngap* heterozygous and WT mice at adult ages (*t*-test of slopes following linear regression, $p > 0.05$) (Clement et al. 2012; Komiyama et al. 2002).

Presynaptic function was also examined in CA1 of young adult *Syngap* heterozygous and WT slices. No detectable differences (ANOVA, $p > 0.05$) were observed in the PPR at any inter-pulse interval: 20 ms (WT 1.80 ± 0.16%, $n = 13$; Het 1.91 ± 0.21%, $n = 11$), 50 ms (WT 1.53 ± 0.06%, $n = 13$; Het 1.67 ± 0.15%, $n = 11$) and 100 ms (WT 1.41 ± 0.04%, $n = 13$; Het 1.40 ± 0.07%, $n = 11$).

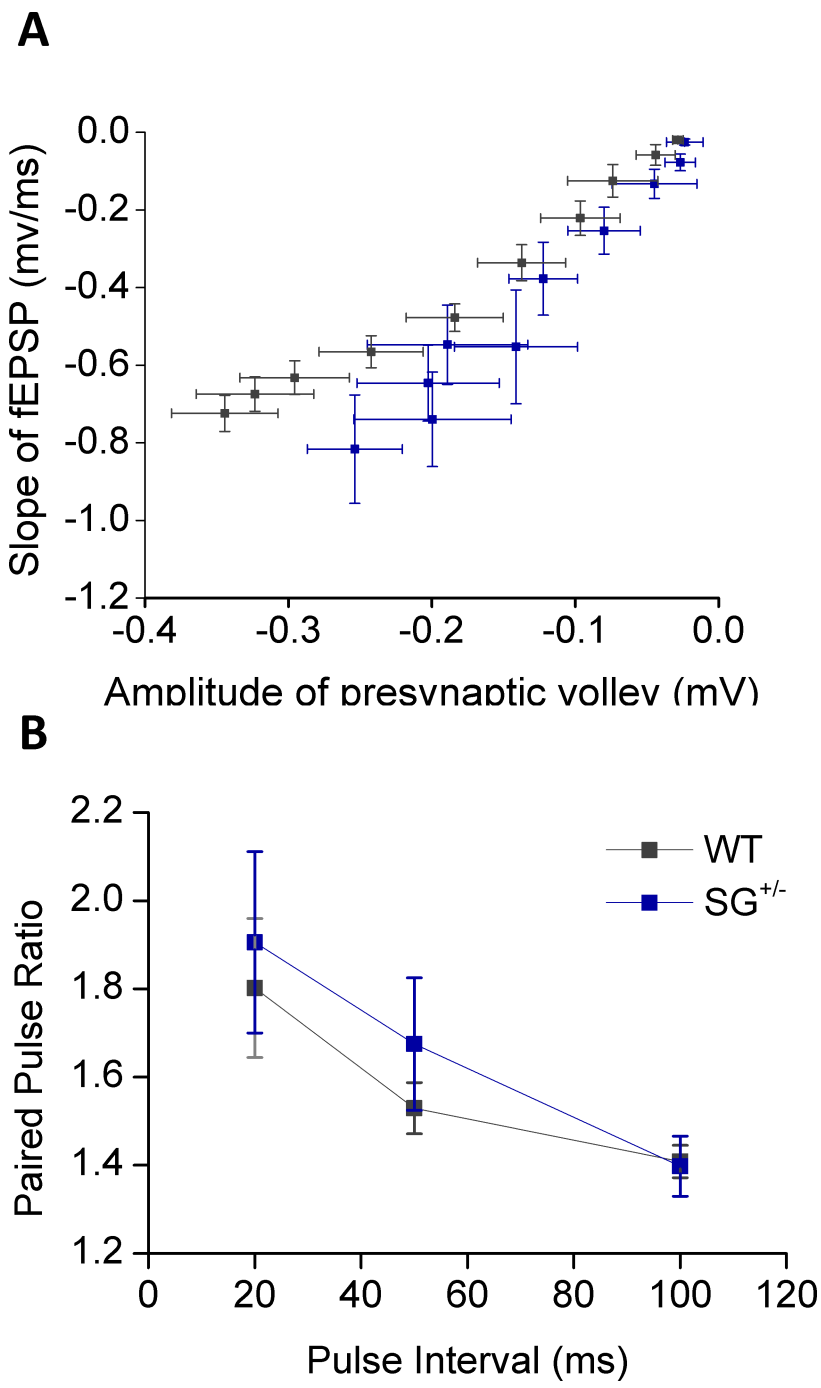


Figure 3.10 Basal synaptic transmission is unaltered in the hippocampus of *Syngap* heterozygous mice. (A) Input-output (IO) curves were generated by plotting the fibre volley amplitude and slope of fEPSPs against the slope of the fEPSPs elicited by increasing the pulse duration from 20-200 µs. I/O function was intact in the hippocampus of *Syngap* heterozygous (*SG* $^{+/-}$, Het) mice compared to WT litters (*t*-test of slopes following linear regression, $p > 0.05$). (B) The application of two concurrent pulses with an inter-pulse interval of 20, 50 and 100 ms to the Schaffer collateral pathway generated similar paired pulse facilitation in WT and *Syngap* het mice at all three inter-pulse intervals (ANOVA $p > 0.05$).

3.6.4 Basal protein synthesis levels are elevated in *Syngap* heterozygous mice

So far the electrophysiological findings in *Syngap* heterozygous mice are comparable to those observed in *Fmr1* KO mice; mGluR-dependent LTD is enhanced and independent of new protein synthesis in area CA1 of the hippocampus. The increased LTD in *Fmr1* KO mice correlates with elevated protein synthesis rates that are regulated by $mGlu_{1/5}$ receptor activation via the Ras-ERK1/2-MAPK signalling cascade. Thus, basal protein rates were examined in hippocampal slices from young adult *Syngap* heterozygous mice to determine whether the independency of mGluR-dependent LTD to new protein synthesis resulted from increased translational rates under steady state conditions. An identical approach was used to that reported in 3.5.4. Consistent with the *Fmr1* KO mice, the incorporation of ^{35}S Met/Cys was significantly increased in dorsal hippocampal slices from *Syngap* heterozygous mice compared to wild-type littermates (WT 100 ± 4%; Het 140 ± 12%; $n = 12$; *t*-test $*p = 0.02$). Furthermore, the increase in ^{35}S incorporation was substantially higher in *Syngap* heterozygous mice compared to *Fmr1* KO mice, 40% in *Syngap* heterozygotes *versus* 20% in *Fmr1* KOs.

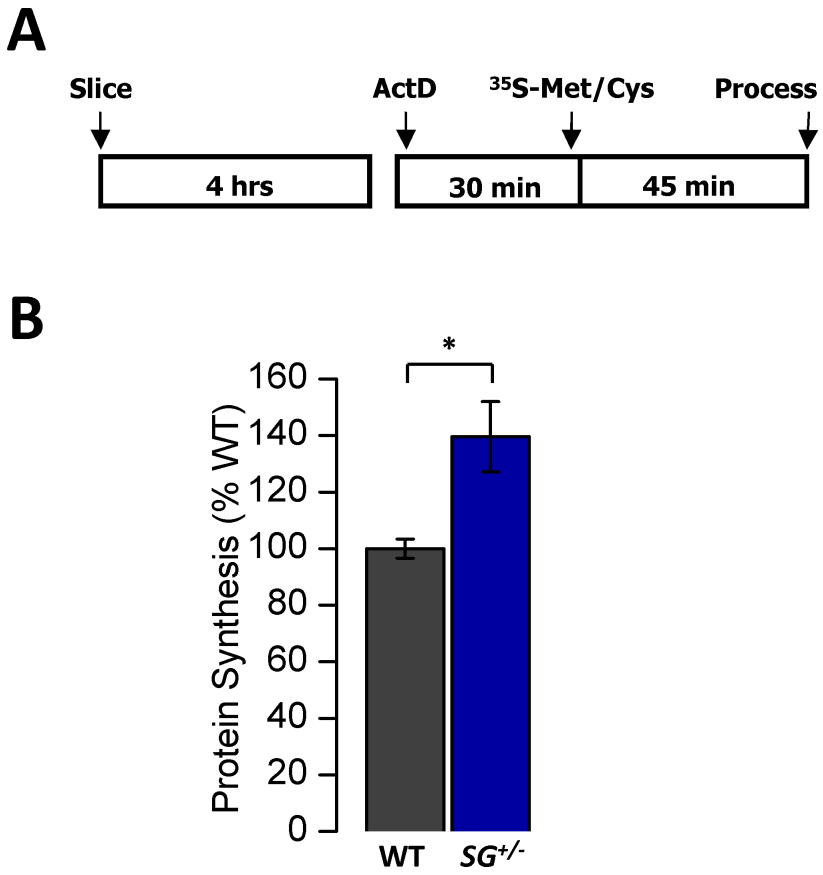


Figure 3.11 Basal protein synthesis rates are elevated in the hippocampus of *Syngap* heterozygous mice. (A) Schematic of experimental timeline for ^{35}[S]-Met/Cys metabolic labeling: slices were recovered in ACSF for 4 hours, incubated in actinomycin D (Act-D), then protein synthesis measured with ^{35}[S]-Met/Cys for 45 mins. (B) Basal protein synthesis levels were significantly elevated in dorsal hippocampal slices from *Syngap* heterozygous (het, *SG* $^{+/-}$) mice relative to WT controls (WT 100 ± 4%; *SG* $^{+/-}$ 140 ± 12%; $n = 12$; *t*-test $*p = 0.02$).

3.7 Results in the *Fmr1*$^{-/y}$ *Syngap* $^{+/-}$Double Mutant

3.7.1 *Syngap1* haploinsufficiency occludes mGluR-mediated LTD in *Fmr1* KO mice

Our findings that two distinct ID/ASD-related mutations causing FXS and *Syngap* haploinsufficiency mice share similar alterations in mGluR-dependent LTD raised the intriguing possibility that they may converge on the same pathophysiological axis. To directly test this hypothesis, we generated double mutant mice by introducing the deletion of *Fmr1* in to the *Syngap* heterozygous mouse. To determine whether *Syngap1* haploinsufficiency exacerbates or occludes the enhanced LTD phenotype observed in *Fmr1* KO mouse, mGluR-dependent LTD was re-examined at CA1 synapses of all four genotypes produced from this cross (Figure 3.12A). Consistent with our previous findings, mGluR-dependent LTD was increased in *Fmr1* KO (*Fmr1* KO 62 ± 5, $n = 10$, $p = 0.08$) and *Syngap* heterozygous (*Syngap* Het 60 ± 4%, $n = 10$, ANOVA $^*p = 0.049$) single mutants compared to WT litters (WT 78 ± 3%, $n = 9$). Similarly mGluR-dependent LTD was found to be significantly enhanced in the *Syngap* heterozygous *Fmr1* KO double mutants relative to WT littermates (Double 59 ± 4; $n = 12$, ANOVA $^*p = 0.02$), however not significantly different from *Fmr1* KO and *Syngap* heterozygous single mutants ($p > 0.05$; Figure 3.12B). As the magnitude of LTD was similar between the double mutant and *Fmr1* KO and *Syngap* heterozygous single mutants it suggests that *Syngap1* haploinsufficiency occludes the increase in DHPG-induced mGluR-dependent LTD caused by the loss of FMRP. This indicates that mutations in *Syngap1* and *Fmr1* converge on the same pathophysiological axis.

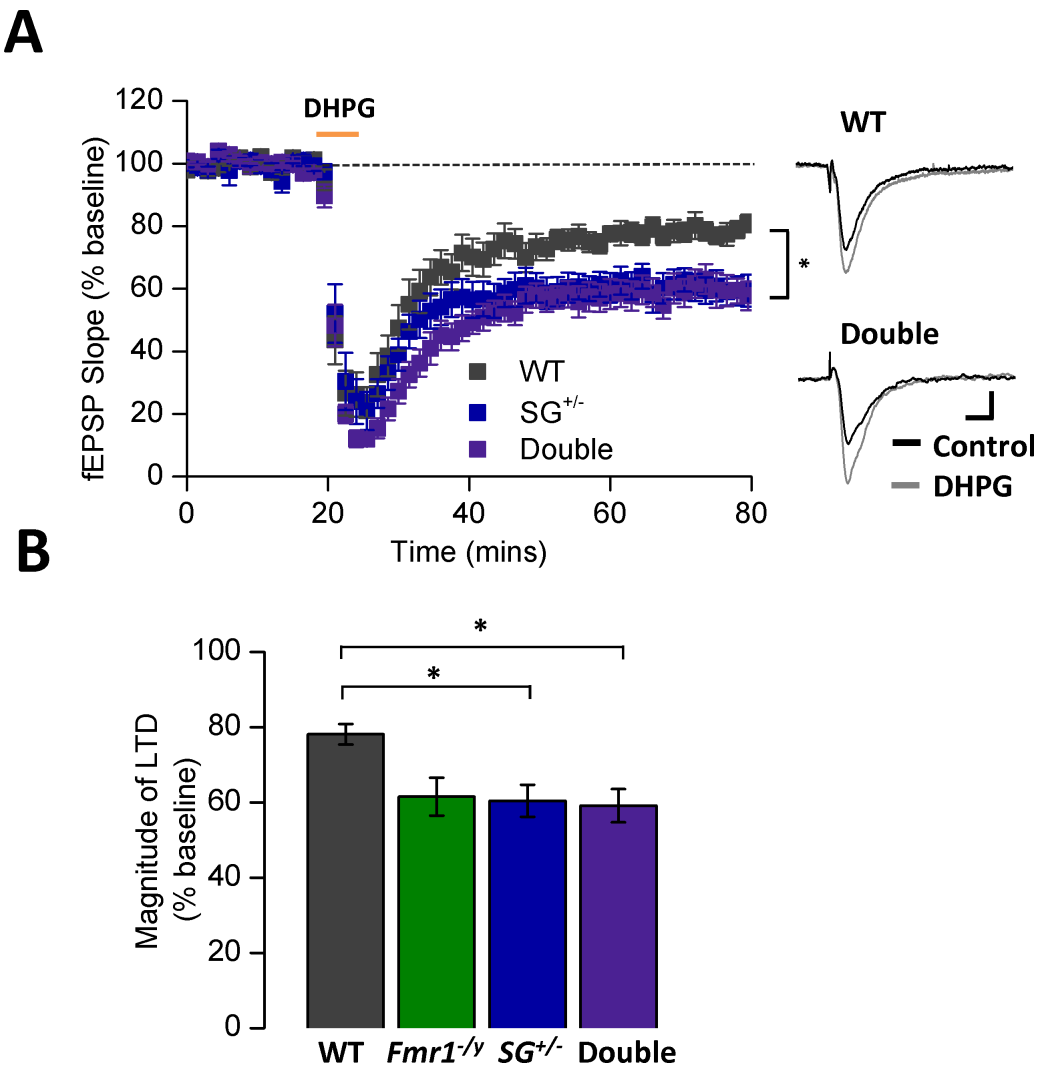


Figure 3.12 *Syngap* haploinsufficiency occludes the increase in mGluR-dependent LTD in the hippocampus in *Fmr1* KO mice. (A) The application of the Gp1 agonist DHPG (5 mins, 100 µM) induced a depression of fEPSPs in $SG^{+/-}$ $Fmr1^{-/y}$ double mutants (59 ± 4%, n = 12) that was significantly greater than the depression observed in WT littermates (WT 78 ± 3%; n = 9, ANOVA *p = 0.02). Yet, the magnitude of LTD observed in the $SG^{+/-}$ $Fmr1^{-/y}$ double mutants was not significantly different from $SG^{+/-}$ (60 ± 4%, n = 10, ANOVA *p = 0.049) and $Fmr1^{-/y}$ (62 ± 5%, n = 10, ANOVA p = 0.08) single mutant mice. (B) Summary of LTD magnitude for all four genotypes. Bar chart represents the mean (± S.E.) depression. Scale bar 200 µV, 10 ms.

3.7.2 DHPG induces a non-saturating level of mGluR-dependent LTD

The introduction of the *Fmr1* mutation into *Syngap* heterozygous mice occluded further depression of synaptic responses induced by the acute application of DHPG (100 μM). However this could arise if the concentration of DHPG used was inducing a saturating level of LTD. To determine whether the finding reported in 3.7.1 resulted from a "floor effect", a subsequent induction of chemical LTD was performed 60 minutes after the first to establish whether DHPG was causing a saturating level of LTD following the first induction (Figure 3.13). In all genotypes, the magnitude of depression was greater following the second application of DHPG: WT (1st 81 ± 3%; 76 ± 6%; $n = 8$, *t*-test $*p = 0.03$); *Fmr1* KOs (1st 73 ± 4%; 2nd 61 ± 5%; $n = 3$, $*p = 0.002$); *Syngap* heterozygous (1st 68 ± 7%; 2nd 51 ± 9%; $n = 5$, $*p = 0.02$) and double mutants (1^{st} 68 ± 11%; 2^{nd} 55 ± 8%; $n = 4$, $p = 0.0996$). These findings indicate that following DHPG-induced mGluR-dependent LTD synapses can still be further depressed by second induction of chemical LTD suggesting that the same intracellular mechanism is utilized in *Fmr1* KO and *Syngap* heterozygous mice to evoke an exaggerated depression of synaptic responses in CA1.

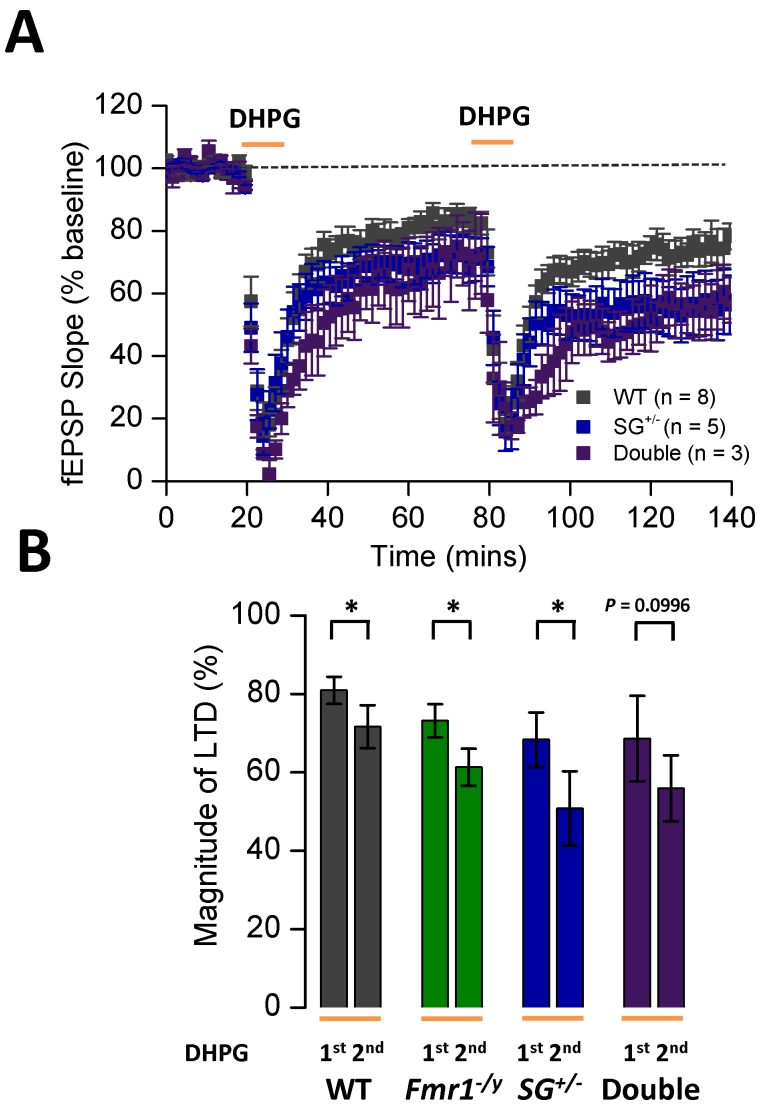


Figure 3.13 DHPG induces a non-saturating level of mGluR-dependent LTD. Extracellular recordings in CA1 region of hippocampal slices from WT, *Syngap* heterozygous (Het, $SG^{+/-}$), *Fmr1* knockout (KO, $Fmr1^{-/y}$) and *Fmr1* KO and *Syngap* heterozygous cross (Double, $Fmr1^{-/y}$ x $SG^{+/-}$). (A) The application of the Gp1 agonist DHPG (5 mins, 100 µM) induced a depression of fEPSPs, that were further reduced following a second application of DHPG (5 mins, 100 µM) 1 hr. after the initial LTD induction. (B) Summary of LTD magnitude for all four genotypes. Bar chart represents the mean (± S.E.) depression of fEPSP relative to pre-drug baseline for all four genotypes after the 1st and 2nd application of DHPG. Significance differences between LTD magnitude induced by the 1st and 2nd application of DHPG were observed in WT (1st 81 ± 3%; 2nd 81 ± 3%; $n = 8$, $*p = 0.03$), $Fmr1^{-/y}$ (1st 73 ± 4%; 2nd 61 ± 5%; $n = 3$, $*p = 0.002$) and $SG^{+/-}$ (1st 68 ± 7%; 2nd 51 ± 9%; $n = 5$, $*p = 0.02$) mice, determined using a paired *t*-test ($p < 0.05$). Although the magnitude of LTD increased between the 1st and 2nd LTD stimulus in the double mutant slices this was not significant (1st 68 ± 11%; 2nd 55 ± 8%; $n = 4$, $*p = 0.0996$).

3.8 Discussion

In this chapter, the physiological properties of Gp1 mGluR-dependent LTD and protein synthesis have been investigated in CA1 of the hippocampus. Furthermore, these $mGlu_{1/5}$ mediated cellular processes have been examined in the context of two highly penetrant forms ID and ASD, FXS and *Syngap1* haploinsufficiency. It is already suspected that distinct genetic causes of ID may share a common pathophysiological axis. However this hypothesis is based on gene ontology databases of known protein function and on the observation that human ID patients and mouse models of ID can share similar phenotypic manifestations. Here, it was directly tested to determine whether two pathogenic mutations of ID, in *Fmr1* and *Syngap1,* share similar abnormalities in synaptic plasticity and protein synthesis by converging on a shared biochemical pathway.

3.8.1 Physiological properties of mGluR-dependent LTD

In CA1 of the hippocampus mGluR-dependent LTD is typically induced either: pharmacologically, by the application of orthosteric agonists of $mGlu_{1/5}$ receptors such as DHPG (Palmer et al. 1997); or synaptically, by repeatedly applying paired-pulses at 1 Hz for 15 minutes to the Schaffer collateral pathway (Huber et al. 2000; Kemp & Bashir 1999). The induction and expression mechanisms of this form of LTD differ over development (Nosyreva et al. 2005) and include both presynaptic and postsynaptic mechanisms.

Induction of mGluR-dependent LTD involve the activation of $mGlu_1$ and $mGlu_5$ receptors, which are predominantly expressed on the postsynaptic side of both excitatory and inhibitory neurones (Nicoletti et al. 2011). At dendritic spines $mGlu_{1/5}$ receptors are found at both perisynaptic and extrasynaptic sites and are activated following prolonged synaptic transmission (Pin et al. 2005). Here they interact with scaffolding proteins, such as Homer, which in turn couple $mGlu_5$ receptors to the GluN2B subunit of the NMDA receptor via a link of scaffolding proteins, which include Shank and PSD-95 (Collett & Collingridge 2004).

Gp1 mGlu receptors are coupled to $G_{q/11}$ heterotrimeric G-proteins whose activity stimulates the hydrolysis of PIP_2 by phospholipase C (PLC) producing IP_3 and DAG. In turn IP_3 and DAG activate Ca^{2+} release from intracellular stores and protein kinase C (PKC) (Fitzjohn & Collingridge 2001; Hermans & Challiss 2001). However, it appears that mGluR-dependent LTD does not use this canonical pathway as the induction of this form of LTD is insensitive to PKC inhibitors (Hou & Klann 2004; Gallagher et al. 2004) and occurs independently of changes in postsynaptic Ca^{2+} (Fitzjohn & Collingridge 2001). Instead, mGluR-dependent LTD is dependent on Ca^{2+}-independent mechanisms that include the PI3K, p38 MAPK and ERK1/2 MAPK intracellular signalling cascades (Hou & Klann 2004; Gallagher et al. 2004; Moult et al. 2008).

The expression of mGluR-dependent LTD at CA1 synapses involves the endocytosis of ionotropic AMPA receptors from the cell surface (Moult et al. 2006; Snyder et al. 2001). In addition to postsynaptic changes, the expression of this form LTD is accompanied by presynaptic alterations that are likely mediated by retrograde signalling. Together, these expression mechanisms result in a long-lasting weakening of synaptic inputs to CA1 pyramidal neurones that is maintained by new protein synthesis at local dendritic sites (Huber et al. 2000).

Electrophysiological recordings in CA1 can capture the depression of synaptic transmission following an LTD inducing stimulus. This is observed as a change in the slope and amplitude of the corresponding fEPSP or EPSC to the same applied stimulus depending on whether extracellular or intracellular recordings are performed. Intracellular recordings of mGluR-dependent LTD commonly use whole-cell recordings, which involve voltage clamping cells to −70 mV and observing a decrease in EPSC amplitude following a 5-minute application of DHPG. In certain brain regions, such as the amygdala, where the dendrites of neurones do not form a uniform layer, intracellular recordings are the only way in which to measure changes in synaptic strength (Suvrathan et al. 2010).

In contrast, the hippocampus has a highly structured and orderly arrangement of cell and dendritic layers. Here extracellular recordings are possible and provide several advantages. Firstly, it measures the summation of postsynaptic potentials generated from a population of CA1 apical dendrites to an evoked stimulus in the presynaptic cell, rather than the response of single cell. Secondly, extracellular recordings leave the internal environment of the neuron in tact whereas and whole-cell recordings involve washing out the internal environment of a cell and replacing it with internal solution. This could disrupt intracellular signalling cascades that are crucial for the induction, expression and maintenance of mGluR-dependent LTD. One way to counteract this disadvantage is to perform perforated patch recordings, which would cause minimal disruption to the internal environment of the target cell. However in this chapter extracellular recordings were exclusively used to measure mGluR-dependent LTD in CA1 synapses of the hippocampus.

To facilitate the induction of mGluR-dependent LTD in hippocampal slices, the $GABA_A$ receptor antagonist picrotoxin (PTX, 50 μM) was added to the ACSF during electrophysiological recordings. PTX eliminated any influence from the inhibitory GABAergic synapses that may be disrupted in mouse models of ID (Adusei et al. 2010; Clement et al. 2012). Furthermore, blocking inhibitory synaptic transmission has been previously shown to increase the overall excitability of the slice, lowering the threshold for induction of mGluR-dependent LTD and inducing a form of LTD that is independent of NMDA receptor activation (Palmer et al. 1997). This suggests that when chemical mGluR-dependent LTD is induced under conditions that increase neuronal excitability then perhaps Ca^{2+} entry is sufficient, without NMDA receptor activation, so NMDA receptor antagonists have little effect on LTD. However, to eliminate any potential confounding factor by NMDA receptor activation, these receptors were blocked during the induction of mGluR-dependent LTD.

In addition, during preparation of hippocampal slices an incision was made through CA3:CA1 boundary. This prevented the generation of epileptiform activity in CA1 following the stimulation of the Schaffer collateral pathway; the probability of which was far greater when inhibitory input to CA1 was blocked with PTX. The occurrence of epileptic activity in CA1 is caused by recurrent excitatory loops in CA3, as axons not only project to CA1 but also to other levels of CA3.

In this chapter, mGluR-dependent LTD was initially evoked both chemically and by synaptic stimulation. Here we observed that the induction of mGluR-dependent LTD with DHPG (>50 μM) or by the application of 900 paired-pulses induced a long lasting form depression of excitatory transmission at Schaffer collateral synapses in young adult mouse. DHPG-induced mGluR-dependent LTD shares a common expression mechanism with PP-LFS (Huber et al. 2001). However, induction of mGluR-dependent LTD with PP-LFS is considered more physiologically relevant, as DHPG-induced mGluR-dependent LTD does not require concurrent stimulation of the presynaptic terminus.

Initial investigations in hippocampal slices from young adult mice utilised both LTD stimulation paradigms. However, it was found that a depression of fEPSPs of 10% or more was not consistently observed using the synaptic induction protocol. It has been previously reported that PP-LFS induces a “reliable” depression of fEPSPs in CA1 of the rat hippocampus (Kemp & Bashir 1997), which may not translate to the mouse. Discussions with other laboratories regarding this form of mGluR-dependent LTD revealed that I was not isolated in observing a high LTD failure rate when using this stimulation paradigm. Two independent laboratories had tried various manipulations of the stimulation protocol to improve LTD success rate. These included: (1) increasing baseline responses to 60-70% of the maximum response, (2) increasing the number of paired pulses from 900 pulses to 1200, or (3) increasing the frequency at which the pulses were delivered to the Schaffer collateral pathway. To try and optimise experimental conditions the stimulation intensity of the current pulse was increased to 70% of the maximum fEPSP but no significant level of improvement was observed to warrant further investigation.

In contrast, DHPG-induced mGluR-dependent LTD consistently induced a depression of synaptic responses, most likely because chemical induction maximises the number of synapses affected and thus induces a more robust form of LTD. This induction protocol also provided several other advantages because: (1) on average DHPG (50-100 μM) induced a greater magnitude of LTD, (2) it would allow us to assess the affect of protein synthesis inhibitors on LTD expression, and (3) this form of stimulation paradigm could be translated to biochemical studies at a later stage. The latter point was of particular importance, as we wanted examined the dependence of mGluR-dependent LTD on new protein synthesis using the translational inhibitor anisomycin, which abolished LTD in WT hippocampal slices. Without a reliable form of LTD, it would have been difficult to assess whether the lack of LTD resulted from a failed LTD expression or the inhibition of new protein synthesis. Thus to progress with our investigations in to mGluR-dependent LTD mechanisms in rodent models of ID, pharmacological LTD induction protocols were solely used.

