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The Role of FMRP in Ethanol-Induced Homeostatic Plasticity in the Hippocampus

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THE ROLE OF FMRP IN ETHANOL-INDUCED HOMEOSTATIC PLASTICITY IN THE HIPPOCAMPUS

by

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KATHRYN BREYEL SPENCER. The role of FMRP in ethanol-induced homeostatic plasticity in the hippocampus.
(Under the direction of L. Judson Chandler).

Exposure to chronic ethanol induces homeostatic alterations in glutamatergic signaling and actin polymerization that may have an important role in the development of ethanol-seeking behaviors. Acute ethanol exposure promotes excitation and dampens inhibition while extended periods of exposure induce long-term adaptations in neuronal function that require new protein synthesis to maintain homeostasis. These adaptations include not only transcription and somatic protein synthesis, but also local dendritic protein translation. One of the major mediators of activity-dependent translation is the mTORC1 signaling pathway and its downstream substrates that include kinases and mRNA-binding proteins, such as p70 S6 kinase 1 (S6K1) and fragile X mental retardation protein (FMRP). FMRP is an mRNA-binding protein that interacts with mRNAs to suppress translation. FMRP also interacts with several different mRNAs that code for proteins that are necessary for synaptic plasticity, and it may also have an important role in regulating ethanol-induced alterations in homeostasis in dendrites and dendritic spines. This dissertation addresses the hypothesis that FMRP is necessary for activity-dependent homeostatic alterations in protein expression and spine morphology following chronic ethanol exposure. First, western blot analysis was used to investigate ethanol-induced alterations in expression of FMRP and proteins that are key mediators of

dendritic excitability. These studies revealed an increase in FMRP phosphorylation as well as alterations in the A-type K+-channel Kv4.2, KChIP3 and NMDA receptor subunits. Further studies examining changes in FMRP interactions with Kv4.2, KChIP3, and NMDA mRNAs showed chronic ethanol-induced changes in FMRP-mRNA binding. Additionally, inhibition of FMRP phosphorylation prevented these alterations in protein expression and FMRP-mRNA interactions following chronic ethanol exposure. Studies included in this dissertation also addressed whether alterations in protein expression are accompanied by changes in actin polymerization and spine morphology. These experiments utilized two different sub-strains of C57BL/6 mice with different polymorphisms in *cyfip2*, a protein regulating actin polymerization that is also implicated in regulation of protein translation. A two-bottle choice/CIE exposure paradigm revealed alterations in ethanol consumption between the two strains as well as differences in ethanol-induced changes in protein expression and spine morphology. Taken together, this dissertation reveals an integral role for FMRP in mediating ethanol-induced alterations in homeostatic protein expression, and that these alterations may influence actin polymerization and drinking behaviors.

Chapter 1

Background and Significance

Impact of ethanol dependence

In many industrialized nations, alcohol is consumed on a daily basis to relieve stress and for its effects on positive mood states (Grant et al 2004). Like coffee or nicotine, it has become a cultural norm at many social events. However, overuse of alcohol presents several issues that have lasting effects on societal as well as individual levels, and alcohol addiction continues to be a major social, economic, and medical burden to communities worldwide. According to the

World Health Organization, alcohol is the third-largest risk factor for health burden, and is the number one risk factor for middle-income countries (Dawson and Grant 1998; Grant et al 2004; Bouchery et al 2011). In the United States, alcohol use disorders are among the most prevalent mental health disorders regardless of ethnicity or socioeconomic status and are the third leading cause of death (Goetze et al 2003; Grant et al 2004). Economically, it has a significant toll on the overall cost to society and the individual. The latest data available for 2006 revealed that alcohol accrued a total cost over $220 billion, owning to loss of productivity, cost to the government, and expenses related to healthcare costs and criminal activity (Bouchery et al 2011).

On the individual level as of 2006, approximately half of adults have a family member with an alcohol use disorders (Boucher et al 2011; Dawson and Grant 2011). Addiction and the development of addictive behaviors involve a complex gene x environment interaction that culminates in a pathology that is costly, poorly understood, and difficult to treat (Goetze et al 2003; Grant 2000). Toxicity from long-term, heavy drinking affects many different body systems and organs from the liver and pancreas to the brain (Grant et al 2004; Bouchery et al 2011). Without a discrete mechanism of action, it is difficult to elucidate the mechanisms driving addictive behaviors and long-term effects of alcohol abuse, and therefore, makes it difficult to treat.

Ethanol's effect on the hippocampus

Ethanol and hippocampal circuitry

Ethanol acts on a number of different organ systems, including the central nervous system. In the brain, ethanol interacts with several brain regions, altering neuronal function resulting in behavioral changes, following both acute and chronic ethanol use (Gulick and Gould 2007; Zorumski et al 2014). These behavioral effects are mediated not only by brain regions associated with reward and executive function, such as the nucleus accumbens (Nac), ventral tegemental area (VTA), and prefrontal cortex (PFC), but also areas mediating emotion and memory, such as the amgydala and hippocampus, respectively **(Figure 1-1)** (Zhou et al 2007; Heinz et al 2009; Bailey et al 2015). The interconnectivity of the hippocampus with other brains regions and its role in learning and memory make this region an important intersection of reward and cognition (Lynch 2003; Adcock et al 2006; Zorumski et al 2014). In other drugs of abuse, differences in hippocampal function mediate not just acquisition of drug-taking, but also alters the time needed to induce reinstatement and relapse (Castilla-Ortega et al 2015, Mague et al 2015). In alcohol abuse disorders, one of the principal cognitive effects is the attenuation of learning and memory (Fadda and Rossetti 1998; Roberto et al 2002; Spanagel 2009). This attenuation can have lasting effects on the individual and a significant impact, both socially and economically (Grant 2000; Grant et al 2004; Bouchery et al 2011).

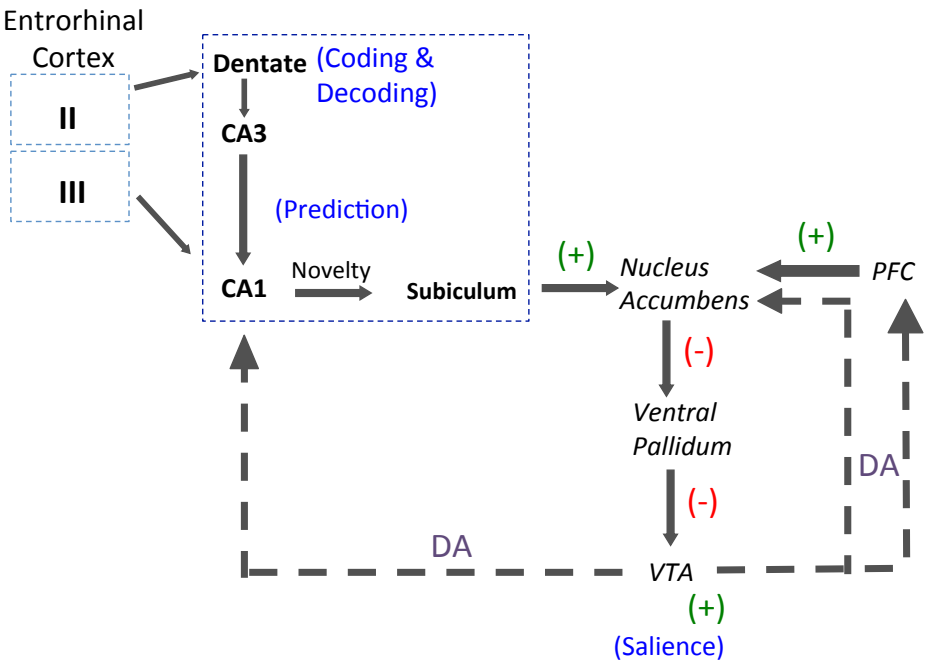

Figure 1-1 Hippocampal VTA Loop: Connection to memory, reward, and salience
Adapted from Heinz et al 2008

The hippocampus is necessary for the formation and encoding of longterm memories. Connections with dopminergic reward circuitry affect ethanol craving. Increasing salience of stimuli associated with ethanol memories may be protective or contribute to relapse.

Ethanol's effect on memory

Acute episodes of binge drinking that are accompanied by a rapid increase in blood ethanol concentration can interfere with hippocampal-mediated episodic memory formation (White et al 2000; Zorumski et al 2014). Following a binge-drinking episode, individuals can participate in salient and emotionally-charged situations in the present, but cannot remember events that occurred shortly after the drinking episode (Miller et al 1978; White et al 2000). Although most

individuals do not sustain long-term complications after a period of prolonged abstinence, some alcoholics do in fact develop permanent disabilities that require lifetime care (Sullivan et al 1995, Makris et al 2008; Zorumski et al 2014). These include memory-related disorders such as ethanol-induced persistent amnesia caused by Wernicke-Korsakoffs syndrome. Although this loss of function was originally attributed to thalamic and mammillary bodies dysfunction, more recent studies implicate the hippocampus (Squire et al 1990, Sullivan and Marsh 2003, Kurth et al 2004, Beresford et al 2006). Specifically, the loss of adult neurogenesis and reduction in functional efficiency of the hippocampus in processing information from other regions (Sullivan and March 2003; Adcock et al 2006; Makris et al 2008). Wernick-Korsakoffs syndrome is the most well-characterized long-term deficit resulting from chronic ethanol exposure, other more subtle deficits in hippocampal-dependent memory may also exist (Walter et al 1980; Beresford et al 2006; Spanagel 2009). In adult men with a history of long-term heavy alcohol use, total hippocampal volume was significantly reduced, and this reduction is independent of total brain or intracranial volumes (Beresford et al 2006; Makris et al 2008). This shrinkage was drastically increased among Wernicke-Korsakoffs patients compared to non-amnesic alcoholics (Walker et al 1980; Agartz et al 1999; Sullivan and Marsh 2003).

The role for the hippocampus in ethanol-induced memory deficits and its contribution to alcohol dependence is also an active area of preclinical alcohol research studies. In nonhuman primates given free access to alcohol, hippocampal volume was inversely correlated with drinking behaviors (Zhou et al

2007; Kroenke et al 2014). Studies in rodent models of alcohol exposure show a decrease in newly formed neurons in adult animals, suggesting in addition to white matter, alcohol use may also alter cell proliferation and survival (Herrera et al 2003; Anderson et al 2012; Talani et al 2013). Behaviorally, in rodent models, alcohol-preferring rats show deficits in spatial learning during the Morris Water Maze task (Santin et al 2000; White et al 2000). Although the hippocampus is not generally thought of as mediating ethanol-mediated behaviors in terms of ethanol-seeking or motivation, its role in salience and memory, along with the long-term effects of chronic alcohol use, indicate an important overall role in ethanol-induced short- and long-term behavioral deficits (Santin et al 2000 Gulick and Gould 2007).

A number of environmental changes may induce alterations in the brain at the behavioral, regional, and cellular level that allow for adaptation and maintenance of homeostasis (Spanagel, 2009; Thomas et al 2013; Bailey et al 2015). In the hippocampus, like other areas of the brain, these cellular alterations involve both pre- and postsynaptic events to remodel synaptic function to fit these environmental changes (Davies et al 1989; Bliss and Collingridge 1993; Bellot et al 2014). This includes reestablishing a balance between excitation and inhibition to prevent hyperexcitability while also preserving proper neuronal function, and actin polymerization and cytoskeletal remodeling to accommodate alterations in cellular activity (Sutton et al 2006; Gal-Ben-Ari et al 2011; Thomas et al 2013; Labno et al 2014; Baily et al 2015). In pyramidal neurons in the hippocampus, this balance includes excitatory glutmatergic systems, inhibitory

GABAergic influences, as well as local inhibitory proteins in axons, dendrites, and dendritic spines (Lei et al 2008; Lei et al 2010; Enoch et al 2012; Korkotian et al 2013).

The hippocampus as a model system

The hippocampus is often used in studies as a model system for exploring the cellular mechanisms driving neuronal activity in different experimental conditions. It is an essential component of learning and memory, and can have significant effects on global brain function (Pastalkova et al 2006; Simons et al 2009). The hippocampus is also one of the brain regions that is highly susceptible to damage, and its function is impaired in many different brain diseases, including addiction, making it a useful region to study several aspects of cellular function (Holopainen 2005; Korkotian et al 2013). The utility of this region as a model system is due to the neuroanatomy, connectivity, and cytoarchitecture (Turner 1959; Hsia et al 1998; Knierim 2002). Although the hippocampus receives input from several brain regions, the main input mechanism lies with the perforant pathway. This is a ‘one way’ circuit through the perforate pathway to the dentate gyrus, then CA3 to the CA1 followed by the major output through the subliculum (Hsia et al 1998; Knierim 2002). *In vivo* models can readily assess hippocampal deficits with behavior paradigms, such as the Morris water maze (Morris 1981; Vorhees and Williams 2006). *In vitro* experiments can address neuronal mechanisms driving these behaviors. In

organotypic hippocampal slice cultures the intra-regional connectivity, and mature cytoarchitecture are preserved, and can be used for several weeks (Stoppini et al 1991; Holopainen 2005**)**. Taken together, these features make the hippocampus a useful tool in elucidating and manipulating the cellular mechanisms driving learning and memory as well as alcohol use disorders.

Glutmatergic signaling and NMDA receptors

NMDA receptor structure and kinetics

Ethanol directly interacts with a number of different cellular mechanisms and proteins, including NMDA receptors, that induce alterations in neuronal homeostasis (Lovinger et al 1990; Hendrickson et al 2004; Nagy 2008). NMDA receptors, along with AMPA and kainate receptors are glutamate receptors that promote excitation in the brain (Keinanen et al 1990; Nakanishi 1992; Dingledine et al 1999). They are heteromeric ligand-gated ion channels that pass both Na^{+} and Ca^{+2} ions, but NMDA receptors are five to ten times more permeable to calcium than sodium (Hume et al 1991; Koh et al 1995; Mori and Mishina 1995; Rosenmund et al 1998). The NMDA receptor itself is composed of two GluN1 subunits and two of GluN2A-D subunits. The GluN1 subunit is necessary for trafficking to the membrane and contains the binding site for the co-agonist glycine or D-serine (Benveniste and Mayer 1991; Mori and Mishina 1995; Dingledine et al 1999; Hawkins et al 2004). GluN2 subunits determine channel

kinetics and contain the glutamate-binding site (Hume et al 1991; Flint et al 1997; Zhang et al 2013; Hansen et al 2014). Each channel has an extracellular domain that contains a modulatory and ligand binding region for association with the agonist and co-agonist, a membrane domain contributes to the channel that conveys the high permeability to calcium, and the extensive cytoplasmic domain that contains residues for direct modification by different kinases to alter channel function and localization (Kuner et al 1996; Fong et al 2002; Hawkins et al 2004; Chen and Roche 2007; Goebel-Goody et al 2009; Zhang et al 2013). These channels may localize to the postsynaptic density or the extrasynaptic space (Groc et al 2006; Goebel-Goody et al 2009; Groc et al 2009; Gladding and Raymond 2011). Composition of the receptor and the stage of brain development dictate the localization (Flint et al 1997; Barria and Malinow 2002; Groc et al 2007; Gladding and Raymond 2011). As the brain develops into adulthood, there is the addition of GluN2A containing NMDA receptors, with GluN2A typically trafficked to the postsynaptic density and GluN2B shuttled to the extrasynaptic space (Ehlers et al 1995; Groc et al 2006; Akashi et al 2009; Groc et al 2009; Gladding and Raymond 2011).

In addition to both an agonist and co-agonist, NMDA receptors also have a voltage-gated Mg^{2+} ion site that blocks the channel pore (Mayer et al 1984; Nowak et al 1984). For activation, the NMDA receptor must bind both glutamate and glycine that is typically released from the presynaptic neuron while the postsynaptic membrane is depolarized to remove the Mg^{2+} block (Mayer et al 1984; Nowak et al 1984; Seeberg et al 1995). Requiring both of these

circumstances to occur pairs pre- and postsynaptic activity, and it is thought to be important in coincidence detection; that is the coincidence of presynaptic glutamate release and postsynaptic depolarization (Bliss and Lomo 1973; Davies et al 1989; Kullman and Nicoll 1992; Markram et al 1997; Lauri et al 2007). The concept of coincidence detection may have an important influence over neuronal information by forming associations between two separate neuronal events that are spatially separated, but temporally close that converge on a common point (Davies et al 1989; Kullmann and Nicoll 1992; Bliss and Collingridge 1993). At the cellular level, the paired mechanism for NMDA receptor activation may represent the persistent activation needed to trigger the strengthening of synapses between two neurons, and may also provide a potential synaptic model for memory in the formed long-term potentiation (LTP) (Bliss and Lomo 1973; Davies et al 1989; Bashir et al 1991; Bliss and Collingridge 2002; Lauri et al 2007).

LTP in the hippocampus

LTP is one of the most prominent hypothesized mechanisms for adaptive synaptic plasticity, and is widely associated in the hippocampus with learning and memory (Bliss and Collingridge 1993; Bliss and Colingridge 2013). As mentioned previously, NMDA receptors are coincidence detectors and have important implications for LTP and learning and memory (Davies et al 1989; Bashir et al 1991; Tsien et al 1996; Luthi et al 2001; Pastalkova et al 2006). This process has

three properties: cooperativity, associativity, and specificity (Bliss et al 1977; McNaughton et al 1978; Levy and Steward 1979; Bliss and Colingridge 1993; Bliss and Collingridge 2013). Cooperativity describes the intensity of the threshold needed to induce LTP (McNaughton et al 1978; Davies et al 1989; Bliss and Collinridge 1993). While weak stimulation of a single pathway is insufficient to produce LTP, it relies on appropriate threshold and timing of the stimulus for LTP induction to occur (Bliss and Lomo 1973; Bliss and Collinridge 1993). Associativity of LTP states that a weak stimulus can be potentiated if a separate, stronger signal converges on a single postsynaptic site (McNaughton et al 1978; Levy and Steward 1979). Lastly, specificity refers to LTP input. That is, LTP is specific for the active synapse, and synapses that are not active at the same time and do not experience the appropriate stimulation will not induce the mechanisms of LTP (Andersen et al 1977; Lynch et al 1977; Bliss and Collinridge 1993). These three properties are all under the assumption that this stimulation occurs on an area of the dendrite that is already depolarized (Bliss and Collinridge et al 1993; Lisman and Spruston 2005)

LTP can be divided into two different phases. The early phase in NMDA-dependent LTP involves the opening of NMDA receptors and an influx of calcium into the postsynaptic neuron, but does not require transcription or new protein synthesis (Alford et al 1993; Bliss and Collingridge 1993; Emptage et al 1999). This influx of calcium is the critical event for the induction of LTP (Alford et al 1993; Spruston et al 1995; Emptage et al 1999; Torras-Garcia 2005). However, activation and calcium influx from NMDA receptors alone may not be enough to

produce LTP. The NMDA receptor antagonist, AP-V inhibits LTP, but treatment with thapsigarin also prevents LTP induction, suggesting that intracellular calcium is also important (Harvey and Collingridge 1992; Mody and MacDonald 1995; Emptage et al 1999).

Throughout this early phase, calcium-induced activation of Ca2+/calmodulin-dependent protein kinase II (CaMKII) leads to increased phosphorylation of NMDA receptors (Gnegy 2000; Hayashi et al 2000; Fong et al 2002; Lee et al 2009). This, in turn, causes an increase in surface-expressed AMPA and increases the sodium permeability of the receptor that alters membrane potential and kinetics (Malinow 2003; Fleming and England 2010; Lisman et al 2012; Luscher and Malenka 2012). After the initial phosphorylation of CaMKII, activation persists due to autophosphorylation (Giese et al 1998; Lucic et al 2008). The late phase of LTP, unlike the early phase, requires transcription of new mRNAs and somatic and dendritic translation of new protein (Krug et al 1984; Frey et al 2001; Adams and Dudek 2005). Inhibition of protein synthesis with the polyribosome inhibitor ansiomycin blocks late-phase LTP, but does not alter LTP induction (Krug et al 1984; Bailey et al 2015).

This switch from early- to late-phase LTP involves the activation of several different intracellular signaling cascades that are needed to shift from the induction to the maintenance phase. These include both transcription and translation factors that are necessary for the new protein synthesis needed during late-phase LTP. Late-phase LTP activates CREB and corresponds with an increase in CRE-mediated gene targets (Riccio and Ginty 2002; Panja et al 2014;

Bailey et al 2015). Additionally, several downstream substrates of the ERK pathway are also upregulated or activated (Kim et al 2005; Ivanov et al 2006; Gladding and Raymond 2011). One of the main pathways responsible for local dendritic translation is also activated, PI3-K, the upstream kinase for Akt and mTORC1, is unregulated in the shift from early to late LTP (Tang et al 1999; Gong et al 2006; Bekinschtein et al 2007; Dibble and Cantley 2015). Blockade of PI3-K blocks the maintenance, but not induction of LTP (Brami-Cherrier et al 2002; Takei and Hiroyki 2014; Dibble and Cantley 2015).

In the hippocampus, the behavioral manifestations of LTP have been heavily investigated. Inhibition of LTP, either early- or late-phase, is associated with blockade of hippocampal-dependent behaviors (Torras-Garcia 2005; Kleykamp et al 2010). Rats injected with AP-V perform poorly on the Morris water maze compared to vehicle controls, and this attentuation of spatial learning corresponds to the blockade of cellular activity associated with LTP (Morris et al 1986, Zalutsky and Nicoll 1990; Hanse and Gustafsson 1992; Tsien et al 1996). However, blockade of NMDA receptors seems particularly important during the acquisition phase of memory. Pretraining on the Morris water maze does not produce inhibition of LTP or impair performance, but lack of experience prevents memory acquisition (Bannerman et al 1995). It also appears that NMDA-dependent LTP is particularly important for certain types of memory. NMDA activation is the main receptor responsible for induction of LTP in most, but not all areas of the brain (Johnston et al1992; Torras-Garcia 2005). Mice with increases in total NMDA receptor protein or increased GluN2B subunit

expression show enhanced LTP (Tang et al 1999; Rinaldi et al 2007; Wang et al 2009). Disruption of LTP via inhibition of NMDA receptor activity leads to normal tone-dependent fear learning that is not dependent on hippocampal function, but impairments of context fear learning, which does rely on the hippocampus (Stiedl et al 2000; Torras-Garcia 2005; Kim et al 2006: Robert and Hunt 2015). Additionally, inhibition of other components downstream of the initial acquisition phase of LTP and learning also impair LTP and hippocampal-dependent memory (Zhao et al 2005; Lynch 2003; Niewoehner et al 2007). Inhibition or deletion of CaMKII produces hippocampal-dependent learning deficits in spatial memory and impairs LTP (Strack et al 2000; Kasai et al 2003; Bliss and Collingridge 2013).

Enhancement of LTP and memory is also associated with alterations in the actin cytoskeleton and spine morphology (Fukazawa et al 2003). LTP causes spine enlargement and is associated with long-term spine stabilization to a mature phenotype (Tolias et al 2005; Hill and Zito 2013; Bellot et al 2014; Cingolani and Goda 2008; Lemphrecht 2014). This change in morphology may have an important role in maintaining the strengthened synapses (Spacek et al 1997; Lynch 2003; Nageri et al 2004; Bellot et al 2014). This change can be characterized by alterations in spine shape, number, and density, and throughout most of the hippocampus, these changes are dependent on NMDA activation and induction of late phase LTP (Engert and Bonhoeffer 1999; Hill and Zito 2013; Lamprecht 2014). Like the electrophysiological properties of LTP, CaMKII is also an important regulator of spine morphology during LTP (Okamoto et al 2007;

Kasai et al 2003). CaMKII mediates several actin-binding proteins, and CaMKII knockout animals or knockdown of CaMKII with shRNA produces deficits in not only LTP and learning, but also induces an immature spine phenotype that is resistant to activity-dependent changes in morphology (Sobczyk et al 1995; Strack et al 2000; Okamoto et al 2007; Cingolani and Goda 2008).

NMDA receptors and chronic ethanol use

Glutamatergic synapses, LTP, and dendritic spines are all important components of hippocampal-mediated behavior and are also altered following chronic ethanol exposure (Lovinger et al 1990; Nestler 2001; Nagy 2011). NMDA receptors in particular are susceptible to ethanol-induced alterations in expression and activity. In the context of alcohol use, these receptors have differential responses to acute and chronic ethanol, and may have an important role in ethanol-induced hyperexcitability (Lovinger 1993; Tsai and Coyle 1998; Gulick et al 2007). Acutely, ethanol dose-dependently inhibits NMDA receptors to rapidly decrease peak-current amplitude and accelerates the rate of current desensitization, decreases open channel probability, and these changes occur in a dose-dependent manner (Lovinger et al 1989; Gass and Olive 2008; Moykkynen and Korpi 2012). Previous studies indicate preferential inhibition for either GluN2A- or GluN2B-containing NMDA receptors (Blevins et al 1997; Nagy 2011). However, this may also be region specific, as other studies indicate preferences for GluN2B-containing receptors (Masood et al 1994; Du et al 2011).

Inhibition of NMDA receptors with MK-801 potentiates acute ethanol-induced impairment in memory tasks (Camarini et al 2000). Although ethanol partially blocks NMDA receptors, this is effective in producing the 'blackouts' seen with acute exposure as well as longer, deficits in LTP and behavioral tasks examining hippocampal-dependent memory (Miller et al 1994; Lukoyanov et al 2000; Kleykamp et al 2010; Bisby et al 2015; Robert and Hunt 2015). In addition, dose-dependent effects on pyramidal cell NMDA suppression correlates with dose-dependent effects on episodic memory in rodent studies (Santin et al 2000; White and Best 2000; Tokuda et al 2007). Rats exposed to ethanol also have impaired performance on hippocampal-dependent memory tasks, such as the Morris water maze (Lukoyanov et al 2000; Schulteis et al 2008; Robert and Hunt 2015).

Clinical studies also show that this effect on NMDA receptors alters not only hippocampal-dependent memory, but also translates into effects in neuronal function. Individuals with long-term ethanol use show a selective increase in mRNA levels for the NMDA glutamate receptor in the hippocampus (Hall and Zador 1997; Birnir et al 2014; Jin et al 2014). Other preclinical studies in rats and mice exposed to chronic intermittent ethanol have shown an increase in surface-expressed GluN2B- containing NMDA receptors and NMDA-mediated current during chronic ethanol exposure (Trevisan et al 1994; Follesa and Ticku 1996; Kumari and Ticku 2000). This increase is characterized by a shift in GluN2B-containing NMDA receptors from the extrasynaptic space into the postsynaptic density (Carpenter-Hyland et al 2004; Akashi et al 2009). Functionally, this may

have an important role in ethanol-induced hyperexcitability and lead to toxicity and neuronal death upon ethanol-withdrawal (Mody and MacDonald 1995; Hendricson et al 2007; Lau and Zukin 2007; Haft et al 2014).

Ethanol's effect on NMDA receptors also effects ethanol-induced excitotoxicity during acute withdrawal after cessation of ethanol use following chronic exposure. Ethanol dependence that results in withdrawal can occur even after short bouts of chronic ethanol abuse (Macey et al 1996; Hall and Zador 1997). This withdrawal syndrome is characterized by both behavioral and cellular effects that increase excitability (Roberto et al 2002; Anderson et al 2012) Repeated bouts of ethanol withdrawal potentiates withdrawal hyperexcitability in the hippocampus and results in 'kindling' of withdrawal seizures (Duka et al 2004; Pawlak et al 2005). Previous studies have found that withdrawal toxicity due to chronic ethanol use is associated with an increase in NMDA receptor expression and function (Roberto et al, 2001, Hendricson et al, 2007). This includes an increase in GluN1 expression in dendritic spines in the CA1 (Pian et al 2010). Activation of NMDA receptors is necessary for seizure activity; blockade of the receptor with AP-V or MK-801 prevents ethanol-induced withdrawal (Chandler et al 1993; Camarini et al 2000; Hendricson et al 2007).

Proteins regulating glutamatergic signaling

Role of Kv4.2 Channels in Neuronal Excitation

In addition to proteins regulating excitatory input, ethanol also alters proteins that balance excitation with inhibition. One important mediator of neuronal excitation in dendrites and dendritic spines is the voltage-gated K+-channel Kv4.2. Kv4.2 is a sub-threshold channel that carries A-type current that is part of the Shal-family of K+-channels (Birnbaum et al 2004; Jerng et al 2004; Leung 2010; Barros et al 2012) Like all A-type K+- channels, Kv4.2 produces an outward, transient K+- current that rapidly inactivates, and inactivation and recovery occur at sub-threshold membrane potential (Jerng et al 2004; Barros et al 2012; Carrasquillo et al 2012). These channels form either homo- or heteromultimeric complexes with the alpha subunits forming the pore of the channel and modulatory beta subunits creating a complete channel. Kv4.2 has 6 transmembrane domains with a conserved P-loop, which is shared by many K+-channels and function in K+ recognition, while the fourth transmembrane domain serves as the voltage sensor (Serodio and Rudy 1998; Orlova et al 2003; Birnbaum et al 2004; Jerng et al 2004; Ren et al 2005**)**.

Shal-family K+- channels are expressed throughout the central nervous system as well as the heart, where they also help to modulate excitation and maintain function **(**Rasmusson et al 1998; Jerng et al 2004; Zhou et al 2004;

Kaufmann et al 2012; Carraquillo et al 2012). Expression of Kv4.2 channels in the hippocampus increases with distance from the soma, and are highly concentrated around dendritic branch points in the extrasynaptic space (Cai et al 2004; Kim et al 2007; Kerti et al 2012, Nester and Hoffman 2011; Kaufmann et al 2012). The location and kinetic properties of these channels make them important regulators of back propagating action potentials, compartmentalization of activity in dendrites, firing frequency, and spike repolarization (Serodio and Rudy 1998; Kim et al 2007; Carrasquillo et al 2012).

Kv4.2 channels also form complexes with auxiliary proteins that modulate channel surface expression and kinetics to form a fully functional channel. Each auxiliary subunit conveys different kinetic properties to the channel. One auxiliary subunit, dipeptidyl-aminopeptidase-like protein 6 (DPP6), interacts with Kv4.2 near the first transmembrane domain, and accelerates channel recovery time (Rhodes et al 2004; Ren et al 2005, Leung 2010; Barros et al 2012; Lin et al 2014; Bezerra et al 2015; Kitazawa et al 2015). Another group of proteins known as K+-channel interacting proteins 1-4 (KChIP 1-4), and are particularly important for Kv4.2 expression and function, especially in the hippocampus, interact with all Shal-family K+- channels (An et al 2000; Lin et al 2004; Li et al 2006; Lin et al 2010; Kunjilwar et al 2013). These proteins determine not only inactivation time, but also rate of depolarization, and promote K+-channel surface expression (Lin et al 2004; Menegola et al 2006; Ruiz-Gomez et al 2006; Lin et al 2010; Norris et al 2010). Although these channels do not require these auxiliary components for surface expression, Kv4.2 must interact with at least one of two subtypes of

proteins for the channel to function (Rhodes et al 2004; Li et al 2006; Attali et al 2009; Lin et al 2010; Norris et al 2010; Kitazawa et al 2015).

In addition to auxiliary subunits, Kv4.2 activity is also mediated through post-translational modifications that fine tune channel function based on intra- or extracellular changes in activity (Anderson et al 2000; Jerng et al 2004; Barros et al 2012). Phosphorylation of Kv4.2 at different sites by different kinases alters different components of channel function, and these changes are also determined by cellular location, such as distance from the soma (Varga et al 2004; Hammond et al 2008; Kerti et al 2012; Nestor and Hoffman, 2012). Phosphorylation by CaMKII increases Kv4.2-mediated A-type current through an increase in Kv4.2 surface expression (Varga et al 2004; Labna et al 2014). Kv4.2 has three potential regulatory sites for phosphorylation via the ERK/MAPK pathway (Adams et al 2000; Schrader et al 2002; Schrader et al 2006; Lin et al 2010). At one threonine site, phosphorylation decreases current (Adams et al 2000; Schrader et al 2006; Labno et al 2014). At another site, Kv4.2 current is increased, but this requires the inclusion of KChIP3 specifically in the macromolecular complex (Schrader et al 2006; Kim et al 2007; Kunjilwar et al 2013). As previously mentioned, Kv4.2 distribution varies, depending on distance from the soma (Cai et al 2004; Kerti et al 2012, Nester and Hoffman 2011; Kaufmann et al 2012). This distance also dictates turnover rate of the channel. Increasing the distance also increases the turnover rate through phosphorylation by PKA, and this turnover rate is activity-dependent (Schrader et al 2002; Hammond et al 2008, Monaghan et al 2008; Nester and Hoffman 2011).

