Contributions of Metabotropic Glutamate Receptors to the Pathophysiology of Autism

by

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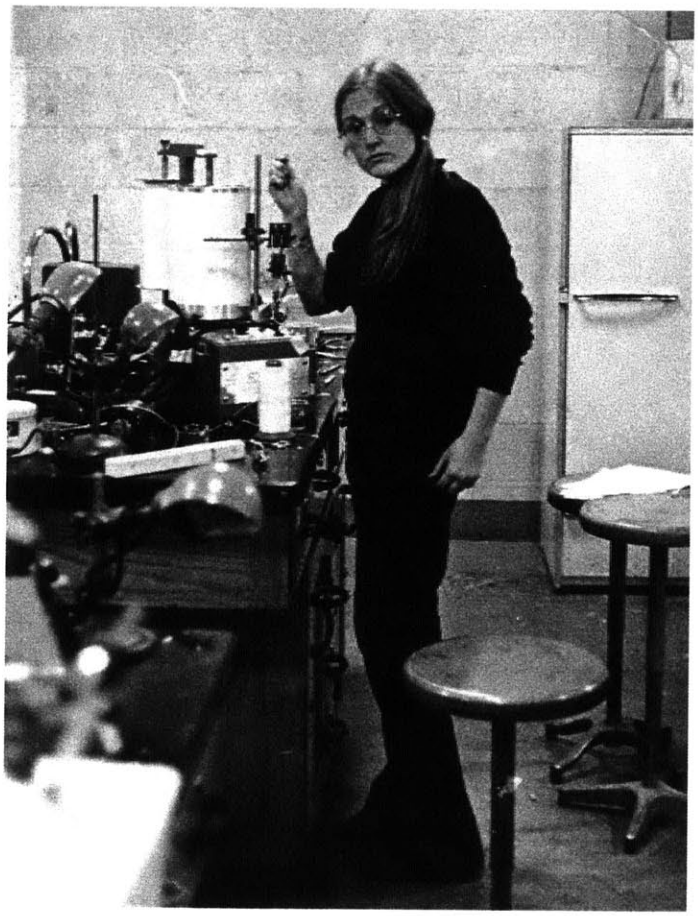

Dedicated to my mom,
the hardest working scientist I know

Contributions of Metabotropic Glutamate Receptors to the Pathophysiology of Autism

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Benjamin D. Auerbach

Submitted to the Department of Brain and Cognitive Sciences on May 29, 2013
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Neuroscience

Abstract

Autism spectrum disorder (ASD) is a complex and heterogeneous disorder, and in the vast majority of cases the etiology is unknown. However, there are many syndromes of known genetic origin that have a high incidence of autism. These highly penetrant syndromic forms of autism offer a unique opportunity for the study of ASD because animal models can be readily engineered to carry the same genetic disruption. Animal models are crucial for understanding neurological disorders at the biological level, and while these monogenic disorders are relatively rare, their animal models are likely to prove indispensable in identifying common pathogenic pathways in ASD and associated intellectual disability (ID). As evidence accumulates from genetic and molecular studies, autism is increasingly being regarded as a disease of the synapse. In particular, a preponderance of genes associated with ASD appear to regulate the synaptic signaling pathways necessary for the proper control of neuronal protein synthesis. Here, we test the hypothesis that many ASDs may result from alterations in synaptic protein synthesis by examining neuronal translation in the mouse models of fragile X (FX) and tuberous sclerosis (TSC), the two leading inherited causes of ASD. Specifically, we determined if altered synaptic protein synthesis downstream of metabotropic glutamate receptor 5 (mGluR5) is a shared disruption in these disorders, and therefore may ultimately contribute to the pathophysiology of ASD in general. First, we show that multiple aspects of mGluR-mediated protein synthesis are altered in the mouse model of FX, suggesting that exaggeration of these processes may account for the diverse phenotypes associated with the disorder. Next, we demonstrate that disruptions in the mGluR pathway do not appear to be limited to this FX, as there is diminished synaptic protein synthesis and mGluR-LTD in a mouse model of TSC as well. This suggests that genetically heterogeneous causes of ASD and ID may produce similar deficits through bidirectional deviations in mGluR-mediated protein synthesis. Finally, we address the mechanisms by which mGluR activation is coupled to protein synthesis, which may elucidate novel avenues for the next generation of mGluR-based therapies for the treatment of ASD.

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Table of Contents

Chapter 1
Synaptic pathophysiology in autism and autism-related disorders 15

1.1 Introduction 17
1.1.1 Phenotypic diversity in autism and ASD 17
1.1.2 Genetics of autism and ASD 18
1.2 The pathophysiology of ASD 20
1.2.1 Developmental verses ongoing pathology in ASD 20
1.2.2 Synaptic dysfunction in ASD 21
1.2.3 Synaptic structural proteins associated with ASD 22
1.2.4 Synaptic signaling is disrupted in ASD 24
1.3 The role of protein synthesis in neurons 25
1.3.1 Translational regulation of synaptic plasticity 25
1.3.2 Local synaptic control of protein synthesis 27
1.3.3 Pathways regulating neuronal translation are disrupted in ASD 28
1.4 Dissecting the role of metabotropic glutamate receptors in ASD 31

Chapter 2
Multiple facets of metabotropic glutamate receptor-mediated protein synthesis are disrupted in the mouse models of fragile X 41

2.1 Abstract 43
2.2 Introduction 45
2.2.1 Regulation of protein synthesis by FMRP 45
2.2.2 The mGluR theory of fragile X 46
2.2.3 mGluR-dependent priming of LTP 47
2.3 Results 48
2.4 Discussion 49
2.4.1 mGluR-dependent translation regulates proteins required for both LTD and LTP 50
2.4.2 Loss of FMRP decouples mGluR-dependent priming of LTP from protein synthesis 51

2.4.3 Implications for the pathophysiology of fragile X 52
2.5 Methods 55
2.5.1 Animals 55
2.5.2 Hippocampal slice preparation 55
2.5.3 Electrophysiology 55
2.5.4 Reagents 56

Chapter 3
Mutations causing syndromic autism define an axis of synaptic pathophysiology 67

3.1 Abstract 69
3.2 Introduction 71
3.2.1 Tuberous sclerosis complex 71
3.3 Results 73
3.3.1 Excessive mTOR signaling suppresses the protein synthesis required for mGluR-LTD 73
3.3.2 Augmentation of mGluR5 rescues synaptic and behavioral impairments in *Tsc2*$^{+/-}$ mice 75
3.3.3 Mutations in *Fmr1* and *Tsc2* cancel one another on a functional axis 76
3.4 Discussion 77
3.4.1 Mechanisms of mTOR-dependent suppression of translation 78
3.4.2 Regulation of gene-specific translation 80
3.4.3 Functional significance of gene-specific translation 82
3.4.4 Role of mGluR dysfunction in TSC and ASD 83
3.5 Methods 85
3.5.1 Animals 85
3.5.2 Electrophysiology 85
3.5.3 Metabolic labeling of new protein synthesis 86
3.5.4 Immunoblotting 87
3.5.5 Immunoprecipitation 87
3.5.6 Contextual fear conditioning 87
3.5.7 Reagents 88

Chapter 4
β-arrestin2 signaling mediates mGluR-dependent ERK activation and long term synaptic depression 103

4.1 Abstract 105
4.2 Introduction 107
4.2.1 Signaling pathways that couple mGluRs to protein synthesis 108
4.2.2 β-arrestin-mediated signaling 110
4.3 Results 112
4.4 Discussion 114
4.4.1 Implications for mGluR signaling and LTD 115
4.4.2 Implications for ASD treatment 117
4.5 Methods 119
4.5.1 Animals 119
4.5.2 Electrophysiology 119
4.5.3 Immunoblotting 120
4.5.4 Reagents 120

Chapter 5
Implications and future directions 131

5.1 Introduction 133
5.2 Contribution of mGluRs to the synaptic pathophysiology of autism 134
5.3 Regulating protein synthesis at the synapse 137
5.3.1 Somatic verses synaptic translation 137
5.3.2 Multiple pools of mRNA are differentially translated in neurons 138
5.3.3 Identifying the two pools 140
5.3.4 Functional distinction between ERK and mTOR regulated protein synthesis 142
5.4 Synaptic pathophysiology beyond post-synaptic protein synthesis 144
5.5 Synaptic dysfunction in relation to the autistic phenotype 145
5.6 Concluding remarks 148

References 15

List of figures and tables

Table 1.1 – Genes associated with high risk for ASD 35

Figure 1.1 – Highly penetrant mutations associated with autism converge at the synapse 37

Figure 1.2 – General and gene specific mechanisms for translation initiation and elongation 39

Figure 2.1 – The mGluR theory of fragile X 57

Figure 2.2 – DHPG application facilitates subsequently induced LTP 59

Figure 2.3 – DHPG-induced priming of LTP does not require protein synthesis in the *Fmr1* KO mouse 61

Figure 2.4 – Summary of DHPG-induced priming of LTP and its protein synthesis dependency in wild-type and *Fmr1* KO mice 63

Figure 2.5 – Model for role of FMRP in mGluR-dependent priming of LTP 65

Figure 3.1 – *Tsc2*$^{+/-}$ mice have a specific deficit in mGluR-LTD 89

Figure 3.2 – The protein synthesis-dependent component of mGluR-LTD is absent in *Tsc2*$^{+/-}$ mice 91

Figure 3.3 – Presynaptic component of DHPG-induced LTD 93

Figure 3.4 – Excessive mTOR activity suppresses the protein synthesis required for mGluR-LTD 95

Figure 3.5 – Positive modulation of mGluR5 reverses synaptic and behavioral deficits in *Tsc2*$^{+/-}$ mice 97

Figure 3.6 – Genetic cross of *Tsc2*$^{+/-}$ and *Fmr1* KO mice rescues synaptic and behavioral impairments present in both single mutants 99

Figure 3.7 – Models to account for opposing alterations in mGluR responses in *Tsc2*$^{+/-}$ and *Fmr1* KO mice 101

Figure 4.1 – Activation of mGluR5 leads to divergent signaling cascades 121

Figure 4.2 – mGluR-mediated ERK phosphorylation is attenuated in *Arrb2* mutants 123

Figure 4.3 – The Akt-mTOR pathway is not recruited by mGluR activation and is unaltered in *Arrb2* mutants 125

Figure 4.4 – Loss of the protein synthesis-dependent component of mGluR-LTD in *Arrb2* mutants 127

Figure 4.6 – Normal basal synaptic function and NMDAR-dependent LTP in *Arrb2* mutants 129

Figure 5.1 – Defining an axis of synaptic pathophysiology in autism 151

Chapter 1

Synaptic pathophysiology in autism and autism-related disorders

1.1 Introduction

Autism spectrum disorder (ASD) is a severe neurodevelopmental condition characterized by social disability, communication impairment, and repetitive/restricted behavior. It is one of the most common neurodevelopmental disorders, affecting up to 1 in 100 people (Baird et al., 2006; Newschaffer et al., 2007), and the direct and indirect medical costs associated with autism are estimated to be more than $35 billion per year and rising (Ganz, 2007). In spite of this high prevalence and substantial societal cost, effective therapeutic interventions for autism are woefully lacking. The most common pharmacological treatments include antidepressants, stimulants, and antipsychotics (Oswald and Sonenklar, 2007). The main goal of these current treatments is to alleviate behavioral symptoms that interfere with an individual's daily functioning, such as seizures, sleep disturbances, irritability, and hyperactivity (Broadstock et al., 2007). However, most of these treatments are associated with their own adverse side effects. Educational and behavioral interventions are also commonly applied to ASD management (Myers and Johnson, 2007), however the evidence for the effectiveness of these therapies is currently lacking and more data from randomized controlled trials is needed to properly assess their efficacy (Warren et al., 2011). Currently there are no treatments directed at the core disturbances of the disorder.

Developing effective treatments for autism and other psychiatric illnesses requires understanding of their underlying pathophysiology. At this time, there is no known unifying mechanism for ASD at the molecular, cellular or systems level. There are no therapies that address the core disturbances in autism because we do not understand the cause of these disturbances; the complexity of the disorder makes it difficult to study. This largely stems from the highly heterogeneous nature of autism with regards to both its genetic underpinnings and behavioral manifestations.

1.1.1 Phenotypic diversity in autism and ASD

An individual is defined as having ASD if they meet the diagnostic criteria for a combination of 2 or more of a triad of symptoms: social disability, communication impairment, and repetitive/restricted behavior (American Psychiatric, 2003). However, beyond this broad definition there is an extreme degree of clinical heterogeneity in ASD. There are striking differences in the expression of these core symptoms across the autistic population–ranging from

mild to severe (Bailey et al., 1996). Additionally, there is much variability in the severity of impairment between core disturbances within in an individual with ASD. Hence, autism represents a spectrum of disorders spanning a broad continuum of severity and heterogeneity in regards to phenotype (Abrahams and Geschwind, 2008). Compounding this phenotypic heterogeneity is the fact that these symptoms are not static, but emerge with development and progressively change over time. Furthermore, ASD often co-occurs with other neurological conditions, such as intellectual disability (ID) and seizure disorders, which affect up to 70% and 25% of individuals with autism, respectively (Tuchman and Rapin, 2002). Other common comorbid conditions include obsessive-compulsive disorder, attention deficit or hyperactivity disorder, or gait and motor disorders (Levy et al., 2009). Thus, the core symptoms of ASD are variably expressed, change over development, and are compounded by other related comorbidities.

This phenotypic complexity has not only impeded the diagnosis of ASD, but has raised questions about the diagnosis itself. There is debate as to the degree to which different aspects of ASD signify: (1) entirely distinct entities; (2) disorders that have overlapping foundations; or (3) different variants of one underlying disease (Geschwind and Levitt, 2007). It is difficult to define these diagnostic boundaries because the pathogenic mechanisms of ASD are largely unknown. In order to address these fundamental questions, research has recently focused on defining the genetic etiology of ASD in hopes of understanding the disorder at its core. With the rapid advance in human genetics and gene sequencing technology, there is an increasing availability of genome-wide data for ASD (Abrahams and Geschwind, 2008). This information has greatly advanced our knowledge of the pathophysiology of autism and has allowed for this disorder to be examined at a molecular level.

1.1.2 Genetics of Autism and ASD

Genetic risk factors are important in the causation of all major psychiatric disorders (Kendler, 2005). ASD is among the most highly heritable psychiatric disorders, with mono- and dizygotic twin studies estimating the heritability of ASD at over 90% and high occurrence rates among siblings and other relatives (Bailey et al., 1996; Risch et al., 1999). Despite this strong heritability, the genetics underlying autism are astoundingly complex. Recent gene association and whole-genome linkage studies have implicated over one hundred genes and genetic loci in

autism and ASD (Abrahams and Geschwind, 2008; Betancur, 2011; Freitag et al., 2010). Despite this strong genetic component, identifying causative genetic factors for ASD has remained elusive. Microscopically visible chromosomal alterations have been reported in ~ 5% of ASD cases; the most frequent abnormalities are 15q11–q13 duplications, and deletions of 2q37, 22q11.2 and 22q13.3 (Betancur, 2011). Recent whole-genome microarray analyses have discovered submicroscopic deletions and duplications, called copy number variations (CNVs), affecting many loci and including *de novo* events in 5%–10% of ASD cases (reviewed in (Betancur, 2011)). ASD can also result from mutations of single genes involved in autosomal dominant, autosomal recessive and X-linked disorders (Table 1.1). It is now known that defined mutations, genetic syndromes, and *de novo* CNVs account for 10-20% of ASD cases, however none of these known causes individually account for more than 1-2% of ASD cases (Abrahams and Geschwind, 2008; Geschwind and Levitt, 2007). Indeed, in the vast majority of instances the etiology of ASD remains unknown.

Broadly speaking, the genetic etiology of ASD can be delineated into two categories: (1) rare, highly penetrant single gene mutations that have a large causal effect; or (2) common inherited functional variants that individually present a small risk factor but cumulatively can have profound impact (Campbell et al., 2009; Levitt and Campbell, 2009). While evidence suggests this latter multifactorial etiology may account for the majority of cases of ASD, it is the rare, highly penetrant mutations associated with autism that have provided the best experimental platform for studying autism at the molecular and cellular levels. In particular, the study of syndromic forms of ASD has been influential in our understanding of ASD pathophysiology.

Syndromic autism refers to genetically well-defined disorders in which ASD is observed at higher than expected frequencies (Table 1.1). The most common of these syndromes (*GENE)* associated with ASD is fragile X (*FMR1*), accounting for 2-5% of cases of ASD. Other monogenic disorders associated with ASD include tuberous sclerosis (*TSC1*, *TSC2*), neurofibromatosis (*NF1*), Angelman syndrome (*UBE3A*), Rett syndrome (*MECP2*) and *PTEN* mutations in patients with macrocephaly and autism (Table 1.1) (Betancur, 2011). Syndromic forms of autism with highly penetrant single-gene mutations offer a unique opportunity for the study of ASD because animal models can be readily engineered to carry the same genetic disruption. Animal models are crucial for understanding neurological disorders at the biological level, and while these monogenic disorders are relatively rare, their animal models are likely to

prove indispensable in identifying common pathogenic pathways in ASD. Although genetic mutations associated with ASD will undoubtedly manifest differently at the behavioral level in animals and humans, it is reasonable to assume that disruptions in elementary neuronal functions are likely to be shared across species.

While animal models of syndromic autism have become a powerful tool for studying the pathophysiology of ASD at a mechanistic level, it is important to realize that none of these etiologies is specific to autism. Each syndrome encompasses a variable proportion of individuals with and without autism, and each possesses their own unique physiological symptoms. Therefore, it is critical to define similarities and differences across these syndromes in order to determine which underlying disruptions are specific to ASD. Cross comparison of animal models for syndromic disorders and rare mutations associated with autism has the potential to uncover shared molecular pathways/processes that may be central to autism pathophysiology. Specifically, if there is a shared pathogenesis between these disorders, then it is reasonable to assume the same pathogenic mechanism may be associated with autism of unknown etiology. Not only will this aid our understanding of how these diseases are, or are not, related to each other, but it will help determine if treatments developed for one disease may also be effective for others, including idiopathic autism. The studies in this thesis were designed to determine if there is shared molecular dysfunction in the two leading inherited causes of ASD, fragile X (FX) and tuberous sclerosis (TSC).

1.2 The pathophysiology of ASD

Studies from animal models of autism, combined with advances in human genetic research, present the best approach to addressing fundamental questions about the nature of ASD. Below, the current thinking about the pathophysiology of ASD is briefly reviewed.

1.2.1 Developmental verses ongoing pathology in ASD

The early onset of ASD symptoms highlights the importance of development in the pathophysiology of autism. Changes in brain development are undoubtedly important in autism etiology, exemplified by a high prevalence of macrocephaly in children with ASD (Courchesne et al., 2004). However, in the majority of cases of ASD, neuropathological studies point to only minor and inconsistent abnormalities in brain size (Schumann et al., 2004). The delayed post-

natal onset of ASD symptoms may lend insight into how brain development is altered. The period in which ASD symptoms present themselves typically coincides with the time in which synaptic sculpting (i.e. synapse formation, synapse pruning and myelination) takes place. It has been suggested that autism may arise from more subtle deficits in brain development required for establishing proper synaptic connectivity, such as neuronal migration and axon pathfinding (Geschwind and Levitt, 2007). In particular, alterations in postnatally-regulated features of dendritic development may be central to the pathogenesis of ASD (Zoghbi and Bear, 2012). Indeed, evidence from both human and animal studies have found that there is a preponderance of long, thin, immature dendritic spines in many forms of autism, suggesting arrested synaptic development (Minshew and Williams, 2007).

The developmental nature of ASD emphasizes the need for early treatment intervention. However, recent studies in animal models has shown that postnatal genetic and/or pharmacological manipulations can reverse many behavioral symptoms, even when treatment begins in adulthood (Ehninger et al., 2008a; Ehninger et al., 2008b; Guy et al., 2007; Michalon et al., 2012). This presents the exciting possibility that, with the proper interventions, both the pathophysiological and accompanying cognitive deficits of ASD might be ameliorated even in adulthood. Furthermore, it suggests that the underlying molecular perturbations in ASD not only affect development but may continue to influence behavior throughout an individual's lifetime. Therefore, it is important to not only understand how synaptic development is altered in ASD, but by what molecular mechanisms, as this will provide therapeutic targets not only important for early intervention but that may remain valuable throughout an individual's lifetime.

1.2.2 Synaptic dysfunction in ASD

As evidence accumulates from genetic and molecular studies, autism is increasingly being regarded as a disease of the synapse (Gilman et al., 2011; Kelleher and Bear, 2008; Zoghbi, 2003; Zoghbi and Bear, 2012). A preponderance of highly penetrant mutations associated with autism are in genes that are critical regulators of synaptic structure and function (Table 1.1). Correspondingly, one of the most consistent findings in animal models of ASD is disrupted synaptic function. Common deficits include altered synapse number and strength, aberrant dendritic size and shape, and disrupted synaptic plasticity (Zoghbi and Bear, 2012). Furthermore, studies of post-mortem human brain tissue have found the presence of aberrant

dendritic structure and/or number in the brains of autistic patients (Hinton et al., 1991; Minshew and Williams, 2007; Purpura, 1974). Intriguingly, it seems that deficits in synaptic structure and function can be related to higher-level phenotypes seen in ASD and related disorders. Disrupted synaptic function may lead to altered plasticity, which in turn may underlie the cognitive deficits seen in these disorders. Altered synaptic number and function will affect the balance between excitatory and inhibitory connections, which may lead to seizures, hyperactivity, and disruptions in sensory processing. As research continues on the human genetics and animal models of autism, a picture is emerging where there is a convergence of diverse genetic and molecular perturbations on a common dysfunction that may account for the numerous deficits seen in ASD. For all the variability in its genetic origins and phenotypic expression, synaptic dysfunction appears to be the bottleneck through which autism pathology runs.

1.2.3 Synaptic structural proteins associated with ASD

Some of the earliest clues linking ASD to synaptic function were the discovery of mutations in cell adhesion molecules (CAMs) and scaffold proteins that are essential to synaptic integrity. Neuroligins are postsynaptic CAMs that mediate synapse formation between neurons by interacting with their presynaptic counterparts, neurexins. While rare, mutations in neuroligin3 (*NLGN3*) and neuroligin4 (*NLGN4*) (Jamain et al., 2003; Laumonnier et al., 2004; Lawson-Yuen et al., 2008), as well as neurexin1 (*NRXN1*) and neurexin2 (*NRXN2*) have been shown to be highly penetrant risk factors for autism (Table 1.1, Figure 1.1) (Kim et al., 2008; Zahir et al., 2008; Zweier et al., 2009). The neurexin-neuroligin trans-synaptic complex organizes the pre- and postsynaptic compartments through interactions with various synaptic scaffolding molecules. A prominent family of synaptic scaffolding molecules are the SH and Ankyrin-domain-containing proteins (Shanks) (Sheng and Kim, 2000).

Shanks interact with other scaffolding proteins at the synapse to link multiple receptors and signaling pathways, thereby regulating synaptic structure and function (Sheng and Kim, 2000). Of particular interest is the finding that Shanks can specifically interact with neuroligins via SAPAPs (Figure 1.1). Mutations in both *SHANK2* and *SHANK3* have also been implicated in autism (Berkel et al., 2010; Durand et al., 2007; Kim et al., 2008; Yan et al., 2005). In particular, haploid deficiency in *SHANK3* has been consistently identified as a causative factor in Phelan-McDermid syndrome (PMS) (Bonaglia et al., 2006; Wilson et al., 2003), a developmental

disorder with high rates of autism resulting from a microdeletion of chromosome 22q13 (Table 1.1) (Phelan and McDermid, 2012). As more genome-wide association studies are performed for ASD, several other genes encoding for synaptic adhesion and scaffolding proteins have been shown to be associated with ASD (Table 1.1). This suggests that there is an interconnected network of structural proteins that modulate synaptic signaling, and perturbations at many points along this network can result in the disrupted synaptic function implicated in autism.

Genetic studies in mice demonstrate that neuroligns, neurexins and Shanks are integral for proper synaptic function. Deletion of *Nlgn1* or *Nlgn2* respectively results in impairment of excitatory or inhibitory synaptic transmission, consistent with their localization (Chubykin et al., 2007). Studies have also shown that mice lacking neurexins have decreased spontaneous and evoked neurotransmitter release (Missler et al., 2003). Interestingly, neither neuroligin or neurexin deletion results in alterations of synaptic number or structure, suggesting that while they are essential for synaptic function, they are not involved in the initial formation of synapses –an interesting observation in light of the developmental context of ASD symptoms. In animal models, overexpression of *Shank1* or *3* results in increased maturation and size of dendritic spines and can even induce the formation of functional spines in neurons that typically do not possess them (Roussignol et al., 2005; Sala et al., 2001). Conversely, deletion of *Shank1* or *3* results in reduced spine density and decreased synaptic transmission (Hung et al., 2008; Peca et al., 2011). Thus, altered expression or function of these proteins consistently results in excessive or diminished excitatory synaptic transmission, leading to subsequent alterations in the balance of excitation and inhibition.

Behavioral studies in these mutant mice provide evidence for a relationship between alterations in synaptic function and ASD pathogenesis. *Nlgn3* and *Nlgn4* knock out (KO) mice display several autistic-like behaviors, including impaired ultrasonic vocalization, altered social interactions and/or impaired social memory (Jamain et al., 2008; Radyushkin et al., 2009). Several different mutations of *Shank3* result in behavioral deficiencies relevant to ASD as well, such as excessive grooming and stereotyped behaviors, increased anxiety, and decreased social interactions (Bozdagi et al., 2010; Peca et al., 2011; Wang et al., 2011). Of particular interest, mice engineered to carry a specific mutation of *Nlgn3* associated with ASD display both altered synaptic transmission and impaired social interactions, demonstrating that recapitulation of mutations that cause autism in humans also disrupt synaptic function and behavior in mouse

models (Tabuchi et al., 2007). This mutation appears to actually increase inhibitory synaptic functions, suggesting that both loss-of-function and gain-of-function mutations in neuroligins can result in autistic-like behaviors in mice. Consistent with this idea, both deletions and duplications spanning *SHANK3* have also been reported in patients with ASD or ID (Durand et al., 2007; Okamoto et al., 2007). This suggests that *bidirectional* changes in synaptic function may contribute to the ASD phenotype.

The notion that some ASDs may be regarded as a disease of the synapse is a major conceptual advance in the understanding of autism pathophysiology (Zoghbi, 2003; Zoghbi and Bear, 2012). Yet major questions remain regarding how synaptic dysfunction arises, and what the nature of these disruptions are. Despite the fact that numerous genes implicated in ASD are involved in synaptic function, there is much heterogeneity to their exact role at the synapse (Table 1.1, Figure 1.1). While studies on synaptic adhesion and scaffolding proteins have been influential in directing attention to the synapse as a common pathology in ASD, mutations in genes directly involved in synaptic formation and stabilization are still exceedingly rare (Abrahams and Geschwind, 2008). However, studies on these rare mutations have highlighted the importance of activity-dependent synaptic signaling in the pathophysiology of ASD. In particular, mutations in these scaffolding and adhesion molecules possess a commonality – disruption in glutamatergic signaling.

1.2.4 Synaptic signaling is disrupted in ASD

A defining feature of synapses is their ability to undergo activity-dependent changes. These changes are mediated by synaptic receptors that act on a complex array of signaling cascades (Figure 1.1). At excitatory synapses, two glutamate receptors have been strongly implicated: (1) the calcium-permeable N-methyl-D-aspartate ionotropic receptor (NMDARs); and (2) the G_q-coupled (group 1) metabotropic glutamate receptor (mGluR1 and mGluR5). Synaptic adhesion and structural proteins organize these glutamate receptors at the postsynaptic density (PSD), thereby regulating synaptic transmission and signaling (Sudhof, 2008; Tu et al., 1999). Deletion/mutation of *Nlgn1* or *Shank3* in mice not only alters synaptic strength, but disrupt the function of NMDARs and mGluRs as well (Bangash et al., 2011; Baudouin et al., 2012; Peca and Feng, 2012; Varoqueaux et al., 2006). Alterations in synaptic activity have also been shown to rapidly change the composition of the PSD (Ehlers, 2003) and activation of the

signaling cascades downstream of these glutamatergic receptors can directly influence synapse strength, number, and development (Barnes and Slevin, 2003; Kelleher et al., 2004). Thus, there is a reciprocal relationship between activity-dependent signaling and structure at the synapse.

Importantly, many genes characterized by high penetrance for ASD are involved in the signaling pathways downstream of glutamate receptor activation (Table 1.1, Figure 1.1) (Kelleher and Bear, 2008; Levitt and Campbell, 2009; Peca and Feng, 2012). In particular, the synaptic signaling pathways necessary for the regulation of neuronal protein synthesis appear to be regulated by genes associated with ASD (Table 1.1, Figure 1.1) (Kelleher and Bear, 2008). As discussed below, activity-dependent synaptic mRNA translation is essential for normal synaptic function and development (Kelleher et al., 2004). Thus, despite the heterogeneity in their function, there may be a common thread amongst the genes implicated in ASD. We propose that altered activity-dependent regulation of neuronal protein synthesis is a prominent convergence point in the synaptic pathophysiology of ASD.

1.3 The role of protein synthesis in neurons

The ongoing synthesis of new proteins is a fundamental process essential for the function and survival of all cells. The importance of proper translational regulation is underscored by the high degree of conservation in the cellular pathways that govern protein synthesis across species and cell types (Rhoads, 1999). While translational regulation is a ubiquitous process in all cells, the functional consequences of this protein synthesis varies by cell type, as different cells perform different functions. Neurons are a particularly distinctive type of cell and as such, the requirements for and consequences of protein synthesis are unique in many respects. Aside from their ability to propagate action potentials, two defining features of neurons are their inability to undergo cell division and their dramatic compartmentalization. These features greatly define how protein synthesis is regulated in neurons, and what functions it serves.

1.3.1 Translational regulation of synaptic plasticity

In the majority of cell types, stimulating protein synthesis is typically associated with the regulation of cell division and growth (Mathews, 2000). However, aside from a few specific areas within the brain, mature neurons are fully differentiated and no longer divide. Neurons

therefore utilize protein synthesis for different processes essential for ongoing neuronal function. One of the most important functions of neurons that requires translation is the maintenance of long-lasting changes in synaptic efficacy, i.e. synaptic plasticity (Kelleher et al., 2004). Synaptic plasticity is the ability of neurons to alter the strength and/or number of their synaptic connections in an experience-dependent manner. These changes in synaptic efficacy are thought to underlie long-term changes in neural circuits and thus modify behavior. In particular, synaptic plasticity is thought to be the cellular/molecular correlate of learning and memory, as they share many of the same mechanisms for expression, including the requirement of *de novo* protein synthesis (Flexner et al., 1963; Gkogkas et al., 2010).

The hippocampus has long been implicated in playing an important role in memory formation and has been used as a model system for the study of synaptic plasticity and its protein synthesis-dependency (Morris et al., 2003). Although not limited to this structure, much of our understanding of the mechanisms which support experience-dependent synaptic plasticity have come from studies in the hippocampus. Long-term potentiation (LTP) and long-term depression (LTD) are well-characterized forms of synaptic plasticity associated with learning and memory. While these persistent changes in synaptic strength can be induced by a variety of manipulations and their expression mechanisms are diverse, the long-term maintenance of most forms of LTP and LTD requires the synthesis of new proteins (Gkogkas et al., 2010). Interestingly, it seems that many of the signaling pathways that regulate cell division and growth in other cell types have been adapted for the regulation of protein-synthesis dependent plasticity in neurons (see section 1.3.3) (Kelleher et al., 2004). Alteration of synaptic strength is an energy intensive process, and it has been suggested that competition for translation machinery may be a limiting factor in the maintenance of long-term plasticity (Fonseca et al., 2004; Govindarajan et al., 2011). The maintenance of long-term changes in synaptic strength is also associated with alterations in the structure and/or number of synapses (Tada and Sheng, 2006). Thus, the proper regulation of protein synthesis in neurons is essential for synaptic plasticity, and disruptions in the signaling pathways that regulate translation will undoubtedly affect synaptic structure and function.

1.3.2 Local synaptic control of protein synthesis

Another aspect of neurons that makes the regulation of translation unique is their high degree of compartmentalization. Most neurons have elaborate dendritic processes consisting of thousands of synapses with well-defined patterning that are located distally from the cell soma. One of the hallmarks of synaptic plasticity is input-specificity, whereby changes in synaptic strength induced at a particular set of synapses do not spread to other synapses. This poses a unique challenge for the cell-wide synthesis of new proteins to be properly transported only to synapses undergoing plastic changes. Traditionally, transcription and translation were thought to be tightly coupled processes that occur in the cell soma (Kelleher et al., 2004). Therefore, translation was originally thought to play a permissive role in synaptic plasticity. Recently however, it has been shown that many messenger (m)RNAs are trafficked to dendrites, where the required translation machinery is also present (Steward and Levy, 1982), suggesting an additional role for local synaptic control of protein synthesis (Steward and Schuman, 2001). This compartmentalization of translation is intriguing as it provides a mechanism for maintaining the input-specificity of long-lasting changes in synaptic efficacy that require new protein synthesis.

While the roles of somatic transcription and translation in synaptic plasticity have been well-characterized (Kandel, 2001a), the importance of synaptically localized translation has only recently been explored (Sutton and Schuman, 2006). Indeed, activity-dependent translation of pre-existing dendritic mRNA at the synapse is necessary for the expression of multiple forms of synaptic plasticity (Bradshaw et al., 2003; Cracco et al., 2005; Huber et al., 2000; Kang and Schuman, 1996; Miller et al., 2002; Smith et al., 2005). This is evidenced by their dependence on translation but not transcription, and the ability to maintain these modifications via new translation in dendrites isolated from the parental soma (Huang et al., 2005; Huber et al., 2000; Kang and Schuman, 1996; Vickers et al., 2005). Many proteins necessary for the maintenance of synaptic plasticity and dendritic spine formation/remodeling have been shown to be locally synthesized at the synapse (Lee et al., 2005; Tiruchinapalli et al., 2003) and activation of local protein synthesis has been shown to affect spine morphology (Vanderklish and Edelman, 2002). Thus, the local regulation of translation may be particularly important for the proper function of synapses. Interestingly, the mRNA for many of the synaptic scaffolding molecules implicated in autism are present at the synapse and undergo activity-dependent local translation (Bockers et

al., 2004; Lee et al., 2005; Todd et al., 2003), thereby directly linking activity-dependent synaptic translation and disrupted synaptic function in ASD.