During chemical induction of LTD, the activation of Gp1 mGlu receptors with DHPG (50 – 100 μM) leads to an initial reduction in synaptic transmission that is caused by a transient membrane depolarisation (TMD). This TMD results from an increase in the membrane input resistance that occurs independently of changes in fEPSPs and leads to increased neuronal firing (Brager & Johnston 2007). It is a associated with a decrease in I_h that could reduce inactivation of T- and N- type calcium channels thus increasing neuronal excitability and preventing the loss of neuronal output following an LTD stimulus (Zho et al. 2002; Brager & Johnston 2007; Brager et al. 2012). This alteration in membrane excitability is short-lived and membrane resistance and resting membrane potential return back to baseline level 30 minutes after DHPG washout.

In contrast, fEPSPs only partially recover producing a plateau depression, which fails to return to baseline an hour after DHPG application. In fact, mGluR-dependent LTD, induced by 100 μM DHPG, can persist for several hours indicating that DHPG-induced mGluR-dependent LTD causes a long lasting reduction in excitatory synaptic transmission at CA1 synapses. A single application of DHPG (100 μM) does not induce a saturating level of mGluR-dependent LTD at CA1 synapses as a further application of DHPG, 60 mins after the first, led to a significantly greater level of depression. Although not shown in this study, mGluR-dependent LTD is saturable, as no further depression is observed following a third application of DHPG (Huber et al. 2001).

Furthermore the magnitude mGluR-dependent LTD induced by DHPG shows a dose-dependent relationship as lowering the Gp1 agonist concentration reduced the magnitude but not the duration of the LTD, consistent with previous studies (Palmer et al. 1997; Huber et al. 2001). In WT hippocampal slices, the induction of chemical LTD with 30 μM DHPG led to an initial TMD of synaptic responses, however fEPSPs failed to stabilise at a depressed level and instead returned to baseline or showed a depression of less than 10%. At 100 μM LTD induction was significantly elevated compared to baseline responses. Thus, our findings from WT mice revealed that DHPG concentrations of 50 μM or more ensure a long lasting depression of synaptic responses.

The expression of mGluR-dependent LTD in young adult mice involves both a presynaptic and postsynaptic mechanism (Fitzjohn et al. 2001; Huber et al. 2001; Snyder et al. 2001; Nosyreva 2005). In this study, PPRs were examined in the same population of synapses before and after DHPG application to examine whether mGluR-dependent LTD expression at CA1 synapses involves a presynaptic change. Stimulation of the Schaffer collateral axons with two current pulses in quick succession, with an inter-pulse interval of 50 ms led to an increase in the slope and amplitude of the second fEPSP compared to the first. The potentiation of the second pulse is due to an increase in the presynaptic Ca^{2+} concentration from the residual Ca^{2+} left over from the initial pulse, which increases the probability of vesicle release from the presynaptic terminal (Zucker 1989).

In WT slices, it was observed that the application of DHPG significantly increased PPRs, which occurred in parallel with a depression of fEPSPs. Increases in the PPRs following an LTD-inducing stimulus have been previously reported at CA1 synapses (Fitzjohn et al. 2001), and is also observed in other brain regions (Choi & Lovinger 1997). The magnitude of the PPR is inversely proportional to the presynaptic release probability (Manabe et al. 1993). This suggests that mGluR-dependent LTD may lead a decrease in the release probability of glutamate from the presynaptic terminal. This could be explained by postsynaptic alterations being communicated back to the presynaptic terminus via a retrograde signalling mechanism. Potential retrograde messengers include 12-lipoxygenase metabolites of arachidonic acid (Feinmark et al. 2003). Likewise an alternative mechanism by which an LTD inducing stimulus could result in an increase in the PPR is via the preferential loss of AMPA receptors from synapses where the probability of release is greater leading to the activation of low probability synapses (Poncer & Malinow 2001). Thus to strengthen this finding that chemical LTD causes a presynaptic change, the rate of blockade of NMDA receptor mediated responses to MK-801 could have been examined, as well as the frequency and amplitude of mEPSCs, before and after DHPG-induced LTD.

Changes in synaptic strength also involve a postsynaptic mechanism, which results in the insertion or removal or GluA1- and GluA2/3-containing AMPA receptors from the postsynaptic terminal. Although measurements of AMPA receptor surface expression were not directly measured here, it is reported that the expression of DHPG-induced LTD also involves the internalisation of AMPA receptors from the cell surface (Snyder et al. 2001) (Nosyreva 2005), via a mechanism that is Ca^{2+} independent and most likely involves MAPK signalling pathways, which are examined further in Chapter 4. DHPG-induced decreases in AMPA receptor surface expression is developmentally regulated as it only observed in hippocampal slices prepared from adolescent ages (P21-28) not at immature synapses (P8-15) (Nosyreva 2005). Furthermore, the induction of mGluR-dependent LTD at more mature synapses leads to a decrease in the expression of AMPA receptors at the cell surface that is dependent on new protein synthesis, as preincubation of hippocampal slices in translational inhibitors prior to LTD induction failed to show a reduced AMPA receptor surface expression 60 minutes after DHPG treatment (Nosyreva et al. 2005).

This indicates that the persistent depression of excitatory transmission following Gp1 $mGlu_{1/5}$ receptor activation is dependent on rapid mRNA translation, highlighting the dependence of mGluR-dependent LTD on new protein synthesis. It has previously been reported that Gp1 mGlu receptor activation induces new protein synthesis of pre-existing mRNA at local dendritic sites (Huber et al. 2000). Consistent, with Nosyreva et al. (2006) it was shown that the pharmacological inhibition of mRNA translation with anisomycin resulted in a reduced or total loss of LTD expression an hour after induction in young adult hippocampal slices.

The dependence of mGluR-dependent LTD on mRNA translation is developmentally regulated as mGluR-dependent LTD in neonatal hippocampal slices is insensitive to protein synthesis inhibitors (Nosyreva et al. 2005). This could mean that the expression of mGluR-dependent LTD at immature synapses is governed solely by presynaptic changes, which persists in to adulthood but is accompanied by a reduction in AMPA receptor surface expression and a dependence on new protein synthesis. Thus in the adolescent brain there appears to be greater tractability in

regulating in long-term synaptic strength at Schaffer collateral/CA1 synapses. Interestingly, it is observed in this study that mGluR-dependent LTD is not always fully abolished in the presence of translational inhibitors. As changes in paired-pulse facilitation are not dependent on new protein synthesis, it could mean that the residual LTD is mediated by presynatically.

It is hypothesized that the proteins rapidly synthesized in response to $mGlu_{1/5}$ activation are involved in the endocytosis of AMPA receptors from the cell surface or AMPA receptor trafficking following endocytosis. Thus, these newly synthesized proteins have been termed "LTD proteins". Several of these proteins have been identified, based on their expression profiles following $mGlu_{1/5}$ receptor activation. Their involvement in changing the functional properties of synapses is beginning to be unravelled and is discussed later on.

3.8.2 LTD and protein synthesis downstream of $mGlu_{1/5}$ receptor activation is disrupted in the mouse model of FXS

In this chapter, DHPG-induced mGluR-dependent LTD was investigated in the *Fmr1* KO mouse. FXS is caused by a CGG expansion mutation in the *Fmr1* gene, which encodes the RNA binding protein FMRP. FMRP is rapidly synthesized in response to Gp1 mGlu receptor activation and is hypothesized to negatively represses mRNA translation at dendritic sites (Weiler et al. 1997). The link between $mGlu_{1/5}$ signalling and FXS has already been previously studied, however it was important to determine whether abnormalities in $mGlu_{1/5}$ signalling could be reproduced in the *Fmr1* KO mouse that was bred on a different strain background. The modest enhancements in mGluR-dependent LTD in the *Fmr1* KO mice is not always observed (Hou et al. 2006; Ronesi & Huber 2008). Likewise, in WT rodents the protein synthesis dependency of mGluR-dependent LTD it not always present (Moult et al. 2008). Thus, it needed to be established whether the $mGlu_{1/5}$-mediated pathology associated with FXS could be reproduced in our mouse model of FXS.

Here, it was shown that DHPG-induced mGluR-dependent LTD is enhanced in the hippocampus of *Fmr1* KO mice relative to WT littermates, reproducing the original findings reported by Huber et al. (2002). This excessive LTD in *Fmr1* KO hippocampal slices occurred in the absence of any significant alterations in AMPA receptor mediated synaptic transmission indicating that basally synapses are functionally intact. In contrast, under Gp1 mGlu receptor stimulated conditions there is an excessive weakening of synapses in the *Fmr1* KO brain. In has been shown previously in culture and through biotinylation studies in slices, that enhanced mGluR-dependent LTD arises from the excessive removal of AMPA receptors from the postsynaptic terminal leading to an exaggerated depression of synaptic responses in the *Fmr1* KO brain.

Also we observed that mGluR-dependent LTD in the hippocampus of *Fmr1* KO mice remained excessively depressed in the presence of the translational inhibitor anisomycin, indicating that this form of synaptic plasticity occurs independently of new protein synthesis. This suggests that the proteins involved in the removal of AMPA receptors from the postsynaptic sites are already sufficiently present in the *Fmr1* KO hippocampus to allow mGluR-dependent LTD to proceed without the need for new protein synthesis.

In this study basal protein synthesis rates were directly measured in the hippocampus of *Fmr1* KO mice and were found to be elevated by 20% in comparison to WT littermates. The magnitude of this increase is in close agreement with other *in vitro* studies in the hippocampus of *Fmr1* KO mice (Osterweil et al. 2010; Dölen et al. 2007; Michalon et al. 2012). Furthermore, *in vivo* studies have revealed that increased basal protein synthesis rates are elevated in several other brain regions (Qin et al. 2005a) but show a selectivity indicating that FMRP may regulate mRNA translation in a spatially-dependent manner.

The major components of the protein synthesis machinery, which include ribosomes translation factors and mRNAs are present in dendritic shafts and spines suggesting that there is local protein synthesis, that is independent of transcription in the cell soma, which may be mediating long-lasting forms of synaptic plasticity, such as mGluR-dependent LTD (Sutton & Schuman 2006). At these dendritic sites FMRP is associated with mRNAs, RNA granules and translating polyribosomes. FMRP functions as an RNA binding protein, repressing translation of a subset of mRNAs (~4% all known mRNAs) encoding proteins that are involved in synaptic maturation and plasticity (Darnell et al. 2011). At local dendritic sites it is thought to regulate activity-dependent protein synthesis, functioning to repress mRNA translation primarily at postsynaptic sites following $mGlu_{1/5}$ receptor activation. *Fmr1* mRNA itself is rapidly translated following $mGlu_{1/5}$ receptor activation (Weiler et al. 1997), and interacts with many of the transcripts that are translated following an mGluR-dependent LTD stimulus (Bassell & Warren 2008).

The electrophysiological phenotypes observed in the *Fmr1* KO, reported here and elsewhere, support the hypothesis that FMRP plays a prominent role in mGluR-dependent protein synthesis and plasticity. Our findings indicate that FMRP functions to suppress steady-state levels of mRNA translation. The exact mechanism by which FMRP represses the translation of its target mRNAs is not clear, however there is evidence to suggest it may be via targeting initiation through interactions with: non coding RNA BC1 protein, which could increase FMRP binding to target mRNAs (Zalfa et al. 2003); or CYFIP1, a EIF4E binding protein which competes with EIF4G for EIF4E (Napoli et al. 2008). There is also evidence to suggest that FMRP may target a post-initiation step, as FMRP associated with translating polyribosomes (Stefani 2004).

Further investigations into protein synthesis in *Fmr1* KOs revealed that mRNA translation is saturated downstream of constitutive $mGlu_{1/5}$-ERK1/2 activity (Osterweil et al. 2010). In *Fmr1* KO mice, the excessive basal protein synthesis rates are rescued by inhibiting $mGlu_5$, Ras or ERK1/2 (Michalon et al. 2012; Osterweil et al. 2010; Osterweil et al. 2013). This indicates that in the *Fmr1* KO brain, protein

synthesis rates are not decoupled from $mGlu_{1/5}$ activation because if this was the case, inhibition of $mGlu_{1/5}$ would have no effect on the elevated protein synthesis rates in the *Fmr1* KO mouse.

Efforts have been made to identify potential target mRNAs of FMRP. Through UV cross-linking studies, FMRP was found to interact with several hundred mRNAs (Darnell et al. 2011). Many of these transcripts are implicated in synaptic functions that are disrupted in the *Fmr1* KO brain. These include mRNAs encoding: cytoskeletal associated protein (Arc) (Zalfa et al. 2003; Chowdhury et al. 2006), amyloid precursor protein (APP) (Westmark & Malter 2007), microtubule-associated protein 1B (MAP-1b) (Davidkova & Carroll et al. 2007) and striatal-enriched protein tyrosine phosphatase (STEP) (Zhang et al. 2008), components of AMPA receptor endocytosis machinery or involved in AMPA receptor internalization; the catalytic subunit of PI3K p-110 and PIKE-L (Sharma et al. 2010; Gross et al. 2010), components of $mGlu_{1/5}$ mediated downstream signalling; PSD-95 (Todd et al. 2003), involved in anchoring and stabilising glutamatergic receptors at the cell surface.

The majority of these transcripts are up regulated in response to $mGlu_{1/5}$ receptor stimulation. In contrast, the same targets appear to be overabundant and no longer up regulated in an activity-dependent manner in the *Fmr1* KO brain. Thus it is hypothesized that the surplus of these LTD proteins lead to excessive AMPA receptor internalization in response to $mGlu_{1/5}$ activation, eliminating the requirement for new protein synthesis and causing an exaggerated depression of synaptic responses.

3.8.3 Core deficits observed in *Fmr1* KO mice translate to the rat model of FXS

The generation of the rat model of FXS has enabled us to investigate whether the core pathophysiological phenotypes in the *Fmr1* KO mouse translate to another rodent specie (Hamilton et al. 2014). Here we report that cellular processes associated with Gp1 mGlu receptor signalling, including DHPG-induced LTD and basal protein synthesis rates, are elevated in hippocampal slices prepared from young adult *Fmr1* KO rats (P25-32). It has not been previously shown whether $mGlu_{1/5}$ receptor phenotypes associated with the loss of FMRP are shared between two independent mammalian species. Here it is reported that the *Fmr1* KO rats exhibit increased basal protein synthesis rates alongside altered Gp1 mGluR-dependent synaptic plasticity in the hippocampus. These findings further validate the mGluR theory of FXS and suggest a strong evolutionary link between FMRP and $mGlu_{1/5}$ receptor signalling. Thus, it would be interesting to determine whether pharmacological strategies employed in correcting deficits in the *Fmr1* KO mouse are efficacious in the *Fmr1* KO rats.

The rat model of FXS will also be a useful tool in studying more complex behavioural paradigms as rats display a richer social behavioural repertoire and level of communication compared to mice. This will be particular important when assessing the social and communication deficits in genetic models of ID and ASD, such as FXS and *Syngap1* haploinsufficiency, where these phenotypes are prominent features of the disorder. Also, there are greater similarities in the physiology of rats and humans, so the rat model of FXS may provide a more sensitive tool in evaluating the potential therapeutic treatments for ID and ASD. Rats are also preferentially used by pharmaceutical companies for many aspects of drug development including investigating the potential side effects of a compound and how it will be metabolized in humans (Gilby & O'Brien 2013). Thus the rat model of FXS will be an important preclinical model of ID.

3.8.4. The mouse model of *Syngap1* haploinsufficiency mimics the hippocampal pathophysiology associated with FXS.

One of the key questions that need to be addressed in the ID and ASD field is whether genetically distinct causes of ID share similar phenotypic manifestations at the cellular and molecular level. Here electrophysiological and biochemical studies were employed to examine another genetic cause of ID that results from mutations in *SYNGAP1*. *SYNGAP1* encodes the synaptic GAP protein, SynGAP that regulates Ras-mediated signalling. There is now a substantial body of evidence that link genetic mutations in Ras-associated proteins to neurodevelopmental disorders. Furthermore, there is evidence to suggest that elevated protein synthesis rates in the *Fmr1* KO mouse result from a hypersensitivity of the translation machinery to $mGlu_{1/5}$ signalling through Ras-ERK1/2-MAPK signalling pathway (Osterweil et al. 2010; Osterweil et al. 2013). In *Syngap* heterozygous mice both Ras and ERK1/2 are hyperactive in the hippocampus (Komiyama et al. 2002; Carlisle et al. 2008; Ozkan et al. 2014), which we hypothesized could lead to similar phenotypic manifestations, in terms of $mGlu_{1/5}$ receptor-mediated processes, such as mGluR-dependent LTD and protein synthesis at local dendritic sites. Thus the aim of this chapter was to determine whether mutations in *Syngap1* share similar dysfunction in Gp1 mGluR-dependent LTD and protein synthesis.

In this thesis, it is shown for the first time that mGluR-dependent LTD is enhanced in the hippocampus of young adult *Syngap* heterozygous mice, which is observed at various concentrations of DHPG (30, 50 and 100 μM). Furthermore, inhibition of mRNA translation with anisomycin fails to abolish mGluR-dependent LTD in hippocampal slices from *Syngap* heterozygous mice indicating that this form of plasticity is no longer reliant on new protein synthesis at CA1 synapses. These findings suggest that SynGAP may be one of several intracellular components linking $mGlu_{1/5}$ receptor activation to mRNA translational machinery, which governs the persistent removal of AMPA receptors from the cell surface upon $mGlu_{1/5}$ receptor activation. Thus, SynGAP may function as a modulator of synaptic strength at CA1 synapses.

At the PSD SynGAP is bound to PSD-95 and SAP102 and interacts with the GluN2B subunit of the NMDA receptor (Kim et al. 1998). Thus it is ideally positioned to relay synaptic activation of both ionotropic and metabotropic glutamate receptors to the initiation of intracellular events via Ras-mediated signalling cascades. Consistent with this hypothesis, it is reported that NMDA receptor mediated forms of synaptic plasticity are impaired in the hippocampus of *Syngap* heterozygous mice (Komiyama et al. 2002; Kim et al. 2003; Carlisle et al. 2008; Ozkan et al. 2014). As mentioned previously, CAMKII lies upstream of SynGAP and phosphorylates this protein at three sites, increasing its GAP activity (Oh et al. 2004). Once activated, SynGAP specifically binds and inactivates the Ras family of G-proteins that are required for the expression and maintenance mGluR-dependent LTD (Gallagher et al. 2004; Hou & Klann 2004).

Interestingly, inhibition of CAMKII by KN-62, facilitates DHPG-induced mGluR-dependent LTD at CA1 synapses (Schnabel 1999). One possible explanation of this finding is that in the presence of KN-62, CAMKII can no longer phosphorylate and activate SynGAP. This would mean that upon an LTD inducing stimulus, loss of CAMKII activity would leave SynGAP in its dormant state, which could consequently prolong the activity of Ras and its downstream targets due to the loss of inactivation by SynGAP. This could lead to the excessive removal of AMPA receptors from the postsynaptic terminal, potentially via protein synthesis dependent mechanism, leading to enhanced mGluR-dependent LTD.

In support of SynGAPs role in AMPA receptor endocytosis, it has been observed in hippocampal cultures that overexpression of SynGAP suppresses AMPA receptor surface expression (Rumbaugh et al. 2006). Furthermore excitatory synaptic transmission is elevated in the hippocampus of *Syngap* heterozygous mice at P14 suggesting that during early development SynGAP normally restricts AMPA receptor accumulation at the postsynaptic terminal, during a development period (<P21) where synapses gradually acquire AMPA receptors and increase their synaptic strength (Clement et al. 2012). This coincides with the peak expression of

SynGAP levels in this hippocampus, and may indicate a time-point at which synapses are most vulnerable to reduced SynGAP expression.

Thus *Syngap1* mutations lead to a derepression of synapse maturation leading to abnormally strong excitatory transmission that is functionally similar to adult-like levels of synaptic strength. By P21 basal synaptic transmission in WT synapses reach the same level of strength as *Syngap* heterozygous mice so there are no longer detectable differences in I/O function suggesting that at more mature synapses the expression of AMPA receptors is similar between *Syngap* heterozygous mutants WT mice (Clement et al. 2012). In this study, glutamatergic synaptic transmission in *Syngap* heterozygous mice at P25-32 was found to be intact, in agreement with previous reports (Komiyama et al. 2002; Clement et al. 2012).

The comparable electrophysiological findings between *Syngap* heterozygous and *Fmr1* KO mice lead us to predict that basal protein synthesis rates may too be elevated in the hippocampus of *Syngap* heterozygous mice. If so, this could account for our findings that mGluR-dependent LTD no longer requires new protein synthesis when SynGAP expression is reduced. In agreement with our prediction, basal protein synthesis rates are elevated in the hippocampus of the *Syngap* heterozygous mice. This result is consistent with increased protein synthesis rates in *Syngap* KD cortical cultures, which is accompanied by an increase in synaptic strength from increases in the expression of GluA1 containing AMPA receptors at the cell surface (Wang et al. 2013). However our findings reflect the consequence of reduced SynGAP expression, rather than total loss, and thus model *Syngap* haploinsufficiency more closely. Although it is unknown whether specific proteins are overexpressed, one would hypothesize that proteins involved in the removal of AMPA receptors are the most likely candidates. This would explain why *Syngap1* haploinsufficiency results in an excessive LTD, and would alleviate the need for new protein synthesis to maintain depression of synaptic responses.

In *Fmr1* KO mice elevated protein synthesis rates lie downstream of the ERK1/2 MAPK signalling pathway, a pathway involved in the initiation of cap-dependent translation. Upstream of ERK1/2, is Ras, whose activity is regulated by synaptic GTPase activating proteins, such as SynGAP and neurofibromin (Ye & Carew 2010). SynGAP reduces Ras activity by catalysing the hydrolysis of active Ras (GTP-bound) to its inactive form (GDP-bound). Thus when SynGAP expression is reduced, through loss of single functional copy of *Syngap1,* one would predict that the amount of time Ras remains in its active state is prolonged. Consequently this could lead to the persistent activation of cap-dependent translation via hyperactivity of Ras-mediated signaling cascades that include ERK1/2-MAPK and PI3K-Akt-mTOR signaling leading to excessive translation of proteins involved in the expression and maintenance of long-lasting forms of synaptic plasticity.

Interestingly, the magnitude of this increase in basal protein synthesis rates in *Syngap* heterozygous mice was far greater than that observed in *Fmr1* KO mice. This may suggest that SynGAP regulates the translation of a greater subset of transcripts in the hippocampus than FMRP, which is known to bind to approximately 4% of mRNAs (Darnell et al. 2011). Thus increased protein synthesis levels may also be the core deficits underlying the pathophysiology of *Syngap1* haploinsufficiency and it will be interesting to see whether the hippocampal deficits presented here can be corrected by the same pharmacological strategies used in mouse model of FXS.

It should be noted by that an increase in ^{35}S Met/Cys incorporation in hippocampal slice homogenates from *Syngap* heterozygous mice compared WT littermates could also reflect a reduction in the rate of protein degradation rather than an increase in protein synthesis. Both protein synthesis and degradation are important for synaptic plasticity and need to work in an orchestrated manner to regulate normal synaptic function (Tai et al. 2008; Steward & Schuman 2003). One way to test this alternative hypothesis would be via a pulse-chase method, in which slices are radiolabelled for 45 minutes before being transferred back to fresh ACSF then slices collected at various time intervals to quantify the rate of ^{35}S Met/Cys breakdown between *Syngap* heterozygous and WT mice.

Based on these initial findings that *Syngap* heterozygous and *Fmr1* KO single mutants have a shared pathophysiology in the hippocampus, we wanted to directly test the hypothesis that these two genetically distinct causes of ID share a common pathophysiological axis. Thus a double transgenic was generated to investigate whether SynGAP and FMRP converge on the same molecular signalling mechanism that is involved in the expression of mGluR-dependent LTD. If SynGAP and FMRP utilised distinct pathways to modulate this form of synaptic plasticity then the magnitude of this depression would be further augmented in the *Syngap* heterozygous x *Fmr1* KO cross. However, if SynGAP and FMRP converge on the same cellular mechanisms then *Syngap1* haploinsufficiency would occlude the increase in mGluR-dependent LTD by the *Fmr1* KO mutation. In the double mutant cross, mGluR-dependent LTD was enhanced compared to WT littermates but there was no further increase in magnitude of LTD than observed in the *Fmr1* KO and *Syngap* heterozygous single mutants.

To confirm that the magnitude of mGluR-dependent LTD in the double mutants was not caused by a "floor effect", it was determined whether DHPG was inducing a saturating level of LTD for all four genotypes. Here it was observed that following a second application of DHPG, the magnitude of LTD was further increased in WT, *Fmr1* KO and *Syngap* heterozygous mice, with double mutants showing a trend towards significance. This would suggest that DHPG induced LTD in double mutant is not saturated, thus there was the potential for further depression.

3.9 Summary

Thus to summarise, the data presented in this chapter demonstrate that two genetically distinct causes of ID, FXS and *Syngap1* haploinsufficiency, share overlapping dysfunctions in cellular processes that are mediated by $mGlu_{1/5}$ receptors. Both $mGlu_{1/5}$ receptor-mediated synaptic plasticity and protein synthesis are exaggerated in the hippocampus of *Syngap* heterozygous and *Fmr1* KO mice. Together these findings suggest that FMRP and SynGAP may suppress steady-state levels of basal protein synthesis at CA1 synapses and in effect modulate AMPA receptor internalisation during periods of synaptic activation. Furthermore, it is shown that FMRP and SynGAP mediate their effects on synaptic strength via a common intracellular signalling cascade, most likely via Ras-ERK1/2 MAPK signalling, suggesting that mutations in *Fmr1* and *Syngap1* may share a common pathophysiological axis that results in excessive protein synthesis rates and exaggerated mGluR-dependent LTD. Thus *Syngap1* lies on the same side of the pathological spectrum of synaptic function as *Fmr1*. This raises the intriguing possibility that synaptic abnormalities reported in the mouse model of *Syngap1* haploinsufficiency may be amenable to the same therapeutic strategies employed in the *Fmr1* KO mouse.

Chapter 4

Biochemical Analysis of *Syngap*$^{+/-}$ Mice

4.1 Key findings

1.) Western blot analysis reveals altered ERK1/2, Akt and S6 signalling under steady-state conditions in the hippocampus of *Syngap* heterozygous mice.

2.) Protein synthesis rates downstream of $mGlu_5$ receptor activation are saturated in the hippocampus of *Syngap* heterozygous mice.

3.) In contrast, western blot analysis of hippocampal homogenates and synaptoneurosome preparations reveal no significant differences in Ras mediated signalling or in the expression levels of "LTD proteins" in *Syngap* heterozygous mice.

4.2 Introduction

In Chapter 3 it was revealed that two monogenic causes of ID, FXS and *Syngap1* haploinsufficiency result in elevated basal protein synthesis in the hippocampus. Cellular functions, such as long-lasting forms of synaptic plasticity (e.g. mGluR-dependent LTD, L-LTP) rely on the translation of specific proteins during periods of synaptic activity (Huber et al. 2000; Frey et al. 2003). Consistent with increases in translational rates, mGluR-dependent LTD at CA1 synapses was found to be enhanced and no longer dependent on new protein synthesis in *Fmr1* KO and *Syngap* heterozygous mice. Furthermore investigations in the *Fmr1* KO × *Syngap* heterozygous double mutants revealed that this exaggerated depression of excitatory synaptic transmission following an LTD–inducing stimulus likely involved the same intracellular signalling mechanism. These findings suggest that mutations in the *Fmr1* and *Syngap1* gene may converge on a common pathophysiological axis that is mediating increased protein synthesis rates in mouse models of FXS and *Syngap1* haploinsufficiency.

Overall protein synthesis rates play an important role in neuronal function and must be tightly regulated. The process of mRNA translation, involves three main stages: initiation, elongation and termination. Translation factors and intracellular cascades target various components of this process and can determine gene expression in an activity-dependent manner. Upon $mGlu_{1/5}$ receptor activation, both ERK1/2 and mTOR pathways stimulate cap-dependent mRNA translation by targeting components involved in initiation, the major rate-limiting step of mRNA translation (Banko et al. 2006; Ronesi & Huber 2008).

The initiation of translation begins with the recognition of the 5' mRNA cap by eukaryotic initiation factor 4E (eIF4E) (Richter & Sonenberg 2005). This leads to the formation of the translation initiation complex, EIF4F that also includes EIF4A and EIF4G. Once the EIF4F complex is bound to the 5' end of the mRNA, the small ribosomal subunit is recruited to the capped 5' end of the mRNA transcript, where it traverses the 5' untranslated region (UTR) in a 5'-3' direction to identify the initiation codon AUG. Once initiation is complete, elongation factors are recruited to the ribosomal mRNA complex leading to the commencement of polypeptide

elongation and the production of newly synthesized proteins.

Both ERK1/2 and mTOR signalling initiate translation by triggering the phosphorylation of eIF4E and 4E-BP, allowing the formation of EIF4F complex (Banko et al. 2006; Ronesi & Huber 2008; Richter & Sonenberg 2005; Proud 2007). In addition $mGlu_{1/5}$ receptor activation of mTOR and ERK1/2 can stimulate translation by phosphorylating S6K1/2 and RSK, respectively, which activate the ribosomal protein S6 (Antion et al. 2008a). In turn S6 can phosphorylate and activate EIF4B, which potentiates EIF4A activity increasing EIF4F formation (Shahbazian et al. 2006). Furthermore S6 can increase the translation of a specific subset of 5'TOP mRNAs, which encode all ribosomal protein subunits and translation initiation factors, such as elongation factor 1A (EF1A), that enables a neuron to increase its overall translation capacity (Antion et al. 2008a; Figure 1.7).