Kv4.2 channels, LTP, and learning

Kv4.2 channels also have an important influence over NMDA receptors in controlling hyperexcitability, especially in the hippocampus. Deletion of Kv4.2 in knockout animals or through viral knockdown increases the expression of GluN2B-containing NMDA receptors and the induction of LTP (Chen et al 2006, Kaufmann et al 2012; Lugo et al 2012). Conversely, an increase in A-type current through activation of Kv4.2 channels decreases GluN2B-containing NMDA receptors (Kaufmann et al 2012; Korkotian et al 2013). This increase in Kv4.2 activation is dependent on calcium influx through NMDA receptors specifically and the activity of CaMKII, and can be blocked by inhibition of GluN2B- but not GluN2A- containing NMDA receptors (Jung et al 2008, Lei et al 2010, Kaufmann et al 2012; Labna et al 2014). NMDA receptors may also mediate Kv4.2 expresssion. Downregulation of Kv4.2 channels is dependent on GluN2B-containing NMDA receptors (Lei et al 2008; Lei et al 2010). As such, Kv4.2 may remodel synapses and this balance between excitation an inhibition may have an influential role in ethanol-induced hyperexcitability.

This effect on GluN2B-containing NMDA receptors may have an important role in altering learning and hippocampal-dependent behaviors, such as spatial memory and temporal lobe epilepsy (Leung et al 2010, Kaufmann et al 2012, Labna et al 2014). Kv4.2 channels in the hippocampus are of great interest due to their role in regulating NMDA-mediated hyperexcitability and LTP (Lei and Xu 2008; Lei et al 2010). Previous studies have shown both deficient and enhanced

LTP in Kv4.2 knockout animals (Chen et al 2006; Kaufmann et al 2012). These studies revealed that deletion of Kv4.2 results in deficits in the learning phase of the Morris water maze, and these deficits are not attributed to differences in GABAergic function (Lugo et al 2012). Blockade of Kv4.2 also impairs performance on the radial arm maze task, and this performance is restored after channels are fully functional (Labna et al 2014). In addition, learning hippocampal-dependent memory tasks have been shown to promote an increase in expression of Kv4.2 mRNA in dendrites and dendritic spines (Petrecca et al 2000; Gross et al 2011; Trucket et al 2012). Interestingly, this is not associated with an increase in total protein expression, but rather may ensure maintenance of protein expression and activity-dependent changes throughout the learning process (Ruschenschmidt et al 2006; Trucket et al 2012; Labno et al 2014).

At the cellular level, it is hypothesized that Kv4.2 channels alter long, but not short term plasticity (Andrasfalvy et al 2008; Truchet et al 2012). Their influence over NMDA-dependent LTD may modulate signal to noise ratios to optimize the appropriate synaptic connections to facilitate learning and memory and fine-tune excitability in neuronal networks (Lei and Xu 2008; Labno et al 2014). Selective increases or decreases in Kv4.2 expression or function in certain populations of neurons may be key for proper function in the learning and memory network, and may be an essential component of hippocampal-dependent memory tasks and cognition (Shen et al 2008; Prince and Ring 2011; Truchet et al 2012). Due to its role in excitability, Kv4.2 is also an important mediator of other hippocampal-dependent behaviors and diseases. Formation of

Kv4.2 complexes and proper channel function are necessary for proper integration of synaptic signaling and activity-induced neuroplasticity (Lin et al 2004; Jung et al 2008; Leung 2010; Labna et al 2014).

As a therapeutic target in neurodegenerative disorders, blockade of Kv4.2 prevents the loss of K+, decreases intracellular apoptotic processes, and decreases neuronal cell death (Ruschenschmidt et al 2006; Leung et al 2010). Temporal lobe epilepsy is associated with long-term decreases in Kv4.2, and this change in expression parallels the acquisition of recurrent seizures (Monaghen et al 2008; Su et al 2008). Therapeutic agents that enhance channel opening are effective in altering epileptic seizures and decreasing interictal activity (Lugo et al 2008; Aronica et al 2009; Leung et al 2010). Animal models with both pilocarine- or kainic acid- induced seizures, show a long-term decrease in surface-expressed Kv4.2 channels (Jung et al 2008, Su et al 2008). Kv4.2 knockout mice have increased pilocarpine-induced seizures and are prone to excitotoxic neuronal death (Barnwell et al 2009).

KChIP3's regulation of Kv4.2

As mentioned above, Kv4.2 channels require auxiliary proteins for optimal channel function. Both DPPX and KChIPs interact with Kv4.2, but KChIP interactions with the channel have a larger effect on channel kinetics and function (An et al 2004; Lin et al 2004; Rhodes 2004; Callsen et al 2005; Kitazawa et al 2015). KChIPs are EF- hand calcium sensors, and subtypes 1-4 contain 4

calcium sensor domains on the conversed C-terminus and a divergent N-terminus (Spreafico et al 2001; Callsen et al 2005; Barghann et al 2008; Woo et al 2008; Mikhaylova et al 2011). Of particular interest in regulating excitability and activity-dependent hippocampal function is KChIP3. Each KChIP has its 'preferred' binding partner, and has a somewhat discrete regional and cellular expression pattern (Rhodes et al 2004; Xiong et al 2004; Menegola et al 2006). Although, KChIP2 is the most widely expressed of the four subtypes, based on results from co-immunoprecipitation studies, KChIP3 is the preferential binding partner of Kv4.2 (Xiong et al 2004; Han et al 2006; Menegola et al 2006). The difference in the N-terminus of the four KChIPs is responsible for the variation in Shal-family channel kinetics with different KChIPs (Callsen et al 2005; Barghann et al 2008; Raghuram et al 2012). Phosphorylation of KChIP3 induces KChIP3-Kv4.2 interaction, but this alone is not enough to induce Kv4.2 to be trafficked to the membrane, and palmitoylation of KChIP3 is also required for localization to the surface (Takimoto 2002).

Functionally, KChIP3 binds to Kv4.2 as an intracellular auxiliary unit to convey specific gating properties, channel kinetics, and promote surface expression (Shibata et al 2003; Menegola et al 2006; Woo et al 2008; Norris et al 2010). The conserved C-terminus of the KChIPs, in addition serving as the calcium-binding region, also interact with the alpha pore-forming subunit Kv4.2 (Spreafico et al 2001; Shibata et al 2003; Han et al 2006). However, the N-terminus gives different properties to the channel (Hopkins et al 1994; Callsen et al 2005; Han et al 2006). KChIP3 interaction with Kv4.2 produces more rapid

depolarization and faster inactivation, compared with other KChIP subtypes (Kunjilwar et al 2004; Norris et al 2010). Decreasing KChIP3 expression through viral knockdown decreases Kv4.2 channel surface expression and Kv4.2-mediated A-type current, even in the presence of KChIP2 (Menegola et al 2006; Norris et al 2010). Likewise, decreasing Kv4.2 expression in the hippocampus is also associated with a reduction in KChIP3 expression (Menegola and Trimmer 2006). The other auxiliary subunit, DPP6, does not produce a surface-expressed channel with optimal function without KChIP3 interaction (Norris et al 2010). They retain the ability to pass current, but are significantly reduced in the absence of KChIPs. However, KChIP3 interaction alone with Kv4.2 channels, results in a current that is lower in amplitude than that of a channel possessing both DPP6 and KChIP3 (Norris et al 2010; Kitazawa et al 2015). Functionally, this interaction between KChIP3 and Kv4.2 has important implications in behavior and pathology. KChIP3 knockout animals have decreased Kv4.2-mediated A-type current as well as decreased performance on memory tasks such as novel object recognition (Wu et al 2008; Alexander et al 2009).

In addition to its role with Kv4.2, KChIP3 was also discovered in two other fields of research. It was independently discovered as downstream regulatory element antagonist modulator (DREAM) and calsenilin (Buxbaum et al 1998; Carrión et al 1999). DREAM in a transcriptional regulator that binds to downstream regulatory elements (DRE) to suppress transcription (Carrion et al 1999; Mellstrom et al 2001; Mellstrom et al 2014). The C-terminus of DREAM serves as the DRE-DREAM interaction domain with DRE, and binding of calcium

induces dimerization of DREAM and translocation to the nucleus (Carrion et al 1999; Osawa et al 2001; Woo et al 2008; Ramachandran et al 2012). In the nucleus, DREAM must drop off the bound calcium to all three EF-hand domains (Craig et al 2002; Woo et al 2008; Mellstrom et al 2014). DREAM has also been shown to be an important regulator of a number of different mRNAs, including prodynorphin and cFos (Spreafico et al 2012; Alexander et al 2009). Mice overexpressing a calcium insensitive form of DREAM (tg-DREAM), have increased levels of prodynorphin compared to knockouts, and have an response to activity-dependent changes in cFos protein expression (Spreafico et al 2001; Dierssen and Naranjo 2012).

Due to its role as a dynorphin regulator, DREAM was intensively studied as a potential therapeutic target for chronic pain (Cheng et al 2002; Costigan and Woolf 2002, Cheng and Penninger 2004). DREAM knockout mice have a higher pain tolerance compared to control mice that accompany this increase in dynorphin expression (Cheng et al 2002). However, the complexity of its involvement in other activity-dependent mechanisms, and its diffuse nuclear and somatodendritic expression pattern makes it difficult to manipulate as a pharmacological target for a phenotype that is limited to only one of its functions (Cheng and Penninger 2004; Woo et al 2008; Alexander et al 2009). Although all four KChIP proteins have a conserved C-terminus and are capable of binding DRE sequences, none of the other subtypes localize to the nucleus. In yeast-two hybrid studies, when any of the four subtypes are expressed in the nucleus, all show DRE sequence interactions and serve as a transcriptional repressor

(Pruunslid and Timmusk 2005; Raghuram et al 2012). However, animal models show knockdown of KChIP3, not the other three subtypes, alter mRNAs whose corresponding gene contains the specified DRE sequence (Woo et al 2008; Pruunslid and Timmusk 2012).

In addition to DREAM, KChIP3 was also described separately as calsenilin. Calsenilin interacts with presenilin, the active subunit of the enzyme gamma amlyase, which synthesizes beta amyloid (Buxbaum et al 1998; Lilliehook et al 2003). Through interaction with this subunit, calsenilin regulates the rate of conversion and the type of beta amyloid made (Fontan-Lozano et al 2009; Craig et al 2013). The role of calsenilin in beta amyloid production has lead to an interest in its role in the pathology of Alzheimer's disease (Dong-Gyu et al 2004; Alexander et al 2009; Craig et al 2013). Calsenilin also interacts with other intracellular mediators to regulate apoptosis and intracellular Ca^{2+} release from the endoplasmic reticulum (Leissring et al 2000; Lilliehook et al 2002).

Localization of KChIP3 within the cell is important in regulating cellular processes and behavior. In animals overexpressing a calcium-insensitive KChIP3 it is unable to act as a transcription factor, and as a result there is less localization to the nucleus (Dierssen and Naranjo 2012). While these animals show normal LTP, they display enhanced contextual fear conditioning (Wu et al 2010, Alexander et al 2009). However, in patients with Alzheimer's disease, KChIP3 expression is elevated which may be associated with a decline in cognitive function as well as an increase in apoptosis (Lilliehook et al 2002; Craig et al 2013). In the context of epilepsy, there is a decrease in KChIP3 in the

hippocampus of post mortem seizure patients (Hong 2003). These conflicting pathologies indicate that localization and regulation of KChIP3 is highly dependent on intracellular signaling pathways that intricately coordinate and balance the function of KChIP3.

Role of KChIP3 in regulating NMDA receptor function

In the context of hyperexcitability and hippocampal-dependent deficits in behavior, KChIP3 is a potentially important component of the balance between excitation and inhibition. In addition to its role in promoting Kv4.2 expression and function, KChIP3 also modulates glutamate receptor trafficking. KChIP3 interacts with the GluN1 subunit of NMDA receptors to decrease surface expression and NMDA-mediated current (Zhang et al 2010). Additional research also indicates KChIP3 may interact with GluN2B as well, and may preferentially modulate GluN2B-containing NMDA receptors (Zhang et al 2010; Wu et al 2010). This interaction with GluN1 is calcium-dependent. The binding of each EF-hand calcium sensor to a calcium ion results in a progressive reduction of current (Zhang et al 2010; Wang and Wang 2012). Other studies in the calcium insensitive overexpressing KChIP3 mice indicate that even in the absence of calcium, KChIP3 can still inhibit NMDA receptors. However, this occurs through interaction with the anchor protein PSD-95, and does not alter NMDA-mediated current, but instead decreases surface expression (Wu et al 2010; Wang and Wang 2012). As a result, KChIP3 can not only alter cellular function though the

regulation of ion channels to dampen excitation, but also directly interact with glutamate receptors to further decrease intracellular calcium levels and glutamatergic receptor function.

KChIP3 may have an important role in ethanol-induced hyperexcitability in the hippocampus. Its dual role in regulating both NMDA and Kv4.2 surface expression and function creates the possibility that KChIP3 is part of a homeostatic mechanism that balances local excitation and inhibition during chronic ethanol exposure.

Activity-dependent translation in dendrites

Different proteins at glutamatergic synapses are important for the balance of excitation and inhibition, and to maintain homeostasis during activity-dependent alterations in the intra- and extracellular environment (Sutton et al 2006). Long-term maintenance of neuronal activity requires the induction of more permanent mechanisms that induce transcription and translation of new protein in dendrites and dendritic spines in an activity-dependent manner (Gardiol et al 1999; Kang and Schuman 2004; Holt and Bullock 2009; Zukin et al 2009; Dieck et al 2014).

Review of translation

In the brain, translation of new protein is a key step in synaptic plasticity and in the processing and retention of required information (Cajigas et al 2010; Darnell and Richter 2012; Holt and Schuman 2013). Typical activity-dependent translational regulation is necessary not only to maintain proper synaptic function, but also has important behavioral consequences (Sutton and Schuman 2006; Costa-Mattioli et al 2009; Holt and Schuman 2013). Translation in dendrites and dendritic spines, like axonal or somatic translation, is cap-dependent and relies upon the unwinding of the 5' cap for protein synthesis (Levy et al 1991; Steward and Schuman 2001; Sutton and Schuman 2006). The process is divided into three steps: initiation, elongation, and termination **(Figure 1-2)**.

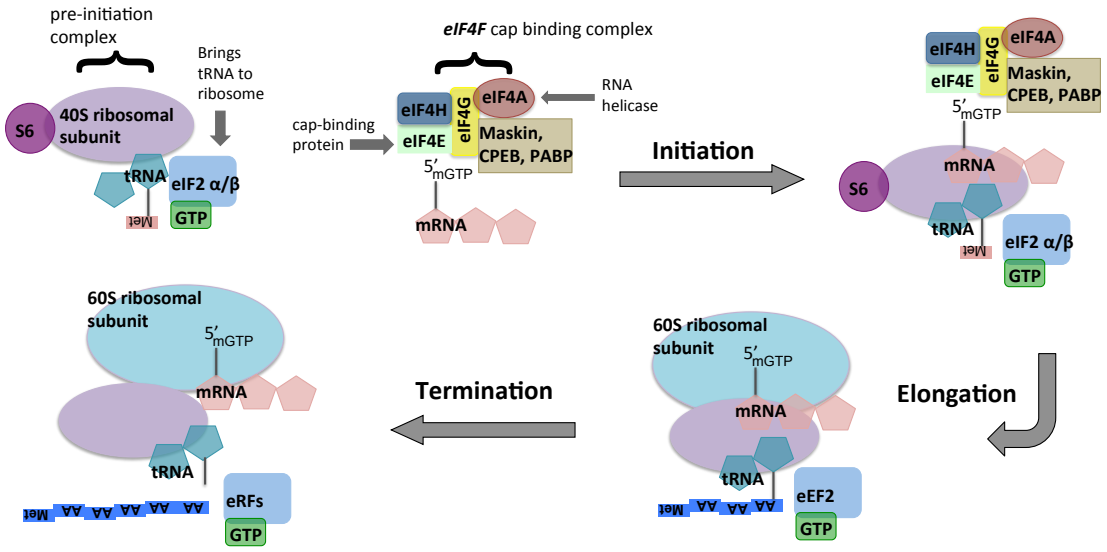

Figure 1-2 Overview of Translation

Translation begins with the assembly of the initiation and pre-initiation complexes consisting of initiation factors that bring together all the components necessary to unwind the mRNA and bring the correct corresponding amino acid. After assembly of the complexes the first amino acid, methionine, begins the protein. The 60S ribosomal subunit is recruited to continue the addition of amino acids to the change brought by the tRNA and mediated by elongation factors. Finally, termination releases the finished protein from the ribosome.

Initiation is the most well characterized step in protein synthesis, and is also the rate-limiting step for translation due to the number of components necessary for the pre-initiation and initiation complexes (Nakamoto and Kalokfsky 1966; Davis and Squire 1984; Holz et al 2005; Pestova et al 2007). This assembly involves recruitment of eukaryotic initiation factors (eIFs) and coordinated activity of different ribosomal subunits. After trafficking to the synapse, the eIF4E binds the 5' cap of the mRNA (Levy et al 1991; Pinkstaff et al 2001; Scheper and Proud 2002; Pestova et al 2007). Next, eIF4G acts a scaffolding protein for assembly of the rest of the initiation complex (Oberer et al 2005; Hinnebusch and Lorsch 2012). The last initiation factor in this complex is eIF4A, which is the active

helicase that unwinds structures in the 5' untranslated region of the mRNA to facilitate translation (Tsokas et al 2005; Sonenbergy and Dever 2003). Collectively these three initiation factors eIF4E, -G, and -A along with eIF4H are known as eIF4F once they are assembled into an active complex (Sonenberg and Dever 2003; Oberer et al 2005; Hoeffer et al 2013). After assembly of eIF4F, another initiation factor, eIF3 recruits the 40S small ribosomal subunit to the assembled complex (Hinnebusch 2006). This subunit acts as a scaffold for appropriate alignment and initial association of the tRNA with the other components assembled for initiation (Colombo et al 1968; Erzberger et al 2014; Korostelev 2014).

After assembly of the entire complex, the 40S subunit scans the untranslated region as it unwinds until it reaches the start codon, AUG, which codes for methionine (Colombo et al 1968; Revel et al 1968; Hussain et al 2014). tRNA is responsible for bringing the appropriate amino acid to the polyribosome based on the mRNA triplet base pairings. This methionyl- tRNA serves as a specialized initiator for translation. GTP-bound eIF2 brings the methionyl-tRNA to the 40S subunit (Clark and Marcker 1966; Revel et al 1968; Cheung et al 2007; Erzberger et al 2014; Hussain et al 2014). Hydrolysis of GTP bound to eIF3 to GDP signals for the dissociation of the small 40S subunit, which is replaced by the large 60S ribosomal subunit (Holland et al 2004; Hinnebusch and Lorsch 2012; Gamalinda et al 2014).

Following initiation with the assembly of the necessary components and the addition of the first amino acid, the rest of the mRNA is translated into protein

through elongation. Elongation is a widely conserved process that is mediated by eukaryotic elongation factors (eEFs) (Hinnebusch and Lorsch 2012; Sala 2014). Like the final stage of initiation, this involves tRNAs bringing the appropriate amino acid to the ribosome for addition on the translated protein (Dresios et al 2006). The elongation factor eEF1A in GTP-bound form separates the amino acid from the tRNA. However, this process requires the GEF eEF1B to maintain a certain rate of function for activity-dependent translation to occur (Rogers et al 2001; Kapp and Lorsch 2004; Cao et al 2014). After each amino acid addition, the large 60S subunit slides down the mRNA via hydrolysis of the GTP bound to eEF2 (Ryazanov and Davydova 1989; Kapp and Lorsch 2004; Heise et al 2014).

The final step in protein translation is termination. This step is not well studied, and the components and the exact process are not completely understood, compared to that of the two previous steps. However, like initiation and elongation, termination also utilizes separate proteins that are eukaryotic release factors (eRFs) for release of the finished peptide (Zhouravlev et al 1995; Kapp and Lorsch 2004). Once the assembled polyribosome encounters the consensus stop code, UAG, hydrolysis of GTP-bound to eRFs releases the finished peptide and promotes disassembly of the current active complex to allow the components to return to the pool of available subunits (Dever and Green 2012).

Most protein synthesis occurs in the soma, and the proteins are then transported to different neuronal processes via molecular motors (Kanai et al 2004). However, more recent studies have followed lines of research focusing on

the role of local dendritic protein synthesis and its relevance to synaptic plasticity, particularly in the hippocampus (Kang and Schuman 1996; Asaki et al 2003; Govindarajan et al 2011). While most of this production is somatic, dendritic protein translation is necessary, in some circumstances, to maintain long-term activity-dependent changes (Huber et al 2000; Martin et al 2000; Asaki et al 2003; Sutton and Schuman, 2006; Govindarajan et al 2011). Elimination of dendritic translation through mutation of the mRNA dendritic targeting element produced deficits in the spatial recognition and contextual learning (Kang and Schuman 1996; Morris et al 2002; Sutton and Schuman; 2006). Typically, this local translation involves a highly coordinated series of events with not only mRNAs, but also assembly of the polyribosome for translation and intracellular regulatory processes each component (Liu-Yesucevitz et al 2011; Parysan et al 2011; Korostelev 2014). Regulation of translation can occur at each of the three stages.

Due to its function as the rate-limiting step and as the first point of contact for mRNAs, the initiation step has several different components that alter both the initiation and rate of translation at the polyribosome (Rogers et al 2001; Dobrikov et al 2013). One of the most well-studied proteins involved in translation is the cap-binding protein eIF4E (Raught and Gingras 1999; Rogers et al 2001; Scheper and Proud 2002; Sonnenberg and Dever 2003; Gkogkas et al 2013; Sonenberg and Hinnbusch 2009). This is the first protein to interact with mRNAs and represents the first element in the rate-limiting step (Parysan et al 2013;. Phosphorylation of eIF4E decreases the affinity for capped mRNA, which in turn,

slows the rate of translation (Raught and Gingras 1999; Waskiewicz et al 1999). This protein also interacts with 4 eukaryotic initiation-binding proteins (4E-BPs). These regulate translation by binding to eIF4E and inhibiting interaction with the 5' cap (Levy et al 1991; Rogers et al 2001). This includes 4E-BP2, nuclear FMRP interacting protein (NuFIP) and cytoplasmic interacting proteins (Cyfips) that convey long-term inhibition of total protein expression. These act as linker proteins between the polyribosome and larger macromolecular complexes that often include other proteins and noncoding RNAs to not only block the cap-binding protein but to stall out the polyribosome to prevent cap-independent translation (Bardoni et al 2003; Napoli et al 2008; Pathania et al 2014). Although these act as translational repressors, they are important in the coordination of activity-dependent protein synthesis. Knockout or dysfunction of these proteins leads to aberrant synaptic plasticity, and deficits in hippocampal-dependent learning tasks (Banko et al 2007; Liu-Yesucevitz and 2011; Santini et al 2012; Pathania et al 2014). Activity of the alpha helicase is another component of initiation that affects the rate of translation. The poly(A)-binding protein (PABP) interacts with the 3' end to circularize the mRNA (Le et al 2000; Atkins et al 2004; Kahvejian et al 2005). This enhances unwinding of the helix and drastically enhances the rate of translation (Atkins et al 2004; Dieck et al 2014).

The process of mRNA and protein elongation is regulated through phosphorylation of elongation factors. Binding of GTP to eEF1A is relatively slow without the GEF activity of eEF1B (Tsokas et al 2005; Cao et al 2014). Phosphorylation of either of these elongation factors by PKC or casein kinase

alters the rate at which elongation of the protein occurs (Price et al 1991; Redpath and Proud 1993). Phosphorylation by PKC decreases the ability of eEF1A to bind GTP and slows translation (Redpath and Proud 1993; Ryazanov et al 1988; Tsokas et al 2005). However, phosphorylation of either protein by casein kinase enhances the rate of translation (Price et al 1991). The larger ribosomal subunit 60S slides down the mRNA and connects the appropriate amino acids brought by the tRNA (Gamalinda et al 2014). This is controlled by eEF2, which is subject to both acute and longer-term regulation (Ryazanov and Davydova 1989). Acutely, calcium-activated eEF2 kinase (eEF2K) prevents hydrolysis of GTP by eEF2, and inhibits movement of the larger subunit down the mRNA (Ryazanov et al 1988). However, eEF2K is only activated by acute influx of calcium at the synapse; inhibition lasts around 30 minutes following the influx of calcium (Ryazanov and Davydova 1989; Sala 2014). Dephosphorylation of eEF2 by PP2A occurs after activation of other intracellular signaling pathways (Sonenberg and Dever 2003; Im et al 2009).

Although little is known about the process of termination of translation, this is also regulated by phosphorylation of release factors that alter GTP hydrolysis and the rate of release. However, more regulatory steps occur during the first two steps, initiation and elongation, where mRNA stability, assembly of the complex, and rate of synthesis are affected.

Another important component regulating translation does not necessarily alter the rate of translation, but rather the mRNAs that are translated. mRNA-binding proteins are a large class of proteins that can form macromolecular

complexes to regulate mRNA interaction with the polyribosome. These proteins are important in trafficking, localization, and stability of mRNAs after transcription (Zalfa et al 2006; Bramham and Wells 2007; Bolognani and Perrone-Bizzozero 2008). These proteins contain multiple binding regions for RNAs; however, each region interacts with a specific mRNA sequence. This gives these proteins the ability to bind to several different targets while also giving target specificity (Jones 2003; Shan et al 2003). They are often regulated by post-translational modifications, with the three main types being phosphorylation, arginine methylation, and small ubiquitin-like modification (SUMO) (Glisovic et al 2008). These change activity and RNA binding affinities, induce the formation of ribonucleoprotein complexes (RNP), and creates conformational changes that alter mRNA binding and transport (Shan et al 2003; Darnell and Richter 2012; Hinnebusch and Lorsch 2012). Many of these proteins, like fragile X mental retardation protein (FMRP) are translational repressors that prevent interaction with the ribosome (Laggerbauer et al 2001; Darnell et al 2011). However other proteins such as the Staufen family of RNA-binding proteins, are important in processed mRNA transport down microtubules and into dendrites (Heraud-Farlow and Kiebler 2014). Other proteins, such as Smaug, are important in RNA destabilization and degradation (Tadros et al 2007; Bologani and Perrone-Bizzozero 2008; Gotez and Wahle 2015. However, these proteins also form RNP complexes that contain mRNAs and multiple mRNA-binding proteins to properly regulate trafficking, localization, stability, and translation activity (Braham and Wells 2007; Bologani and Perrone-Bizzozero 2008; Darnell and Richter 2012).

NMDA receptors and dendritic translation

Translation of new protein is a finely tuned process with several regulatory elements that alter not only assembly of the complex and mRNA binding, but also the rate at which mRNAs are translated. In dendrites, the process of local translation is an essential component of the maintenance of adaptive activity-dependent changes to neuronal activity (Ostroff et al 2002; Costa-Mattioli et al 2009; Holt and Schuman 2013). NMDA receptors are a key component for initiating activity-dependent translation, and NMDA-dependent processes, such as NMDA-dependent LTP, require protein synthesis to occur (Marin et al 1997; Im et al 2009; Costa-Mattioli et al 2009). These receptors activate different downstream components that alter local translation in different ways, and regulate all three stages of protein synthesis.

During initiation, several components of the initiation complex are subject to activity-dependent regulation through phosphorylation of different kinases in order adjust the rate of translation to the current cellular conditions. *In vivo* and *in vitro* studies have outlined a role for NMDA-dependent ERK activation in altering specific components of the initiation complex. Downstream substrates MAP kinase signal interacting kinases (Mnk1 and Mnk2) phosphorylate the cap-binding protein eIF4E (Raught and Gingras 1999; Perkinton et al 2002). This phosphorylation enhances activity and the general rate of translation by pulling mRNAs more quickly to the ribosome. Activation of ERK and Mnk kinases via NMDA receptor activity and this subsequent increase in eIF4E activity promotes

new protein synthesis to sustain late phase LTP in the mouse hippocampus (Knauf et al 2001; Perkinton et al 2002; Carriere et al 2011). Mutation at the eIF4E phosphorylation does not alter the induction of LTP, but hippocampal neurons do not maintain LTP activity (Topisirovic et al 2004; Im et al 2009). As previously mentioned, PI3-K is activated during the transition from early to late phase LTP, and induces ERK-independent translational changes via the mTORC1 pathway (Perkinton et al 2002; Shahbazian et al 2006). This regulatory pathway alters several downstream components that are necessary to coordinate activity-dependent translation.

During the elongation phase of translation, activation of NMDA receptors continues to regulate the rate of protein synthesis. However, unlike initiation, this regulation occurs during early phase LTP as well as late phase (Im et al 2009). The eEF2 kinase eEF2K operates via a calcium-dependent mechanism that facilitates binding to eEF2 to slow GTP hydrolysis and the rate of translation (Browne and Proud 2004; Im et al 2009). This paradoxical coupling of both inhibition and activation of translation at different stages represents a finely regulated process that facilitates the necessary cellular functions to accommodate the different requirements of both early and late phase LTP (Im et al 2009). Although NMDA receptor activation enhances the initiation process, it inhibits elements that are downstream to slow translation. However, this inhibition is transient and spatially restricted (Ryazanov and Davydova 1989; Martin et al 2000; Govindarajan et al 2011). Persistent activation of NMDA receptors produces transient blockade of translation via eEF2 inhibition, despite the

presence of calcium at the synapse (Marin et al 1997; Browne and Proud 2004; Im et al 2009). Additionally, eEF2K phosphorylation is restricted to the activated synapses and does not alter translation outside of dendritic spines (Ryazanov and Davydova 1989).

This initial repression of translation may coordinate LTP in different ways. It may serve as a mechanism to allow coordinated mRNA transport and synaptic arrangement to produce the necessary protein needed to maintain LTP (Costa-Mattioli et al 2009; Sossin and Lacaille 2010; Thomas et al 2013; Bailey et al 2015). This may allow for mRNA-specific inhibition of translation, rather than a blanket, general blockade of all translational mechanisms (Doench and Sharp 2004; Bramham and Wells 2007, Dieck et al 2014). At the same time, general protein translation is repressed following NMDA receptor activity, and the expression of certain locally translated proteins, such as CaMKII, are increased (Pinkstaff et al 2001; Asaki et al 2003; Costa-Mattioli et al 2009).

NMDA-dependent LTP is characterized by not only alterations in synaptic activity, but also the production of new protein (Kapp and Lorsch 2004; Klann and Richet 2007; Kim et al 2013). As such, studies have confirmed that NMDA receptor activity is important in the regulation and coordination of activity-dependent translational mechanisms. This process involves several components of the translation machinery that both promote and inhibit local activity-dependent translation (Klann and Richet 2007). With defined deficits in NMDA-dependent LTP in the hippocampus resulting from chronic ethanol exposure, translational

mediators and components controlling the rate of translation are likely candidate proteins for homeostatic regulation in CIE.

mTORC1 translational pathway

Local regulation of dendritic translation requires the convergence and coordination of several different proteins to allow for fluid, effective activity-dependent translation. However, many of these components, those that both promote and inhibit translation, are part of a common regulatory pathway. In dendrites and dendritic spines, one of the most well characterized pathways regulating activity-dependent translation is the Akt-mTORC1 pathway (Brown et al 1995; Gingras et al 2001; Dibble and Cantley 2015). This is a large, diverse pathway with downstream components that control dendritic and somatic translation, and both promote and inhibit protein translation through activation of different substrates **(Figure 1-3)** (Browne and Proud 2003; Holz et al 2005).