1.3.3 Pathways regulating neuronal translation are disrupted in ASD

In order to fully understand the potential role of local protein synthesis in normal brain function and disease pathophysiology, it is imperative to understand the molecular mechanisms by which synaptic activity governs this process. While the involvement of neuronal protein synthesis in the maintenance of synaptic plasticity and memory has been known for decades (Flexner et al., 1963), only recently have we begun to elucidate the requisite upstream signaling pathways. Two intracellular signaling cascades are prominently implicated in the regulation of neuronal protein synthesis: (1) the mammalian target of rapamycin (mTOR) pathway; and (2) the extracellular signal-regulated kinase (ERK) pathway (Figure 1.1). Recent work has established that the ERK and mTOR signaling pathways couple synaptic activity to the translational machinery during both protein synthesis-dependent LTP and LTD (reviewed in (Kelleher et al., 2004)).

Initiation is typically considered the rate-limiting step for protein synthesis, and therefore serves as a major target for translational control (Richter and Sonenberg, 2005). Both the mTOR and ERK pathways can stimulate global translation by regulating components of initiation (Figure 1.2A) (reviewed in (Costa-Mattioli et al., 2009)). Initiation factors (eIFs) recognize the 5' cap of an mRNA and promote the formation of a ribosomal complex that can scan for the initiation codon to begin translation (Figure 1.2A). The mTOR and ERK pathways can regulate this step by phosphorylation of eIF4E binding proteins (4E-BPs). Under basal conditions, 4E-BPs bind and sequester eIF4E to inhibit translation. When phosphorylated, they release eIF4A allowing for the initiation of translation (Figure 1.2A).

The mTOR pathway can also regulate initiation through activation of the p70 ribosomal protein S6 kinases (S6Ks) leading to S6 and eIF4B phosphorylation, however the relevance of S6K activation in stimulating translation remains unclear (Hay and Sonenberg, 2004). The ERK pathway has also been shown to regulate translation by phosphorylation of several components of the initiation complex, including the ribosomal protein S6 and eIF4B via ribosomal S6 kinases (RSKs) as well as eIF4E via MAPK-interacting kinase (MNK) activation (Figure 1.1, 1.2A) (reviewed in (Costa-Mattioli et al., 2009)). MNK-dependent phosphorylation of eIF4E decreases

its affinity for the cap structure yet is still positively correlated with translation rates. It has been hypothesized that eIF4E phosphorylation is associated with translation of a specific subset of mRNAs as opposed to bulk translation (Costa-Mattioli et al., 2009), suggesting that the ERK pathway can regulate translation in a gene-specific manner. Both pathways can also regulate translation at the elongation step, where elongation factors (eEFs) promote the binding of an amino acid bearing transfer (t)RNA to their corresponding mRNA codons and catalyze the synthesis of polypeptide chains (Figure 1.2B). The major regulatory step for elongation is phosphorylation of elongation factor 2 (eEF2) by its kinase (eEF2K), which suppresses elongation (Mathews, 2000). RSK and S6K activation have been shown to inhibit the eEF2K, thus relieving eEF2 of this suppression and promoting elongation (Figure 1.2B).

The relative contributions of the ERK and mTOR pathways to the regulation of protein synthesis have been difficult to disentangle. Both pathways have been shown to converge on many of the same targets, and it has been suggested that they can have a synergistic effect on translation (Banko et al., 2006). Consistent with this idea, the activation of many receptors has been shown to recruit both the ERK and mTOR pathways (Rozengurt, 2007; Sengupta et al., 2010). However, in many cases, one pathway is activated preferentially over the other, demonstrating that these pathways can also diverge and operate in parallel (Clerk et al., 2006; Weng et al., 2001). Furthermore, there are many instances of cross-talk between the two pathways, both inhibitory and promoting, suggesting a complex relationship between their signaling (Mendoza et al., 2011). Thus ERK and mTOR represent parallel yet interconnected pathways for regulating protein synthesis, involving many positive and negative feedback mechanism that result in several points of convergence and divergence.

Intriguingly, a number of mutations associated with high rates of autism fall along the pathways that regulate neuronal protein synthesis (Table 1.1, Figure 1.1) (Kelleher and Bear, 2008). Tuberous sclerosis (TSC) is a neurodevelopmental disorder with a high prevalence of ASD (25-60%) caused by mutations in either the *TSC1* or *TSC2* gene (see Chapter 3) (Wiznitzer, 2004). TSC1 and 2 form a complex that inhibits the rapamycin-sensitive mTOR-raptor complex (mTORC1) and deletion of *TSC1* or *2* leads to enhanced mTORC1 activity (Kwiatkowski and Manning, 2005). An upstream regulator of mTORC1, the phosphoinositide-3 kinase (PI3K), has also been linked to ASD. Loss-of-function mutations in the *PTEN* phosphatase, a negative regulator of PI3K (Georgescu, 2010), are responsible for a family hamartoma-tumor syndromes

associated with ASD (up to 20%) (Butler et al., 2005). Neurofibromatosis 1 (NF1) is another neurodevelopmental disorder with abnormally high rates of ASD caused by mutations in the NF1 gene (Boyd et al., 2009). The NF1 gene product, neurofibromin, is an inhibitor of the small GTPase Ras, which is a critical upstream regulator of the ERK pathway (Williams et al., 2009). Several other components of the Ras-ERK pathway are also risk factors for both syndromic and idiopathic ASD (Table 1.1, Figure 1.1), suggesting that the ERK pathway may also be central to ASD pathophysiology (Betancur, 2011; Samuels et al., 2009).

The observation that many mutations in the ERK and mTOR pathways are independently associated with ASD strongly implicates these pathways in the pathogenesis of ASD. The role of these pathways in neuronal protein synthesis and synaptic plasticity suggests that defects in translational regulation may represent a shared mechanism underlying synaptic dysfunction, and consequently contribute to ASD pathophysiology. The role of neuronal translation in autism pathology is further underscored by disorders that directly impact protein synthesis rates or protein levels at the synapse (Table 1.1, Figure 1.1). Fragile X (FX), the leading inherited cause of ASD, results from the loss of the fragile X mental retardation protein (FMRP) (Pieretti et al., 1991; Verkerk et al., 1991). FMRP is an mRNA binding protein that represses translation, and exaggerated synaptic protein synthesis in the absence of FMRP is generally regarded as pathogenic in this disorder (see Chapter 2) (Garber et al., 2008). Angelman syndrome is thought to result from the loss of UBE3A (Kishino et al., 1997; Matsuura et al., 1997), an E3 ubiquitin ligase essential for the degradation of proteins in neurons and therefore likely to modify synaptic protein levels (Greer et al., 2010). Rett syndrome is caused by loss-of-function mutations in the methyl-CpG binding protein 2 (MeCP2), which can function as both a transcriptional activator and repressor, thereby bidirectionally altering neuronal mRNA and, subsequently, protein levels (Moretti and Zoghbi, 2006).

The disruptions observed in the above disorders suggest a convergence of diverse molecular triggers on a common disease-causing pathway: synaptic protein synthesis. While syndromic forms of ASD have pointed to disrupted synaptic protein synthesis in its pathophysiology, it is interesting to speculate that this dysregulation may be involved in many idiopathic forms of autism as well. The regulation of translation is exquisitely complex; thus, there are many regulatory points along this pathway that when altered could disrupt proper neuronal function. An intricate system like this may be vulnerable to an accumulation of multiple

genetic perturbations that on their own have minimal effect, similar to the multifactorial genetic etiology thought to underlie many forms of ASD.

The notion that many ASDs may result from alterations in synaptic protein synthesis is a testable hypothesis and this thesis will determine if, and how, neuronal translation is altered in two prominent mouse models of ASD, the *Fmr1* KO and *Tsc2*$^{+/-}$ mouse. Activity-dependent gene expression is essential for proper synaptic function, neural circuit performance, and cognitive and behavioral competency. If there is indeed a shared disruption of synaptic protein synthesis in these mouse models of ASD, it suggests that this dysregulation may ultimately contribute to the cognitive deficits associated with ASD, and that targeting this process may serve a potential treatment not only for FX and TSC, but also for ASD of unknown etiology.

1.4 Dissecting the role of metabotropic glutamate receptors in ASD

If altered neuronal protein synthesis is indeed a shared deficit in ASD it suggests a host of potential therapeutic targets. In fact, targeting the ERK and mTOR pathways has recently shown promise in the treatment of several ASD-associated disorders (Ehninger et al., 2008a; Li et al., 2005; Osterweil et al., 2013). However, many components of the pathways that regulate neuronal protein synthesis are ubiquitously expressed in all cell types and are important for basic processes such as cell cycling and growth (Cargnello and Roux, 2011; Tee and Blenis, 2005). Thus, global manipulation of these pathways may cause potentially severe side effects. The ideal target would be a neuronal receptor-based therapeutic that specifically modulates synaptic signaling while maintaining the proper activity-dependent properties of the synapse.

The activity-dependent translation of pre-existing mRNAs at the synapse is mediated by many signals, including growth factors and neuromodulators, acting on a variety of receptors (Steward and Schuman, 2001). As mentioned above, the two most prominent regulators of cell signaling at excitatory synapses are the NMDAR and group1 (Gp1) mGluRs, and these receptors have been strongly implicated in the regulation of synaptic protein synthesis as well (Gkogkas et al., 2010). Due to their wide expression pattern and post-natal abundance, these glutamate receptors are promising therapeutic targets for regulating synaptic protein synthesis. Gp1 mGluRs, in particular the mGluR5 subtype, is a widespread and potent regulator of local dendritic protein synthesis (Job and Eberwine, 2001; Weiler and Greenough, 1993), and mGluR-dependent translation has been show to play a role in many forms of synaptic plasticity (Pfeiffer

and Huber, 2006). There is also a well-developed class of pharmacological agents that modulate mGluR function in receptor subtype-specific and activity-dependent manners (Conn et al., 2009) (see Chapter 4). While there are many processes that can modulate synaptic protein synthesis, we propose that targeting mGluRs, specifically mGluR5 (the predominant subtype in the forebrain and hippocampus (Masu et al., 1991)), is a promising treatment strategy for ASD due to their wide-expression, amenability to drug targeting, and prominent role in regulating dendritic protein synthesis.

A particularly compelling example of a form of plasticity requiring local translation is mGluR-dependent LTD (mGluR-LTD) in the CA1 region of the hippocampus. Activation of Gp1 mGluRs, either synaptically by paired-pulse low-frequency stimulation (PP-LFS) or chemically with the selective agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG), results in a persistent decrease in synaptic strength that is mechanistically distinct from classical NMDAR-dependent LTD (Oliet et al., 1997). NMDAR-LTD can be maintained for several hours solely by post-translational modifications; in contrast, the maintenance of mGluR-LTD requires rapid protein synthesis within minutes of its induction (Hou et al., 2006; Huber et al., 2000). This protein synthesis is likely to be synaptic in nature, as mGluR-LTD can still be induced in the apical dendrites of CA1 pyramidal neurons even if they are physically severed from the cell body layer (Huber et al., 2000). mGluR-LTD is expressed, in part, by the removal of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors from synapses, which also requires rapid *de novo* translation (Snyder et al., 2001). Furthermore, activation of group 1 mGluRs rapidly stimulates protein synthesis in hippocampal slices (Osterweil et al., 2010), and specifically in dendrites and synaptoneurosomes (Job and Eberwine, 2001; Weiler and Greenough, 1993). Thus, mGluRs are not only a promising target for the modulation of synaptic protein synthesis, but mGluR-LTD is a sensitive functional read-out of dendritic translation as well. In this sense, mGluR-LTD can be used as a tool for monitoring alterations in synaptic protein synthesis levels resulting from mutations associated with ASD.

We have proposed that mGluR signaling is an avenue to both manipulate and monitor dendritic translation rates. However, several lines of evidence suggest that alterations in mGluR-dependent synaptic protein synthesis may *specifically* contribute to the pathophysiology of ASD. First, many of the mutations associated with autism fall along the pathways required for mGluR-mediated protein synthesis (Figure 1.1). Both the mTOR and ERK pathways have been shown

to couple mGluRs to translation machinery and have been implicated in the expression of mGluR-LTD (Gallagher et al., 2004; Hou and Klann, 2004). Second, a major function of the structural proteins disrupted in ASD is to anchor glutamate receptors at the synapse (Figure 1.1) (Sala et al., 2001), and deletion of these proteins has explicitly been shown to disrupt mGluR function in several contexts (Bangash et al., 2011; Baudouin et al., 2012; Ronesi and Huber, 2008; Verpelli et al., 2011; Wan et al., 2011). Third, it is well-established that exaggerated mGluR5 function is causally linked to the pathophysiology of FX (see Chapter 2) (Bear et al., 2004). Findings from the FX field of study have been particularly influential in demonstrating the importance of mGluR function in ASD and the potential of mGluR modulators for treatment (Krueger and Bear, 2011). Finally, human genetic studies have found that the protein products of many genes associated with ASD are either targets of FMRP or are embedded in the mGluR signaling pathway (Figure 1.1) (Iossifov et al., 2012; Kelleher Iii et al., 2012). In fact, a recent study has identified the gene encoding mGluR5 (*GMR5*) itself as a risk factor for ASD (Skafidas et al., 2012). Thus, mGluR-mediated protein synthesis may be a common molecular pathway disrupted in ASD, further supporting mGluR5 as a promising target for treatment.

The studies presented in this thesis are designed to dissect the nature and expanse of disrupted mGluR function in the pathophysiology of ASD. In the next chapter, we demonstrate that multiple consequences of mGluR-stimulated protein synthesis are altered in the mouse model of FX, and thus may generally contribute to the pathogenesis of FX. In Chapter 3, we show that disruptions in this pathway are not limited to FX, but that mGluR function is altered, albeit in a surprising manner, in a mouse model of TSC, another prevalent disorder associated with ASD. Results from this study suggest there is an optimal range of synaptic protein synthesis and that deviations in either direction can adversely affect neuronal function. Furthermore they suggest that opposing perturbations in synaptic function may manifest similarly in cognitive impairment and autistic traits. In the final chapter, we address the mechanisms by which mGluR activation is coupled to protein synthesis, which may elucidate novel avenues for the next generation of mGluR-based therapies for the treatment of ASD.

Gene	Function	Diagnosis	Citations
SHANK 3	Synaptic Scaffold	Phelan-McDermid syndrome	(Durand et al., 2007; Gauthier et al., 2009; Moessner et al., 2007)
SHANK 2	Synaptic Scaffold	ASD	(Berkel et al., 2010)
DLGAP2	Synaptic Scaffold	ASD	(Marshall et al., 2008)
SAPAP2	Synaptic Scaffold	ASD	(Pinto et al., 2010)
HOMER1	Synaptic Scaffold	ASD	(Kelleher Iii et al., 2012)
NLGN3	Synaptic CAM	ASD	(Jamain et al., 2003)
NLGN4	Synaptic CAM	ASD	(Jamain et al., 2003; Laumonnier et al., 2004; Lawson-Yuen et al., 2008)
NRXN1	Synaptic CAM	ASD	(Kim et al., 2008; Zahir et al., 2008)
NRXN2	Synaptic CAM	ASD	(Glessner et al., 2009)
CNTNAP2	Synaptic CAM	ASD	(Alarcon et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Zweier et al., 2009)
CNTN4	Synaptic CAM	ASD	(Glessner et al., 2009; Morrow et al., 2008; Roohi et al., 2009)
TSC1	mTOR/PI3K pathway	Tuberous Sclerosis	(Wiznitzer, 2004)
TSC2	mTOR/PI3K pathway	Tuberous Sclerosis	(Wiznitzer, 2004)
PTEN	mTOR/PI3K pathway	Cowden Syndrome	(Butler et al., 2005)
NF1	Ras/ERK pathway	Neurofibromatosis type 1	(Rosser and Packer, 2003)
SYNGAP1	Ras/ERK pathway	ASD	(Pinto et al., 2010)
HRAS	Ras/ERK pathway	Costello syndrome	(Kerr et al., 2006)
RAF1	Ras/ERK pathway	Noonan syndrome	(Narumi et al., 2007)
MEK1/2	Ras/ERK pathway	Costello syndrome, Noonan syndrome	(Kerr et al., 2006; Narumi et al., 2007)
FMR1	Translation repressor	Fragile X Syndrome	(Garber et al., 2008)
MECP2	Chromatin Remodeling	Rett Syndrome	(Amir et al., 1999)
UBE3A	Ubiquitination	Angelman Syndrome	(Glessner et al., 2009)
MEF2C	Transcription factor	ASD	(Le Meur et al., 2010)
RFWD2	Ubiquitination	ASD	(Glessner et al., 2009)

Table 1.1 – Genes associated with high risk for ASD. Validated genes with highly penetrant (although rare) mutations associated with ASD (Toro et al., 2010). This includes many syndromic forms of ASD. Orange corresponds to genes involved in synaptic structure and formation, blue corresponds to genes involved in synaptic signaling, and yellow corresponds to genes involved with regulation of mRNA and protein abundance (see Figure 1.1).

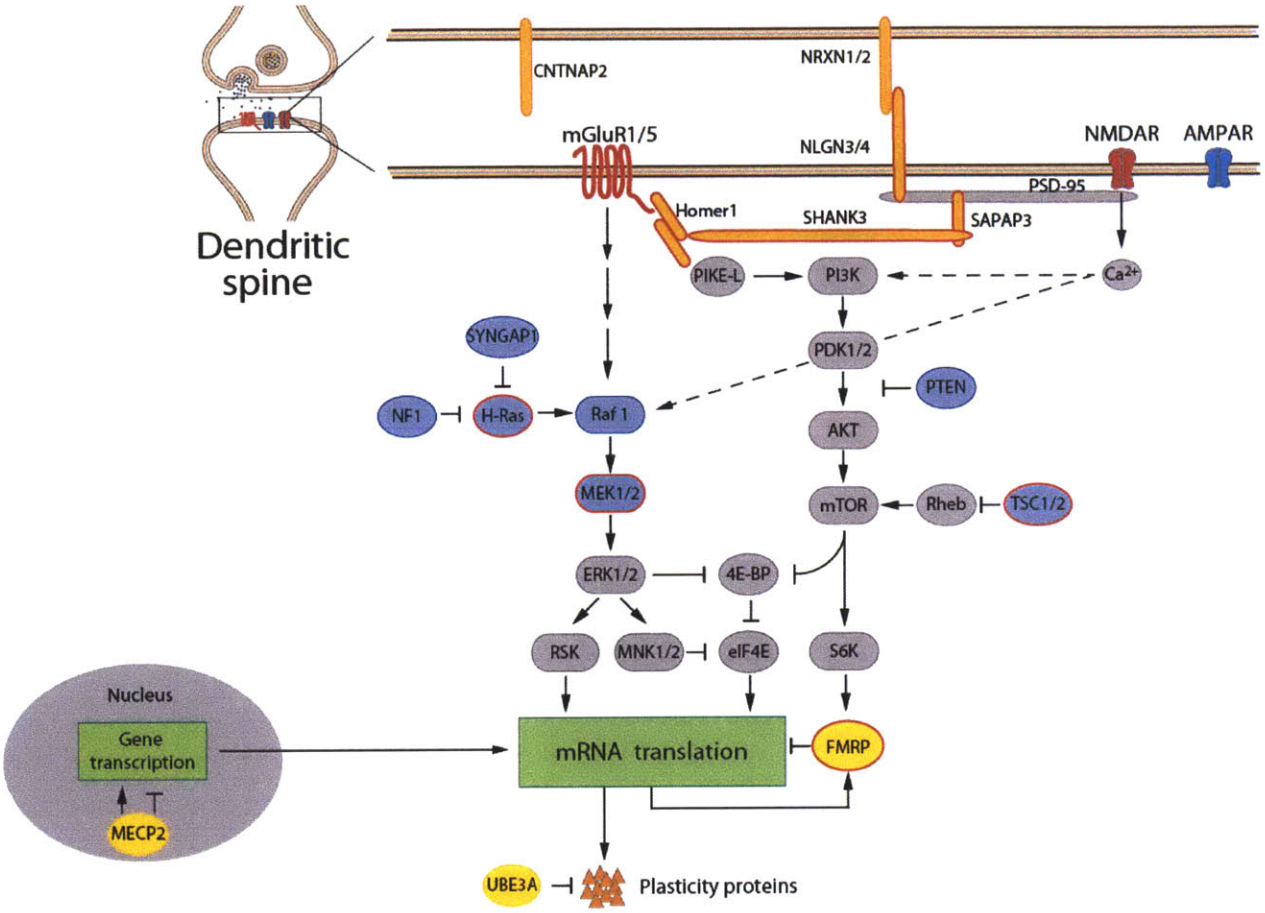

Figure 1.1 – Highly penetrant mutations associated with autism converge at the synapse. Recent genetic evidence suggests that many genes associated with ASD are involved in the regulation of synaptic function, and in particular, the regulation of local mRNA translation at the synapse. While not exhaustive, the function of these genes can be classified into several groups: synaptic structural and cell adhesion molecules (orange), synaptic signaling molecules (blue), or molecules involved in regulating mRNA and protein abundance (yellow). Molecules outlined in red have been shown to specifically affect mGluR function. See Table 1.1 for more details. Abbreviations: 4E-BP, eIF4E binding protein; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Ca^{2+}, intracellular calcium; eIF4E, eukaryotic initiation factor 4E; ERK1/2, extracellular signal-regulated kinase 1/2; FMRP, fragile X mental retardation protein; MeCP2, methyl-CpG binding protein 2; MEK, Mitogen-activated protein kinase kinase; mGluR, metabotropic glutamate receptor; MNK1/2, MAP kinase-interacting kinase 1/2; mTOR mammalian target of rapamycin, NF1, neurofibromatosis 1; NLGN neuroligin; NMDAR, *N*-methyl-D-aspartate receptor; NRXN, neurexin; PI3K, phosphatidylinositide 3-kinase, PIKE-L, PI3K enhancer long isoform; PSD-95, postsynaptic density protein 95; PKD1/2, phosphoinositide-dependent kinase 1/2; PTEN, phosphatase and tensin homolog; RSK, ribosomal s6 kinase; SAPAP3, SAP90/PSD-95-associated protein; SHANK, SH3 and multiple ankyrin repeat domains protein; SYNGAP, synaptic Ras GTPase-activating protein; TSC, tuberous sclerosis complex; UBE3A, Ubiquitin-protein ligase E3A.

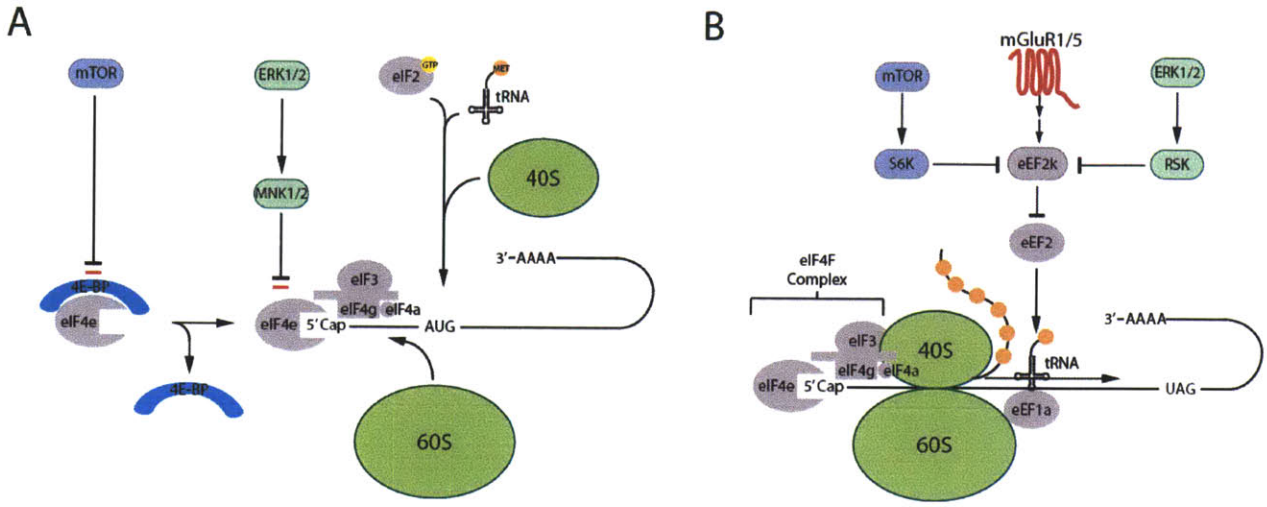

Figure 1.2 – General and gene-specific mechanisms for translation initiation and elongation. (A) Formation of the eIF4F initiation complex requires eIF4E binding to the 5' cap of an mRNA, which then recruits the large 60S ribosomal subunit through an interaction with eIF4G. 4E-BP sequesters eIF4E, preventing formation of the initiation complex. mTOR phosphorylates 4E-BP, decreasing its affinity for eIF4E, and thereby promoting formation of the initiation complex and translation. The ERK pathway can directly phosphorylate eIF4E via its downstream effector MNK, which may decrease general translation rates but enhance translation of specific mRNA (Costa-Mattioli et al., 2009). eIF2 associates with the small 40S ribosomal subunit in its GTP-bound form and the initiator methionyl-tRNA (Met-tRNA), forming a ternary complex that can recognize the AUG initiation codon and begin translation. Phosphorylation of the α subunit of eIF2 inhibits this formation and causes a decrease in general translation initiation, but once again may increase translation of some mRNA. While cap-dependent initiation is considered the general way that translation is mediated, this gene-specific translation may be mediated by initiation via upstream open reading frames (uORFs) on the 5' untranslated region (UTR) or internal ribosome entry sequences (IRES) (see Chapter 3) (Costa-Mattioli et al., 2009). **(B)** Once translation is initiated, polypeptide elongation is promoted by the elongation factors eEF1 and eEF2. eEF1α is required for peptide (orange circle) containing tRNA entry into the ribosome. eEF2 catalyzes the translocation of the ribosome on the mRNA after peptide bond formation. The major mechanism for regulating elongation is phosphorylation of eEF2 by its kinase (eEF2K), which results in a decrease in general translation. Both the mTOR and ERK pathways have been implicated in regulating elongation by inhibiting eEF2K, however the relationship is complex (Mathews, 2000). Interestingly, mGluR activation has been shown to activate eEF2K, potentially through its interaction with Homer, and suppress general elongation while enhancing translation of a specific subset of mRNA (see Chapter 3) (Park et al., 2008). Upon recognition of the UAG stop codon, termination factors promote release of the polypeptide chain from the mRNA and ribosome. This entire process is typically performed on mass scale, with multiple ribosomes rapidly and repeatedly translating an mRNA simultaneously in polyribosomal complex.

Chapter 2

Multiple facets of metabotropic glutamate receptor-mediated protein synthesis are disrupted in the mouse model of Fragile X

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2.1 Abstract

Fragile X (FX), the most common inherited form of intellectual disability and ASD, is caused by the loss of the fragile X mental retardation protein (FMRP). FMRP is a negative regulator of local mRNA translation downstream of group 1 metabotropic glutamate receptor (Gp1 mGluR) activation. In the absence of FMRP there is excessive mGluR-dependent protein synthesis, resulting in exaggerated long-term synaptic depression (LTD) in area CA1 of the hippocampus. Understanding disease pathophysiology is critical for development of therapies for FX, and the question arises of whether it is more appropriate to target excessive LTD or excessive mGluR-dependent protein synthesis. Priming of long-term potentiation (LTP) is a qualitatively different functional consequence of Gp1 mGluR-stimulated protein synthesis at the same population of CA1 synapses where LTD can be induced. Therefore, we determined if LTP priming, like LTD, is also disrupted in the *Fmr1* KO mouse. We found that mGluR-dependent priming of LTP is of comparable magnitude in WT and *Fmr1* KO mice. However, while LTP priming requires acute stimulation of protein synthesis in WT mice, it is no longer protein synthesis dependent in the *Fmr1* KO. These experiments show that the dysregulation of mGluR-mediated protein synthesis seen in *Fmr*1 KO mice has multiple consequences on synaptic plasticity, even within the same population of synapses. Furthermore, it suggests that there is a bifurcation in the Gp1 mGluR signaling pathway, with one arm triggering synaptic modifications such as LTP priming and LTD, and the other stimulating protein synthesis that is permissive for these modifications.

2.2 Introduction

Fragile X (FX) is the most common inherited form of intellectual disability (ID) and the leading known genetic cause of autism (Garber et al., 2008). A third of FX individuals are diagnosed with autism, accounting for 2-5% of the autistic population (Garber et al., 2008). The disease is typically caused by expansion of a CGG triplet repeat sequence upstream of the *FMR1* gene that results in transcriptional silencing and consequent loss of the fragile X mental retardation protein, FMRP (Garber et al., 2008; Pieretti et al., 1991). A key advance in our understanding of FX was the isolation of the *FMR1* gene and subsequent generation of the *Fmr1* knockout (KO) mouse (1994). This mouse model has been cardinal to our understanding of the molecular mechanisms of FX and the function of FMRP in the brain. Studies of the *Fmr1* KO mouse have demonstrated the role of aberrant synaptic protein synthesis in the pathophysiology of FX, and have specifically implicated disrupted mGluR-mediated plasticity and protein synthesis. Furthermore, these studies have highlighted the potential for modulation of mGluR5 signaling in the treatment of FX and, potentially, other autism-related disorders.

2.2.1 Regulation of protein synthesis by FMRP

Several lines of evidence suggest a role for FMRP in the regulation of synaptic protein synthesis. First, FMRP is an RNA-binding protein and a repressor of translation. FMRP associates with mRNAs through one of three RNA-binding domains (Ashley et al., 1993; Siomi et al., 1993), in some cases in conjunction with adaptor proteins (El Fatimy et al., 2012; Napoli et al., 2008). There is evidence that FMRP can repress translation both by blocking initiation and elongation (Bhakar et al., 2012; Santoro et al., 2012). Second, FMRP has been shown to associate with polyribosomes throughout the neuron, including dendrites and spines (Antar et al., 2004; Feng et al., 1997), and many mRNA targets of FMRP are dendritically localized and are important for the regulation of synaptic plasticity and dendritic structure (Brown et al., 2001; Feng et al., 1997; Khandjian et al., 2004; Laggerbauer et al., 2001; Lu et al., 2004; Todd et al., 2003; Weiler et al., 2004; Zalfa et al., 2003). Third, basal protein synthesis rates are significantly elevated in the brains of *Fmr1* KO mice (Dolen et al., 2007; Osterweil et al., 2010; Qin et al., 2005). Moreover, synaptoneurosomes obtained from *Fmr1* KO brains have increased protein levels of many FMRP targets, such as Map1b, CaMKIIα and Arc, as well as higher levels of these mRNAs in polyribosomal fractions. This suggests that the excess protein synthesis is

synaptic in nature (Zalfa et al., 2003). Finally, FMRP is required for the proper expression of synaptic plasticity that requires local synaptic translation, specifically downstream of mGluR activation.

Activation of group 1 (Gp1) mGluRs has been shown to trigger dendritic mRNA translation, including the synthesis of FMRP (Weiler and Greenough, 1993, 1999). In the hippocampus, one functional consequence of activating Gp1 mGluRs is induction of long-term synaptic depression (LTD) at the Schaffer collateral-CA1 synapse (Huber et al., 2001; Oliet et al., 1997; Palmer et al., 1997), expressed in part by a loss of postsynaptic AMPA-type glutamate receptors (Snyder et al., 2001; Waung et al., 2008) (see Chapter 1). In wild-type (WT) rats and mice, mGluR-stimulated protein synthesis is obligatory for stable expression of LTD (Huber et al., 2000; Huber et al., 2001). This led to the investigation of mGluR-LTD in *Fmr1* KO mice, under the assumption that FMRP was important for the expression of LTD. Surprisingly, LTD magnitude is enhanced rather than deficient in the *Fmr1* KO mouse, possibly due to exaggerated protein synthesis (Huber et al., 2002). Consistent with this interpretation, mGluR-LTD in the *Fmr1* KO mouse also no longer requires acute stimulation of protein synthesis, presumably due to constitutive overexpression of "LTD proteins" (Hou et al., 2006; Nosyreva and Huber, 2006).

2.2.2 The mGluR theory of fragile X

Understanding disease pathophysiology is critical for development of therapies for the treatment of FX. The above studies suggest that mGluRs and FMRP normally act in functional opposition to maintain an optimal level of synaptic protein synthesis – mGluR activation drives protein synthesis and FMRP represses this protein synthesis (Figure 2.1A) (Bear et al., 2004). In the absence of FMRP, mGluR-dependent protein synthesis is left unchecked, resulting in runaway protein synthesis and exaggerated LTD. One way to correct for this is to decrease mGluR5 activity. Indeed, either pharmacologically or genetically reduction of mGluR can reverse both the excessive mGluR-LTD (Figure 2.1B) and protein synthesis in *Fmr1* KO mice. As LTD mechanisms are believed to be important for sculpting synaptic connections during postnatal development, a reasonable conjecture is that exaggerated LTD could be pathogenic in FX (Huber et al., 2002). Moreover, increased LTD in hippocampal area CA1 could contribute specifically to the cognitive impairment that is characteristic of this disease. Thus, targeting

impaired synaptic efficacy (e.g. by modulating AMPAR function) has been suggested as a potential therapy for FX (Lynch et al., 2008).

While mGluR-LTD is one of the most-well characterized Gp1 mGluR-mediated processes in the hippocampus, their activation can have a myriad of cellular and synaptic effects. These include changes in excitability, synaptic structure, and maintenance of plasticity (Francesconi et al., 2004; Lee et al., 2002; Neyman and Manahan-Vaughan, 2008; Vanderklish and Edelman, 2002). Importantly, many of these changes are dependent upon rapid, *de novo* protein synthesis (Merlin, 1998; Raymond et al., 2000). It is also true that the FX phenotype is multifaceted. Despite its simple genetic origin, the disorder consists of a remarkably diverse set of behavioral and neurological symptoms, including delayed cognitive development, seizures, anxiety, movement disorders, and altered dendritic structure (Reiss and Hall, 2007).

Therefore, it is possible that exaggeration of the multiple consequences of mGluR-mediated protein synthesis may contribute to the many diverse characteristics of FX. It has now been shown that decreasing mGluR5 activity not only reverses the excessive mGluR-LTD and protein synthesis in *Fmr1* KO mice, but also a constellation of phenotypes associated with FX, suggesting these synaptic impairments are a causal factor in the pathophysiology of FX (Dolen et al., 2007). Furthermore, the fact that many of the behavioral and synaptic dysfunctions can be reversed acutely by pharmacological antagonism of mGluR5 has important clinical implications (McBride et al., 2005; Michalon et al., 2012). In fact, preliminary human clinical trials using drugs that inhibit mGluR5 have shown promise in the treatment of some of the symptoms associated with FX (Hagerman et al., 2012). The question arises then of whether it is more appropriate to target excessive LTD and impaired AMPA receptor function (Lynch et al., 2008), or excessive mGluR-dependent protein synthesis (Bear, 2005; Bear et al., 2004).