In CA1 of the hippocampus mGluR-dependent LTD is reliant on both ERK1/2 and mTOR activation, both of which have been shown to play an important role in the expression and maintenance of LTD and the regulation of mRNA translation (Gallagher et al. 2004; Hou & Klann 2004; Banko et al. 2006; Ronesi & Huber 2008). It is suspected that $mGlu_{1/5}$ receptors stimulate the synthesis of proteins, which participate in AMPA receptor endocytosis and trafficking, resulting in a depression of excitatory transmission. For this reason they are referred to as "LTD proteins" (Huber et al. 2000).

In *Fmr1* KO mutants, there is an overabundance of "LTD proteins" which include but are not limited to PSD-95 (Todd et al. 2003), APP (Westmark & Malter 2007), EF1A (Huang et al. 2005), MAP1b (Westmark & Malter 2007) and Arc (Chowdhury et al. 2006; Waung et al. 2008). Several of these proteins are directly implicated in the internalisation of AMPA receptors and their overexpression is thought to underlie the excessive reduction in synaptic strength following $mGlu_{1/5}$ receptor activation. For these reasons both the ERK1/2 and mTOR pathways have been extensively studied in mouse model of FXS where $mGlu_{1/5}$ mediated synaptic plasticity and protein synthesis are exaggerated.

It is reported that elevated basal protein synthesis in the *Fmr1* KO hippocampus are corrected by targeting the Ras-ERK1/2 signalling pathway indicating that increased translational rates under steady-state conditions may lie downstream of Ras-ERK1/2-MAPK signalling, rather than PI3K-Akt-mTOR signalling (Osterweil et al. 2010; Osterweil et al. 2013). The same study found that activity levels of ERK1/2 and mTOR signalling were intact and $mGlu_{1/5}$ activation caused no further increase in elevated protein synthesis rates in the *Fmr1* KO brain (Osterweil et al. 2010). Thus at rest mRNA translation is excessive and saturated downstream of $mGlu_5$ receptor activation in the *Fmr1* KO brain, resulting from a hypersensitivity of the mRNA translational machinery to Ras-ERK1/2 signalling due to the loss of translational repression by FMRP.

In *Syngap* heterozygous mice, it has been reported that Ras and ERK1/2 activity levels are increased in the hippocampus (Carlisle et al. 2008; Komiyama et al. 2002; Ozkan et al. 2014), leading us to hypothesize that elevated protein synthesis rates in *Syngap* heterozygous mice arise from the hyperactivity of the Ras-ERK1/2 MAPK signalling pathway and moreover that they are saturated downstream of $mGlu_5$ receptor activation. In this chapter this hypothesis is tested, by measuring the phosphorylation status of key signalling components of the ERK1/2 and mTOR signalling cascades in hippocampal extracts from *Syngap* heterozygous mice. In addition, protein synthesis rates are measured in the presence DHPG, at a concentration that led to excessive mGluR-dependent LTD in the hippocampus of *Syngap* heterozygous mice. Furthermore the expression levels of key synaptic proteins, including "LTD proteins" that are involved in glutamatergic synaptic transmission, are quantified in hippocampal homogenates and synaptoneurosome preparations to determine whether steady-state levels are also increased in *Syngap* heterozygous mice. Thus this chapter sets out to examine the molecular mechanisms that may underlie elevated basal protein synthesis rates and enhanced mGluR-dependent LTD in the hippocampus of *Syngap* heterozygous mice.

4.3 Results

4.3.1 Basal levels of phosphorylated ERK, Akt and S6 are elevated in the hippocampus of *Syngap* heterozygous mice

In Chapter 3 direct measurements of mRNA translation using a metabolic labelling approach revealed that protein synthesis rates were elevated in the dorsal hippocampus of *Syngap* heterozygous mice (P25-32). This is accompanied by enhanced mGluR-dependent LTD that is no longer dependent on new protein synthesis. At the synapse, SynGAP binds and inactivates Ras family members, which converge on intracellular signalling cascades that include ERK1/2 and mTOR. Both of these pathways are linked to the initiation of cap-dependent mRNA translation, which is crucial for the expression of mGluR-dependent LTD at CA1 synapses (Gallagher et al. 2004; Hou & Klann 2004). In *Syngap* heterozygous mutants, where SynGAP expression is reduced by 50%, Ras and ERK1/2 activity are reportedly increased in the hippocampus (Komiyama et al. 2002; Carlisle et al. 2008). Thus we suspect that the abnormal $mGlu_{1/5}$ mediated phenotypes might arise from increased levels of Ras activation that could consequently lead to the hyperactivation of downstream signalling components that mediate mRNA translation.

To test this hypothesis steady-state levels of phosphorylated and total ERK1/2 were quantified through western blot analysis using infrared fluorescence in the same dorsal hippocampal slices where elevated protein synthesis rates were measured (Figure 4.1A). In agreement with existing findings, levels of phosphorylation at sites Thr^{202}/Tyr^{204} on ERK1/2 were elevated in hippocampal slices from *Syngap* heterozygous mice compared to WT littermates (Komiyama et al. 2002; Ozkan et al. 2014). This was observed in the absence of any detectable differences in total ERK1/2 protein levels (phospho/total ERK1/2: WT 100 ± 7%; Het 162 ± 15%; $n = 8$, *t*-test $*p = 0.003$; phospho/total ERK1: WT 100 ± 7%; Het 176 ± 22%; $n = 8$, *t*-test $*p = 0.01$; phospho/total ERK2: WT 100 ± 10%; Het 200 ± 24%; $n = 8$, *t*-test $*p = 0.004$).

To determine whether mTOR signalling was also hyperactive, basal levels of phosphorylated and total Akt and p70 S6 kinase were quantified in hippocampal slices from *Syngap* heterozygous mice. Phosphorylated Akt was found to be significantly elevated in *Syngap* heterozygous slices compared to WT controls with no change in total Akt levels (p-Akt/Akt: WT 100 ± 11%; Het 124 ± 8%; $n = 7$, *t*-test $*p = 0.02$; Figure 4.1B). However using an antibody that recognised the phosphorylation site Thr^{389} on p70 S6 kinase failed to provide a detectable fluorescent signal in hippocampal slices.

One downstream target of both activated ERK1/2 and mTOR is the S6 ribosomal protein, a subunit of the small 40S ribosome (Nygård & Nilsson 1990). S6 is phosphorylated at several serine residues (Ser^{235}, Ser^{236}, Ser^{240}, Ser^{244} and Ser^{247}) by two classes of protein kinases, S6K1/2 and RSK that are in turn activated by mTOR and ERK1/2 signalling respectively (Roux et al. 2007). Phosphorylation of S6 was quantified using two antibodies that recognize S6 when dually phosphorylated at either $Ser^{235/236}$ ($S6^{235/236}$) or $Ser^{240/244}$ ($S6^{240/244}$). In *Syngap* heterozygous mice, phospho-$S6^{235/236}$ levels were significantly elevated compared to WT littermates with no change in the levels of total S6 ($pS6^{235/236}$/S6: WT 100 ± 7%; Het 121 ± 8%; $n = 8$, *t*-test $*p = 0.03$; Figure 4.1C). In contrast there were no detectable differences observed in phospho-$S6^{240/244}$ between *Syngap* heterozygous and WT mice ($pS6^{240/244}$/S6: WT 100 ± 4%; Het 106 ± 7%; $n = 8$, *t*-test $p > 0.05$; Figure 4.1D) indicating that under steady state conditions S6 is overactive in the *Syngap* heterozygous brain, in a site-specific manner.

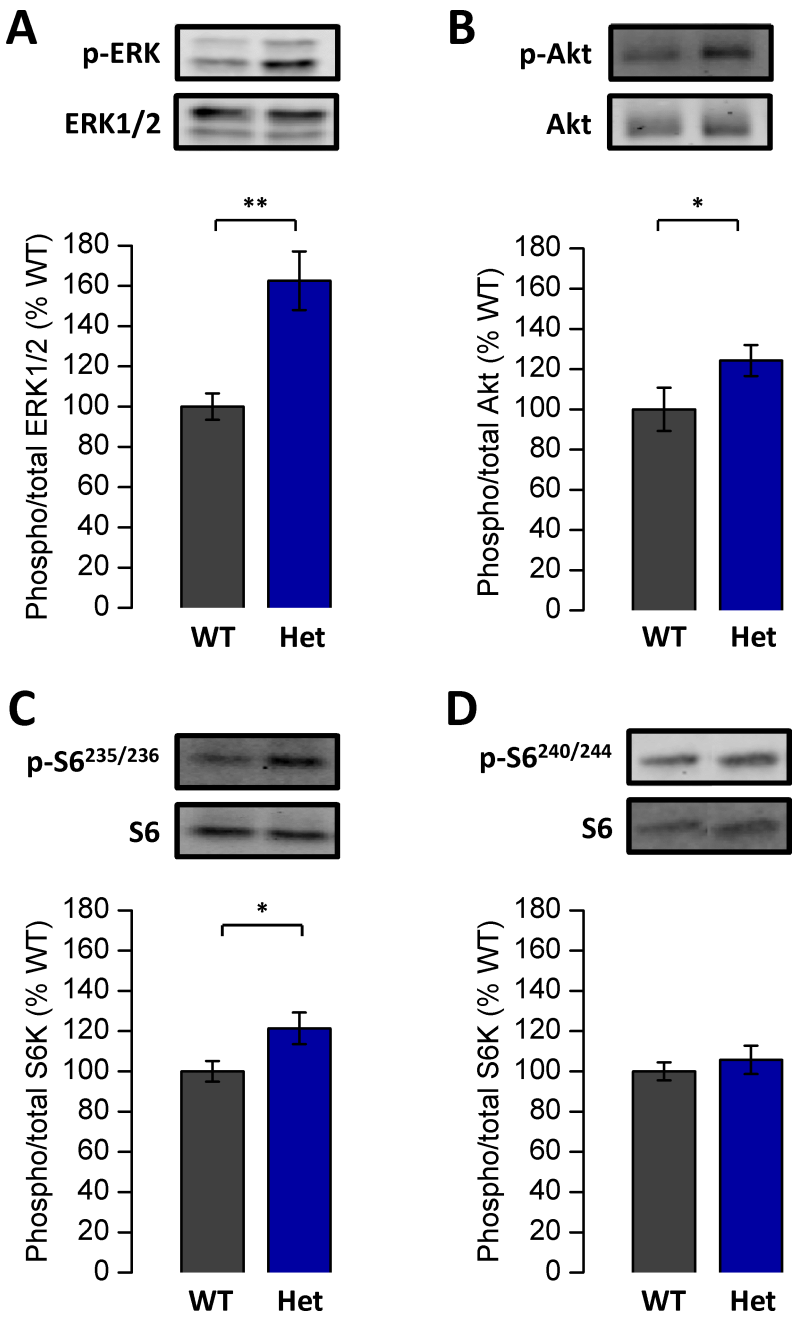


Figure 4.1 Western blots reveal activity levels of key signalling proteins are elevated in hippocampal slices from *Syngap* heterozygote mice. Basal activation states of ERK1/2, Akt and S6 were quantified in hippocampal slices from *Syngap* heterozygous mice (het, $SG^{+/-}$) and expressed as a percentage of their WT pair. (A) Levels of phosphorylated ERK1/2 to total ERK1/2 were increased in *Syngap* heterozygous mice compared to WT littermates (Phospho/total ERK1/2: WT 100 ± 7%; $SG^{+/-}$ 162 ± 15%, n = 9, t-test *p = 0.004). (B) Similarly phosphorylated forms of Akt/total Akt were elevated in hippocampal slices from *Syngap* het mice compared WT littermates (Phospho/total Akt: WT 100 ± 11%; $SG^{+/-}$124 ± 8%, n = 7, t-test *p = 0.001). (C) The basal activation state of S6 at $Ser^{235/236}$ was also significantly increased in *Syngap* heterozygous mice compared to WT controls ($Ser^{235/236}$/S6: 100 ± 5%; $SG^{+/-}$ 121 ± 8%, n = 8, t-test *p = 0.03), whilst phosphorylation of S6 at $Ser^{240/244}$ was indistinguishable from WT littermates ($Ser^{240/244}$/S6: 100 ± 5%; $SG^{+/-}$ 121 ± 8%; n = 8, t-test $p > 0.05$).

4.3.2 mGluR-dependent protein synthesis is saturated in the hippocampus of *Syngap* heterozygous mice

In *Syngap* heterozygous mice, the activation of Gp1 mGlu receptors with DHPG induces a depression of synaptic responses that is greater in magnitude than WT littermates and no longer sensitive to protein synthesis inhibitors. These findings suggest that the proteins required for the stabilizing LTD expression, which are normally synthesized in response to $mGlu_{1/5}$ receptor activation, are already present at *Syngap* heterozygous synapses. In the *Fmr1* KO brain, DHPG failed to increase elevated protein synthesis rates in hippocampal slices suggesting that mRNA translation is saturated downstream of $mGlu_{1/5}$ receptor activation (Osterweil et al. 2010).

Here, we investigated whether translational rates downstream of $mGlu_{1/5}$ receptor activation are elevated in *Syngap* heterozygous under experimental where exaggerated mGluR-dependent LTD is obtained. The application of an mGluR-inducing LTD stimulus, DHPG (100 μM; 5 mins), significantly increased translational rates in WT hippocampal slices by ~40% when normalised to WT vehicle treated slices (WT veh 100 ± 5%; WT DHPG 138 ± 14%; $n = 6$; $*p = 0.008$; Figure 4.2). However measurements of ^{35}S incorporation in hippocampal slices from *Syngap* heterozygous revealed that DHPG failed to significantly increase elevated protein synthesis levels further in hippocampal slices from *Syngap* heterozygous mice (Het veh 135 ± 7%; Het DHPG %, 148 ± 15% $n = 6$, $p = 0.43$). These findings suggest that in WT slices $mGlu_{1/5}$ activation leads to a significant increase in mRNA translation, whilst in *Syngap* heterozygous slices DHPG has no effect on protein synthesis rates because they are already saturated downstream of $mGlu_5$ receptor activation under basal conditions.

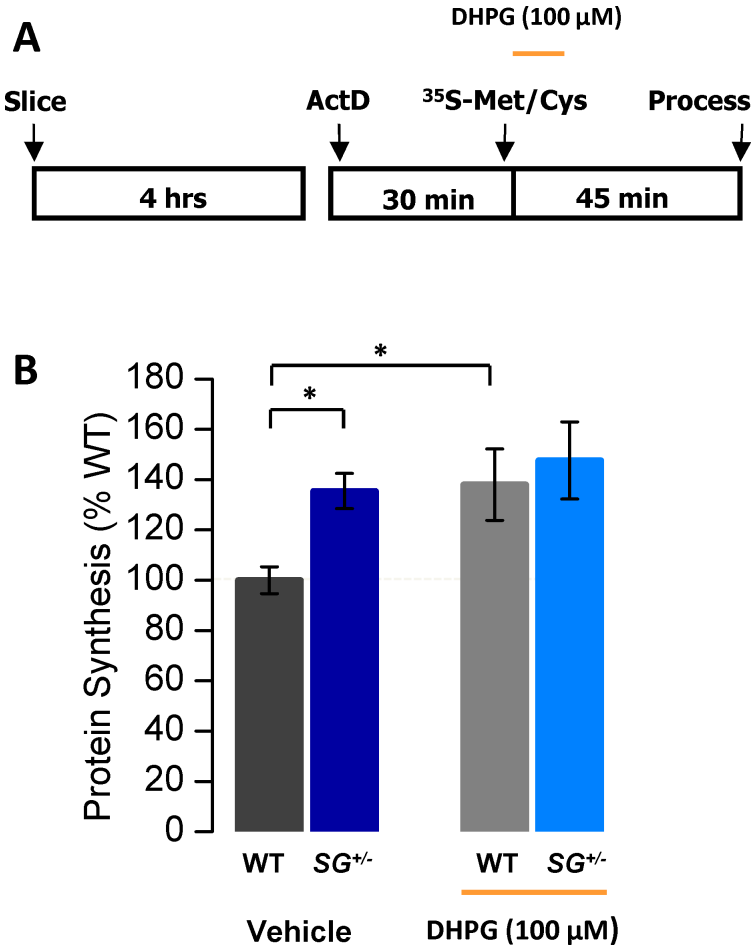


Figure 4.2 Protein synthesis rates are saturated downstream of mGlu$_{1/5}$ receptor activation in *Syngap* heterozygous mice. (A) Schematic of experimental timeline for ^{35}[S]-Methionine/Cysteine metabolic labeling in the presence of DHPG (100 µM). (B) Protein synthesis levels were significantly elevated in vehicle (veh) treated dorsal hippocampal slices from *Syngap*$^{+/-}$ (het, *SG* $^{+/-}$) *versus* wild type mice (*SG* $^{+/-}$ veh: 135 ± 7%; WT veh: 100 ± 7%; $n = 6$; ANOVA *$p = 0.02$). DHPG treatment significantly increased protein synthesis rates in WT slices but did not further increase ^{35}S incorporation in *Syngap* heterozygous mice (WT DHPG: 138 ± 14%; *SG* $^{+/-}$ DHPG: 148 ± 15%; ANOVA treatment *$p = 0.008$, $n = 6$).

4.3.3 No detectable differences in protein expression levels of key synaptic proteins in hippocampal homogenates from *Syngap* heterozygous mice

In *Fmr1* KO mice, elevated protein synthesis rates are accompanied by increases in steady state levels of synaptic proteins, which include CAMKII, Arc, and PSD-95 (Todd & Malter 2002; Chowdhury et al. 2006; Roux et al. 2007; Waung et al. 2008; Zalfa et al. 2003). Here the expression levels of a specific subset of synaptic proteins were quantified in hippocampal homogenates from *Syngap* heterozygous and WT mice (P25-32). The aim of this experiment was to examine whether key synaptic proteins, implicated in $mGlu_{1/5}$ receptor signalling and known to be overabundant at *Fmr1* KO synapses, are overexpressed in the hippocampus of *Syngap* heterozygous mutants.

Using an alternative method of detection, enhanced chemiluminescence (ECL), it was observed that protein expression levels of PSD-95, CAMKII and Arc, were not significantly different in hippocampal homogenates from *Syngap* heterozygous and WT controls (Figure 4.3). Similarly, no detectable genotype differences were observed in the expression of glutamatergic receptor subunits in hippocampal homogenates. These included the expression of $mGlu_5$ receptors ($mGlu_5$: WT 100 ± 5%; Het 80 ± 10%; n = 4, t-test p = 0.16), AMPA receptor subunits GluA1 and GluA2 (GluA1: WT 100 ± 5%; Het 90 ± 14%; n = 4, t-test p = 0.89; GluA2: WT 100 ± 19 %; Het 78 ± 15%; n = 4, t-test p = 0.23), and the GluN2A and GluN2B subunits of the NMDA receptor (GluN2A: WT 100 ± 8%; Het 98 ± 10%; n = 5, t-test p = 0.83; GluN2B: WT 100 ± 3%; Het 102 ± 9%; n = 5, t-test p = 0.83). However, high levels of variability were observed in the expression of certain proteins, such as GluA2, which would require a greater n to determine any significant phenotypic differences in protein expression.

4.3.4 $mGlu_5$ receptor expression is downregulated in hippocampal synaptoneurosomes preparations from *Syngap* heterozygous mice

Synaptoneurosome preparations prepared from *Syngap* heterozygous and WT hippocampi were probed for the same subset of proteins (Figure 4.4). There were no

significant differences in the expression profiles of PSD-95, CAMKII or Arc in synaptoneurosomes prepared from *Syngap* heterozygous and WT hippocampi. Similarly no significant differences were observed in protein expression of NMDA receptor subunits GluN2A or GluN2B (GluN2A: WT 100 ± 15%; Het 76 ± 15%; n = 5, t-test p = 0.17; GluN2B: WT 100 ± 17%; Het 92 ± 28%; n = 5, t-test p = 0.80), or AMPA receptor subunits GluA1 or GluA2 (GluA1: WT 100 ± 7%; Het 83 ± 11%; n = 5, t-test p = 0.20; GluA2: WT 100 ± 23%; Het 77 ± 18%; n = 5, t-test p = 0.27), however again there was a significant level of variability observed. Interestingly, the only significant finding was that levels of $mGlu_5$ receptor expression were significantly lower in *Syngap* synaptoneurosome preparations ($mGlu_5$: WT 100 ± 10%; Het 66 ± 5%; n = 5, t-test $*p$ = 0.003).

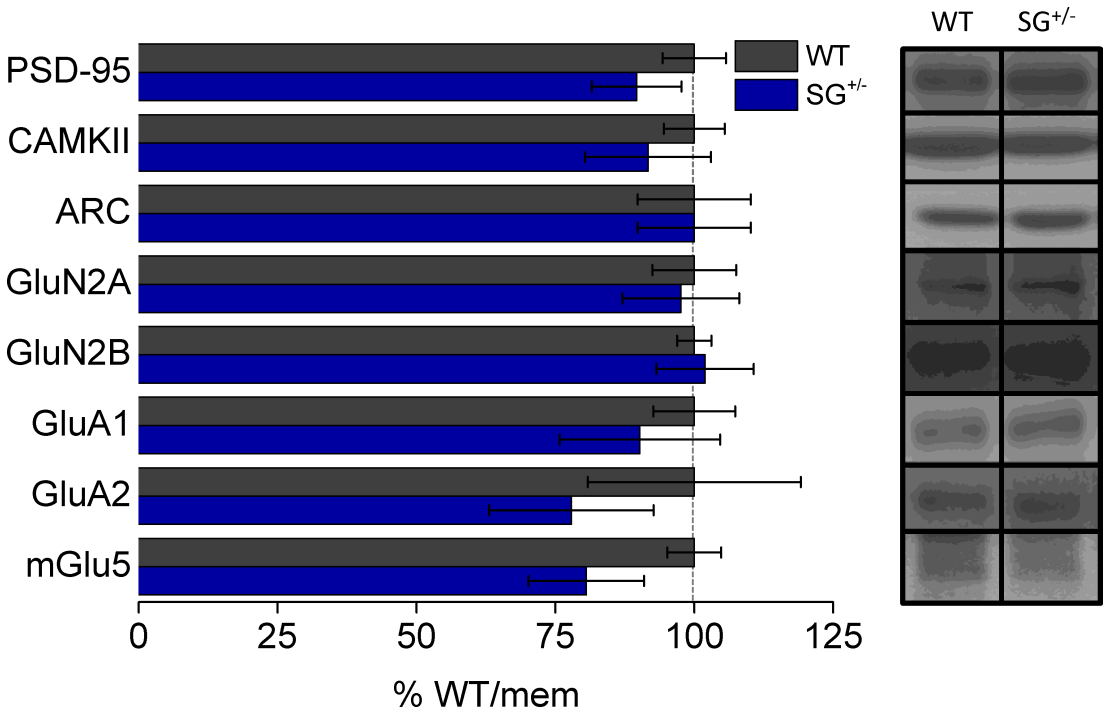


Figure 4.3 Western blots of PSD protein expression levels in hippocampal homogenates from *Syngap* heterozygous and WT mice. (A) Bar plot showing protein expression levels in whole hippocampal homogenates from *Syngap* heterozygous (het, $SG^{+/-}$) mice, quantified by western blotting and expressed as a percentage of wild-type (WT) pair. For all proteins examined no significant differences were observed between *Syngap* heterozygous and WT mice.

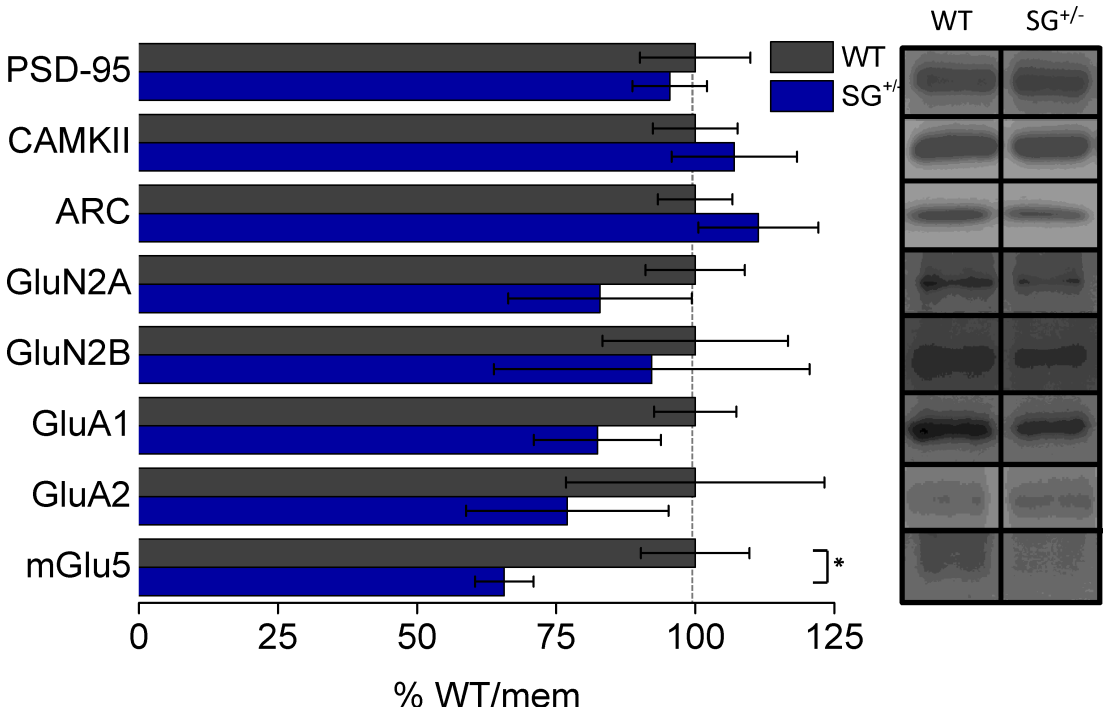


Figure 4.4 Western blots of PSD protein levels in hippocampal synaptoneurosome preparations from *Syngap* heterozygote and WT mice. (A) Bar plot showing protein expression levels in whole hippocampal homogenates from *Syngap* heterozygous (het, $SG^{+/-}$) mice, quantified by western blotting and expressed as a percentage of wild-type (WT) pair. The only significant difference observed was in $mGlu_5$ receptor expression (WT 100 ± 10%; $SG^{+/-}$ 66 ± 5%; $n = 5$, $*p = 0.008$).

4.3.5 Western blot analysis of hippocampal homogenates and synaptoneurosome preparations do not reveal elevations in ERK1/2 or mTOR signalling

Next, we investigated whether the elevation in basal levels of ERK1/2 and Akt measured in *Syngap* heterozygous hippocampal slices could be reproduced in hippocampal homogenates and synaptoneurosome preparations at the same developmental time point (P25-32) via ECL methodology. Here protein phosphorylation levels of several components of the ERK and mTOR signalling cascades were quantified in hippocampal tissue that had been rapidly dissected and immediately frozen from *Syngap* heterozygous and WT mice. Contradicting our previous findings in hippocampal slices, no significant differences were observed in any of components of the Ras-mediated signalling cascade in either hippocampal homogenates (Figure 4.5), or synaptoneurosome preparations from *Syngap* heterozygous *versus* WT mice (Figure 4.6).

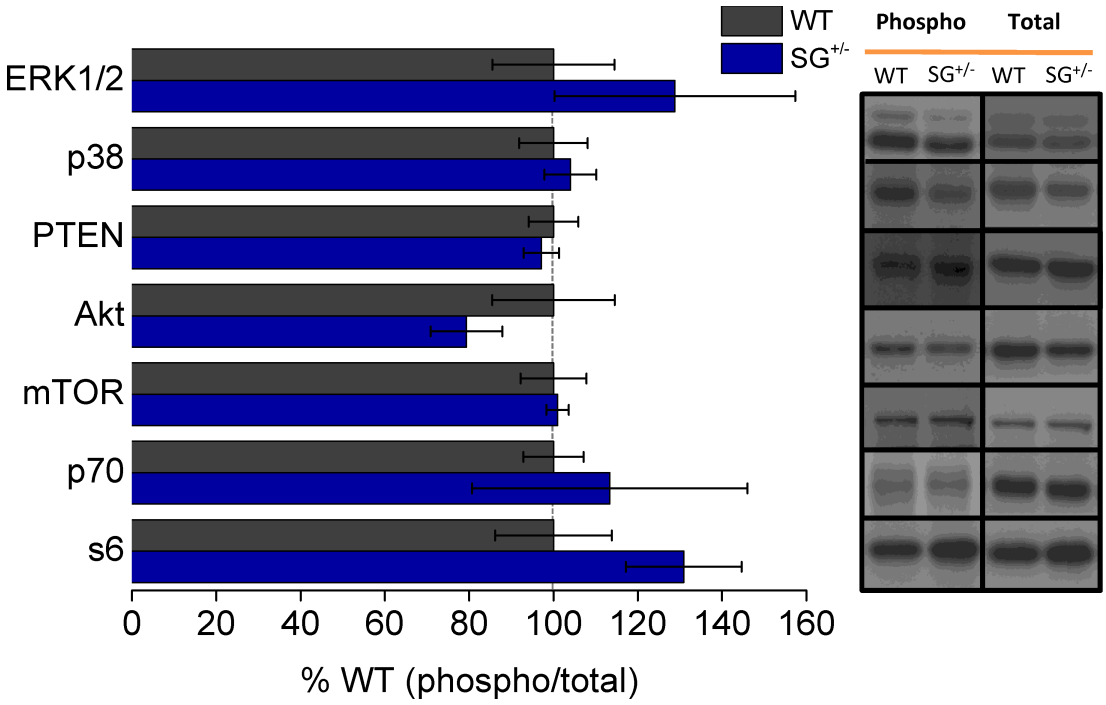


Figure 4.5 Western blots of intracellular signalling proteins in hippocampal homogenates from *Syngap* heterozygote and WT mice. Bar plot relative protein expression levels in whole hippocampal homogenates from *Syngap* heterozygous (het, $SG^{+/-}$) mice, quantified by western blotting and expressed as a percentage of wild-type (WT) pair. No significant were detected between genotypes.