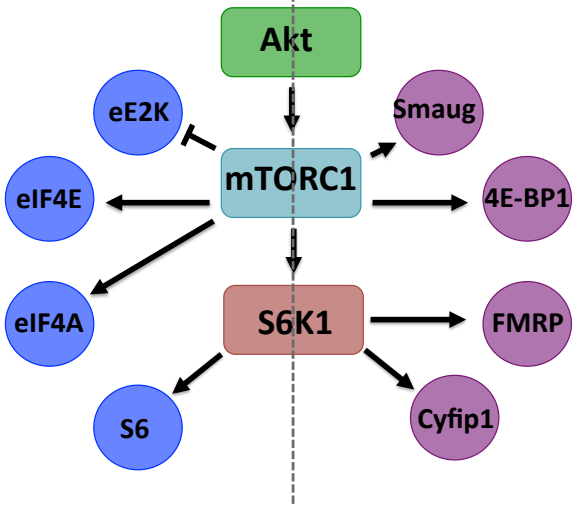

Figure 1-3 mTORC1 signaling

mTORC1 coordinates translation by activating different downstream substrates that are necessary for translation and those that repress translation. Regulated activation of both categories of substrates are important for dendritic *de novo* protein synthesis.

Phosphorylation of phosphoinositide 3-kinase (PI3-K) initiates activity of protein kinase B (Akt). In turn, Akt phosphorylates the 2164 threonine residue to induce assembly mTORC1. Active mTORC1 involves the addition of the catalytic subunit Raptor to the complex (Kim et al 2002; Dibble and Cantley 2015). Following raptor assembly into the complex, the p70 ribosomal protein S6 kinase 1 (S6K1) is phosphorylated (Brown et al 1995; Kim et al 2002). Like mTORC1, S6K1 has a myriad of downstream proteins that control translation in the soma, axons, dendrites, and dendritic spines that both promote and inhibit activity-dependent translation, depending on the localization and function in the neuron (Burnett et al 1998; Gingras et al 2001; Holz et al 2005; Im et al 2009; Ma and Blenis 2009). Some of these substrates include the cap-binding protein eIF4E,

the alpha-helicase eIF4A, and the ribosomal subunit S6; these are all components of the active polyribosome that are necessary for translation (Hara et al 1997; Hara et al 1998; Gingras et al 2001; Ma and Blenis 2009). However, S6K1 also activates other substrates including mRNA-binding proteins like FMRP, that serve as a translational repressors, Smaug, which facilitates mRNA degradation, and Cyfip1, which is a 4E-BP that halts all mRNA association with the polyribosome (Burnett et al 1998; Czaplinski and Singer 2006; Ma and Blenis 2009; Chen and Joseph 2015). Activation of these downstream components follows discrete regulatory mechanisms that tailor activation of each component to the particular change in activity to compensate in both a short- and long-term fashion (Holz et al 2005; Ma and Blenis 2009). Not all downstream proteins are activated simultaneously or even phosphorylated at all during mTORC1 or S6K1 activation (Holz et al 2005).

mTORC1 activity is regulated through a number of different mechanisms, and controls a number of activity-dependent cellular functions in addition to translation including cell growth and apoptosis. In addition to mTORC1, Akt also activates mTORC2 (Urbanska et al 2012). These two complexes differ in their assembly, activity, and sensitivity to rapamycin. mTORC1 is composed of the mTOR and mTOR-associated protein (Ma and Blenis 2009). The recruitment of the catalytic subunit Raptor activates the complex to phosphorylate downstream substrates (Ma and Blenis 2009; Urbanska et al 2012). While dephosphorylation causes dissociation of this subunit and ceases activity; exactly how it differentially regulates activity of each of its downstream substrates is not known.

NMDA-dependent regulation of mTORC1

In addition to ERK/Mnk regulation of translation, NMDA receptor activity also regulates mTORC1 activity to control local translation. In this context, it triggers the translation of mTORC1 substrates including p70 ribosomal S6 kinases 1 and 2 as well as p90 ribosomal S6 kinases 1 and 2 (Rsk1 and 2)(Lenz and Avruch 2005; Gong et al 2006; Carriere et al 2011). This is completely independent of ERK activity and is blocked by treatment with rapamycin (Carriere et al 2011; Dibble and Cantley 2015). Additionally, activation of other downstream mTORC1 substrates can regulate initiation and elongation. eIF4E and other components of the initiation complex are regulated in an mTORC1-dependent manner (Hara et al 1997; Hara et al 1998; Gingas et al 2001; Shahbazian et al 2006). This alteration in activity of mTORC1 substrates corresponds to an increase in S6K1 phosphorylation and activity (Hara et al 1998; Holz et al 2009; Fenton et al 2011). Other studies also suggest the nature of mTORC1 activity in NMDA-dependent regulation of translation (Marin et al 1997; Lenz and Avruch 2005; Gong et al 2006; Meng et al 2013). Stimulation of NMDA receptors and subsequent activation of mTORC1 and S6K1 leads to the suppression of specific substrates that are key in balancing dendritic excitation, such as Kv1.1 (Raab-Graham et al 2006; Meng et al 2013). Treatment with rapamycin abolished this NMDA-induced decrease in Kv1.1. Additionally, treatment with rapamycin abolishes late-, but not early-phase LTP in the hippocampus, suggesting that its activation is key for synthesis of new that are

necessary to maintain but not intiate LTP (Raab-Graham et al 2006; Bekinschtein et al 2007; Russo et al 2013). In the context of ethanol-seeking behaviors, mTORC1 activation is necessary for the reduction in ethanol-seeking behaviors in rats treated with the NMDA receptor antagonist, ketamine (Sabino et al 2013).

mTORC1 in dendritic spines and its role in behavior

The role of mTORC1 as a translational regulatory pathway is well studied. However, long-term differences or dysfunction of dendritic translation is associated with differences in dendritic spine density and morphology (Hoeffer et al 2012; Bowling and Klann 2014; Tang and Sulzer 2014). In animal models of autism spectrum disorders, hyperactive mTORC1 is correlated with aberrant spine morphology compared to controls (Huber et al 2002; Bowling and Klann 2014). However, the connection between mTORC1-mediated translation and actin polymerization and spine formation is not well characterized, but there are studies showing correlations between alterations in mTORC1 activity and alterations in dendritic spines (Hoeffer et al 2012; Tang and Sulzer 2014). Neurodegenerative disorders that exhibit decreases in mTORC1 activity also show deficits with activity-dependent maintenance of actin polymerization and differences in spine formation (Urbanska et al 2012; Takei and Hiroyki 2014; Tang and Sulzer 2014)

Due to its well-characterized role in protein translation, mTORC1 is also studied in behaviors that have previously been shown to rely on LTP. Inhibition of

mTORC1 via treatment with rapamycin in the dorsal hippocampus prevented consolidation and reconsolidation of contextual fear conditioning (Bekinschtein et al 2007). Inhibitory avoidance tasks significantly increased mTORC1 and S6K1 phosphorylation and activity. Inhibition of mTORC1 prevented this increase in S6K1 phosphorylation and hippocampal-dependent learning (Im et al 2009; Fenton et al 2011).

Previous studies have shown that long-term exposure to ethanol preferentially up or down regulates specific proteins at glutamatergic synapses. In particular, proteins necessary for NMDA receptor signaling, such as CaMKII and PSD-95, are upregulated (Barak et al 2013; Sabino et al 2013). Additional components of the mTORC1 pathway that are key for appropriate, proficient activity-dependent translation, such as 4E-BP1 and S6K1, exhibit enhanced activity following chronic self-administration of ethanol (Nesta et al 2010; Barak et al 2013). Treatment with rapamycin not only inhibited these cellular changes in protein expression, it also prevents reinstatement of ethanol-seeking behaviors during cue-induced reinstatement. This inhibition of reinstatement was present after two weeks of rapamycin treatment (Barak et al 2013).

Included in the mTORC1 pathway are downstream components of S6K1 that serve as mRNA-binding proteins (Zalfa et al 2006; Chen and Joseph 2015). As previously discussed, these proteins, although translational repressors, are essential components for coordinated translation. Of these, the translational repressor fragile X mental retardation protein (FMRP) has previously been shown to have a key role in regulating *de novo* protein synthesis in response to changes

in activity at glutamatergic synapses (Zalfa et al 2006; Darnell et al 2011; Henry et al 2011; Darnell and Klann 2013; Fernandez and Bagni 2013).

Role of FMRP in activity-dependent translation

FMRP in dendritic translation

The mRNA-binding protein FMRP is of particular importance in the regulation of local translation in dendrites and dendritic spines (Bardoni et al 2001; Darnell et al 2011; Zalfa et al 2006). FMRP is a translational repressor that binds to mRNAs and prevents mRNA interaction with the cap-binding protein eIF4E (Laggerbauer et al 2001; Fernandez and Bagni 2013; Sala et al 2014). It is one of the necessary components for activity-dependent translation in dendrites in the hippocampus and is also needed to maintain homeostatic basal protein levels (Bardoni et al 1997; Henry 2011; Fernandez and Bagni 2013). It has been most well studied in the context of neuronal development and fragile X syndrome, and there are fewer studies investigating the role of FMRP in typical activity-dependent neuronal function (Sutherland 1979; Weiler et al 2004; Henry 2011; Schaeffer et al 2012). FMRP is encoded by the *fmr1* gene that is highly conserved throughout the animal kingdom, from *drosophila* to human (Bardoni et al 1997; Laggerbauer et al 2001; Usdin and Kumari et al 2015). It codes for seven different isoforms of FMRP, with isoform 6 being the most commonly expressed. However, it is of note that each isoform varies in the mRNA binding

regions and potential differences in mRNA binding and activity have not been characterized (Usdin and Kumari et al 2015).

Structurally, FMRP contains several binding regions for both coding and noncoding RNAs as well as proteins that regulate the function of FMRP and residues that undergo post-translational modification to mediate FMRP activity (Schaeffer et al 2012; Adams-Cioaba et al 2010). For regulation of mRNA translation, FMRP contains two KH domains as well as an RGG rich segment **(Figure 1-4)** (Bardoni et al 1997; Adams-Cioaba et al 2010; Chen and Joseph 2015; Usdin and Kumari 2015). These regions contain ubiquitous sequences that allow FMRP to interact with 3-4% of total mRNAs in hippocampal pyramidal neurons (Jones 2003; Darnell et al 2012). These include proteins that modulate the balance of excitation and inhibition such as Kv4.2, KChIP3, and the NMDA receptor subunits (Darnell et al 2011, Gross et al 2011; Jan et al 2011; Lee et al 2011). However, each mRNA binding region tends to interact with specific sequences on mRNAs. This allows FMRP to have specificity for specific mRNAs while multiple regions maximizes binding partners (Brown et al 1995; Bardoni et al 1997; Jones 2003; Darnell et al 2011; Schaeffer et al 2012)**.** FMRP activity is mediated by phosphorylation at three different serine residues, S496, S499, and S503, located in the RGG-rich region (Bardoni et al 1997, Ceman et al 2003; Edbauer et al 2010). Of interest in the context of mRNA activity is S499 (Bartley et al 2014). This is a highly conserved site for post-translational modification that alters FMRP's mRNA binding activity (Jones et al 2001; Bhattacharya et al 2012; Bartley et al 2014). Increases in phosphorylation at S499 via S6K1 increases

FMRP activity (Ceman et al 2003; Holz et al 2005; Fenton and Grant 2011; Bhattacharya et al 2012; Bartley et al 2014). This increase in activity causes an increase in mRNA-FMRP interaction and therefore a decrease in protein synthesis of that mRNA (Laggerbauer et al 2001; Ceman et al 2003; Darnell and Klann 2013; Usdin and Kumari 2015).

FMRP also interacts with various proteins, noncoding RNAs, and miRNAs that regulate its function. Two proteins, nuclear FMRP interacting protein (Nufip) and cytoplasmic FMRP interacting protein 1 (Cyfip1), bind with FMRP to serve as linker proteins of larger macromolecular complexes that may not only regulate translation, but also mRNA localization and stability (Bardoni et al 2003; Napoli et al 2008; Pathania et al 2014). In dendrites, Cyfip1 interacts with FMRP at sites adjacent to the mRNA-binding KH regions. Once bound to FMRP, Cyfip1 also interacts with the eIF4E to prevent all translation at that ribosome (Napoli et al 2008). This binding with the polyribosome may also serve to regulate the translation of specific mRNAs. As seen in **Figure 1-4**, FMRP has different binding regions for mRNAs and Cyfip1, and may interact with both Cyfip1 and mRNA simultaneously (Zalfa et al 2006; Fernandez and Bagni 2013; Usban and Kumari 2015). If so, the interaction of Cyfip1 and FMRP with the ribosome may also serve to coordinate translation by holding certain mRNAs near ribosomes for quick *de novo* protein synthesis (Zalfa et al 2006; Napoli et al 2008). FMRP also binds noncoding RNAs, such as BC1 that interact with the ribosome to inhibit translation (Zalfa et al 2003; Napoli et al 2008).

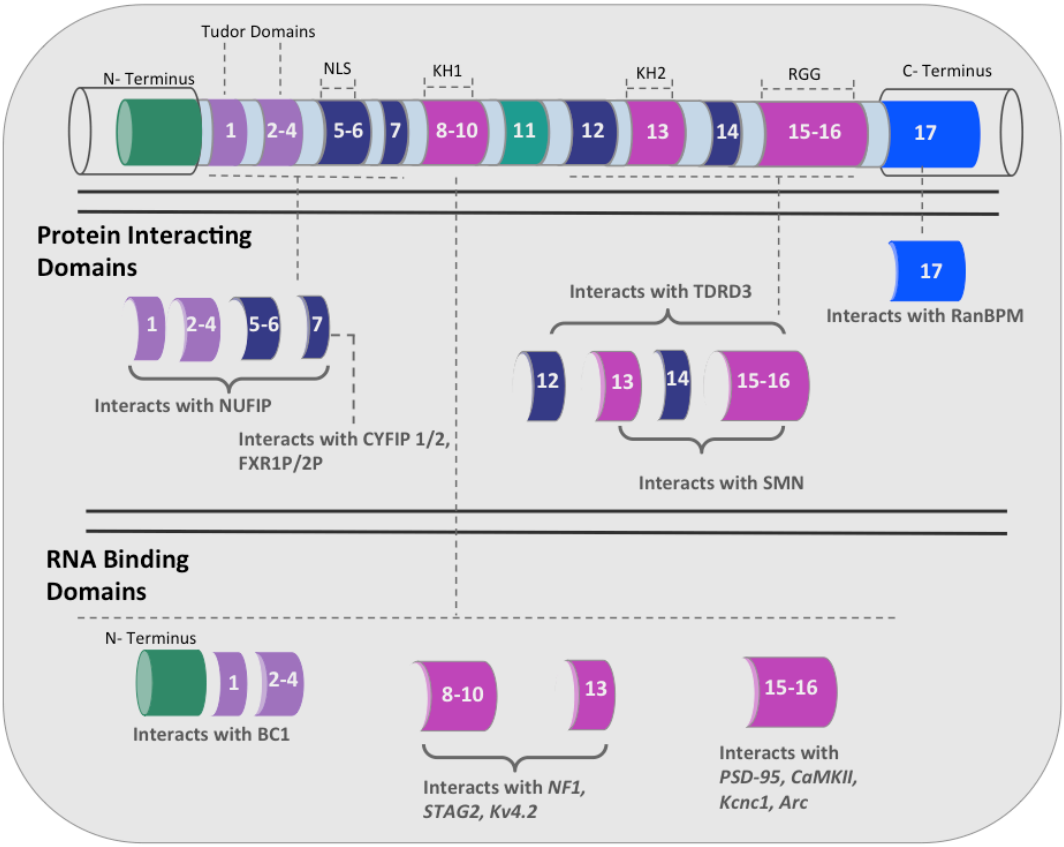

Figure 1-4 The structure of FMRP
Adapted from Fernandez and Bagni 2013

The structure of FMRP allows for specific binding with particular motifs in certain mRNAs while multiple binding regions maximizes potential binding partners. FMRP also has different regions for protein-protein interactions that have a pivotal role in determining FMRP function.

In addition to translation, FMRP may also regulate RNA trafficking at the synapse. Increases in phosphorylation at S503 increases FMRP binding to Dicer-processed miRNAs (Ceman et al 2003; Jin et al 2004). Dicer cuts pre-miRNAs

into shorter strands for insertion into the RNA-induced silencing complex (RISC) (Hayashi-Takagi et al 2010). In this capacity, FMRP aids Dicer by interacting with processed miRNAs through one of the KH domains and acts as an acceptor protein to localize and traffic the miRNA to the other RISC components (Jin et al 2004; Zalfa et al 2006; Hayashi-Tagaki et al 2010). Outside of this capacity, there is some controversy as to whether FMRP alters trafficking of mRNAs or holds them at specific synapses in the dendrite. Studies from different groups have produced contradictory results. In *fmr1-/-* mice, two different studies in the same preparation have reported opposite results for the same mRNA. One study implicated FMRP in Kv4.2 mRNA trafficking (Gross et al 2011). However, another study examining the FMRP-mRNA binding domains showed that mutation of the binding site on either FMRP or Kv4.2 mRNA did not produce any alteration in mRNA trafficking down the dendrite (Lee et al 2011). However, it was postulated that mRNAs may still be trafficked down the dendrites, but without FMRP interaction these mRNAs are unstable (Gross et al 2011).

FMRP and synaptic plasticity

As an mRNA-binding protein, FMRP has numerous targets of interest related to maintenance of homeostasis and glutamatergic signaling. These include PSD-95, CaMKII, Arc, Kv4.2, KChIP3, and NMDA receptor subunits (Brown et al 1995; Muddashetty et al 2007; Jones et al 2012; Niere et al 2012; Darnell et al 2013). FMRP is also implicated in regulation of specific mRNAs that

are translated with relatively low efficiency. These are 5'- TOP mRNAs, which are mRNAs that contain an unusual terminal oligopyramidine tract at the 5' untranslated region (Levy et al 1991; Jefferies et al 1997). Most of the proteins directly involved in the assembly of the initiation and translation complexes, as well as several proteins necessary for regulation of ribosome assembly, are TOP mRNAs (Levy et al 1991; Brown et al 1995; Zalfa et al 2006). These mRNAs are transcripted, transported down into neuronal processes where they are sequestered in inactive ribonucleoprotein complexes (Bardoni et al 2003; Antar et al 2005; Zalfa et al 2006). The association of these proteins with active polyribosomes is significantly lower than other proteins whose mRNAs are trafficked out to the synapse under basal conditions (Levy et al 2991; Jefferies et al 1997). Typically, in non-neuronal cell types, activation of these mRNAs involves trafficking back to active translation sites in response to growth factor stimulation (Bardoni et al 2003; Castets et al 2005). However, in mature neurons, this also occurs in response to activity-dependent changes in synaptic plasticity. Another aspect of these mRNAs that is very different from other transcripts is that these mRNAs have an almost 'all or none' translational efficiency (Levy et al 1991; Aloni et al 1992; Jefferies et al 1997). Under basal state conditions, practically none of these mRNAs are translated, but, after synaptic stimulation, the overwhelming majority of the mRNAs present near active synapse are trafficked to active polyribosomes for translation. Due to this sudden shift in translation, FMRP may play a significant role in tethering these proteins near active polyribosomes in dendrites and dendritic spines to stabilize the mRNA

transcript and promote immediate translation as needed (Levy et al 1991; Aloni et al 1992). Translation of these mRNAs is also almost exclusively dependent on mTORC1/S6K1 activation. Treatment with rapamycin almost completely ablates *de novo* protein synthesis of these mRNAs, even with strong synaptic stimulation or growth factors (Jefferies et al 1997). Since these TOP mRNAs are transcripts of necessary components in all three stages of translation, such as eIF4E, eIF4A, eEF2, and eRF2, dysregulation of translation of these mRNAs directly alters the ability to the facilitate protein translation and maintain changes in synaptic plasticity, such as LTP (Levy et al 1991; Brown et al 1995; Schaeffer et al 2012).

FMRP is a key mediator of activity-dependent translation of numerous mRNAs in dendrites and dendritic spines (Ramocki and Zoghbi 2008). These mRNAs include several that code for proteins that are essential in mediating synaptic excitability (Brown et al 1995; Jones 2003; Darnell et al 2013). In disorders characterized by hyperexcitability, such as fragile X syndrome and epilepsy, dysfunctional FMRP creates an aberrant basal state and dysregulated activity-dependent translation (Krueger and Bear 2011; Vislay et al 2012). Fragile X syndrome is characterized by the expansion of the regulatory CpG region that recruits transcriptional regulators that promote hypermethylation and silencing of the gene (Bardoni et al 1997; Usdin and Kumari 2015). Therefore, many individuals with fragile X syndrome completely lack FMRP (Sutherland 1979; Laggerbauer et al 2001). This lack of FMRP leads to increases in basal state protein levels, including Kv4.2 and KChIP3 (Brown et al 1995; Darnell et al 2011; Gross et al 2011; Schaeffer et al 2012). In individuals with fragile X syndrome

and in *fmr1-/-* mice, synaptic plasticity is limited and while induction of activity-dependent mechanism is unaffected, these processes cannot be maintained. Although FMRP is a translational repressor, it has an important role in coordinating and fine-tuning mRNA interactions to produce the necessary increases in protein expression to maintain activity-dependent processes, such as LTP or LTD (Ostroff et al 2002; Niere et al 2012; Maurin et al 2014). Complete lack of FMRP has greater effects on LTD compared to LTP, but this effect may be age-dependent, vary by brain region, and depend on the knockout mouse model (Eadie et al 2010; Niere et al 2012). In the nucleus accumbens, adult *fmr1-/-* mice display impaired NMDA-dependent plasticity (Neuhofer et al 2015). Additionally, in more recent mouse models of fragile X, NMDA-dependent LTP is also impaired in the hippocampus (Mittman 2009; Eadie et al 2010)

FMRP may also control excitability by altering the polyribosome itself. As a regulator of 5' TOP mRNAs, FMRP activity alters protein expression of the obligatory subunits for active ribosomes (Levy et al 1991). Lack of FMRP in animal models has shown deficiencies in activity-dependent translation of these proteins. Although basal protein expression levels remain unchanged compared to control animals, these mRNAs are trafficked to the ribosome with low efficiency during activity-dependent synaptic plasticity, despite relatively normal levels of other mRNA- binding proteins (Levy et al 1991; Jefferies et al 1992). This suggests that FMRP not only regulates proteins that directly mediate synaptic function, but also those necessary for maintenance of synaptic plasticity.

Studies evaluating protein translation during epileptigenesis show that, although there is no change in total protein expression of FMRP, there is aberrant regulation of FMRP activity. Proteins that balance local glutamatergic inputs, such as Kv4.2 and KChIP3, are downregulated in both clinical patients and mouse models of temporal lobe epilepsy (Muddashetty et al 2007; Lee et al 2011; Fernandez and Bagni 2013; Maurin et al 2014). In some of these mouse models, FMRP total protein expression remains unaffected, but trafficking and FMRP-mRNA interactions are altered compared to controls, indicating a role for FMRP in activity-dependent protein translation in epilepsy (Mittman 2009; Lee et al 2011; Neuhofer et al 2015).

Due to its previously described role in regulating basal protein levels and activity-dependent translation of proteins altered by chronic ethanol exposure, FMRP is a likely candidate for mediating ethanol-induced hyperexcitability in the hippocampus. Blockade of upstream components of FMRP prevent the ethanol-induced alterations in protein expression. In other pathologies characterized by hyperexcitability, these alterations in protein expression and upstream kinases correspond to alterations in FMRP expression and activity.

FMRP and actin dynamics

Alterations in protein expression are often accompanied by changes in actin polymerization and spine morphology. Patients with fragile X syndrome who lack FMRP, have increased density of immature stubby dendritic spines and a

significant decrease in long/thin spines (Hoeffer et al 2012). In *drosophila*, FMRP interacts with a Cyfip protein that is a regulator of actin polymerization and synapse development (Schenck et al 2003; Henry 2011; Zhao et al 2013). In rodent models, *fmr1-/-* also display impaired spine development (Dictenberg et al 2008; Henry 2011). Conversely, *Cyfip+/-* mice show a behavioral phenotype characterized by deficits in hippocampal-dependent learning and increases in basal protein expression, similar to *fmr1-/-* mice (Antar et al 2003; Pathania et al 2014; Han et 2015). However, this connection between spine development and protein translation has not been well characterized. Cyfip proteins provide a potential link to activity-dependent actin polymerization and translational changes associated with synaptic plasticity (Lee and Jan 2012).

Spine morphology and activity-dependent translation

Overview of actin dynamics

Regulation of actin dynamics and morphology of dendritic spines involves a coordinated, highly regulated activity-dependent mechanism that balances the acting polymerization with functional changes at the synapse.

Actin cytoskeletal dynamics are essential to maintain proper neuronal function. One particular regulatory element is the WAVE complex. The WASP-family veropolin homologous protein (WAVE) is found in nearly all cells, including neurons, and is active in a number of different cellular processes (Bompard

2004; Stradal et al 2004; Kim et al 2006; Pollitt and Insall 2009). The WAVE complex consists of WAVE1, Arp2/3, Hem1, Abi1, and either Cyfip1 or 2. While the complex is inactive when completely assembled, dissociation of Cyfip/Hem1 allows for its activation via Arp2/3 nucleation of actin filaments. When assembled into the WAVE complex, Cyfip blocks the Arp2/3 activating domain (VCA region) and prevents activity-dependent actin polymerization. In order to facilitate actin polymerization, Arp2/3 must have a free VCA region and be activated by an F-actin filament (Smith and Rong 2004; Stradal et al 2004; Kim et al 2006).

Arp2/3-mediated polymerization of G-actin to F-actin filaments is a four step process. The first step is dissociation of the Cyfip/Hem1 inhibitory complex, which exposes the VCA region (Kim et al 2006; Pollitt and Insall 2009). The next step requires the uninhibited Arp2/3 protein to bind to a preformed actin filament. Following filament binding, Arp2/3 tethers a free actin monomer to the existing filament. The final step is elongation of the actin chain with the addition of other actin monomers. Initiation of this process of actin polymerization is ATP-dependent (Suetsugu et al 1999; Innocenti et al 2004; Kim et al 2006). Hydrolysis of ATP bound to Arp2 serves as a timing mechanism to promote dynamic actin networks that can easily and readily respond quickly to activity-dependent changes. ATP hydrolysis is stimulated by interaction with either the actin monomer or filament (Smith and Rong 2004; Innocenti et al 2004). Therefore, hydrolysis may occur before the polymerization step is complete. However, the rate of nucleation of actin monomers matches the rate of ATP hydrolysis. Phosphate dissociation does not immediately induce disassembly of the F-actin,

but does allow for factors such as cofilin that facilitate the breakdown of filamenteous actin into actin monomers to interact with the newly formed chain (Kasai et al 2003; Okamoto et al 2007; Pilpel and Segal 2005; Bellot et al 2014).

The role of Cyfip in activity-dependent spine changes

Dissociation of Cyfip from the WAVE complex is the first step that is necessary for WAVE activation and subsequently actin polymerization (Kim et al 2006; Bellot et al 2014). Previous studies have shown a role for Rac1 signaling in regulating WAVE-mediated actin polymerization. However, Rac1 does not directly interact with the WAVE complex (Miki et al 1998; Rao and Craig 2000; Tolias et al 2005). More recent studies have shown that Rac1 interacts with Cyfip to facilitate dissociation and disinhibition of the VCA region **(Figure 1-5)**. GTP-bound Rac1 induces Cyfip/Hem1 to dissociate from the rest of the WAVE complex (Miki et al 1998: Tolias et al 2005; Bongmba et al 2011**)**. This dissociation relieves the Cyfip-mediated block on the VCA region of Arp2/3 (Machesky et al 1999; Eden et al 2002; Choi Et al 2005). Following this disinhibition, Arp2/3 can polymerize actin monomers to filamentous actin, and hydrolysis of GTP allows for Cyfip to reassociate with WAVE and prevent further cytoskeletal remodeling via WAVE. The binding of GTP Rac1 requires GEF activity. However, it is unclear which GEF is involved in this process, and how it is regulated. Unlike many Rac1-mediated processes, this is independent of Rho A activity and it is not affected by Cdc4 activation (Suetsugu et al 1999; Eden et

al 2002; Tolias et al 2005). Regulation of spine morphology and actin dynamics may have significant functional implications. Decreases in protein translation are associated with immature spine development and impairment of synaptic plasticity. This difference in spine morphology may have a contribution to the synaptic compartmentalization and the development or maintenance of LTP (Dahl et al 2003; Soderling et al 2003; Maurin et al 2014).

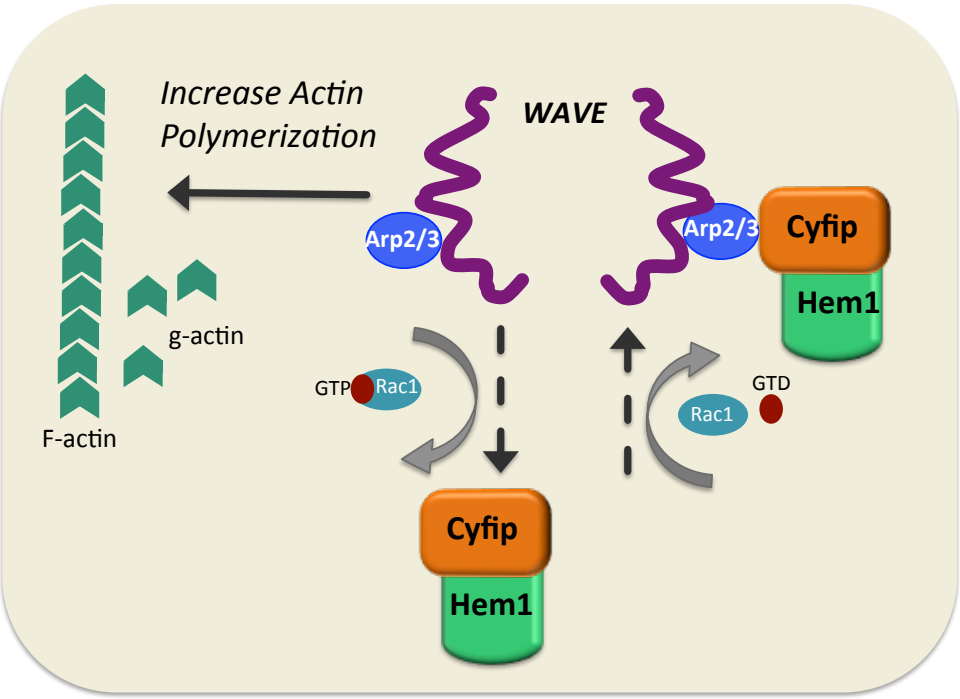

Figure 1-5 Cyfip-mediated actin polymerization through the WAVE complex

Regulation of the WAVE complex that increases actin polymerization occurs through binding of GTP-bound Rac1**.** This causes dissociation of Cyfip/Hem1 complex that blocks the VCA active region on Arp2/3 and therefore, disinhibition and actin polymerization. Hydrolysis of GTP allows for Cyfip reassociation with WAVE.

As mentioned above, the inactive WAVE complex contains either Cyfip1 or 2. These two proteins, although 88% homologous, are encoded by different genes, and have a key role in dendritic translation and actin dynamics (Antar et al 2003; Billuart et al 2003; Schenck et al 2003; Abebkhouhk and Bardoni 2014). Yeast-two hybrid studies show both Cyfip proteins were found to interact with FMRP (Napoli et al 2008). However, *in vivo* studies have shown preferential interaction of Cyfip1 with FMRP over Cyfip2 (Antar et al 2003). Cyfip2 was found

to localize to the WAVE complex and alter actin polymerization over protein translation (Antar et al 2003; Bongmba et al 2011; Abekhoukh et al 2014). Clinical studies show that aberrant function of both proteins may produce similar phenotypes. Unregulated Cyfip1 mRNA is present in some patients with autism spectrum disorders, which is often a comorbid diagnosis with fragile X syndrome (Sutherland 1979; Turner et al 1980; Hoeffer et al 2012). Additionally, some clinical populations with autism spectrum disorder or fragile X syndrome also have increased Cyfip2 mRNA and protein expression (Hoeffer et al 2012). This suggests that protein translation and actin dynamics may be connected, and that alterations in either process produces a similar pathology.