2.2.3 mGluR-dependent priming of LTP

The phenomenon of LTP priming, first described by Abraham and colleagues in rats (Cohen and Abraham, 1996; Cohen et al., 1998; Raymond et al., 2000), offers an interesting opportunity to distinguish between the two above alternatives. Normally, weak high-frequency stimulation (HFS) elicits modest long-term synaptic potentiation (LTP) at the Schaffer collateral-CA1 synapse. However, if Gp1 mGluRs are first stimulated briefly with a low concentration of the selective agonist R,S-dihydroxyphenylglycine (DHPG; 10 μM), then the LTP produced by

subsequent HFS is augmented substantially. Like mGluR-LTD induced by higher DHPG concentrations, LTP priming in WT rats is abolished by inhibitors of mRNA translation, but not by inhibitors of transcription. Thus, LTD and LTP priming are qualitatively different functional consequences of Gp1 mGluR-stimulated protein synthesis at the Schaffer collateral-CA1 synapse. In the current study we ask if LTP priming, like LTD, is also disrupted in the *Fmr1* KO mouse.

We find, first, that priming of LTP results from weak activation of Gp1 mGluRs with DHPG in mouse CA1, as previously reported in rats. Second, although the effect of LTP priming is quantitatively similar in *Fmr1* KO and WT mice, it is blocked by a protein synthesis inhibitor only in WT animals. These findings suggest that proteins overexpressed in FX are not restricted to "LTD proteins", as they apparently include those required for LTP priming as well. These findings also indicate that there is a post-translational component of mGluR-dependent LTP priming. Instead of serving as a trigger for LTP priming (or LTD), dendritic protein synthesis may rather serve as a gate for synaptic plasticity that normally opens only in response to an mGluR-signaling event. In fragile X, it appears that this gate is perpetually open due to excessive basal protein synthesis and overexpression of proteins that are normally rate-limiting for these forms of synaptic modification.

2.3 Results

In order to confirm that there is facilitation of LTP by prior Gp1 mGluR activation in mice (as has been demonstrated in rats), we first established a tetanization protocol that produced a sub-saturable level of LTP and has been shown to be amenable to priming (Cohen et al., 1998; Mellentin et al., 2007). Brief HFS (1-s 100-Hz) produced a modest but reliable level of LTP one hour post tetanus in slices from both WT and *Fmr*1 KO mice (WT: 111.2 ± 2.1%, n = 9; KO: 113.8 ± 3.1%, n = 9; Figure 2.2). As has been reported previously, there was no significant difference in the basal level of hippocampal LTP in *Fmr*1 KO compared to WT mice ($p = 0.51$) (Godfraind et al., 1996; Paradee et al., 1999).

We then replicated the previously reported mGluR-dependent priming of LTP in slices obtained from WT mice (Cohen et al., 1998; Mellentin et al., 2007). The Gp1 mGluR agonist DHPG (10 μM) was bath applied to slices for 10 minutes after a stable 20 minute baseline

recording period. DHPG application produced a transient depression of synaptic responses that recovered to baseline levels after a 30 minute washout. The same tetanus protocol as above (1-s, 100-Hz) now produced a significantly larger magnitude of LTP compared to unprimed slices (unprimed: 111.2 ± 2.1%, n = 9; primed: 123.9 ± 3.8%, n = 10; p = 0.012; Figure 2.2A). These findings in mice are consistent with those previously reported in rats (Cohen et al., 1998; Mellentin et al., 2007).

We next characterized the effect of DHPG application on subsequent LTP in *Fmr*1 KO mice. As was the case in WT animals, the DHPG priming protocol also enhanced LTP in slices from *Fmr*1 KO mice (unprimed: 113.8 ± 3.1%, n = 9; primed: 133.7 ± 6.7%, n = 11; p = 0.016; Figure 2.2B). However, there was no significant difference in the magnitude of facilitation seen in primed *Fmr*1 KO slices as compared to primed WT slices (p = 0.22).

Finally, we examined the role of protein synthesis in DHPG induced priming in both WT and *Fmr*1 KO mice. As expected (Raymond et al., 2000), a brief application of the protein synthesis inhibitor cycloheximide (CHX; 60 μM, 30 minutes) completely abolished DHPG induced priming in WT slices (unprimed: 117.5 ± 7.0%, n = 7; primed: 118.6 ± 6.0%, n = 9; p = 0.94; Figure 2.3A). However, this same treatment had no effect on DHPG induced priming in slices from *Fmr*1 KO mice (unprimed: 118.5 ± 7.0%, n = 7; primed: 149.6 ± 11.0%, n = 8; p = 0.035; Figure 2.3B). These results show that while the magnitude of LTP enhancement induced by DHPG priming is not quantitatively different in *Fmr*1 KO mice, induction of priming is qualitatively different in that it no longer requires the synthesis of new proteins (Figure 2.4).

2.4 Discussion

In this study we characterized mGluR-dependent priming of LTP in the FX background. In WT mice we confirmed previous reports that brief application of the Gp1 mGluR agonist DHPG at a low dose enhances the magnitude of subsequent LTP, and that this priming of LTP is protein synthesis dependent. In the *Fmr1* KO we determined that although mGluR-dependent priming of LTP is not significantly enhanced as compared to WT, it no longer requires acute protein synthesis at the time of induction. These experiments provide valuable insight into the nature of the underlying pathophysiology of FX, as well as provide details for the mechanisms of LTP priming.

The results of this study are threefold: (1) The proteins downstream of mGluR activation that are regulated by FMRP are not limited to LTD proteins but rather are able to mediate bidirectional changes in synaptic strength, as the removal of FMRP affects not only LTD but also LTP; (2) mGluR-stimulated protein synthesis can be decoupled from mGluR-mediated plasticity because mGluR activation is still necessary to trigger LTP priming in the *Fmr1* KO even though it no longer requires *de novo* protein synthesis; and (3) The dysregulation of mGluR-mediated protein synthesis seen in *Fmr*1 KO mice has multiple consequences on synaptic plasticity, even within the same population of CA1 synapses, suggesting that alterations in many mGluR-mediated processes may contribute to the FX phenotype, not just LTD.

2.4.1 mGluR-dependent translation regulates proteins required for both LTD and LTP

Although our goal was to determine if the impact of excessive protein synthesis in area CA1 of *Fmr1* KO mice is exclusive to mGluR-LTD, the results also have implications for the mechanisms of LTP priming. There have been several studies exploring the induction mechanisms of mGluR induced priming (Cohen et al., 1999; Cohen et al., 1998; Mellentin et al., 2007), yet little is known about how the facilitation is achieved. While priming depends on availability of newly synthesized proteins, the exact identity of the proteins required has not been explored. *Fmr*1 KO mice may prove to be a valuable tool in this regard.

The fact that bidirectional changes in synaptic plasticity are altered in Fmr1 KO mice suggests two possibilities for the nature of FMRP targets. First, FMRP may control the synthesis of many proteins, some of which are required for LTD and others that are necessary for LTP maintenance. This is supported by the notion that FMRP regulates the synthesis of hundreds of proteins, many of which are involved in synaptic plasticity (Darnell et al., 2011). It is interesting to note that some proteins that are traditionally thought to be important in LTP but not LTD, such as CaMKII, are targets of FMRP (but see (Mockett et al., 2011)). The fact that priming no longer requires protein synthesis in the *Fmr*1 KO mouse suggests that the proteins required for the priming effect are already present. It may be possible to identify candidate proteins that are necessary for priming by comparing their basal expression levels in *Fmr*1 KO mice with basal and primed protein levels in WT controls.

Alternatively, there may be a common set of proteins that gate bidirectional changes in synaptic strength mediated by mGluRs. Conceptually, these plasticity gatekeepers could be

proteins involved in AMPAR cycling. It may be that these proteins are required to stabilize a certain cycling pattern, and whether this is removal or insertion of AMPARs depends on the activity or signals present at a particular synapse. Although speculative at this point, the protein Arc is an interesting candidate gating molecule. It is normally expressed at low levels, but can be rapidly synthesized in response to Gp1 mGluR activation (Park et al., 2008; Waung et al., 2008) and has been reported to be overexpressed in the *Fmr1* KO (Zalfa et al., 2003). It is known to interact with the molecular machinery responsible for AMPA receptor cycling through the synaptic membrane (Chowdhury et al., 2006; Shepherd et al., 2006) and has been implicated in both LTP and LTD (Park et al., 2008; Plath et al., 2006; Waung et al., 2008). Finally, the absence of Arc renders synapses virtually immutable by experience or deprivation, at least in visual cortex (McCurry et al.).

2.4.2 Loss of FMRP decouples mGluR-dependent priming of LTP from protein synthesis

The simplest model for priming is that Gp1 mGluR signaling facilitates subsequent LTP induction by directly stimulating protein synthesis. However, priming and protein synthesis are decoupled in the *Fmr1* KO. Priming still results from mGluR activation in the KO (Figure 2.2), but via a mechanism that operates without acute stimulation of protein synthesis above basal levels (Figure 2.3). This finding suggests a model, illustrated in Figure 2.5, in which signaling from Gp1 mGluRs bifurcates, with one arm triggering priming and the other stimulating protein synthesis that is permissive for synaptic modifications, including LTP priming and LTD. In WT mice and rats, the protein synthesis "gate" is closed under basal conditions so no priming (or LTD) is possible without concurrent mGluR stimulation of mRNA translation. In the KO, however, increased basal protein synthesis leaves the gate open so that the varied consequences of mGluR activation are determined solely by post-translational modifications. The fact that the priming trigger can be dissociated from the protein synthesis gate in *Fmr*1 KO mice could be exploited in future studies to distinguish these bifurcating pathways, as only interventions that disrupt the trigger pathway would be effective at blocking LTP priming in these mice. Thus, in addition to serving as a valuable disease model, *Fmr*1 KO mice are also useful for dissecting the diverse mechanisms of Gp1 mGluR signaling and for understanding the role of mGluRs in normal brain function.

It is interesting to note that while priming and LTD share the requirement for rapid protein synthesis, it seems their induction mechanisms differ. mGluR-LTD may not require canonical G_q-dependent signaling, as it is not sensitive to inhibitors of PLCβ or PKC and does not require intracellular Ca^{2+} increases (Gladding et al., 2009; Schnabel et al., 1999) (see Chapter 4). Conversely, mGluR-dependent priming of LTP both requires PLCβ activation and intracellular Ca^{2+} release from ryanodine receptors (Cohen et al., 1998; Mellentin et al., 2007). Thus, mechanistically distinct forms of mGluR-mediated plasticity can be evoked at the same synapses, and the direction of plasticity may depend on the type of signal received. It has previously been shown that there is a wide time-window (up to three hours) in which the priming effect can be achieved (Raymond et al., 2000). mGluR-stimulated protein synthesis might increase the general capacity of synapses to undergo bidirectional changes in synaptic strength. It could be that strong mGluR activation preferentially evokes LTD and stimulates the local synthesis of plasticity proteins, while weaker activation may preferentially induce mechanisms required for LTP priming. The result would be a rearrangement in the inputs that a cell preferentially responds to, which may contribute to some forms of learning (Clem and Huganir, 2010; Xu et al., 2009).

2.4.3 Implications for the pathophysiology of fragile X

To develop appropriate therapies for FX it is important to understand the exact nature of its pathophysiology. Specifically, the question arises of whether it is more appropriate to target excessive LTD or excessive mGluR-dependent protein synthesis. Previous work from our lab has shown that genetic or pharmacological reduction of mGluR5 expression corrects a wide variety of phenotypes examined in these mice (Dolen et al., 2007; Michalon et al., 2012). In this regard, it is remarkable that simply reducing the activity of one protein can have such a profound effect on the constellation of symptoms associated with FX. However, Gp1 mGluR activation has been shown to have a myriad of cellular and synaptic effects, many which require *de novo* protein synthesis, and there is an intriguing similarity between many of the symptoms observed in FX and the processes regulated by mGluR dependent protein synthesis. Here we demonstrated a novel phenotype in *Fmr1* KO mice which suggests that the dysregulation of mGluR-dependent protein synthesis is central to the pathogenesis of FX.

Altered regulation of mGluR-dependent priming of LTP may contribute to the cognitive impairment seen in FX, which is one of the defining characteristics of the syndrome. Recent studies have shown that competition for translational machinery or newly synthesized proteins may be a limiting factor for the number of synapses that can undergo long lasting-changes in synaptic efficacy (Fonseca et al., 2004; Govindarajan et al., 2011). This competition may serve as an important checkpoint, so that only a subset of synapses undergo stabilization. While the ability to have long-lasting changes in synaptic efficacy is undoubtedly beneficial, it may be possible to have too much of a good thing. FX could be a case in point, where extraneous synapses are maintained, contributing to the cognitive deficits seen in the disorder.

The mGluR theory of fragile X posits that exaggerated responses to Gp1 mGluR activation are responsible for multiple aspects of the disease phenotype (Bear et al., 2004). A key assumption is that FMRP negatively regulates varied responses triggered by mGluR-stimulated protein synthesis. Findings that mGluR-dependent LTP priming in hippocampal area CA1, epileptogenesis in CA3 (Chuang et al., 2005), and LTD in both CA1 (Huber et al., 2002; Nosyreva and Huber, 2006) and cerebellum (Koekkoek et al., 2005) are all dysregulated in the *Fmr1* KO mouse provide considerable support for this proposal.

2.5 Methods

2.5.1 Animals

*Fmr*1 mutant mice (Jackson Labs) were bred on the C57Bl/6J clonal background. In an effort to reduce variability due to rearing conditions, all experimental animals were bred from *Fmr*1 heterozygote mothers, group housed (animals weaned to solitary housing were excluded), and maintained on a 12:12 hr. light:dark cycle. The Institutional Animal Care and Use Committee at MIT approved all experimental techniques.

2.5.2 Hippocampal slice preparation

Transverse hippocampal slices (350 μm thick) were prepared from 6-10 week old mice in ice-cold dissection buffer containing (in mM): NaCl 87, Sucrose 75, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 0.5, $MgCl_2$ 7, Ascorbic acid 1.3, and D-glucose 10 (saturated with 95% O2/5% CO2). Immediately following slicing the CA3 region was removed. Slices were recovered in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 3.5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$ 2, $MgCl_2$ 1, and D-glucose 10 (saturated with 95% O2/5% CO2) at room temperature for at least 3 h prior to recording.

2.5.3 Electrophysiology

Field recordings were performed in a submersion chamber, perfused with ACSF (2-3 ml/min) at 30 °C. Field excitatory postsynaptic potentials (fEPSPs) were recorded in CA1 *stratum radiatum* with extracellular recording electrodes filled with ACSF. Baseline responses were evoked by stimulation of the Schaffer collaterals at 0.033 Hz with a 2-contact cluster electrode (FHC) using a 0.2 ms stimulus yielding 40-60% of the maximal response. Priming was induced by applying 10 μM DHPG for 10 minutes (Mellentin et al., 2007). Pairs of primed and unprimed slices were recorded simultaneously. LTP was induced with a 1-s 100-Hz tetanus. Protein synthesis was inhibited by applying 60 μM cycloheximide (CHX) for 30 minutes as follows: 15 minutes of pretreatment during baseline recording, 10 minutes during DHPG application, and 5 minutes post DHPG application; or during the equivalent time of baseline recording in unprimed slices.

fEPSP recordings were filtered at 0.1 Hz - 1 kHz, digitized at 10 kHz, and analyzed using pClamp9 (Axon Instruments). The initial slope of the response was used to assess changes in synaptic strength. Data were normalized to the baseline response and are presented as group means ± SEM. LTP was measured by comparing the average response 55-60 minutes post tetanus to the average of the last 5 minutes of baseline. ANOVA and unpaired t-tests were used to determine statistically significant differences, unless otherwise specified. Experiments used aged-matched and interleaved WT and *Fmr*1 KO mice. For all experiments the experimenter was blind to genotype.

2.5.4 Reagents

R,S-DHPG was purchased from Tocris Biosciences (Ellisville, MO). All other reagents were purchased from Sigma (St. Louis, MO). Fresh bottles of DHPG were prepared as a 100x stock in H_20, aliquoted, and stored at -80°C. Fresh stocks were made once a week. CHX was prepared at 100x stock in H_20 daily. These stocks were diluted in ACSF to achieve final concentration.

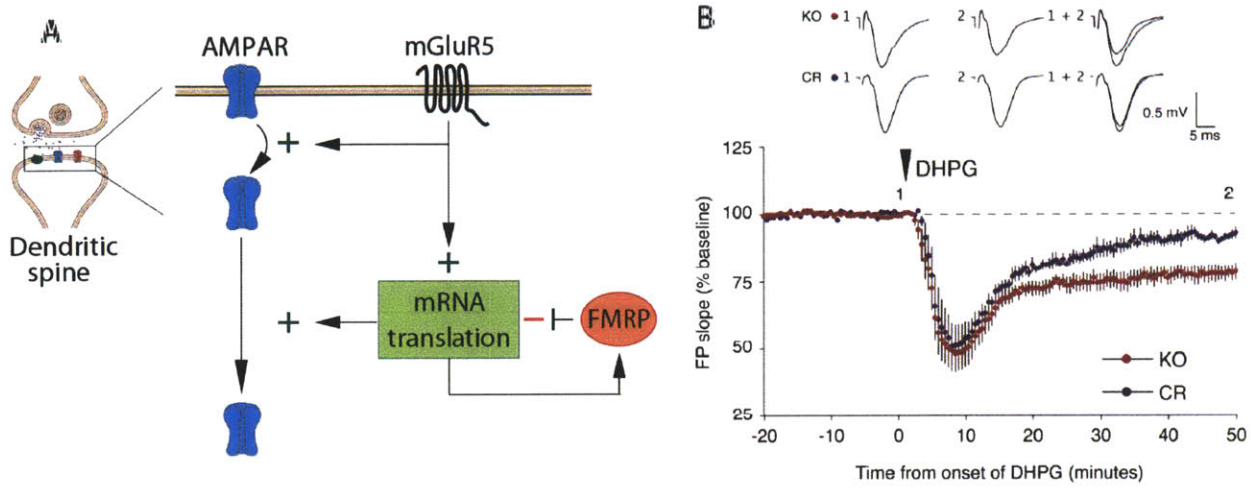

Figure 2.1 – The mGluR theory of fragile X. (A) FMRP and mGluR5 impose opposite regulation on the local mRNA translation required for mGluR-LTD expression. In the absence of FMRP, there is excessive protein synthesis and exaggerated LTD. One way this can be ameliorated is by decreasing mGluR5 activity. **(B)** Significant effect of mGluR5 gene dosage on LTD in Fmr1 KO mice. 50 μM DHPG was applied for 5 min (arrow) to slices from Fmr1 KO (KO) and $Grm5^{+/-}$ x Fmr1 KO (CR) animals (n = 14 and 11 slices from 6 and 5 mice for KO and CR, respectively). Average field EPSP slopes 45-50 min after DHPG were significantly different from 5 min averages immediately prior to DHPG in both genotypes (% baseline: KO = 78.1 ± 3.1 %, P = 0.000005; CR = 91.6 ± 1.4 %, P = 0.00007; paired t-test). However, reduction of *Grm5* gene dosage by 50% significantly decreases the magnitude of LTD in CR relative to Fmr1 KO mice (n = 5 and 6 animals, respectively, P = 0.0058, unpaired t-test). Representative field potential traces (averages of 10 sweeps) were taken at times indicated by numerals. Parts of this figure are previously published in (Dolen et al., 2007).

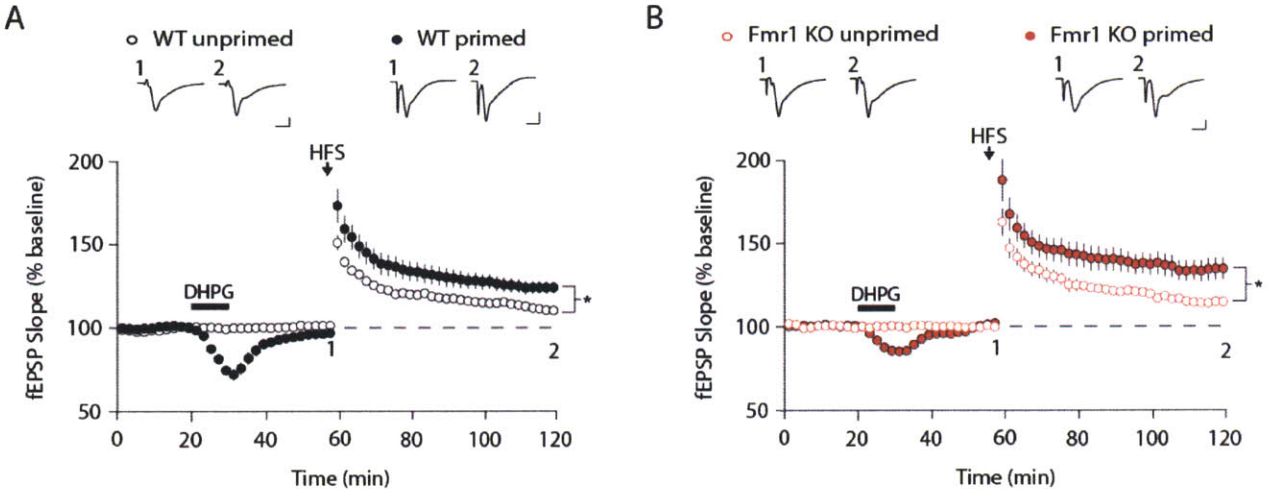

Figure 2.2 – DHPG application facilitates subsequently induced LTP. fEPSPs were recorded from the CA1 region of hippocampal slices from either **(A)** wild-type (WT) or **(B)** *Fmr*1 KO mice. After 1 hour of baseline recording, unprimed slices were administered a 1-s 100-Hz tetanus (indicated by arrow) which induced a modest level of LTP in both WT and KO slices (WT: 111.2 ± 2.1%, n = 9 slices from 9 animals, open black circles; KO: 113.8 ± 3.1%, n = 9 slices from 8 animals, open red circles). In both genotypes, a 10 minute priming application of the Gp1 mGluR agonist DHPG (10 μM, black bar) significantly enhanced the magnitude of subsequent LTP induced using this same 100-Hz tetanus (WT: 123.9 ± 3.8%, n = 11 slices from 11 animals, closed black circles, $p < 0.02$; KO: 133.7 ± 6.7%, n = 11 slices from 10 animals, closed red circles, $p < 0.02$). Representative field potential traces (average of 10 sweeps) were taken at times indicated by numerals. Scale bars equal 0.5 mV, 5 ms. Error bars represent SEM.

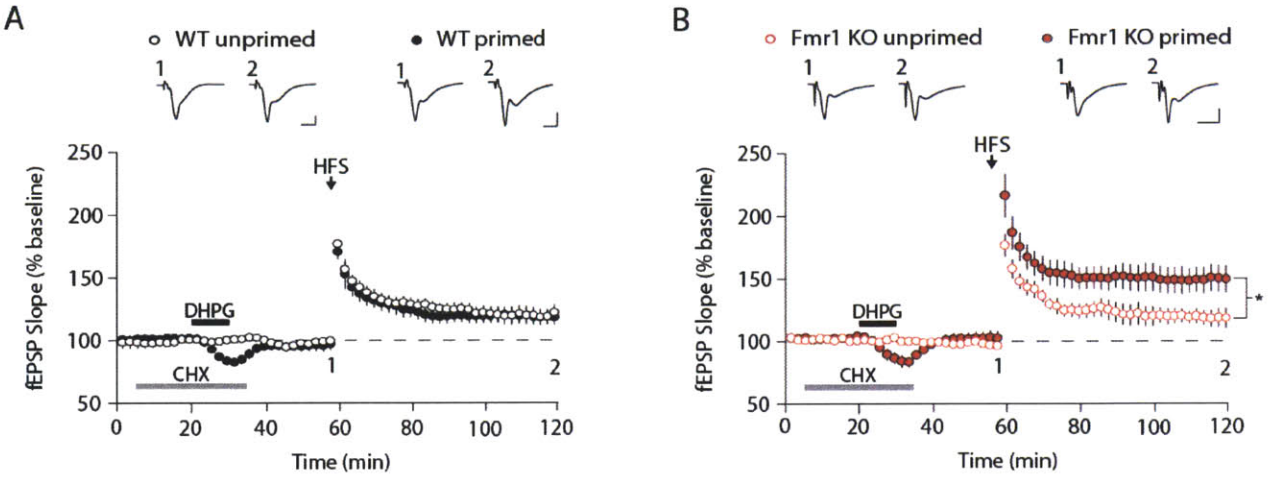

Figure 2.3 – DHPG-induced priming of LTP does not require protein synthesis in *Fmr*1 KO mice. Delivery of the protein synthesis inhibitor cycloheximide (60 μM, 30 min; CHX, gray bar) before and during DHPG priming prevented facilitation of LTP in slices from wildtype mice (**A**; unprimed: 117.5 ± 7.0%, n = 7 slices from 6 animals, open black circles; primed: 118.6 ± 6.0%, n = 8 slices from 6 animals, closed black circles; $p = 0.94$), however, this treatment had no effect on DHPG induced priming in slices from *Fmr*1 KO mice (**B**; unprimed: 118.5 ± 6.1%, n = 7 slices from 6 animals, open red circles; primed: 149.6 ± 11.0%, n = 8 slices from 7 animals, closed red circles; $p < 0.05$). Representative field potential traces (average of 10 sweeps) were taken at times indicated by numerals. Scale bars equal 0.5 mV, 5 ms. Error bars represent SEM.

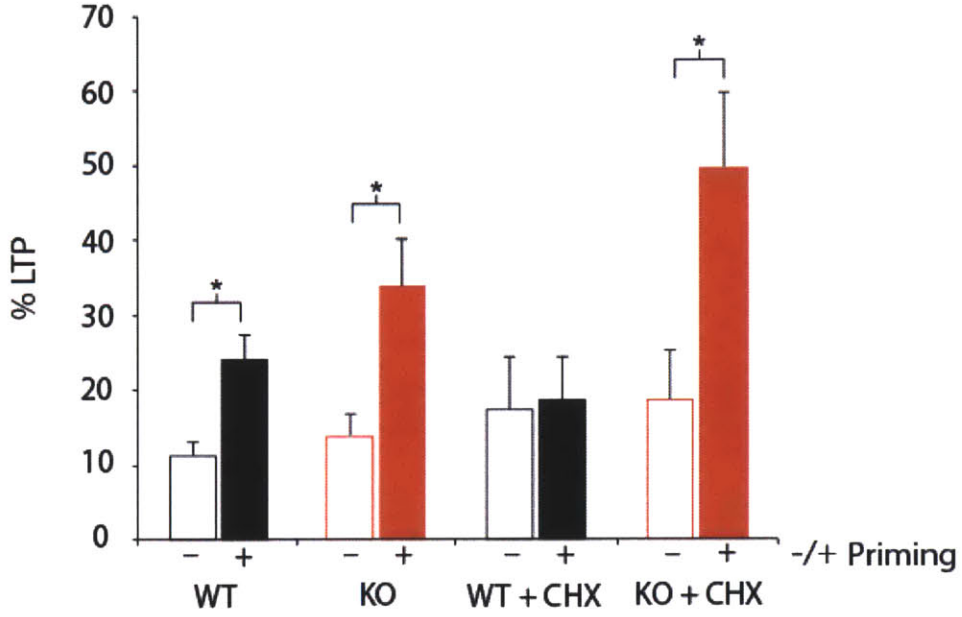

Figure 2.4 – Summary of DHPG-induced priming of LTP and its protein synthesis dependency in wild-type and *Fmr*1 KO mice. Bar graphs represent the average percent LTP observed 55-60 minutes post tetanus. Wild-type (WT) unprimed: open black, wild-type primed: closed black, *Fmr*1 KO (KO) unprimed: open red, *Fmr*1 KO primed: closed red. Asterisks denote significant differences (unpaired student's t-test, $p < 0.05$).

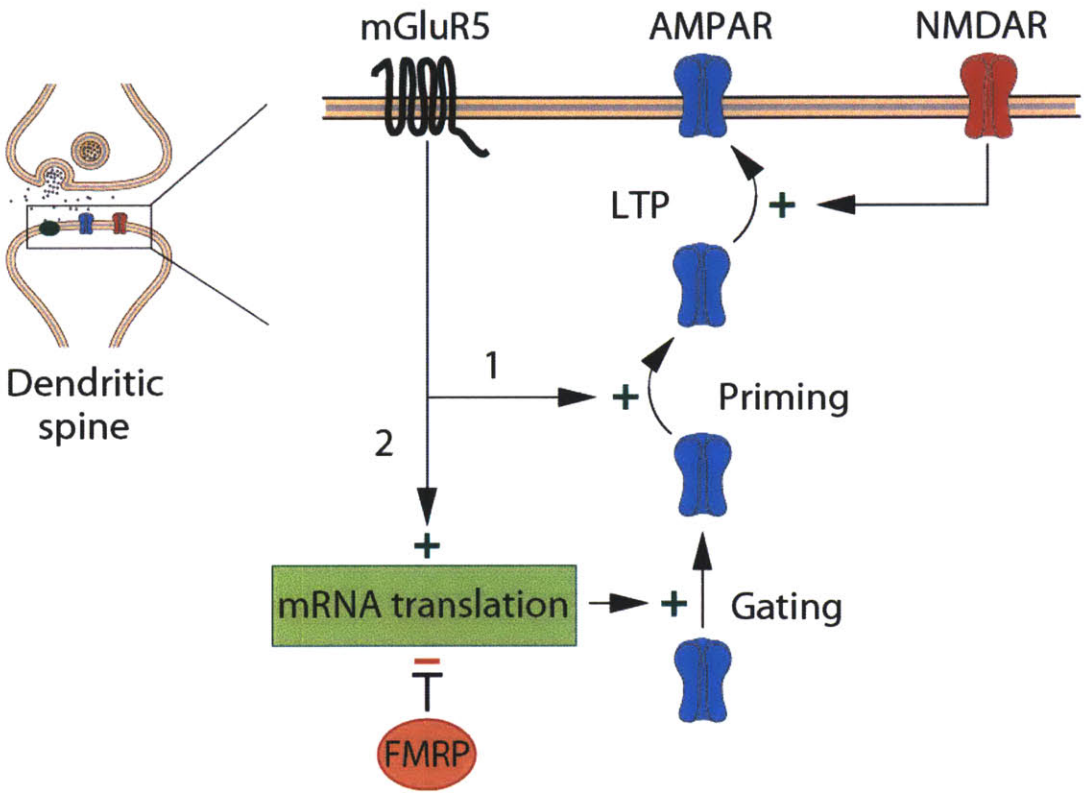

Figure 2.5 – Model for role of FMRP in mGluR-dependent priming of LTP. The finding that LTP priming by mGluR activation occurs in the *Fmr1* KO without a need for acute protein synthesis suggests a bifurcation in the signaling pathway. The priming step (1) occurs in response to mGluR activation via a mechanism involving posttranslational modification of synaptic proteins (possibly the AMPA receptor itself). In WT animals, priming is not possible without (2) concurrent mGluR activation of mRNA translation and synthesis of protein(s) that gate plasticity. In the absence of the translational repressor FMRP, the gating proteins are constitutively overexpressed, rendering priming no longer sensitive to protein synthesis inhibitors. The identity of the hypothetical gating proteins remains to be determined.

Chapter 3

Mutations causing syndromic autism define an axis of synaptic pathophysiology

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3.1 Abstract

Tuberous sclerosis complex and fragile X are genetic diseases characterized by intellectual disability and autism. Because both syndromes are caused by mutations in genes that regulate protein synthesis in neurons, it has been hypothesized that excessive protein synthesis is one core pathophysiological mechanism of intellectual disability and autism. Using electrophysiological and biochemical assays of neuronal protein synthesis in the hippocampus of *Tsc2*$^{+/-}$ and *Fmr1* KO mice, we show that synaptic dysfunction caused by these mutations actually falls at opposite ends of a physiological spectrum. Synaptic, biochemical and cognitive defects in these mutants are corrected by treatments that modulate metabotropic glutamate receptor 5 in opposite directions, and deficits in the mutants disappear when the mice are bred to carry both mutations. Thus, normal synaptic plasticity and cognition occur within an optimal range of metabotropic glutamate receptor-mediated protein synthesis, and deviations in either direction can lead to shared behavioural impairments.

3.2 Introduction

Greater than 1% of the human population has an autism spectrum disorder (ASD), and it has been estimated that up to 70% of those with ASD also have intellectual disability (ID) (Newschaffer et al., 2007). In the large majority of cases, the cause is unknown. However, genetically defined syndromes with increased prevalence of autism and ID offer an opportunity to understand the brain pathophysiology that manifests as ASD and ID, and this knowledge can suggest potential therapies. A case in point is fragile X syndrome (FX), caused by silencing of the *FMR1* gene and loss of the protein product, FMRP. Studies of the *Fmr1* knockout (KO) mouse revealed that in the absence of FMRP, protein synthesis is increased downstream of metabotropic glutamate receptor 5 (mGluR5). Multiple consequences of mGluR-mediated protein synthesis are altered in *Fmr1* KO mice (Chapter 2). Likewise, diverse mutant phenotypes in FX animal models have been corrected by genetic or pharmacological inhibition of mGluR5, and preliminary human clinical trials using drugs that inhibit mGluR5 have shown promise (Krueger and Bear, 2011). Because several other syndromic forms of ASD and ID are associated with mutations of genes that regulate mRNA translation at synapses, it has been hypothesized that altered synaptic protein synthesis might contribute generally to the autistic phenotype (Chapter 1) (Kelleher and Bear, 2008). The aim of the current study was to test the hypothesis that a mutation responsible for another genetic syndrome associated with ASD and ID—tuberous sclerosis complex (TSC)—produces abnormalities in synaptic protein synthesis and plasticity similar to FX. If this were the case, treatments developed for one disorder might be beneficial for the other, and possibly for autism and ID more broadly.

3.2.1 Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is a neurodevelopmental disorder characterized by central nervous system dysfunction (Prather and de Vries, 2004). The choice of TSC was guided by several considerations: Like FX, (1) TSC is a single-gene disorder with core symptoms of ASD and ID; (2) the affected gene(s) lie in a signaling pathway that couples cell surface receptors to mRNA translation; (3) there are well validated mouse models of the disease, and (4) some mutant phenotypes in these mouse models have responded to pharmacological treatments that affect protein synthesis (Ehninger et al., 2008a; Meikle et al., 2008; Onda et al., 2002).