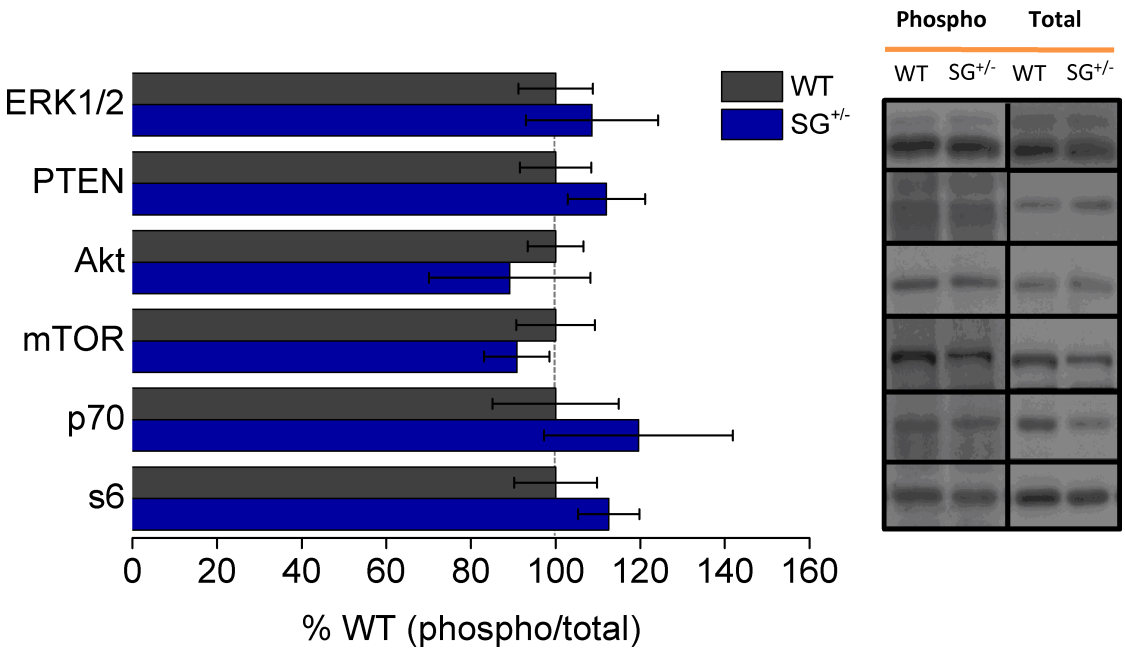


Figure 4.6 Western blots of intracellular signalling proteins in hippocampal synaptoneurosome preparations from *Syngap* heterozygote and WT mice. Bar plot relative protein expression levels in whole hippocampal synaptoneurosomes from *Syngap* heterozygous (het, $SG^{+/-}$) mice, quantified by western blotting and expressed as a percentage of wild-type (WT) pair. No significant were detected between genotypes.

4.4 Discussion

In Chapter 3 it was shown that mutations in two ID-related genes, *Fmr1* and *Syngap1,* lead to similar phenotypic manifestations in the hippocampus of *Fmr1* KO and *Syngap* heterozygous mice. These findings suggest that both FMRP and SynGAP form part of the same intracellular signalling cascade that link $mGlu_{1/5}$ receptor activation to mRNA translation. Thus this chapter was dedicated to investigating the intracellular signalling mechanisms that may be disrupted in *Syngap* heterozygous mice, which could consequently lead to excessive protein synthesis and enhanced mGluR-dependent LTD.

The activation of Gp1 mGlu receptors at CA1 synapses in the hippocampus stimulates the translation of pre-existing mRNA transcripts at local dendritic sites (Weiler et al. 1997; Huber et al. 2000). Here, $mGlu_1$ and $mGlu_5$ receptors do not signal through the canonical pathway associated with $G_{q/11}$ coupled GPCRs but instead utilize Ca^{2+}-independent signalling mechanisms to target the initiation and elongation phase of translation. The two major signalling cascades that couple $mGlu_{1/5}$ receptors to the translational apparatus are the ERK1/2-MAPK and PI3K-Akt-mTOR pathways (Banko et al. 2006; Ronesi & Huber 2008a). Both of these signalling cascades are activated by $mGlu_{1/5}$ receptors and are crucial for long-lasting forms of synaptic plasticity (Gallagher et al. 2004; Hou & Klann 2004). Thus ERK1/2 and mTOR signalling have been extensively studied in the mouse model of FXS and were the primary focus in this study.

4.4.1 Hyperactivity of ERK1/2 and Akt lead to elevated protein synthesis rates in *Syngap* heterozygous mice

Although $mGlu_{1/5}$ receptor mediated synaptic plasticity and protein synthesis are aberrant in the *Fmr1* KO mouse, until recently there was no evidence to directly link either the ERK1/2 or mTOR pathway to elevated protein synthesis rates in the hippocampus. However, a recent study by Osterweil et al. (2010) directly measured mRNA translational rates in *Fmr1* KO mice and quantified activation states of key signalling components of the ERK1/2 and mTOR signalling cascade under similar

conditions in which exaggerated LTD was observed. Here elevated protein synthesis rates in *Fmr1* KOs were not accompanied by the hyperactivation of either pathway but were caused by a hypersensitivity of the translational machinery to $mGlu_{1/5}$ signalling. Furthermore, the same study found that inhibitors of ERK1/2, but not mTOR, normalized elevated protein synthesis rates to WT levels suggesting that the saturation of mGluR-dependent mRNA translation in the *Fmr1* KO brain was downstream of the ERK1/2-MAPK pathway.

By employing a similar experimental approach it was shown in this chapter that elevated protein synthesis rates in the hippocampus of *Syngap* heterozygous mice are accompanied by significant increases in the activation state of both ERK1/2 and Akt. Although a more detailed biochemical analysis of these two pathways is required, the hyperactivity of ERK1/2 and Akt are consistent with SynGAPs role as a negative regulator of Ras activity. Ras family members include Ras, Rap1 and Rap2. Both Ras and Rap1 can activate the ERK1/2 and mTOR pathways through a distinct series of phosphorylation steps (Ye & Carew 2010). The $mGlu_5$ receptor can activate the PI3K-Akt-mTOR through its interaction with homer (Rong et al. 2003), or through activation of Ras. The mechanism by which $mGlu_5$ receptors are linked to Ras mediated signalling, including activation of ERK-MAPK pathway, is not known. However there is evidence to suggest that it may involve scaffolding proteins, such as the β-arrestins or the kinase suppressor of Ras1 (KSR) (Shalin et al. 2006; DeWire et al. 2008).

Substrates of both ERK1/2 and mTOR signalling are known to be involved in the translational control of gene expression, and include: Mnk1, S6 protein kinases, eIFs, 4E-BPs and eukaryotic elongation factor 2 (Richter & Sonenberg 2005). Here the activation state of an S6K kinase substrate, ribosomal protein S6, was examined in in the hippocampus of *Syngap* heterozygous mice. S6 is the subunit of the small 40S ribosome that directly interacts with mRNA, tRNA and translational initiation factors (Nygård & Nilsson 1990). Western blot analysis revealed that phosphorylation levels of S6 at $Ser^{235/236}$ were elevated in hippocampal slices from *Syngap* heterozygous mutants, whilst phosphorylation at $Ser^{240/244}$ was unaffected. S6 is phosphorylated at

several serine residues ($Ser^{235/236}$, $Ser^{240/244}$ and Ser^{247}) by two classes of protein kinases, S6K1/2 and RSK, which are activated by mTOR and ERK1/2 respectively (Frödin et al. 2000). Ras signalling is specifically linked the phosphorylation of S6 at $Ser^{235/236}$ but not $Ser^{240/244}$ (Richter & Sonenberg 2005; Roux et al. 2007). In contrast, mTOR signalling via S6K1/2 leads to the phosphorylation of S6 at all five serine sites (Roux & Blenis 2004; Pende et al. 2004).

In addition to cap-dependent translation, the activation of S6 is associated with the translation of 5'TOP mRNAs that encode translation initiation factors and ribosomal protein subunits (Antion et al. 2008a). Thus hyperactivation of S6 may increase the translation capacity of hippocampal neurones exacerbating protein synthesis rates in *Syngap* heterozygous mice under basal conditions. Interesting, DHPG-induced increases in S6 phosphorylation and 5'TOP mRNAs persist in the absence S6K1/2 (Antion et al. 2008b). Furthermore the same study reported that *S6K2* KOs exhibit enhanced mGluR-dependent LTD. This may point towards a mechanism, in which $mGlu_{1/5}$ signals through RSK to activate S6, rather than S6K1/2 to initiate mGluR-dependent mRNA translation. Alternatively, S6K2 activation could lead to the functional repression of translation during an LTD stimulus. Thus, one potential signalling mechanism that may result in excessive basal protein synthesis rates in the hippocampus of *Syngap* heterozygous mice may involve Ras signalling through ERK1/2 to activate RSK which phosphorylates S6 at $Ser^{235/236}$ leading to 5'TOP mRNA translation as well as the initiation of cap-dependent translation via the activation of EIF4B or Mnk1 (Shahbazian et al. 2006; Banko et al. 2006; Figure 4.7).

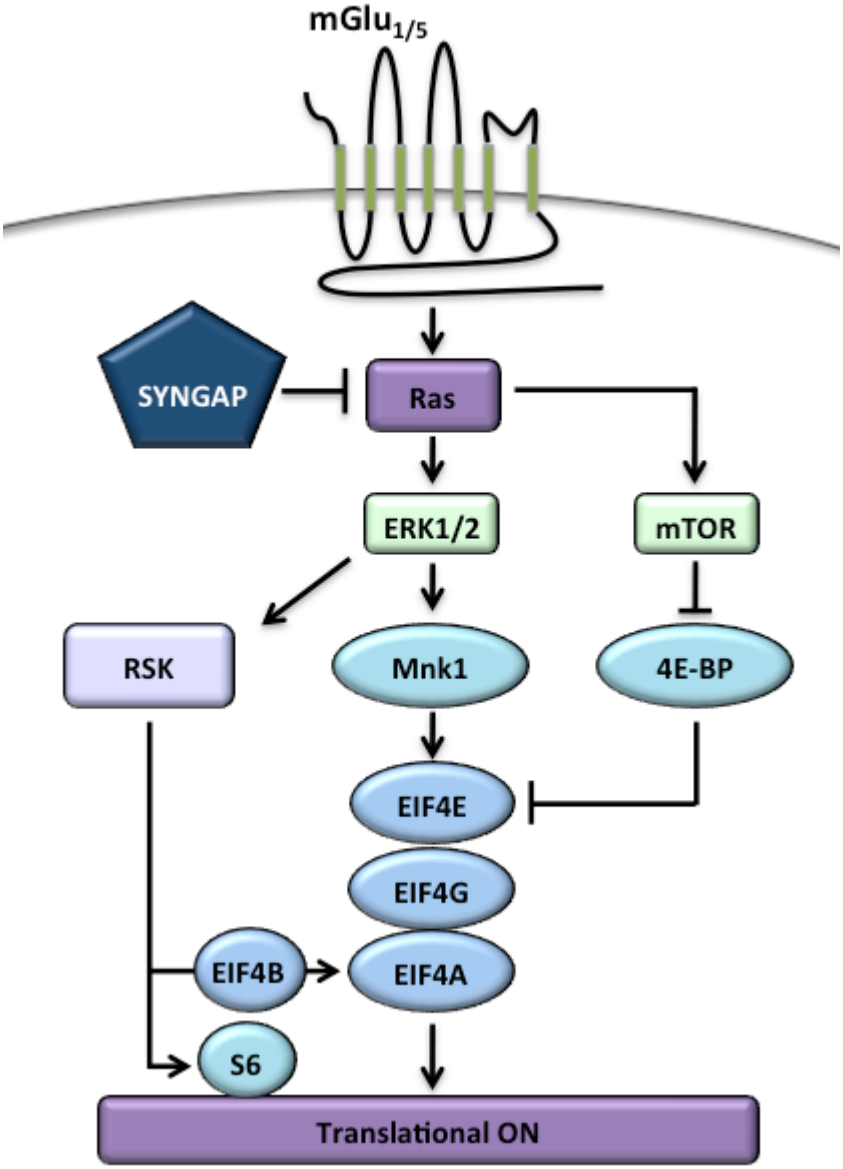


Figure 4.7 mGluR-dependent mRNA translation in the hippocampus. Activation of Gp1 mGlu receptors leads to the activation of Ras signalling. Targets of Ras include both ERK1/2 and PI3K-Akt-mTOR signalling cascade. ERK1/2 can initiate mGluR-dependent translation by targeting the p90 S6K, RSK, which targets ribosomal protein S6, which is linked to the 5TOP mRNA translation. In addition RSK can target EIF4B, which potentiates the activity EIF4A, facilitating the formation of the EIF4F complex leading to the initiation of cap-dependent translation. In response to mTOR, Ras may also signal through mTOR, which targets 4E-BP, leading to the release of EIF4E, which binds to EIF4G. Once EIF4E is bound to the EIF4F, Mnk1 phosphorylates EIF4E through activation of ERK leading to the initiation of cap-dependent translation.

Another downstream target of ERK1/2 and mTOR is 4E-BP (Kelleher et al. 2004; Banko et al. 2006). Similarly to *S6K2* KOs, the genetic deletion of *4E-BP2* also results in elevated mGluR-dependent LTD that is sensitive to U0126 but insensitive to rapamycin (Antion et al. 2008b). This suggests that mTOR may lie upstream of 4E-BP2, signalling through this protein to regulate mGluR-dependent LTD (Figure 4.7). Previously it has been shown that inhibition of mTOR, not ERK1/2, prevents DHPG-induced phosphorylation 4E-BP2 (Banko et al. 2006). At the synapse 4E-BP family members are bound to eIF4E inhibiting the formation of the EIF4F complex and preventing the initiation of mRNA translation (Richter & Sonenberg 2005). Consequently, the loss of 4E-BP is thought to lead to an increase in the availability of EIF4E, permitting EIF4E to bind EIF4G, leading to the formation of the EIF4F initiation complex. Once EIF4E is bound to EIF4G, Mnk1 can phosphorylate this protein, in an ERK-dependent manner, and initiate cap-dependent translation (Banko et al. 2006). Thus increases in basal protein synthesis rates in *Syngap* heterozygous mice could also arise through ERK1/2 and mTOR inhibiting 4E-BP.

Collectively, these intracellular signalling cascades may contribute to elevated translational rates in the hippocampus of *Syngap* heterozygous mice. In the mouse model of FXS many of symptoms are thought to arise from an increase in synaptic protein synthesis, which could be applicable to the mouse model of *Syngap* haploinsufficiency. Importantly, increased protein synthesis rates in *Fmr1* KO mice can be restored in adulthood (Dölen et al. 2007; Osterweil et al. 2010; Osterweil et al. 2013). This raises the intriguing possibility that pharmacologically strategies used in the treatment of FXS, may also correct the hippocampal pathophysiology we observe in *Syngap* heterozygous mice following symptom onset.

4.4.2. Translational rates are saturated downstream of $mGlu_5$ receptor activation in *Syngap* heterozygous mice

The activation of Gp1 mGlu receptors with DHPG, at a concentration that induces a persistent depression of synaptic responses, did not cause a further increase in mRNA translational rates in *Syngap* heterozygous hippocampal slices. This suggests that genetic reduction of SynGAP expression mimics and occludes the effect of DHPG on protein synthesis. Thus it appears that in the hippocampus of *Syngap* heterozygous mice mRNA translational rates downstream of $mGlu_{1/5}$ activation are saturated and no longer regulated in an activity-dependent manner.

In CA1 of the hippocampus the expression of mGluR-dependent LTD is dependent on cap-dependent translation to maintain reduced AMPA receptor expression at the postsynaptic terminal (Huber et al. 2000). Thus it was hypothesized that proteins normally up regulated in response to $mGlu_{1/5}$ receptor activation may be present in the *Syngap* heterozygous brain at rest. However, western blot analysis of hippocampal extracts from *Syngap* mutant mice revealed that there were no significant differences in the expression of PSD-95, CAMKII and Arc. This was unexpected as elevated protein synthesis rates in the hippocampus of *Syngap* heterozygous mice are accompanied by the enhanced levels of mGluR-dependent LTD, which we predicted would arise from the overexpression of proteins that facilitate AMPA receptor removal.

One explanation for these apparently conflicting findings is that there may also be alterations in protein degradation in the hippocampus of *Syngap* heterozygous mice. For mGluR-dependent protein expression to be consistent between *Syngap* heterozygous and WT mice, when translational rates are increased in *Syngap* mutants, newly synthesized proteins would have to be broken down at a similar rate to which they were made. This could indicate that the protein degradation pathway is also hyperactive under basal conditions in the *Syngap* heterozygous brain. However if protein synthesis and degradation rates were matched this wouldn't explain why LTD is enhanced and independent of new protein synthesis, unless mGlu receptor activation puts a brake on protein degradation. Inhibition of the proteasome

degradation pathway would lead to a rapid build up of "LTD proteins" at CA1 synapses due to enhanced mRNA translation rates in the *Syngap* heterozygous brain. The sudden overabundance of "LTD proteins" could contribute to exaggerated AMPA receptor removal. In order to test this idea, mRNA translational rates could be measured in the presence of a proteasome inhibitor, such as Mg132, and western blot analysis performed on the same slice homogenates. This will determine whether the proteasome degradation pathway impacts basal protein synthesis rates in both WT and *Syngap* heterozygous mice and allow quantification of proteins overtranslated in the *Syngap* heterozygous brain.

Another possibility is that targets of "LTD proteins", which include components of the AMPA receptor endocytotic machinery, are somewhat hypersensitive to their expression. This could occur if ERK1/2 and/or mTOR activation have additional functional effects at the synapse, for example priming proteins that are involved in the mGluR-dependent depression of synaptic responses (e.g. STEP, MAP1b, Arc). As observed in *Fmr1* KO and *Syngap* heterozygous mice, increases in basal protein synthesis only impact synapse function when $mGlu_{1/5}$ receptors are activated. Basally there is no reduction in AMPA receptor content at the synapse as measured by I/O function and western blot analysis. This suggests that a stimulus is still required in order to trigger AMPA receptor removal, even though in *Fmr1* KO mice there is an overabundance of "LTD proteins". Thus it will be important to access whether inhibitors of ERK or mTOR impress on mGluR-dependent LTD in the hippocampus of *Syngap* heterozygous mice. It is already known from Chapter 3 that this form of synaptic plasticity is insensitive to translation inhibitors in the *Syngap* heterozygous brain, thus if ERK1/2 and mTOR are altering synapse function solely through mRNA translation then they are unlikely to effect the expression of LTD in *Syngap* heterozygous hippocampal slices.

In addition we detected no significant differences in the activation states of proteins that compose the ERK1/2 or mTOR pathway in hippocampal homogenates or synaptoneurosomes from *Syngap* heterozygous mice. This data was in stark contrast to findings observed in hippocampal slice homogenates. This could arise from a

caveat associated with our experimental approach because protein expression levels were not quantified in conditions where elevated protein synthesis and mGluR-dependent LTD were observed. Quantification of Ras-mediated signalling components were initially performed in single dorsal hippocampal slices, which were left to recover for at least 4 hours following tissue preparation and quantified using infrared fluorescence. Previously Osterweil et al. (2010) has shown that protein synthesis rates in the hippocampus require a recovery period of at least 4 hours before mRNA translational rates stabilize. In contrast, here western blot analysis was performed on whole hippocampi that were rapidly dissected and processed. This method of tissue preparation may only capture early post-mortem differences in protein metabolism, rather than alterations in protein expression under stable slice conditions where electrophysiological recordings were performed (Osterweil et al. 2010).

This may explain why we did not observe significant increases in ERK1/2 or mTOR signalling or in "LTD protein" expression in hippocampal extracts from *Syngap* heterozygous mice. The only significant difference observed was in the expression of $mGlu_5$ receptors, which were markedly reduced in *Syngap* heterozygous synaptoneurosomes but not in homogenate. This may suggest total $mGlu_5$ expression in the hippocampus is unaffected but there is a reduction of $mGlu_5$ receptors at the postsynaptic terminal. Altered $mGlu_5$ receptor levels have also been detected in PSD fractions prepared from *Fmr1* KO mice with no detectable change in total $mGlu_5$ receptor expression in forebrain homogenates (Giuffrida et al. 2005). These findings may reflect a compensatory effect due to the functional consequences $mGlu_5$ receptor activation imposes at *Syngap* heterozygous and *Fmr1* KO synapses. Furthermore, our findings that GluN2A and GluN2B were unchanged in the *Syngap* hippocampal extracts is in agreement with a previous study, which found no significant differences in NMDA receptor expression in the hippocampus of mature *Syngap* heterozygous mice (Komiyama et al. 2002).

In retrospect, quantification of $mGlu_5$-related proteins, particularly of those encoded by cap- and TOP- mRNAs, in metabolically stable slices would have allowed a more comparable assessment of protein expression under conditions where we observe abnormal mGluR-dependent synaptic plasticity and protein synthesis. Although this would be possible for highly abundant proteins, such as PSD-95 and CAMKII, it may not be feasible for low abundant proteins such as p70S6K unless multiple hippocampal samples were pooled together. As previously mentioned, when quantifying phospho-p70S6K in single hippocampal slice homogenates, using infrared fluorescence, there was no quantifiable signal. Thus experimental conditions need to be optimised to obtain a robust readout of protein expression in order to examine the activation status of key signalling proteins implicated in the pathophysiology of FXS and *Syngap1* haploinsufficiency. Furthermore it should be noted that western blot analysis only captures alterations in protein expression that differ by 20% or more (Personal communication Professor Kind). Thus, perhaps this method of quantification is not sensitive enough to identify proteins overtranslated in *Syngap* heterozygous brain. An alternative approach could be to use a translating ribosome affinity protocol (TRAP) assay, to determine which mRNA transcripts are bound to the actively transcribing polyribosomes in the hippocampus of *Syngap* heterozygous mice under basal conditions. This would allow the identification of potential pathogenic proteins that may contribute to the underlying synaptic function dysfunction associated with *Syngap1* haploinsufficiency.

4.5 Summary

Although phenotypically, *Syngap* heterozygous and *Fmr1* KO mice share a similar hippocampal pathophysiology, data presented in this chapter reveals that there are clear distinctions between these two models of ID at the molecular level. Firstly, in the *Fmr1* KO hippocampus elevations in basal protein synthesis were observed in the absence of any alterations in either ERK1/2 or mTOR signalling. Yet the deletion of *Fmr1* occluded any further increase in mRNA translation induced by Gp1 mGlu receptor activation and inhibitors of either $mGlu_5$ or the Ras-ERK1/2 signalling pathway (but not mTOR) corrected increased basal protein synthesis raised (Osterweil et al. 2010; Osterweil et al 2013). These findings indicated that increased translational rates in *Fmr1* KO mice resulted from a hypersensitivity of the mRNA translational machinery to constitutive $mGlu_5$–ERK1/2 signalling that was direct effect of the loss of translational repression in the absence of FMRP. In this chapter the same experimental conditions, as used in *Fmr1* KO mice, revealed an increase in the activity of ERK1/2 and Akt in the hippocampus of *Syngap* heterozygous mice suggesting that elevations in translational rates may arise from hyperactivity in ERK1/2 and mTOR signalling under steady state conditions. Thus, there is a possibility that inhibition of ERK1/2 or mTOR signalling may restore elevated protein synthesis rates in the hippocampus of *Syngap* heterozygous mice.

Chapter 5

Rescue of the Hippocampal Pathophysiology in the Mouse Model of *Syngap1* Haploinsufficiency

5.1 Key findings

1.) Elevated protein synthesis rates in *Syngap* heterozygous mice are selectively reduced to WT levels by acute inhibition of either $mGlu_5$, Ras or ERK1/2.

2.) U0126 & Lovastatin lower overactive ERK1/2 and $S6^{235/236}$ phosphorylation in *Syngap* heterozygous hippocampal slices.

3.) U0126 & rapamycin reduce mGluR-dependent LTD in *Syngap* heterozygous mice via a protein synthesis independent mechanism.

4.) Rapamycin reduces elevated protein synthesis rates, normalizes elevated mGluR-dependent LTD and restores the protein synthesis dependency of this form of synaptic plasticity.

5.2 Introduction

Altered $mGlu_{1/5}$ receptor signalling appears to be a shared dysfunction in the mouse model of FXS and *Syngap1* haploinsufficiency. In Chapters 3 and 4 it was observed that *Syngap* heterozygous mice share a similar hippocampal pathophysiology to FXS mutants, including enhanced mGluR-dependent LTD and increased translational rates that are saturated downstream of $mGlu_{1/5}$ receptor activation. In *Fmr1* KO mice elevated basal protein synthesis rates are observed in the absence of altered ERK1/2 or mTOR signalling, suggesting that mRNA translational apparatus are hypersensitive to constitutive $mGlu_5$ receptor activity due to the loss of steady-state translational repression by FMRP. In contrast, both ERK1/2 and mTOR signalling appear overactive in the *Syngap* heterozygous mutants when examined under similar experimental conditions. This suggests that increased translation rates, associated with *Syngap1* haploinsufficiency, may arise from a hyperactivity of Ras-mediated intracellular signalling.

Normally SynGAP binds and inactivates Ras family members suppressing the activation of downstream signalling cascades that include both ERK1/2 and mTOR (Ye & Carew 2010). However in *Syngap* heterozygous mice, where SynGAP expression is reduced by at least 50%, there is a loss of Ras inactivation that consequently leads to the overactivation of ERK1/2 and mTOR signalling under steady-state conditions. At CA1 synapses both ERK1/2 and mTOR can trigger the initiation of cap-dependent and 5'TOP mRNA translation by regulating translation factors and RNA binding proteins (Banko et al. 2006; Ronesi & Huber 2008; Antion et al. 2008a). They are both activated in response to $mGlu_{1/5}$ receptor stimulation and play a crucial role in the expression and maintenance of mGluR-dependent LTD (Hou & Klann 2004; Gallagher et al. 2004).

Based on this evidence, overactivation of ERK and mTOR signalling may underlie increased translational rates and enhanced mGluR-dependent LTD that is observed in the *Syngap* heterozygous brain. In *Fmr1* KO mice, elevated protein synthesis rates are restored to WT levels by inhibitors of either $mGlu_5$, Ras or ERK1/2, but not mTOR (Dölen et al. 2007; Osterweil et al. 2010; Osterweil et al. 2013; Michalon et al. 2012). Thus the focus of this chapter is to assess whether the hippocampal pathophysiology associated with *Syngap1* haploinsufficiency can be reversed using similar pharmacological strategies known to be effective at correcting hippocampal-based phenotypes in mouse models of ID (Figure 5.1).

CTEP a highly specific & long-lasting negative allosteric modulator of the $mGlu_5$ receptor

Recently CTEP, a novel, long lasting (K_d = 1.8 nM for mouse) and orally bioavailable NAM of $mGlu_5$ was developed. CTEP is 1000 times more selective for the $mGlu_5$ receptor than its predecessor MPEP (Lindemann et al. 2011). Furthermore, unlike MPEP and fenobam, which are extremely short acting (half-life approximately 15 minutes in the brain), a single-dose of CTEP administered every 48 hours achieves consistent receptor occupancy of 81%. In *Fmr1* KO mice, chronic treatment with CTEP starting at 4-5 weeks corrected: learning and memory deficits, hyperactivity to sensory stimulus, elevated locomotor activity, increased dendritic spine density, increased AGS susceptibility, excessive mGluR-dependent LTD and elevated protein synthesis (Michalon et al. 2012).

Lovastatin reduces Ras-ERK signalling

Similarly, lovastatin is a promising therapeutic strategy for the correction of synaptic dysfunction associated with mouse models of ID. Previous work in *NF1* heterozygous mice has shown that lovastatin, an inhibitor of HMG-CoA reductase, rescues cognitive abnormalities and impaired LTP associated with the hyperactivity of Ras (Li et al. 2005). In *Fmr1* KO mice, lovastatin corrected elevated protein synthesis rates and exaggerated mGluR-dependent LTD in the hippocampus along

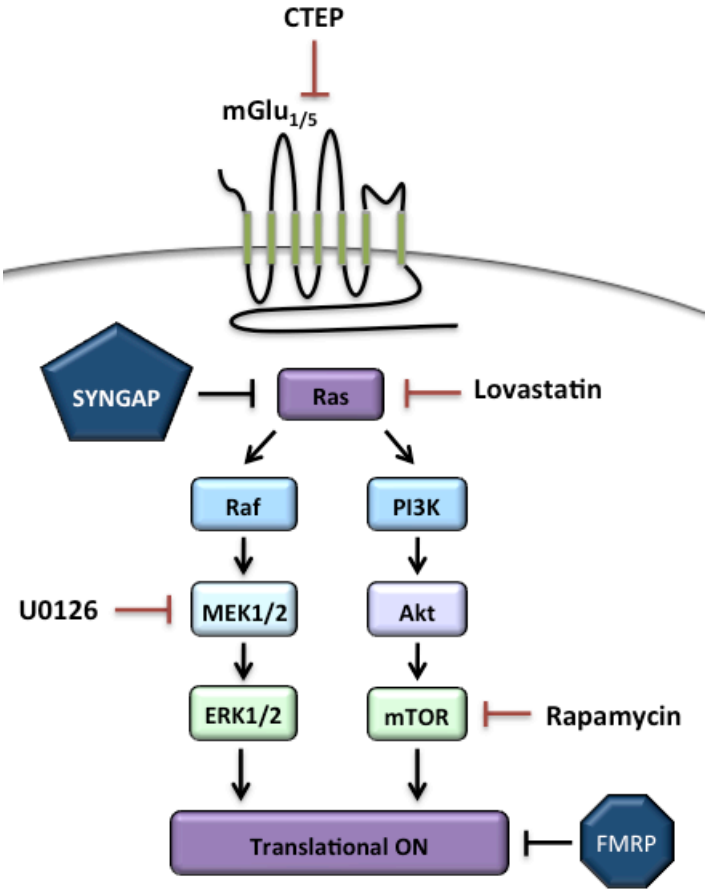


Figure 5.1 CTEP, lovastatin, U0126 & rapamycin target components of $mGlu_5$ receptor signalling that converge on mRNA translation. $mGlu_5$-Ras-mediated signalling can be reduced in the hippocampus by targeting various components of the intracellular signalling cascade. CTEP selectively targets $mGlu_5$, lovastatin causes a subtle reduction in Ras activity, U0126 inhibits MEK1/2 reducing ERK1/2 signalling whilst rapamycin targets mTOR. Both ERK and mTOR signalling converge on the translational apparatus that initiate mRNA translation.

with *in vivo* and *in vitro* epilepsy phenotypes (Osterweil et al. 2013). Lovastatin indirectly reduces Ras activity by targeting the mevalonate pathway involved in farnesylating the C-terminal tail of Ras, which is required for Ras to translocate to the plasma membrane where it is activated and functional (Li et al. 2005). Thus in *Syngap* heterozygotes where SynGAP expression is reduced, lovastatin may provide a potential pharmacological tool to reduce Ras activity.