Alterations in actin dynamics and spine morphology occur as a result of activity-dependent changes at glutamatergic synapses. However, like protein translation, changes in spine morphology involves a highly coordinated pathway with several components that regulate actin polymerization. As a key regulator of the WAVE complex, Cyfip2 was recently found to have a role in drug-induced alterations in spine morphology. Kumar et al found a naturally occurring polymorphism between two lines of commercially available C57BL/6 animals. This polymorphism did not alter total Cyfip2 expression, rather it changed the stability of the protein. In the C57BL/6N animals, Cyfip2 showed a decreased half-life compared to C57BL/6J mice. This created a higher turnover in protein, and these animals also showed decreased spine density in the nucleus accumbens. This decrease in density was due to a decrease in both mature long/thin spines, and immature stubby spines. Additionally, these 6N did not

show the typical behavioral responses to acute or chronic cocaine administration. Passive injection of cocaine did not elicit the increase in locomotor activity in 6N animals that was seen with 6J mice. 6N mice were also resistant to reinstatement following a cocaine prime. This is likely due to the Cyfip2 polymorphism, as experiments using a knockin on a C57BL/6J background produced the same results (Kumar et al 2013).

Atlerations in spine morphology may have a direct or indirect role in modulating protein translation and cellular function. Translation and actin dynamics might represent a coupled mechanism that must occur in tandem to have functional changes in synaptic plasticity.

Summary

Ethanol affects many areas of the brain including reward circuitry and memory systems, which include the hippocampus. While acute exposure to ethanol dampens excitation while promoting inhibition, chronic ethanol exposure induces activity-dependent changes to accommodate the persistent decrease in excitation (Carpenter-Hyland and Chandler 2006; Zhou et al 2007; Enoch et al 2012; Zorumski et al 2014). In dendrites and dendritic spines, this is characterized by an increase in excitation and a decrease in local inhibition. However, the mechanisms driving these changes are not fully understood.

It has previously been shown that chronic ethanol increases NMDA receptor activity and expression. But, regulation of excitatory inputs in dendritic spines

also includes proteins that mediate inhibition. Of the proteins regulating excitation in dendrites and dendritic spines, Kv4.2 and its auxiliary protein KChIP3 are viable candidates for ethanol-induced homeostatic changes in inhibition. Kv4.2 is an A-type K+- channel that is one of the main regulators of backpropagating action potentials and excitotoxicity (Kim et al 2007; Kaufmann et al 2012; Labno et al 2014). Other disorders characterized by hyperexcitability show decreases in Kv4.2 expression and function (Hong et al 2003; Monaghan et al 2008; Aronica et al 2009). Additionally its interacting protein KChIP3 not only enhances inhibition by promoting Kv4.2 surface expression and function, but also inhibits NMDA-mediated current and protein expression (Kunjilwar et al 2004; Lin et al 2004; Norris et al 2010; Zhang et al 2010; Wang et al 2012). These proteins exhibit inverse activity-dependent changes in expression and function. As NMDA receptors are activated, Kv4.2 expression decreases. Taken together, Kv4.2 and KChIP3 may have an influential role in mediating the homeostatic changes induced by neurons in the hippocampus to balance increased NMDA receptor expression and function.

Previous studies have shown hippocampal-dependent behaviors rely on the synaptic mechanism of LTP to execute learned behaviors that involve long-term memory, and this process may be altered by chronic ethanol exposure. Maintenance of LTP requires the induction of *de novo* protein synthesis in dendrites and dendritic spines (Pastalkova et al 2006; Govindarajan et al 2011; Hill et al 2013). The process of new protein synthesis relies on coordinated activity to increase or decrease expression of specific proteins. The factors in

dendrites and dendritic spines that mediate this activity-dependent translation have not been identified, they are components of the mTORC1 pathway and its downstream substrate FMRP is a likely candidate protein (Ma and Bliss 2009; Hoeffer 2012; Nesta 2014). As an mRNA-binding translational repressor, FMRP interacts with several mRNAs whose proteins are altered following chronic ethanol exposure, as well as the proteins of interest including Kv4.2, KChIP3, and NMDA receptor subunits (Jones 2003; Darnell et al 2012; Schaeffer et al 2012). FMRP is also one of the key proteins mediating activity-dependent changes in protein homeostasis and is necessary for maintenance of basal protein levels (Antar et al 2005; Zalfa et al 2006; Henry 2011; Darnell and Richter 2012). However, the role FMRP may have in regulating specific mRNAs during chronic ethanol exposure is completely uncharacterized. Studies in this dissertation seek to determine whether FMRP expression or activity is altered following chronic exposure, and if these changes correspond to alterations in FMRP-mRNA binding.

Long-term changes in protein expression at glutamatergic synapses is also accompanied by changes in dendritic spines. Disorders with disrupted translational regulation, such as fragile X syndrome show aberrant spine distribution and morphology (Jones 2003; Antar et al 2005; Castets et al 2005; Dictenberg et al 2008; Han et al 2015). It is not clear how these two processes are connected and how ethanol may alter both to maintain neuronal homeostasis. Cyfip2 is not only an interacting protein of FMRP, but also is necessary for appropriate actin polymerization (Pilpel and Segal 2005; Kim et al 2006; Chen et

al 2014). As seen with translational deficits, dysfunction of Cyfip2 induces alterations in protein translation, and these alterations correspond to resistance in developing drinking behaviors (Nesta et al 2012; Barak et al 2013; Han et al 2015). Therefore, the question remains: how does chronic ethanol facilitate activity-dependent homeostatic changes in dendrites and dendritic spines? **The overarching hypothesis of this dissertation is that FMRP is necessary for activity-dependent homeostatic changes in protein expression and spine morphology following chronic ethanol exposure (Figure 1-6)**

Specific Aims

Specific Aim 1: Test the hypothesis that exposure to chronic ethanol alters downstream substrates of FMRP at dendritic spines. As key regulators of excitability and plasticity, Kv4.2, KChIP3, and NMDA receptor subunits are likely targets for homeostatic changes in protein expression to reestablish balanced neuronal signaling. These proteins are also downstream targets of FMRP, one of the main mediators of activity-dependent translation in dendrites and dendritic spines in the hippocampus. Studies under this aim will establish the nature of activity-dependent homeostatic alterations in protein expression following chronic ethanol exposure, and examine how ethanol may alter FMRP expression and function to drive these changes.

Specific Aim 2: **Test the hypothesis that inhibition of FMRP phosphorylation prevents ethanol-induced translational changes in the hippocampus.** Although ethanol may alter FMRP activity through changes in phosphorylation, total protein expression, or binding partners, it may not be an essential component of ethanol-induced homeostasis. As a potential major regulatory element in the translational mechanism of ethanol-induced changes, it is necessary to determine the nature of FMRP-mediated changes in the hippocampus during chronic ethanol exposure. The hypothesis tested under this aim is whether blockade of FMRP activity alter ethanol-induced, homeostatic changes in protein expression.

Specific Aim 3: **Test the hypothesis that ethanol-induced alterations in FMRP-mediated translation are accompanied by changes in spine morphology.** Previous studies have shown that alterations in FMRP-mediated protein expression are accompanied by changes in dendritic spine morphology. These alterations appear to be mediated by Cyfip2. A naturally occurring genetic polymorphism in two different C57BL/6 mouse lines provides the opportunity to examine the connection between actin polymerization and protein translation. Experiments conducted under this aim will discern whether these two processes are linked in chronic ethanol exposure of if they can occur independently of one another.

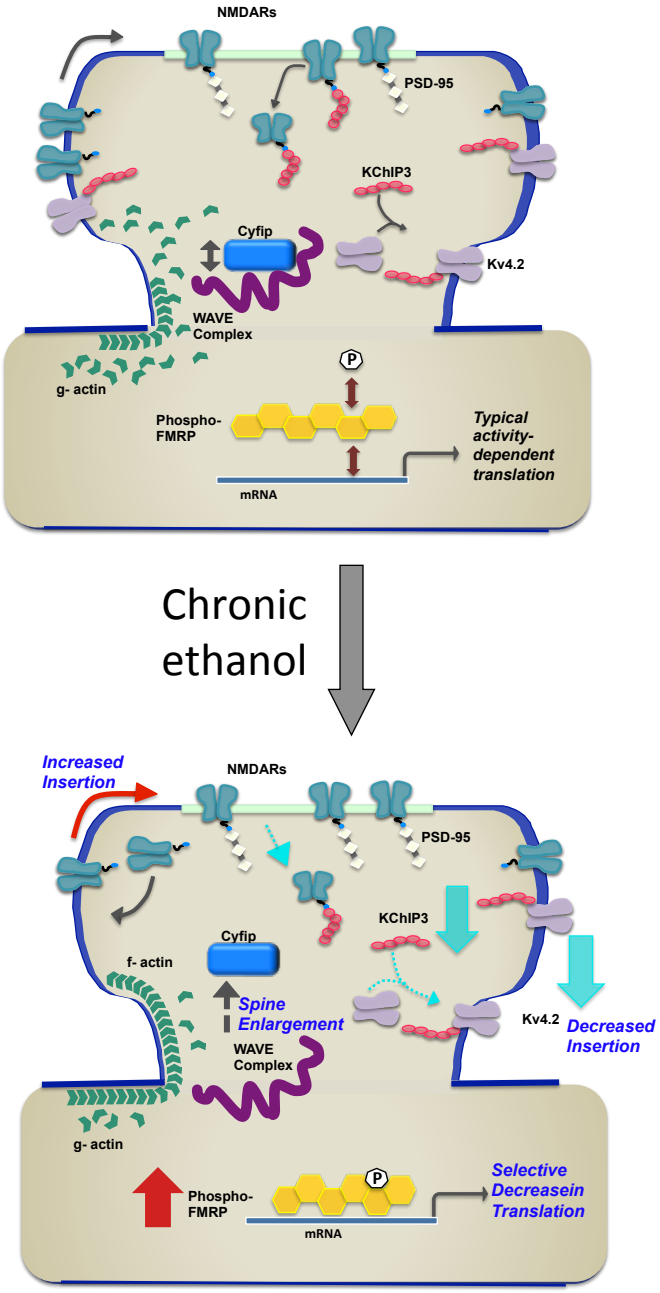

Figure 1-6 Overall hypothesis for alterations resulting from chronic exposure to ethanol.

Regulation of synaptic plasticity involves alterations in FMRP phosphorylation and protein translation that produce adaptive changes in function. These changes in translation also correspond to alterations in spine morphology via changes in WAVE activity. It is hypothesized that chronic ethanol exposure induces alterations in FMRP expression or activity that cause selective decreases in proteins mediating synaptic inhibition while also enhancing excitation. This alterations induce changes in spine morphology via alterations in Cyfip2.

Chapter 2

Exposure to chronic ethanol alters downstream substrates of FMRP at dendritic spines

Background and Significance

Alterations at glutamatergic synapses in dendrites and dendritic spines in the hippocampus during exposure to chronic ethanol function to reestablish neuronal homeostasis (Calabrese et al 2006; Ramocki and Zoghbi 2008; Caljigas et al 2010; Haft et al 2014). Evidence suggests that during chronic ethanol exposure, prolonged inhibition of NMDA receptors induces homeostatic mechanism to maintain proper neuronal function (Lovinger et al 1990; Kumari and Ticku 2000; Carpenter-Hyland et al 2004; Hendrickson et al 2004; Tokuda et

al 2007; Nagy 2008; Gladding and Raymond 2011) This long-term exposure requires a less transient, more lasting change to balance excitation and inhibition (Kumari and Ticku 2000; Jung et al 2008; Nagy 2008; Pian et al 2010). However, the nature of these changes in the hippocampus, and how they are achieved are not well understood.

Disorders defined by hyperexcitable states such as epilepsy and fragile X syndrome provide evidence of a role not only for NMDA receptors, but also inhibitory proteins responsible for dampening local excitatory inputs (Ruschenschmidt et al 2006; Monaghan et al 2008; Henry 2011; Fernandez and Bagni 2008; Meng et al 2013; Russo et al 2013). In pyramidal neurons in the hippocampus, the A-type K^+- channel Kv4.2 and its auxiliary protein KChIP3 are two of the main proteins that regulate glutamatergic inputs and balance excitation (Lin et al 2004; Kim et al 2007; Carrasquillo et al 2012; Kunjilwar et al 2013; Labno et al 2014). As some of the main regulators of excitability in dendrites and dendritic spines in the hippocampus, these proteins are important for balancing local glutamatergic inputs with the intracellular environment to prevent hyperexcitability and neurotoxicity (Ruiz-Gomez et al 2006; Kim et al 2007; Andrasfalvy et al 2008; Barnwell et al 2009; Carrasquillo 2013)

Maintaining long-term changes in homeostasis, in addition to alterations in function, it also likely involves adjusting local protein expression. This requires alterations in signaling cascades regulating protein synthesis and the translational machinery itself to make the necessary adjustments to glutamatergic function (Krug et al 1984; Klann and Richter 2007; Mercaldo et al

2009; Sossin and Lacaille 2010; Kim et al 2013; Thomas et al 2013). Previous studies indicate a potential role for substrates in the mTORC1 translational pathway and one of its downstream components, the mRNA-binding translational repressor protein, FMRP (Weiler et al 2004; Raab-Graham 2006; Costa-Mattioli et al 2009; Ma and Blenis 2009; Darnell and Klann 2013). Treatment with the mTORC1 inhibitor, rapamycin, blocks FMRP-mediated decreases in Kv4.2 expression in the hippocampus and prevents homeostatic increases in other proteins at glutamatergic synapses, namely PSD-95 and CaMKII, in rat models of chronic ethanol exposure (Gross et al 2011; Lee et al 2011; Barak et al 2013; Takei and Hiroyki 2014).

FMRP expression and function is an important modulator of activity-dependent changes in homeostatic signaling as well as overall basal protein levels for a number of downstream targets, including those affected by ethanol (Ramocki and Zoghbi 2008; Cajigas et al 2010; Henry et al 2011; Darnell et al 2013). As such, FMRP likely contributes to the homeostatic mechanism inducing intracellular changes to compensate for the long-term effects of ethanol at glutamatergic synapses. However, how FMRP alters translation, and which proteins regulated by FMRP are altered by chronic ethanol exposure are not known.

Studies presented in this chapter focused on determining which targets of FMRP are altered by chronic ethanol exposure in the *in vivo* CIE mouse model and *in vitro* organotypic hippocampal slice cultures. These studies also examined the effect of chronic ethanol on FMRP activity and total protein levels in the

hippocampus and investigate whether these ethanol-induced changes in protein expression are consistent with alterations in FMRP-mRNA interactions during long-term ethanol exposure.

Methods

Organotypic hippocampal slice culture ethanol exposure

Hippocampal slice cultures were prepared from P6-P8 Sprague-Dawley rats as described in Mulholland et al 2014. In brief, pups were euthanized using either ice or isofluorane, sacrificed, and both hippocampi dissected out on cold dissecting media (500 ml Eagle's Basal, 25 mM HEPES, 100 µg/ml Streptomycin, 2 mM Glutamax). Next hippocampi were sliced into 400 µm coronal sections and placed with four slices per well onto an organotypic cell culture membrane in culture media (50 ml Dissecting media, 36 mM glucose, 25% v/v Earle's Balanced Salt Solution, and 25% v/v heat inactivated horse serum). Cultures were incubated with 7.5% CO_2 at 37 ^{o}C in culture media for at least eight days, and media was changed every 4 days. Following eight days of incubation, slices were treated with 75 mM ethanol in regular culture media or media alone in sealed vapor chambers. All experiments adhered to NIH Animal Care Guidelines and were approved through the IACUC.

Protein Assay

Organotypic hippocampal slice cultures or hippocampal lystates from CIE-treated mice were sonicated in 2% LDS. A BCA protein assay kit (Pierce) was

used to determine total protein content. Following sonication, standards with a blank (0, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0) and samples at 1:10 ratio with LDS were loaded in triplicate in a 96 well plate. Once complete, 9.8 ml of Reagent A and 200 µl of Reagent B were mixed, and 200 µl of the combined solution was added to each well. The plate was then incubated in the dark at 37 ^{o}C for 30 minutes. The concentration for each well was calculated using the Bio-Rad 550 MicroPlate Reader.

Western blot analysis

Protein samples were mixed using the NuPage Invitrogen western system and then heated at 70 ^{o}C for 10 min. Proteins were separated via electrophoresis on a 7.5% Bis-Tris gel, transferred onto a PVDF membrane using the Bio-Rad semi-dry transfer, and a reversible Swift total protein stain was used to evaluate loading and for normalization of the data. Following the total protein stain, membranes were blocked in 4% non-fat milk, and incubated in primary antibody at 4 ^{o}C overnight. Primary antibodies used for these experiments are phosphorylated S499 FMRP (1:1000), FMRP total protein (1:2000), KChIP3 (1:500), Kv4.2 (1:1000), GluN1 (1:3000), and GluN2B (1:3000). After primary antibody incubation, membranes were incubated in secondary antibody, KChIP3 goat anti-rabbit and Kv4.2, GluN1, and GluN2B goat anti-mouse, for 1 hour at room temperature. After secondary antibody incubation, membranes were exposed to enhanced chemillumiscience using ChemicDoc MP Imaging System.

Co-immunoprecipitation (co-IP) and RT-qPCR

Following treatment with ethanol, PF-4708671 (PF) or ethanol and PF-4708671 concurrently (PF + EtOH), cultures were processed for either western blot analysis or co-IP and RT-qPCR. Co-IP with mRNA solutions were adapted from the procedures described by Lee et al 2011 and the protocol of the Pierce Co-IP Kit. Using the resin and coupling spin columns from the Pierce Co-IP kit, 6 µg of FMRP antibody was used for every mg of beads, with 6-7 mg of beads per spin column used for resin pre-clearing and antibody immobilization. AminoLink coupling resin was added to the spin column followed by coupling buffer washes (10 mM NaH_2PO_4, 150 mM NaCl in nuclease free water). Antibody for coupling was added to the spin columns in coupling buffer and incubated at room temperature for 120 minutes while slowly rotating. Following antibody coupling incubation, quenching buffer (1 M Tris-HCl) was added to ensure proper coupling. Hippocampal lysates were also pre-cleared with coupling buffer for 1 hr prior to co-IP. Slice cultures were homogenized with a needle and syringe with RNase and protease inhibitors in homogenization buffer (1.28 M sucrose, 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, and 1% NP-40, with ~ 2 ug/ml of RNasin Plus inhibitors, and 1X cOmplete Mini EDTA-Free Protease Inhibitor). Hippocampal lysates were cleared with control agarose resin (80 µl for every 1 mg of lysate).

For lysate clearing, new columns were coated with coupling buffer, then lysate and control resin were incubated in the column for 1 hour at 4 ^{o}C with gentle mixing. Flow-through by centrifugation provided cleared lysate for co-IP experiments. After antibody and lysate preclearing, co-IP with mRNA was performed (all steps performed 4 ^{o}C). Antibody-cleared spin columns were rinsed with wash buffer (0.1M PBS, 200 mM NaCl, and 2µl/ml of RNase Plus and 1X protease inhibitor,), pre-cleared lysate added to spin column, and incubated overnight at 4 ^{o}C. Columns were then rinsed with wash buffer, and FMRP-mRNAs were eluded from the column with Elution Buffer from Pierce kit, and mRNAs were then dissociated from FMRP with a TRIzol- chloroform extraction (Lee et al 2011). In brief, 200 µl of chloroform per 1 ml of TRIzol was added to each tube (1 ml TRIzol for every 75 mg of tissue), and incubated for 3 min at room temperature. After incubation, samples were centrifuged at 12,000 *xg* for 15 min at 4 ^{o}C. The upper aqueous phase containing the mRNA was then transferred to a separate tube.

mRNA was purified using the Qiagen RNeasy Mini Kit and nonspecific cDNA was transcribed using the Applied Biosystems High Capacity RNA- to –cDNA Kit (4387406). The reverse transcription reaction used a total volume of 20 µl at 37 ^{o}C for 60 minutes, 95 ^{o}C for 5 minutes, and a 4 ^{o}C hold (Bio-Rad C1000 thermocycler). For qPCR, a Sybr Green qPCR kit on a Bio-Rad CFX 96 thermocycler was used with an initial denaturation of 2 min at 94 ^{o}C followed by 40 cycles with a 15 sec denaturation at 94 ^{o}C and 1 min of annealing and

extension at 60 ^{o}C and a 4 ^{o}C hold. All primer sequences used are shown in **Table 1**. A schematic of the co-IP protocol is provided in **Figure 2-1.**

mRNA	Primer Sequence
GAPDH	5'- AAGGCTCATGACCA 3'- CAGGGATGATGTTCT
HPRT	5' TTGGATACAGGCCAGACTTTGTT 3' CTGAAGTACTCATTATAGTCAAGGGCATA
FAAH	5'- ATGAACCCGTGGAAGCCCTC 3'- CGCCGATGTCAGTGCCTAAAC
GluN1	5'- CTCTAGCCAGGTCTACGCTATCC 3'- GACGGGGATTCTGTAGAAGCCA
GluN2B	5'- CTGGAGTTCTGGTTCCTTACTG 3'- ATTCTCCTATCTTGCCCGGA
KChIP3	5' CACCTATGCACACTTCCTCTTCA 3' ACCACAAAGTCCTCAAAGTGGAT
Kv4.2	5'-GCCTTCGTTAGCAAATCTGG 3' GTGACATAAGGACACTGGG

Table 1. Primer sequences for RT-qPCR.

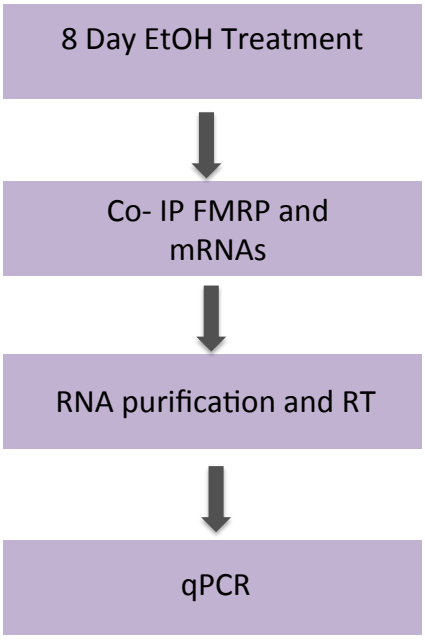

Figure 2-1 Co-IP Experimental Paradigm.

Organotypic hippocampal slice cultures are treated using an 8-day exposure paradigm. Following exposure, FMRP is immunoprecipitated with mRNAs. The purified samples then undergo a nonspecific reverse transcription and a quantitative PCR with cDNA-specific primers.

Chronic intermittent ethanol

Male C57BL/6J mice, approximately 9 weeks of age at the start of the experiment, were housed in a climate-controlled vivarium with a 12-hour light/dark cycle. Beginning approximately 3 hours into the dark cycle, mice were exposed to either ethanol or air vapor for 14 hours, followed by an 8-hour withdrawal period. This cycle of intermittent ethanol exposure was repeated for four days. Following the last 14-hour exposure period of a 4-day cycle, animals experienced a 72-hour withdrawal. All animals included in the study completed four of these weeklong cycles of CIE exposure and withdrawal.

Statistical Analysis

Western blots were quantified using ImageJ software (National Institutes of Health). Density of the appropriate band for each antibody was measured. Background for each lane was subtracted from the band density and variations in protein sample loading were normalized with a total protein stain, as previously mentioned. Student t-tests were used to test for significant differences between control and ethanol groups. Four-day timepoints are paired t-tests; samples from each group for each *n* were taken from the same animal.

RT-qPCR experimental cDNAs were normalized against GAPDH for each sample. Student t-tests were again used to evaluate significant differences between the fold change for control and ethanol groups.

Results

Homeostatic changes in protein expression at glutamatergic synapses

Acute ethanol decreases NMDA receptor function and local excitatory inputs to dendritic spines in the hippocampus. In contrast, prolonged exposure to ethanol induces mechanism to counteract the chronic inhibition and maintain proper excitatory function (Lovinger et al 1990; Kumari and Ticku 2000; Nagy 2008; Korkotian et al 2013; Talani et al 2013). However, the nature of these changes has not been characterized.

To evaluate changes in protein expression at glutamatergic synapses, western blot analysis was used to define differences in NMDA receptor subunit expression in chronic intermittent ethanol (CIE)-exposed mice. Whole hippocampal homogenates were tested for GluN1 and GluN2B expression. As shown in **Figure 2-2,** in mice exposed to CIE, hippocampal tissue showed an increase in total GluN1 receptor expression as well as increases in GluN2B expression (*GluN1*: *t* test, $t(20)= 2.146$, * $p< 0.05$, $n= 11$; *GluN2B*: *t* test, $t(20)= 2.132$, * $p< 0.05$, $n=11$).

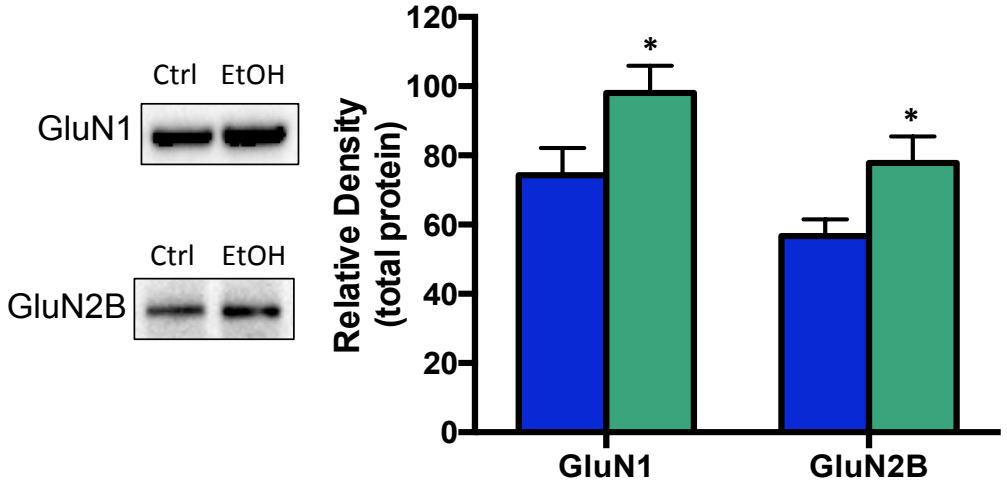

Figure 2-2 Ethanol-induced changes in NMDA receptor subunits in the mouse hippocampus.

Chronic intermittent ethanol exposure increased both GluN1 and GluN2B total protein expression in the mouse hippocampus (*n*= 11 , *p< 0.05).

In addition to changes in NMDA glutamate receptors, balance in excitation also involves alterations in proteins that mediate hyperexcitability; this includes the A-type K^+-channel Kv4.2 and its auxiliary protein KChIP3. Again, western blot was used to evaluate changes in Kv4.2 and KChIP3 protein expression in the same CIE mouse model. Immunoblot analysis of total protein expression showed a decrease in Kv4.2 expression **(Figure 2-3)**. Blots for KChIP3 also revealed a decrease in protein expression, suggesting ethanol alters both excitatory and inhibitory proteins to maintain neuronal homeostasis (*Kv4.2*: *t* test, $t(16)=2.121$, *p<0.05, $n = 9$; *KChIP3*: *t* test, $t(18)=2.123$, *p<0.05, $n= 10$).

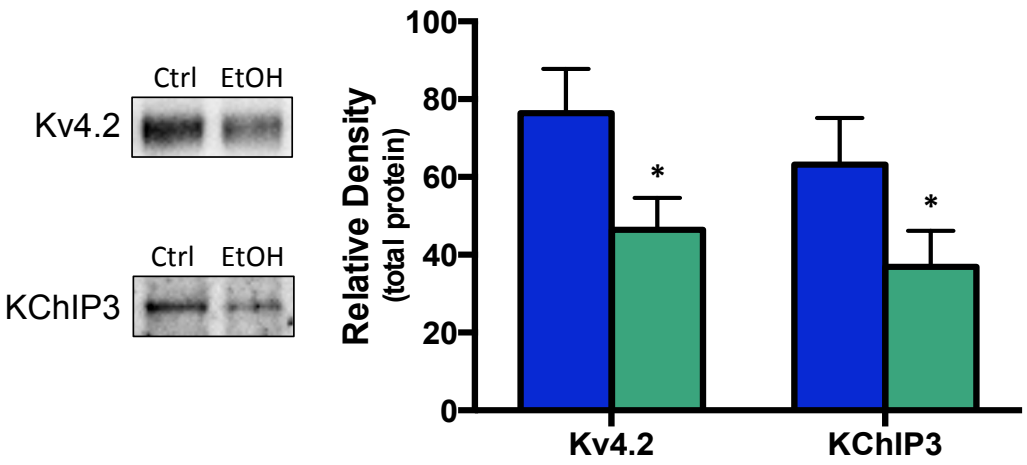

Figure 2-3. Alterations in Kv4.2 and KChIP3 after chronic intermittent ethanol exposure.

Mouse hippocampal tissue from ethanol-treated animals showed a decrease in Kv4.2 and KChIP3 expression (*n*= 10, *p< 0.05).

In order to further investigate the mechanisms driving these changes, protein expression in the organotypic hippocampal slice culture model was evaluated to confirm consistency with the CIE *in vivo* paradigm. As before, using western blot, NMDA receptor subunit expression was measured by western blot. At a dose of 75 mM ethanol, GluN1 protein levels were increased, thus replicating the observations obtained in CIE- exposed mice. This increase occurred after 8 days of exposure, but it was not seen with an acute exposure period of 24 hours with the same dose. Although there was a slight increase in GluN1 subunit expression after 4 days of exposure, this was not significant **(Figure 2-4**; *GluN1*: 24 hr: *t* test, *t*(4)=1.217, p= 0.2903, *n*= 5; 4-day: *t* test, *t*(3)=2.822, p= 0.0667, *n*= 6, 5; 8-day: *t* test, *t*(10)=2.997, *p<0.05, *n*= 6). In addition to GluN1, GluN2B protein levels were also measured. A time-course

treatment with 75 mM ethanol also induced an increase in GluN2B. Similar to GluN1, this increase was not seen after 24 hours of ethanol exposure, but was significantly increased following both 4- and 8-day exposure paradigms **(Figure 2-4**; *GluN2B*: 24 hr: *t* test, $t(4)=1.295$, p= 0.1325, *n*= 5; 4-day: *t* test, $t(4)=2.796$, *p<0.05, *n*= 5; 8-day: *t* test, $t(12)=2.612$, *p<0.05, *n*= 7).

As with the *in vivo* model, western blot was also used to evaluate changes in total protein levels of Kv4.2 and KChIP3. These proteins also mimicked results from CIE animals and exhibited a decrease in protein expression after both 4 and 8 days of ethanol exposure. Time course experiments revealed that these changes were limited to the longer exposure periods of 4 and 8 days **(Figure 2-5**; *Kv4.2*: 24 hr: *t* test, $t(4)=0.2454$, p= 0.4091, *n*= 5; 4-day: *t* test, $t(4)=2.960$, *p<0.05, *n*= 5; 8-day: *t* test, $t(12)=2.174$, *p<0.05, *n*= 7; *KChIP3*: 24 hr: *t* test, $t(4)=1.405$, p= 0.1163, *n*= 5; 4-day: *t* test, $t(5)=3.588$, *p<0.05, *n*= 5,6; 8-day: *t* test, $t(10)=3.016$, *p<0.05, *n*= 6).