The disease is caused by heterozygous mutations in the genes encoding TSC1 (also known as hamartin) or TSC2 (also known as tuberin) proteins that together form the TSC1/2 complex. TSC1/2 acts to inhibit Rheb, a Ras family GTPase with high specificity for mTOR within a protein complex called mTORC1 (Figure 1.1) (Kwiatkowski and Manning, 2005). Rheb activation of mTORC1 can stimulate mRNA translation and cell growth, and excessive mTORC1 activation is believed to be pathogenic in TSC (Ehninger et al., 2009). TSC is characterized by the growth of hamartomas that are thought to result from inactivation of the functional allele within the tumor cells (loss of heterozygosity) (Carbonara et al., 1994; Green et al., 1994). Although some neurological manifestations of TSC are thought to be related to tumor growth in the cerebral cortex, others, including cognitive impairment and autism, have been proposed to result from abnormal signaling at synapses (de Vries and Howe, 2007). Consistent with this idea, mice engineered to carry heterozygous loss-of-function mutations in *Tsc1* or *Tsc2* have been shown to have hippocampus-dependent learning and memory deficits without having tumors in the brain or seizures (Ehninger et al., 2008a; Goorden et al., 2007). Here we chose the *Tsc2*$^{+/-}$ mouse model because *TSC2* mutations are more common and produce a more severe phenotype in humans (Cheadle et al., 2000), and this animal model is in widespread use (Ehninger et al., 2008a; Nie et al., 2010; Onda et al., 1999; Young et al., 2010). Of particular significance, postnatal treatment of *Tsc2*$^{+/-}$ mice with the mTORC1 inhibitor rapamycin was previously shown to ameliorate hippocampal memory impairments suggesting the exciting possibility that some aspects of TSC, like FX, might be amenable to drug therapy (Ehninger et al., 2008a).

While dysregulated mTOR activity is generally regarded as pathogenic in TSC, it is unclear how this relates to the cognitive impairments associated with the disorder. A prominent hypothesis is that synaptic dysfunction in TSC relates to increased protein synthesis in response to elevated mTORC1 activity (Hoeffer and Klann, 2010). Signaling via mTORC1 has been suggested to contribute to the coupling of mGluR5 to protein synthesis and, although still controversial, it has been proposed that elevated mTOR activity might also be a cause of elevated protein synthesis in the *Fmr1* KO mouse (Sharma et al., 2010). A sensitive electrophysiological read-out of local mRNA translation in response to mGluR5 activation is long-term synaptic depression (LTD) in area CA1 of the hippocampus (Huber et al., 2000; Huber et al., 2001). Indeed, it was exaggerated LTD in the *Fmr1* KO mouse that led to the mGluR theory of FX

(Chapter 1) (Bear et al., 2004; Huber et al., 2002). Therefore, to test the hypothesis of a shared pathophysiology between TSC and FX, we first examined mGluR-LTD in the hippocampus of male *Tsc2*$^{+/-}$ mice.

3.3 Results

3.3.1 Excessive mTOR signaling suppresses the protein synthesis required for mGluR-LTD

LTD was induced by activation of group 1 (Gp1) mGluRs (mGluR 1 and 5) with the selective agonist DHPG ((R,S)-3,5-dihydroxyphenylglycine) in hippocampal slices (Huber et al., 2001). Unexpectedly, we discovered that DHPG-induced LTD was deficient rather than enhanced in the hippocampus of *Tsc2*$^{+/-}$ mice, as compared to WT controls (Figure 3.1A). A similar deficit was observed when mGluR-LTD was induced by patterned electrical stimulation of Schaffer collateral synapses (Figure 3.1B). In agreement with a previous report (Ehninger et al., 2008a), basal synaptic transmission in CA1 appeared normal in the *Tsc2*$^{+/-}$ mice, indicating that the impairment in mGluR-LTD is not due to general disruption of synaptic function (Figure 3.1D,E). Moreover, there was no difference in the magnitude of the NMDA receptor-dependent form of LTD between WT and *Tsc2*$^{+/-}$ mice (Figure 3.1C) demonstrating that the deficit is specific to mGluR-LTD, as these same synapses are able to undergo activity-induced depression via a different mechanism. To test the possibility of a general disruption in Gp 1 mGluR function, we examined DHPG-induced phosphorylation of extracellular signal-regulated kinase (ERK), a common measure of Gp1 mGluR signaling and a critical step for mGluR-mediated protein synthesis and LTD (Gallagher et al., 2004; Osterweil et al., 2010). Basal ERK phosphorylation and DHPG-induced increases in ERK phosphorylation are unaltered in *Tsc2*$^{+/-}$ mice (Figure 3.1F). These results suggest that the deficit in mGluR-LTD seen in the *Tsc2*$^{+/-}$ hippocampus is not due to a global dysregulation of synaptic function or Gp 1 mGluR signaling.

mGluR-LTD in area CA1 of the hippocampus is expressed via two independent mechanisms: reduced probability of presynaptic glutamate release (Fitzjohn et al., 2001; Mockett et al., 2011; Nosyreva and Huber, 2005) and reduced expression of postsynaptic AMPA receptors (Luscher and Huber, 2010; Nosyreva and Huber, 2005). In WT animals, the postsynaptic modification is known to require immediate translation of mRNAs available in the dendrites of hippocampal pyramidal neurons (Huber et al., 2000; Snyder et al., 2001).

Accordingly, we found that LTD in WT mice at the age range examined (postnatal day (P) 25-30) is reliably reduced by the protein synthesis inhibitor cycloheximide (60 μM; Figure 3.2A). The presynaptic component of LTD was monitored by measuring paired-pulse facilitation (PPF), which showed a persistent increase following DHPG that reflects reduced probability of glutamate released at the presynaptic terminal (Figure 3.3) (Fitzjohn et al., 2001; Mockett et al., 2011; Nosyreva and Huber, 2005). Changes in PPF were not inhibited by cycloheximide (Figure 3.2C; Figure 3.3), suggesting that residual LTD in the presence of the drug is expressed presynaptically. While LTD was reduced in *Tsc2*$^{+/-}$ mice, the persistent PPF change after DHPG was no different than in WT, suggesting a deficient postsynaptic modification (Figure 3.2C; Figure 3.3). Indeed, unlike WT, cycloheximide treatment had no effect on LTD in the *Tsc2*$^{+/-}$ animals (Figure 3.2B). These data suggest a selective loss of the protein synthesis-dependent component of LTD in the mutant mice.

These electrophysiological results in the *Tsc2*$^{+/-}$ hippocampus stand in stark contrast to the *Fmr1* KO mouse in which mGluR-LTD is exaggerated (Huber et al., 2002). In the FX mouse model, increased LTD correlates with an increased rate of basal mRNA translation downstream of mGluR5. Therefore we were compelled to examine protein synthesis in hippocampal slices from the *Tsc2*$^{+/-}$ mouse as previously described for the *Fmr1* KO mouse (Osterweil et al., 2010). Consistent with the mGluR-LTD findings, we found a small but significant decrease in ^{35}S-methionine/cysteine incorporation into protein under basal conditions in the hippocampus of *Tsc2*$^{+/-}$ mice (Figure 3.2D). This finding suggested the possibility that protein(s) required for mGluR-LTD are deficiently translated in the hippocampus of *Tsc2*$^{+/-}$ mice. To test this idea we examined levels of Arc, a plasticity related protein that is rapidly synthesized in response to Gp 1 mGluR activation and is required for mGluR-LTD (Park et al., 2008; Waung et al., 2008). Interestingly, we found that Arc expression is decreased in *Tsc2*$^{+/-}$ hippocampal slices (Figure 3.2E). To determine whether this decrease was due to diminished translation, we measured the amount of newly-synthesized Arc in *Tsc2*$^{+/-}$ slices by performing immunoprecipitation experiments on metabolically-labeled slices (see Methods) (Osterweil et al., 2010). Examination of the ^{35}S-incorporated fraction revealed a significant reduction in Arc translation in the hippocampus of *Tsc2*$^{+/-}$ mice (Figure 3.2F). These results suggest that mGluR-LTD is deficient in the *Tsc2*$^{+/-}$ hippocampus due to a decrease in the translation of the proteins required to stabilize LTD, including Arc.

As in the human disease, the germ line mutation in *Tsc2* can have myriad secondary consequences on neural development that could contribute to the observed LTD and protein synthesis phenotypes. To test the hypothesis that the deficient mGluR-LTD seen in *Tsc2*$^{+/-}$ mice is a specific consequence of unregulated mTOR activity, we examined the effects of the mTORC1 inhibitor rapamycin. We found that acute rapamycin treatment (20 nM) restored mGluR-LTD in the *Tsc2*$^{+/-}$ mice to WT levels (Figure 3.4A), while this same treatment had no effect on mGluR-LTD or its protein synthesis dependency in slices from WT mice (Figure 3.4D,E). This rescue is due specifically to the recovery of the protein synthesis-dependent component of LTD, as the effect of rapamycin in *Tsc2*$^{+/-}$ mice was eliminated in the presence of cycloheximide (Figure 3.4B). The same rapamycin treatment also restored basal protein synthesis rates in *Tsc2*$^{+/-}$ hippocampal slices back to WT levels (Figure 3.4C). The simple model that best fits the data is that unregulated mTOR activity caused by the *Tsc2*$^{+/-}$ mutation suppresses the protein synthesis that is required for mGluR-LTD (Figure 3.5A).

3.3.2 *Augmentation of mGluR5 rescues synaptic and behavioral impairments in* Tsc2$^{+/-}$ *mice*

In the *Fmr1* KO model of FX, excessive mGluR-LTD and hippocampal protein synthesis can be corrected by reducing signaling via mGluR5 (Figure 2.1) (Dolen et al., 2007; Michalon et al., 2012; Osterweil et al., 2010). We therefore wondered if the opposite approach of potentiating mGluR5 signaling with a positive allosteric modulator (PAM) could be beneficial in this model of TSC (Figure 3.5A). PAMs are compounds that do not activate mGluR5 directly but act on an allosteric site to potentiate physiological activation of the receptor (Conn et al., 2009). Indeed, we found that pretreatment of hippocampal slices with the mGluR5 PAM 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB (Kinney et al., 2005)) restored the magnitude of mGluR-LTD in *Tsc2*$^{+/-}$ mice to WT levels (Figure 3.5B). The rescue of LTD appears to be due specifically to recovery of the protein synthesis-dependent component because the effect of CDPPB was completely eliminated by cycloheximide (Figure 3.5C). Consistent with this conclusion, CDPPB treatment also restored basal protein synthesis levels (Figure 3.5D) and rescued the deficit in Arc synthesis in the *Tsc2*$^{+/-}$ mice (Figure 3.5E). Thus, allosteric augmentation of mGluR5 signaling can overcome the inhibitory effect of unregulated mTOR activity on the synaptic protein synthesis that supports LTD.

In an important recent study, cognitive impairments in the *Tsc2*$^{+/-}$ mice were shown to be significantly improved by treating the animals with the mTORC1 inhibitor rapamycin (Ehninger

et al., 2008a). In light of our electrophysiological and biochemical findings, we wondered if a similar amelioration would be observed with the mGluR5 PAM. A robust phenotype was reported to be an impairment in the ability of the *Tsc2*$^{+/-}$ mice to distinguish between familiar and novel contexts in a fear conditioning paradigm. Advantages of this paradigm are the learning occurs in one trial making it amenable to acute drug treatment, and the memory is hippocampus-dependent (Frankland et al., 1998). Although a requirement for CA1 LTD *per se* has not been established, contextual fear discrimination does depend on both mGluR5 (Lu et al., 1997) and new protein synthesis at the time of training (Stiedl et al., 1999). In this assay, mice are first exposed to a distinctive context in which they receive an aversive foot shock. The next day, context discrimination is tested by dividing the animals into two groups; one is placed in the familiar context associated with the shock, and the other is placed in a novel context (Figure 3.5F). Context discrimination is assessed by measuring the time the animals express fear by freezing in each context. Although the WT mice clearly discriminate between contexts, the *Tsc2*$^{+/-}$ mice do not (Ehninger et al., 2008a) (Figure 3.5G). To test the effect of augmenting mGluR5 signaling, mice from both genotypes were injected i.p. with CDPPB (10 mg/kg) 30 minutes prior to training. Although this treatment had no effect in the WT mice, it was sufficient to correct the deficit in context discrimination observed in the *Tsc2*$^{+/-}$ mice. These results show that augmentation of mGluR5 signaling is beneficial at the behavioral level in *Tsc2*$^{+/-}$ mice and that disrupted mGluR5 function may be relevant to cognitive impairments associated with TSC.

3.3.3 Mutations in Fmr1 *and* Tsc2 *cancel one another on a functional axis*

Contrary to our initial hypothesis we found that mutations causing FX and TSC, two disorders associated with autism and ID, show mirror symmetrical alterations in protein synthesis-dependent LTD and have beneficial responses to treatments that modulate mGluR5 in opposite directions (Figure 3.6A). These findings raised the intriguing possibility that these two mutations could cancel one another on this functional axis. To test this hypothesis, we introduced an *Fmr1* deletion into the *Tsc2*$^{+/-}$ background by crossing *Tsc2*$^{+/-}$ males with *Fmr1*$^{+/-}$ females (Figure 3.6B). This approach also enabled us to compare directly with WT the effects of the *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mutations in littermates reared under identical conditions. As expected, mGluR-LTD was diminished in *Tsc2*$^{+/-}$ mice and excessive in the *Fmr1* KO mice, as compared to WT (Figure 3.6C,D). However, mice harboring both mutations showed mGluR-LTD that was indistinguishable from WT (Figure 3.6C,D).

While *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mutations cause opposite alterations in mGluR-LTD and protein synthesis, the human disorders they are associated with have similar neurological and cognitive phenotypes. Might opposite deviations in synaptic function lead to shared cognitive impairments? To examine this question, we compared context discrimination in the *Tsc2*$^{+/-}$ and *Fmr1* KO mice and discovered that indeed they do share a deficit in this measure of memory (Figure 3.6E). Remarkably, instead of being exacerbated, this memory deficit was erased in the double mutants (Figure 3.6E). These results suggest that the opposing synaptic deviations seen in *Tsc2*$^{+/-}$ and *Fmr1* KO mice may manifest similarly at the behavioral level, as introducing both mutations not only reverses the disruptions of synaptic plasticity but rescues this memory impairment as well.

3.4 Discussion

LTD and protein synthesis downstream of mGluR5 have attracted attention in the context of several diseases, most notably FX (Luscher and Huber, 2010). In the *Fmr1* KO mouse model, basal protein synthesis is elevated and LTD is exaggerated downstream of an mGluR5 signaling pathway involving ERK (Osterweil et al., 2010). In Chapter 2 we demonstrated multiple consequences of mGluR-mediated protein synthesis are affected in *Fmr1* KO mice, suggesting that this disruption could contribute to the constellation of phenotypes associated with FX. Indeed, partial inhibition of mGluR5 or ERK corrects multiple aspects of fragile X in animal models (Dolen et al., 2010; Krueger and Bear, 2011; Michalon et al., 2012; Osterweil et al., 2013). Recent data suggest that the mTOR signaling pathway is also constitutively overactive in the *Fmr1* KO mouse (Sharma et al., 2010), but the relevance to exaggerated protein synthesis and altered synaptic function has been controversial.

mGluR activation has been shown to recruit both the ERK and mTOR pathways, and disentangling the contributions of each to mGluR-dependent LTD and protein synthesis has been difficult. The current findings show that inhibition of mTOR with rapamycin does not disrupt mGluR-LTD in WT mice or its protein synthesis dependency. However, this same treatment rescues LTD and protein synthesis rates in the *Tsc2*$^{+/-}$ mice, suggesting that increased synaptic mTOR activity suppresses the protein synthesis required for LTD in these animals (Figure 3.5A). These results are supported by two other recent studies demonstrating disrupted mGluR-LTD in

different but complimentary mouse models of TSC (Bateup et al., 2011; Chevere-Torres et al., 2012). The results from Bateup et al. are particular enlightening, as they showed that sparse *in vivo* knockdown of *Tsc1* results in deficient LTD, demonstrating that this disruption is not only acute but cell autonomous as well. The idea that reduced protein synthesis is a causative factor in the observed deficit in synaptic plasticity is supported by the finding that pharmacological rescue with both rapamycin and CDPPB is abolished by cycloheximide, and the observation that Arc is deficiently translated in the *Tsc2*$^{+/-}$ mice. There is good evidence that Arc is one protein that normally must be synthesized to support mGluR5-dependent forms of long-term plasticity (Park et al., 2008; Waung et al., 2008).

3.4.1 Mechanisms of mTOR-dependent suppression of translation

Precisely how excess mTOR activity suppresses synthesis of plasticity proteins downstream of mGluR activation remains to be determined. An intriguing hypothesis is that the effect of elevated mTOR on protein synthesis may be mediated through FMRP (Figure 3.7A). It has been shown that post-translational modifications of FMRP can regulate translational repression (Bhakar et al., 2012). Phosphorylation has been suggested to stall ribosomal translocation while maintaining the association of FMRP with mRNA, thus inhibiting translation of FMRP targets (Ceman et al., 2003; Muddashetty et al., 2011). Recent work has attempted to determine the phosphatases and kinases that may regulate FMRP downstream of mGluR activation. One model proposes that mGluR5 stimulation initially results in activation of the protein phosphatase 2A (PP2A), which dephosphorylates FMRP, resulting in de-repression of protein synthesis (Narayanan et al., 2007) (Figure 3.7A). However, FMRP is rapidly re-phosphorylated in an mTOR-dependent manner via S6 kinase (S6K), suppressing translation once again (Narayanan et al., 2008; Santoro et al., 2012). Thus, if mTOR is chronically over-activated, as is the case when TSC1/2 function is disrupted, this could lead to hyperphosphorylation of FMRP, resulting in the translational suppression of FMRP targets required for LTD (Figure 3.7A). This model is supported by a recent study demonstrating that local translation of the potassium channel Kv4.2 is suppressed via mTOR-dependent phosphorylation of FMRP (Lee et al., 2011). The fact that deleting FMRP in the *Tsc2*$^{+/-}$ background rescues both protein synthesis rates and LTD levels also lends some support to this model. However, if increased phosphorylation of FMRP was solely responsible for the

diminished protein synthesis and LTD in *Tsc2*$^{+/-}$ mice, then the double mutants should phenocopy *Fmr1* KO mice rather than resemble WT animals. Therefore, additional mechanisms are likely involved in mTOR-mediated suppression of protein synthesis.

The above scenario demonstrates that translation is a tightly regulated process that requires precise timing of all its components. mTOR is known to promote initiation of translation and numerous studies have demonstrated increased phosphorylation of mTOR targets (e.g. 4E-BP1 and S6K) in *Tsc2* deficient cells (Kwiatkowski and Manning, 2005). There is also good evidence to suggest that phosphorylation of FMRP can stall elongation (Bhakar et al., 2012). The mTOR-dependent phosphorylation of FMRP may normally serve as a checkpoint to limit runaway protein synthesis (Figure 3.7A). However, when mTOR is constitutively active, the timing of this feedback loop is disrupted, resulting in stalled elongation even when initiation is being promoted. In this way, chronically overactive mTOR signaling may counter-intuitively result in decreased protein synthesis rates. A prediction of this model is that there will be a higher percentage of stalled ribosomes in *Tsc2* deficient neurons, particularly those that are also associated with FMRP.

An alternative, although not mutually exclusive, model is that mTOR stimulates translation of an unrelated pool of mRNAs not regulated by mGluRs (Figure 3.7B) (Bear et al., 2004; Ehninger et al., 2009; Park et al., 2008). The fact that mGluR-LTD is altered in opposite directions in *Tsc2*$^{+/-}$ and *Fmr1* KO mice (3.6A), and that both deviations are corrected in the double mutants, suggests that the pool comprising LTD proteins is differentially regulated by FMRP and TSC1/2 (Figure 3.7B). A potential mechanism for this "push-pull" regulation of translation is cap- verses non-cap-dependent protein synthesis. mTOR activation is known to recruit the components required for 5'cap-dependent translation, which is sometimes regarded as global or general translation (Hay and Sonenberg, 2004) (Figure 1.2A). However, there are alternative mechanisms for translation initiation that does not require ribosomal binding to the 5'cap and can result in gene-specific translation. Some mRNAs have an internal ribosomal entry site (IRES) in the 5' UTR that allows the ribosome to directly scan for the initiation codon and initiate translation (Mathews, 2000). Interestingly, many mRNA that are FMRP targets and are translated downstream of mGluR activation have this IRE sequence, such as Arc, Map1b, CaMKIIα (Pinkstaff et al., 2001). Thus, mRNA translation can be regulated in a global or gene-specific manner. It has been suggested that the regulation of gene-specific translation is

particularly important for synaptic plasticity and memory consolidation in the hippocampus (Jiang et al., 2010). The differentiation between TSC-mTOR-dependent translation and mGluR-ERK-FMRP-dependent translation may be defined by the mechanism of initiation.

3.4.2 Regulation of gene-specific translation

Regulation of the initiation factor eIF2α is a well-characterized mechanism for modulating both global and gene-specific translation (Costa-Mattioli et al., 2009). eIF2α facilitates binding of the initiator methionyl-tRNA to the 40S ribosomal subunit (Figure 1.2A). Phosphorylation of eIF2α disrupts formation of this complex, inhibiting general translation while paradoxically resulting in the upregulation of a specific subset of mRNA containing upstream open reading frames (uORFs) (Costa-Mattioli et al., 2009). Increasing (Jiang et al., 2010) or decreasing (Costa-Mattioli et al., 2005) eIF2α phosphorylation in the hippocampus has been shown to bidirectionally modify synaptic plasticity and memory consolidation, suggesting this mechanism could be important for proper neuronal function. Interestingly, these changes are not thought to be the result of altered global translation but rather due changes in the translation of the uORF-containing mRNA for the transcription factor ATF4, a negative regulator (Karpinski et al., 1992) of CRE-dependent transcription critical for memory consolidation (Silva et al., 1998).

eIF2α is not directly regulated by the mTOR pathway, but rather is regulated by a set of kinases that respond to various cellular stressors (Mathews, 2000). However, it has been suggested that dysregulated protein synthesis and disturbances in nutrient signaling caused by uncontrolled mTOR signaling might lead to endoplasmic reticulum (ER) stress in TSC1/2-deficient cells (Ozcan et al., 2008). Indeed, *Tsc2* deficient neurons have been shown to have an increased ER stress response (Di Nardo et al., 2009) and deletion of *Tsc2* results in increased phosphorylation of eIF2α by its kinase PERK (Ozcan et al., 2008). Thus, loss of *Tsc2* may result in a negative feed back loop that results in the suppression of general translation by eIF2α but an increase in translation of uORF containing mRNA. However, while it has been shown that complete absence of TSC1/2 function results in increased ER stress and eIF2α phosphorylation, this has not been demonstrated in the *Tsc2*$^{+/-}$ animals used in this study. In fact, direct comparison of *Tsc2* lacking tumor cells (resulting from loss of heterozygosity) and neighboring *Tsc2*$^{+/-}$ cells revealed increased ER stress specifically in the tumor cells (Ozcan et al., 2008),

suggesting that heterozygous deletion may not be sufficient to activate the stress response pathway.

Another potential mechanism for differential translation involves the regulation of the elongation factor eEF2. Inhibition of eEF2 results in a general blockage of translation elongation (Figure 1.2B). However, it has been suggested that by doing so, poorly initiated transcripts benefit from the increased concentration of free translation initiation factors, including several FMRP targets known to be regulated by mGluR activation (Park et al., 2008). Phosphorylation of eEF2 in response to mGluR5 activation has shown to promote translation of LTD proteins, such as Arc and MAP1b (Park et al., 2008). Conversely, activation of the mTOR pathway inhibits eEF2 kinase activity, thereby decreasing eEF2 phosphorylation, which may suppress the LTD pool and favor translation of targets downstream of mTOR (Costa-Mattioli et al., 2009; Mathews, 2000). Thus, recruiting the mTOR pathway can stimulate synthesis of a subset of proteins that mutually inhibits the translation of another through either the regulation of initiation (eIF2α) or elongation (eEF2). Determining how the components of the eIF2α and eEF2 pathways are altered in *Tsc2*$^{+/-}$ mice, and if the protein targets downstream of these pathways are increased or decreased, will lend insight into how translation is disrupted in the *Tsc2*$^{+/-}$ animals.

Even given that mTOR and ERK modulate different pools of proteins, it still seems counter-intuitive that excessive mTOR signaling would lead to decreased total levels of protein synthesis if it regulates global translation. However, it is important to remember that because neurons are non-dividing cell, their protein make-up is different than most cell types. mTOR signaling is thought to selectively regulate the expression of mRNA containing 5' terminal oligoprymidine (TOPs) motifs (Thoreen et al., 2012). TOP-containing mRNA includes many components required for cell differentiation, growth, and proliferation, including components of the translational machinery itself (Hay and Sonenberg, 2004). In this way, stimulation of TOP-containing mRNA can subsequently increase the overall capacity for translation, and it is through this mechanism that mTOR is thought to most effectively exert its control over translation rates. Thus, while mTOR is regarded as a stimulator of global protein synthesis, its regulation of translation is actually quite complex, and probably restricted to expression of a specific subset of genes. An important distinction of TOP-dependent translation is that it is significantly suppressed in non-dividing cells (Mathews, 2000). Therefore, in neurons, translation of this pool of mRNA may represent a smaller percentage of overall protein synthesis. In this way,

promotion of the mTOR regulated pool, as seen in *Tsc2* deficient neurons, may actually lead to decreased overall protein synthesis rates.

3.4.3 Functional significance of gene-specific translation

If there are indeed two pools of mRNA that are differentially translated by the TSC-mTOR and the mGluR5-ERK-FMRP pathways, it suggests there is likely a difference in the functional consequences of their synthesis. In addition to deficient mGluR-LTD, a recent report has demonstrated the *Tsc2*$^{+/-}$ mice have a reduced threshold for the induction of protein synthesis-dependent LTP. It was shown that a weak LTP-inducing stimulation that normally produces a protein synthesis-independent, transient early-phase potentiation (E-LTP) is converted into persistent late-phase LTP (L-LTP) without the requirement of new protein synthesis. L-LTP normally requires the synthesis of new proteins, suggesting that the proteins required for the maintenance of LTP are already present in *Tsc2*$^{+/-}$ mice.

Interestingly, the eEF2 kinase KO mouse displays a strikingly similar phenotype—deficient mGluR-LTD and enhanced L-LTP (Park et al., 2008)— suggesting mTOR-dependent regulation of eEF2 is indeed a potential mechanism for switching between mGluR- and mTOR-regulated mRNA pools. Reconstituting the inhibition of elongation normally imposed by eEF2 kinase with a low concentration of CHX was shown to rescue mGluR-LTD in the eEF2 kinase KO. This treatment may be similarly effective in *Tsc2*$^{+/-}$ mice if the suppression of mGluR-LTD is indeed due to mTOR-dependent activation of eEF2. In contrast, increasing eIF2α phosphorylation results in deficient L-LTP (Jiang et al., 2010), suggesting this may not the manner by which mTOR regulates protein synthesis in the *Tsc2*$^{+/-}$ mice.

The above studies suggest a simple hypothesis: TSC1/2, mTOR, and eEF2 kinase regulate a pool of mRNA required for synaptic strengthening (LTP) while mGluR5, ERK and FMRP control the synthesis of proteins required for synaptic weakening (LTD) (Figure 3.7B). There is some evidence that LTP is deficient in the hippocampus of *Fmr1* KO mice (Hu et al., 2008; Lauterborn et al., 2007; Lee et al., 2011; Meredith et al., 2011; Shang et al., 2009), however others have found no difference in the threshold for LTP (Godfraind et al., 1996; Zhang et al., 2009). Moreover, in Chapter 2 we demonstrated that FMRP-regulated protein synthesis does play a role in some forms of LTP maintenance. Thus, the differential translation of mRNA at the synapse is likely to be more complicated than simply LTP vs. LTD. However this model

provides a framework for testable hypotheses that may lend more insight into how protein synthesis is regulated at the synapse and what functions it serves. Determining which proteins are under control of TSC1/2 and FMRP will be instrumental to our understanding of the different functions these two pools may serve. Proteomic comparison of the *Tsc2*$^{+/-}$ x *Fmr1* KO cross mice provide an excellent means to this end, as they allow for examination of the protein make-up of the same synapses in littermate mice carrying the *Fmr1* mutation, the *Tsc2* mutation, or both, under identical experimental conditions.

3.4.4 Role of mGluR dysfunction in TSC and ASD

The current findings also suggest a new treatment for behavioral deficits associated with TSC. Previous studies in the *Tsc2*$^{+/-}$ mouse raised the exciting possibility that cognitive aspects of the disorder might be ameliorated with rapamycin, even when treatment is begun in adulthood (Ehninger et al., 2008a). Our data show that an mGluR5 PAM may be similarly effective. While rapamycin has been used clinically, it is problematic for chronic treatment because of its strong immunosuppressive properties and potential for harmful side-effects (Tsai et al., 2013). Furthermore, studies have shown that some beneficial effects of rapamycin are short-lived and symptoms return as soon as treatment is terminated (Bissler et al., 2008). Thus, positive modulation of mGluR5 may be a useful compliment or alternative to rapamycin treatment in TSC. The benefits of mGluR5 PAMs are that they have higher receptor specificity, enhance activity in a physiologically relevant manner, and most importantly, they specifically target the synaptic mechanisms that are likely responsible for the cognitive and behavioral impairments in TSC. Future studies are needed to determine the efficacy of mGluR5 treatment on other aspects of the disorder, such as tumor growth and seizures.

TSC and FX represent two leading genetic risk factors for ASD and ID (Fombonne, 2003). Although great strides have been made in identifying genetic variations that correlate with non-syndromic autism, there is little known about ASD pathophysiology—knowledge that is essential for developing effective therapies. Our test of the hypothesis that the *Fmr1* KO and *Tsc2*$^{+/-}$ mouse models of FX and TSC have a shared synaptic pathophysiology revealed instead that they are at opposite ends of a spectrum: the *Fmr1* mutation causes exaggerated synaptic protein synthesis and LTD that are corrected by inhibition of mGluR5 (Dolen et al., 2007; Michalon et al., 2012), whereas the *Tsc2* mutation causes diminished synaptic protein synthesis

and LTD that are corrected by augmentation of mGluR5 (Figure 3.6A). Moreover, the opposing effects of these mutations balance one another at synaptic and behavioral levels in the double mutant. This finding is interesting in light of recent discoveries that gain- and loss-of-function mutations in individual genes, such as *MECP2*, can often yield syndromes with overlapping features, such as epilepsy, cognitive impairment, and ASD (Ramocki and Zoghbi, 2008). Our findings reveal that even genetically heterogeneous causes of ASD and ID may produce similar deficits by bidirectional deviations from normal on a common functional axis.

The results presented here not only provide further insight into ASD etiology, but also to the treatment of ASD. The present results suggest that altered mGluR activity may indeed be a common autism pathology, further supporting the notion for mGluR5-based therapies. However, the relationship between this molecular perturbation and the clinical manifestation of ASD is complex, as we found that opposing alterations in mGluR-mediated protein synthesis may manifest as similar cognitive impairments. The important implication is that therapies designed to correct one cause of ASD are not likely to be effective for all other causes, and might well be deleterious. It will be critical to understand where a patient lies on the spectrum of synaptic function to choose an appropriate therapy for ASD and other psychiatric disorders.

3.5 Methods

3.5.1 Animals

Tsc2$^{+/-}$ male and female mutant mice on the C57Bl/6J clonal background were bred with C57Bl/6J WT partners to produce the WT and *Tsc2*$^{+/-}$ offspring used in this study. For genetic rescue experiments, heterozygous *Tsc2* male mice (*Tsc2*$^{+/-}$) were bred with heterozygous *Fmr1* females (*Fmr1* x^{+}/x^{-}), both on the C57Bl/6J clonal background, to obtain F1 male offspring of four genotypes: wild type (*Tsc2*$^{+/+}$, *Fmr1*$^{+/y}$), *Fmr1* KO (*Tsc2*$^{+/+}$, *Fmr1*$^{-/y}$), *Tsc2* Het (*Tsc2*$^{+/-}$, *Fmr1*$^{+/y}$), and Cross (*Tsc2*$^{+/-}$, *Fmr1*$^{-/y}$) (Figure 3.6.B). All experimental animals were age-matched male littermates, and were studied with the experimenter blind to genotype and treatment condition. Animals were group housed and maintained on a 12:12 hr. light:dark cycle. The Institutional Animal Care and Use Committee at MIT approved all experimental techniques.

3.5.2 Electrophysiology

Acute hippocampal slices were prepared from P25-35 animals in ice-cold dissection buffer containing (in mM): NaCl 87, Sucrose 75, KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 0.5, $MgSO_4$ 7, Ascorbic acid 1.3, and D-glucose 10 (saturated with 95% O_2 / 5% CO_2). Immediately following slicing the CA3 region was removed. Slices were recovered in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$ 2, $MgCl_2$ 1 and D-glucose 10 (saturated with 95% O_2/5% CO_2) at 32.5°C for ≥3 hours prior to recording.

Field recordings were performed in a submersion chamber, perfused with ACSF (2-3 ml/ min) at 30 °C. Field EPSPs (fEPSPs) were recorded in CA1 *stratum radiatum* with extracellular electrodes filled with ACSF. Baseline responses were evoked by stimulation of the Schaffer collaterals at 0.033 Hz with a 2-contact cluster electrode (FHC) using a 0.2 ms stimulus yielding 40-60% of the maximal response. fEPSP recordings were filtered at 0.1 Hz - 1 kHz, digitized at 10 kHz, and analyzed using pClamp9 (Axon Instruments). The initial slope of the response was used to assess changes in synaptic strength. Data were normalized to the baseline response and are presented as group means ± SEM. LTD was measured by comparing the average response 55-60 minutes post DHPG application to the average of the last 5 minutes of baseline.

The input output function was examined by stimulating slices with incrementally increasing current and recording the fEPSP response. Paired pulse facilitation was induced by applying two pulses at different interstimulus intervals. Facilitation was measured by the ratio of the fEPSP slope of stimulus 2 to stimulus 1. NMDAR-dependent LTD was induced by delivering 900 test pulses at 1 Hz. mGluR-LTD was induced by applying R, S-Dihydroxyphenylglycine (R,S-DHPG, 50 μM) or S-Dihydroxyphenylglycine (S-DHPG, 25 μM) for 5 minutes, or by delivering 1200 pairs of pulses (with a 50 ms interstimulus interval) at 1 Hz. In some experiments slices were incubated with the protein synthesis inhibitor cycloheximide (60 μM) for 30 minutes as follows: 20 minutes during baseline recording, 5 minutes during DHPG application and 5 minutes post DHPG application. For mGluR PAM experiments, slices were pretreated with CDPPB (10 μM) or DMSO control for 30 minutes in same manner as above, either in the presence of cycloheximide or control ACSF. For rapamycin experiments, slices were pretreated with rapamycin (20 nM) or DMSO control, with or without cycloheximide, for at least 30 minutes prior to recording and throughout the entire experiment. Significance was determined by two-way ANOVA and *post-hoc* Student's t-tests. Statistics were performed using each animal as an "n", with each animal represented by the mean of 1-4 slices. All experiments were performed blind to genotype and include interleaved controls for genotype and treatment.