Direct inhibition of ERK & mTOR activity

Downstream of Ras lies ERK1/2 and mTOR signalling, which can be targeted with U0126 and rapamycin respectively. U0126 inactivates the MAPK signalling pathway by selectively targeting the upstream activator of ERK1/2, MEK1/2 (Duncia et al. 1998). Previously, U0126 has been shown to correct elevated protein synthesis rates in the hippocampus of *Fmr1* KO mice. Rapamycin targets mTORC1 complex that consists of mTOR in complex with Raptor and LST8/GβL.

This chapter will set out to examine whether the cellular deficits observed in the hippocampus of young adult *Syngap* heterozygous mice can be restored pharmacologically following phenotypic onset. These findings will establish whether alterations in hippocampal pathophysiology associated with *Syngap1* haploinsufficiency are due to an ongoing overactivation of $mGlu_5$-Ras signalling, which if downregulated can restore normal synaptic function. If successful, then this study will provide further evidence to support the hypothesis that mutations in *Fmr1* and *Syngap1* converge on the same pathophysiological axis and would raise the intriguing possibility that therapeutics currently in clinical trials for the treatment of FXS may also be beneficial to patients with mutants in *SYNGAP1*.

5.3 Results

In *Syngap* heterozygous mice, mGluR-dependent LTD is enhanced and insensitive to protein inhibitors. This is accompanied by hyperactive ERK1/2 and mTOR signalling and elevated basal protein synthesis rates that are saturated downstream of $mGlu_5$ receptors. To assess whether these hippocampal deficits are amenable to pharmacological rescue, a biochemical approach was initially taken to determine whether these compounds could restore elevated protein synthesis rates in the *Syngap* heterozygous brain. Hippocampal slices from juvenile (P25-32) *Syngap* heterozygous and WT mice were prepared and translational rates were quantified by measuring ^{35}S incorporation in the presence of either a vehicle (DMSO) or one of the following inhibitors: CTEP (10 μM), lovastatin (50 or 100 μM), U0126 (5 μM) or rapamycin (20 nM). Slices were preincubated in actinomycin D and either vehicle/inhibitor for 30 minutes and then protein synthesis rates were measured for a further 45 minutes in the presence of vehicle or inhibitor, initially at a drug concentration that was shown to be effective in the *Fmr1* KO mice (Figure 5.2).

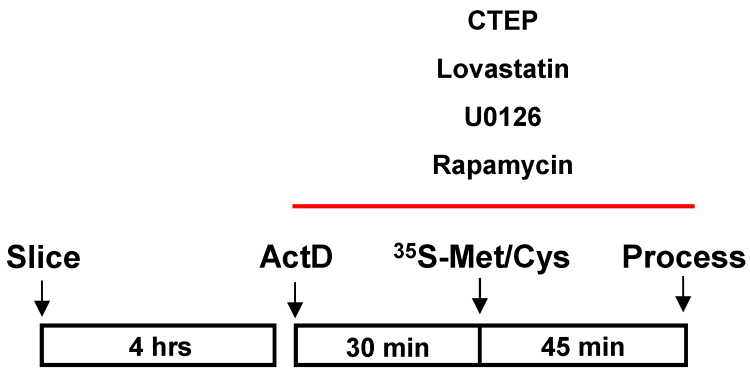


Figure 5.2 Metabolic labelling of hippocampal slices from *Syngap* heterozygous mice in the presence of inhibitors. Experimental timeline for ^{35}[S]-Met/Cys metabolic labeling in the presence of CTEP, lovastatin, U0126 and rapamycin, which target either $mGlu_5$ receptors, Ras, ERK1/2 and mTOR respectively. Dorsal hippocampal slices are left to recover for 4 hours in ACSF, new transcription is blocked with actinomycin D (Act D, 25 μM) ± inhibitor for 30 minutes before slices are transferred to ^{35}S Met/Cys protein labelling mix ± inhibitor for 45 minutes.

5.3.1 Inhibition of $mGlu_5$ receptors reduces elevated basal protein synthesis rates in the hippocampus of *Syngap* heterozygous mice

As altered $mGlu_5$ receptor signalling appears to be a crucial component of the pathogenesis underlying *Syngap1* haploinsufficiency, we examined whether the acute application of CTEP, a highly selective NAM of $mGlu_5$, could restore elevated protein synthesis rates in the hippocampus of *Syngap* heterozygous mice. Hippocampal slices from young adult *Syngap* heterozygous and WT mice were preincubated in CTEP (10 μM) and protein synthesis rates were directly measured by quantifying ^{35}S incorporation. In WT slices, basal protein synthesis levels were unaffected by CTEP treatment indicating that $mGlu_5$ receptor activity does not contribute to steady-state rates of mRNA translational (WT veh 100 ± 3%; WT CTEP 106 ± 5 %, $n = 9$; Figure 5.3). Consistent with findings in *Fmr1* KO mice (Osterweil et al. 2010); CTEP normalized elevated translational rates in the hippocampus of *Syngap* heterozygous mice (Het veh 125 ± 4%; Het CTEP 102 ± 8% $n = 9$; ANOVA genotype $p = 0.06$). Thus, it appears that increased translational rates lie downstream of constitutive $mGlu_5$ receptor activation in *Syngap* heterozygous mutants.

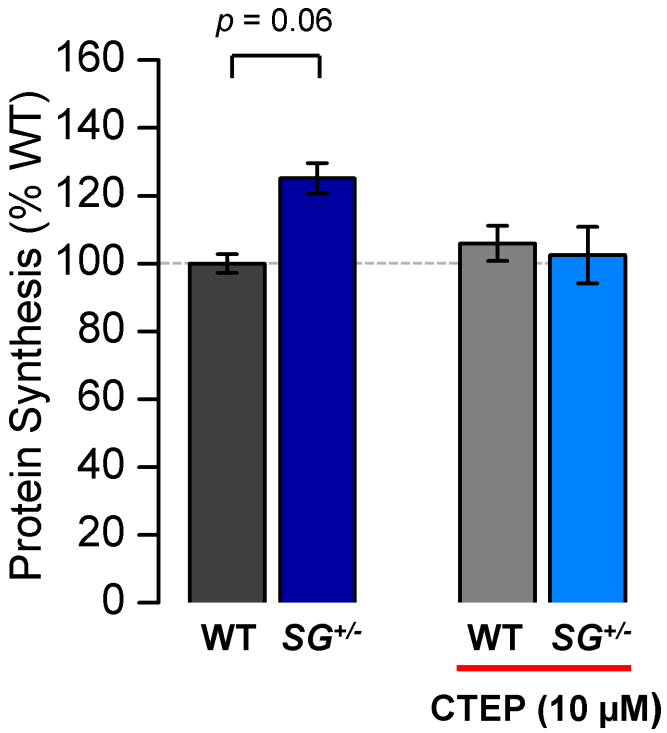


Figure 5.3 CTEP corrects elevated protein synthesis in the hippocampus of *Syngap* heterozygous mice. In vehicle treated slices protein synthesis rates are elevated in dorsal hippocampal slices from *Syngap* heterozygous mice compared to wild-type (WT) littermates. In the presence of CTEP, increased protein synthesis levels are reduced to WT levels (WT: veh 100 ± 3%, 10 µM 106 ± 5%; $SG^{+/-}$: veh 125 ± 4%; 10 µM 102 ± 8%; ANOVA veh genotype $p = 0.06$, $n = 9$).

5.3.2 Lovastatin normalises excessive protein synthesis rates in the hippocampus of *Syngap* heterozygous mice

Both the ERK1/2-MAPK and PI3K-Akt-mTOR signalling cascades converge on translational factors and RNA bindings proteins that initiate cap-dependent and 5'TOP mRNA translation, in response to $mGlu_{1/5}$ receptor activation (Banko et al. 2006; Antion et al. 2008). At the top of these intracellular signalling cascades lies Ras. Similarly to *NF1* mutants, Ras activity is increased in two-fold in forebrain homogenates from *Syngap* heterozygous mice, indicating that it may also be a key component of pathophysiological axis underlying *Syngap1* haploinsufficiency (Li et al. 2005; Carlisle et al. 2008). Furthermore we observed that substrates of Ras signalling, including ERK1/2 and Akt were significantly elevated in hippocampus of *Syngap* heterozygous mice and were accompanied by elevated protein synthesis rates that were saturated downstream of $mGlu_5$ receptor activation.

In the mouse model of NF1 and FXS, inhibitors of Ras activity (e.g. farnesyl thiosalicyclic acid and lovastatin) restored hippocampal pathophysiology, cognitive deficits and abolished epileptogenesis (Li et al. 2005; Osterweil et al. 2013). Thus we hypothesized that downregulation of Ras activity with the acute application of lovastatin may restore protein synthesis rates in the hippocampus of *Syngap* heterozygous mice. To test this hypothesis, hippocampal slices were pre-incubated in lovastatin during metabolic labelling. Here it was observed that 50 μM lovastatin caused a small, albeit significant, reduction of translational rates in *Syngap* heterozygous slices (WT: veh 100 ± 2%, 50 μM lovastatin 102 ± 12%; Het: veh 128 ± 5%; 50 μM lovastatin 115 ± 3%; ANOVA genotype $*p = 0.01$, $n = 4$; Figure 5.4A), therefore, the concentration of lovastatin was increased from 50 μM to 100 μM. Incubation of hippocampal slices in 100 μM lovastatin normalized protein synthesis rates to a level that was indistinguishable from WT controls (WT: veh 100 ± 4%, 100 μM 92 ± 7; Het: veh 142 ± 9%, 100 μM 106 ± 9%; ANOVA genotype $*p = 0.0007$, treatment $*p = 0.006$; $n = 7$; Figure 5.4B) suggesting increased translation rates lie downstream of Ras signaling in the *Syngap* heterozygous mice.

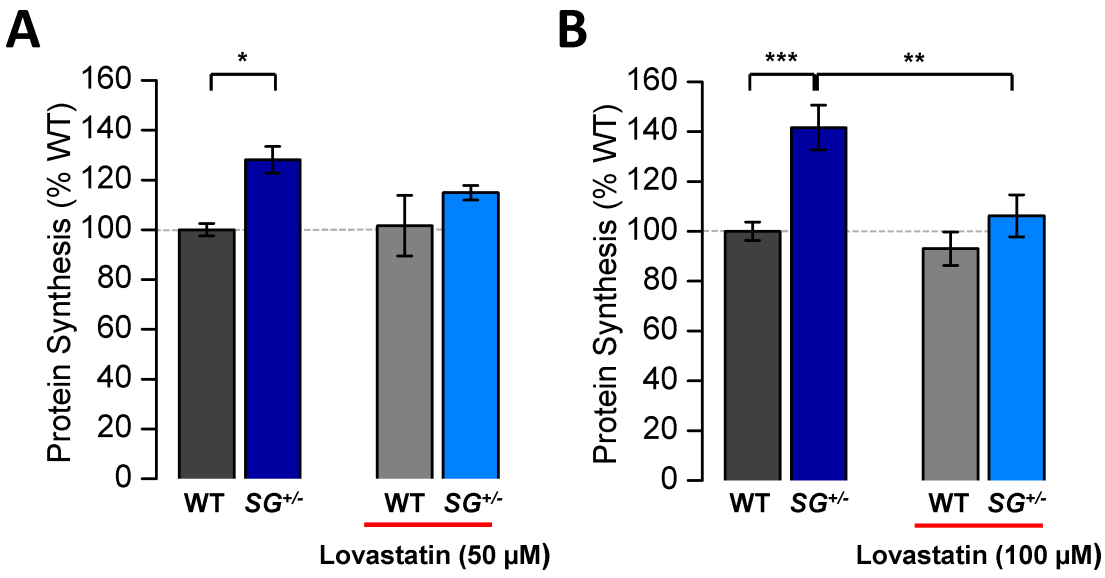


Figure 5.4 Lovastatin corrects elevated protein synthesis in *Syngap* heterozygous mice. (A) In the presence of lovastatin (50 µM), elevated protein synthesis rates were subtly reduced but not corrected to WT levels in dorsal hippocampal slices from *Syngap* heterozygous (het, $SG^{+/-}$) mice (WT: veh 100 ± 2%, 50 µM 102 ± 12%; $SG^{+/-}$: veh 128 ± 5%; 50 µM 115 ± 3%; ANOVA genotype *$p = 0.01$, $n = 4$). (B) Increasing the concentration of lovastatin (100 µM) normalised excessive translational rates to WT levels in hippocampal slices from $Syngap^{+/-}$ mice (WT: veh 100 ± 4%, 100 µM 92 ± 7; $SG^{+/-}$: veh 142 ± 9%, 100 µM 106 ± 9%; ANOVA genotype *$p = 0.0007$, treatment *$p = 0.006$; $n = 7$).

5.3.3 Lovastatin reduces ERK1/2 activity in the hippocampus of *Syngap* heterozygous mice

To examine whether lovastatin elicits its actions via Ras-ERK1/2 signalling, phosphorylated and total ERK1/2 and $S6^{235/236}$ were quantified in a subset of hippocampal slices that underwent metabolic labelling. Western blot analysis of hippocampal slices, treated with either vehicle or lovastatin (100 μM), revealed that lovastatin significantly reduced ERK1/2 activation in both WT and *Syngap* heterozygous mice (phospho/total ERK1/2: WT veh 100 ± 12%; WT 100 μM 67 ± 6%; Het veh 154 ± 30%; Het 100 μM 56 ± 4%; ANOVA treatment **p* =0.005; Figure 5.5A; phospho/total ERK1: WT veh 100 ± 20%; WT 100 μM 83 ± 23%; Het veh 171 ± 38%; Het 100 μM 130 ± 7%; phospho/total ERK2: WT veh 100 ± 29%; WT 100 μM 123 ± 34%; Het veh 137 ± 20%; Het 100 μM 152 ± 49%). However, $S6^{235/236}$/S6 was only significantly reduced in *Syngap* heterozygous slices, not WT slices ($S6^{235/236}$/S6: WT veh 100 ± 13%, WT 100 μM 79 ± 8%; Het veh 109 ± 7%; Het 100 μM 66 ± 4%; ANOVA treatment **p* =0.003, *n* = 5; Figure 5.5B). In *Syngap* heterozygous mice the reduction in $S6^{235/236}$/S6 was caused by a reduction in $S6^{235/236}$ phosphorylation and by an increase in total S6. These findings indicate in the presence of lovastatin, the remaining levels of active ERK1/2 in WT hippocampal slices are sufficient to maintain $S6^{235/236}$ phosphorylation at control levels. Whilst in *Syngap* heterozygous hippocampal slices Ras activity is required to maintain phosphorylation levels of $S6^{235/236}$ under steady states conditions.

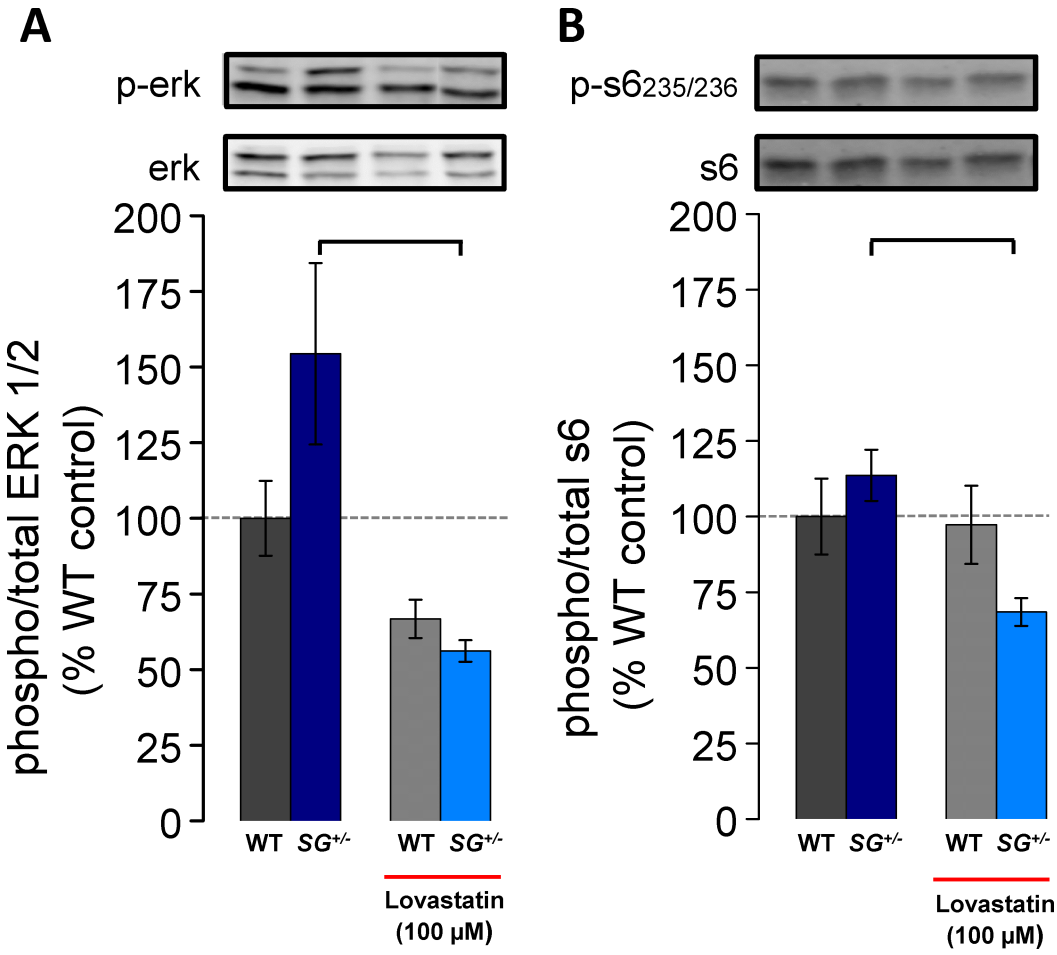


Figure 5.5 Lovastatin inhibits ERK1/2 signalling. (A) Lovastatin downregulates ERK1/2 activity in the hippocampus of *Syngap* heterozygous and WT mice (pERK/ERK: WT veh 100 ± 12%; WT 100 µM 67 ± 6%; $SG^{+/-}$ veh 154 ± 30%; $SG^{+/-}$ 100 µM 56 ± 4%; ANOVA treatment **p* = 0.005, *n* = 4). (B) In *Syngap* heterozygous mice $S6^{235/236}$ activity is also significantly reduced ($pS6^{235/236}$/S6: WT veh 100 ± 13%; WT 100 µM 109 ± 7; $SG^{+/-}$ veh 79 ± 8%, $SG^{+/-}$ 100 µM 66 ± 4%; ANOVA treatment **p* =0.003, *n* = 5).

5.3.4 U0126 corrects excessive protein synthesis rates in the hippocampus of *Syngap* heterozygous mice

So far our findings indicate that inhibiting Ras with lovastatin significantly reduces elevated ERK1/2 activity and restores increased translational rates in the hippocampus of *Syngap* heterozygous mice. In *Fmr1* KO mice elevated protein synthesis rates lie downstream of Ras-ERK1/2 activity, not mTOR, and are corrected with either lovastatin or U0126 (Osterweil et al. 2010; Osterweil et al. 2013). To determine whether lovastatin is mediating its effect via downregulation of ERK1/2 signalling, an inhibitor of ERK1/2s upstream activator was used to examine whether inhibition of ERK1/2 signalling could also restore protein synthesis rates to normal levels. Metabolic labelling was performed in hippocampal slices from WT and *Syngap* heterozygous mice in the presence of 5 μM U0126. Here it was observed that inhibition of ERK1/2 signalling with U0126 (5 μM) was sufficient to correct elevated protein synthesis rates to WT levels (WT: veh 100 ± 4%, 5 μM 99 ± 8%; Het: veh 130 ± 9%, 5 μM 94 ± 9%; ANOVA genotype $*p = 0.018$, treatment $*p = 0.03$, genotype x treatment $*p < 0.05$; $n = 9$; Figure 5.6). Thus it appears that the elevated protein synthesis rates in *Syngap* heterozygous mutants are mediated via increased Ras-ERK1/2 signaling.

Interestingly U0126 does not affect basal protein synthesis rates in WT mice. Similarly in *Syngap* heterozygous mice, abolishing ERK1/2 activity only reduces the elevated component, without affecting normal levels of protein synthesis. These findings suggest the Ras-ERK1/2 signalling cascade is not crucial for regulating basal protein synthesis rates. Thus in the hippocampus, translational rates are being mediated via an alternative pathway.

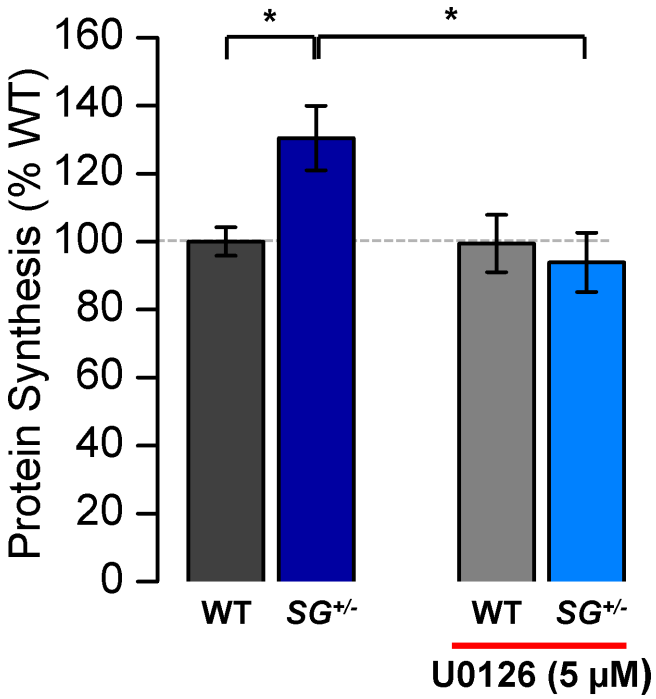


Figure 5.6 U0126 normalises elevated protein synthesis rates in *Syngap* heterozygous mice. In vehicle treated slices, protein synthesis rates were significantly elevated in dorsal hippocampal slices from *Syngap* heterozygous slices. In the presence of U0126 (5 µM) increased protein synthesis levels are reduced to WT levels (WT: veh 100 ± 4%, 5 µM 99 ± 8%; $SG^{+/-}$: veh 130 ± 9%, 5 µM 94 ± 9%; ANOVA genotype **p* = 0.018, treatment **p* = 0.03, genotype x treatment **p* = 0.05; *n* = 9).

5.3.5 U0126 abolishes elevated ERK1/2 signalling in *Syngap* heterozygous mice

To confirm that U0126 at 5 μM was effectively inhibiting ERK1/2 activity in *Syngap* heterozygous and WT slices, phosphorylated and total ERK1/2 and $S6^{235/236}$ were quantified biochemically. Western blot analysis of hippocampal slice homogenates revealed U0126 (5 μM) completely abolishes ERK1/2 activation in both *Syngap* heterozygous and WT slices (phospho/total ERK1/2: WT veh 100 ± 9%; WT U0126 8 ± 1%; Het veh 171 ± 9%; $SG^{+/-}$ U0126 11 ± 1%; ANOVA genotype $*p = 3 \times 10^{-7}$, treatment $*p = 2 \times 10^{-13}$, $n = 4$, Figure 5.7A; phospho/total ERK1: WT veh 100 ± 3%; WT U0126 17 ± 7%; Het veh 157 ± 28%; $SG^{+/-}$ U0126 23 ± 5 %; phospho/total ERK2: WT veh 100 ± 7%; WT U0126 8 ± 2%; Het veh 220 ± 18%; $SG^{+/-}$ U0126 11 ± 3%). Similarly $S6^{235/236}$ activity was significantly reduced in hippocampal slices WT and *Syngap* heterozygous ($S6^{235/236}$/S6: WT veh 100 ± 12%, WT U0126 31 ± 7%; Het veh 136 ± 9%; Het U0126 36 ± 5%; ANOVA genotype $*p$ =0.007, treatment $*p = 1 \times 10^{-8}$, $n = 4$; Figure 5.7B). Thus U0126 (5 μM) significantly reduces phosphorylation of both ERK1/2 and $S6^{235/236}$ activity suggesting that MEK1/2 signals through ERK1/2 to phosphorylates $S6^{235/236}$. Down regulation of ERK1/2 and $S6^{235/236}$ corrects elevated protein synthesis rates in the hippocampus of *Syngap* heterozygous mice suggesting that Ras-ERK1/2, possibly via $S6^{235/236}$, contribute to excessive protein synthesis levels in the hippocampus of *Syngap* heterozygous mice.

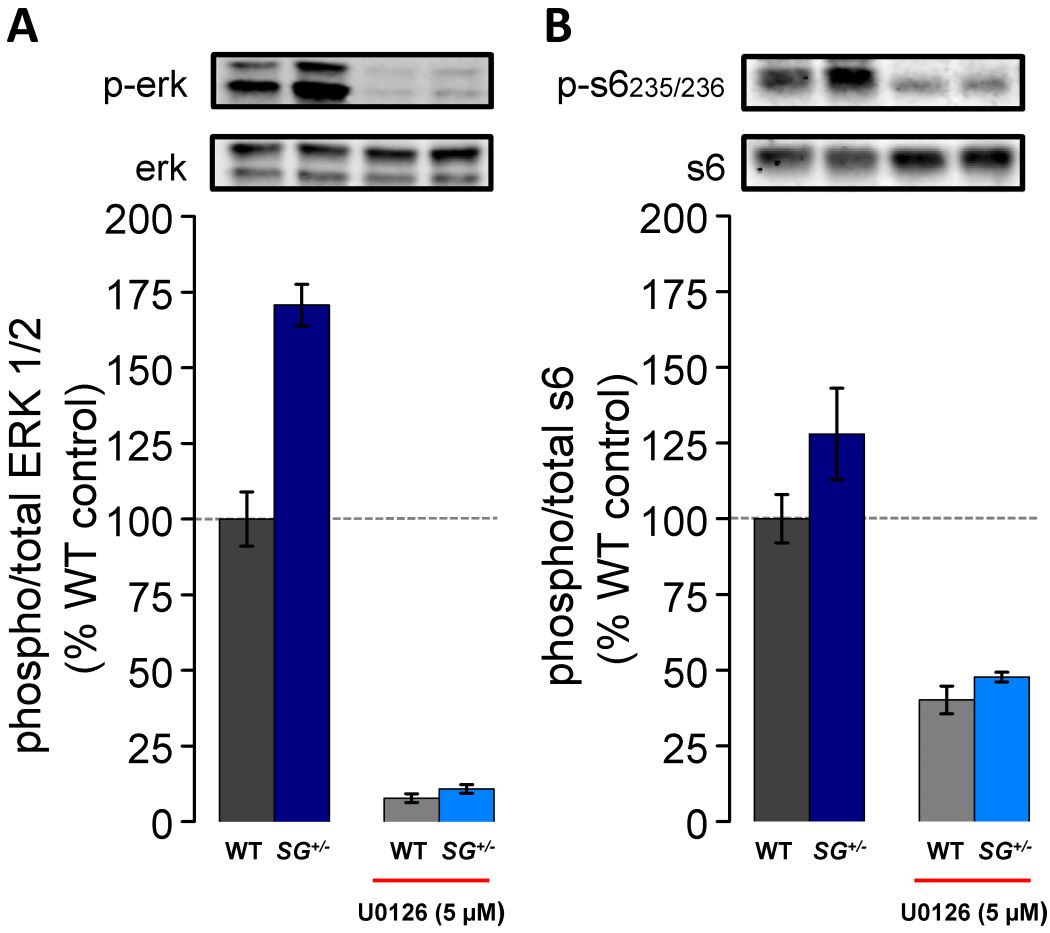


Figure 5.7. U0126 inhibits ERK1/2 and S6 activation. Western blot analysis of phosphorylated and total ERK1/2 and $S6^{235/236}$ levels in *Syngap* heterozygous ($SG^{+/-}$) and wild type (WT) hippocampal slices treated with either vehicle (DMSO) or U0126 (5 µM). (A) In vehicle treated slices ERK1/2 activity is significantly elevated in *Syngap* heterozygous slices compared to WT controls. In the presence of U0126 ERK1/2 activity is abolished in both WT and *Syngap* heterozygous mice (pERK/ERK: WT veh 100 ± 9%; WT U0126 8 ± 1%; $SG^{+/-}$ veh 171 ± 9%; $SG^{+/-}$ U0126 11 ± 1%; ANOVA genotype $*p = 3 \times 10^{-7}$, treatment $*p = 2 \times 10^{-13}$; $n = 4$). (B) $S6^{235/236}$ activity is significantly elevated in vehicle treated *Syngap* heterozygous slices compared to WT mice. In the presence of U0126 $S6^{235/236}$ activity is significantly reduced in both WT and *Syngap* heterozygous slices ($pS6^{235/236}$/S6: WT veh 100 ± 12%, WT U0126 31 ± 7%; $SG^{+/-}$ veh 136 ± 9; $SG^{+/-}$ U0126 36 ± 5%; ANOVA genotype $*p = 0.007$, treatment $*p = 1 \times 10^{-8}$; $n = 4$).