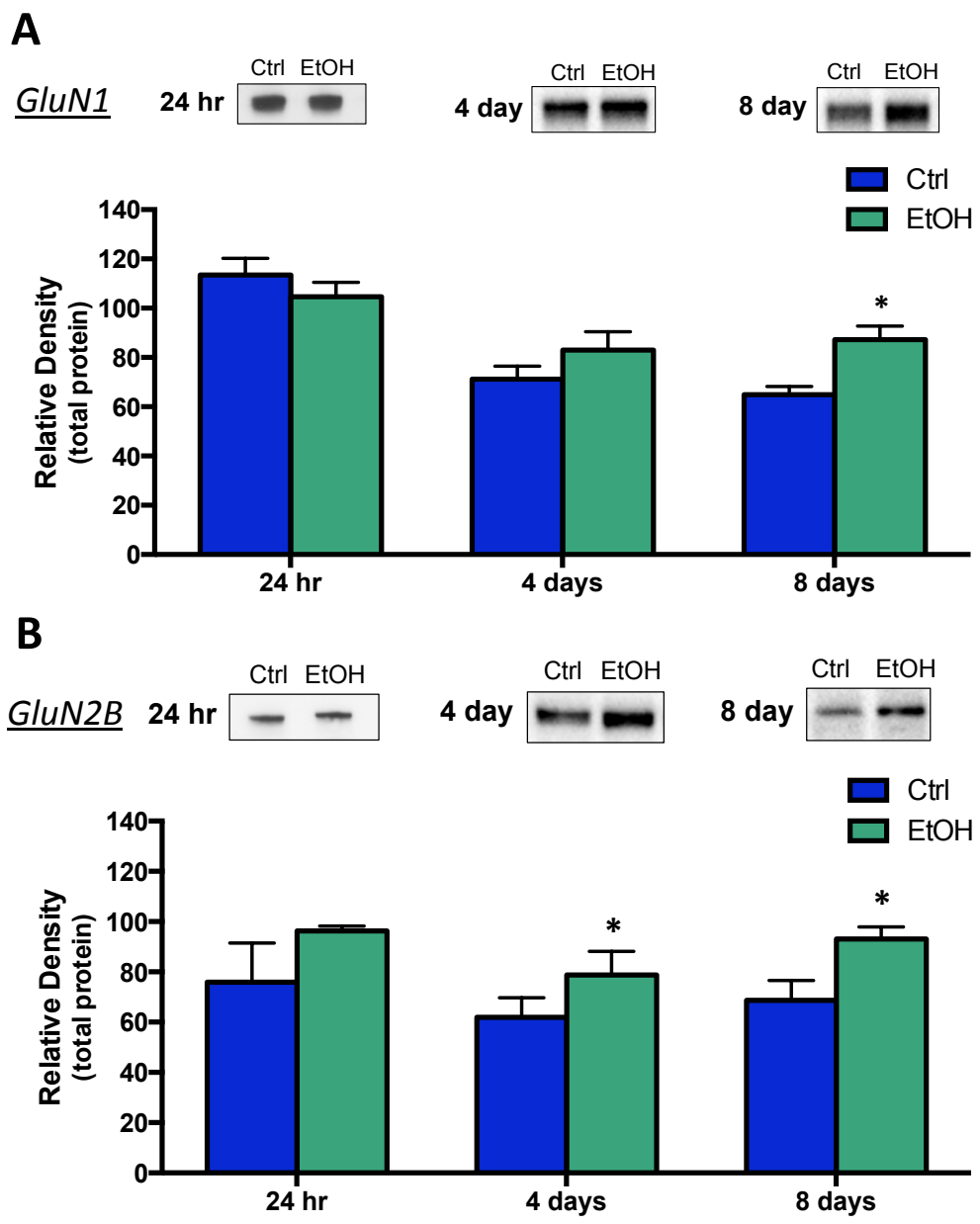

Figure 2-4. Time course of changes in NMDA receptor subunits following acute and chronic ethanol exposure in hippocampal slice cultures.

A. GluN1 expression was significantly increased after 8 days of ethanol exposure, but not 4 days or 24 hours (*n*= 6, 5 $p< 0.05$). **B.** Expression of GluN2B was significantly increased at both 4 and 8 days, but not after 24 hours (*n*= 6, 5 $p< 0.05$).

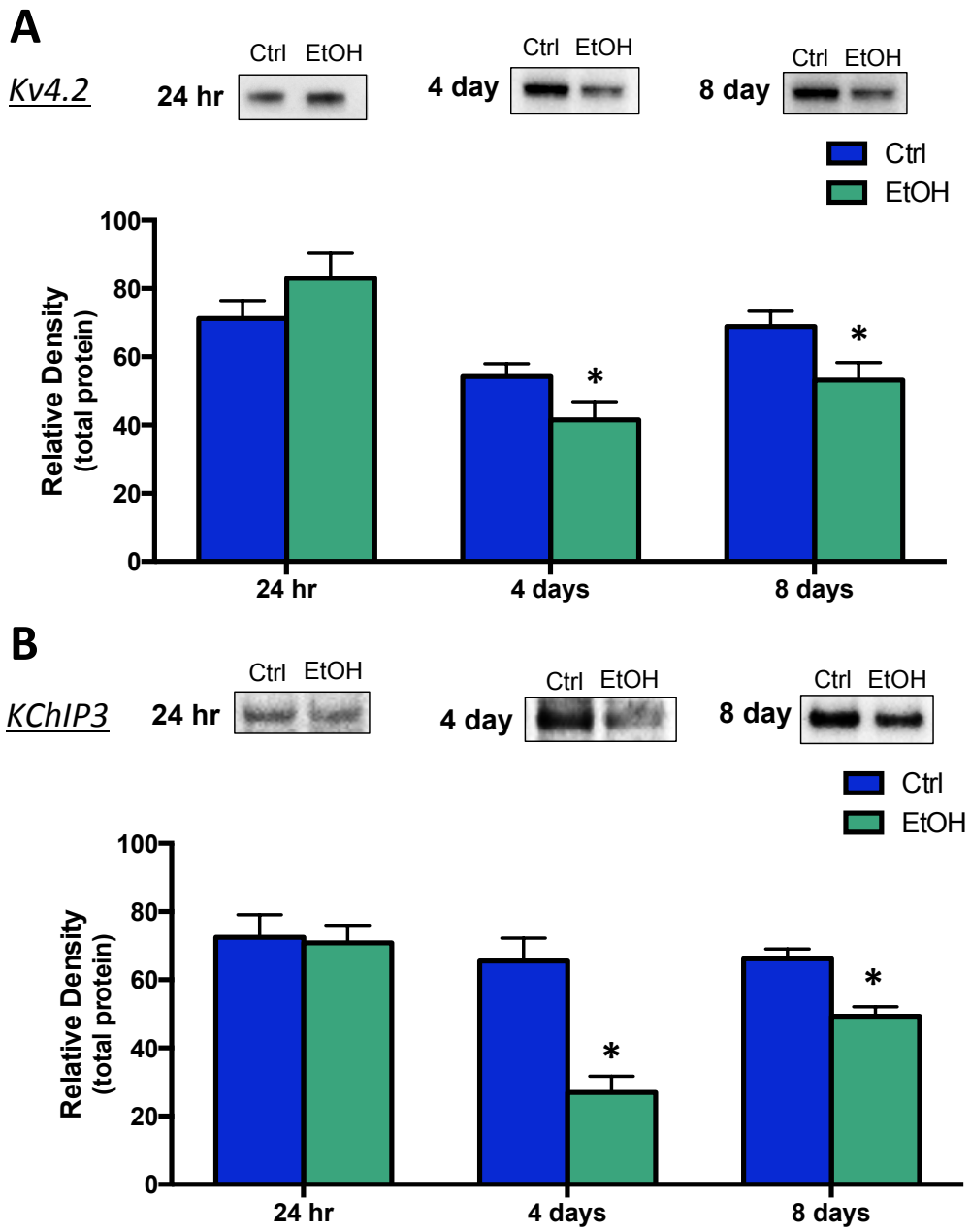

Figure 2-5. Ethanol-induced changes in Kv4.2 and KChIP3 expression in organotypic hippocampal slice cultures.

Both Kv4.2 (**A)** and KChIP3 (**B)** total protein expression was significantly decreased following both 4 and 8 days of ethanol exposure, but no change was seen after 24 hours (*n*= 5, 5, 6 *p< 0.05).

Ethanol-induced changes in FMRP expression and function

Chronic ethanol drives homeostatic changes at glutamatergic synapses to maintain optimal excitatory signaling. However, the mechanism underlying these changes remains unknown. One potential candidate in this process is the mRNA-binding protein FMRP. FMRP has previously been shown to regulate translation of Kv4.2, KChIP3, and NMDA receptor subunits (Jones 2003; Henry et al 2011; Darnell et al 2011). Increases in phosphorylation of FMRP at S499 are associated with an increase in FMRP-mRNA interaction, and therefore, a decrease in protein expression (Laggerbauer et al 2001; Ceman et al 2003; Zalfa et al 2006; Darnell and Klann 2013; Bartley et al 2014).

To evaluate total and phosphorylated FMRP protein expression, western blot analysis was used in CIE-exposed mouse hippocampal tissue. A phospho-specific antibody for S499 revealed an increase in expression of phospho-FMRP protein in the ethanol-exposed mouse hippocampus compared to controls. However, there was no change in total FMRP protein levels **(Figure 2-6**; *phospho-FMRP: t* test, $t(40)=2.382$, *p<0.05, n= 21; *FMRP*: *t* test, $t(35)=0.0997$, p=0.9211, n= 18,19). In the *in vitro* model culture system, western blot was also used to determine changes in phosphorylated and total FMRP protein following different ethanol exposure time points. Similar to changes observed following *in vivo* exposure, there was an increase in phosphorylated FMRP in cultures treated with ethanol, but again no change in total FMRP protein expression. However, unlike observations with NMDA receptor subunits proteins, this

increase was seen acutely after only 24 hours of exposure and persisted throughout the longer exposure periods of 4 and 8 days **(Figure 2-7**; *phospho-FMRP*: 24 hr: *t* test, $t(6)=2.474$, *p<0.05, n= 6; 4-day: *t* test, $t(3)=2.848$, *p<0.05, n= 5, 6; 8-day: *t* test, $t(5)=3.149$, *p<0.05, n= 6; 8-day+24 hr withdrawal: *t* test, $t(6)=2.635$, *p<0.05, n= 6). Additionally, 8 days of ethanol exposure followed by 24 hours of acute withdrawal produced the opposite effect, with a significant decrease in phosphorylated FMRP protein while total protein levels remain unchanged **(Figure 2-7** *FMRP*: 24 hr: *t* test, $t(4)=0.2356$, p= 0.4191, n= 5; 4-day: *t* test, $t(4)=0.2356$, p= 0.4191, n= 5; 8-day: *t* test, $t(5)=0.7408$, p=0.4921, n= 6; 8 –day+24 hr withdrawal: *t* test, $t(6)=0.8217$, p=0.4427, n= 6). This increase was represented by an increase in phosphorylated FMRP, and not a general increase in total protein expression **(Figure 2-8**; *t* test, $t(5)=2.672$, *p<0.05, n= 3).

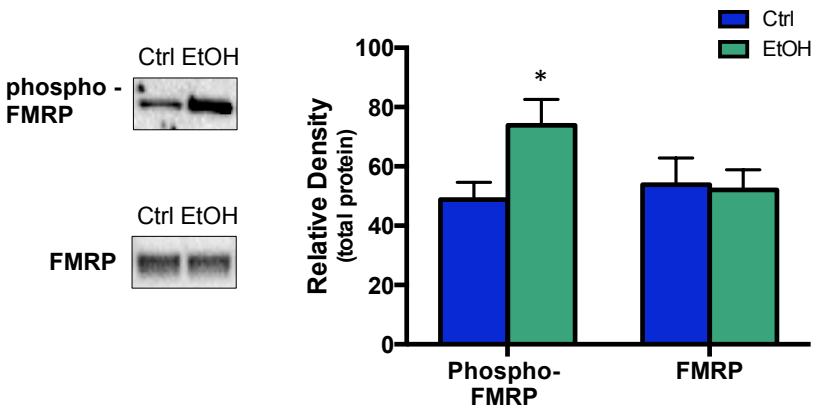

Figure 2-6 Chronic intermittent ethanol-induced differences in phosphorylated and total FMRP protein in the mouse hippocampus.

Exposure to chronic ethanol increased phosphorylated FMRP levels, but did not change total protein. (n= 21, 18, 19 * p< 0.05)

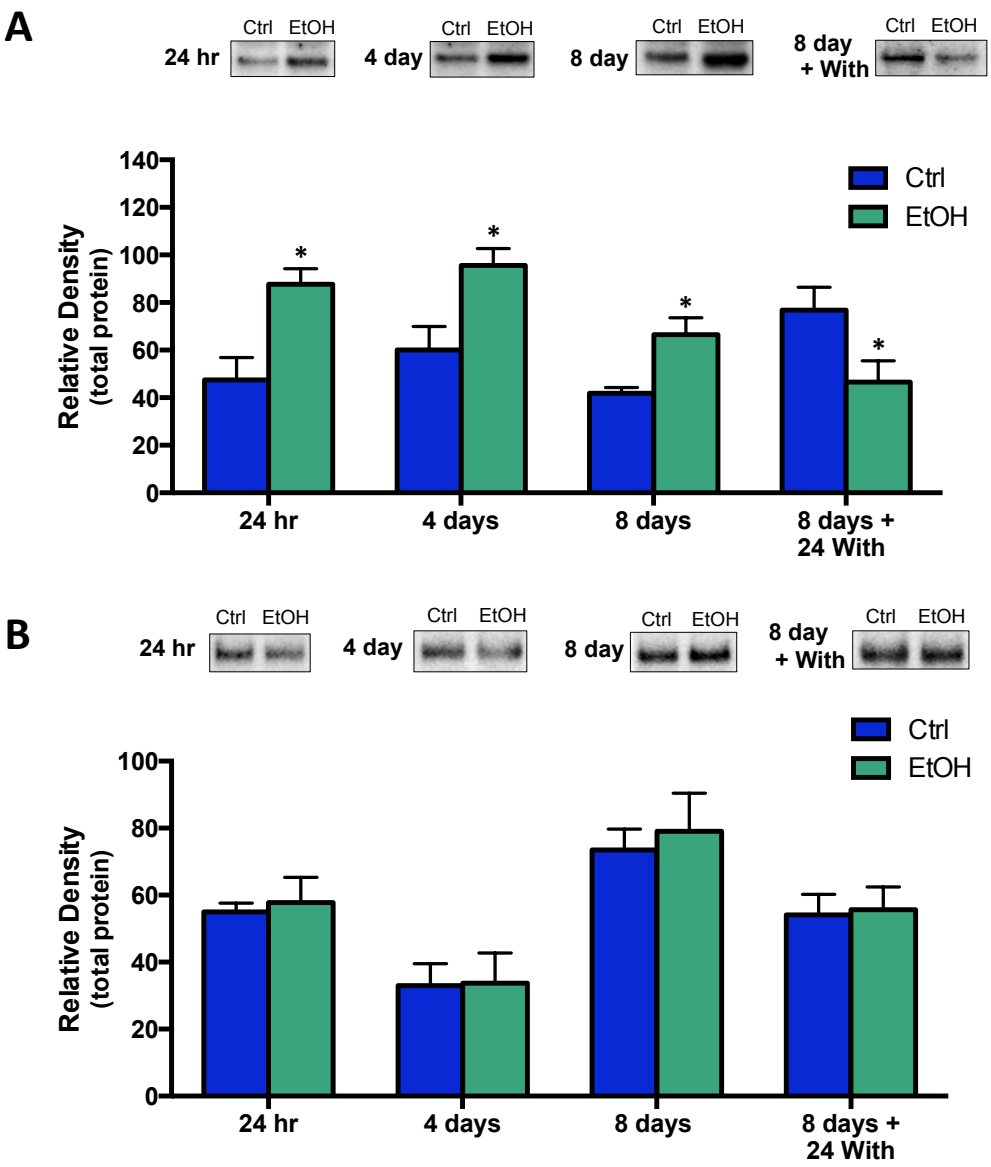

Figure 2-7 Time course of phosphorylated and total FMRP expression in hippocampal slice cultures.

A. Phosphorylated FMRP was increased at three timepoints after ethanol exposure: 24 hours, 4 days, and 8 days. However following 8 days of exposure and a 24 hour withdrawal period, phosphorylated FMRP protein was significantly decreased (n= 5,6 * $p< 0.05$). **B.** Unlike phosphorylated FMRP protein expression, total protein levels remained unchanged during all three exposure time points and following acute withdrawal (n= 5,6 * $p< 0.05$).

FMRP-mRNA interactions

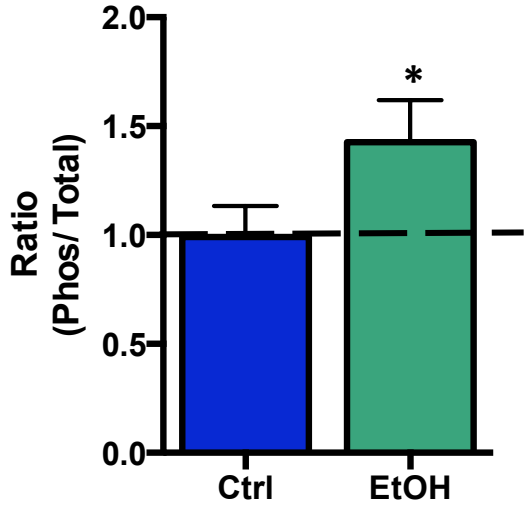

Figure 2-8 Ratio of phosphorylated FMRP to total protein.

Chronic ethanol exposure increased the ratio of phosphorylated to total FMRP protein levels in hippocampal slice cultures. (*n*= 5, 6 * p< 0.05).

Although ethanol may increase FMRP phosphorylation at S499, the mRNAs interacting with FMRP under control and ethanol-treated conditions influence whether FMRP mediates the changes in expression of these proteins. For these experiments, organotypic hippocampal slice cultures was used to investigate changes in FMRP- mRNA binding under control and ethanol-exposed conditions. Using co-IP and RT-qPCR, FMRP with bound mRNAs were pulled down, purified, and amplified with cDNA-specific primers **(Figure 2-1)**. Previous studies in rat models of chronic ethanol have confirmed increases in PSD-95 protein expression (Barak et al 2013). As one of the first, and most widely researched FMRP targets, PSD-95 mRNAs were used as confirmation that mRNA binding to FMRP was preserved using this paradigm, and western blot analysis was used to as a positive control to confirm successful pull down of FMRP **(Figure 2-9**; *PSD-95*: *t* test, $t(5)=2.672$, *p<0.05, *n*= 3). It is important to note that decreased FMRP-mRNA associations

are indicative of an increase in translation, and therefore, an increase in protein expression. In the hippocampal slice cultures, PSD-95 mRNA binding to FMRP is significantly decreased following 8-day ethanol exposure. This suggests that FMRP may also mediate expression of other proteins implicated in ethanol-induced homeostatic changes.

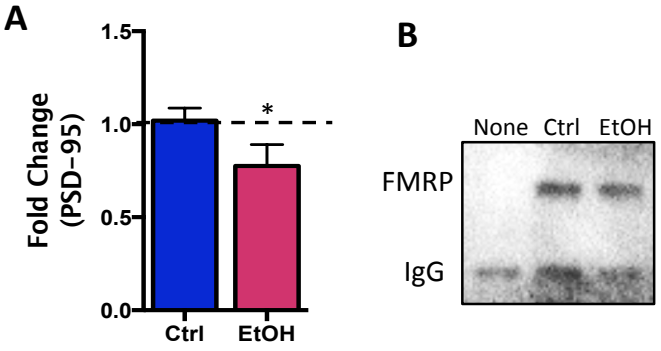

Figure 2-9 Successful pull down of mRNA with FMRP.

A. A significant decrease in PSD-95 mRNA pulled down with FMRP (*n*= 4, *p < 0.05). **B.** Western blot of pooled samples pulled down using the FMRP antibody. Samples incubated with the antibody only and no protein showed only the IgG band. However, both control and ethanol- treated groups showed bands for FMRP and IgG (*n*= 4).

Examination of mRNAs coding for NMDA receptor subunits in the hippocampus indicated no change in GluN1 in ethanol-exposed slices compared to controls. However, analysis of GluN2B show a decrease in FMRP-GluN2B mRNA binding **(Figure 2-10**; *GluN1*: *t* test, *t*(7)=0.9546, p=0.3716, *n*= 8; *GluN2B*: *t* test, *t*(8)=2.402, *p<0.05, *n*= 9; *Kv4.2*: *t* test, *t*(8)=2.340, *p<0.05, *n*= 9; *KChIP3*: *t* test, *t*(7)=2.561, *p<0.05, *n*= 6). In addition to these mRNAs, others coding for proteins that influence inhibitory signaling were also measured, including Kv4.2 and KChIP3. Co-IP and subsequent RT-qPCR showed an increase in FMRP binding to both Kv4.2 and KChIP3 mRNAs. This increase in association indicates an increase in FMRP- mRNA interaction, and therefore an increase in inhibition.

This also suggests a decrease in Kv4.2 and KChIP3 mRNA translation and protein expression.

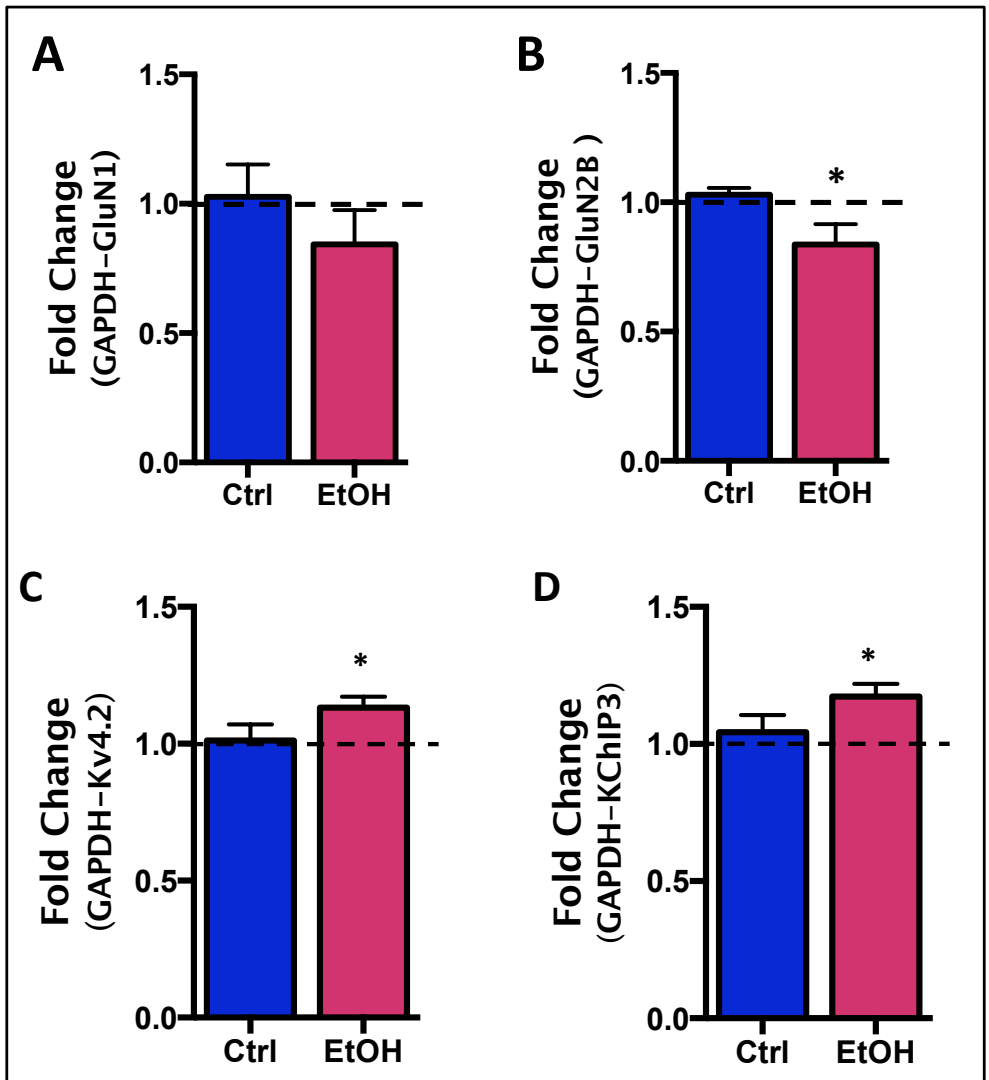

Figure 2-10 Ethanol-induced alterations in FMRP-mRNA interactions in the hippocampus.

A. GluN1 mRNAs were not significantly decreased compared to controls (*n*= 8, *p >0.05). **B.** Unlike GluN1, The level of GluN2B mRNA co-immunoprecipitated with FMRP were significantly increased following ethanol exposure (*n*= 9, * p< 0.05). **C.** Kv4.2 mRNAs pulled down with FMRP were significantly increased following 8 days of ethanol (*n*= 9, * p< 0.05). **D.** Similar to Kv4.2, mRNAs of KChIP3 were also significantly increased as a result of chronic ethanol exposure (*n*= 6, * p< 0.05)

Discussion

The main findings of this chapter are that chronic ethanol exposure induces homeostatic, bidirectional changes in protein expression in dendrites and dendritic spines that may function to reestablish the balance between excitation and inhibition. Furthermore, these changes in protein translation correspond to alterations in FMRP activity and mRNA interactions. These results suggest that restoring homeostasis during exposure to chronic ethanol is a multifaceted, coordinated effort that requires adjustments in both excitation and inhibition. However, components of these systems may share a common translational pathway with FMRP, and increased FMRP activity may be an essential component of ethanol-induced changes in protein expression.

Previous results have shown that long-term ethanol exposure promotes excitation at glutamatergic synapses and dampens inhibition (Lovinger 1990; Hendricson et al 2007; Korkotian et al 2013; Zorumski et al 2014). This increase in glutamatergic signaling is not limited to increases in NMDA receptors, and likely involves a reduction in local inhibitory proteins in dendrites and dendritic spines. Results presented in this chapter confirm a chronic ethanol-induced increase in GluN1 and GluN2B subunit expression in the hippocampus following both *in vitro* and *in vivo* chronic ethanol exposure. Previous studies from our lab indicate that this increase in NMDA receptor expression is specific for GluN2B-containing NMDA receptors, and GluN2A expression remains unchanged (Carpenter-Hyland et al 2004). Functionally, this shift may have important

implications in altered plasticity and excitability in response to chronic ethanol exposure. Previous studies in Huntington's disease, fragile X syndrome, and epilepsy have shown that specifically GluN2B-containing NMDA receptors are important for the development of this hyperexcitable phenotype, and suppression of GluN2B dampens NMDA-mediated cell death in hippocampal primary cultures (Monaghan et al 2008; Lei et al 2010; Russo et al 2013). Taken together with these previous functional studies, in GluN2B-containing NMDA receptors may have a key contribution to the increased excitability induced by chronic ethanol exposure.

These results also suggest an important role for Kv4.2 and the auxiliary protein KChIP3 in mediating ethanol-induced hyperexcitability in the hippocampus. Results presented here confirm other studies from our lab found that shows a decrease in Kv4.2 channel expression following chronic ethanol exposure. Additionally, previously published work demonstrated a decrease in A-type current following chronic ethanol that is not attributed to other A-type K^+-channels, such as Kv1.4 (Mulholland et al 2014). As one of the main influences dampening excitatory inputs, Kv4.2 has a well-defined role in epileptogenesis and hyperexcitability in the hippocampus (Hong et al 2003; Monaghan et al 2008; Aronica et al 2009; Barnwell et al 2009). It is also of note, that Kv4.2 may have an important role in NMDA-mediated hyperexcitability. Interestingly, increases in GluN2B-containing NMDA receptors is associated with decreases Kv4.2-mediated A-type current as well as Kv4.2 expression (Kim et al 2007; Lei et al 2008; Lei et al 2010). As such, Kv4.2 likely has an integral role in maintaining

homeostatic increases in excitability and reduction in Kv4.2 expression that may reflect a coupled homeostatic response with the increase in GluN2B expression.

In addition to a reduction in expression of Kv4.2, our results also show a decrease in KChIP3 protein expression. Importantly, KChIP3 is integral in fine-tuning Kv4.2 channel kinetics and is necessary for Kv4.2 channel surface expression (Shibata et al 2003; Lin et al 2004; Menegola et al 2006 Ruiz-Gomez et al 2006). Previous studies in our lab have shown that this ethanol-induced decrease in expression of Kv4.2 channels is limited to surface-expressed protein (Mulholland et al 2014). Taken together, this suggests that the decrease in KChIP3 contributes to the decreased surface expression of Kv4.2. In addition to Kv4.2, KChIP3 may also contribute to the increased excitation resulting from chronic exposure. KChIP3 interacts either directly with the GluN1 or GluN2B subunit to decrease NMDA surface expression, or indirectly by binding to PSD-95 and decreasing NMDA-mediated current (Zhang et al 2010; Wang et al 2012). Therefore, this ethanol-induced decrease in KChIP3 may have an important role in both excitation and inhibition during chronic ethanol exposure.

These data also provide insight into how ethanol may alter neuronal homeostasis by mediating local translation in dendrites and dendritic spines. Ethanol exposure resulted in an increase in FMRP phosphorylation at S499 in both acute and long-term exposure paradigms with high dose ethanol. However, ethanol-induced changes in other proteins of interest were not seen after a short-term 24-hour exposure period, only after a longer 8-day exposure. This increase in FMRP phosphorylation after acute ethanol exposure may reflect changes in

other FMRP-mediated translational mechanism for short-term adaptations. Studies in seizure disorders, such as epilepsy, have shown that although there is an increase in FMRP after 30 minutes. However, more sustained changes in protein expression of its binding partners may occur between 2 and 48 hours after the initial induction of seizure activity (Price and Ring 2011; Meng et al 2013; Russo et al 2013). Conversely, phosphorylation at S499 was significantly decreased following a 24-hour withdrawal period after an 8-day ethanol treatment. Acute withdrawal periods following long-term exposure are characterized by increased NMDA-mediated hyperexcitability that can lead to withdrawal-induced seizures (Hall and Zador 1997; Duka et al 2004; Hendricson et al 2007). This decrease in phosphorylation may be indicative of the need for an immediate shift in translational mechanisms or protein expression to correct the imbalance caused by an acute withdrawal of ethanol. Taken together, these results suggest that FMRP may mediate both short-term and long-term, homeostatic changes in the hippocampus during ethanol exposure.

Increases in FMRP phosphorylation as a result of ethanol exposure are indicative of changes in activity. However, FMRP may regulate different aspects of translation, and it has a multitude of interacting partners in dendrites and dendritic spines (Laggerbauer et al 2001; Ascano et al 2012; Darnell and Richter 2012). Therefore, it is informative to discern whether there is a difference in the mRNAs corresponding to the proteins of interest bound to FMRP during control and ethanol-treated conditions. Results in these studies revealed changes in FMRP- mRNA binding as a result of 8-day ethanol exposure. These experiments

show an increase in Kv4.2 and KChIP3 mRNAs bound to FMRP and a decrease in GluN2B, but not GluN1 following long-term ethanol treatment. These data suggest that FMRP directly modulates Kv4.2, KChIP3, and GluN2B translation during ethanol exposure. As previously mentioned, the composition of NMDA receptor subunits may affect not only NMDA-mediated hyperexcitability, but also Kv4.2 expression and function (Lei et al 2008; Kaufmann et al 2012). In these experiments, ethanol affects GluN2B, but not GluN1 FMRP-mRNA binding. During chronic ethanol exposure, FMRP may have a more pronounced effect on NMDA channel function by modulating subunit expression and therefore composition, rather than affecting overall NMDA receptor expression.

Experiments in this chapter have confirmed that multiple factors contribute to the maintenance of homeostasis at glutamatergic synapses. Increases in NMDA receptor expression promote excitability and glutamatergic signaling, while decreases in Kv4.2 and KChIP3 dampen excitation. Additionally both of these mechanisms may be mediated by FMRP activity. Further experiments presented in the next chapter will define whether FMRP activity is necessary for ethanol-induced alterations in protein expression.

Chapter 3

Inhibition of FMRP phosphorylation prevents ethanol-induced translational changes in the hippocampus

Background and Significance

FMRP protein expression and activity are important in maintaining basal protein levels and regulating activity-dependent translation in dendrites and dendritic spines (Bardoni et al 1997; Antar et al 2005; Dictenberg et al 2008; Henry et al 2011). The absence of FMRP causes an increase in basal protein levels as well as dysregulated synaptic plasticity in both clinical studies and *fmr1*-

/- mouse models (Hagerman and Strafstorm 2009; Gross et al 2011; Krueger and Bear 2011; Lee et al 2011; Maurin et al 2014; Chen and Joseph 2015). The role of FMRP in regulating cellular function throughout development and in certain pathological states is relatively well characterized. However, how FMRP maintains basal protein levels and activity-dependent translation in a typically functioning adult brain is unknown. Studies in Chapter 2 of this dissertation demonstrated ethanol-induced changes in FMRP phosphorylation and alterations in FMRP-mRNA interactions, supporting the suggestion that chronic ethanol exposure induces an increase in FMRP activity. These results also implicated certain mRNA targets of FMRP in ethanol-induced synaptic plasticity that are key mediators of local excitation in dendritic spines, including Kv4.2, KChIP3, and NMDA receptor subunits. These changes in protein expression represent longer, homeostatic adaptations that are not induced by short-term exposure, even at high doses, and this longer exposure period causes significant changes in FMRP-mRNA interactions for these proteins of interest. Although FMRP activity is altered by chronic ethanol treatment, it is important to determine if these changes in FMRP phosphorylation and mRNA binding are necessary for the homeostatic alterations in protein expression, and to investigate how ethanol might induce these changes in FMRP function. Translation in dendrites and dendritic spines is a highly coordinated process that involves several components that are discretely regulated in order to maintain proper function at the synapse (Kapp and Lorsch 2004; Weiler et al 2004; Sossin and Lacaille 2010; Hinnebusch and Lorsch 2012).