3.5.3 Metabolic labeling of new protein synthesis

Performed as described by Osterweil *et al* (Osterweil et al., 2010). Briefly, 500 μm slices were recovered for 4 h in 32.5°C ACSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2 $CaCl_2$, saturated with 95% O_2 and 5% CO_2), incubated for 30 min with 25 μM ActD ± rapamycin (20 nM) or CDPPB (10 μM), and transferred to fresh ACSF ± drug with 10 μCi/ml ^{35}S-Met/Cys (Perkin Elmer) for another 30 min. After labeling, slices were homogenized, and labeled proteins isolated by TCA precipitation. Samples were read with a scintillation counter and also subjected to a protein concentration assay (Bio-Rad). Final data were expressed as counts per minute (CPM) per μg protein, normalized to the ^{35}S-Met/Cys ACSF used for incubation, and the average incorporation of all samples analyzed in that experiment. For autoradiography, homogenized slices were processed for SDS PAGE, transferred to nitrocellulose, stained for total protein using the Memcode staining kit (Pierce), and ^{35}S-incorporated proteins visualized with the aid of a phosphorimager (Fujifilm).

3.5.4 Immunoblotting

Immunoblotting was performed according to established methods using primary antibodies to Arc (Synaptic Systems), p-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology) or ERK1/2 (Cell Signaling Technology). ERK1/2 phosphorylation was measured by densitometry (Quantity One), and quantified as the densitometric signal of p-ERK1/2 divided by the ERK1/2 signal in the same lane. To quantify Arc expression, the densitometric signal of Arc was divided by the total protein signal (determined by Memcode staining) in the same lane.

3.5.5 Immunoprecipitation

Hippocampal slices (5-8 per animal) were metabolically labeled with 50 μCi/ml ^{35}S-Met/Cys for 3 h, and immunoprecipitation (IP) performed on yoked WT and *Tsc2+/-* slices essentially as described previously(Osterweil et al., 2010). Briefly, slices were homogenized in IP lysis buffer (Pierce) plus protease inhibitors (EMD Biosciences), spun at 16,000 x g, and supernatants pre-cleared with protein A/G sepharose. To avoid contamination of the Arc signal with IgG heavy chain, immunoprecipitation was performed using columns of monoclonal Arc antibody (a generous gift from P. Worley) crosslinked to protein A/G sepharose (Pierce Crosslink IP Kit). Immunoprecipitated Arc was resolved on SDS PAGE gels, transferred to nitrocellulose, and exposed to a phosphorimager screen for 2-3 weeks. The same membranes were then immunoblotted for Arc. For each sample, the ratio of ^{35}S-incorporated : total was calculated by dividing the density of the band seen by autoradiography to the density of band seen by immunoblot (in the same lane). To ensure the specificity of Arc IPs, lysates from metabolically labeled hippocampal slices were incubated with either mouse monoclonal anti-Arc or non-immune mouse IgG, and IP experiments performed as described above. Immunoblot analysis reveals that Arc is significantly enriched in anti-Arc IPs versus IgG IPs from the same lysates (t-test IgG vs. Arc *p = 0.002; n = 5 animals). Additionally, autoradiographs confirm the absence of ^{35}S-incorporated protein in the IgG IP.

3.5.6 Contextual fear conditioning

6-12 week old WT, *Tsc2*$^{+/-}$, *Fmr1*$^{-/y}$, and Cross (*Tsc2*$^{+/-}$ x *Fmr1*$^{-/y}$) mice were fear conditioned to the training context with one 0.8 mA shocks (2 sec) as described by Ehninger *et al* {Ehninger,

2008, 18568033}. The mice were allowed 3 minutes to explore context before conditioning and were removed 15 sec after the shock was given and returned to home cage. Conditioned fear response was assessed 24 hours later by a trained observer blind to condition, measuring the percentage of time spent freezing during the test period (3 min session). To determine context specificity of the conditioned response, mice trained at the same time were separated into two groups: one group was tested in the same training context and the other tested in a novel context. This novel context was created by varying: distal cues, odor (2% acetic acid vs. 70% ethanol), floor material (plastic vs. metal bars), and lighting (red vs. white) of the testing apparatus. For rescue experiments, animals received a single injection of CDPPB (10 mg/kg, i.p.) 30 minutes prior to training session.

3.5.7 Reagents

(R,S)-3,5-dihydroxyphenylglycine (R,S-DHPG) was purchased from Tocris Biosciences (Ellisville, MO) and (S)-3,5-dihydroxyphenylglycine (S-DHPG) was purchased from Sigma (St. Louis, MO). Fresh bottles of DHPG were prepared as a 100x stock in H_2O, divided into aliquots, and stored at -80°C. Fresh stocks were made once a week. Rapamycin (EMD Biosciences, San Diego, CA) was prepared at 10 mM stock in DMSO and stored at -80°C. Final concentration of rapamycin was 20 nM in < 0.01% DMSO. Cycloheximide (Sigma) was prepared daily at 100x stock in H_2O. For slice experiments, 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB, EMD Biosciences) was prepared daily at 75 mM stock in DMSO with 0.5% bovine serum albumin (BSA) and diluted in ACSF to achieve final concentration of 10 μM in < 0.1% DMSO. For *in vivo* experiments, CDDPB was suspended in a vehicle consisting of 20% (2-Hydroxypropyl)-(R)-cyclodextrin in sterile saline. All other reagents were purchased from Sigma.

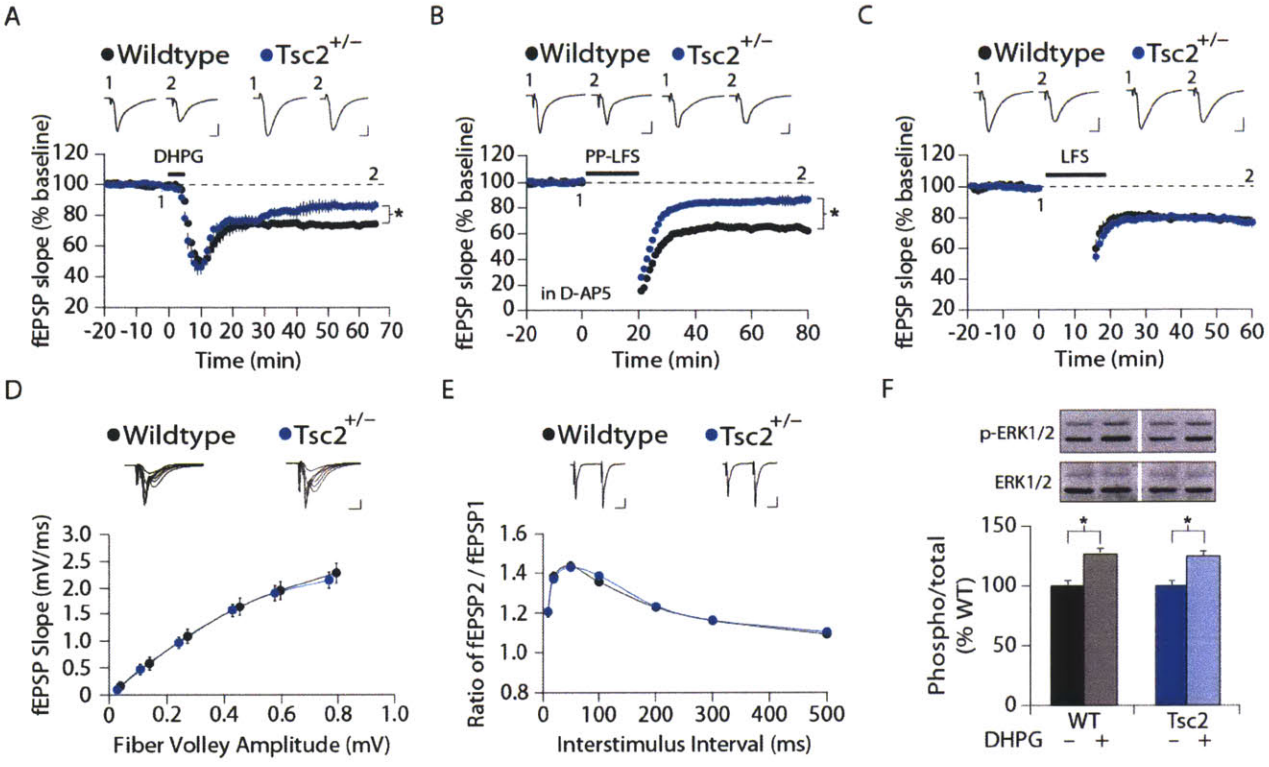

Figure 3.1 – *Tsc2*$^{+/-}$ mice have a specific deficit in mGluR-LTD

(A) DHPG induces significantly less LTD in slices from *Tsc2*$^{+/-}$ mice as compared to slices from littermate WT mice (74.3 ± 1.4%, n = 5 animals, 10 slices; *Tsc2*$^{+/-}$: 86.3 ± 3.1%, n = 6 animals, 12 slices; *p = 0.004). **(B)** Synaptically-induced mGluR-LTD, elicited by delivering pairs of pulses (50 ms interstimulus interval) at 1 Hz for 20 minutes (PP-LFS, 1200 pulses) in the presence of the NMDA receptor antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 μM), is also deficient in slices from *Tsc2*$^{+/-}$ mice (WT: 65.1 ± 2.1%, n = 3 animals, 9 slices; *Tsc2*$^{+/-}$: 85.0 ± 2.5%, n = 4 animals, 11 slices; *p = 0.003). **(C)** The magnitude of NMDA receptor-dependent LTD evoked by low frequency stimulation (LFS, 900 pulses at 1 Hz) does not differ between genotypes (WT: 79.8 ± 1.6%, n = 4 animals, 6 slices; *Tsc2*$^{+/-}$: 79.4 ± 1.9%, n = 6 animals, 6 slices; p = 0.610). **(C)** Basal synaptic transmission (plotted as fEPSP amplitude against presynaptic fiber volley amplitude) does not differ between genotypes. Scales bars equal 0.5 mV, 5 ms. Error bars represent SEM. **(D)** Paired pulse facilitation is normal across several inter-stimulus intervals (20, 30, 50, 100, 200, 300, 500 ms) in *Tsc2*$^{+/-}$ mice. Scale bars equal 0.5 mV, 20 ms for representative field potential traces. Error bars represent SEM. **(E)** Hippocampal slices were stimulated with 50 μM DHPG for 5 min, and ERK1/2 activation (phosphorylation) assessed via immunoblot (WT: 100.0 ± 6.1%, WT DHPG: 119.6 ± 5.5%, *Tsc2*$^{+/-}$: 97.5 ± 5.6%, *Tsc2*$^{+/-}$ DHPG: 116.2 ± 3.9%; ANOVA: genotype p = 0.623, treatment *p = 0.0008, genotype x treatment p = 0.923; n = 9 animals). Results reveal that DHPG significantly increases ERK1/2 activation in both WT (*p = 0.040) and *Tsc2*$^{+/-}$ (*p = 0.003). Error bars represent SEM.

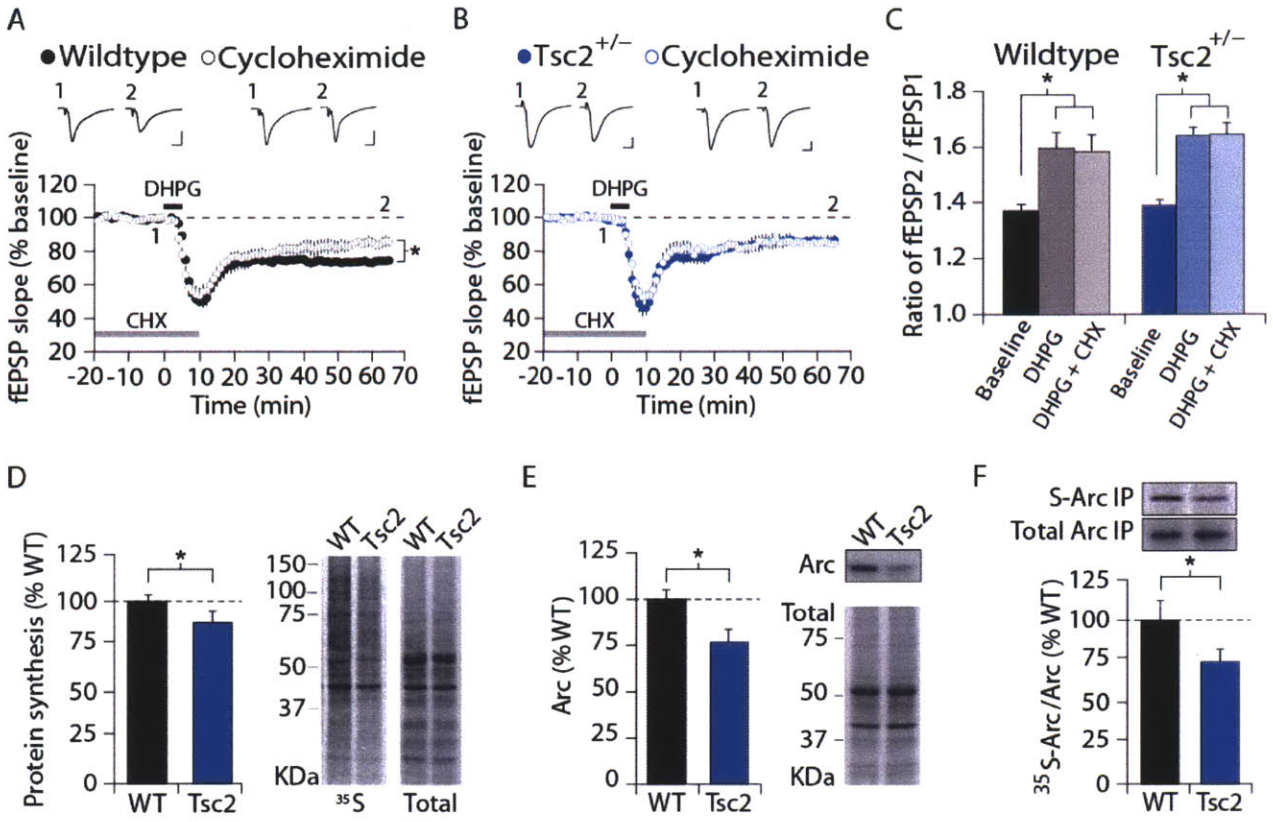

Figure 3.2 – The protein synthesis-dependent component of mGluR-LTD is absent in *Tsc2*$^{+/-}$ mice. (A) LTD is significantly attenuated by pretreatment with the protein synthesis inhibitor cycloheximide (CHX, 60 μM, gray bar) in slices from WT animals (control: 74.3 ± 1.4%, n = 5 animals, 10 slices; CHX: 85.2 ± 2.8%, n = 4 animals, 7 slices; *p = 0.014). **(B)** CHX treatment has no effect on DHPG-LTD in slices from *Tsc2*$^{+/-}$ mice (control: 86.3 ± 3.1%, n = 6 animals, 12 slices; CHX: 85.3 ± 3.2%, n = 4 animals, 7 slices, p = 0.796). ANOVA: genotype *p = 0.041, treatment p = 0.089, genotype x treatment *p = 0.045. **(C)** Presynaptic LTD is not affected by genotype or CHX (see also Figure 3.3). DHPG significantly increased PPF in slices from both WT and *Tsc2*$^{+/-}$ mice (PPF with a 50 ms inter-stimulus interval: WT baseline: 1.37 ± 0.02, WT DHPG: 1.59 ± 0.06, n = 5 animals, 9 slices, *p = 0.003; *Tsc2*$^{+/-}$ baseline: 1.39 ± 0.02, *Tsc2*$^{+/-}$ DHPG: 1.64 ± 0.03, n = 5 animals, 9 slices, *p = 0.001) and this effect was not blocked by CHX (WT DHPG + CHX: 1.58 ± 0.06, n = 7 animals, 11 slices, p = 0.89; *Tsc2*$^{+/-}$ DHPG + CHX: 1.64 ± 0.04, n = 6 animals, 7 slices, p = 0.94). **(D)** Metabolic labeling of hippocampal slices reveals a significant reduction of basal protein synthesis in *Tsc2*$^{+/-}$ mice (WT: 100.0 ± 3.1%, *Tsc2*$^{+/-}$: 88.2 ± 3.3%, n = 13 animals; *p = 0.043). Differences in protein synthesis are exemplified by representative autoradiograph and total protein stain of the same membrane. **(E)** Immunoblotting experiments show that Arc expression is significantly reduced in *Tsc2*$^{+/-}$ hippocampal slices (WT: 100.0 ± 4.7%, *Tsc2*$^{+/-}$: 76.6 ± 6.4%, n = 12 animals; *p = 0.005). **(F)** Arc translation was measured by metabolic labeling of hippocampal slices, followed by immunoprecipitation of Arc. Comparison of the ratios of ^{35}S-incorporated : total Arc reveals a significant reduction in Arc translation in the *Tsc2*$^{+/-}$ hippocampus (WT: 100.0 ± 11.5%, *Tsc2*$^{+/-}$: 74.7 ± 6.8%, n = 19 animals; *p = 0.0498).

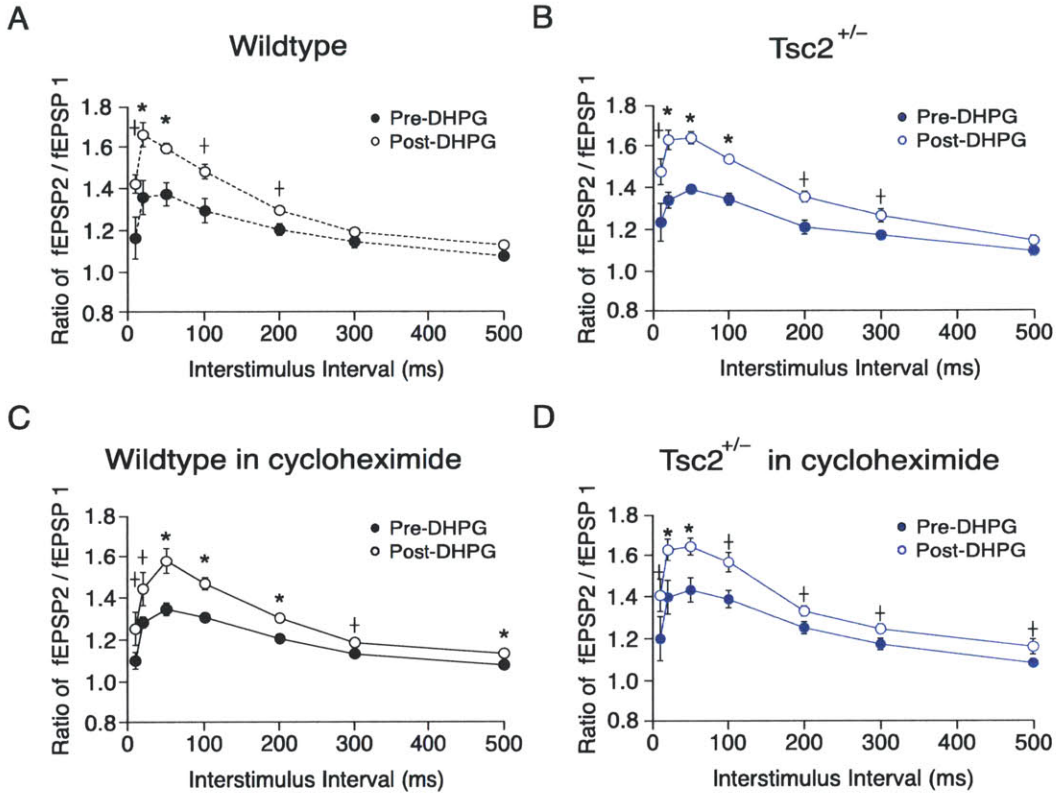

Figure 3.3 – Presynaptic component of DHPG-induced LTD. Pairs of stimulation at several different inter-stimulus intervals were delivered during the baseline period and 60 minutes post DHPG application in slices either pretreated with CHX or control ACSF. **(A,B)** DHPG significantly increased paired pulse facilitation (PPF) in slices from both wild-type **(A)** and *Tsc2*$^{+/-}$ mice **(B)** across many inter-stimulus intervals (WT, n = 5 animals totaling 9 slices; *Tsc2*$^{+/-}$, n = 5 animals totaling 9 slices; *$p < 0.01$, $^{\dagger}p < 0.05$). **(C,D)** The enhancement of PPF by DHPG is not affected by the protein synthesis inhibitor cycloheximide (WT, n = 7 animals totaling 11 slices; *Tsc2*$^{+/-}$, n = 6 animals totaling 7 slices; *$p < 0.01$, $^{\dagger}p < 0.05$). There was no difference in paired pulse ratio between wild-type and *Tsc2*$^{+/-}$ mice at baseline, post DHPG, or post DHPG + CHX. Error bars represent SEM.

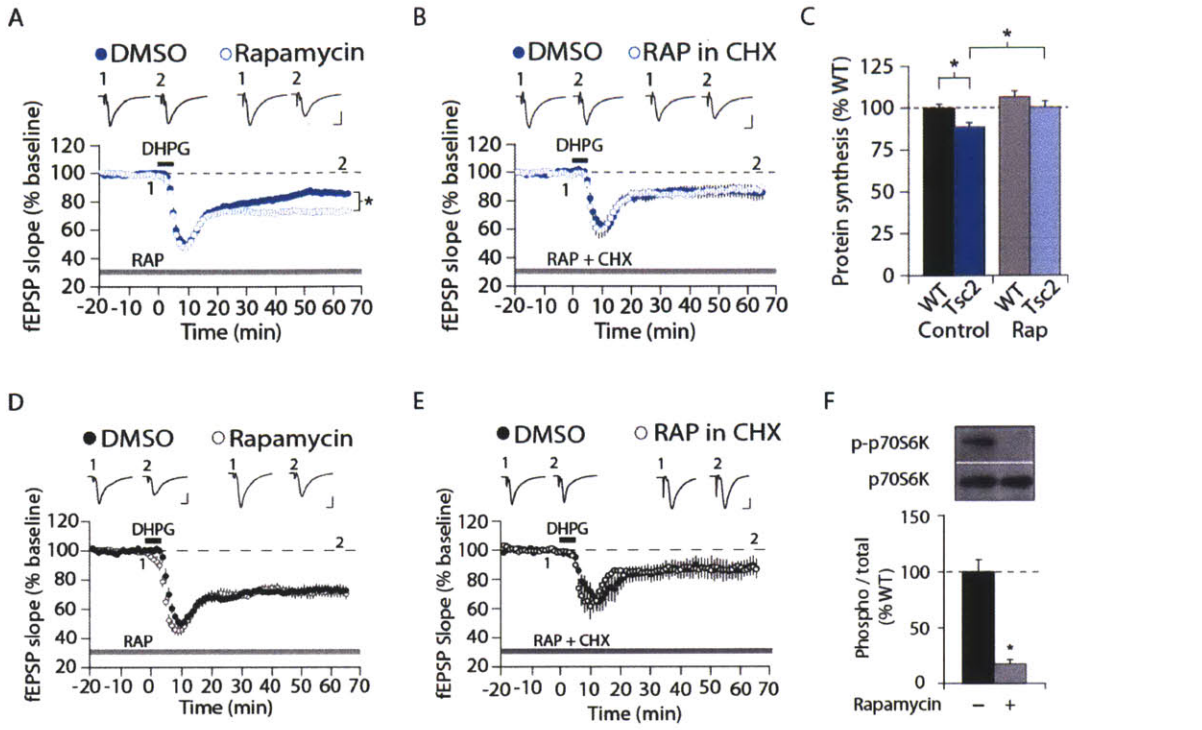

Figure 3.4 – Excessive mTOR activity suppresses the protein synthesis required for mGluR-LTD. (A) Pretreatment of slices with the mTORC1 inhibitor rapamycin (RAP, 20 nM, gray bar) significantly enhances DHPG-induced LTD in slices from *Tsc2*$^{+/-}$ mice (DMSO: 85.7 ± 2.1%, n = 8 animals, 17 slices; RAP: 72.9 ± 1.8%, n = 7 animals, 18 slices; *p = 0.002). **(B)** The rescue by rapamycin of DHPG-induced LTD in *Tsc2*$^{+/-}$ mice is prevented by the protein synthesis inhibitor cycloheximide (DMSO: 87.1 ± 4.7%, n = 6 animals, 10 slices; RAP: 88.1 ± 2.4%, n = 7 animals, 9 slices; p = 0.796). ANOVA: rapamycin treatment *p = 0.043, cycloheximide treatment *p = 0.004, rapamycin x cycloheximide *p = 0.018. **(C)** Metabolic labeling experiments show that rapamycin (20 nM) normalizes protein synthesis in the *Tsc2*$^{+/-}$ hippocampus to WT levels (WT DMSO: 100.0 ± 2.5%, WT RAP: 106.5 ± 3.6%, *Tsc2*$^{+/-}$ DMSO: 88.8 ± 2.6%, *Tsc2*$^{+/-}$ RAP: 100.4 ± 3.9%; ANOVA: genotype *p = 0.008, treatment *p = 0.006, genotype x treatment p = 0.430; t-test: WT vs. *Tsc2*$^{+/-}$ DMSO *p = 0.003; WT vs. *Tsc2*$^{+/-}$ RAP p = 0.344; *Tsc2*$^{+/-}$ DMSO vs. RAP *p = 0.037; n = 22 animals). Error bars represent SEM. **(D)** Pretreatment of slices from WT mice with rapamycin (RAP, 20 nM, gray bar) has no effect on DHPG-induced LTD in hippocampal slices from WT animals (DMSO: 73.2 ± 3.3%, n = 7 animals totaling 12 slices; RAP: 71.9 ± 4.1%, n = 7 animals totaling 12 slices; p = 0.807). Representative field potential traces (average of 10 sweeps) were taken at times indicated by numerals. Scale bars equal 0.5 mV, 5 ms. Error bars represent SEM. **(E)** Co-application of cycloheximide and rapamycin in WT slices still attenuates LTD (DMSO+CHX: 88.2% ± 11.8%, n = 3 animals totaling 5 slices; RAP+CHX: 87.4% ± 12.6%, n = 2 animals totaling 4 slices) **(F)** Rapamycin treatment robustly downregulates mTORC1 activity. Recovered hippocampal slices were incubated ± 20 nM rapamycin for 1 hour, then homogenized and processed for SDS PAGE. mTORC1 activity was assessed by measuring the phosphorylation of p70S6K (at Thr389), the direct substrate of mTORC1. Western blotting confirms that rapamycin robustly reduces p70S6K activation (control 100 ± 9%, rapamycin 15 ± 4%, *p = 0.0001; n = 13 animals). Error bars represent SEM.

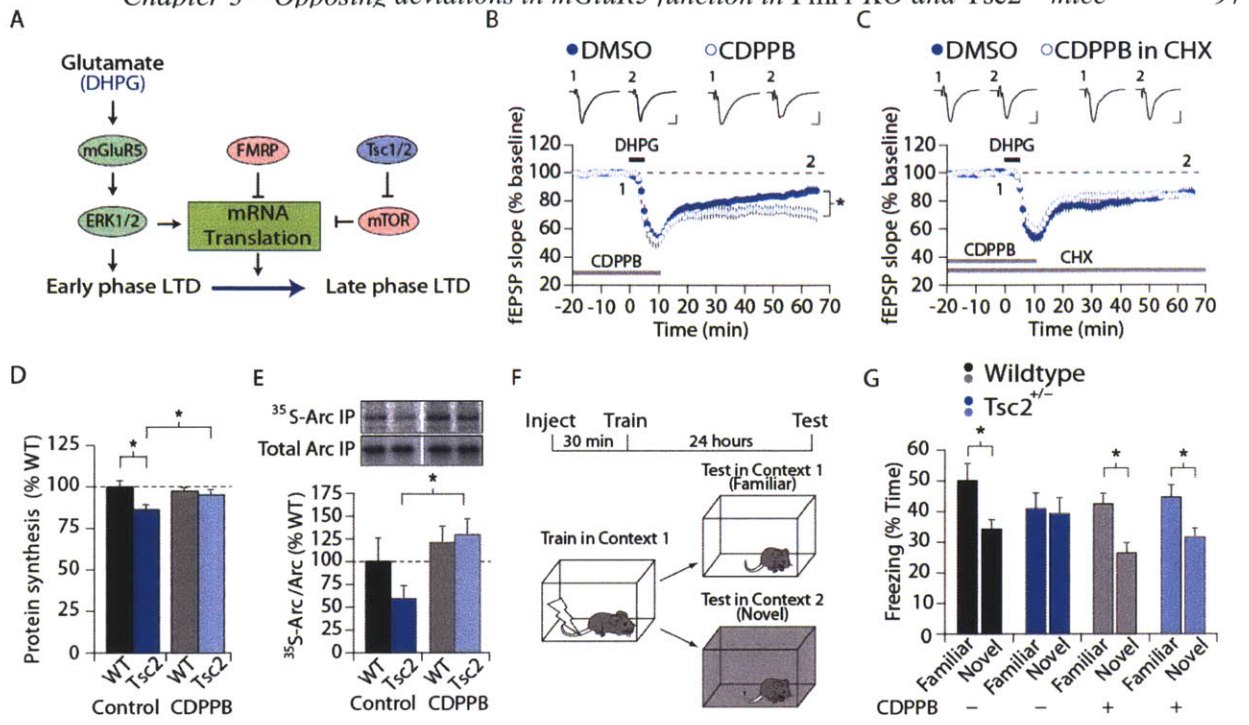

Figure 3.5 – Positive modulation of mGluR5 reverses synaptic and behavioral deficits in *Tsc2*$^{+/-}$ mice. **(A)** Model to account for effects of *Tsc2*$^{+/-}$ and *Fmr1*$^{-/y}$ mutations on mGluR5- and protein synthesis-dependent LTD. This model predicts that this impairment can be overcome either by inhibiting mTOR with rapamycin or by augmenting mGluR5 signaling with CDPPB, an mGluR5 PAM. **(B)** Pretreatment of slices from *Tsc2*$^{+/-}$ mice with CDPPB (10 μM, gray bar) significantly enhances DHPG-induced LTD (DMSO: 86.4 ± 2.5%, n = 8 animals, 13 slices; CDPPB: 71.7 ± 3.9%, n = 7 animals, 12 slices; *p < 0.001). **(C)** CDPPB treatment fails to enhance DHPG-induced LTD in *Tsc2*$^{+/-}$ mice when co-applied with the protein synthesis inhibitor cycloheximide (DMSO: 89.0 ± 4.4% n = 8 animals, 10 slices; CDPPB: 83.9 ± 2.1%, n = 7 animals, 9 slices; p = 0.64). ANOVA: CDPPB treatment *p = 0.008, CHX treatment p = 0.087, CDPPB x CHX *p = 0.034. **(D)** CDPPB (10 μM) restores protein synthesis in the *Tsc2*$^{+/-}$ hippocampus to WT levels (WT DMSO: 100.0 ± 3.2%, WT CDPPB: 97.2 ± 1.9%, *Tsc2*$^{+/-}$ DMSO: 86.1 ± 2.7%, *Tsc2*$^{+/-}$ CDPPB: 94.9 ± 3.0%; ANOVA: genotype *p = 0.006, treatment p = 0.275, genotype x treatment *p = 0.041; t-test: WT vs. *Tsc2*$^{+/-}$ DMSO *p = 0.012; WT vs. *Tsc2*$^{+/-}$ CDPPB p = 0.538; *Tsc2*$^{+/-}$ DMSO vs. CDPPB *p = 0.049; n = 17 animals). **(E)** CDPPB exposure significantly increases Arc translation in the *Tsc2*$^{+/-}$ hippocampus (WT DMSO 100.0 ± 28.2%, WT CDPPB 121.0 ± 21.2%, *Tsc2*$^{+/-}$ DMSO 59.2 ± 7.0%, *Tsc2*$^{+/-}$ CDPPB 129.4 ± 20.3%; ANOVA genotype p = 0.554, treatment *p = 0.0094, genotype x treatment p = 0.114; t-test: *Tsc2*$^{+/-}$ DMSO vs. CDPPB *p = 0.026; n = 6 animals). Error bars represent SEM. **(F)** Experimental design of context discrimination task. **(G)** WT mice display intact memory by freezing more in the familiar context than the novel context (Black bars; Familiar: 50 ± 7.7%, n = 12; Novel: 34.1 ± 3.2%, n = 14; *p = 0.003). A single injection of CDPPB (10 mg/kg, i.p.) 30 minutes prior to training has no effect on WT context discrimination (Familiar: 42.3 ± 3.7%, n = 12; Novel: 26.4 ± 3.6%, n = 12; *p = 0.005). Control *Tsc2*$^{+/-}$ mice display a significant impairment in context discrimination (Blue bars; Familiar: 40.9 ± 5.3%, n = 11; Novel: 39.3 ± 5.2%, n = 14; p = 0.501), but this deficit is corrected by a single injection of CDPPB (Familiar: 44.5 ± 4.3%, n = 11; Novel: 31.6 ± 3%, n = 12; *p = 0.034). Error bars represent SEM.