5.3.6 U0126 reduces mGluR-dependent LTD in the hippocampus of *Syngap* heterozygous mice

It has been previously shown that inhibition of ERK1/2 signalling with U0126 (Gallagher et al. 2004) abolishes mGluR-dependent LTD at CA1 synapses. Upon activation of $mGlu_1$ and $mGlu_5$ receptors, ERK1/2 activity is significantly increased along with the its downstream targets Mnk1, EIF4E and $S6^{235/236}$ (Banko et al. 2006), which imitate cap-dependent and 5'TOP mRNA translation that is crucial for the maintenance of long lasting forms of synaptic plasticity, which include mGluR-dependent LTD (Huber et al. 2000).

As elevated protein synthesis rates were corrected by U0126 (5 μM) in the hippocampus of *Syngap* heterozygous mice, we wanted to determine electrophysiologically whether the same treatment could restore exaggerated mGluR-dependent LTD. Extracellular recordings were performed in CA1 where it was observed that U0126 (5 μM) had no significant effect on basal synaptic transmission with baseline responses remaining consistent throughout an 80 minutes recording in the presence of this compound (Figure 5.13A). Hippocampal slices form WT and *Syngap* heterozygous mice were preincubated in either vehicle or U0126 (5 μM) for at least 20 minutes prior to inducing LTD with the Gp1 mGluR agonist DHPG (50 μM). In WT mice, U0126 (5 μM) had no significant effect on the magnitude of mGluR-dependent LTD (WT: veh 77 ± 3%, $n = 18$; U0126 86 ± 6%, $n = 8$, t-test $p = 0.16$; Figure 5.7A). In contrast, U0126 (5 μM) significantly reduced exaggerated mGluR-dependent LTD in *Syngap* heterozygous mice (Het: veh 62 ± 5%, $n = 15$; U0126 83 ± 6%, $n = 6$; t-test $^*p = 0.04$; Figure 5.8B) to a level that was not significantly different from WT vehicle treated slices or baseline responses. These findings support the biochemical data and indicate that restoring elevated protein synthesis rates in *Syngap* heterozygous mice normalizes enhanced mGluR-dependent LTD in the hippocampus of *Syngap* heterozygous mice.

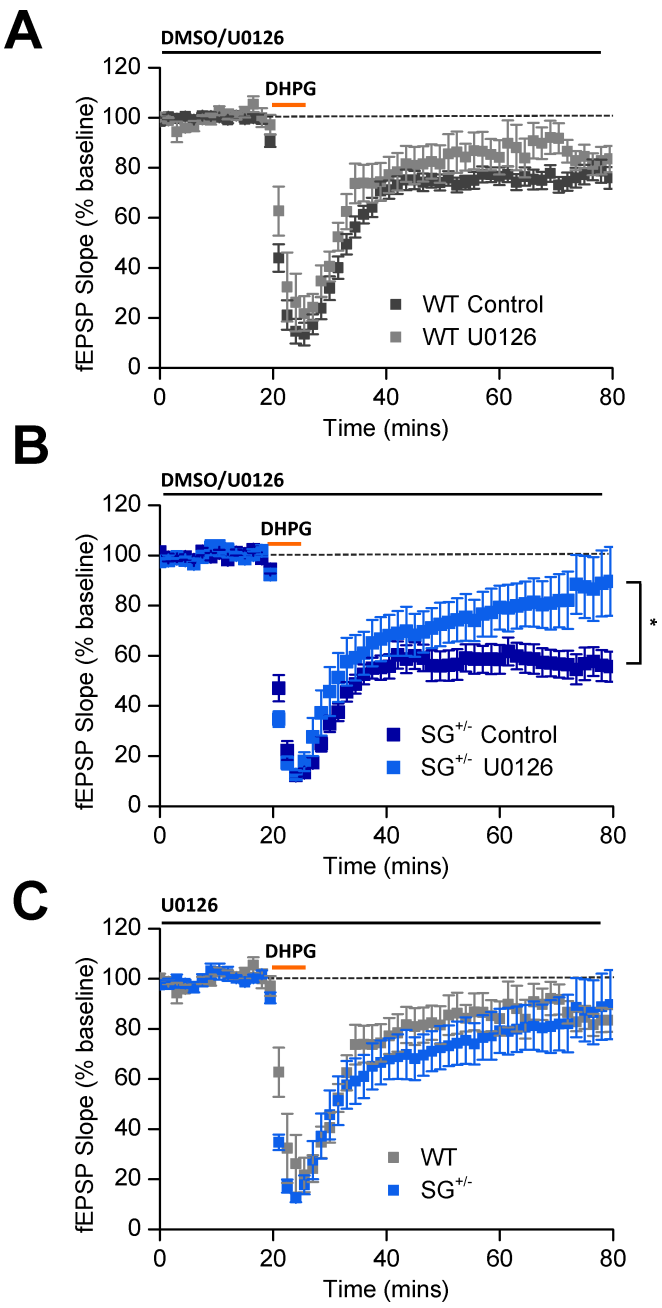


Figure 5.8 U0126 reduces enhanced mGluR-dependent LTD in *Syngap* heterozygous mice. Extracellular recordings were performed in CA1 of hippocampal slices from *Syngap* heterozygous (Het, $SG^{+/-}$) and wild type (WT) mice. (A) Induction of mGluR-dependent LTD (50 µM) in the presence of U0126 (5 µM) did not significantly affect the magnitude of LTD in WT hippocampal slices (WT: veh 77 ± 3%, n = 18; U0126 85 ± 6%, n = 8, t-test p = 0.16). (B) In *Syngap* heterozygous mice, U0126 reduced the magnitude of mGluR-dependent LTD in the hippocampus of *Syngap* heterozygous to a level not significantly different from WT or baseline responses ($SG^{+/-}$: veh 62 ± 5%, n = 15; U0126 83 ± 6%, n = 10; t-test *p = 0.01) (C) Comparison of mGluR-dependent LTD in the presence of U0126 in WT and *Syngap* heterozygous mice.

In addition, we examined the effect of U0126 5 μM on mGluR-dependent LTD using a higher concentration of DHPG (100 μM). Although statistically U0126 (5 μM) had no significant effect on mGluR-dependent LTD in WT mice, a comparison of LTD versus predrug baseline revealed that LTD was no longer significantly different from baseline (Figure 5.9A). Consistent with Gallagher et al. (2004) increasing the concentration of U0126 to 20 μM completely abolished mGluR-dependent LTD in WT slices (WT: veh 74 ± 6%, $n = 12$; 5 μM 87 ± 7%, $n = 5$; 20 μM 100 ± 6%, $n = 7$; ANOVA *$p = 0.02$; Figure 5.9B). In *Syngap* heterozygous mice, 5 or 20 μM U0126 had no significant effect on mGluR-dependent LTD (Het: veh 56 ± 5%, $n = 14$; 5 μM 70 ± 9%, $n = 7$; 20 μM 72 ± 3%, $n = 5$; ANOVA $p > 0.05$; Figure 5.9 C & D). Comparison of mGluR-dependent LTD (100 μM) in the presence of U0126 (5 & 20 μM) between *Syngap* heterozygous and WT mice revealed that LTD was no longer significantly different between genotypes at 5 μM U0126, but not at 20 μM U0126 (*t*-test $p = 0.006$; Figure 5.10 E & F).

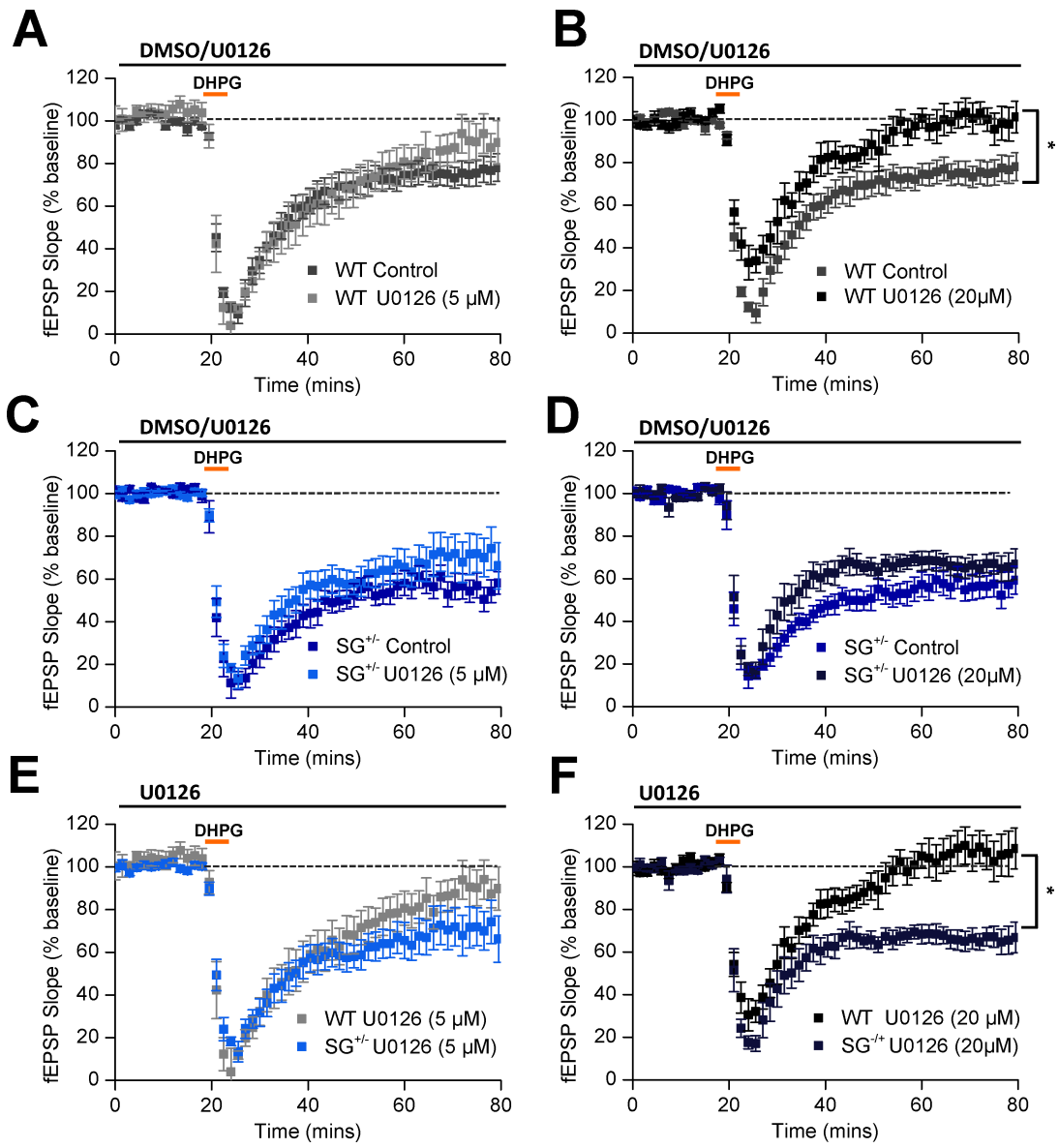


Figure 5.9 U0126 reduces enhanced mGluR-dependent LTD in *Syngap* heterozygous mice. Extracellular recordings were performed in CA1 of hippocampal slices from *Syngap* heterozygous (Het, $SG^{+/-}$) and wild type (WT) mice. (A) Induction of mGluR-dependent LTD (100 µM) in the presence of 5 µM U0126 did not significantly affect the magnitude of LTD in WT hippocampal slices but LTD was no longer significantly difference from baseline whilst 20 µM U0126 significantly reduced LTD compared to vehicle treated slices (WT: veh 74 ± 6%, n = 12; 5 µM 87 ± 7%, n = 5; 20 µM 100 ± 6%, n = 7; ANOVA *p = 0.02). (C, D) U0126 (5 µM and 20 µM) had no significant effect on the magnitude of LTD compared to vehicle treated slices (B) In *Syngap* heterozygous mice, U0126 reduced the magnitude of mGluR-dependent LTD in the hippocampus of *Syngap* heterozygous mutants to a level not significantly different from WT or baseline responses ($SG^{+/-}$: veh 56 ± 5%, n = 14; 5 µM 70 ± 9%, n = 7; 20 µM 72 ± 3%, n = 5; ANOVA p > 0.05). (C) Comparison of mGluR-dependent LTD in the presence of U0126 in WT and *Syngap* heterozygous mice.

5.3.7 Rapamycin restores elevated protein synthesis rates in the hippocampus of *Syngap* heterozygous mice

In response to $mGlu_{1/5}$ receptor activation the mTOR signaling pathway initiates translation of new protein synthesis at CA1 synapses (Banko et al. 2006). In *Syngap* heterozygous mice, basal levels of phosphorylated Akt are elevated compared to WT littermates. To examine whether altered mTOR signaling contributes to excessive protein synthesis rates in the hippocampus of *Syngap* heterozygous mice, translational rates were measured in the presence of the mTOR inhibitor rapamycin (20 nM). Again, we observed a significantly elevation in ^{35}S incorporation in vehicle-treated *Syngap* heterozygous hippocampal slices compared to WT controls (*t*-test $*p = 0.04$). Rapamycin (20 nM) had no significant effect on basal protein synthesis levels in WT slices, however inhibition of mTOR reduced protein synthesis rates in *Syngap* heterozygous slices to a level that was not significantly different from WT controls (WT: veh 100 ± 3%, 20 nM 106 ± 7%; Het: veh 124 ± 6%, 20 nM 109 ± 8%; ANOVA genotype $*p = 0.04$; $n = 8$; Figure 5.10).

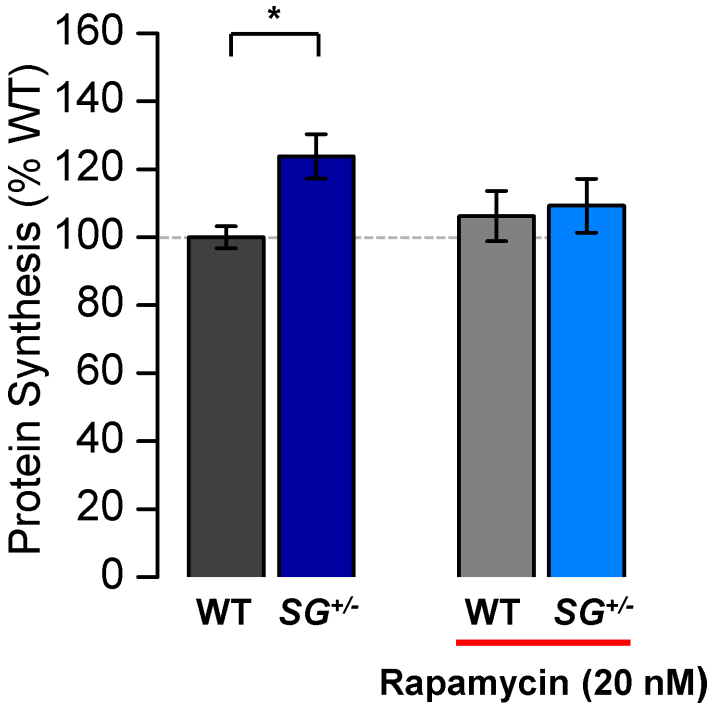


Figure 5.10 Rapamycin reduces elevated protein synthesis rates in *Syngap* heterozygous mice. In vehicle treated slices, protein synthesis rates were significantly elevated in dorsal hippocampal slices from *Syngap* heterozygous slices. In the presence of rapamycin (20 nM) exaggerated protein synthesis levels are reduced to a level that is not significantly different from WT values (WT: veh 100 ± 3%, 20 nM 106 ± 7%; $SG^{+/-}$: veh 124 ± 6%, 20 nM 109 ± 8%; ANOVA genotype *p = 0.04; n = 8).

5.3.8 Rapamycin corrects elevated mGluR-dependent in the hippocampus of *Syngap* heterozygous mice

At CA1 synapses mGluR-dependent LTD requires both PI3K and mTOR activity to be sustained for at least 1 hour following LTD induction (Hou & Klann 2004; Sharma et al. 2010). $mGlu_5$ receptors can couple to the mTOR pathway via the scaffolding protein Homer, and also through activating Ras G-proteins. Together Homer and Ras can regulate mTOR signaling through the activation of PI3K. In Chapter 4 the activation state of Akt was significantly elevated in *Syngap* heterozygous hippocampal slices compared to WT littermates. This is accompanied by an increase in translational rates in the hippocampus of *Syngap* heterozygous mutants that is sensitive rapamycin.

To determine whether elevated mTOR signaling contributes to enhanced mGluR-dependent LTD at CA1 synapses in the hippocampus of *Syngap* heterozygous, slices were preincubated in the mTOR inhibitor rapamycin (20 nM) for at least 20 minutes prior to the induction of mGluR-dependent LTD (DHPG 50 μM). Using extracellular recordings in CA1 it was observed that rapamycin had no significant effect on basal synaptic transmission with baseline responses remaining consistent throughout an 80 minutes recording in the presence of this compound (Figure 5.13B). In WT slices, rapamycin had no significant effect on the magnitude of mGluR-dependent LTD compared to vehicle treated slices (WT control: 77 ± 3%, $n = 18$; WT rapamycin 85 ± 3%, $n = 7$, *t*-test $p = 0.16$; Figure 5.11A). Although this finding is consistent with Auerbach et al. (2011), there are several other studies that report rapamycin inhibits mGluR-dependent LTD in WT slices (Hou & Klann 2004; Sharma et al. 2010). In *Syngap* heterozygous slices rapamycin prevented exaggerated mGluR-dependent LTD that result in a magnitude of LTD that was comparable to WT levels (*Syngap* control 62 ± 5%, $n = 15$; Het rapamycin 83 ± 6%, $n = 10$; *t*-test $*p = 0.01$; Figure 5.11B). Thus inhibition of mTOR restores elevated mGluR-dependent LTD in *Syngap* heterozygous mice to a magnitude that is indistinguishable from WT controls ($p = 0.342$; Figure 5.11C).

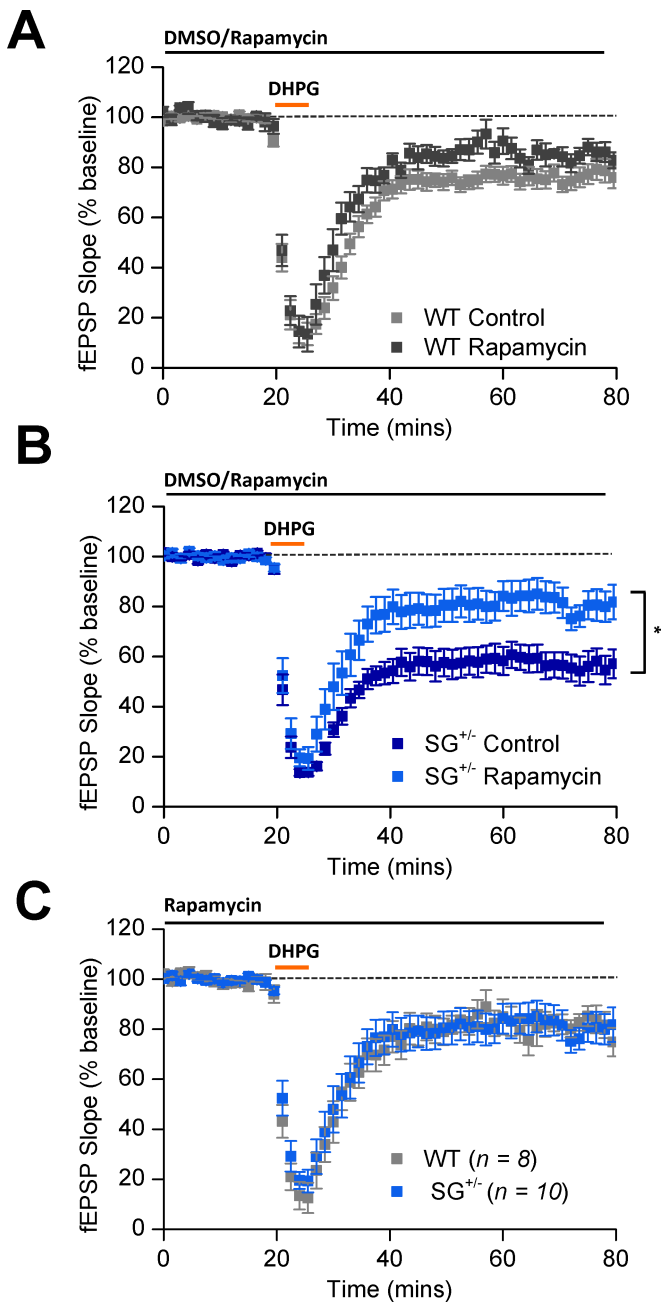


Figure 5.11 Rapamycin normalises elevated mGluR-dependent LTD in *Syngap* heterozygous mice. Field excitatory postsynaptic potentials (fEPSPs) were recorded at 30 ◦C in CA1 region of hippocampal slices from *Syngap* heterozygous (Het, $SG^{+/-}$) and WT littermates. (A) In WT mice, the mTOR inhibitor (20 nM) did not affect the magnitude of DHPG-induced (5 mins, 50 µM) mGluR-dependent LTD in WT hippocampal slices (WT control: 77.0 ± 3%, n = 18; WT rapamycin 85 ± 3%, n = 7, t-test p = 0.157). (B) In contrast, rapamycin significantly reduced the magnitude of mGluR-dependent LTD in *Syngap* het slices ($SG^{+/-}$ control 62 ± 5%, n = 15; $SG^{+/-}$ rapamycin 83 ± 6%, n = 10; t-test *p = 0.01). (C) Comparison of mGluR-dependent LTD in the presence of rapamycin in hippocampal slices from WT and *Syngap* heterozygous mice. In the presence of rapamycin the magnitude of mGluR-dependent LTD in *Syngap* mutants is indistinguishable from WT mice (t-test p = 0.76).

5.3.9 Inhibition of mTOR restores protein synthesis dependency to mGluR-dependent LTD in *Syngap* heterozygous mice

In *Syngap* heterozygous mice, increases in mGluR-dependent LTD are accompanied with qualitative differences; this form of LTD is no longer sensitive to protein synthesis inhibitors. This suggests that the proteins required for the expression and maintenance of this form of plasticity are already present at CA1 synapses in the *Syngap* heterozygous brain. As rapamycin reduced elevated protein synthesis rates and normalized mGluR-dependent LTD to WT levels, this led us to hypothesize that inhibition of mTOR in *Syngap* heterozygous hippocampal slices may also restore the protein synthesis dependency of this form LTD in mutant slices.

Although corrections of mGluR-dependent LTD in mouse models of ID have been reported (Osterweil et al. 2013), these studies have failed to show whether the protein synthesis dependent component of this form of LTD is reestablished. Here we examined whether rapamycin would return protein synthesis-dependence to LTD. Hippocampal slices from *Syngap* heterozygous mice were incubated in ACSF containing rapamycin (20 nM) plus vehicle for 10 minutes and either remained in this solution or swapped to ACSF containing rapamycin (20 nM) and the translational inhibitor anisomycin (20 μM). Consistent with our findings in 5.3.7, preincubation of *Syngap* heterozygous slices in rapamycin (20 nM) reduced the magnitude of mGluR-dependent LTD in *Syngap* heterozygous slices (Het rapamycin + vehicle 74 ± 8%, $n = 3$; Figure 5.12A). Furthermore, in the presence of both rapamycin (20 nM) and anisomycin (20 nM) the expression of mGluR-dependent LTD (50 μM DHPG) was inhibited (Het rapamycin + anisomycin: 97 ± 7%, $n = 5$) to a level that wasn't significantly different from baseline (*t*-test $p = 0.72$). These findings are summarized in Figure 5.12B and suggest that inhibition of mTOR may restore elevated mGluR-dependent LTD and the protein synthesis dependency of mGluR-dependent LTD in *Syngap* heterozygous mice.

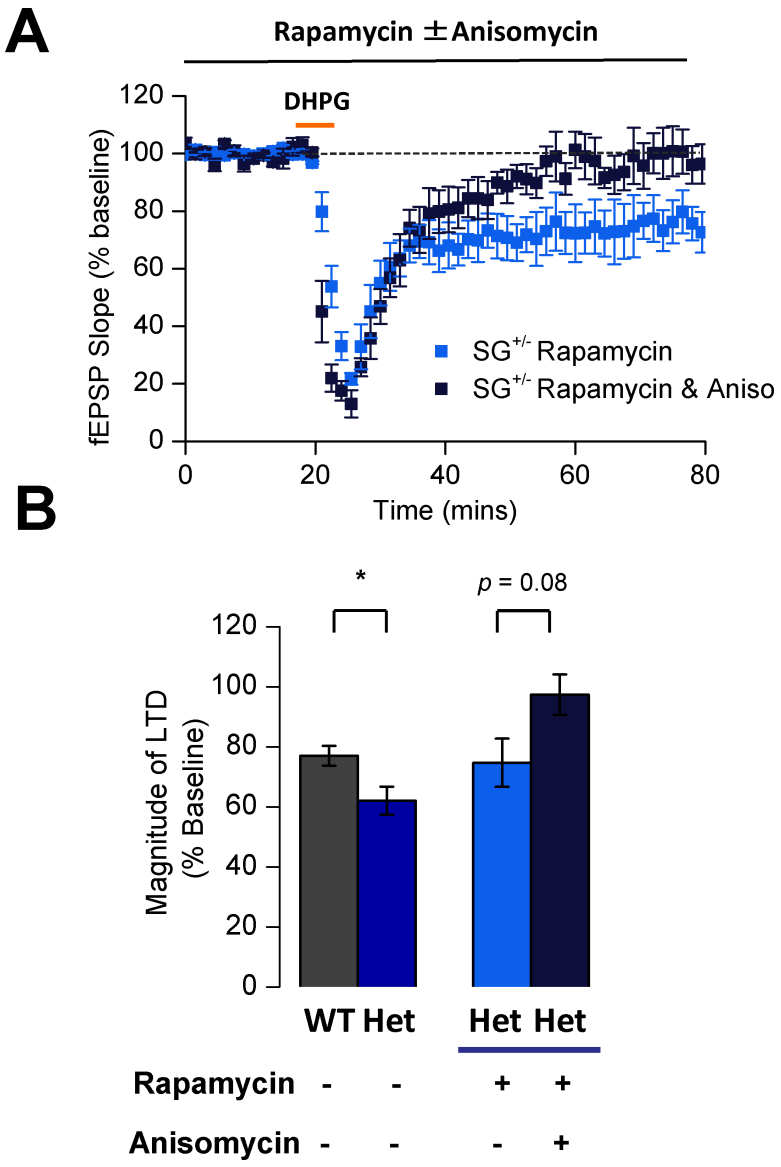


Figure 5.12 Rapamycin restores protein synthesis dependency to mGluR-dependent LTD in *Syngap* heterozygous mice. (A) Extracellular recordings in CA1 reveal that in *Syngap* heterozygous (Het, $SG^{+/-}$) hippocampal slices, treatment with rapamycin (20 nM) reduces enhanced mGluR-dependent LTD (50 µM DHPG) to WT levels and restores the protein synthesis dependency of mGluR-dependent LTD. (B) Bar chart represents the mean (± S.E.) depression of fEPSP relative to pre-drug baseline (Het: rapa + veh 74 ± 8%, $n = 3$; rapa + aniso: 97 ± 7%, $n = 5$, *t*-test treatment $p = 0.08$). (B) Comparison of mGluR-dependent LTD induced by 50 µM in the presence of rapamycin (20 nM) ± DMSO or anisomycin (20 µM) to mGluR-dependent LTD (50 µM) in WT and *Syngap* heterozygous hippocampal slices.

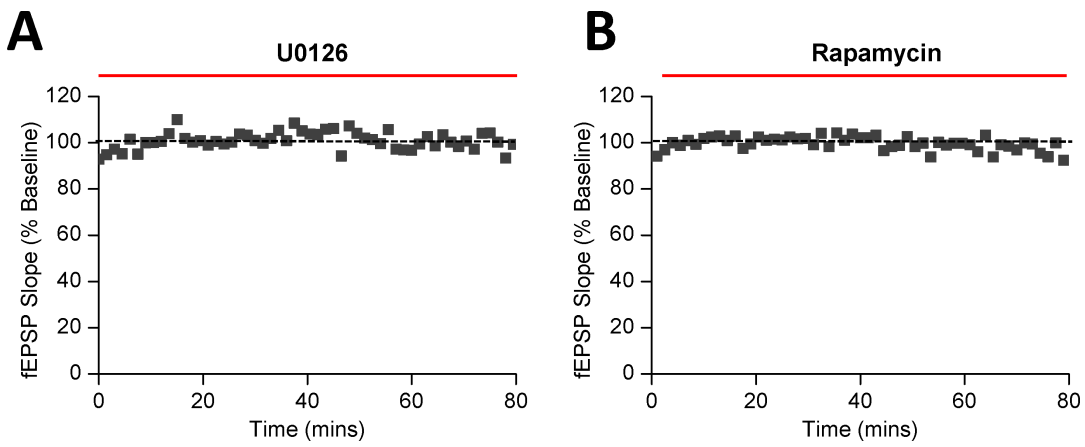


Figure 5.13 Baseline responses obtained in the presence of U0126 and rapamycin. Extracellular recordings reveal stable baseline responses for 80 minutes in the presence of either U0126 (5 μM; A) or rapamycin (20 nM; B).