One of the main intracellular pathways regulating local dendritic translation is the mTORC1 signaling cascade. Studies from other groups have demonstrated an important role for other mTORC1 substrates, namely p70 ribosomal S6 kinase 1 (S6K1), in mediating changes in protein expression following chronic ethanol exposure *in vivo* (Holz et al 2005; Nesta 2010; Barack et al 2013). Results from these studies revealed disruption of mTORC1 activity during ethanol treatment prevents ethanol-seeking behaviors and memory deficits (Nesta et al 2010; Barak et al 2013; Nesta et al 2014). Rats exposed to chronic intermittent ethanol have significantly fewer active lever presses in a standard reinstatement paradigm when treated with the mTORC1 inhibitor rapamycin (Nesta et al 2010; Barak et al 2013) Additionally, rapamycin also blocked ethanol-induced changes in expression of other proteins in dendritic spines that are regulated by FMRP (Nesta et al 2010; Barak et al 2013).

Although these results suggest FMRP and its upstream mediators may play an integral role in mediating translational changes during chronic ethanol exposure, it is unclear if this change in activity is necessary for ethanol-induced translation. Since mTORC1 and its substrates comprise one of the main pathways regulating protein synthesis in response to changes in synaptic activity, components of this pathway, including S6K1 and FMRP, are likely mediators of ethanol-induced synaptic plasticity. During CIE, increased S6K1 activity via mTORC1 activation may regulate increased FMRP phosphorylation at S499. This increase in phosphorylation induces binding to select mRNAs to decrease translation of these specific proteins while other FMRP-mRNA interactions are

reduced, resulting in increased protein expression **(Figure 3-1).** Studies presented in this chapter investigated whether increased FMRP phosphorylation is necessary for ethanol-induced changes in protein expression, and whether these changes in phosphorylation are mediated through S6K1 activity.

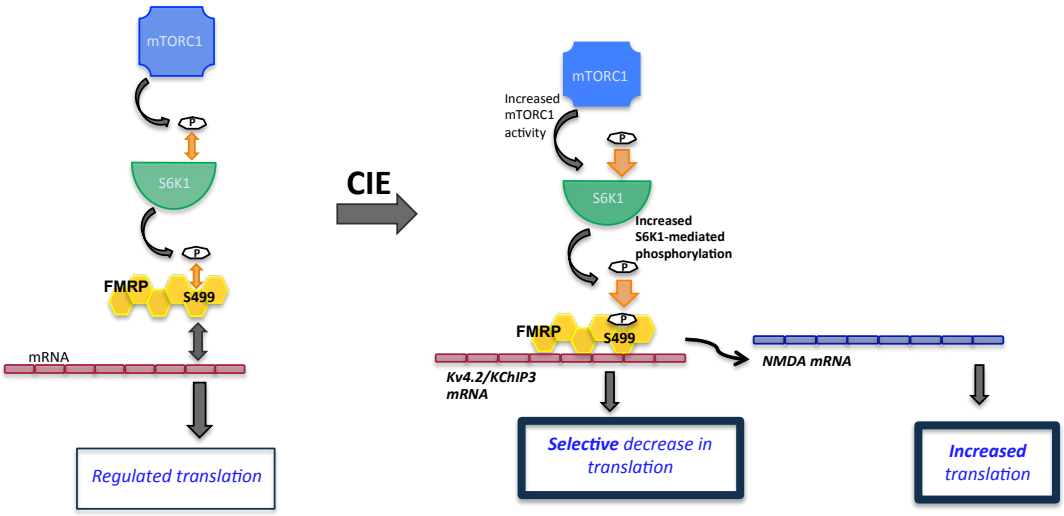

Figure 3-1 CIE-induced changes in FMRP signaling.

S6K1 phosphorylates FMRP in an activity-dependent manner to maintain the necessary levels of protein translation. According to Barak et al 2013, ethanol exposure causes increased mTORC1 and S6K1 activity. S6K1-mediated phosphorylation of FMRP at S499 decreases expression of certain proteins while disinhibiting mRNAs.

Methods

Organotypic hippocampal slice culture ethanol exposure

Hippocampal slice cultures were prepared from P6-P8 Sprague- Dawley rats, as described in Chapter 2. After plating hippocampal slice cultures were incubated in culture media for at least eight days before treatment. All

experiments adhered to NIH Animal Care Guidelines and were approved through the IACUC. Slice cultures were exposed to 75 mM ethanol, 6 uM PF-4708671 (PF), or both for 8 days.

For S6K1 inhibition, a dose-response curve determined that 6 μM PF was the concentration that inhibited FMRP phosphorylation, but did not alter other downstream substrates. Therefore, this concentration was used in subsequent experiments. The inhibitor, PF-4708671 was added to culture media daily concurrent with ethanol exposure. After exposure, slices were scraped from the culture membrane, sonicated in 2% LDS, and prepared for either western blot analysis, or co- immunoprecipitation and RT-qPCR. *See Chapter 2 for extended methods.*

Protein Assay

Organotypic hippocampal slice cultures were sonicated in 2% LDS. A BCA Pierce Protein Assay Kit was used to determine total protein content as described in Chapter 2.

Western blot analysis

Both mouse hippocampal tissue and organotyipc hippocampal slice cultures were sonicated in 2% LDS. Protein samples were separated using electrophoresis, transferred to a PVDF membrane, and a reversible Swift total protein stain was used for normalization. Primary antibodies used were phosphorylated S499 FMRP (1:1000), KChIP3 (1:500), Kv4.2 (1:1000), GluN1

(1:3000), and GluN2B (1:3000). After primary antibody incubation, membranes were incubated in secondary antibody: phosphorylated FMRP and KChIP3 in goat anti-rabbit and FMRP, Kv4.2, GluN1, and GluN2B in goat anti-mouse for 1 hour at room temperature. After secondary antibody incubation, membranes were exposed to an enhanced chemillumiscence and imaged with a ChemicDoc MP Imaging System (Bio-Rad, Hercules, CA). *See Chapter 2 for extended methods.*

Co- immunoprecipitation (co-IP) and RT-qPCR

Following treatment with ethanol, PF, or both, cultures were processed for either western blot analysis or co-IP and RT-qPCR. Co-IP with mRNA solutions were adapted from Lee et al 2011 and Pierce co-IP Kit. For co-IP, 6 µg of FMRP total protein antibody was used for every mg of beads, with 6-7 mg of beads per spin column. AminoLink coupling resin was added to spin column followed by coupling buffer washes. Antibody for coupling was added to the spin columns in coupling buffer and incubated at room temperature for 120 minutes with slow rotation. Hippocampal lysates were also pre-cleared prior to co-IP. After antibody and lysate preclearing, co-IP with mRNA was performed (all steps kept at 4 °C). Columns were then rinsed with wash buffer, and FMRP-mRNAs were eluded from column with Elution Buffer from the Pierce kit, and mRNAs were dissociated from FMRP with a TRIzol- chloroform extraction (Lee et al 2011). Next, mRNA was purified using the Qiagen RNeasy Mini Kit and nonspecific cDNA was transcribed using the Applied Biosystems High Capacity RNA- to –cDNA Kit. For

qPCR, the Sybr Green qPCR kit was used on a Bio-Rad CFX 96 thermocycler with an initial denaturation of 2 min at 94 ^{o}C followed by 40 cycles with a 15 sec denaturation at 94 ^{o}C and 1 min of annealing and extension at 60 ^{o}C and a 4 ^{o}C hold. All experimental data were normalized to GAPDH. The primer sequences used are presented in Table 1; Chapter 2. *Refer to Chapter 2 for detailed methods.*

Statistical Analysis

Experiments with control and either PF or Veh groups were analyzed with a student t-test with significance $p < 0.05$. For experiments with S6K1 inhibitor (PF-4708671) and concurrent ethanol treatment, a one-way ANOVA was used to determine significance.

Results

Inhibition of specific S6K1 substrates

In the previous chapter, experiments addressed whether ethanol alters FMRP protein expression and activity. Results presented here address how ethanol may alter FMRP phosphorylation, and whether this increase in phosphorylation is necessary for the homeostatic changes in protein expression following chronic ethanol exposure. Specifically, these experiments investigate whether S6K1 phosphorylates FMRP at S499, and if blockade of FMRP

phosphorylation through S6K1 inhibition will prevent ethanol-induced changes in FMRP-mRNA binding and protein expression. Importantly, treatment with the 6 μM dose of the S6K1 inhibitor, PF-4786071, did not significantly alter other necessary downstream components of the S6K1 translational pathway. These include S6, which is required for assembly of the pre-initiation complex, and eIF4E, the mRNA cap-binding protein that is necessary to begin active translation **(Figure 3-2**; *phospho-S6*: *t* test, *t*(4)=0.3946, p= 0.7133 *n*= 3; *eIF4E*: *t* test, *t*(4)=1.081, p= 0.3403 *n*= 3; *GluN1*: *t* test, *t*(4)=0.8478, p= 0.4443 *n*= 3; *GluN2B*: *t* test, *t*(4)=0.2354, p= 0.8255 *n*= 3; *Kv4.2*: *t* test, *t*(4)=0.04028, p= 0.9698 *n*= 3; *KChIP3*: *t* test, *t*(4)=0.3239, p= 0.7623 *n*= 3).

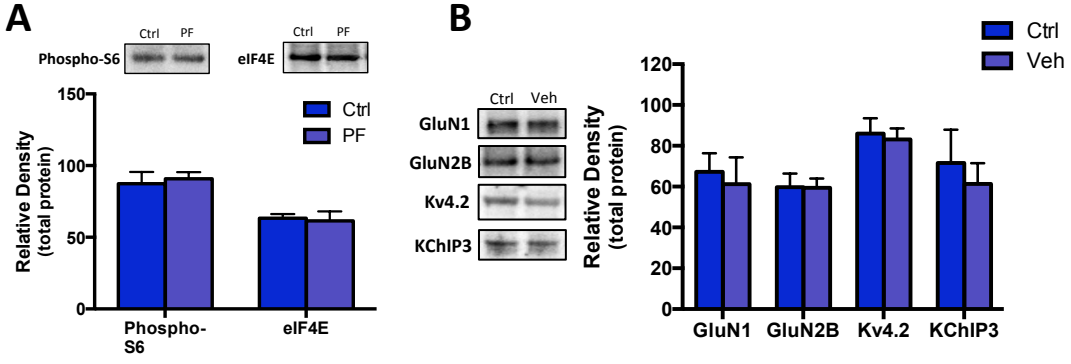

Figure 3-2 Under control conditions blockade of S6K1 did not alter expression of downstream substrates.

A. Long-term treatment with PF-4708671 did not significantly alter key substrates of S6K1 that are necessary for protein translation (*n*= 3, p >0.05). **B.** Vehicle (DMSO) treatment alone did not alter expression of the proteins of interest with the 8-day paradigm (*n*= 3, p >0.05).
Ctrl: Untreated slices; **PF:** S6K1 inhibitor; **Veh:** DMSO

Organotypic hippocampal slice cultures and western blot were used to first characterize the S6K1-specific inhibitor (PF) in both ethanol-treated and untreated neuronal cultures. In an 8-day ethanol exposure paradigm, treatment with PF or concurrent PF + ethanol treatment significantly decreased FMRP phosphorylation levels compared to ethanol only. However, neither group varied significantly compared to controls **(Figure 3-3**; *phospho-FMRP*: one-way ANOVA, $F(3,12)= 6.174$ $p= 0.0062$, Tukey post hoc, *p<0.05, $n= 5$).

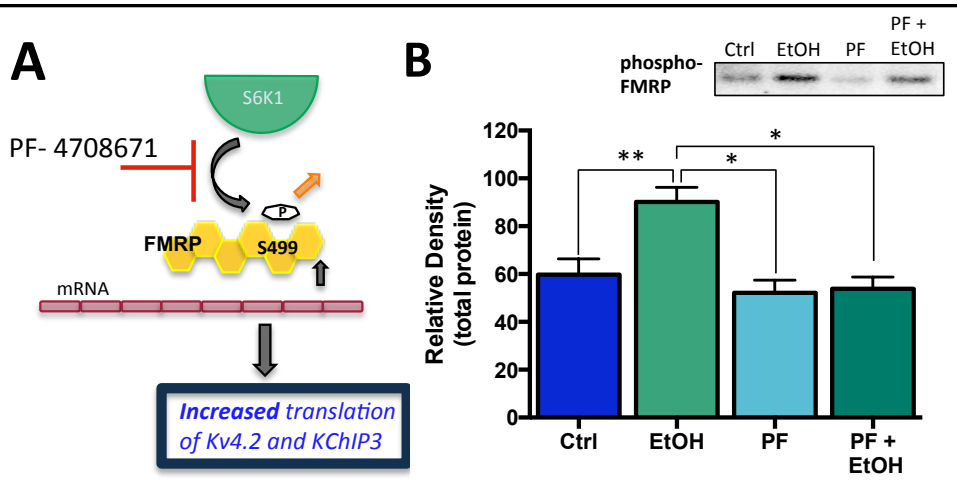

Figure 3-3 Inhibition of FMRP phosphorylation through blockade of S6K1 activity.

A. The S6K1 inhibitor disrupts S6K1 activity, but does not alter upstream regulators including mTORC1. **B.** Treatment with an S6K1 inhibitor, PF-4786071, decreased FMRP phosphorylation levels ($n= 5$, * p< 0.05, ** p< 0.001).
Ctrl: Untreated slices; **EtOH:** Ethanol treatment only; **PF:** S6K1 inhibitor only; **PF+ EtOH:** S6K1 inhibitor and ethanol treatment.

Effect of S6K1 inhibition on protein expression

Using the same 8-day ethanol exposure paradigm, the effects of exposure to the S6K1 inhibitor were evaluated for ethanol-induced alterations in NMDA receptor subunits. Ethanol treatment alone produced the expected effect of an increase in GluN1 subunit expression **(Figure 3-4**; *GluN1*: one-way ANOVA, $F(3,12)= 3.324$ $p= 0.0567$, Tukey post hoc*,* *p<0.05, $n= 5$). However, PF alone and PF with 75 mM ethanol did not significantly decrease GluN1 protein levels compared to ethanol alone.

GluN2B total protein expression was also evaluated using western blot. Similar to GluN1, there was a significant increase in protein expression with ethanol treatment only. Addition of PF to the culture media alone did not significantly change expression levels from either control or ethanol-treated tissue. When PF and ethanol were added to hippocampal slices in combination, GluN2B protein expression decreased significantly compared to ethanol- treated tissue, but did not show a significant change compared to controls **(Figure 3-4** *GluN2B*: one-way ANOVA, $F(3,12)= 5.665$ $p= 0.0.0272$, Tukey post hoc*,* *p<0.05, $n= 5$).

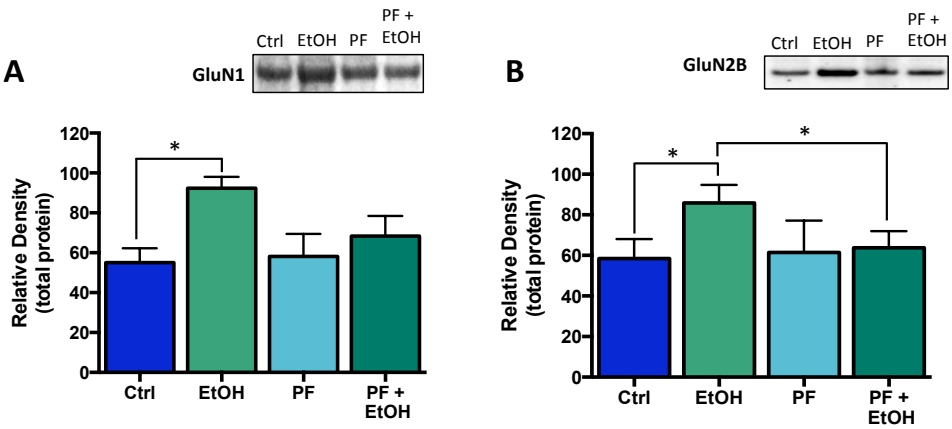

Figure 3-4 Inhibition of FMRP phosphorylation alters ethanol-induced changes in NMDA receptor subunit expression.

A. Inhibition of FMRP phosphorylation did not significantly alter ethanol-induced changes in GluN1 receptor expression (*n*= 5, * p< 0.05). **B**. Decreased FMRP phosphorylation significantly prevented the ethanol-induced increase in GluN2B expression. (*n*= 5, * p< 0.05). **Ctrl:** Untreated slices; **EtOH:** Ethanol treatment only; **PF:** S6K1 inhibitor only; **PF+ EtOH:** S6K1 inhibitor and ethanol treatment.

In addition to NMDA receptor subunits, Kv4.2 and KChIP3 protein levels were also investigated with inhibition of FMRP phosphorylation. Consistent with results from Chapter 2, ethanol alone caused a significant decrease in expression of both Kv4.2 and KChIP3 **(Figure 3-5**; *Kv4.2*: one-way ANOVA, $F(3,12)= 5.509$ $p= 0.0148$, Tukey post hoc, *p<0.05, $n= 5$; *KChIP3*: one-way ANOVA, $F(3,12)= 6.174$ $p= 0.0.146$, Tukey post hoc, *p<0.05, $n= 5$). However, this decrease in Kv4.2 and KChIP3 expression was prevented by exposure to PF. Additionally, KChIP3 protein levels with S6K1 inhibition alone were significantly decreased compared to ethanol alone, but were not different compared to control slices. Kv4.2 expression with S6K1 inhibition, however, was unchanged compared to both controls cultures and ethanol-treated slices.

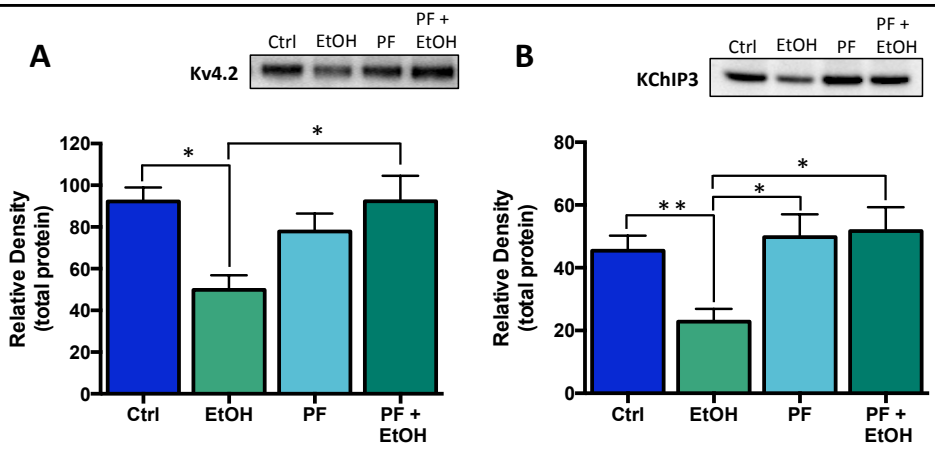

Figure 3-5 Ethanol-induced changes in Kv4.2 and KChIP3 expression blocked by decreased FMRP phosphorylation.

A. Treatment with an S6K1 inhibitor blocked the reduction in Kv4.2 channel expression induced by chronic ethanol exposure (*n*= 5, * p< 0.05). **B**. Decreased FMRP phosphorylation via S6K1 inhibition increases KChIP3 expression in ethanol- treated, PF alone, and PF with concurrent ethanol treatment. (*n*= 5, * p< 0.05).
Ctrl: Untreated slices; **EtOH:** Ethanol treatment only; **PF:** S6K1 inhibitor only; **PF+ EtOH**: S6K1 inhibitor and ethanol treatment.

Alterations in FMRP-mRNA ethanol-induced interactions following S6K1 inhibition

Changes in protein expression with inhibition of FMRP phosphorylation via decreases in S6K1-mediated activity is consistent with a potential role for FMRP in translational changes during chronic ethanol-induced plasticity. To further examine direct FMRP-mRNA interactions, we again used co-immunoprecipitation of FMRP-mRNA complexes followed by RT-qPCR analysis of GluN1, GluN2B, Kv4.2, and KChIP3. As shown in **Figure 3-6**, these results corresponded to the changes in protein expression. GluN1 mRNA bound to FMRP was not significant following ethanol treatment alone, confirming observations in Chapter 2. In the

western blot experiments with PF treatment, none of the other treatment groups, PF or PF + ethanol, were significantly different compared to controls or ethanol alone. However, as seen in previous experiments, GluN2B mRNA bound to FMRP in ethanol-treated cultures was significantly reduced compared to controls alone. Treatment with the S6K1 inhibitor blocked ethanol-induced decreases in GluN2B mRNAs. PF alone did not significantly alter mRNA levels compared to either control or ethanol alone **(Figure 3-6**; *GluN1*: one-way ANOVA, $F(3,12)= 0.3996$ $p= 0.7558$, $n= 5$; *GluN2B*: one-way ANOVA, $F(3,15)= 4.232$ $p= 0.0235$, Tukey post hoc*,* *p<0.05, $n= 6$).

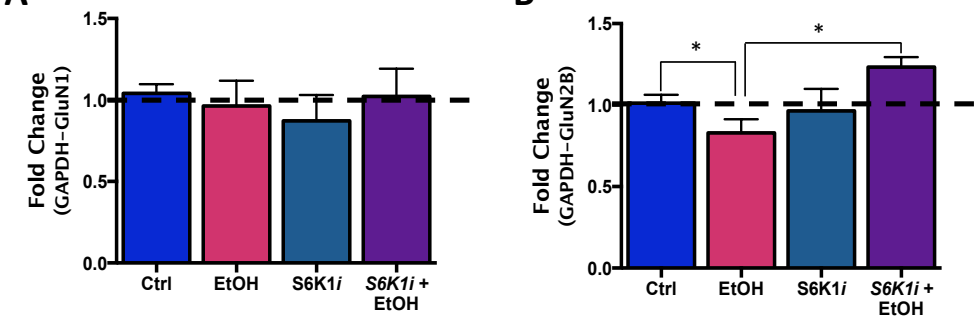

Figure 3-6 Changes in NMDA receptor subunit mRNA interactions with FMRP with S6K1 inhibition during chronic ethanol exposure.

A. Inhibition of S6K1 and reduction of FMRP phosphorylation did not alter FMRP-mRNA trafficking for GluN1 subunits alone or with ethanol treatment ($n= 6$, * $p< 0.05$). **B**. Inhibiting FMRP phosphorylation with PF treatment prevented ethanol-induced decreases in FMRP-GluN2B interactions. ($n= 6$, * $p< 0.05$).
Ctrl: Untreated slices; **EtOH:** Ethanol treatment only; **PF:** S6K1 inhibitor only; **PF+ EtOH:** S6K1 inhibitor and ethanol treatment.

In addition to NMDA receptor subunits, other mRNAs bound to FMRP that are affected by chronic ethanol exposure were tested. Kv4.2 mRNA co-precipitated with FMRP was increased following an 8-day ethanol treatment, replicating previous observations **(Figure 3-7**; *Kv4.2*: one-way ANOVA, $F(3,12)= 3.630$ $p= 0.0351$, Tukey post hoc, *p<0.05, $n= 5$). Treatment with the S6K1 inhibitor alone did not significantly change protein levels compared to either control or ethanol-treatment tissue. Addition of PF blocked ethanol-induced increases in Kv4.2 mRNA and FMRP binding.

Expression of the Kv4.2 auxiliary protein KChIP3 was also evaluated. As with Kv4.2, the addition of ethanol to the culture media caused an increase in KChIP3 mRNAs interacting with FMRP, again consistent with results presented Chapter 2. Importantly, treatment with PF in combination with ethanol prevented the ethanol-induced increase in interactions **(Figure 3-7**; *KChIP3*: one-way ANOVA, $F(3,12)= 4.465$ $p= 0.0252$, Tukey post hoc, *p<0.05, $n= 5$). While PF alone did not cause any change in protein expression compared to control tissue, there was a significant decrease mRNAs compared to controls.

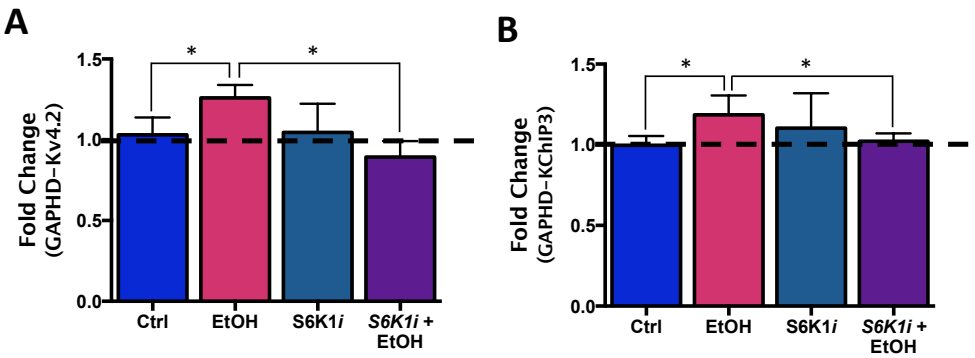

Figure 3-7 Ethanol-induced changes in FMRP-mRNA interactions of inhibitory proteins.

A. Treatment with PF-4708671 prevented ethanol-induced increases in Kv4.2 mRNA binding to FMRP (*n*= 5, * p< 0.05). **B**. Like Kv4.2, KChIP3 mRNAs bound to FMRP were also decreased following concurrent treatment of ethanol and PF. (*n*= 5, * p< 0.05).
Ctrl: Untreated slices; **EtOH:** Ethanol treatment only; **PF:** S6K1 inhibitor only; **PF+ EtOH:** S6K1 inhibitor and ethanol treatment.

Discussion

Data presented in this chapter demonstrated that ethanol-induced increases in FMRP phosphorylation at S499 underlie ethanol-induced homeostatic changes in protein expression in the hippocampus. These studies also suggest a mechanism by which ethanol causes these increases in FMRP phosphorylation through activation of S6K1.

Results from Chapter 2 suggest that FMRP is an integral component of activity-dependent translation by increases in phosphorylation of S499 of FMRP, changes in protein expression of downstream stream targets, and through differences in FMRP-mRNA interactions in control and ethanol-treated

hippocampal slices. Studies in this chapter addressed whether this increase in FMRP phosphorylation is necessary for ethanol-induced homeostatic changes NMDA receptors, Kv4.2, and KChIP3 protein expression, and whether blockade of FMRP phosphorylation will prevent these changes. Studies characterizing other disorders defined by hyperexcitability have shown that aberrant FMRP function dysregulates both basal state and activity-dependent translation, and that knockdown of FMRP in dissociated cultures corrects this phenotype (Jeon et al 2012; Russo et al 2013; Takei and Hiroyki 2014). Consistent with this, results presented here show that inhibition of FMRP phosphorylation can prevent ethanol-induced changes in both mRNA trafficking and protein expression of the GluN2B subunit and inhibitory proteins Kv4.2 and KChIP3. This suggests that FMRP phosphorylation is not only an integral component of synaptic plasticity in dendrites and dendritic spines, but that it is necessary for local activity-dependent alterations in protein expression.

As with previous results, FMRP phosphorylation did not affect GluN1 protein expression or mRNA binding. These studies indicate that FMRP phosphorylation at S499 is not required for ethanol-induced increases in GluN1 protein expression, and this is mediated by other activity-dependent mechanisms. Studies examining fragile X syndrome and epilepsy suggest that K^+- channel deficits may contribute more to the hyperexcitable phenotype than NMDA receptor deficits (Henry 2008; Mercaldo et al 2009; Gross et al 2011; Lee et al 2011). As such, in chronic ethanol exposure, FMRP may have a greater impact on proteins that are mediators of glutamateric signaling in dendritic spines, rather

than NMDA receptor expression directly. Other pathways implicated in alcohol use disorders, such as ERK and MAPK, are also key regulators of GluN1, and may have a greater influence on GluN1 translation (Perkinton et al 2002; Ivanov et al 2006). Our results also revealed that FMRP appears to play a significant role in modulating GluN2B subunit expression, suggesting that FMRP have a more influential role in NMDA receptor function through regulation of NMDA subunit expression rather than total protein expression of the receptor itself. Further studies investigating both signaling pathways are necessary to discern how NMDA receptors are regulated and trafficked during chronic ethanol exposure.

An important finding in these studies is that ethanol-induced changes in FMRP phosphorylation and activity are mediated by S6K1, and are likely part of the mTORC1 pathway. Work from other labs have shown that S6K1 phosphorylation is increased following chronic ethanol exposure, and that this change in phosphorylation is essential to produce the long-term cellular and behavioral phenotype induced by heavy ethanol exposure (Nesta et al 2010; Barak et al 2013; Sabino et al 2013) Phosphorylation at S499 is not completely dependent on S6K1 in all activity-dependent mechanisms, and phosphorylation at this site may also be regulated by other mTORC1 substrates. Previous studies examining local dendritic protein translation have manipulated mTORC1 and showed changes in FMRP, rather than altering S6K1 directly. Results presented here implicate S6K1 specifically as the upstream regulator for activity-dependent increases in ethanol-induced FMRP phosphorylation at S499. This provides

insight into how ethanol may induce these activity-dependent changes in FMRP and its binding partners.

Experiments in these studies used a pharmacological intervention directed at the upstream kinase S6K1 to alter FMRP phosphorylation, rather than direct manipulation of the FMRP protein. The S6K1 inhibitor PF-4706871 affected some, but not all substrates in the translational pathway. It is important to note, that certain key proteins necessary for translation, namely S6 and eIF4E, were not altered by the dose used in these experiments. Additionally, other mechanism may compensate for low S6K1 activity, such as S6K2 and other kinases in the RSK family including p90 ribosomal proteins RSK1 & 2 (Urbanaska et al 2012). This inhibitor also did not interfere with the ability of mTORC1 to phosphorylate S6K1, or any of the other downstream components, rather its action appears to be limited to S6K1 (Pearce et al 2010). Limiting inhibition to S6K1 was of particular importance for these sets of studies to ensure proper assembly of the polyribosome and to preserve the ability of neurons to induce activity-dependent translation.

Although *fmr1-/-* mice are available, the developmental phenotype that includes important cellular changes in activity, differences in circuitry and synaptic development, and the behavioral deficits in knockout animals as adults makes them a difficult model system in the context of these experiments (Zhao et al 2005; De Rubeis and Bagni 2011; Sidorov et al 2013). The goal of these studies was to determine whether FMRP has a role in the homeostatic changes at glutamatergic synapses using typical activity-dependent mechanisms. With the

established cellular and behavioral phenotype, *fmr1-/-* mice do not have typical activity-dependent translational mechanisms that include differences in basal Kv4.2 protein levels, and hippocampal-dependent memory tasks, and are not as useful for these studies (Gross et al 2011; Lee et al 2011; Darnell and Richter 2012).

FMRP and S6K1 have previously been shown to be important in regulating hyperexcitability in the hippocampus (Hara et al 1998; Lenz and Avruch 2005; Fenton and Gout 2011; Lee et al 2011). Studies in this chapter reveal that not only is FMRP activity altered through S6K1-mediated phosphorylation in response to chronic ethanol exposure, but also that this increase in phosphorylation is necessary for ethanol-induced changes in translation. Blockade of FMRP phosphorylation also prevented alterations in mRNA trafficking and protein expression. In addition to differences protein translation, alterations in S6K1 and FMRP-mediated signaling pathways have been shown to alter spine morphology and actin polymerization (Calabrese et al 2006; Bongmba et al 2011; Bowling and Klann 2014). Experiments in the next chapter will address whether differences in spine morphology are linked to changes in protein expression and drinking behaviors.