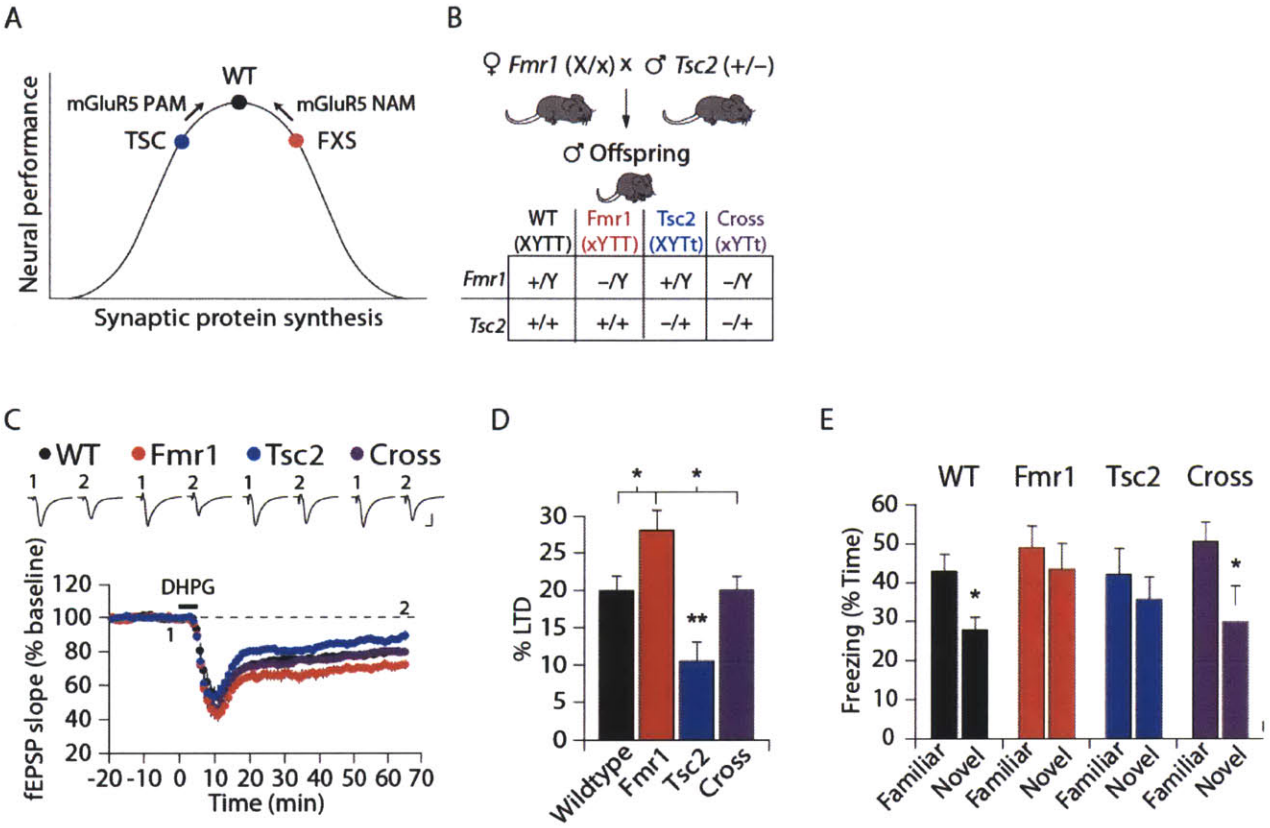

Figure 3.6 – Genetic cross of *Tsc2*$^{+/-}$ and *Fmr1* KO mice rescues synaptic and behavioral impairments present in both single mutants. **(A)** The data suggest that mutations in TSC and FXS cause opposing deviations in synaptic function which impairs neuronal performance and respond to opposite alterations in mGluR5 signaling(Bear et al., 2008; Kelleher and Bear, 2008). These results raise the possibility that introducing both mutations to a mouse may normalize aspects of neural function. **(B)** Genetic rescue strategy. Heterozygous *Tsc2* male mice (*Tsc2*$^{+/-}$) were bred with heterozygous *Fmr1* females (*Fmr1* x^{+}/x^{-}) to obtain male offspring of four genotypes: wild type (*Tsc2*$^{+/+}$, *Fmr1*$^{+/y}$), *Fmr1* KO (*Tsc2*$^{+/+}$, *Fmr1*$^{-/y}$), *Tsc2* Het (*Tsc2*$^{+/-}$, *Fmr1*$^{+/y}$), and Cross (*Tsc2*$^{+/-}$, *Fmr1*$^{-/y}$). **(C)** DHPG-induced LTD is significantly decreased in slices from *Tsc2*$^{+/-}$ mice (*p = 0.002) and significantly increased in slices from *Fmr1*$^{-/y}$ mice (*p = 0.017), as compared to WT slices. DHPG-LTD in slices from *Tsc2*$^{+/-}$ x *Fmr1*$^{-/y}$ mice is comparable in magnitude to WT slices (p = 0.558). (WT: 78.9 ± 2.1%, n = 7 animals, 17 slices; *Fmr1*: 71.2 ± 2.7%, n = 7 animals, 21 slices; *Tsc2*: 89.5 ± 2.6%, n = 7 animals, 15 slices; Cross: 77.4 ± 1.8%, n = 9 animals, 19). **(D)** Summary of LTD data. Bar graphs represent percent decrease from baseline in fEPSP (average of last 5 minutes of recording ± SEM); *p < 0.05, **p < 0.01. **(E)** Both mutations cause a deficit in context discrimination that is rescued in the double mutant. WT mice (Familiar: 42.9 ± 4.6%, n = 11; Novel: 27.8 ± 3.4%, n = 12; *p = 0.024) , *Fmr1*$^{-/y}$ mice (Familiar: 49.0 ± 5.6%, n = 11; Novel: 43.5 ± 6.7%, n = 12; p = 0.483), *Tsc2*$^{+/-}$ (Familiar: 42.1 ± 6.8%, n = 12; Novel: 35.6 ± 6.0%, n = 12; p = 0.395) and *Tsc2*$^{+/-}$ x *Fmr1*$^{-/y}$ mice (Familiar: 50.5 ± 5.2%, n = 11; Novel: 29.8 ± 5.2%, n = 11; *p = 0.011). Error bars represent SEM.

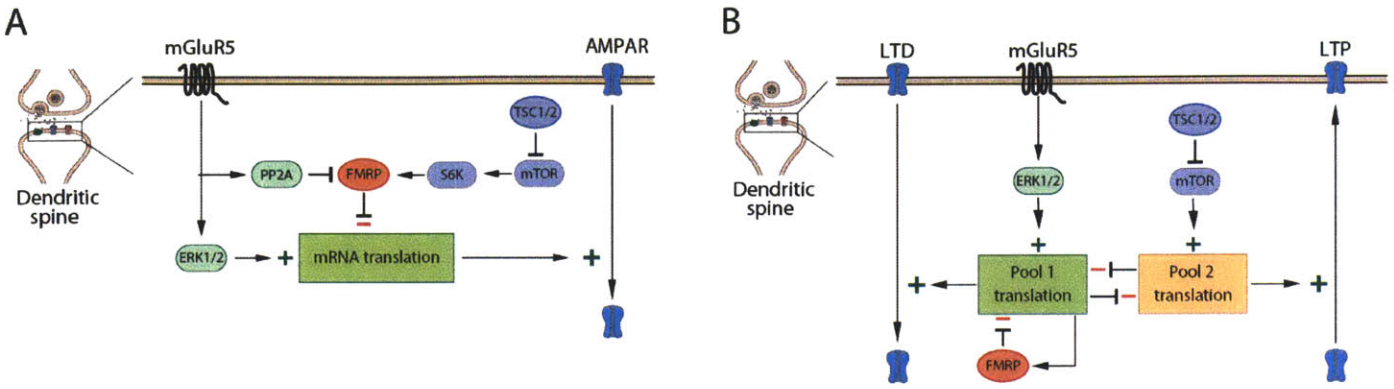

Figure 3.7 – Models to account for opposing alterations in mGluR responses in *Tsc2*$^{+/-}$ and *Fmr1* KO mice. (A) Hyperphosphorylation of FMRP model. Phosphorylation of FMRP is thought to enhance its translation repressing abilities. mTOR is known to regulate FMRP phosphorylation via is downstream effector S6K. In *Tsc2*$^{+/-}$ mice, the tonic inhibition of mTOR normally imposed by TSC1/2 is absent, resulting in chronic over-activation of mTOR. This excessive mTOR activation may lead to hyperphosphorylation of FMRP and suppress the synthesis of FMRP targets, subsequently leading to deficient mGluR-LTD and decreased protein synthesis rate. Some predictions of this model would be increased phoso-FMRP levels in *Tsc2*$^{+/-}$ mice and restoration of LTD and protein synthesis levels in these mice by expression of a phospho-resistance FMRP. Activation of mGluR5 has been demonstrated to de-phosphorylate FMRP, suggesting that mGluR5 PAM treatment may restore LTD and protein synthesis rates by enhancing the activity of PP2A. **(B)** Two pools model. Two pools of mRNA exist at the synapse whose translation is mutually exclusive. mGluR-mediated ERK activation results in synthesis of proteins required for LTD and regulated by FMRP (Pool 1). mTOR regulates synthesis of a separate, competing pool of proteins required for expression of L-LTP that is regulated by TSC1/2 (Pool 2). Consistent with this idea, de-repression of Pool 1 (by deletion of *Fmr1*) results in exaggerated LTD while de-repression of Pool 2 (by reduction in *Tsc2*) results in enhanced LTP. Potential mechanisms for mutual inhibition may involve differential regulation of initiation and/or elongation by the ERK and mTOR pathway or competition for translation machinery.

Chapter 4

β-arrestin2 signaling mediates mGluR-dependent ERK activation and long term synaptic depression

4.1 Abstract

Evidence from genetic and molecular studies has demonstrated that altered synaptic protein synthesis downstream of metabotropic glutamate receptor 5 (mGluR5) may be a common disruption in autism spectrum disorders (ASD). This work has suggested a variety of targets, one of the most promising being direct manipulation of mGluR5 activity. However, there is a concern that global manipulation of mGluR5 will also affect processes that are unrelated to autism pathophysiology, resulting in side effects that will sharply limit the utility of this approach. Ideally, pharmacological treatments should specifically interfere with the signaling pathways that regulate protein synthesis leaving other arms of signaling unaffected. In this study, we determined if β-arrestin signaling is a critical link between mGluR5 activation and mGluR-mediated protein synthesis and synaptic plasticity. β-arrestins are adaptor proteins that are important for the regulation of G-protein coupled receptors (GPCRs), such as mGluR5, and recently have been shown to be directly involved in a novel form of GPCR signaling that is independent of G-protein activation. Specifically, β-arrestins have been shown to recruit the extracellular regulated kinase (ERK) pathway required for mGluR-dependent protein synthesis and synaptic plasticity. We found that mGluR-mediated ERK activation was severely disrupted in β-arrestin 2 heterozygous and homozygous KO mice. The consequences of this disruption were functionally relevant, as the protein synthesis-dependent component of mGluR-LTD was also absent in these mice. Thus, targeting β-arrestin-mediated signaling may be a way to specifically modulate dysregulated protein synthesis associated with ASD, potentially leading to the next generation of selective drugs for the treatment of ASD.

4.2 Introduction

In Chapter 2 we demonstrated that many processes that rely on mGluR-mediated protein synthesis are exaggerated in the *Fmr1* KO mouse, including mGluR-LTD, and it has been suggested that this exaggeration may account for the diverse phenotypes associated with fragile X (FX) (Bear et al., 2004). While the role of aberrant neuronal protein synthesis and mGluR5 activity in the pathophysiology of FX is well characterized, we demonstrated in Chapter 3 that disruptions in the mGluR pathway do not appear to be limited to this disorder. In a mouse model of tuberous sclerosis complex (TSC), the *Tsc2*$^{+/-}$ mouse, there is diminished synaptic protein synthesis and mGluR-LTD, suggesting that genetically heterogeneous causes of autism spectrum disorders (ASD) and intellectual disability (ID) may produce similar deficits through bidirectional deviations in mGluR-mediated protein synthesis (Figure 3.6). Recent studies have now demonstrated disrupted mGluR function in numerous animal models of syndromic and non-syndromic ASD (Bangash et al., 2011; Baudouin et al., 2012; Chen et al., 2011; Huber et al., 2002; Phelan and McDermid, 2012), and human genetic studies have found that the protein products of many genes embedded in the mGluR signaling pathway, including mGluR5 itself, are associated with ASD (Iossifov et al., 2012; Kelleher Iii et al., 2012) (Skafidas et al., 2012). Thus, there is an abundance of evidence to suggest that mGluR-mediated protein synthesis is a common molecular pathway disrupted in ASD, making mGluR5 an attractive target for the treatment of ASD.

Positive and negative allosteric modulators (PAMs and NAMs) of mGluR5 represent a promising class of drugs for the treatment of ASD. Allosteric modulators are drugs that do not directly activate or inhibit a receptor, but rather modulate the receptors' response to endogenous activation, thereby modulating activity in a physiologically relevant way (Conn et al., 2009). Furthermore, because these molecules do not bind to the orthosteric ligand binding site, which is highly conserved between receptor subtypes, allosteric modulators with high subtype specificity can be developed (Conn et al., 2009). Studies in the mouse models of FX and TSC have already demonstrated the potential of using allosteric modulators of mGluR5 as a treatment for ASD. For example, chronic treatment of *Fmr1* mice with the mGluR5 NAM CTEP was shown to correct not only mGluR-LTD and protein synthesis, but also a host of behavioral phenotypes (Michalon et al., 2012). In line with this view, preliminary human clinical trials using mGluR5 NAMs have shown promise in the treatment of some symptoms associated with FX (Hagerman

et al., 2012). In the previous chapter, we demonstrated that augmentation of mGluR5 with the PAM CDDPB rescues biochemical, electrophysiological and behavioral impairments in *Tsc2*$^{+/-}$ mice. Thus, positive modulators of mGluR5 may prove to be similarly beneficial in patients with TSC.

Stimulating protein synthesis, however, is only one of the many functions of mGluR5 signaling. mGluR5 activation has a diverse array of cellular effects, including 2nd messenger recruitment, mobilization of intracellular calcium (Ca^{2+}) stores, modulation of ion channels, and the synthesis of endocannabinoids (Hermans and Challiss, 2001). Therefore, global manipulation of mGluR5 activity, such as with the PAMs and NAMs described above, is likely to affect some or all of these processes. Since evidence suggests that a primary pathogenic culprit in ASD is altered protein synthesis, not mGluR signaling *per se*, therapies for ASD should, ideally, specifically target mGluR-mediated protein synthesis while leaving these other cellular processes unaffected. Indeed, there are reports that mGluR5 antagonism can result in unwanted side effects (Olive, 2009) and these could jeopardize the success of clinical trials. Therefore, it is of utmost importance to understand the mechanisms which couple mGluR5 activation to protein synthesis in order to develop more selective therapeutic interventions in ASD.

4.2.1 Signaling pathways that couple mGluRs to protein synthesis

Although mGluR5 is a well-established activator of protein synthesis (Job and Eberwine, 2001; Osterweil et al., 2010; Weiler and Greenough, 1993), it remains uncertain how activation of the receptor regulates this process. Canonically, mGluR5 signaling occurs through the G_q-dependent activation of phospholipase Cβ (PLCβ), which hydrolyzes phosphoinositides (PI) leading to increases in diacyl glycerol (DAG), which subsequently activates protein kinase C (PKC) and D (PKD), and inositol triphosphate (IP3), whose receptor activation leads to intracellular Ca^{2+} release (Figure 4.1A)(Abe et al., 1992). However, the role of this canonical pathway in mGluR-mediated protein synthesis and LTD is unclear. While mGluR-LTD is deficient in G_q knock out (KO) mice (Kleppisch et al., 2001), acute inhibition of PLCβ or PKC does not affect mGluR-LTD or protein synthesis (Mockett et al., 2011; Schnabel et al., 1999). Furthermore, mGluR-LTD is not Ca^{2+}-dependent as it can be evoked in the presence of Ca^{2+} chelators, with depletion of intracellular Ca^{2+} stores, and even in a Ca^{2+} free medium (Fitzjohn et

al., 2001; Gladding et al., 2009). Thus, mGluR-mediated protein synthesis may be mediated in a G-protein independent manner.

On the other hand, mGluR activation has been shown to couple the ERK and mTOR pathways (Figure 1.1), whose activation has proven to be crucial for mGluR-dependent protein synthesis and LTD (Gallagher et al., 2004; Hou and Klann, 2004; Osterweil et al., 2010). Targeting the ERK and mTOR pathways has been investigated as a potential avenue for treatment in several ASD-associated disorders, and general inhibitors of mTOR or ERK have indeed shown promise in some animal models (Ehninger et al., 2008a; Li et al., 2005; Osterweil et al., 2013). However, the mTOR and ERK pathways are ubiquitously activated in all cell types and are important for basic processes such as cell cycling and growth (Cargnello and Roux, 2011). Therefore, as with mGluR5-targeted therapies, manipulating this pathway may cause potentially severe side effects (Tsai et al., 2013). The ideal pharmacological treatment would be a neuronal receptor (e.g. mGluR5) based therapeutic agent that specifically interferes with the signaling pathways that regulate protein synthesis while leaving the other arms of signaling unaffected. Thus, it is imperative to understand the mechanisms that couple mGluR5 activation to mTOR and ERK signaling in order to identify novel therapeutic targets in ASD.

Recent evidence has suggested that mGluR5 activation is coupled to the mTOR pathway through interactions with the post-synaptic scaffolding protein Homer (Ronesi and Huber, 2008). Homer can recruit the small GTPase phosphoinositide-3 kinase enhancer (PIKE) to directly activate PI3K, leading to subsequent activation of the mTOR pathways (Figure 1.1). Disrupting mGluR-Homer interactions specifically inhibits DHPG-induced PI3K and mTOR activation without affecting ERK signaling, suggesting that manipulation of this interaction may be a specific way to regulate mGluR-dependent mTOR activation. However, studies in the *Tsc2*$^{+/-}$ mouse (presented in Chapter 3) suggest that mTOR activation may actually suppress the synthesis of LTD proteins (Figure3.5a). Therefore, disrupting this interaction may actually lead to excess protein synthesis downstream of mGluR activation.

Consistent with this idea, mGluR5 is less associated with Homer in *Fmr1* KO mice and there is deficient mGluR-mediated mTOR activation (Ronesi et al., 2012). Increasing mGluR-Homer interactions rescues some aspects of exaggerated protein synthesis in these mice, once again suggesting that increasing mTOR activation may negatively regulate the protein synthesis exaggerated in *Fmr1* KO mice. However, restoring mGluR-Homer interactions in *Fmr1* KO

mice does not rescue exaggerated LTD or excess levels of specific LTD proteins, such as Arc and Map1b (Ronesi et al., 2012). Therefore, alterations in mGluR-mTOR signaling are likely to be a secondary consequence of disrupted protein synthesis in the *Fmr1* KO mice. Thus, while increasing mGluR-mediated mTOR activation via Homer interactions may be a beneficial avenue for therapy in an indirect manner, it does not directly target the dysregulated protein synthesis that is likely to be pathogenic.

The ERK pathway is a well-established mediator of mGluR-dependent protein synthesis and LTD (Gallagher et al., 2004; Osterweil et al., 2010), and manipulation of several components of this pathway has proved to be therapeutic in the mouse model of FX. Previous work has shown that there is a hypersensitivity to ERK signaling in *Fmr1* KO mice, and inhibition of this pathway ameliorates excessive protein synthesis in these animals (Osterweil et al., 2010). Furthermore, mild inhibition of Ras, an upstream regulator of ERK (Figure 1.1), ameliorates several FX phenotypes in *Fmr1* KO mice at the molecular, cellular, and behavioral level (Osterweil et al., 2013).

Despite the strong support for the role of the ERK activation in mGluR-mediated protein synthesis, how mGluR5 may couple to the ERK signaling cascade is poorly understood. While it is known that G_q-protein activation can result in ERK phosphorylation via PKC signaling (Ueda et al., 1996), mGluR activation in the hippocampus has been shown to recruit the ERK pathway even in the presence of PLC inhibitors (Ronesi et al., 2012). Moreover, the fact that inhibition of PKC or PLCβ does not disrupt mGluR-mediated protein synthesis (Mockett et al., 2011) or LTD (Gallagher et al., 2004; Schnabel et al., 1999) suggests this G-protein dependent pathway is not required for mGluR-mediated, ERK-dependent protein synthesis. Therefore, it is likely that mGluR5-mediated ERK activation is achieved via a G-protein independent mechanism. The aim of this study is to determine if β-arrestins are the critical link between mGluR5 signaling and subsequent activation of the ERK pathway, and thus a way to specifically target mGluR-mediated protein synthesis and synaptic plasticity.

4.2.2 β-arrestin-mediated signaling

β-arrestins are adaptor proteins that are important regulators of GPCR signaling and cycling. Recently they have been shown to be directly involved in a novel form of GPCR signaling that is independent of G-protein activation and which has distinct biochemical and

functional consequences (Shenoy and Lefkowitz, 2011). The binding of β-arrestins to GPCRs is a requisite step for agonist-induced desensitization and internalization, resulting in the termination of G-protein signaling (Figure 4.1B) (Shenoy and Lefkowitz, 2011). However, by acting as a scaffold, β-arrestins can also recruit signaling molecules to the receptor, leading to the G-protein independent activation of these signaling pathways (Figure 4.1C) (Pierce and Lefkowitz, 2001). In this way, β-arrestins can dissociate GPCR signaling from its G-protein counterpart, inhibiting canonical G-protein pathways while concurrently activating distinct G-protein-independent signaling cascades (Figure 4.1C). Interestingly, one of the most prominent pathways recruited by this β-arrestin-mediated signaling is the ERK pathway (Daaka et al., 1998; Luttrell et al., 2001).

β-arrestins can provide a scaffold for Raf, MEK, ERK, and MNK (DeWire et al., 2008; Luttrell et al., 2001), suggesting their binding to GPCRs may regulate mRNA translation via the ERK pathway (Figure 4.1C). In fact, β-arrestins have been shown to directly mediate protein synthesis in an ERK-dependent manner (DeWire et al., 2008). This suggests the intriguing possibility that protein synthesis downstream of mGluR5 is mediated by β-arrestin-dependent ERK activation, and therefore dissociable from other G-protein-dependent mGluR processes (Figure 4.1C). If there is indeed this bifurcation in mGluR5 signaling, targeting the β-arrestin pathway may be a way to specifically modulate the dysregulated protein synthesis and accompanying synaptic and behavioral deficits observed in ASD without affecting other mGluR-mediated processes. Importantly, there is a well-developed class of pharmacological agents that preferentially target β-arrestin signaling over concurrent G-protein signaling, or *vice versa*—the so called 'biased ligands' (Violin and Lefkowitz, 2007). Therefore, if β-arrestins are critically involved in mGluR-mediated protein synthesis, it may be possible to develop more targeted therapeutic agents for the treatment of ASDs without the side effects of current receptor-based therapies or broad-spectrum signaling inhibitors.

There are two known neuronal isoforms of β-arrestin, β-arrestin1 and 2. As of yet, there is no systematic way to identify which β-arrestins bind to which GPCRs as both isoforms associate with a variety of classes of GPCRs (DeWire et al., 2007). Currently, it is unknown which of the isoforms, or both, associate with mGluR5 (De Blasi et al., 2001). However, there were several reasons we chose to explore the role of β-arrestin2 in mGluR-mediated protein

synthesis and plasticity over β-arrestin1. First, β-arrestin2 is more promiscuous, binding to a larger number of GPCRs (Kohout et al., 2001). Additionally, while both isoforms have been shown to mediate cell signaling independent of G-protein activation, there is stronger evidence to support the role of β-arrestin2 in modulating ERK activation and in directly stimulating protein synthesis, particularly downstream of G_q-coupled receptors like mGluR5 (DeWire et al., 2007; DeWire et al., 2008). Thirdly, β-arrestin2 is the more highly expressed isoform in the hippocampus (Attramadal et al., 1992)(http://mouse.brain-map.org). Therefore, in the experiments described in this chapter, we characterized the effect of genetically reducing β-arrestin2 on mGluR function in the hippocampus. However, this does not preclude the involvement of β-arrestin1 in regulating mGluR function, particularly in other brain areas (Dhami and Ferguson, 2006). We found that mGluR-mediated ERK activation was severely disrupted in β-arrestin2 heterozygous (*Arrb2*$^{+/-}$) and homozygous (*Arrb2*$^{-/-}$) KO mice. The consequences of this disruption were functionally relevant, as the protein synthesis-dependent component of mGluR-LTD was also absent in these mice. The results presented here demonstrate that ERK activation downstream of mGluR5 is dependent upon β-arrestin-mediated signaling, and thus may be a fundamental link between mGluRs and the dysregulated protein synthesis in ASD.

4.3 Results

We first tested the hypothesis that β-arrestin2 is required for mGluR-induced ERK activation in the hippocampus (Figure 4.2). Hippocampal slices from wild-type (WT), *Arrb2*$^{+/-}$, and *Arrb2*$^{-/-}$ mice were stimulated with the selective mGluR1/5 agonist (S)-dihydroxyphenylglycine (DHPG, 25 uM) for 5 minutes, and levels of ERK phosphorylation were assessed immediately, 15 minutes, and 30 minutes following DHPG treatment (Figure 4.2A). We found no difference in basal ERK phosphorylation (pERK) or total protein levels between slices obtained from WT and *Arrb2* mutant mice (Figure 4.2B,C). DHPG treatment induced a rapid and robust increase in pERK within 5 minutes in WT slices that was sustained for at least 15 minutes post-DHPG stimulation (Figure 4.2A). In contrast, DHPG-induced ERK activity was markedly attenuated in slices from both *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice as compared to WT, with pERK levels returning to baseline levels within 15 minutes for both mutants (Figure 4.2A). The

residual ERK activation seen in the *Arrb2* mutants is likely due to G-protein mediated signaling (Wei et al., 2003).

β-arrestins have been shown to mediate several signaling cascades in addition to ERK, including the AKT-mTOR pathway that has been implicated in the regulation of protein synthesis and mGluR-LTD (DeWire et al., 2007; Gladding et al., 2009). There was no difference in phosphorylation of Akt, mTOR, or its downstream target p70 S6 kinase between the WT and the *Arrb2* mutants (Figure 4.3). Furthermore, mGluR activation did not recruit the mTOR pathway and reduction of *Arrb2* did not affect stimulated activity of this pathway (Figure 4.3). This is consistent with our previous results demonstrating mTOR signaling is not required for the expression of mGluR-LTD (Figure 3.4).

ERK activation is required for mGluR-dependent protein synthesis and mGluR-LTD. As expected, the same DHPG stimulation that increased pERK levels in WT slices also resulted in significant LTD (Figure 4.4 A,D). This WT mGluR-LTD was significantly reduced when DHPG was applied in the presence of the protein synthesis inhibitor cycloheximide (CHX, 60μM; Figure 4.4 A,D), demonstrating its protein synthesis dependency. In slices from *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice, however, mGluR-LTD was significantly decreased as compared to WT levels and the magnitude of mGluR-LTD was unaffected by CHX treatment (Figure 4 B,C). As with our previous studies, the residual LTD remaining in *Arrb2* deficient or CHX treated slices is likely due to changes in presynaptic function (Figure 3.3). The data presented here demonstrate that the protein synthesis-dependent component of mGluR-LTD is absent in *Arrb2*$^{-/-}$ mice. Moreover, they suggest that β-arrestin 2 plays a prominent role not only in mediating mGluR-dependent ERK activation but LTD as well.

There have been few studies to date examining the effect of β-arrestin deletion on synaptic function. Determining whether synaptic function is affected by β-arrestin 2 deletion is critical for interpreting the phenotypes observed in mGluR-mediated plasticity. We found there was no difference in basal synaptic transmission, as measured by input-output relationship, or presynaptic function, as measured by paired-pulse ratio (Figure 4.5 A,B). This suggests that there are no gross alterations in synaptic function that may account for the deficiency in mGluR-mediated plasticity observed in the *Arrb2* mutants. Furthermore, we found no difference in the magnitude of NMDAR-dependent LTD (Figure 4.5C), which is a mechanistically distinct form of LTD that does not require ERK activation or protein synthesis for its initial maintenance.

This demonstrates that the disruption of mGluR-LTD is not due to a global disruption in the ability of *Arrb2* deficient synapses to undergo activity-dependent depression.

4.4 Discussion

In this study, we examined the role of β-arrestin 2 in mGluR signaling in the hippocampus. We found that mGluR-dependent ERK activation is deficient in *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice. No differences were found in the recruitment of other signaling pathways, namely the mTOR pathway, suggesting that β-arrestin 2 specifically couples mGluR activation to the ERK cascade. The disruption in ERK activation appears to have functional consequences as well, as *Arrb2* deficiency was accompanied by diminished mGluR-LTD, likely due to the loss of the protein synthesis-dependent component of this form of plasticity. As ERK activation is a robust upstream indicator and LTD is a sensitive downstream consequence of mGluR-mediated protein synthesis, these results suggest that β-arrestin 2 may couple mGluR activation to mRNA translation. However, more work must be done to fully characterize β-arrestin signaling downstream of mGluR function before these implications can be fully realized.

While β-arrestins have been shown to mediate GPCR-dependent cell signaling that is independent of G-protein activation, they also act as important regulators of agonist-induced GPCR desensitization and internalization (Figure 4.1). Therefore genetic deletion of *Arrb2* is likely to affect G-protein-dependent signaling downstream of mGluR5 activation in addition to disrupting β-arrestin-mediated processes. Determining whether canonical mGluR signaling is affected by *Arrb2* deletion is critical for proper interpretation of the cellular phenotypes observed in this study. It will therefore be essential to examine G-protein-dependent mGluR-mediated processes, such as PI turnover, Ca^{2+} mobilization or PKD phosphorylation in the *Arrb2* mutants. PKD phosphorylation is a particularly promising assay, as it is PLC- and PKC-dependent but also specific to mGluR5 activation (Krueger et al., 2010).

β-arrestin 2 regulates the function of many GPCRs (Pierce and Lefkowitz, 2001) and therefore germ line deletion of *Arrb2* likely affects many processes. The fact that mGluR-mediated ERK activation and LTD were specifically disrupted in *Arrb2* mutants, without the alteration of basal ERK signaling or synaptic function, suggests that β-arrestin 2 directly mediates mGluR function. However, more work is required to explicitly demonstrate β-

arrestin2-mGluR5 interaction. Acute manipulation of this coupling may lend greater insight to the role of β-arrestin 2 in mGluR signaling. It will be important in future studies to specifically delineate between G-protein- and β-arrestin-dependent signaling in mGluR function. Ideally this could be achieved by pharmacological agents with biased signaling (see below), however it is also possible to engineer G_q-coupled receptors that have biased activation towards either G-protein (Lan et al., 2009) or β-arrestin signaling (Wei et al., 2003). These mutant receptors would be able to distinguish the relative contributions of G-protein and β-arrestin signaling to mGluR function in the hippocampus and would help define the mechanisms of mGluR-mediated protein synthesis and LTD.

4.4.1 Implications for mGluR signaling and LTD

Activation of several neuronal GCPRs has been shown to recruit β-arrestin-dependent signaling (Del'guidice et al., 2011; Zheng et al., 2008), including several mGluR subtypes (Emery et al., 2012; Gu et al., 2012). However, to our knowledge, this is the first demonstration that β-arrestin 2 is involved in mGluR5-dependent signaling in the hippocampus. An intriguing implication of this result is that receptor internalization is required for mGluR-mediated ERK activation and LTD (Figure 4.1C). This may impose some distinctive qualities onto mGluR-mediated ERK signaling, providing novel insights into mGluR function and the mechanisms of mGluR-LTD.

β-arrestin-mediated signaling has several distinct temporal and spatial aspects compared to traditional G-protein-dependent signaling, and this may influence the nature of mGluR5 coupling to the ERK cascade and protein synthesis machinery. In most cases, G-protein-dependent ERK activation is rapid and succinct. Peak levels of ERK activity occur within 2 minutes of receptor activation and return to baseline levels by 10 minutes (Ahn et al., 2004). Conversely, β-arrestin-dependent signaling typically has a slower onset, as it is recruited to terminate G-protein signaling, and results in more sustained ERK activation (Ahn et al., 2004). mGluR-LTD is thought to rely on rapid translation of mRNA at the synapse, and it has been estimated that new protein synthesis is required within minutes of receptor activation (Huber et al., 2000). The results presented here reveal a deficiency in DHPG-induced ERK activity in as soon as 5 minutes following mGluR activation, suggesting β-arrestin-dependent ERK activation is sufficiently rapid to mediate the protein synthesis requirements of mGluR-LTD.

β-arrestin-dependent ERK signaling is also spatially distinct from its G-protein-dependent counterpart. ERK activated by G-proteins generally accumulates in the nucleus, regulating transcription factors such as Elk-1 (Tohgo et al., 2002). In contrast, ERK signaling mediated by β-arrestins is generally excluded from the nucleus and instead confined to the cytoplasmic compartment (Ahn et al., 2004). This difference in localization likely results in distinct physiological consequences for ERK activation mediated by β-arrestins compared to G-proteins. Indeed, overexpression of β-arrestins inhibits G_q-dependent Elk-1 activation in the nucleus, while simultaneously enhancing cytosolic ERK signaling (Luttrell et al., 2001). The restriction of β-arrestin-activated ERK to the cytosol indicates that it is particularly well-placed to mediate dendritic mRNA translation downstream of mGluR activation. In fact, β-arrestin-mediated signaling is known to regulate protein synthesis in an ERK-dependent manner (DeWire et al., 2008). Further experiments will be required to determine if hippocampal mGluR-mediated β-arrestin signaling follows this same spatially localized pattern. If it does, modulation of β-arrestin-dependent mGluR signaling may be a way to specifically manipulate the synaptic protein synthesis required for LTD and implicated in the pathophysiology of ASD.

β-arrestins have been shown to scaffold a wide variety of proteins (Shenoy and Lefkowitz, 2011). Aside from moderating signal cascades, β-arrestin-mGluR interactions may regulate additional processes, which may provide some novel insight into the mechanisms of LTD. As we have demonstrated, there are two independent mechanisms for the expression of mGluR-LTD: reduced probability of presynaptic glutamate release (Fitzjohn et al., 2001; Mockett et al., 2011; Nosyreva and Huber, 2005) and reduced expression of postsynaptic AMPA receptors (Luscher and Huber, 2010; Nosyreva and Huber, 2005). Even within this postsynaptic LTD there is a further segregation in mechanism. While rapid protein synthesis is required to stabilize AMPAR internalization, there is an initial endocytosis that is not protein synthesis-dependent (Snyder et al., 2001). Recent evidence suggests that LTD results in lateral diffusion of AMPARs that are then internalized from a perisynaptic location (Lu et al., 2007; Petrini et al., 2009), which is also where mGluRs are most highly expressed (Lujan et al., 1996). It is possible then that β-arrestins may scaffold mGluRs with local AMPARs, thus concurrently internalizing AMPARs with mGluRs, and thereby regulating both the initial protein synthesis-independent AMPAR endocytosis as well the subsequent protein synthesis-dependent component via ERK

activation. Analysis of the mGluR-β-arrestin complex and associated proteins may help uncover mechanisms of mGluR-dependent AMPAR internalization yet to be elucidated.

4.4.2 Implications for ASD treatment

Alterations in hippocampal protein synthesis and mGluR-LTD have previously been utilized to gain insight into the pathophysiology of several mouse models of autism (Auerbach et al., 2011; Huber et al., 2002; Michalon et al., 2012). In particular, increases in protein synthesis and LTD downstream of mGluR5 are thought to be pathogenic in *Fmr1* KO mice (Chapters 2). In Chapter 3 we demonstrated there were decreases in these processes in the *Tsc2*$^{+/-}$ mice and, remarkably, introducing both *Fmr1* and *Tsc2*$^{+/-}$ mutations restored proper LTD levels (Chapter 3). The LTD phenotype in the *Arrb2* mutants is strikingly similar to that previously described in *Tsc2*$^{+/-}$ mice (Chapter 3), suggesting genetic reduction of *Arrb2* levels may be similarly effective in normalizing mGluR-mediated protein synthesis in *Fmr1* KO mice. If this is the case, it is a strong indicator that β-arrestin 2 signaling may be causally linked to the cellular processes disrupted in ASD and that manipulation of β-arrestin 2 may be effective at treating the core disturbances in FX, and possibly other forms of ASD.