5.4 Discussion

The mouse model of *Syngap1* haploinsufficiency closely phenocopies the hippocampal plasticity deficits reported in the *Fmr1* KO mouse including: enhanced mGluR-dependent LTD, which is independent of new protein synthesis; and elevated basal protein synthesis rates, that are saturated downstream of $mGlu_5$ receptor activation. Dissimilarly to *Fmr1* KO mice ERK1/2, Akt and S6 activity are elevated in hippocampal slice homogenates, indicating that the *Syngap1* haploinsufficiency may arise from hyperactivity, rather than a hypersensitive response, to Ras-mediated signalling (Osterweil et al. 2010). Irrespective of this, loss of FMRP and reduced SynGAP expression result in similar phenotypic outcomes in the hippocampus via a common mechanism, which led us to hypothesize that they may be amenable to same pharmacological strategies shown to correct the hippocampal pathophysiology in the mouse model of FXS. To test this hypothesis, biochemical and electrophysiological investigations were repeated in the presence of inhibitors that target $mGlu_5$, Ras or their downstream targets, to determine whether the synaptic pathophysiology could be restored in the mouse model of *Syngap1* haploinsufficiency.

5.4.1 Elevated protein synthesis rates in *Syngap* heterozygous mice lie downstream of $mGlu_{1/5}$ receptors and Ras-mediated signalling

CTEP, U0126 and lovastatin all normalised elevated protein synthesis rates to WT levels in *Syngap* heterozygous hippocampal slices, whilst rapamycin led to a reduction in basal protein synthesis. Together these findings suggest that increased translational rates in the hippocampus of *Syngap* heterozygous mice lie downstream of hyperactivate Ras-signalling signalling. It is well documented that the ERK1/2 and mTOR pathway converge on various components of the translational apparatus that initiate mRNA translation in response to synaptic activity (Figure 5.14). However our findings suggest that in the *Syngap* heterozygous brain, ERK1/2 and mTOR signalling may be overactive in response to constitutive $mGlu_5$ receptor activation, stimulating mRNA translation under basal conditions.

In WT mice, the acute application of DHPG leads to an increase in translational rates suggesting that $mGlu_5$ receptors play a major role in activity-dependent protein synthesis. Activation of $mGlu_{1/5}$ receptors is correlated with increases in phosphorylation levels of Mnk1, S6 and the cap-dependent translation initiation factor EIF4E, which are dependent on ERK1/2 activation (Banko et al. 2006; Antion et al. 2008). Furthermore $mGlu_5$ receptor activity is associated with mTOR-induced phosphorylation of 4E-BP, which increases EIF4E availability for EIF4F complex formation (Banko et al 2006). Thus in the *Syngap* heterozygous brain where ERK1/2 and mTOR are hyperactive under steady state conditions it is likely these two pathways are increasing translation by overactivating downstream targets including Mnk1, EIF4E and 4E-BP.

In this chapter it was observed that both U0126 and lovastatin normalised protein synthesis rates to WT levels in the hippocampus of *Syngap* heterozygous mice and this was accompanied by a significant reduction in increased ERK1/2 phosphorylation. This suggests that elevated protein synthesis rates in the hippocampus of *Syngap* heterozygous mice may arise through ERK-induced mRNA translation. One possible mechanism by which lovastatin and U0126 reduce translation might be via targeting ERK-induced Mnk1 phosphorylation of EIF4E halting the initiation of cap-dependent mRNA translation.

In addition rapamycin, and perhaps lovastatin and CTEP, may reduce protein synthesis rates in the hippocampus of *Syngap* heterozygous mice by inhibiting PI3K-Akt-mTOR signalling, which targets 4E-BP in response to $mGlu_5$ receptor activation (Banko et al. 2006). The interaction between 4E-BP and EIF4E prevents the assembly of the EIF4F complex that is crucial for the initiation of mRNA translation (Raught & Gingras 1999). Thus inhibition of PI3K-Akt-mTOR may conserve the 4E-BP-eIF4E interaction reducing EIF4E availability. Moreover this will preclude Mnk1 phosphorylation of EIF4E, which can only elicit its actions once eIF4E is bound to eIF4F assembly.

In addition both the ERK1/2-MAPK and PI3K-Akt-mTOR signalling pathway target the ribosomal S6 kinases (Antion et al. 2008a). ERK1/2 activity is coupled to RSK family members, whilst mTOR signalling targets S6K1/2, both of which lead to the activation of eIF4B (Shahbazian et al. 2006). This initiation factor stimulates EIF4F activity by potentiating the RNA helicase activity of EIF4A, that together with EIF4G and EIF4E form the EIF4F complex required for cap-dependent mRNA translation (Roux & Blenis 2004). Both RSK and S6K1/2 also target the ribosomal S6 protein, which is also associated with the 5'TOP mRNA translation (Antion et al. 2008a).

A previous study has shown that $mGlu_{1/5}$ activation increases S6 phosphorylation, which persists in the absence of S6K1/2, and leads to an increase in expression of a 5'TOP mRNA transcript EF1A (Antion et al. 2008). Thus inhibition of ERK1/2 and mTOR may reduce elevated protein synthesis rates by not only preventing the initiation of cap-dependent translation but also 5'TOP mRNA translation. This could reduce the synthesis of proteins required for the expression and maintenance of mGluR-dependent LTD, and reduce translational competence in neuronal cells by preventing the synthesis of ribosomal subunits and translation factors in the *Syngap* heterozygous brain (Huber et al. 2000; Antion et al. 2008a).

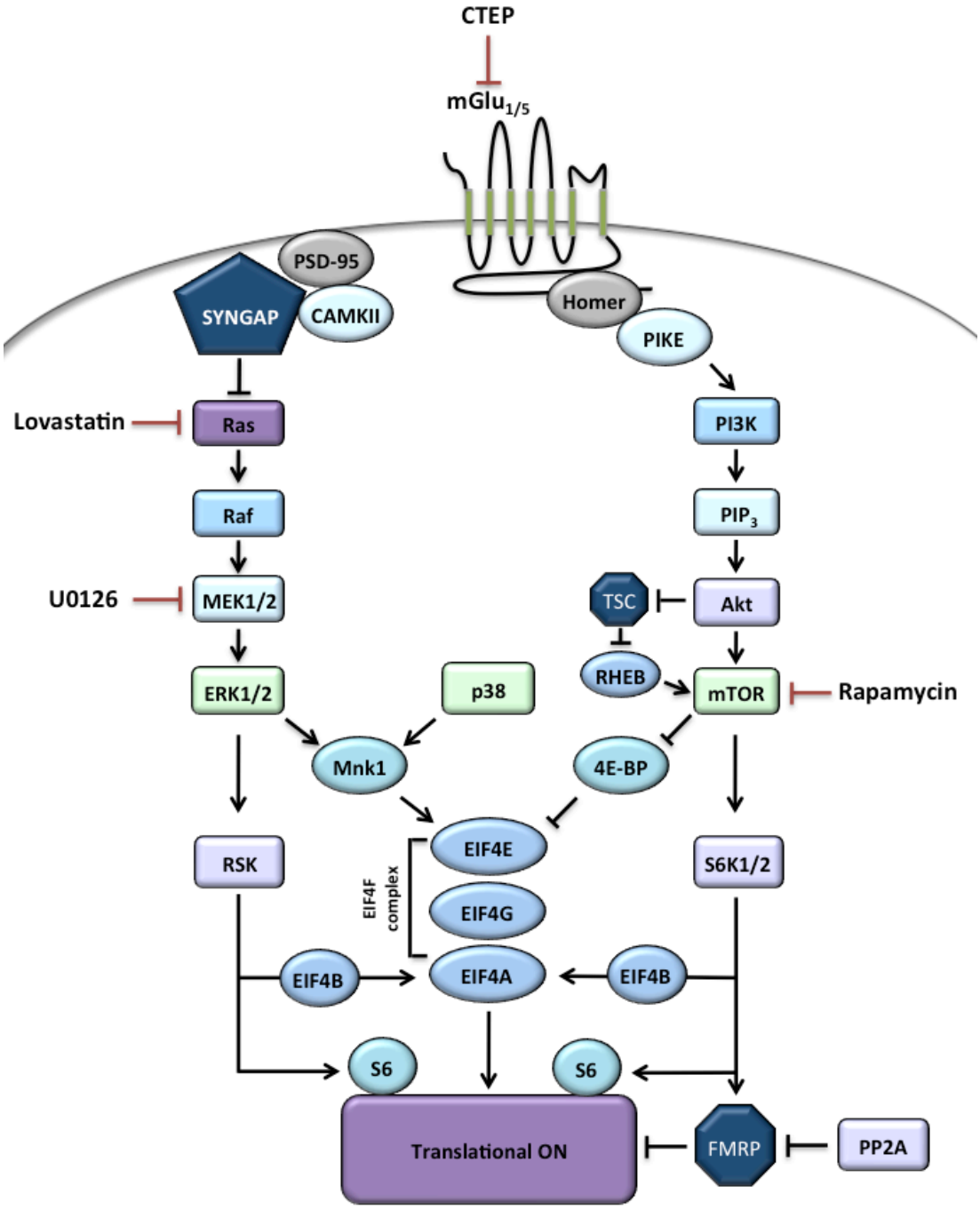


Figure 5.14. CTEP, lovastatin, U0126 and rapamycin reduce translational rates in the *Syngap* heterozygous brain. The potential intracellular signalling mechanisms targeted by inhibitors of $mGlu_5$ receptors, Ras, ERK and mTOR.

5.4.2 Basal protein synthesis rates may be independent of ERK1/2 and PI3K activity in the hippocampus of WT mice

In WT hippocampal slices neither CTEP, U0126, lovastatin or rapamycin treatment significantly affected basal protein synthesis rates consistent with previous findings (Osterweil et al. 2010; Michalon et al. 2012; Osterweil et al. 2013). This could suggest: (1) constitutive activity of $mGlu_5$, Ras, ERK1/2 or PI3K do not regulate mRNA translation under steady state conditions, or (2) ERK1/2 and mTOR signalling are able to compensate for one another when either cascade is inhibited. To test this, protein synthesis rates would have to be quantified in the presence of both U0126 and rapamycin to determine whether ERK1/2 and mTOR signalling can counteract each other to maintain mRNA translation at a constant rate, or if neither are essential for the maintenance of basal protein synthesis.

A previous study has reported that p38 MAPK signalling controls the phosphorylation levels of Mnk1 and EIF4E under steady-state conditions (Bank et al. 2006). Inhibition of p38 MAPK significantly reduced the basal phosphorylation levels of Mnk1 and EIF4E, whilst U0126 treatment had no significant effect. However activation of $mGlu_{1/5}$ receptors triggers an increase in the phosphorylation levels of both Mnk1 and EIF4E, which are abolished by U0126 (Banko et al. 2006). This suggests that at rest p38 MAPK activity controls activity levels of Mnk1 and EIF4E, however under stimulated conditions $mGlu_{1/5}$ receptors signals through ERK1/2 and Mnk1 to target eIF4E, facilitating EIF4F complex formation and the initiation of mRNA translation that is crucial for the expression and maintenance of mGluR-dependent LTD in young adult mice (Huber et al. 2000; Huber et al. 2001).

5.4.3 MEK1/2 maintains steady-state levels of ERK and S6

Here it was observed that inhibition of MEK1/2 with U0126 (5 μM) dramatically reduces basal phosphorylation levels of ERK1/2 and $S6^{235/236}$ by ~90% and ~70%, respectively, in both WT and *Syngap* heterozygous hippocampal slices. This indicates that MEK1/2 signals through ERK1/2 to phosphorylate $S6^{235/236}$, under steady-state conditions. Comparatively, lovastatin caused a more subtle reduction in phosphorylation levels of ERK1/2, whilst $S6^{235/236}$ levels were not significantly affected in WT slices suggesting that either the residual activity of ERK1/2 was sufficient to maintain normal levels of $S6^{235/236}$ activity or there was compensation from an alternative pathway.

Ras can target S6 by either activating the ERK1/2 or mTOR signalling cascades. Although the function of S6 is not firmly established, phosphorylation levels of this protein are correlated with increased protein synthesis rates including translation of 5' TOP mRNA translation (Antion et al. 2008). To determine whether this form of mRNA translation is upregulated in the *Syngap* heterozygous brain, proteins encoded by 5'TOP mRNA transcripts (e.g. EF1A) would need to be quantified in hippocampal slice homogenates to see if expression levels were upregulated in the *Syngap* heterozygous brain. This would determine whether this particular form of mRNA translation is contributing to hippocampal pathophysiology associated with *Syngap1* haploinsufficiency. Although U0126 and lovastatin significant inhibited ERK1/2 and or S6 signalling neither had a significant effect on basal protein synthesis rates. Thus in agreement with Banko et al. (2006) it appears that MEK1/2-ERK1/2-S6 pathway is not the predominant mechanism mediating steady-state translational rates at CA1 synapses.

5.4.4 U0126 abolishes mGluR-dependent LTD in *Syngap* heterozygous mice

Our electrophysiological recordings reveal that although preincubating WT hippocampal slices in U0126 (5 μM) does not lead to a significant reduction in mGluR-dependent LTD, the magnitude of LTD is not always significantly different from baseline. Thus our findings are not conclusive as to the impact of 5 μM U0126 on mGluR-dependent LTD. Only when the concentration of U0126 was raised to 20 μM was mGluR-dependent LTD completely abolished. As U0126 (5 μM) is sufficient to block the majority of ERK1/2 activity, this raises the question as to whether higher concentrations of U0126 are targeting additional pathways that lead to the full inhibition of mGluR-dependent LTD or are just causing a greater reduction in the activation states of ERK1/2s downstream targets such as $S6^{235/236}$. To address this question, western blot analysis of ERK1/2 and its downstream targets need to be examined in hippocampal slices preincubated in both 5 and 20 μM U0126.

5.4.5 Inhibitors of mTOR and ERK may correct mGluR-dependent LTD via a mechanism independent of new protein synthesis

In *Syngap* heterozygous mice, elevated mGluR-dependent LTD (50 μM) was dramatically reduced in the presence of U0126, to a level not significantly different from WT controls or baseline responses. This indicates that ERK1/2 signalling contributes to elevated mGluR-dependent LTD levels at CA1 synapses. However, it also highlights that ERK1/2 is regulating synaptic strength through a protein synthesis independent mechanism, as our previous findings in Chapter 3 showed that the translational inhibitor anisomycin has no significant effect on the magnitude of mGluR-dependent LTD. Therefore, in additional to U0126 normalising protein synthesis rates to WT levels, it is also targeting an alternative mechanism that is independent of new protein synthesis and facilitates AMPA receptor insertion at the PSD. This is plausible considering the numerous substrates of ERK1/2 (Roskoski 2012).

Although not demonstrated in this thesis, it is reported that lovastatin corrects elevated mGluR-dependent LTD in *Fmr1* KO mice to WT levels (Osterweil et al. 2013). Similarly to U0126, lovastatin must be mediating its affects via a mechanism that is independent of new protein synthesis as mGluR-dependent LTD in *Fmr*1 KO mice is insensitive to translational inhibitors.

Furthermore electrophysiological investigations revealed that rapamycin corrects elevated mGluR-dependent LTD to WT levels. Inhibition of mTOR also restores the protein synthesis dependency of this form of LTD, as the residual LTD expression is inhibited by anisomycin. This is an important finding, as it has not been previously shown whether any of the pharmacological strategies used in the treatment of FXS or TSC actually restore this core phenotype. As stated above, because the translational inhibitor anisomycin has no significant effect on the magnitude of LTD in *Syngap* heterozygous mice, rapamycin is also reducing mGluR-dependent LTD via a mechanism that is independent of mRNA translation. Intriguingly, inhibition of mTOR signalling reverts mGluR-dependent LTD to a state where it is reliant on the synthesis of new proteins in order to be sustained.

In the *Fmr1* KO brain, enhanced mGluR-dependent LTD occurs independently of new protein synthesis (Huber et al. 2002). This thought to arise from an overabundance of "LTD proteins" under steady state conditions, which are sufficient to maintain decreases in cell surface AMPA receptor expression following an LTD stimulus. In support of this hypothesis, the expression levels of proteins synthesized in response to $mGlu_{1/5}$ receptor activation are increased and no longer regulated in an activity dependent manner in *Fmr1* KO mice (Todd et al. 2003).

If "LTD proteins" were overabundant in *Syngap* heterozygous mice, then one possible mechanism that U0126 and rapamycin may be inhibiting mGluR-dependent LTD is by facilitating the degradation of "LTD proteins" at postsynaptic sites in response to $mGlu_{1/5}$ activation. Loss of these proteins would now revert mGluR-dependent LTD back to a state where it is dependent on new protein synthesis in order to maintain the persistent removal of AMPA receptors. One potential way to

access the effects of U0126 and rapamycin on protein degradation would be to induce mGluR-dependent LTD in the presence of U0126/rapamycin and a proteasome inhibitor, such as MG-132. If U0126 and mTOR were eliciting their effects via this pathway then in the presence of MG-132 they would no longer inhibit mGluR-dependent LTD at CA1 synapses.

5.4.6 Pharmacological intervention following phenotypic onset restores $mGlu_5$ receptor function in the hippocampus of *Syngap* heterozygous mice

Together these findings in *Syngap* heterozygous mice are consistent with those obtained in *Fmr1* KO mice. Downregulation of constitutive $mGlu_5$-Ras mediated signalling rescues the hippocampal disruptions in the mouse model of *Syngap1* haploinsufficiency providing further support to our initial hypothesis that mutations in *Syngap1* and *Fmr1* converge on a common pathophysiological axis. Thus this study has identified key therapeutic targets for the treatment of the hippocampal pathophysiology associated with *Syngap1* haploinsufficiency, which include $mGlu_5$, Ras, ERK1/2 and mTOR. This study also raises the intriguing possibility that current therapeutics undergoing clinical trials for the treatment of FXS, such as $mGlu_5$ antagonists and lovastatin, may be beneficial in treating patients with mutations in *SYNGAP1*. Although treatment of *Syngap1* haploinsufficiency pharmacologically is unlikely to replace the function of protein expression, it may compensate and reduce phenotypic expression of a subset of cognitive symptoms.

Furthermore the data presented in this chapter reveal that acute pharmacological interventions *in vitro*, which follow phenotypic onset in young adult mice, restore synaptic dysfunction in the *Syngap* heterozygous mice. Thus certain phenotypes associated with *Syngap1* haploinsufficiency are reversible in the *Syngap* heterozygous brain. This is in agreement with several other studies that have observed phenotypic reversal in mouse models of neurodevelopmental disorders in adulthood, after commencement of phenotypic manifestations, including in FXS (Dölen et al. 2007; Osterweil et al. 2013; Henderson et al. 2012), NF1 (Costa et al. 2002; Li et al. 2005), TSC (Ehninger & Silva 2011; Meikle et al. 2008; Zeng et al.

2007), Angelman syndrome (van Woerden et al. 2007) and Rett syndrome (Guy et al. 2007).

In addition, data in this chapter challenges a recent paper by Clement et al. (2012) that suggests phenotypic rescue is limited to critical period (~P14), as complete restoration of gene function in adult mutants failed to correct behaviour or cognitive endophenotypes in *Syngap* heterozygous mice, confining *Syngap1* haploinsufficiency to being a disorder of neurodevelopment. However, this thesis demonstrates that synaptic dysfunction in the adult brain are restored by reducing the activity of SynGAP's downstream effectors suggesting that SynGAP plays a role in regulating synaptic plasticity at the mature synapse. This means *Syngap1* haploinsufficiency may not strictly be a neurodevelopmental disease but also a disorder of neuroplasticity and neuromaintenance.

Chapter 6

Conclusion

6.1 Final Discussion and Future Experiments

SYNGAP1 haploinsufficiency is a debilitating neurodevelopmental disorder caused by autosomal *de novo* mutations in the *SYNGAP1* gene leading to the loss of one functional copy of *SYNGAP1*. Genetic screening of small cohorts of ID patients have revealed that the prevalence of *SYNGAP1* mutations in the ID population could be as high as 4%, which would make *SYNGAP1* haploinsufficiency one of the most common causes of sporadic and non-syndromic ID (Hamdan et al. 2011). Clinical examination of patients with *SYNGAP1* mutations has revealed that this disorder is characterised by moderate to severe ID with a high incidence of other comorbidities including epilepsy and ASD (Klitten et al. 2011; Vissers et al. 2010; Berryer et al. 2012; Carvill et al. 2013; Pinto et al. 2010).

At present current therapeutic strategies used in the treatment of ID do not target the underling cause of the disorder, rather specific behavioural symptoms, such as hyperactivity, attention deficits, anxiety and irritable/aggressive behaviours (Berry-Kravis 2014). To treat the core deficits that underlie ID targeted pharmacotherapies need to be developed. This requires a greater understanding of the molecular mechanisms that underlie the learning and behavioural deficits associated with mutations in ID-related genes. Thus the generation of mouse models of ID have provided a valuable neurobiological tool to characterise protein function and the consequence of its loss in the mammalian brain.

So far this has led to great advancements in the study of FXS with the identification of key cellular processes that are disrupted in the *Fmr1* KO brain. Furthermore key intracellular mechanisms that are responsible for synaptic dysfunction have been identified, thus providing potential targets for therapeutic intervention in FXS. However, it remains unclear how *Syngap1* haploinsufficiency impacts key synaptic events that underlie cognitive function in the mammalian brain. In this thesis it was hypothesized that the hyperactivity of Ras mitogen protein kinase signalling pathway in *Syngap* heterozygous mice may disrupt cellular mechanisms that underlie learning and memory deficits associated with *Syngap1* haploinsufficiency.

Based on the evidence from the mouse model of FXS that abnormalities in $mGlu_{1/5}$ receptor mediated synaptic plasticity and protein synthesis lie downstream of Ras-ERK1/2-MAPK signalling cascade, we hypothesized that *Syngap* heterozygous mice might share similar pathophysiological deficits in the hippocampus. Using electrophysiological and biochemical techniques, it was shown in this thesis that mGluR-dependent LTD and basal protein synthesis rates were disrupted in young adult *Syngap* heterozygous mice, consistent with the mouse model of FXS. In addition it was directly shown that mutations in *Syngap1* and *Fmr1* converge on a common pathophysiological axis that appears to trigger hippocampal abnormalities in the mouse model of FXS and *Syngap1* haploinsufficiency. Furthermore, it was found that pharmacological tools used in the treatment of FXS restore hippocampal function in *Syngap* heterozygous mice.

The Role of Syngap1 in the Developing Brain

There is now a convergence of findings that have begun to elucidate the function of SynGAP in the mammalian brain. It appears that during development, SynGAP has a profound effect on postsynaptic function at glutamateric synapses. At early ages SynGAP modulates a critical period of synapse maturation, limiting excitatory gain by restricting AMPA receptor insertion at immature synapses (Clement et al. 2012). Similarly, at this time point LTP is impaired, likely the consequence of saturated AMPA receptor accumulation at synapses, which prevents further increases in synaptic strength (Ozkan et al. 2014).

Although by juvenile ages basal synaptic transmission stabilizes and is consistent between both WT and *Syngap* heterozygous mice, our data suggests that SynGAP plays a pivotal role in repressing basal mRNA translational rates that underlie long-lasting forms of synaptic plasticity. It appears that in the post-adolescent brain *Syngap* heterozygous mice display enhanced Gp1 mGluR-dependent LTD at CA1 synapses that is no longer reliant on new protein synthesis.

At the PSD SynGAP is bound to scaffolding proteins PSD-95 and SAP-102 and interacts with the GluN2B subunit of the NMDA receptor, so is ideally positioned to relay synaptic activity to intracellular events (Chen et al. 1998; Kim et al. 1998). It is likely that SynGAP is mediating its effects at glutamatergic synapses via its synaptic GAP activity. Upon activation by CAMKII, SynGAP binds and inactivates several small G-proteins including Ras, Rap1, Rap2 and Rab5 (Oh et al. 2004; Tomoda et al. 2004; Ye & Carew 2010). The Ras mitogen protein kinases initiate transcription, translation and phosphorylation of synaptic proteins, which play a pivotal role in synaptic plasticity and are crucial for cognitive function (Krab et al. 2008; Ye & Carew 2010). Thus, genetic mutations in components of Ras signalling, known as RASopathies are linked to ID (Cesarini et al. 2009).

In the *Syngap* heterozygous brain, where SynGAP expression is dramatically reduced, phenotypic manifestations may arise from derepression of basal levels of Ras and its downstream substrates that include ERK1/2-MAPK and PI3K-mTOR-Akt. Together the hyperactivation of these signalling cascades lead to the initiation of cellular processes that would normally lie dormant under steady-state conditions. In agreement, it appears that the genetic reduction of *Syngap* expression leads to the derepression of ERK1/2, Akt and S6 at rest. These proteins are components of cascades that mediate the initiation of mRNA translation through various effectors and it seems likely that overactivation of these proteins underlies the excessive translational rates in the hippocampus of *Syngap* heterozygous mice. In support of this hypothesis, inhibitors of Ras and MEK1/2 activity significantly reduced the activation state of ERK1/2 and S6, normalising protein synthesis rates in the hippocampus of *Syngap* heterozygous mice.

Furthermore protein synthesis rates were saturated and were no longer regulated in an activity-dependent by $mGlu_{1/5}$ receptor activation the *Syngap* heterozygous brain. Consequently, it appears that there may be a downregulation of $mGlu_{1/5}$ receptor expression in hippocampal synaptoneurosomes from *Syngap* heterozygous mutants, perhaps a compensatory effect to counteract exaggerated mGluR-dependent signalling. Interestingly, increased basal protein synthesis rates in the hippocampus

of *Syngap* heterozygous mice did not appear to have a functional affect at the synapse until $mGlu_{1/5}$ receptors were activated. However upon $mGlu_{1/5}$ activation there was a disproportionate level of synaptic depression likely mediated by the excessive removal of AMPA receptors from the postsynaptic surface. In addition, our findings suggest that ERK and mTOR signalling may have dual functionality at the synapse mediating translation rates as well as AMPA receptor removal.

To elucidate the exact mechanisms underlying excessive translational rates and AMPA receptor endocytosis in the hippocampus of *Syngap* heterozygous mutants, the activation states of a greater number of ERK1/2 and PI3K substrates need to be quantified under steady-state conditions, which was beyond the scope of this thesis. Furthermore, although in this study there were no detectable differences in expression levels of a subset of "LTD proteins", this needs to be re-examined in hippocampal slices that have undergone the same experimental conditions which revealed the electrophysiological and biochemical phenotypes associated with *Syngap1* haploinsufficiency.

Basal protein synthesis rates: a potential biomarker for intellectual disability

This thesis has also shown that increased protein synthesis rates are consistently reproducible *in vitro* in *Syngap* heterozygotes and *Fmr1* KO mice and this phenotype also translates to the rat model of FXS. Furthermore *in vivo* measurements have revealed that there is a global increase in protein synthesis in the *Fmr1* KO brain (Qin et al. 2005a). This raises the intriguing possibility that measurements of cerebral protein synthesis in human patients could be used as a reliable biomarker for the disease (Bishu et al. 2009).

This is of particular importance as certain genetic causes of ID share striking similar behavioural manifestations yet have opposing biochemical and cellular phenotypes (e.g. *Fmr1* KO *versus TSC* heterozygotes; Auerbach et al. 2011). These findings have emphasized the need for optimal levels of synaptic protein synthesis. Furthermore, depending on where the mutation lies on the pathophysiological axis of

ID will determine treatment outcome i.e. PAM of $mGlu_5$/mTOR inhibitors or NAM of $mGlu_5$/Ras-ERK1/2 inhibitors. For example, therapeutic strategies that are beneficial for FXS could potentially exacerbate symptoms in the *TSC* heterozygous mutants and vice versa because protein synthesis rates lie on opposite ends of the spectrum. Thus it is important to establish how ID-related mutations affects synaptic plasticity and protein synthesis in order to establish the most effective therapeutic intervention i.e. PAM or NAM of $mGlu_5$ (Auerbach et al. 2011). However interestingly, *Syngap1* haploinsufficiency was amenable to both mTOR and ERK inhibitors.

Therapeutic targets in the mouse model of Syngap haploinsufficiency

The identification of cellular processes that are dysfunctional in the *Syngap* heterozygous brain, which can be partially/fully restored *in vitro* following phenotypic onset in young adult mice have elucidated potential therapeutic targets for the treatment of *Syngap1* haploinsufficiency. These targets include $mGlu_5$, Ras, ERK1/2 and mTOR. Downregulation of $mGlu_5$, Ras and ERK1/2 signalling rescued elevated protein synthesis rates in the hippocampus of *Syngap* heterozygotes, whilst enhanced and protein synthesis independent mGluR-dependent LTD was restored by an inhibitor mTOR. It appears that ERK1/2 also plays an important role in the expression and maintenance of mGluR-dependent LTD at CA1 synapses. However in order to determine whether it can restore mGluR-dependent LTD to WT levels perhaps a more subtle reduction in ERK1/2 signalling is required, which might be achieved by using an inhibitor of Ras, such as lovastatin.