Chapter 4

Ethanol-induced alterations in FMRP-mediated translation are accompanied by changes in spine morphology

Background and Significance

Data presented in the previous chapters focused on the role of ethanol-induced alterations in FMRP-mediated translation in the hippocampus of proteins associated with homeostatic changes at glutamatergic synapses. However, changes in protein expression are often accompanied by alterations in spine morphology and actin polymerization (Fukazawa et al 2003; Kasai et al 2003;

Lebeau et 2011; Chen et al 2014). Following chronic ethanol exposure, previous studies have observed an increase in actin clusters and enlargement of dendritic spines (Carpenter-Hyland and Chandler 2006; Zhou et al 2007; Brigman et al 2010). In a number of developmental disorders, dysregulation of translational mechanisms results in alterations in dendritic spine shape and distribution (Krueger and Bear 2011; Hoeffer et al 2012; De Rubeis et al 2013; Pathania et al 2014; Han et al 2015; Neuhofer et al 2015). In fragile X syndrome and *fmr1-/-* animals, loss of FMRP resulted in an increase in spine density that is largely due to an increase in stubby spines (Krueger and Bear 2011; Hoeffer et al 2012; Neuhofer et al 2015).

Several mechanisms may regulate spine development and actin dynamics in the hippocampus. Of interest to these studies is the WAVE complex protein termed cytoplasmic FMRP interacting protein 2 (Cyfip2). As a binding partner of FMRP as well as a member of an actin regulatory complex, Cyfip2 may serve as a link between local activity-dependent translational changes and alterations in spine morphology (Smith and Rong 2004; Zhao et al 2013; Abekhoukh and Bardoni 2014). Fragile X patients exhibit an increase in Cyfip2 expression in the hippocampus, and *fmr1-/-* animal models also show increased Cyfip2 expression that accompanies the increase in immature stubby spines (Castets et al 2004; Hoeffer et al 2012). Cyfip2 regulates actin polymerization through direct interaction with the WAVE complex and inhibits WAVE activity and F-actin formation (Smith and Rong 2004; Pilpel and Segal 2005; Zhao et al 2013; Chen et al 2014). As illustrated in **Figure 4-1**, the binding of GTP-bound Rac1 to Cyfip2

induces dissociation between WAVE and Cyfip2, allowing for disinhibition and an active WAVE complex (Castets et al 2005; Zhao et al 2013; Chen et al 2014).

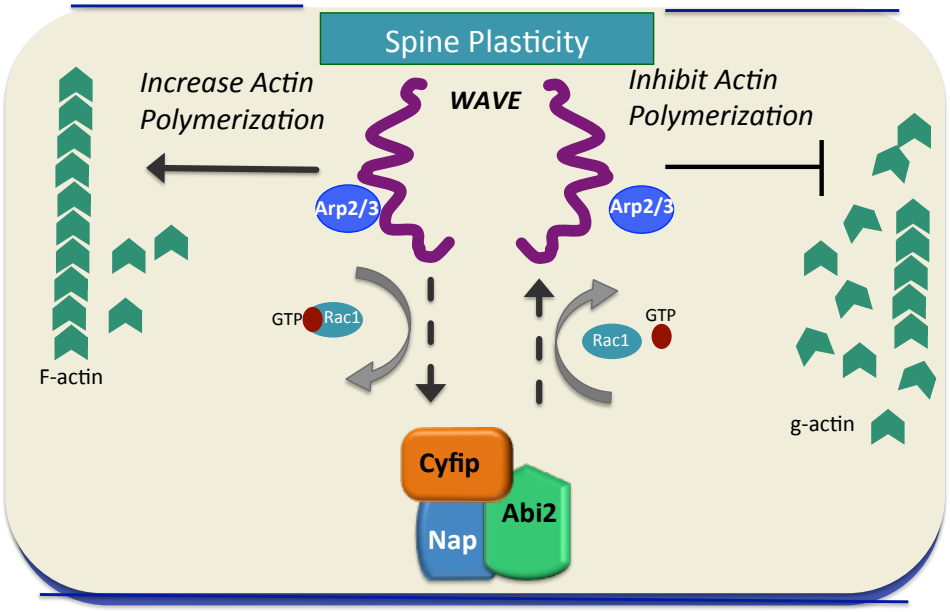

Figure 4-1 Cyfip2-mediated actin polymerization

Cyfip2 directly interacts with the WAVE complex to inhibit actin polymerization and spine formation. GTP-bound Rac1 causes disassociation between cyfip2 and WAVE to increase f-actin. The unbinding of Rac1 allows cyfip2 to bind WAVE and inhibit actin polymerization

Recent work by Kumar et al 2013 demonstrated a potential role for Cyfip2 in regulating the morphological and behavioral changes resulting from drug abuse. In this study, a SNP polymorphism in the Cyfip2 gene of two substrains of C57BL/6 mice were identified in which a serine found in the C57BL/6J mice was substituted with a phenylalanine in the C57BL/6N mice (Kumar et al 2013) It was further observed that this SNP is found in all commercially available mice of the

6N substrain **(Figure 4-2).** This polymorphism codes for an unstable form of Cyfip2, and it was hypothesized that this instability disrupts proper coordinated, activity-dependent changes in spine morphology. Additionally, 6N animals were shown to be resistant to acute cocaine-induced increases in locomotor sensitization, displayed fewer active lever presses, and did not show reinstatement. Spine analysis showed an overall decrease in density, and a highly significant decrease in long/thin spines in the nucleus accumbens (Kumar et al 2013).

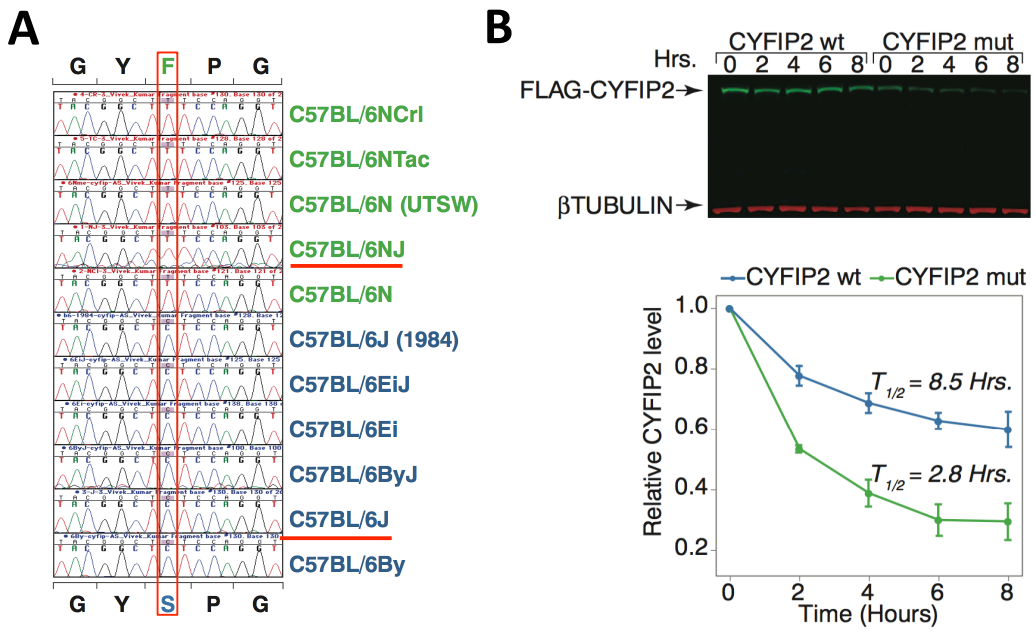

Figure 4-2 *Cyfip2* SNP change between different C57BL/6 mouse lines *from Kumar et al, 2014*

A. Single nucleotide substitution in the C57BL/6N mice compared to 6J. **B**. SNP does not alter total protein expression, but is less stable with a shorter half-life.

Studies presented in this chapter aim to discern whether ethanol-induced alterations in local protein translation are accompanied by changes in spines morphology. Using two commercially available substrains of C57BL/6J or /6NJ, these experiments may provide insight into how chronic ethanol alters both protein expression and spine morphology in an activity-dependent manner.

Methods

Animals

Animals used in these studies consisted of two different substrains of C57BL/6 mice: C57BL/6J and C57BL/6NJ. Both lines were obtained from Jackson Labs and will be referred to as 6J and 6N, respectively. Twenty-four male mice per genotype (48 animals total) arrived at 9 weeks of age, and were allowed to acclimate to the animal vivarium for 2 weeks. All animals were singly housed on a 12 hour light/dark cycle. Lights were off from 11am -11pm. Animals had access to food and water in the home cage throughout the entire experiment. Cages were changed and animals weighed weekly at least 48 hours after the end of the last 24-hour session. All experimental procedures were approved by the Animal Care and Use Committee and the National Institutes of Health.

Two-bottle choice and chronic intermittent ethanol vapor exposure

For the two-bottle choice ethanol drinking procedure, mice were divided into two treatment groups: a Naive group that received tap water in both bottles, and an EtOH group that received one bottle containing 15% ethanol and another bottle containing only tape water. Groups represented in the study are 6J Naive, 6N Naive, 6J EtOH, and 6N EtOH. Each bottle was assigned to a specific animal, and bottles were cleaned and refilled between each session, and all bottles were weighed before and after each session. Weight difference was converted to volume using the contributing density of ethanol. Volume was then converted to

grams per kilogram (g/kg). Mice gained and lost weight throughout both baseline drinking and chamber weeks, and the average of weights before and after each drinking week was used to calculate g/kg. Drinking sessions began 1 hour before the dark cycle, with bottle weights taken after 4 and 24 hours. After 4 consecutive 24-hour drinking sessions, bottles were removed, and all animals were provided home cage tap water for 72 hours.

Following four weeks of baseline two-bottle choice drinking, mice then began the chronic intermittent ethanol vapor exposure paradigm. A member of Dr. Howard Becker's lab kindly performed the vapor chamber exposure for these experiments. As illustrated in **Figure 4-3**, mice that received ethanol during the two-bottle choice drinking sessions underwent ethanol vapor exposure for 16 hours followed by an 8 hour withdrawal as described previously in Becker and Lopez 2004, Lopez and Becker 2005, and Griffin et al 2014.

The water only group received air exposure for the 16-hour period and remained ethanol naive. Both groups received IP injections of pyrazole (1mmol/kg) and an ethanol prime injection (1.6 g/kg; 8% w/v). This 16-hour vapor session was followed by an 8-hour withdrawal period, and animals continued this cycle for 4 consecutive days. This was followed by a 72-hour withdrawal period. Blood was taken via retro-orbital bleed on either the 3rd or 4th day of each of the cycles for determination of blood ethanol concentration (BECs). After each week of ethanol exposure and withdrawal, mice began two-bottle choice drinking for 4 days, and then another 72-hour withdrawal period. These two week cycles were repeated 4 consecutive times. Following the last day of two-bottle choice drinking

after the fourth cycle of vapor exposure, mice were euthanized with urethane (1.5g/kg) and were either perfused for spine analysis or brains were taken for western blot analysis.

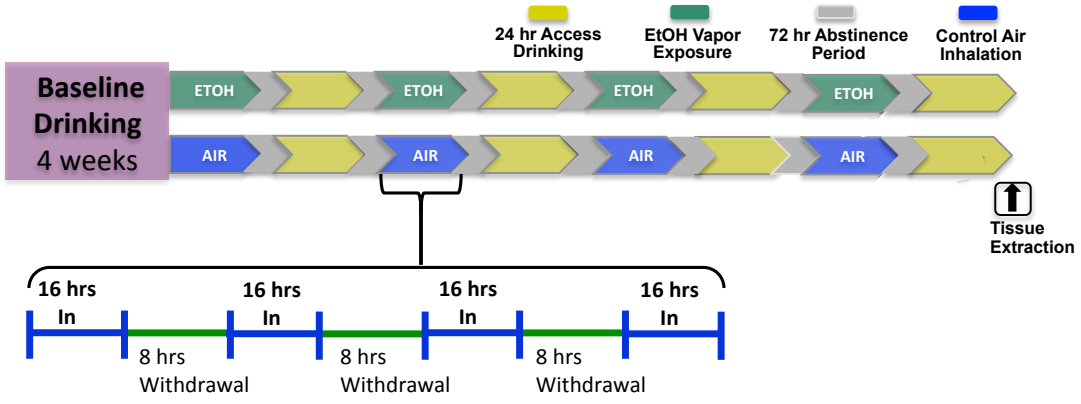

Figure 4-3 Two-Bottle Choice Chronic and Intermittent Ethanol Exposure Paradigm

Mice were divided into two groups: ethanol or water only (naive). Each group had 4 weeks of baseline two-bottle choice drinking, and then 4 weeks of chambers and bottles, alternating for a total of 8 weeks. Chamber exposure consisted of 16 hour-ethanol exposure and an 8 hour withdrawal period. Brains were extracted at the end of the 4th week of two-bottle choice.

Diolistic labeling and spine analysis

Diolistic labeling of slices obtained from fixed brains was used to assess the effects of CIE and active drinking on dendritic spine morphology in the hippocampus as previously described (Kroener et al. 2012). In brief, 6 mice from each of the 4 experimental groups were anesthetized and perfused with 0.1 M phosphate buffer followed by 1.5% paraformaldehyde (PFA) in phosphate buffer and post-fixed in 1.5% PFA for 1 hour at room temperature. Brains were then

kept at 4^{o}C in 0.1 M phosphate buffer until coronal sections of 150 μm were prepared on a vibratome. Tungsten particles (1.7 μm diameter) coated with DiI were delivered diolistically using a Helios Gene Gun (Bio-Rad) fitted with a polycarbonate filter (3.0 μm pore size; BD Biosciences). DiI was allowed to diffuse overnight at 4°C, and the slices were then post-fixed in 4% PFA for 1 hour prior to mounting. Images of the apical dendrites of the CA1 region of the hippocampus were collected in the Z-plane with a stack interval of 0.1 μm. A total of 8 z-stack images of 6-8 dendrites from 8 different cells per animal were collected. AutoQuant (MediaCybernetics, Rockville, MD) was then used to create deconvolved 3-D images. A filament of the dendritic shaft and spines was then created using Imaris XT (Bitplane, Zurich, Switzerland). Dendritic spines were classified as long, mushroom, stubby, or filopodia based on their length and neck and head width, where *L* is spine length, *WH* is spine head width, and *WN* is spine neck width. Long spines were identified as having a $L \geq 0.75$ μm and <3 μm, mushroom spines as a $L < 3.5$ μm, $WH > 0.35$ μm and a $WH > WN$, stubby spines had a $L < 0.75$ μm, and filopodia were identified as having a $L \geq 3$ μm.

Lysate preparation and protein assay

Six animals per group were euthanized and 1.0 mm coronal brains slices were prepared using a standard brain block. From these slices, 2.0 mm bilateral tissue punches of the hippocampus were homogenized by sonication in 4% LDS . Protein concentration was determined by the BCA procedure described in Chapter 2.

Western blot analysis

Western blots were performed as described in Chapter 2. Briefly, following electrophoresis and membrane transfer, a reversible Swift total protein stain was used to evaluate errors in loading and normalization. Following the total protein stain, membranes were blocked in 4% non-fat milk, and incubated in primary antibody at 4^{o}C overnight. Primary antibodies included phospho-S499 S499 FMRP (1:1000), FMRP total protein (1:2000), KChIP3 (1:500), Kv4.2 (1:1000), GluN1 (1:3000), and GluN2B (1:3000). After primary antibody incubation, membranes were incubated in secondary antibody: S499 FMRP, FMRP, and KChIP3 with goat anti-rabbit (1:2000) Kv4.2, GluN1, and GluN2B with goat anti-mouse (1:2000), for 1 hour at room temperature. After secondary antibody incubation, membranes were exposed to an enhanced chemillumiscience with ChemicDoc MP Imaging System (Bio-Rad, Hercules, CA).

Statistical Analysis

Two-bottle choice drinking data was analyzed using SPSS (IBM) with either a one- or two-way repeated measures ANOVA (* $p < 0.05$). Significance for animal weights was examined using student t-test, and the spine data were analyzed with SPSS using a general linear mixed model. Western blots were analyzed with a one-way ANOVA in GraphPad Prism.

Results

Blood Ethanol Concentrations (BECs)

Following baseline two-bottle choice drinking, mice were exposed to four cycles of vapor exposure along with a two-bottle choice 24-hour access paradigm between each week of vapor exposure. As seen in **Figure 4-4**, blood-ethanol levels steadily rose for both groups with each chamber cycle. While the reason for this increase is not clear, no significant differences were found between 6J and 6N animals throughout any cycle (one-way ANOVA, $F(3,66) = 0.2964$, $p > 0.05$ $n = 12$).

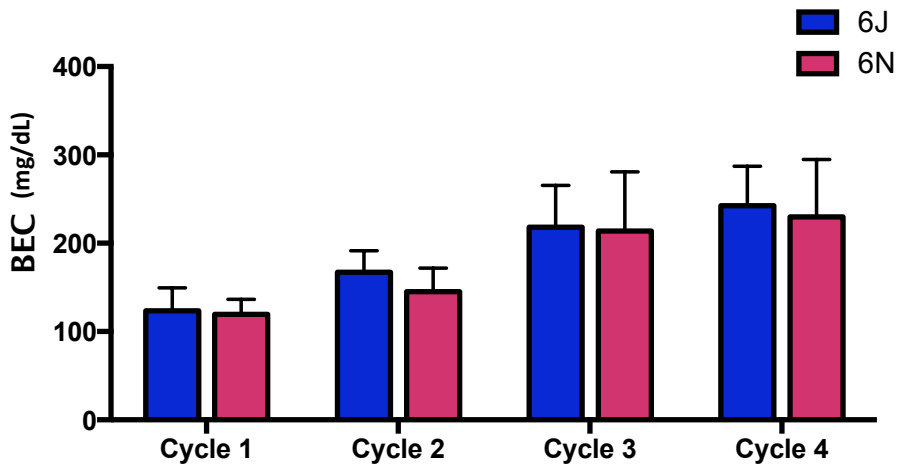

Figure 4-4 Average BEC measurements for each ethanol vapor exposure cycle.

Blood ethanol content (BEC) measurements taken during each vapor chamber cycle show no difference in BECs between 6J and 6N animals (*n*= 6 p >0.05).

Genotype	Cycle 1	Cycle 2	Cycle 3	Cycle 4
6J	123.5 ± 7.48	167.2 ± 7.00	218.3 ± 13.67	242.7 ± 12.83
6N	119.7 ± 4.86	145.3 ± 7.70	214.0 ± 19.29	229.7 ± 18.83

Table 4-1. BECs for each chamber cycle (average ± SEM)

Two-bottle choice 24-hour access

During two-bottle choice drinking, animals were divided into four groups with 6 animals per group as follows: 6J Naive- water only, 6J EtOH- ethanol and water, 6N Naive- water only, and 6N EtOH- ethanol and water. Mice were given free access to bottles for 24 hrs with time points taken after 4- and 24-hours during the two-bottle choice paradigm. Throughout the baseline period and most of the drinking days between ethanol vapor exposures, mice in the 6J EtOH

group exhibited significantly higher ethanol intake (g/kg) in a 24-hour period compared to the 6N EtOH group **(Figure 4-5A,** two-way ANOVA, $F(31, 682)= 1.928$ $p= 0.0020$, Sidak post-hoc, * $p< 0.05$, $n= 12, 11$**).** 6N and 6J groups also differed in their total daily water consumption. Not only did 6N mice consume less ethanol overall, they also consumed more water compared to 6J ethanol-drinking mice **(Figure 4-5B,** two-way ANOVA, $F(31, 682)= 2.811$ $p= 0.0016$, Sidak post-hoc, * $p< 0.05$, $n= 12, 11$**)**. Additionally, although 6N mice consumed less ethanol compared to the 6J group, both 6J and 6N mice exhibited an escalation in drinking following the final week of vapor exposure compared to their respective baseline levels **(Figure 4-5C**, two-way ANOVA; $F(4,15)=5.085$; $p= 0.0163$; Tukey post-hoc * $p< 0.05$, $n= 12, 11$**)**. Therefore, both genotypes showed CIE-induced escalation in ethanol consumption. However, these two sub-strains varied in baseline overall ethanol intake**.** Total water intake for the ethanol-naïve 6J and 6N mice did not significantly vary by genotype. **(Figure 4-6,** two-way ANOVA, $F(31, 682)= 1.286$ $p= 0.1390$, $n= 12$**).**

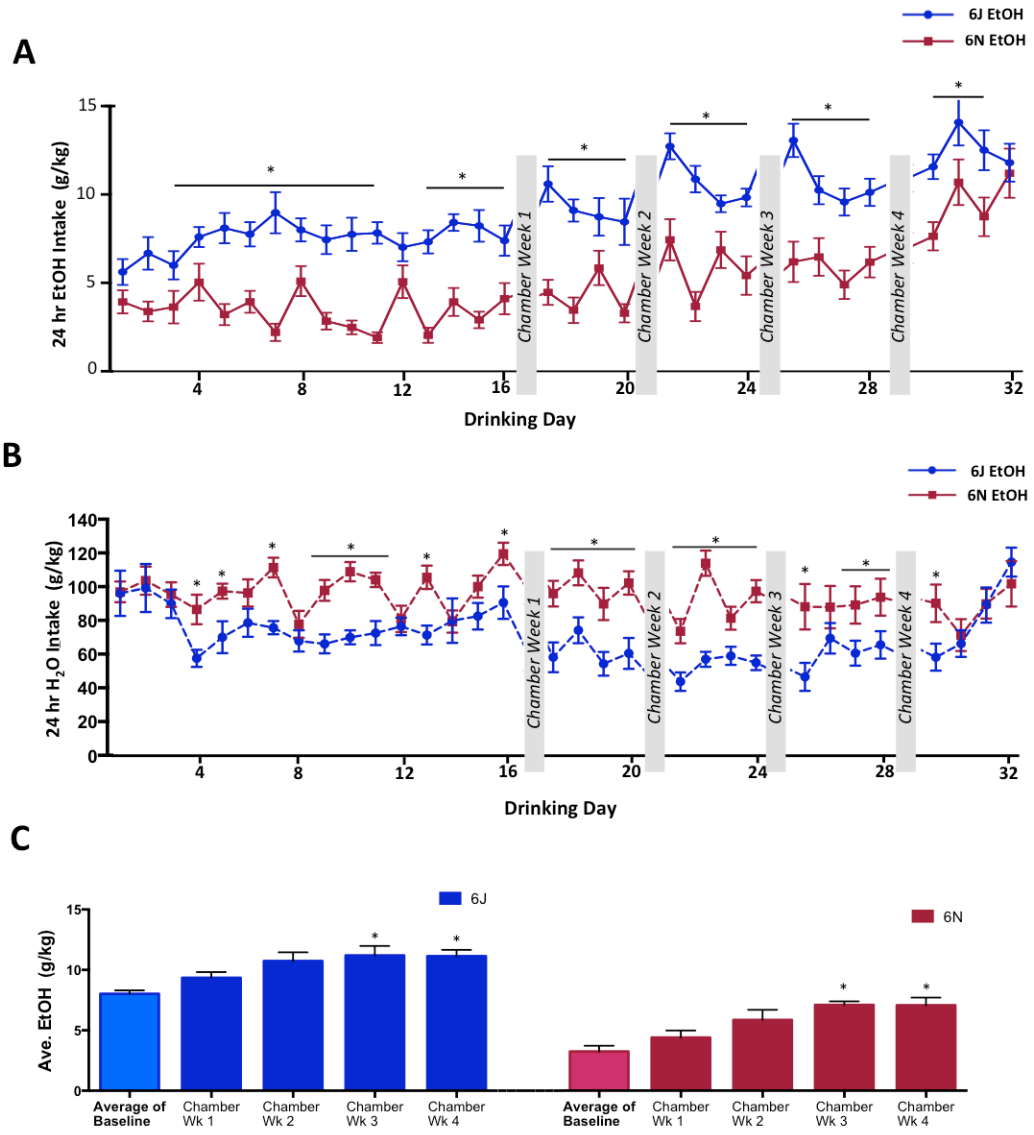

Figure 4-5 Total ethanol and water intake as well as escalation in drinking of the 6J and 6N groups.

A. During each 24-hour drinking period, 6J mice consumed more ethanol compared to 6N animals (*n*= 12, 11 *p< 0.05) **B.** The 6N group consumed more water than the 6J group for several of the 24- hour two-bottle choice drinking days. **C.** Although 6N mice consumed less ethanol than 6J mice, average weekly intake indicates an escalation in drinking following the third and fourth week of vapor chamber exposure compared to baseline for both genotypes (*n*= 12, 11 *p < 0.05; indicates significant difference compared to average of baseline within genotype).

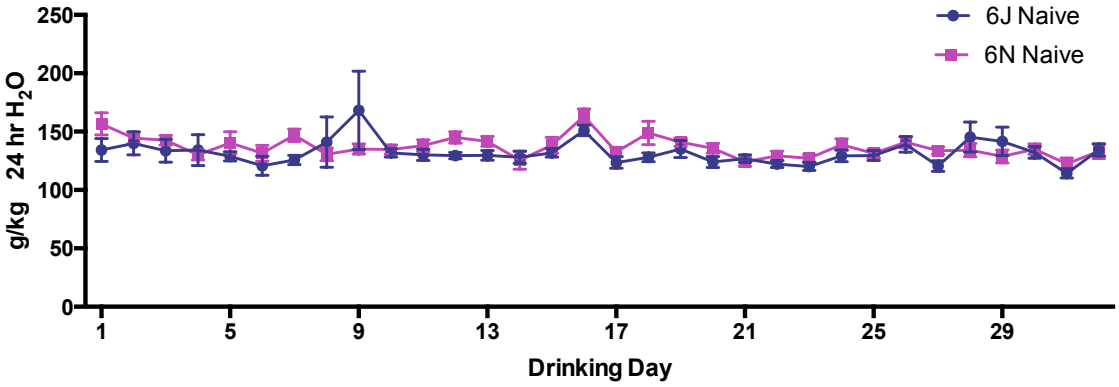

Figure 4-6 Daily water intake for 6J and 6N ethanol-naive mice

There was no significant difference was seen between 6J and 6N ethanol-naive, water only animals (*n*= 12, 11 p> 0.05).

The difference in water consumption between the 6J and 6N genotypes resulted in significantly lower total fluid intake over several 24-hour sessions for the 6J ethanol-drinking mice compared to 6N ethanol animals **(Figure 4-7A,** two-way ANOVA, $F(31, 682)= 3.040$ p= 0.0030, Sidak post-hoc, * p< 0.05, *n*= 12, 11**).** Interestingly during two-bottle choice drinking between chamber exposures as 6J mice increased their ethanol intake, their water consumption decreased**.** Ethanol also accounted for a higher percent of total intake throughout most of the two-bottle choice drinking paradigm **(Figure 4-7B/C**, *B:* two-way ANOVA, $F(31, 682)= 2.820$ p< 0.0001, Sidak post-hoc, * p< 0.05, *n*= 12, 11; *C*: two-way ANOVA, $F(31, 682)= 3.438$ p< 0.0001, Sidak post-hoc, * p< 0.05, *n*= 12, 11**)**. However, the 6N group maintained their water intake throughout the entirety of the paradigm**.**

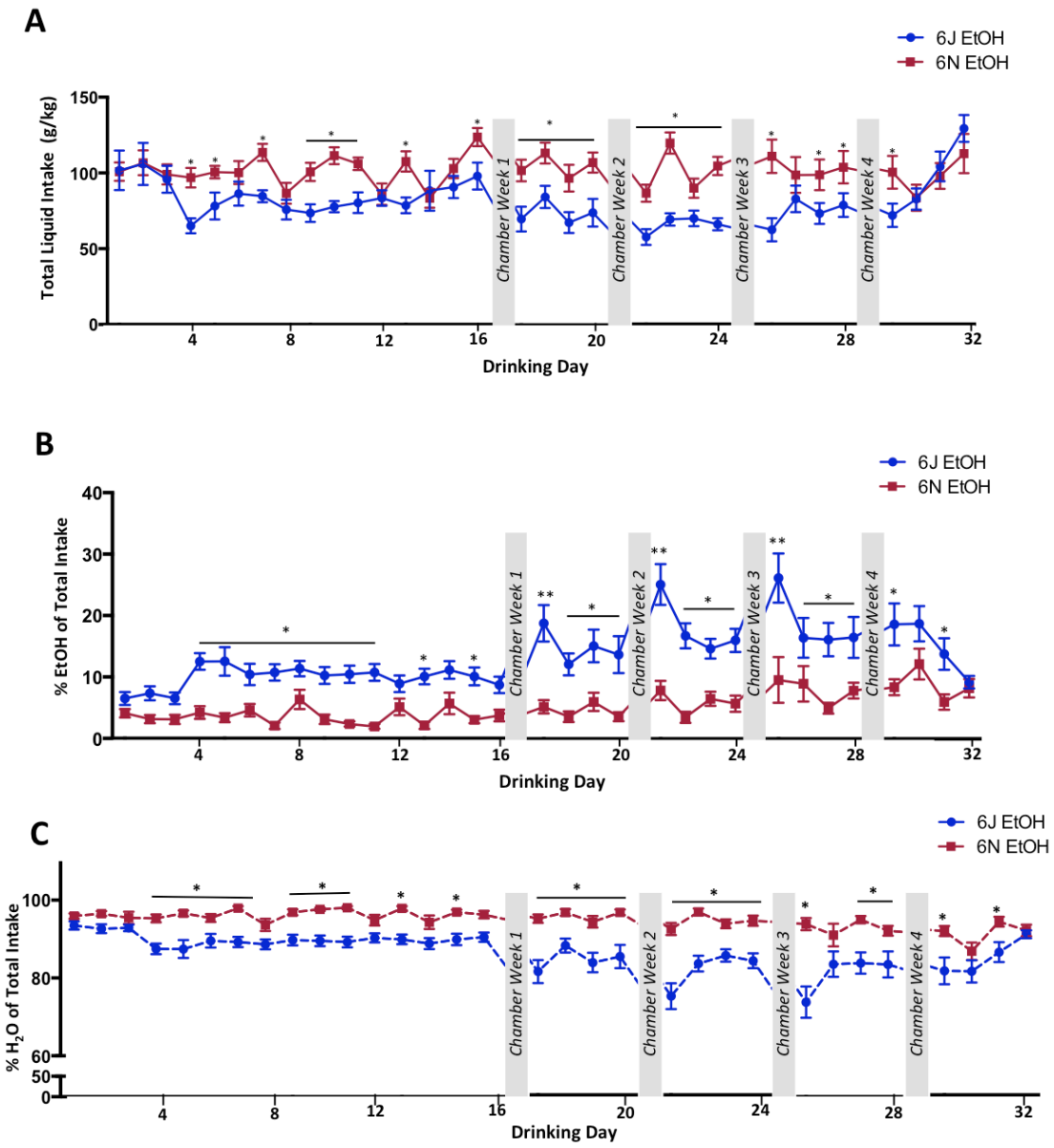

Figure 4-7 Total fluid intake and percent ethanol intake in 6J and 6N mice

A. Since 6N mice consumed more water in a 24 hour period on several days throughout the paradigm, their total fluid intake was higher for several of the two-bottle choice drinking days compared to the 6J group (*n*= 12, 11 * p< 0.05) **B.** 6J mice not only consumed more ethanol by weight, ethanol also accounted for a higher percentage of total liquid intake compared to 6N mice (*n*= 12, 11 * p< 0.05; ** p< 0.001).