GPCRs respond to wide variety of signals and initiate a large number of distinct cellular signaling pathways. This versatility has made GPCRs an attractive target for pharmacological therapies, and over 50% of the current drugs used clinically target these receptors (Insel et al., 2007). The finding that β-arrestin- and G protein-dependent cellular signaling are pharmacologically separable has important implications for our understanding of GPCR signaling and the use of GPCR modulators for the treatment of disease. For some disorders, only one of these two signaling pathways may translate into beneficial physiological effects, while the other could mediate undesirable outcomes (Shukla et al., 2011). The use of mGluR5 modulators for the treatment of ASD may be a case in point. Thus, development of functionally selective (i.e. biased) ligands or allosteric modulators that preferentially target one signaling arm over the other may result in more refined therapeutic interventions for this disorder.

Evidence from genetic and molecular studies suggests that altered regulation of synaptic protein synthesis may be a common dysfunction in ASD. One of the most promising approaches to address this dysfunction in a viable therapeutic manner is the manipulation of

mGluR5 signaling (Chapters 2,3). However, there is still a concern that global manipulation of mGluR5 will affect processes that are unrelated to autism pathophysiology, yielding side effects that will sharply limit the utility of this approach. The results presented here indicate that mGluR activation may be coupled to the pathways required for translation in a β-arrestin-dependent manner. This presents the intriguing possibility that β-arrestin-biased modulation of mGluR5 may be a way to specifically manipulate the signaling pathways that regulate protein synthesis without affecting other mGluR-mediated processes that are unrelated to ASD pathophysiology. There is evidence that mGluR5 allosteric modulators with biased signaling may already exist (Sheffler et al., 2011), and development could eventually be optimized for biased modulation, leading to the next generation of selective drugs for the treatment of ASD.

4.5 Methods

4.5.1 Animals

The *Arrb2* mice were a generous gift from the lab of Dr. Lefkowitz. *Arrb2*$^{+/-}$ male and female mutant mice on the C57Bl/6J clonal background were bred together to produce the WT, *Arrb2*$^{+/-}$, and *Arrb2*$^{-/-}$ offspring used in this study. All experimental animals were age-matched male littermates, and were studied with the experimenter blind to genotype and treatment condition. Animals were group housed and maintained on a 12:12 hr. light:dark cycle. The Institutional Animal Care and Use Committee at MIT approved all experimental techniques.

4.5.2 Electrophysiology

Slices were prepared as described previously (Chapter 3). Acute hippocampal slices were prepared from P28-35 animals in ice-cold dissection buffer containing (in mM): NaCl 87, Sucrose 75, KCl 2.5, NaH2PO4 1.25, NaHCO3 25, CaCl2 0.5, MgSO4 7, Ascorbic acid 1.3, and D-glucose 10 (saturated with 95% O2 / 5% CO2). Immediately following slicing the CA3 region was removed. Slices were recovered in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 5, NaH2PO4 1.23, NaHCO3 26, CaCl2 2, MgCl2 1, and D-glucose 10 (saturated with 95% O2/5% CO2) at 32.5°C for ≥3 hours prior to recording.

Field recordings were performed in a submersion chamber, perfused with ACSF (2-3 ml/ min) at 30 °C. Field EPSPs (fEPSPs) were recorded in CA1 stratum radiatum with extracellular electrodes filled with ACSF. Baseline responses were evoked by stimulation of the Schaffer collaterals at 0.033 Hz with a 2-contact cluster electrode (FHC) using a 0.2 ms stimulus yielding 40-60% of the maximal response. fEPSP recordings were filtered at 0.1 Hz - 1 kHz, digitized at 10 kHz, and analyzed using pClamp9 (Axon Instruments). The initial slope of the response was used to assess changes in synaptic strength. Data were normalized to the baseline response and are presented as group means ± SEM. LTD was measured by comparing the average response 55-60 minutes post DHPG application to the average of the last 5 minutes of baseline.

The input output function was examined by stimulating slices with incrementally increasing current and recording the fEPSP response. Paired pulse facilitation was induced by applying two pulses at different interstimulus intervals. Facilitation was measured by the ratio of the fEPSP slope of stimulus 2 to stimulus 1. NMDAR-dependent LTD was induced by delivering 900 test pulses at 1 Hz. mGluR-LTD was induced by S-Dihydroxyphenylglycine (S-

DHPG, 25 μM) for 5 minutes, or by delivering 900 pairs of pulses (with a 50 ms interstimulus interval) at 1 Hz. In order to determine the protein synthesis dependency of mGluR-LTD, slices were incubated with the protein synthesis inhibitor cycloheximide (60 μM) for at least 10 minutes prior to recording and throughout the entire experiment. The magnitude of LTD was measured by comparing the average response 55-60 minutes post DHPG/PP-LFS/LFS application to the average of the last 5 minutes of baseline. Statistical significance for input-output function, paired-pulse facilitation, and mGluR- or NMDAR-dependent plasticity was determined by two-way ANOVA and post-hoc Student's t-tests. Statistics were performed using each animal as one "n", with each animal represented by the mean of 1-4 slices. All experiments were performed blind to genotype and include interleaved controls for genotype and treatment.

4.5.3 Immunoblotting

Hippocampal slices were prepared and recovered as described above. Sets of slices were stimulated with DHPG (25 μM) for 5 minutes and then processed either immediately, 15 minutes, or 30 minutes after stimulation. Yoked unstimulated slices were also processed to assess basal signaling levels. Immunoblotting was performed according to established methods using primary antibodies to p-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology), p-Akt (Ser473) (Cell Signaling Technology), Akt (Cell Signaling Technology), p-mTOR (Ser2448) (Cell Signaling Technology), mTOR (Cell Signaling Technology), p-p70 s6 kinase (Ser371) (Cell Signaling Technology), and p70 s6 kinase (Cell Signaling Technology). Protein levels were measured by densitometry (Quantity One), and quantified as the densitometric signal of phospho-protein divided by the total protein signal in the same lane. To quantify basal ERK expression, the densitometric signal of ERK was divided by the total protein signal (determined by Memcode staining) in the same lane. Significance was determined by a repeated measures two-way ANOVA between time post-DHPG (-5,0,15,30 min) and genotype. All experiments were performed by an experimenter blind to genotype.

4.5.4 Reagents

(S)-3,5-dihydroxyphenylglycine (S-DHPG) was purchased from Sigma (St. Louis, MO). Fresh bottles of DHPG were prepared as a 100x stock in H_2O, divided into aliquots, and stored at -80°C. Fresh stocks were made once a week. Cycloheximide (Sigma) was prepared daily at 100x stock in H_2O. All other reagents were purchased from Sigma.

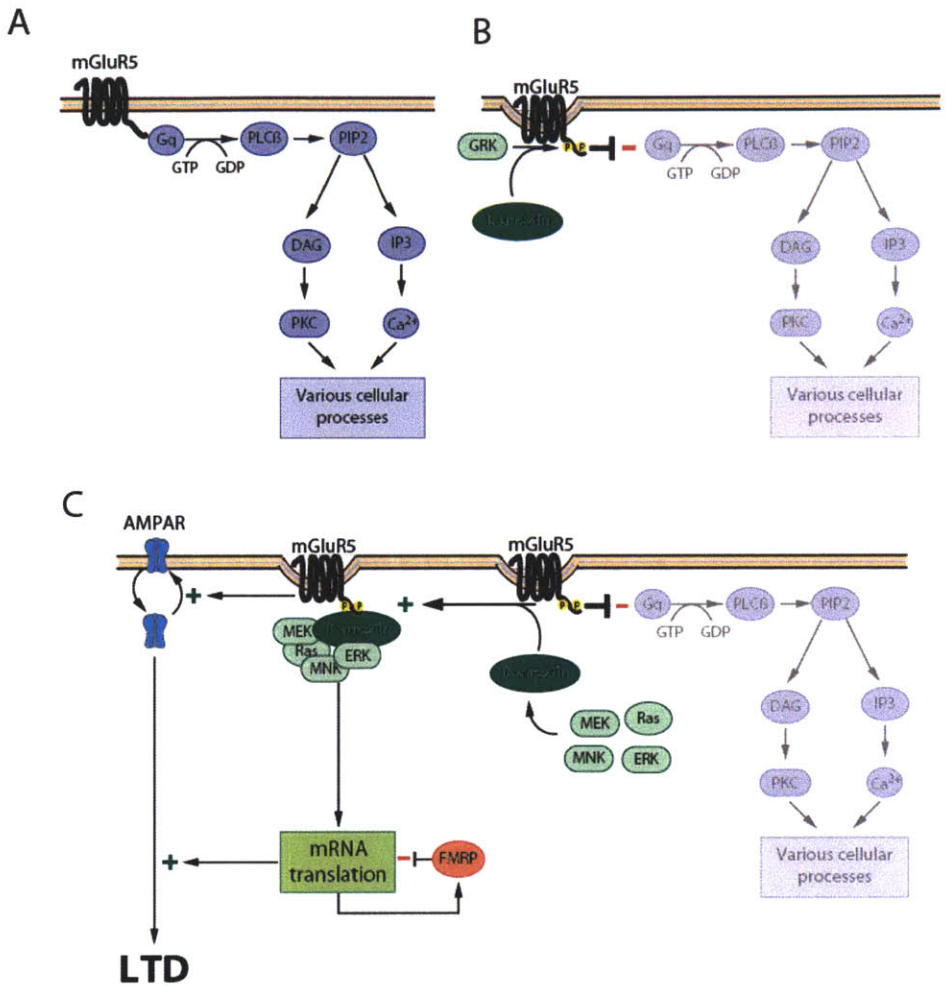

Figure 4.1 -- Activation of mGluR5 leads to divergent signaling cascades. (A) Canonically mGluR5 is coupled to the pertussis toxin-insensitive G-protein α subunit q/11 effector (G_q). G_q activates the enzyme PLCβ, leading to cleavage of PIP2 and increases in PKC activity and intracellular calcium (Ca^{2+}) release, resulting in various cellular processes (see text). **(B)** Classical role for β-arrestins. G-protein signaling is terminated by phosphorylation of mGluR5 and the binding of β-arrestins, which promotes interaction with clatherin and subsequent internalization of receptor. **(C)** New evidence suggests β-arrestins can also act as scaffold for signaling molecules and promote activation of distinct signaling cascades independent of G-protein activation, in particular the ERK pathway. mGluR5-mediated mRNA translation and long term synaptic depression (LTD) is independent from the G_q-protein pathway and may rely on β-arrestin-dependent ERK activation. Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; FMRP, fragile X mental retardation protein; GRK, G-protein receptor kinase; GDP, guanosine diphosphate; GTP, guanine triphosphate; IP3, inositol-1,4,5-triphoshpate; MEK; mitogen activated protein kinase (MAPK) kinase; mGluR5, metabotropic glutamate receptor; MNK, MAP kinase-interacting kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C, PLCβ, phospholipase Cβ;

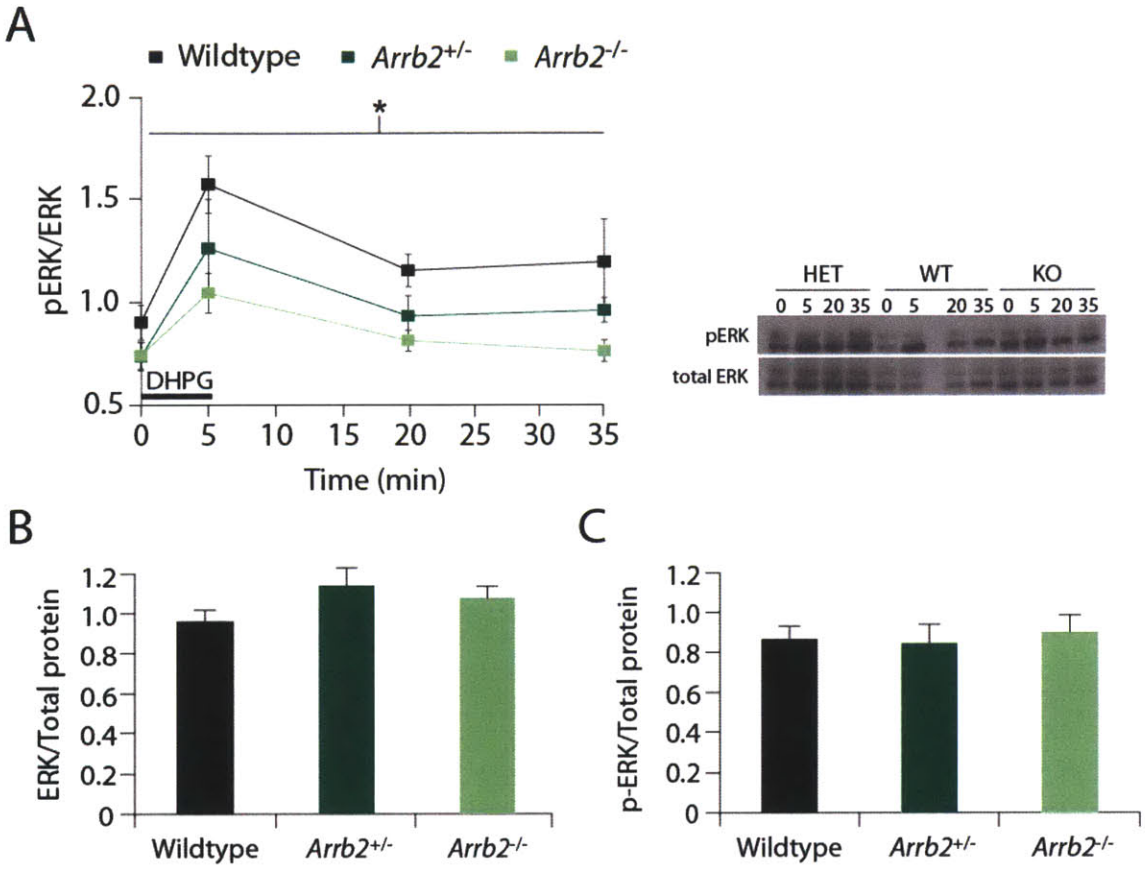

Figure 4.2 – mGluR-mediated ERK phosphorylation is attenuated in *Arrb2* mutants. **(A)** Hippocampal slices were stimulated with 25 μM DHPG for 5 min, and ERK activation (pERK/ERK) assessed via immunoblot before stimulation (time 0; WT: 0.90 ± 0.08, *Arrb2*$^{+/-}$: 0.76 ± 0.06, *Arrb2*$^{-/-}$: 0.74 ± 0.07), immediately after DHPG (WT: 1.57 ± 0.14, *Arrb2*$^{+/-}$: 1.28 ± 0.21, *Arrb2*$^{-/-}$: 1.05 ± 0.10), 15 minutes after DHPG (WT: 1.16 ± 0.08, *Arrb2*$^{+/-}$: 0.92 ± 0.09, *Arrb2*$^{-/-}$: 0.82 ± 0.05) and 30 minutes after DHPG (WT: 1.20 ± 0.21, *Arrb2*$^{+/-}$: 0.95 ± 0.05, *Arrb2*$^{-/-}$: 0.76 ± 0.05; n = 11 for all groups). Two-way repeated measures ANOVA demonstrated there was a significant effect of genotype ($p = 0.031$) and time ($p < 0.001$) but no interaction ($p = 0.981$). There was a significant difference in pERK between WT and *Arrb2*$^{-/-}$ ($p = 0.031$), WT and *Arrb2*$^{+/-}$ ($p = 0.05$), but not *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ ($p = 0.48$). A significant increase in pERK was seen in WT mice at 5 minute ($p = 0.002$), 15 minutes ($p = 0.049$) but not 30 minutes ($p = 0.164$). A significant increase in pERK was seen at 5 minutes in *Arrb2*$^{+/-}$ mice ($p = 0.013$) but not at15 ($p = 0.292$) or 30 (0.181) minutes. There was no significant increase in pERK at 5 ($p = 0.053$), 15 ($p = 0.503$) or 30 ($p = 0.612$) minutes in *Arrb2*$^{-/-}$ mice. Basal levels of **(B)** total ERK (WT: 0.96 ± 0.06, *Arrb2*$^{+/-}$: 1.14 ± 0.08, *Arrb2*$^{-/-}$: 1.08 ± 0.05) and **(C)** phospho-ERK (WT: 0.87 ± 0.06, *Arrb2*$^{+/-}$: 0.85 ± 0.09, *Arrb2*$^{-/-}$: 0.90 ± 0.09) were not significantly different. Error bars represent SEM.

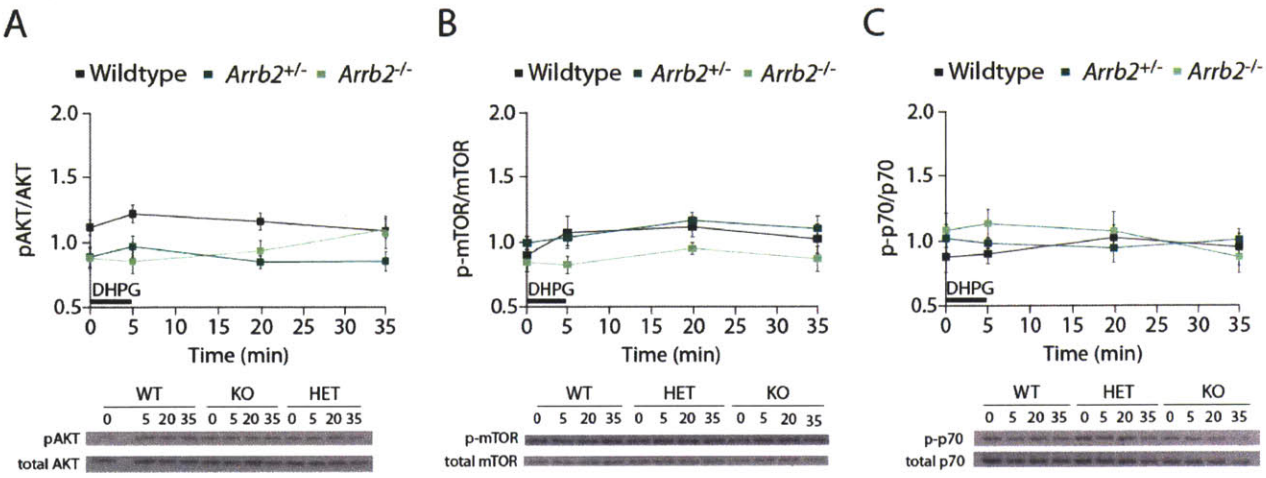

Figure 4.3 – The Akt-mTOR pathway is not recruited by mGluR activation and is unaltered in *Arrb2* mutants. (A) Hippocampal slices were stimulated with 25 μM DHPG for 5 min, and AKT activation (pAKT/total AKT) assessed via immunoblot before stimulation (time 0; WT: 1.07 ± 0.07, *Arrb2*$^{+/-}$: 0.89 ± 0.08, *Arrb2*$^{-/-}$: 0.88 ± 0.08), immediately after DHPG (WT: 1.23 ± 0.10, *Arrb2*$^{+/-}$: 0.97 ± 0.08, *Arrb2*$^{-/-}$: 0.85 ± 0.09), 15 minutes after DHPG (WT: 1.17 ± 0.07, *Arrb2*$^{+/-}$: 0.85 ± 0.05, *Arrb2*$^{-/-}$: 0.93 ± 0.08) and 30 minutes after DHPG (WT: 0.99 ± 0.07, *Arrb2*$^{+/-}$: 0.85 ± 0.08, *Arrb2*$^{-/-}$: 1.10 ± 0.12; n = 11 for all groups). Two-way repeated measures ANOVA demonstrated there was no significant effect of genotype ($p = 0.137$) or time ($p = 0.737$) and no interaction ($p = 0.233$). **(B)** Hippocampal slices were stimulated with 25 μM DHPG for 5 min, and mTOR activation (p-mTOR/total mTOR) assessed via immunoblot before stimulation (time 0; WT: 0.90 ± 0.07, *Arrb2*$^{+/-}$: 0.99 ± 0.05, *Arrb2*$^{-/-}$: 0.86 ± 0.06), immediately after DHPG (WT: 1.07 ± 0.09, *Arrb2*$^{+/-}$: 1.03 ± 0.06, *Arrb2*$^{-/-}$: 0.85 ± 0.06), 15 minutes after DHPG (WT: 1.15 ± 0.06, *Arrb2*$^{+/-}$: 1.16 ± 0.06, *Arrb2*$^{-/-}$: 0.96 ± 0.04) and 30 minutes after DHPG (WT: 1.02 ± 0.10, *Arrb2*$^{+/-}$: 1.10 ± 0.10, *Arrb2*$^{-/-}$: 0.91 ± 0.10; n = 11 for all groups). A two-way repeated measures ANOVA demonstrated there was a significant effect of genotype ($p = 0.024$) but not time ($p = 0.054$) and no interaction ($p = 0.835$). While there was a significant difference between *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice ($p = 0.034$), there was no difference in WT and *Arrb2*$^{-/-}$ ($p = 0.063$) or *Arrb2*$^{+/-}$ ($p = 0.508$). **(C)** Hippocampal slices were stimulated with 25 μM DHPG for 5 min, and p70 activation (phosphor-p70/ total p70) assessed via immunoblot before stimulation (time 0; WT: 0.88 ± 0.12, *Arrb2*$^{+/-}$: 1.02 ± 0.11, *Arrb2*$^{-/-}$: 1.08 ± 0.13), immediately after DHPG (WT: 0.90 ± 0.08, *Arrb2*$^{+/-}$: 0.98 ± 0.04, *Arrb2*$^{-/-}$: 1.13 ± 0.11), 15 minutes after DHPG (WT: 1.02 ± 0.10, *Arrb2*$^{+/-}$: 0.94 ± 0.10, *Arrb2*$^{-/-}$: 1.07 ± 0.15) and 30 minutes after DHPG (WT: 0.95 ± 0.14, *Arrb2*$^{+/-}$: 1.00 ± 0.104, *Arrb2*$^{-/-}$: 0.87 ± 0.12; n = 7 for all groups). Two-way repeated measures ANOVA demonstrated there was no significant effect of genotype ($p = 0.712$) or time ($p = 0.604$) and no interaction ($p = 0.705$). Error bars represent SEM.

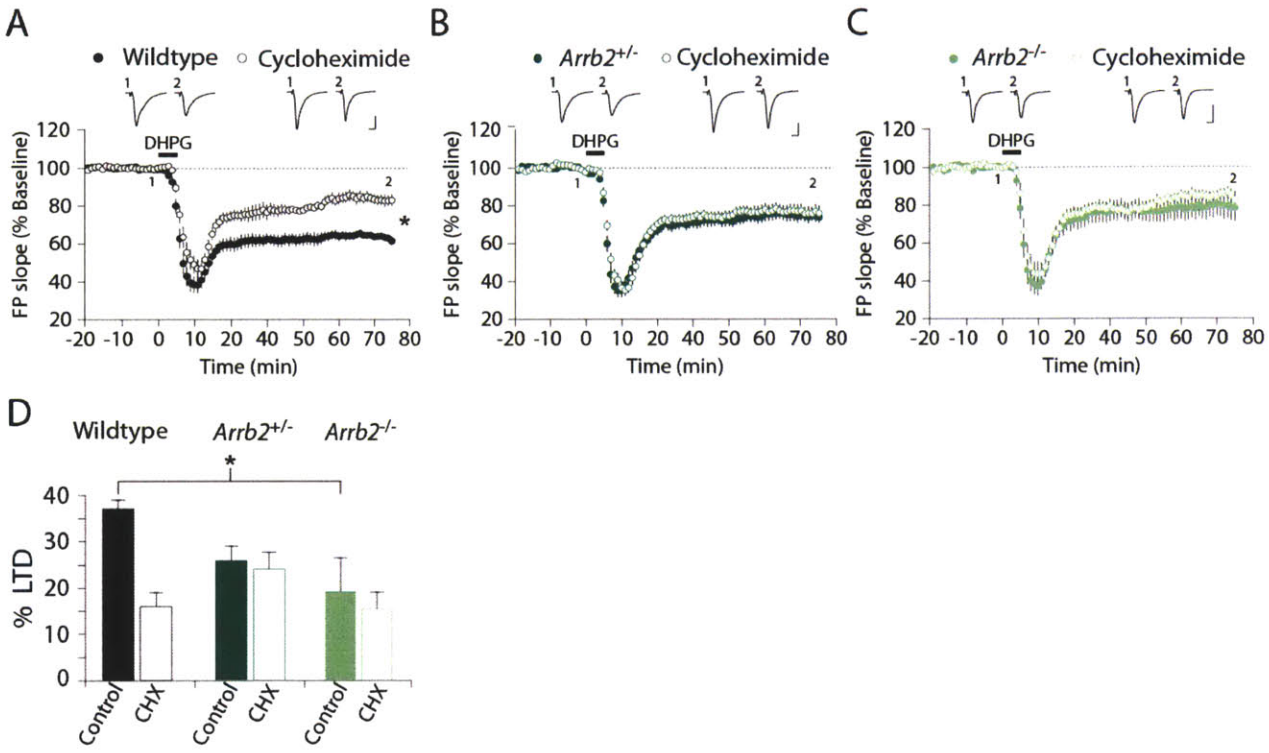

Figure 4.4 – Loss of the protein synthesis-dependent component of mGluR-LTD in *Arrb2* mutants. **(A)** LTD is significantly attenuated by pretreatment with the protein synthesis inhibitor cycloheximide (CHX, 60 μM) in slices from WT animals (control: 63.0 ± 1.9%, n = 13 animals; CHX: 84.1 ± 3.0%, n = 7 animals; *p < 0.001). **(B)** CHX treatment has no effect on DHPG-LTD in slices from *Arrb2*$^{+/-}$ mice (control: 74.1 ± 3.1%, n = 12 animals; CHX: 76.0 ± 3.7%, n = 11 animals; p = 0.719). **(C)** CHX treatment has no effect on DHPG-LTD in slices from *Arrb2*$^{-/-}$ mice (control: 80.8 ± 7.3%, n = 10 animals; CHX: 84.6 ± 3.6%, n = 7 animals; p = 0.1.03). **(D)** DHPG-induced LTD is significantly decreased in *Arrb2* mutants (ANOVA: genotype *p = 0.02, treatment p = 0.002, genotype x treatment p = 0.06; WT vs. *Arrb2*$^{+/-}$ p = 0.075, WT vs. *Arrb2*$^{-/-}$ p = 0.008). Bar graphs represent the average percent LTD observed 55-60 minutes post DHPG. Representative field potential traces (average of 10 sweeps) were taken at times indicated by numerals. Scale bars equal 0.5 mV, 5 ms. Error bars represent SEM.

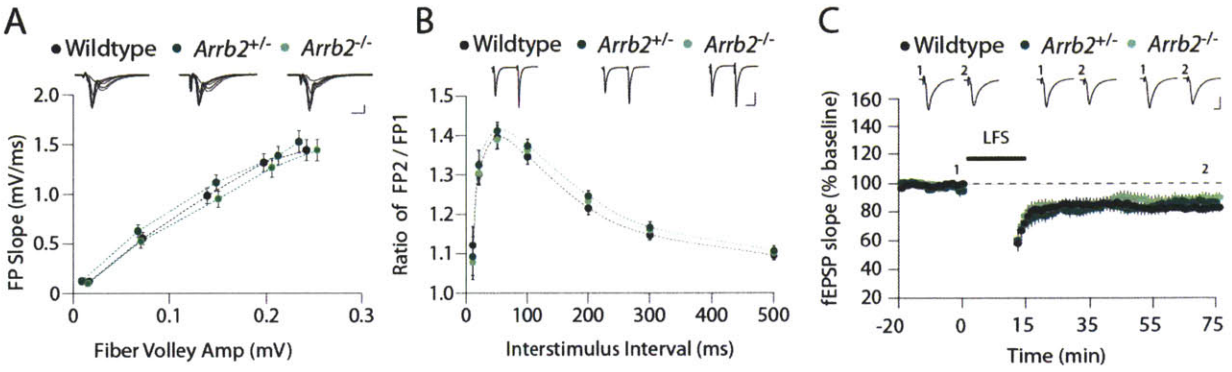

Figure 4.5 – Normal basal synaptic function and NMDAR-dependent LTD in *Arrb2* mutants. **(A)** Basal synaptic transmission (plotted as fEPSP amplitude against presynaptic fiber volley amplitude) does not differ between genotypes. Scale bars equal 0.5 mV, 20 ms for representative field potential traces. **(B)** Paired pulse facilitation is normal across several inter-stimulus intervals (20, 30, 50, 100, 200, 300, 500 ms) in *Arrb2* mutants. Scale bars equal 0.5 mV, 20 ms. **(C)** The magnitude of NMDA receptor-dependent LTD evoked by low frequency stimulation (LFS, 900 pulses at 1 Hz) does not differ between genotypes (WT: 83.9 ± 3.5%, n = 6 animals; $Arrb2^{+/-}$: 87.9 ± 0.4%, n = 8 animals; $Arrb2^{+/-}$: 87.2 ± 0.5%, n = 8 animals p = 0.610). Representative field potential traces (average of 10 sweeps) were taken at times indicated by numerals. Scales bars equal 0.5 mV, 5 ms. Error bars represent SEM.

Chapter 5

Implications and future directions

5.1 Introduction

Our understanding of the etiology of autism has greatly evolved since the disorder was first described by Kanner in the 1940s (Kanner and Eisenberg, 1957). While researchers originally believed that autism resulted from emotional deprivation in infancy, it is now appreciated that autism is a biological disorder rooted in genetic perturbations. Despite this advance, the clinical heterogeneity and complex genetics of autism spectrum disorder (ASD) has made it difficult to untangle its underlying pathophysiology. Many questions remain about the nature of ASD, and there is still disagreement as to whether autism should be considered a large set of related disorders with diverse mechanisms (Geschwind and Levitt, 2007) or a single disorder with a common dysfunction at the cellular or systems level that is variably expressed (Kelleher and Bear, 2008). However, recent evidence from human genetic studies and animal models has converged on synaptic dysfunction as the core of ASD pathology (Gilman et al., 2011; Spooren et al., 2012; Zoghbi, 2003; Zoghbi and Bear, 2012).

While there are undoubtedly many ways for this dysfunction to arise, as evidenced by the hundreds of gene implicated in ASD (Betancur, 2011), it has been proposed here that dysregulation of synaptic protein synthesis may be a common cellular mechanism underlying ASD (Kelleher and Bear, 2008). The studies in this thesis were designed to examine the contribution of synaptic protein synthesis to the pathogenesis of ASD by examining the mouse models of fragile X (FX) and tuberous sclerosis complex (TSC), the two most common inherited causes as ASD. Surprisingly, we found the synaptic pathophysiology of TSC and FX to be mirror images of one another (Figure 3.6A). These results suggest that bidirectional deviations in neuronal protein synthesis may be associated with ASD. This not only supports the general notion that proper synaptic function requires a fine balance of local protein synthesis, but it demonstrates that disruptions in either direction can lead to overlapping behavioral phenotypes. The direction of the dysfunction may matter less, with respect to symptomatology, than the fact that this process has been pushed outside its optimal range. While bidirectional changes in gene dosage have previously been associated with autism (Christian et al., 2008; Okamoto et al., 2007; Vandewalle et al., 2009), this is the first demonstration, to our knowledge, of genetically heterogeneous causes of ASD resulting from opposing alterations at the molecular level. Major questions remain as to how these changes in protein synthesis arise, and where these opposing alterations may converge to result in the complex phenotypes associated with ASD.

5.2 Contribution of mGluRs to the synaptic pathophysiology of autism

While there are many signals that can mediate mRNA translation, we have examined protein synthesis through the lens of group 1 (Gp1) mGluRs, particularly mGluR5, the predominant subtype in the forebrain and hippocampus (Masu et al., 1991). We view the role of mGluRs in the synaptic pathophysiology of ASD as threefold: (1) mGluR-LTD is a sensitive assay for interrogating the status of synaptic protein synthesis in animal models of ASD; (2) the mGluR5 receptor is a potent modulator of synaptic protein synthesis and therefore well situated to be a target for the treatment of ASD; and (3) the downstream proteins regulated by mGluRs may specifically be central to ASD pathology.

Two assays have greatly aided our examination of the role of neuronal protein synthesis in animal models of ASD: metabolic labeling and mGluR-LTD. Biochemical measurement of metabolically labeled amino acid incorporation into acutely-prepared brains slices provides a direct measure of protein synthesis rates. However, the assay does not distinguish somatic verses dendritic translation and lacks information on the functional consequences of these changes. Electrophysiological measurements of mGluR-mediated LTD, on the other hand, is by no means a direct measure of protein synthesis, but it is known to require rapid local translation of pre-existing mRNA at the synapse (Huber et al., 2000). Thus, mGluR-LTD could be considered a sensitive tool for monitoring changes specifically in local dendritic translation.

The studies in this thesis indicate that examination of mGluR-LTD is indeed a reliable indicator of altered protein synthesis. We have shown that *Fmr1* KO and $Tsc2^{+/-}$ mice have opposing alterations in basal hippocampal protein synthesis rates, and this is reflected in their levels of mGluR-LTD (Chapter 3). Furthermore, treatments that modify LTD in these animals also restore proper protein synthesis levels, suggesting that the two are causally linked. These complimentary assays are simple but reliable tools for examining alterations in synaptic protein synthesis. Their use can be extended in future studies to other mouse models of ASD, thereby allowing us to determine the prevalence of dysregulated synaptic protein synthesis in the disorder.

Not only are mGluRs a sensitive tool for monitoring synaptic protein synthesis but they are also a potential way to manipulate translation. mGluR5 is a robust activator of local protein synthesis (Job and Eberwine, 2001; Weiler and Greenough, 1993) that is widely expressed throughout the brain (Masu et al., 1991). Moreover, the mGlu5 receptor is particularly amenable

to pharmacological manipulation with a well-developed class of allosteric modulators that regulate its activity in a subtype specific and activity-dependent manner (Sheffler et al., 2011). Studies in the mouse models of FX and TSC have already demonstrated the potential for allosteric modulators of mGluR5 in the treatment of ASD. For example, a diverse array of mutant phenotypes in FX animal models have now been corrected by pharmacological inhibition of mGluR5 (Michalon et al., 2012). Similarly, in Chapter 3 we demonstrated that augmenting mGluR5 in $Tsc2^{+/-}$ mice rescues deficits at the biochemical, electrophysiological and behavioral levels. In Chapter 4, we illustrated the potential to specifically modulate synaptic protein synthesis downstream of mGluR5 activation, via β-arrestins, without affecting other mGluR5-mediated processes. Thus it may be possible to develop mGluR5-based therapies that are not only physiologically responsive, but also specific to the processes thought to underlie many forms of synaptic pathophysiology in ASD.