It will be interesting to see whether the chronic application of these compounds *in vivo* correct similar phenotypic manifestations. NAMs of $mGlu_5$ receptors, lovastatin and rapamycin can be administered early on in developmental through subcutaneous injections (Michalon et al. 2012). In addition lovastatin can be administered orally, post-weaning, through specially formulated rodent chow (Osterweil et al. 2010). It will also be important to establish whether the pharmacological rescue of molecular and cellular deficits in hippocampus of *Syngap* heterozygous mice extends to

correcting impairments in hippocampal-dependent learning and memory tasks, such as water maze, radial arm maze and elevated T-maze. This will clarify whether the hyperactivity of Ras-mediated signalling is the root cause of the behavioural abnormalities associated with *Syngap1* haploinsufficiency. Furthermore it will determine whether the pharmacological reduction of Ras signalling can restore cognitive function in the mouse model of *Syngap1* haploinsufficiency.

Promising findings from studies in the *NF1* heterozygous mouse have revealed that lovastatin treatment was successful at rescuing impairments in LTP and spatial learning as well as attention deficits (Li et al. 2005). Furthermore administration of lovastatin to *NF1* patients rescued similar abnormalities (Mainberger et al. 2013). In *Fmr1* KO mice, both oral and acute injection of lovastatin has been shown to restore enhanced mGluR-dependent LTD, elevated basal protein synthesis rates and epileptogenesis *in vitro* and *in vivo* (Osterweil et al. 2013). Moreover an open-label trial in FXS patients revealed that the compound was well tolerated and had minimal side effects with patients showing a significant improvement in general ID behaviours and those specifically associated with FXS (Caku et al. 2014). As *Syngap* heterozygous mice closely phenocopy both *Fmr1* KOs and *NF1* heterozygotes, it is tempting to speculate that lovastatin may also successively treat similar phenotypes reported in *Syngap* heterozygous mice and patients with *SYNGAP1* mutations. This compound is already approved for use in children to treat hypercholesterolemia and has a good safety profile. Thus treatment for ID could be started early in development.

Genetic Rescue of Syngap haploinsufficiency

Recently, another novel approach to rescuing the pathology associated with *Syngap1* has been proposed. This involves increasing SynGAP expression during the developmental period of synaptogenesis by targeting transcription factors that regulate the transcription of SynGAP mRNA at excitatory synapses (Aceti et al. 2014). One potential target is the myocyte enhancer factor 2 (MEF2) family of

transcription factors. These transcription factors are highly abundant in the brain where they regulate gene transcription in an activity dependent manner.

Activation of MEF2 family members, in response to neuronal depolarisation or synaptic activity, leads to the transcription of MEF2 target genes that appear to restrict the number of excitatory synapses during development (Flavell et al. 2006; Barbosa et al. 2008; Pfeiffer et al. 2010; Tsai et al. 2012). Importantly recent studies suggest that MEF2 induced gene transcription and synapse elimination may be critical for learning and memory behaviours (Barbosa et al. 2008). Several MEF2-generated transcripts have been identified and include *Arc, PCDH10* (photocadherin 10)*, Ube3A* (E3 ubiquitin-protein ligase) and *Syngap* (Tsai et al. 2012; Flavell et al. 2006; Flavell et al. 2008). Thus, their aim is to determine whether there are any pharmacological agents that will activate MEF2-dependent transcription during early development in order the increase SynGAP expression at excitatory synapses in the *Syngap* heterozygous mice in the hope of restoring cognitive function.

Previous attempts at genetic reversal of *Syngap1* haploinsufficiency have identified a critical period in which *Syngap* must be reintroduced in order to successfully restore behavioural phenotypes (Clement et al. 2012; Ozkan et al. 2014; Aceti et al. 2014). In these studies they observed that the efficacy of postnatal rescue decreased by P21, whilst no improvement was observed in adulthood. Thus, at later stages of development perhaps a pharmacological rescue will be a crucial therapeutic strategy to restore synaptic dysfunction.

Pharmacological rescue of epileptogenesis in the mouse model of Syngap haploinsufficiency

From the list of therapeutic targets identified in this study, it is now important to establish whether they are effective at correcting other phenotypes associated with *Syngap1* haploinsufficiency. Of particular interest to our laboratory is how *SYNGAP1/Syngap1* mutations confer an increased susceptibility to epileptogenesis in both human patients and the mouse model of this disorder. Human patients appear particularly vulnerable to epileptic episodes that begin early on in childhood (Berryer

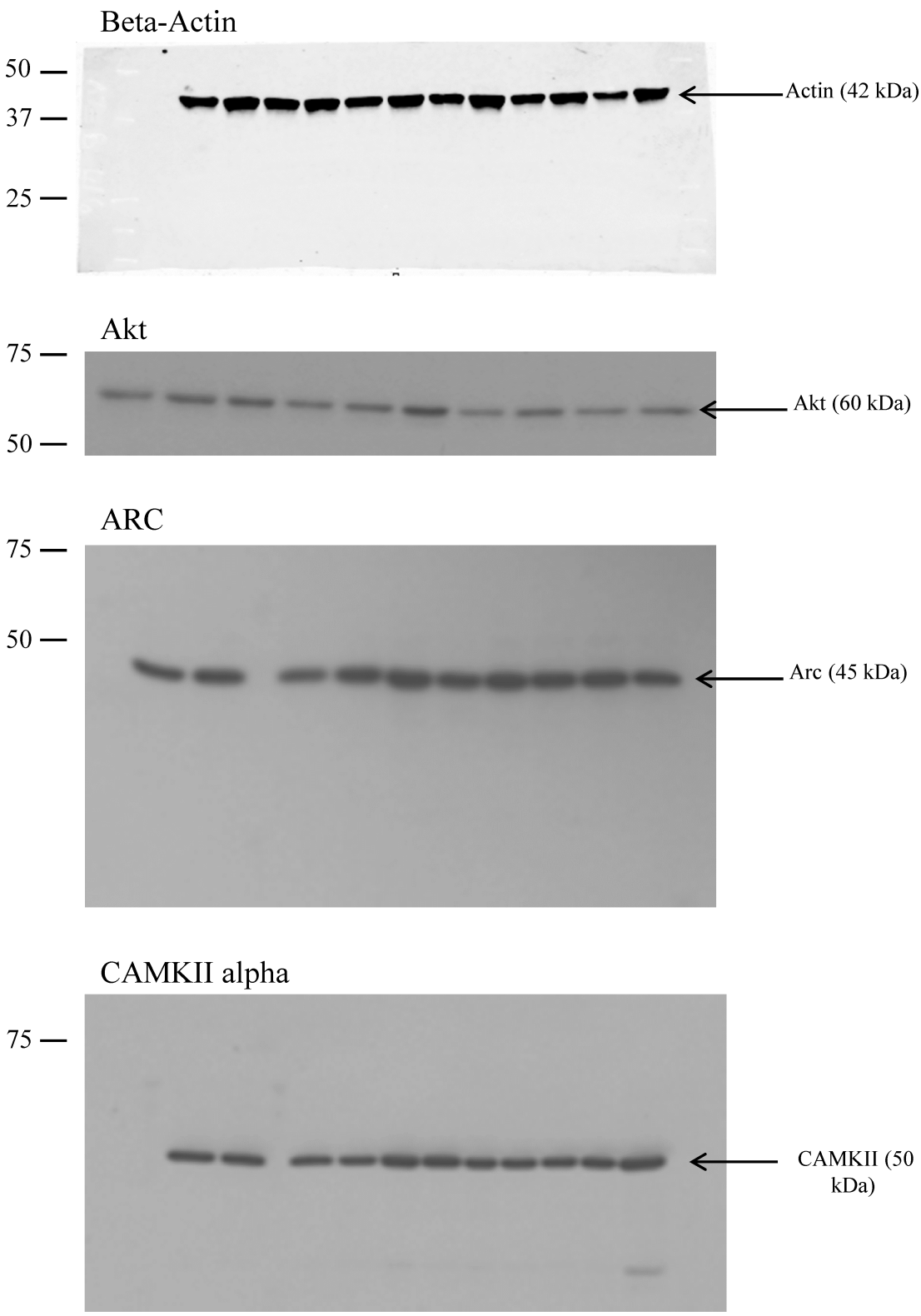
et al. 2012). It is reported that these patients have abnormal EEG patterns that reflect the presence of generalised seizures, which can be recapitulated in rodent models *in vivo* by exposure to high intensity auditory stimuli. Here it has been observed that *Syngap* heterozygous mice have a lower induction threshold for chemically-induced and audiogenic seizures (Guo et al. 2009; Clement et al. 2012).

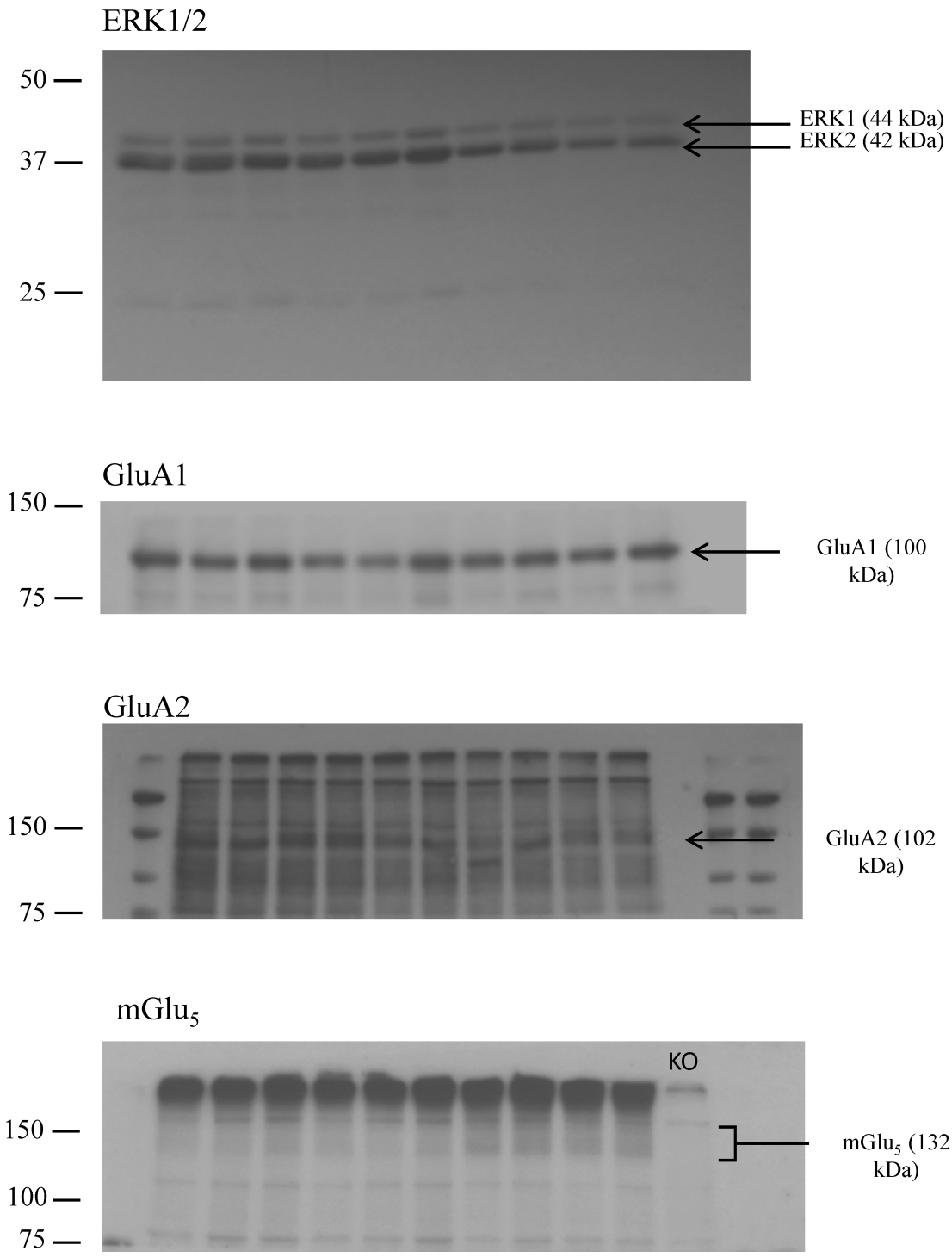
Previous studies has shown that a lasting consequence of $mGlu_5$- and ERK1/2-mediated protein synthesis is the generation of epileptiform activity in CA3 of the hippocampus (Zhao et al. 2004). *Fmr1* KO mice are also sensitive to epileptogenesis and it has been shown that lovastatin prevents the emergence of epileptiform activity *in vitro* and reduces susceptibility to AGS *in vivo* (Osterweil et al. 2013). To assess generalized epilepsy in our mouse model of *SYNGAP1* haploinsufficiency, AGS equipment was set-up according to Osterweil et al. (2010). Based on existing data that *Syngap* heterozygous mice exhibit an increased susceptibility to AGS seizures and from our current findings that $mGlu_5$-Ras-ERK signalling plays an important role in the pathogenesis of *Syngap1* haploinsufficiency, we wanted to determine whether reducing Ras-ERK1/2 mediated signalling through oral administration of lovastatin could prevent the emergence of epileptic seizures (Guo et al. 2009; Clement et al. 2012). Unfortunately due to problems with animal breeding we were unable to demonstrate a pharmacological rescue *in vivo*. However experimental conditions were optimised using a small cohort of *Syngap* heterozygous and WT mice. Thus, it will soon be determined whether $mGlu_5$-Ras signalling is also the predominant pathological mechanism underlying the generalised epilepsy associated with *Syngap1* haploinsufficiency.

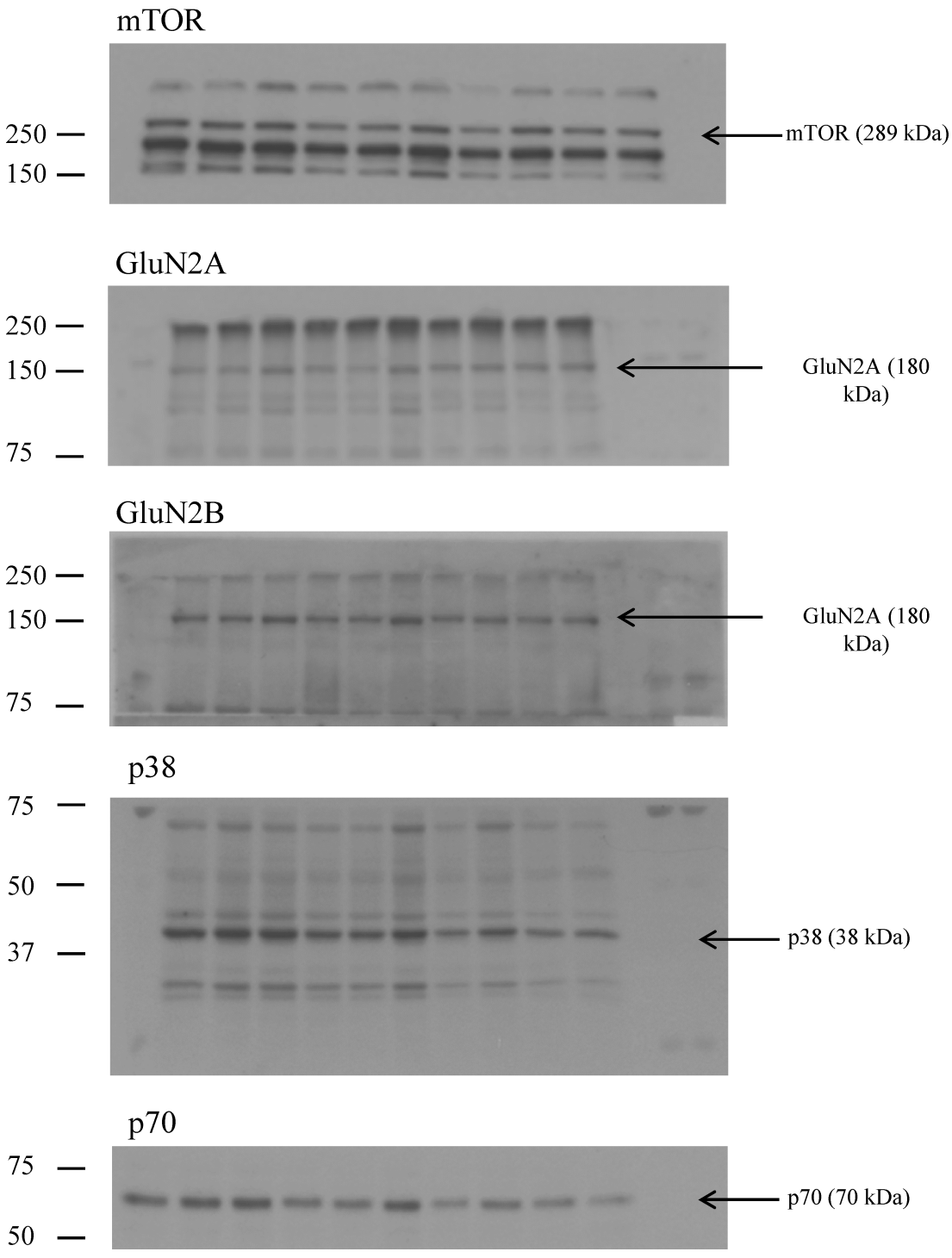
Chapter 7

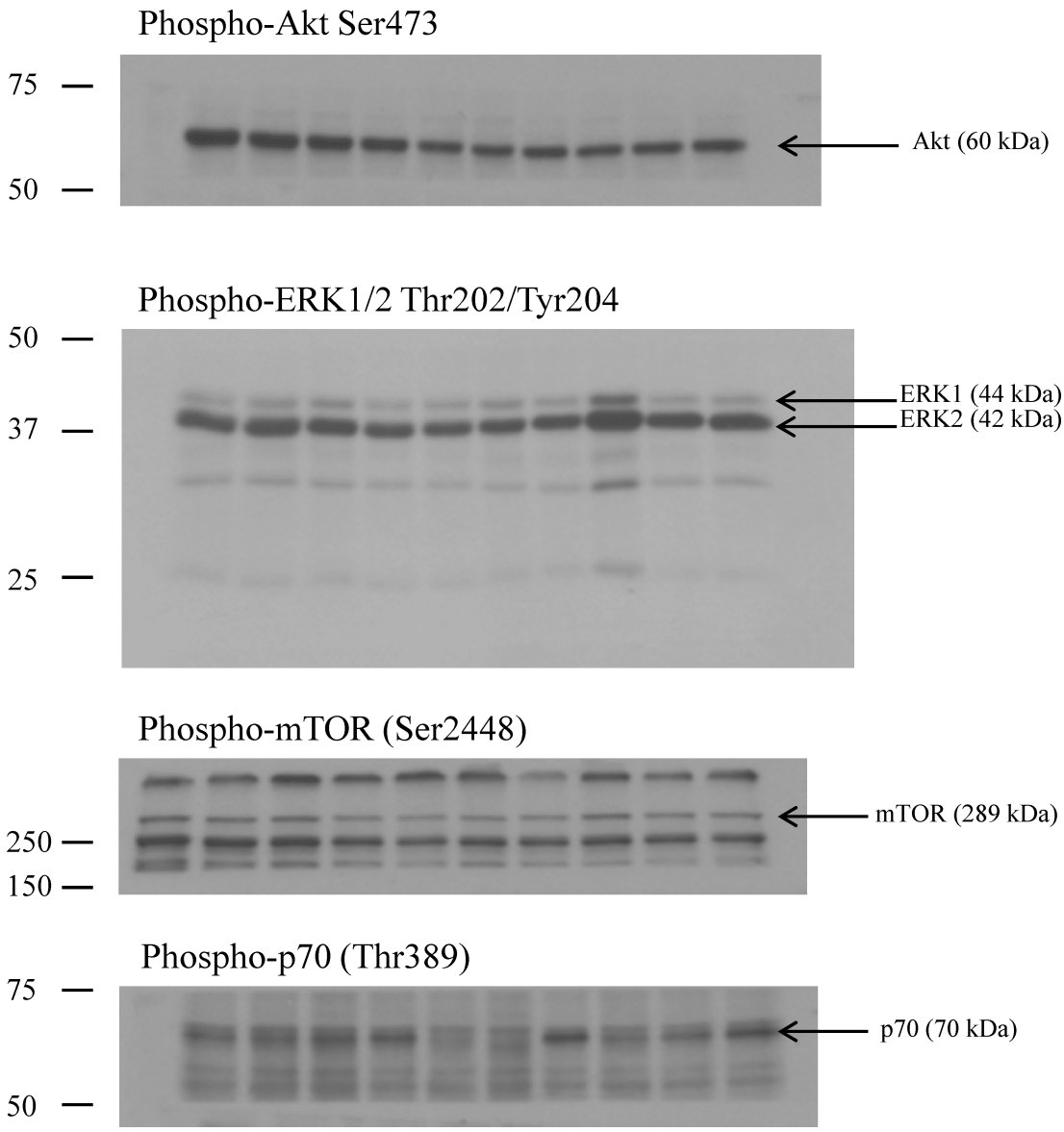
Appendix

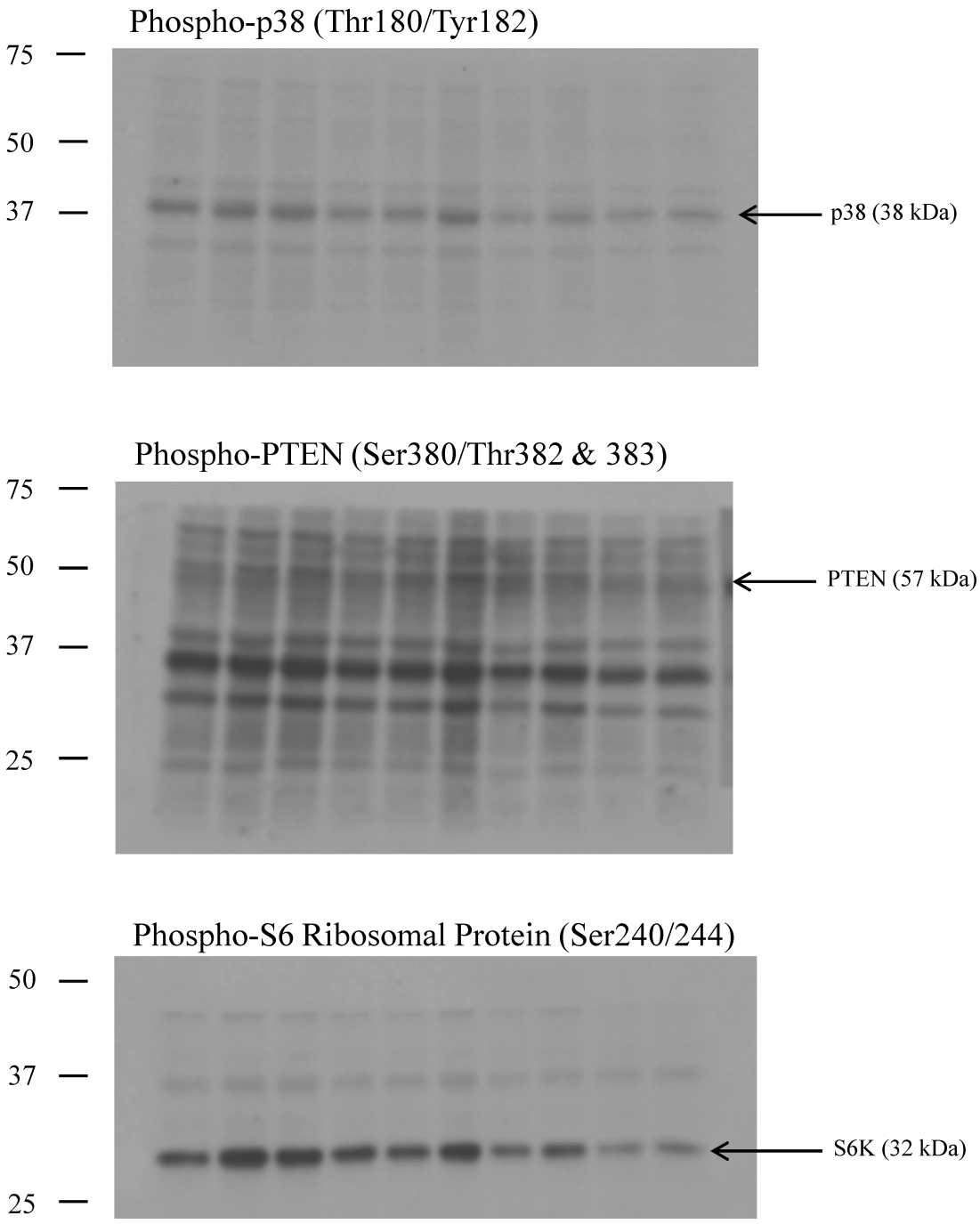
Whole gel images of every anti-protein antibody used within this thesis.

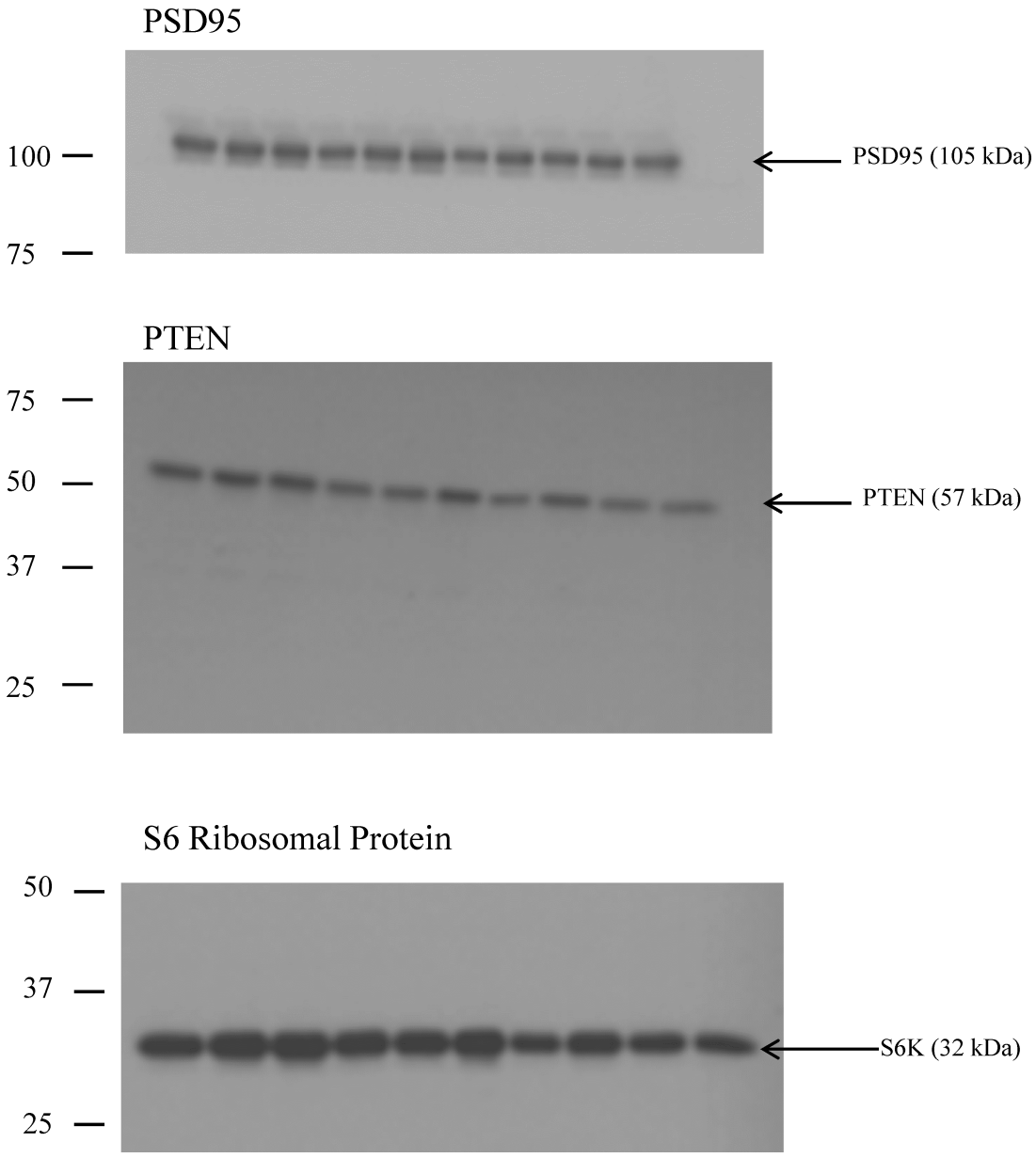



ERK1/2
50
37
25
ERK1 (44 kDa)
ERK2 (42 kDa)
GluA1
150
75
GluA1 (100 kDa)
GluA2
150
75
GluA2 (102 kDa)
mGlu5
KO
150
100
75
mGlu5 (132 kDa)


mTOR
250
150
mTOR (289 kDa)
GluN2A
250
150
75
GluN2A (180 kDa)
GluN2B
250
150
75
GluN2A (180 kDa)
p38
75
50
37
p38 (38 kDa)
p70
75
50
p70 (70 kDa)


Phospho-Akt Ser473
75
50
Akt (60 kDa)
Phospho-ERK1/2 Thr202/Tyr204
50
37
25
ERK1 (44 kDa)
ERK2 (42 kDa)
Phospho-mTOR (Ser2448)
250
150
mTOR (289 kDa)
Phospho-p70 (Thr389)
75
50
p70 (70 kDa)


Phospho-p38 (Thr180/Tyr182)
75
50
37
p38 (38 kDa)
Phospho-PTEN (Ser380/Thr382 & 383)
75
50
37
25
PTEN (57 kDa)
Phospho-S6 Ribosomal Protein (Ser240/244)
50
37
25
S6K (32 kDa)


PSD95
100
75
PSD95 (105 kDa)
PTEN
75
50
37
25
PTEN (57 kDa)
S6 Ribosomal Protein
50
37
25
S6K (32 kDa)

Chapter 8

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