The above evidence demonstrates that controlling mGluR activity is a method for both monitoring and manipulating synaptic protein synthesis. However, mGluR-mediated protein synthesis may be *specifically* disrupted in several forms of ASD. There is considerable support for the notion that dysregulation of mGluR-dependent protein synthesis is central to the pathogenesis of FX (Bhakar et al., 2012). In many ways, it is remarkable that simply reducing the activity of mGluR5 can have such a profound effect on the constellation of symptoms associated with FX. However, mGluR-mediated plasticity is prominent in many brains areas associated with ASD, and in many cases this plasticity requires rapid protein synthesis for its maintenance (Luscher and Huber, 2010). In Chapter 2, we demonstrated that multiple consequences of mGluR-dependent protein synthesis are altered in *Fmr1* KO mice, even within the same population of synapses. Therefore, disruption of mGluR-mediated protein synthesis will likely have diverse consequences across many brain regions. Indeed, modulation of mGluR5 function has been shown to reverse phenotypes in many brain regions in FX, and has even proved beneficial in processes where mGluR activity had previously not been implicated (Dolen et al., 2007; Hays et al., 2011).

Disruption in mGluR function is not limited to FX, however. We have shown that mGluR-LTD and protein synthesis are disrupted in both *Fmr1* KO mice and in a mouse model of TSC, suggesting that genetically heterogeneous causes of ASD may lead to disrupted mGluR function (Chapter 3). Indeed, mGluR dysfunction has now been demonstrated in numerous

other animal models of ASD as well (Bangash et al., 2011; Baudouin et al., 2012; Chen et al., 2011; Phelan and McDermid, 2012). Interestingly, the genes disrupted in many of these animal models are involved with scaffolding mGluRs at the synapse, suggesting that both upstream regulation of mGluRs and the downstream signaling pathways mediated by mGluRs are associated with ASD. Consistent with these animal studies, human genetic data has shown many genes involved in the regulation of mGluR-mediated protein synthesis are associated with ASD, including the gene for mGluR5 itself (Iossifov et al., 2012; Kelleher Iii et al., 2012). Thus, multiple lines of evidence suggest that mGluR-mediated protein synthesis is a common molecular pathway disrupted in ASD.

As was described above, mGluR-LTD is sensitive to alterations in synaptic protein synthesis. Therefore, it is also possible that altered mGluR function in some animal models of ASD is a consequence of dysregulated synaptic protein synthesis by other mechanisms, rather than a cause of dysregulation. Many animal models of ASD associated with disrupted mGluR function exhibit alterations in AMPAR and NMDAR function as well (Bangash et al., 2011; Bozdagi et al., 2010; Ehninger et al., 2008a; Lee et al., 2011; Lee et al., 2008; Won et al., 2012), which may also contribute to deviations in synaptic protein synthesis. Even in FX, where evidence for the pathogenic nature of mGluR5 is most abundant, mGluR activation is unlikely to be the only avenue by which synaptic protein synthesis is disrupted. For example, removal of FMRP occludes TrkB-mediated increases in protein synthesis (Osterweil et al., 2010) and alters other forms of G-protein-coupled receptor (GPCR)-mediated protein synthesis-dependent plasticity (Connor et al., 2011; Volk et al., 2007). Therefore it is likely that multiple signaling pathways can converge to regulate synaptic protein synthesis.

However, as discussed below, neuronal translation is not a uniform entity. There may be specific subsets of mRNA whose translation is relevant to the synaptic pathophysiology of ASD. While mGluRs are not the only way to regulate mRNA translation, the set of proteins controlled by mGluR activation may be particularly important to the pathophysiology of ASD, demonstrated by the fact that they are regulated by both FMRP and TSC1/2. While the breadth of proteins involved in ASD remains to be characterized, examination of mGluR-dependent processes is clearly a useful approach to understanding its synaptic pathophysiology. Furthermore, regulation of mGluR5 may be a way to specifically regulate the pool of proteins

implicated in ASD, and therefore is a promising therapeutic avenue for the treatment of this disorder.

5.3 Regulating protein synthesis at the synapse

It has long been known that the synthesis of new proteins is essential for both neuronal function and memory formation (Kandel, 2001a). However, the idea that mRNA translation could be compartmentalized in neurons was not realized until the observation of polyribosomes in spines by Steward and Levy in 1982 (Steward and Levy, 1982). mRNA localization is an elegant mechanism for spatially restricting gene expression within the neuron, facilitating rapid responses at stimulated synapses while confining biochemical changes to a specific compartment. This compartmentalization therefore allows for multiple layers of control. However, with this increased control comes increased complexity. While it is now widely accepted that many mRNAs localize to dendrites, and that local translation of this dendritic mRNA contributes to many forms of plasticity, major questions remain. For example, it is unclear how synaptic activity regulates dendritic translation and how this local translation is delineated from somatic transcription and translation. Moreover, the identities of the mRNA preferentially translated at the synapse and the functions they may serve have yet to be fully defined. The studies in this thesis begin to elucidate the potential mechanisms for the differential regulation of translation in neurons and define the purposes they may serve.

5.3.1 Somatic verses synaptic translation

The ERK and mTOR pathways are implicated in both translational and transcriptional regulation (Kandel, 2001b; Richter and Klann, 2009; Sweatt, 2004), and it has been difficult to determine how activation of these pathways can differentially regulate synaptic and somatic processes (Kelleher et al., 2004). A recent study demonstrated that BDNF-dependent ERK activation may regulate translation in a compartment-specific manner, as it was shown to specifically activate eIF4E in synaptic fractions while only phosphorylating eEF2 in the cell body (Kanhema et al., 2006). The mTOR pathway has also been shown to undergo compartment specific activation (Belelovsky et al., 2005). Thus, activity-dependent regulation of the translational machinery may be spatially restricted by sequestering signaling cascades into particular subcellular compartments. β-arrestin-mediated signaling is an attractive mechanism

for mediating spatially segregated ERK activation downstream of mGluR5. In Chapter 4, we demonstrated that mGluR-mediated ERK activation requires β-arrestin2. A defining characteristic of β-arrestin-dependent signaling is that it is restricted to the cytosol and does not translocate to the nucleus (Luttrell et al., 2001). Thus, mGluR-dependent ERK signaling may be restrained to the dendritic compartment, thereby specifically regulating local protein synthesis at the synapse.

Another way to selectively isolate translation to a specific compartment is by localizing mRNA-specific translational repressors, such as FMRP. Evidence suggests that FMRP may be required for the regulation of dendritic but not somatic translation; multiple forms of plasticity dependent upon local translation are altered in *Fmr1* KO mice (Huber et al., 2002) (Chapter 2), while transcription-dependent forms are not (Godfraind et al., 1996; Paradee et al., 1999). Consistent with this idea, a recent study found that while removal of FMRP resulted in increased levels of Arc protein in hippocampal dendrites, there was actually a slight decrease in Arc levels in the soma (Niere et al., 2012). This suggests that FMRP's role in the nucleus may be different than its role at the synapse. While mTOR signaling has been shown to specifically regulate dendritic translation, it also has important functions in the nucleus (Hay and Sonenberg, 2004). Thus, while removal of FMRP may specifically alter dendritic protein synthesis, removal of *Tsc2* is likely to affect somatic and synaptic function.

5.3.2 Multiple pools of mRNA are differentially translated in neurons

The results presented here indicate there is a dichotomy in the signaling pathways that regulate neuronal protein synthesis. While the ERK and mTOR pathways are generally thought to stimulate protein synthesis in parallel, or even synergistically (Banko et al., 2006), we found that activation of these pathways can have contrasting effects on hippocampal protein synthesis and plasticity. Specifically, removal of *Fmr1* results in excessive protein synthesis and LTD downstream of mGluR5-ERK signaling, while enhancing mTOR signaling by decreasing *Tsc2* levels results in diminished protein synthesis and LTD (Figure 3.6A). How can activation of the pathways that mediate global translation have such disparate effects on neuronal protein synthesis and protein synthesis-dependent plasticity? In Chapter 3 we proposed that the regulation of translation requires temporal precision and disruption of this activity-dependent timing may lead to unexpected consequences (Figure 3.7A). As discussed above,

compartmentalization of these signaling cascades to distinct cellular domains may result in differential regulation of cellular and synaptic processes. Most interestingly, we have discussed the possibility that the ERK and mTOR pathways may regulate the translation of different pools of proteins whose expression is mutually exclusive (Figure 3.7B).

While both the ERK and mTOR pathways are known to regulate global translation rates, there is also ample evidence demonstrating their involvement in gene-specific translation (Gkogkas et al., 2010). Emerging evidence suggests that gene-specific regulation of translation may be particularly important for proper neuronal function, potentially as a mechanism for delineating between synaptic and somatic processes (Pfeiffer and Huber, 2006). As discussed in Chapter 3, there are multiple mechanisms by which this differential translation can be achieved. However, the most straightforward explanation for the mutually inhibitory effects of mTOR and ERK on translation may simply be competition for a finite resource.

Although difficult to quantify, electron microscopic studies indicate that the number of ribosomes at individual synapses is quite limited (Ostroff et al., 2002). As activated polyribosomes typically translate a single mRNA, at most one or two mRNAs are being translated at the synapse at any given time (Schuman et al., 2006). Indeed, recent functional data has suggested that competition for translation machinery may be the rate-limiting step for protein synthesis-dependent plasticity (Fonseca et al., 2004). In fact, experiments in which late-phase LTP (L-LTP) is induced at a single spine have shown there is competition between two adjacent stimulated spines within a dendritic branch (Govindarajan et al., 2011). Thus, the limiting factor for dendritic translation may be the availability of translational machinery. Under these conditions, increasing initiation probability via the ERK or mTOR pathway will likely not increase overall translational capacity. However, it may be possible to qualitatively alter the nature of the synthesis by modifying aspects of the translational machinery, such as eIF2α or eEF2 (see Chapter 3). Thus, the differential effects on protein synthesis and plasticity exerted by the ERK and mTOR pathways may be explained by their altering the preference of limited translational resources from one pool to another.

More work is required to dissect the distinct contributions of the ERK and mTOR pathways to synaptic function, and how alterations in these pathways may contribute to the pathogenesis of ASD. One thing is certain, however: the regulation of protein synthesis is complicated. Due to this complexity, there are important caveats to the study of protein

synthesis regulation. It is critical to realize that manipulating different aspects of the translational pathway may yield varying results depending on the cellular and experimental context in which they are performed. This is particularly relevant when attempting to translate observations made in mitotic cells to neurons, where the function of protein synthesis is markedly different. The *Fmr1* KO x *Tsc2*$^{+/-}$ cross line may provide a unique opportunity to study the subtleties associated with protein synthesis regulation in the brain, as they allow for examination of mutations that result in opposite changes in neuronal protein synthesis in littermate mice under identical experimental conditions.

5.3.3 Identifying the two pools

The results presented in this thesis suggest that dysregulation of a specific set of proteins, rather than global translation, is critical to the synaptic pathophysiology of ASD. A major obstacle to our understanding of the role of synaptic protein synthesis in ASD pathophysiology is identification of the proteins that are dysregulated. Specifically, determining which proteins are under control of the TSC1/2-mTOR and ERK-FMRP pathways will be instrumental to our understanding of the different functions these two pathways may serve. We have suggested that the protein Arc may be a prototypical member of the FMRP pool (Chapter 2). Arc mRNA is present in dendrites where it is rapidly translated on demand (Shepherd and Bear, 2011). It has been shown to be a target of FMRP (Zalfa et al., 2003) and dendritic expression of this protein is increased in *Fmr1* KO mice (Niere et al., 2012). Furthermore, Arc is required for the expression of mGluR-LTD, and decreasing Arc levels in the *Fmr1* KO mouse reverses their LTD phenotype (Park et al., 2008; Waung et al., 2008). In Chapter 3, we demonstrated that there is diminished Arc synthesis in the *Tsc2*$^{+/-}$ mouse, suggesting that this protein is bidirectionally regulated by FMRP and TSC1/2. The divergence in ERK- and mTOR-dependent regulation of Arc is further demonstrated by a study examining the mechanism for LTP consolidation in the dentate gyrus (Panja et al., 2009), where it was shown that the maintenance of L-LTP requires ERK-dependent synthesis of Arc. Interestingly, while LTP stimulation also recruited the mTOR pathway, it was completely dispensable for the maintenance of L-LTP (Panja et al., 2009), supporting a dominant role for ERK signaling in the regulation of Arc.

Emerging evidence suggests that the neuroligin family of proteins may be potential targets of the mTOR pool. A recent study examined the consequences of excessive eIF4E

activity by overexpressing eIF4E or knocking out 4E-BP2, a specific inhibitor of eIF4E (Figure 1.2A). Inhibition of 4E-BP2 is a major function of the mTOR pathway (Figure 1.2A), thus excessive eIF4E activity is likely to have similar functional consequence to chronic activation of the mTOR pathway as seen in *Tsc2*$^{+/-}$ mice. Consistent with our findings (Chapter 3), excessive eIF4E activity does not result in an overall increase in protein synthesis rates ((Gkogkas et al., 2013) but see (Santini et al., 2013)). However, examination of candidate proteins revealed that there was a specific increase in the synthesis of neuroligins. Mice with excessive eIF4E activity also displayed autistic phenotypes similar to those found in *Tsc2*$^{+/-}$ mice (Young et al., 2010), which could be rescued by decreasing neuroligin 1 levels (Gkogkas et al., 2013). Furthermore, the authors found that a reporter gene fused to the 5' UTR of *Nlgn1* was better translated in cells with excessive mTOR signaling (*Pten*$^{+/-}$ or *Tsc2* KO cells) similar to those with increased eIF4E activity. Thus, while mTOR suppresses the synthesis of proteins downstream of mGluR5 activation, e.g. Arc, it stimulates translation of other proteins associated with autism, namely the neuroligins.

Two other recent studies have demonstrated that neuroligins may act to suppress the mGluR-ERK pool. First, it was shown that deletion of *Nlgn3* results in excessive mGluR-LTD in the cerebellum, suggesting that decreases in neuroligin may promote mGluR function and the expression of LTD. Consistent with this notion, a second study found that neuroligin 1 levels are reduced in the hippocampus and cerebellum of *Fmr1* KO mice, and overexpression of *Nlgn1* specifically rescues social deficits in the *Fmr1* KO mouse (Dahlhaus and El-Husseini, 2010). Thus, neuroligins may be differentially regulated by mGluR5 and mTOR in the opposite manner than Arc. This suggests these proteins exist in separate pools and once again demonstrates the bidirectional modification of proteins associated with ASD.

The idea that mTOR signaling can regulate the synthesis of neuroligins may clarify the plasticity phenotypes seen in *Tsc2*$^{+/-}$ mice. The Neuroligin-Shank complex is important for the anchoring of NMDAR and mGluRs at the synapse (Figure 1.1). Despite this mutual interaction, it seems that disruption of this complex affects NMDAR- and mGluR-mediated plasticity in opposite directions. Deletion of neuroligins in mice results in decreased NMDAR responses and plasticity (Budreck et al., 2013; Sudhof, 2008) but excessive mGluR function (Baudouin et al., 2012). Likewise, a *Shank3* mutation associated with ASD that results in a 90% decrease in Shank3 levels in hippocampus leads to deficient NMDAR-LTP and LTD but enhanced mGluR-

LTD (Bangash et al., 2011). Furthermore, deletion of SAPAP3, which mediates the interaction between neuroligins and Shanks, also results in excessive mGluR5 signaling (Wan et al., 2011). Why does disrupting this synaptic scaffolding complex result in opposing regulation of mGluRs and NMDARs? Results from our β-arrestin study suggest that receptor internalization may be required for mGluR-mediated signaling and LTD (Chapter 4). Thus, *decreasing* mGluR association with the synaptic membrane may actually *increase* mGluR-mediated plasticity by making it more readily internalized by β-arrestin. It is interesting to speculate that there may be increased neuroligin expression in *Tsc2*$^{+/-}$ mice, leading to enhanced retention of NMDA and mGlu receptors at the synapse, which results in exaggerated NMDAR-LTP but deficient mGluR-LTD.

The above examples demonstrate how identification of the proteins dysregulated in models of ASD may lead to a better understanding of how synaptic function is disrupted in the disorder. However, it is unlikely that there are only one or two global "plasticity proteins" – multiple proteins likely regulate different processes in parallel. Ideally, unbiased screens to examine the protein make-up of synapses in mouse models of ASD will illuminate the processes essential for proper synaptic function and those that may be disrupted in autism. To date, these efforts have been hindered by variability and replicability of experimental results. Comparison of the *Fmr1* KO x *Tsc2*$^{+/-}$ cross mice may add another layer of validity to these studies, as proteins that are increased in one mutant, decreased in the other, but normalized in the double mutants are likely to be central to the synaptic processes associated with ASD.

5.3.4 Functional distinction between ERK and mTOR regulated protein synthesis

Proper neuronal function requires synaptic strength to be maintained within an optimal functional range (Figure 3.6A) and this necessitates mechanisms to prevent runaway LTP/LTD. It has been proposed that synapses can modulate the threshold for the induction of plasticity based upon their previous history of activity (Abraham and Bear, 1996). This metaplasticity is a potential way to maintain an optimal range of synaptic strength. There are many examples of reciprocal interactions between the proteins associated with ASD, and it is intriguing to speculate that these proteins may represent a network required for synaptic metaplasticity. For example, we suggested above that neuroligin expression may differentially regulate LTP and LTD. It has also been shown that surface levels of neuroligin 1 are bidirectionally altered by the induction of

LTD or LTP (Schapitz et al., 2010). Indeed, many of the structural proteins involved in regulating glutamate receptor function are also themselves regulated by activation of these receptors (Ehlers, 2003). Many targets of FMRP have been shown to be parts of the translation machinery or involved in the signaling pathways that mediate protein synthesis as well (Darnell et al., 2011).

These structural and signaling proteins may form a self-regulating complex that is constantly assessing synaptic function and directing local translation in order to appropriately constrain synaptic strength within its functional range, potentially by regulating the threshold for LTP and LTD (Bienenstock et al., 1982). Specifically, we have proposed that there are two pools of proteins that regulate synaptic function in an opposite manner: an mGluR-ERK pool required for synaptic weakening (e.g. Arc) and a TSC1/2-mTOR pool required for synaptic strengthening (e.g. Neuroligins). It is easy to imagine that if either of these opposing constraints is lost, the result is an imbalance in synaptic strength and disruption of neuronal function.

While we have proposed that the mTOR and ERK pathways are differentially activated and have divergent functions in neurons, it has been shown that mGluR activation can recruit both of these pathways, begging the question as to how this divergence is achieved. It is possible that different types of mGluR stimulation can activate different pathways. We discussed in Chapter 2 that while mGluR-LTD and LTP priming share a requirement for local translation, the upstream mechanisms are distinct. We suggested that the qualitative nature of mGluR stimulation received may determine LTP vs. LTD. Likewise, different stimulation paradigms may determine if the ERK or mTOR pathway is preferentially activated. The fact that mGluR-mediated ERK activation and LTD may require β-arrestin-dependent internalization of the mGlu receptor suggests that strong activation is required for mGluR-LTD. Prolonged weak activation may preferentially recruit the mTOR pathway via Homer interactions, leading to the synthesis of LTP proteins. Support for this hypothesis comes from the fact that inhibition of mTOR with rapamycin prevents mGluR-dependent enhancement of LTP with a priming-like stimulus (Cammalleri et al., 2003), while it does not disrupt mGluR-LTD, at least in our hands (Figure 3.4). Thus, the strength and duration of mGluR stimulation may preferentially evoke different signaling pathways resulting in opposite changes in synaptic strength. Differences in overall activity levels may also alter which pathway is preferentially activated. These experimental differences may contribute to the conflicting results in the literature.

5.4 Synaptic pathophysiology beyond post-synaptic protein synthesis

Several lines of evidence point to synaptic dysfunction in the pathophysiology of ASD. The results presented here demonstrate that altered dendritic protein synthesis may be a major contributor to this dysfunction, however it is unlikely the only player. While proper post-synaptic regulation of translation is clearly important, there are many other processes that regulate synaptic function. Recent evidence has suggested that protein homeostasis, i.e. maintaining proper synaptic protein levels, is important for synaptic function and plasticity (Cajigas et al., 2010). Consistent with the role of protein synthesis in ASD, several studies have reported many genes that are risk factors for ASD involve the ubiquitin proteasome system that regulates protein degradation (Glessner et al., 2009). The role of the proteasome system in ASD is most directly demonstrated in Angelman syndrome, as mutations in *UBE3A*, an E3 ubiquitin ligase, are thought to be responsible for this disorder (Kishino et al., 1997). Ubiquitination involves activating enzymes (E1), conjugating enzymes (E2), and ligases (E3). Ligation of ubiquitin to a protein directs it to be degraded, and substrate specificity is usually provided by the E3 ligases (Hochstrasser, 1995). Interestingly, a major target of Ube3a in neurons is Arc and deletion of Ube3a results in increased Arc levels at the synapse (Greer et al., 2010). Thus, alterations in synaptic protein synthesis and degradation associated with ASD may converge on the same protein targets.

The proteasome system is also required for the induction of mGluR-LTD and rapid degradation of FMRP is thought to be a potential mechanism for the de-repression of protein synthesis downstream of mGluR5 activation (Hou et al., 2006). Furthermore, proteasome activation has also been shown to regulate a form of mTOR-dependent local translation (Briz et al., 2013). This suggests that there is a reciprocal relationship between mRNA translation and protein degradation – dysregulating one may lead to compensatory changes in the other. Rather than there being an optimal level of synaptic protein synthesis *per se*, it may be that proper synaptic function requires an optimal level of proteins at the synapse, which can be disrupted by altering either translation or degradation. Compensatory changes in the proteasome system in response to alterations in protein synthesis, or *vice versa*, may be just as crucial to the pathophysiology of ASD as the primary dysfunction itself.

Just as coordination between translation and degradation is required for optimal protein levels at the synapse, proper synaptic function also involves the coordination between pre-

synaptic and post-synaptic processes. Studies of neurexins and neuroligins have demonstrated that coordinated pre- and post-synaptic function is crucial for proper synaptic transmission, and that this coordination may be disturbed in ASD. Several studies have shown that many FMRP targets encode presynaptic proteins (Bassell and Warren, 2008; Brown et al., 2001; Darnell et al., 2011), and axonal projections and presynaptic function are altered in *Fmr1* KO mice (Bureau et al., 2008; Hanson and Madison, 2007). The results from a recent study using a novel high throughput cross-linking immunoprecipitation (HITS-CLIP) assay to identify FMRP targets is particularly illuminating (Darnell et al., 2011). Over 800 mRNA binding targets of FMRP were identified and interestingly, presynaptic targets for FMRP were found to be just as numerous as postsynaptic targets. The TSC1/2 complex has been shown to regulate axon formation as well, and there are aberrant axonal projections and abnormal growth cone collapse in $Tsc2^{+/-}$ mice (Choi et al., 2008). This suggests that presynaptic disruptions are also likely to contribute to the pathogenesis of both FX and TSC. In fact, several proteins related to axonal growth and synapse formation have now been identified as potential risk factors for ASD (Alarcon et al., 2008; Morrow et al., 2008; Wang et al., 2009; Weiss et al., 2009).

While the evidence for local protein synthesis in axons or axon terminals in the mature nervous system is still sparse, recent studies have demonstrated that during early axon development and synapse formation local protein synthesis plays an important role in pathway and target selection (Akins et al., 2009; Jung et al., 2012). Thus, dysregulation of local protein synthesis may disrupt presynaptic development as well as postsynaptic plasticity, with the combination resulting in the altered synaptic function and connectivity characteristic of ASD. Furthermore, mRNA profiling of regenerating sensory axons has revealed that the accumulation of specific mRNAs in axons can be increased or decreased in response to different ligands (Willis et al., 2007). Therefore, the protein population of pre-synaptic inputs may be bidirectionally modifiable in a similar manner as post-synaptic inputs.

5.5 Synaptic dysfunction in relation to the autistic phenotype

Genetic and molecular studies have made great strides in identifying synaptic dysfunction as a common pathology in ASD, and this has led to potential therapeutic targets. However, connecting this synaptic pathology to the complex behavioral phenotypes of ASD remains a significant challenge. We have demonstrated that there is an optimal range of protein synthesis

for synaptic function and that deviations too far in either direction are detrimental. While this in itself may not be entirely surprising, the fact that these bidirectional alterations can lead to similar cognitive and behavioral dysfunction was certainly unexpected. This suggests a convergence of these opposing alterations on a common function or process, and highlights the need to examine circuit-level disturbances that may unify these opposing synaptic disruptions.

The work in this thesis has focused on the hippocampus because it is a model system for the study of synaptic plasticity and essential for memory and cognition. However, disruptions associated with autism are by no means limited to this area. Indeed, alterations in synaptic plasticity are present in many brain regions in the *Fmr1* KO mouse (Pfeiffer and Huber, 2009). A recent intriguing study demonstrated that specifically deleting *Tsc1* in the cerebellum resulted in several social behavior deficits, demonstrating the importance of this brain area to the autistic phenotype (Tsai et al., 2012). Many genes implicated in autism have complex expression patterns and therefore may have different effects on different brain regions. An extreme example is Angelman syndrome, where the *UBE3A* gene is maternally imprinted resulting in a very discrete deletion pattern (Kishino et al., 1997). Thus, disruption of the genes associated with ASD may occur in a complex temporal and spatial pattern, which likely contributes to the heterogeneity of the autistic phenotype.

It is tempting to speculate that certain regions may be involved with specific aspects of the ASD phenotype, for example: the hippocampus and cognitive/learning deficits; the striatum and stereotyped behavior; the amygdala and emotional responsiveness; the cerebellum and motor/gait disruption; and primary cortical areas and aberrant sensory processing. However, uncovering the social brain has remained elusive. It is likely that coordinated activity between a network of brain regions is required for the interpretation of complex stimuli that underlies social interaction.

Cognitive and executive functions are thought to rely on the coordinated interactions of a large number of neurons that are distributed within and across different specialized brain areas. Neurophysiological studies have demonstrated that local synchronization of high frequency brain oscillations within a brain area, and global synchrony between brain regions, may be important for this coordination (Uhlhaas and Singer, 2006). It is interesting to speculate that alterations in the synchronization of neural activity may impair communication between brain regions, and it has been suggested that decreased functional connectivity between brain areas may contribute to

the cognitive and behavioral deficits associated with ASD (Geschwind and Levitt, 2007). Supporting this notion, numerous human studies using fMRI and MEG have found altered synchrony in the brains of people with autism, (Cherkassky et al., 2006; Damarla et al., 2010; Just et al., 2004; Khan et al., 2013; Villalobos et al., 2005). Interestingly, alterations in neuronal synchrony have recently been recapitulated in several mouse models of ASD (Penagarikano et al., 2007; Zhang et al., 2008), which will allow for dissection of the underlying cellular process that may contribute to this disruption in functional connectivity.

Alterations in the ratio of cellular excitation to inhibition (E/I balance) is thought to regulate neural synchrony (Sohal et al., 2009; Yizhar et al., 2011), and it has been suggested that disruption in E/I balance may a common pathology in ASD (Gogolla et al., 2009; Rubenstein, 2010; Rubenstein and Merzenich, 2003). Indeed, a recent study has demonstrated that enhancing the E/I ratio in the cortex of mice is sufficient to both alter neuronal oscillations and disrupt social behavior, demonstrating a connection between these processes (Yizhar et al., 2011). Many mouse models of ASD exhibit alterations to excitatory and/or inhibitory synaptic strength (Dani et al., 2005; Etherton et al., 2009; Gibson et al., 2008; Gkogkas et al., 2013; Tabuchi et al., 2007). Interestingly, many of the mutations that disrupt synaptic protein synthesis also result in changes in cellular excitation (Bateup et al., 2011; Gibson et al., 2008; Gkogkas et al., 2013), suggesting that alterations in synaptic protein synthesis can lead to changes in connectivity and *vise versa*.

The maintenance of a healthy E/I balance requires synaptic strength to be maintained within an optimal functional range. Above we discussed the possibility that the proteins dysregulated in ASD may provide an architecture for maintaining this functional range. Thus, disrupting the balance of synaptic protein synthesis in either direction may lead to an imbalance in the E/I ratio, subsequently disrupting functional connectivity between brain regions and impairing the cognitive processes required for complex behaviors like language and social interaction. A more thorough examination of E/I balance and neural synchrony in animal models of ASD, and their relation to previously defined synaptic alterations, is required to determine how synaptic dysfunction may be related to the behavioral phenotypes associated with ASD.

5.6 Concluding remarks

Two technical advances have been instrumental in advancing our understanding of the pathophysiology of ASD: (1) large-scale genomic sequencing that has identified potential risk factors for the disorder; and (2) genetic engineering that has allowed researchers to model these genetic disruptions in animals. The last decade has seen a marked increase in data addressing the genetic nature of ASD and literally hundreds of genes have been identified as potential risk factors (Abrahams and Geschwind, 2008; Betancur, 2011). These studies have provided a framework for identifying the molecular pathways that may be associated with ASD. Furthermore, they have provided targets that can be modeled in animal systems in order to determine the molecular and cellular underpinnings of ASD.

This thesis directly compared animal models of ASD resulting from distinct genetic mutations with the motivation of finding an underlying connection in the molecular and cellular basis of ASD. The data presented here has demonstrated that synaptic protein synthesis, particularly downstream of mGluR5 activation, may be a common pathology in ASD. Specifically, we have shown that there are bidirectional deviations in synaptic protein synthesis in the mouse models of FX and TSC, the two leading inherited causes of ASD (Figure 3.6a). However, there are several additional animal models of ASD that have disruptions in the ERK and mTOR pathways. It will be illuminating to determine where these other models of ASD lie on this axis of synaptic protein synthesis (Figure 5.1).

Based on the results described here, animal models characterized by excessive mTOR signaling, like those with *Pten* mutations, would be predicted to display a similar phenotype to the *Tsc2*$^{+/-}$ mice. Conversely, those characterized by excessive ERK signaling, like the *NF1*$^{+/-}$ mouse, would phenocopy *Fmr1* KO mice. If alterations in synaptic protein synthesis and LTD are indeed found in other animal models of ASD, it would provide further evidence that this is a shared synaptic disruption in ASD and will elucidate potential therapeutic strategies. Interestingly, decreasing ERK signaling with the Ras inhibitor lovastatin has been shown to be beneficial in both the *NF1*$^{+/-}$ (Li et al., 2005) and *Fmr1* KO mice (Osterweil et al., 2013). Likewise, rapamycin treatment has been shown to reverse several cellular and behavioral phenotypes in both *Tsc2*$^{+/-}$ and *Pten*$^{+/-}$ mice (Ehninger et al., 2008a; Zhou et al., 2009). It will be informative to determine the effect of mGluR modulation in these other animal models to determine the scope of this therapeutic approach.

While germ line mutations in ASD-related genes best model the human disorders, it is difficult to distinguish between synaptic disruptions that cause altered brain function and those that are consequences of altered brain development in these animal models. While both are important for understanding disease pathophysiology, it is likely that treatments targeting the primary pathogenic cause(s) of synaptic dysfunction will be more efficacious. A case in point may be the excessive mTOR signaling that has been demonstrated under certain conditions in the *Fmr1* KO mouse. While this would suggest that inhibition of mTOR is a potential therapy for FX, the results presented here suggest the contrary, as a disease characterized by excess mTOR activity (the $Tsc2^{+/-}$ mouse) displayed opposite phenotypes from the *Fmr1* KO mouse. Furthermore, increasing mTOR signaling by decreasing *Tsc2* levels in the *Fmr1* KO mice ameliorated both synaptic and behavioral phenotypes.

It is important to exercise caution when attempting to translate cellular phenotypes in animal models into clinical therapies. Two things must be considered. First, while acute manipulation of ASD associated genes may not replicate the disorder as well as germ line mutations, it is still a useful tool for dissociating the primary pathogenic deficits from secondary or compensatory consequences. To this end, acute deletion of *Fmr1* has been shown to result in exaggerated LTD, and acute re-expression of FMRP rescues this exaggeration in the *Fmr1* KO mouse (Zeier et al., 2009). Acute deletion of *Tsc1* has likewise been shown to result in deficient LTD (Bateup et al., 2011).

Secondly, the consequences of any cellular or molecular phenotypes in animal models of ASD should be validated at the behavioral level before extrapolating to drug therapies. However, it is important to remember that a mouse brain is not a human brain. Deletions of ASD-associated genes in mice are not likely to lead to the same cognitive phenotypes as they do in humans. Despite this, it is likely that these disruptions will have some effect on animal behavior, making behavioral examination still important, particularly for determining the potential efficacy of treatments. Moreover, though the behavioral manifestations in animal models of ASD may not recapitulate the phenotypes seen in humans, it is likely that the effect of these mutations at the cellular and circuit levels will be similar. Determining how synaptic dysfunction may converge at the circuit and system levels, guided by the knowledge gained from genetic and molecular studies, is required to develop a complete understanding of pathophysiology of ASD.

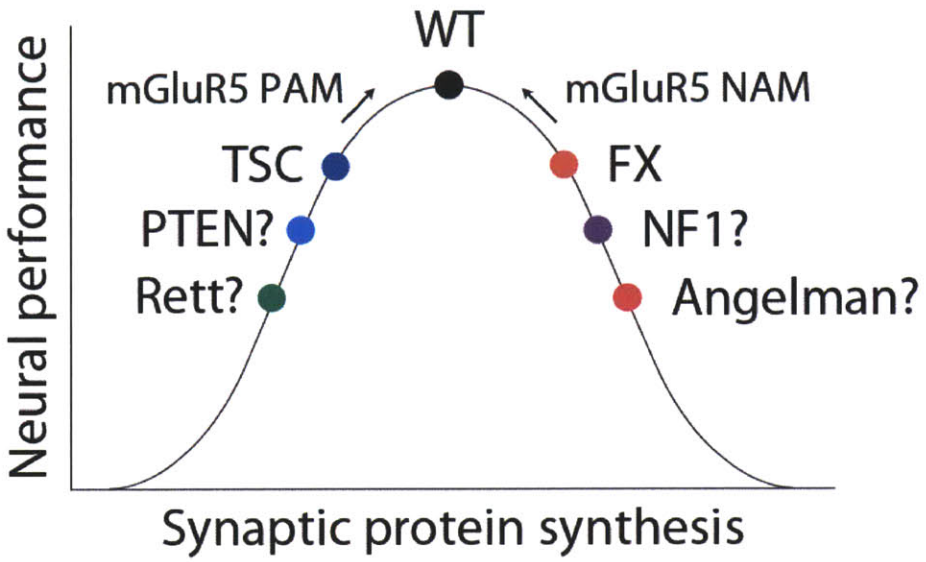

Figure 5.1 – Defining an axis of synaptic pathophysiology in autism. The research in this thesis has indicated that an optimal synaptic function requires a narrow and tightly regulated level of synaptic protein synthesis, and that deviations of protein synthesis in either direction can impair function. Here we have demonstrated that impaired functions in FX caused by excessive local protein synthesis can be corrected by a negative allosteric modulator (NAM) of mGluR5. On the other hand, impaired function in TSC caused by reduced local protein synthesis can be restored by an mGluR5 PAM. Future studies will be required to determine the extent to which dysregulated synaptic protein synthesis is associated with other models of ASD and the effectiveness of mGluR modulators in ameliorating synaptic and behavioral disruptions in these animal models.

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