Although 6J mice consumed more ethanol throughout most of the 24-hour drinking sessions, these studies also examined fluid consumption during the first four hours of each these 24-hour drinking days **(Figure 4-8**, *EtOH*: two-way ANOVA, $F(31, 682)= 2.123$ $p= 0.0023$, Sidak post-hoc, * $p< 0.05$, $n= 12, 11$; *Water:* two-way ANOVA, $F(31, 682)= 7.768$ $p< 0.0001$, Sidak post-hoc, * $p< 0.05$, $n= 12, 11$**)**. After 4 hours of two-bottle choice, the 6J group had significantly higher ethanol consumption for most of the baseline drinking phase compared to 6N mice. However, following chamber exposure periods, 6J and 6N animals maintained similar levels of ethanol intake for the first four hours on most drinking days. As expected, 6N animals had significantly higher water intake compared to 6J mice throughout the entirety of the paradigm. Additionally, following chamber exposure, as 6N mice increased their intake of ethanol, water consumption also increased, a trend that continued throughout the next 20 hours. In general, 6N mice consumed less ethanol and more water than 6J animals. However, unlike the four hours of drinking, there was no escalation in water consumption among the 6N group **(Figure 4-9**, *EtOH*: two-way ANOVA, $F(31, 682)= 2.144$ $p= 0.0004$, Sidak post-hoc, * $p< 0.05$, $n= 12, 11$; *Water:* two-way ANOVA, $F(31, 682)= 2.580$ $p< 0.0001$, Sidak post-hoc, * $p< 0.05$, $n= 12, 11$**)**. Ethanol consumption also remained relatively consistent throughout most of the paradigm for the last 20-hour period. As noted above, due to this increase in water consumption during the first four hours of the drinking sessions, total liquid intake increased in 6N

animals, especially during each bottle week following vapor chamber exposures. 6J ethanol mice, however, remained relatively consistent during this time period.

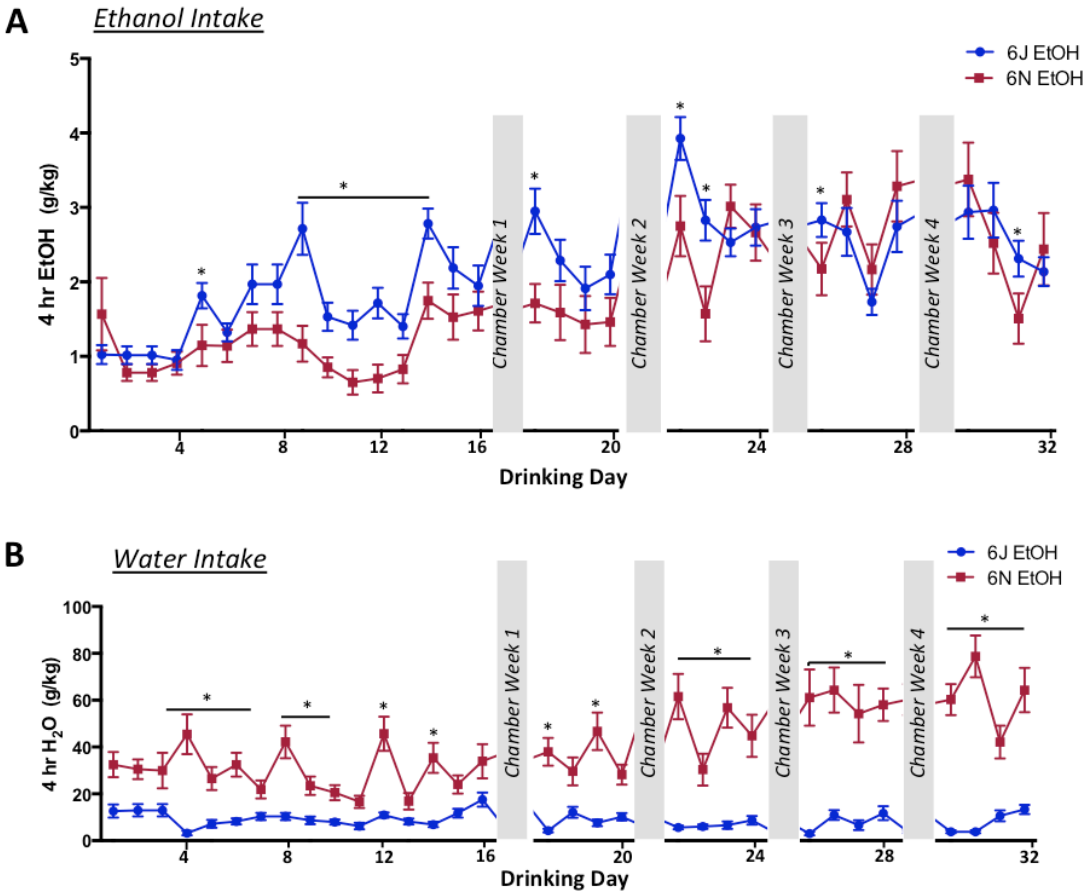

Figure 4-8 Daily ethanol and water consumption for the first 4 hours of each 24 hour session.

A 6J mice consumed more ethanol at each time point for most drinking days throughout baseline. However, the ethanol intake for the 6J groups was significantly increased in only half of the time points following chamber exposure (*n*= 12,11; * p< 0.05) **B.** Water consumption in the 6N group was significantly increased compared to 6J mice throughout the entire paradigm (*n*= 12,11; * p< 0.05).

Throughout two-bottle choice drinking and CIE vapor exposure cycles, body weight for all mice was recorded immediately before and after each cycle. It was observed that both 6N naive and 6N ethanol groups lost weight following each CIE vapor exposure, but gained weight during each two-bottle choice week. Unlike the 6N genotype, minimal fluctuation in weight was observed for the first three cycles of CIE vapor and two-bottle choice drinking in both 6J naive and 6J ethanol mice. Following the fourth and final CIE chamber cycle, however, a decrease of 4-5 grams was observed in both 6J and 6N ethanol mice. Mice in the 6J group had regained this weight at the time of sacrifice following the final two-bottle choice drinking period. However, 6N ethanol mice did not experience any weight gain at the time of sacrifice **(Figure 4-10)**.

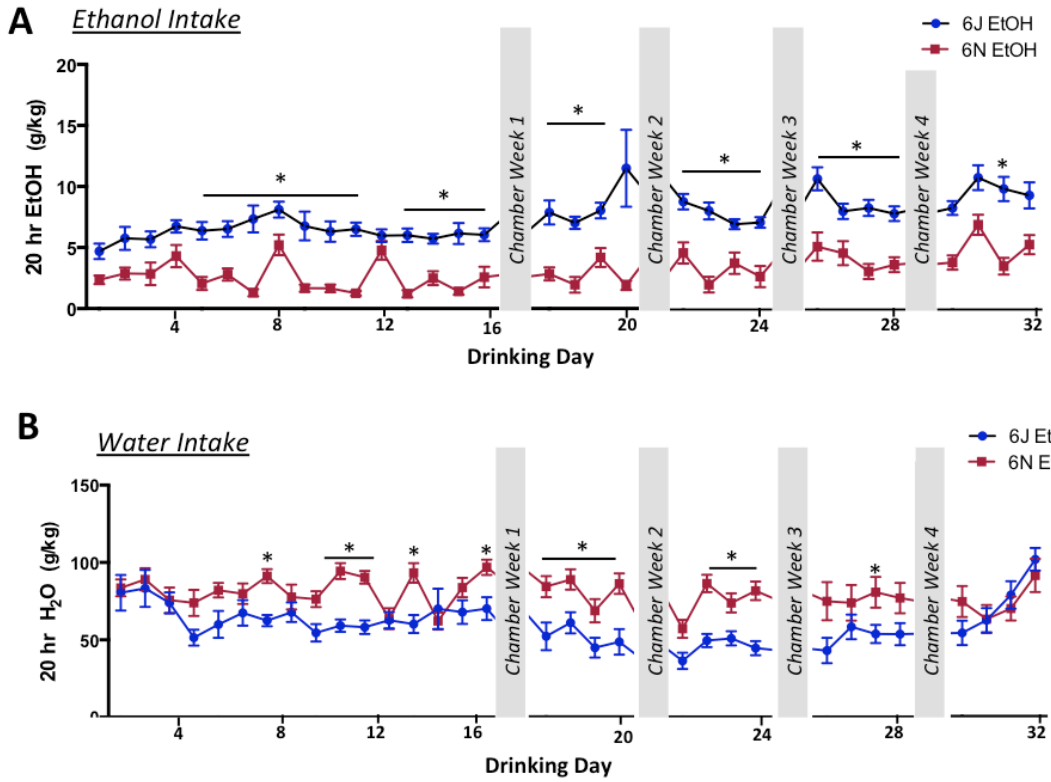

Figure 4-9 Daily ethanol and water consumption for the last 20 hours of each 24 hour session.

A. 6J animals consumed more ethanol throughout the entire 24 hour two-bottle choice paradigm, during both baseline and chamber drinking sessions. (*n*= 12, 11; * p< 0.05) **B.** The 6N ethanol group drank more water during the latter 20 hours for most of the baseline and chamber drinking weeks. (*n*= 12, 11; * p< 0.05)

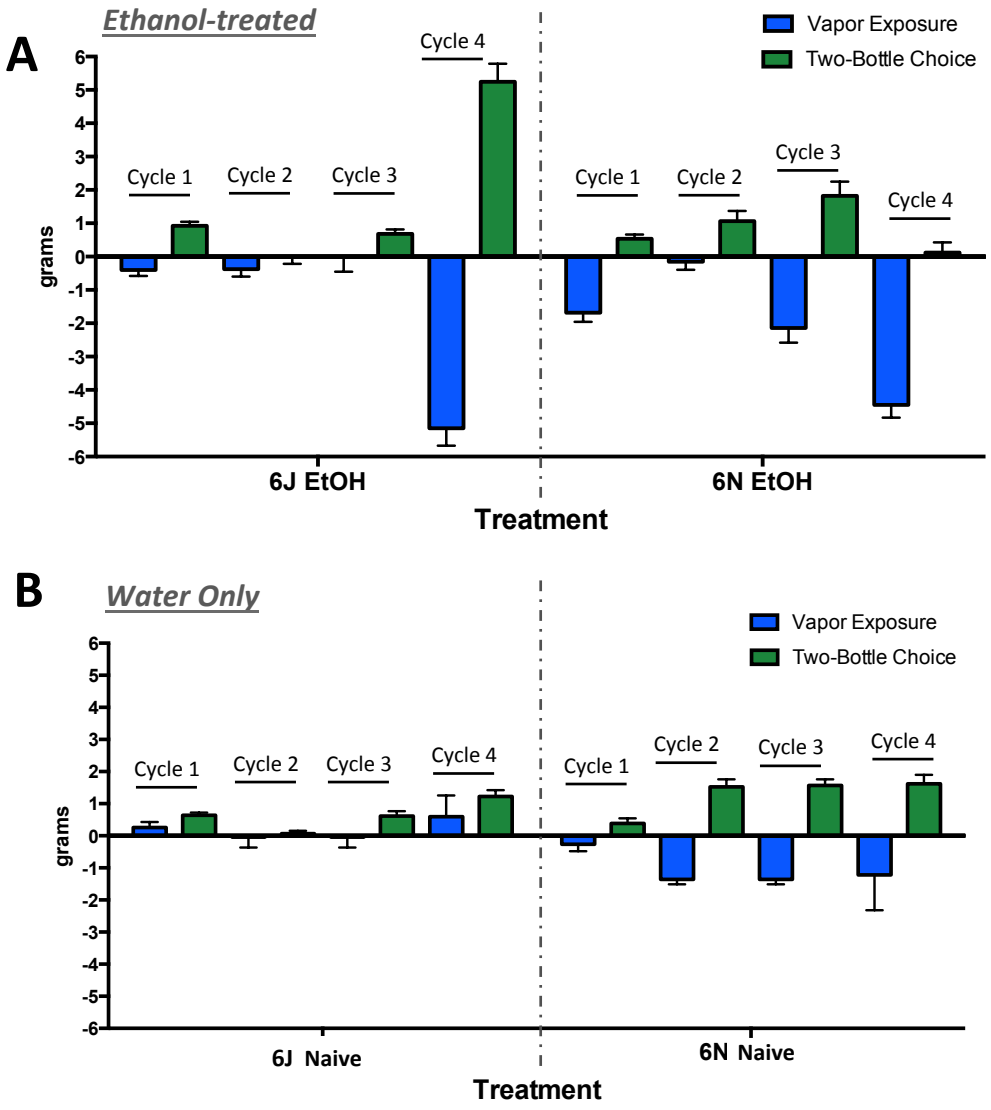

Figure 4-10 Weight differences by ethanol treatment in 6J and 6N mice.

Each bar represents the weight lost or gained during each week of either CIE vapor chamber exposure or two-bottle choice. Therefore, bars represent the change in weight due to either vapor exposure or two-bottle choice.

A. 6J ethanol-treated mice lost and gained small amounts of weight depending on the treatment. Vapor exposure was associated with small decreases in weight while two-bottle choice weeks caused small increases in the 6J ethanol group. However the final vapor exposure cycle induced a larger weight loss in both 6J and 6N ethanol-treated mice. 6J mice regained this weight by time of sacrifice, but 6N mice did not **B.** Weight of mice in the 6J naive group did not fluctuate during the paradigm. However, 6N mice did experiences alterations in weight depending on ethanol cycle.

Changes in protein expression in 6J and 6N mice following chronic ethanol exposure

Data presented in previous chapters of this dissertation have shown that chronic ethanol exposure alters expression of proteins associated with homeostatic plasticity in the hippocampus in both a CIE *in vivo* mouse model and *in vitro* hippocampal slice culture model. In the present chapter, hippocampal tissue from 6J and 6N naive and ethanol-exposed mice were analyzed using western blot for FMRP and other proteins of interest previously shown to be altered by chronic ethanol. Expression of the translational repressor protein FMRP was assessed for total protein and the previously identified phosphorylation site of interest (S499). Consistent with results from previous chapters, blots revealed an increase in phospho-FMRP protein expression in ethanol-exposed 6J mice compared to 6J naive animals. Additionally, the 6J ethanol group exhibited an increase in phosphorylated FMRP compared to both 6N treatment groups. Importantly, the 6N ethanol mice did not show an increase in expression of either phosphorylated or total FMRP protein expression compared to either the 6J or 6N naive groups. As seen in previous studies, total FMRP protein expression did not vary between any of the groups tested **(Figure 4-11**; *phospho-FMRP*: one-way ANOVA, $F(3,20) = 5.152$, $p=0.0084$, Tukey post-hoc * $p< 0.05$ $n= 6$; *FMRP*: one-way ANOVA, $F(3,20)= 0.1105$, $p=0.9528$, Tukey post-hoc $p> 0.05$ $n= 6$**)**. Of note, these data also show no difference between the

6J and 6N naive mice, indicating that the polymorphism in Cyfip2 does not lead to differences in basal levels of phosphorylated or total FMRP protein.

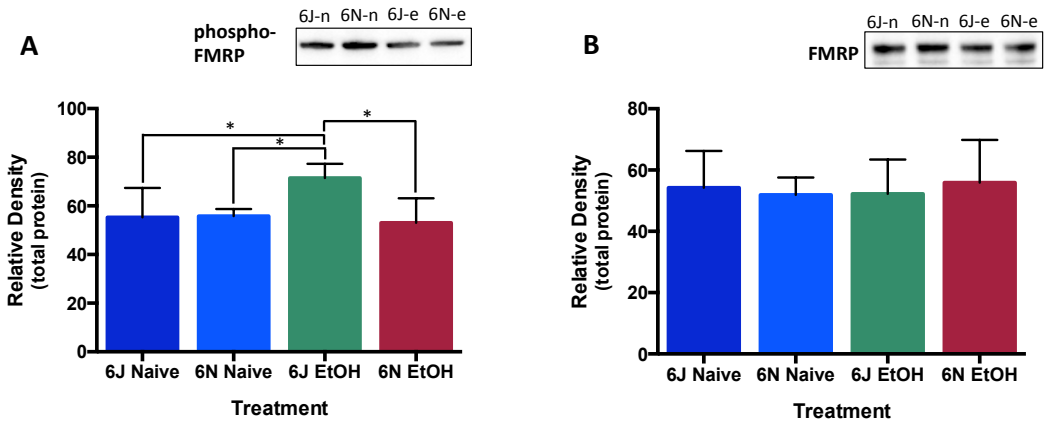

Figure 4-11 Ethanol-induced changes in FMRP phosphorylation and expression in 6J and 6N mice

A. Ethanol caused the expected increase in FMRP phosphorylation in 6J animals. This difference was significant compared to both ethanol and naive 6N groups (*n*= 6, * p< 0.05). **B.** Total FMRP protein expression did not change in either ethanol treated group (*n*= 6, p >0.05).

6J Naive= C57BL/6J water/air only, **6N Naive**= C57BL/6NJ water/air only, **6J EtOH** = C57BL/6J ethanol treated, **6N EtOH**= C57BL/6NJ ethanol treated.

In the next set of experiments, NMDA receptor subunits were evaluated in both genotypes. As observed in Chapter 2, 6J mice exposed to chronic ethanol displayed increased levels of GluN1 protein expression compared to 6J ethanol naive animals **(Figure 4-12**; *GluN1*: one-way ANOVA, F(3,19) = 6.733, p=0.034, Tukey post-hoc * p< 0.05 *n*= 5, 6; *GluN2B*: one-way ANOVA, F(3,20) = 5.844, p=0.0049, Tukey post-hoc * p< 0.05 *n*= 6**)**. Additionally, the 6J ethanol group also showed increases in GluN1 compared to 6N naive mice. There was a slight, non-signficant increase in GluN1 in the 6N ethanol group. GluN2B protein levels were increased with 6J ethanol mice compared to both the 6J and 6N naive group.

However, 6N ethanol-exposed mice did not show any increase in GluN2B levels compared to either control group. Genotypic differences between the two naive groups did not result in any change with either GluN1 or GluN2B protein levels. Like FMRP, this indicates there is no difference in basal expression of either protein.

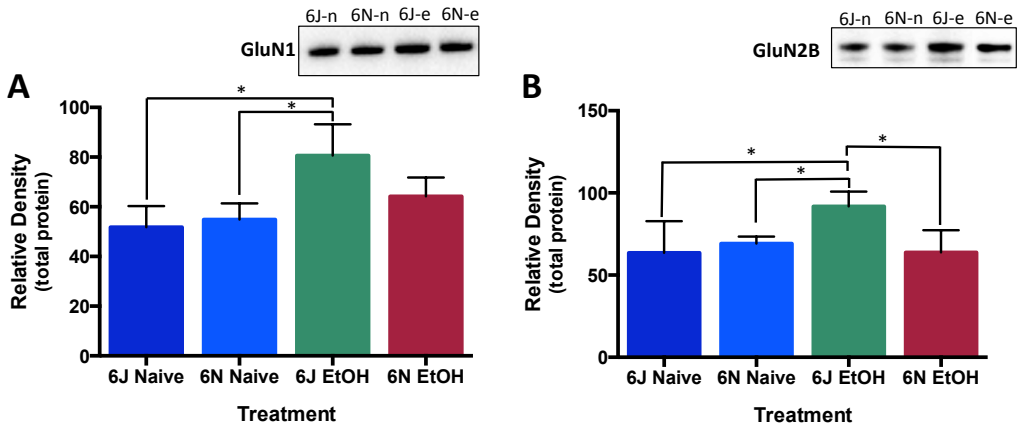

Figure 4-12 Increases in NMDA receptor subunits following ethanol exposure in 6J, but not 6N mice.

A. An exposure paradigm of two-bottle choice with alternating vapor chamber exposures induced the expected increases in 6J mice compared to both 6J and 6N ethanol naive animals (*n*= 6, * p< 0.05). **B.** Ethanol also increased GluN2B subunit expression in 6J animals compared to both ethanol naive groups. However, this was also significantly different from 6N ethanol exposed animals

6J Naive= C57BL/6J water/air only, **6N Naive**= C57BL/6NJ water/air only, **6J EtOH** = C57BL/6J ethanol treated, **6N EtOH**= C57BL/6NJ ethanol treated.

In addition to NMDA receptor subunits, Kv4.2 and KChIP3 protein levels were also assessed in both mouse lines **(Figure 4-13**; *Kv4.2*: one-way ANOVA, F(3,19) = 5.122, p=0.0104, Tukey post-hoc * p< 0.05 *n*= 6, 5; *KChIP3*: one-way ANOVA, F(3,20) = 6.248, p=0.0047, Tukey post-hoc * p< 0.05 *n*= 6**)**. This analysis revealed both Kv4.2 and KChIP3 expression was decreased as a result of ethanol exposure in the 6J mice compared to both 6N and 6J ethanol naive

mice. However, protein levels in 6N ethanol-treated mice remained unchanged compared to both naive groups. Data also show no change between 6J or 6N naive mice. These observations also indicate no baseline differences in expression of these proteins between the two genotypes for either protein.

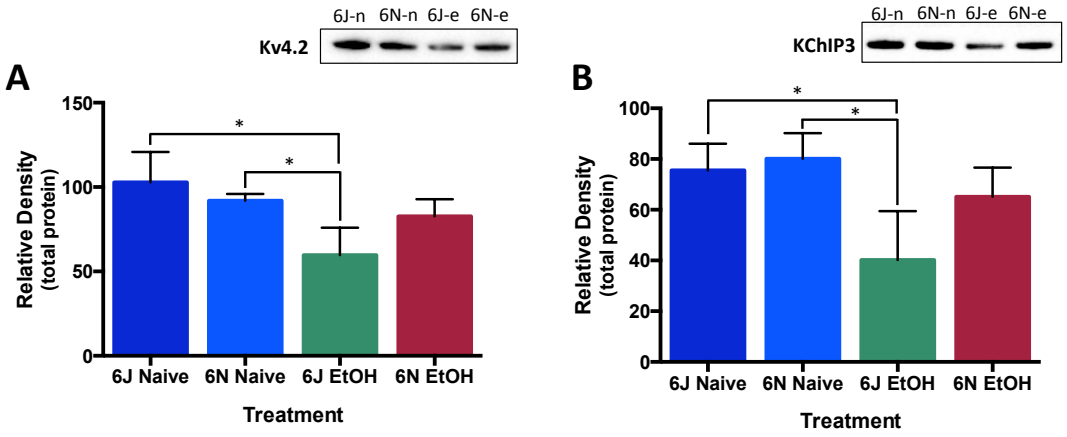

Figure 4-13 Alterations in Kv4.2 and KChIP3 protein expression following two-bottle choice and CIE

A. Repeated vapor chamber and two-bottle choice caused the expected decrease in Kv4.2 in 6J animals compared to ethanol naive 6J and 6N animals (*n*= 6, * p< 0.05). **B.** Ethanol also decreased KChIP3 protein expression compared to both water only groups (*n*= 6, * p< 0.05).

6J Naive= C57BL/6J water/air only, **6N Naive**= C57BL/6NJ water/air only, **6J EtOH** = C57BL/6J ethanol treated, **6N EtOH**= C57BL/6NJ ethanol treated.

Spine morphology in 6J and 6N naive and ethanol-treated mice

It has been previously reported that Cyfip2 function is associated with a reduction in overall spine density in the nucleus accumbens that was largely due to a decrease in thin and stubby spine density (Kumar et al 2013). This decrease in spine density was accompanied by a resistance to cocaine-induced locomotor sensitization and decreased cocaine-seeking behaviors. Previous studies have

also shown that ethanol-induced changes in actin polymerization result in enlargement of dendritic spines, and this enlargement may be dependent on changes in protein expression (Carpenter-Hyland and Chandler 2006; Zhou et al 2007; Cingolani and Goda 2008; Akashi et al 2009). Therefore, the next set of studies examined the effect of ethanol exposure in hippocampal dendritic spine density and morphology between the 6J and 6N mouse lines.

Quantification of the spine density in the CA1 region of the hippocampus yielded results similar to previous finding in the nucleus accumbens (Kumar et al 2013). 6N ethanol naive mice showed baseline differences in spine density compared to the 6J naive group. Exposure to chronic intermittent ethanol caused an increase in total spine density of 6J mice compared to both 6J and 6N naive groups as well as 6N ethanol-exposed animals **(Figure 4-14**; *Total spines*: $F(2,18)= 5.156$, * $p <0.05$, *n*= 6, 5; *Classification of spines*: $F(3,85)= 2.254$, * $p< 0.05$, *n*= 6, 5**)**. Classification of dendritic spines revealed that these alterations in total density were due specifically to changes in long/thin spines. Compared to 6J naive mice, the 6N naive group showed a significant decrease in long/thin spines, suggesting baseline differences in spine density and morphology between the two genotypes. Mice in the 6J ethanol group exhibited an increase in density compared to 6J naive mice, and this increase was also characterized by increased density of long/thin spines.

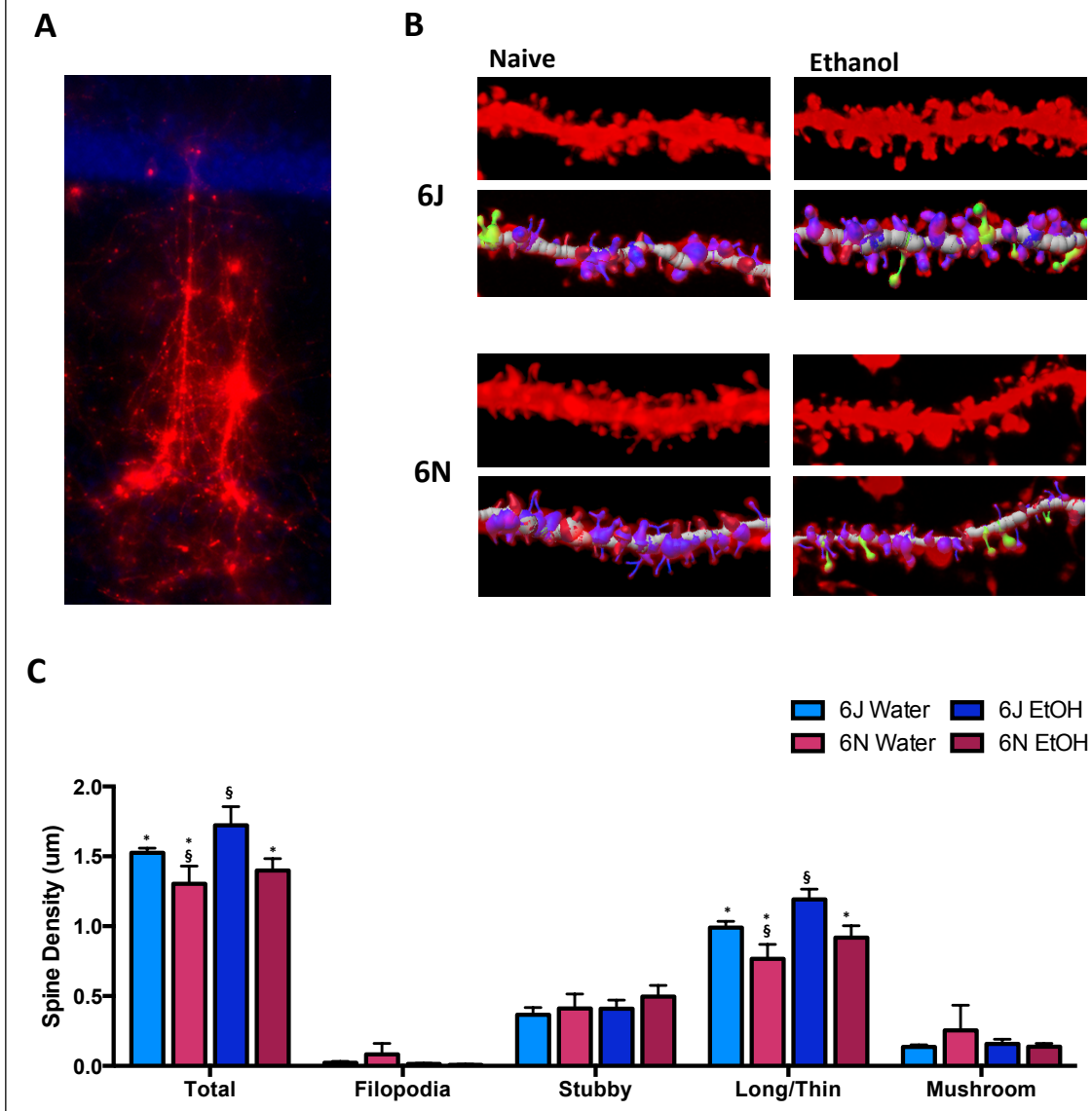

Figure 4-14 Ethanol-induced alterations in dendritic spines in 6J and 6N mice.

A. Representative image of DiI labeled CA1 pyramidal neuron in the hippocampus. Blue channel is a draq5 nuclear stain highlighting the cell body layer. **B.** Raw and analyzed representative images for 6J and 6N naive and ethanol-exposed animals. **C.** Total differences and distribution by spine type for each genotype and condition. Overall 6J ethanol animals showed an increase in density that is largely due to an increase in long/thing spines (n= 6,5 *p< 0.05 significance represents differences compared to 6J EtOH mice, § p< 0.05 significance compared to 6J Naive animals. A total of 6-8 dendrites were used per animal).

Discussion

The results of studies in this chapter revealed two important findings. The first that alterations in Cyfip2 function may correlate to a reduction ethanol consumption. However, the 6N mice carrying the phenylalanine SNP still exhibited CIE-induced escalation in drinking. Secondly, 6N animals do not show baseline differences in proteins implicated in synaptic plasticity, but do not induce mechanisms that allow for homeostatic changes in protein expression and spine morphology. Additionally, similar to previous results reported in the nucleus accumbens, 6N naive mice have baseline differences in spine density and morphology compared to the 6J strain in hippocampal CA1 pyramidal neurons (Kumar et al 2013)

These results further suggest that the polymorphism in the *cyfip2* gene that alters protein stability may have a significant influence over synaptic plasticity in not only spine morphology, but also protein translation and drinking behaviors. Not only did 6N ethanol-exposed animals drink less ethanol, they also consumed more water, and this water intake increased as ethanol consumption increased. Both 6J and 6N animals consumed more ethanol in the first four hour period than the expected daily intake, assuming a constant rate of consumption over 24 hours. 6N ethanol treated animals consumed more in the first four hours, indicating they drink more at the start of the dark cycle, but do not maintain this behavior throughout the 24-hour period. The drop off in consumption seen during the last 20 hours of the session is not likely a result of decreased ethanol

metabolism since BECs did not differ between the two genotypes following CIE exposure. Also, 6N animals did show escalation in drinking during two-bottle choice weeks following the final two weeks of chamber exposure, but did not escalate to the levels achieved by 6J mice. Of note, the 6J animals drinking levels were relatively even throughout the first four hours during both baseline and chamber periods, suggesting that the escalation in drinking did not occur during the first part of the dark cycle, but 6N animals displayed the opposite behavior, increasing ethanol consumption in the first 4 hours.

As with previous chapters, differences in protein expression was also evaluated in naive and ethanol-exposed animals. In mice, decreased Cyfip2 total protein expression has been shown to cause a reduction in basal levels of FMRP total protein as well as cognitive deficits similar to *fmr1-/-*. However, 6N naive mice that show decreased Cyfip2 activity did not exhibit baseline differences in FMRP phosphorylation or total expression, nor did they display any alterations in GluN1, GluN2B, Kv4.2,or KChIP3 expression compared to 6J naive mice. Additionally, ethanol treatment in the 6N group did not induce changes in protein expression. However, the expected increases or decrease in GluN1 and GluN2B or Kv4.2 and KChIP3, respectively, were observed in 6J ethanol mice compared to 6J naive. This suggests that differences in Cyfip2 activity do not affect basal protein expression, but may have an important role in activity-dependent changes in FMRP activity and protein translation. However, further experiments are necessary to investigate the mechanism by which chronic ethanol mediates both Cyfip2 and FMRP in response to alterations in homeostatic plasticity.

The studies investigating structural differences in dendritic spines revealed that 6N mice exhibit a lower density of spines in the hippocampus compared to 6J mice. Analysis of spine type indicated this difference was largely due to a decrease in the density of long/thin spines. This reduction in total density and thin spines is similar to results from studies in the nucleus accumbens (Kumar et al 2013). Ethanol did cause a slight difference in 6N mice, but this did not vary significant from either 6J or 6N naive mice. Again, this difference is due to an alteration in long, thin spines. However, baseline differences in spine density and morphology in the 6N naive mice were not accompanied by alterations in basal protein levels. The 6N group was resistant to ethanol-induced changes in protein expression and spine morphology. This suggests that changes protein translation and actin polymerization that occur in tandem may be limited to activity-dependent plasticity, and that basal protein translation and actin dynamics may be differentially regulated.

Drinking studies in this chapter indicate 6N mice with the polymorphism in *cyifp2* consumed less ethanol that 6J mice. Although 6N mice did escalate their ethanol intake compared to baseline, consumption in the 6N ethanol group was still significantly less than 6J animals for several 24-hour time points. Studies with cocaine with a knock-in of the 6N SNP on a 6J background produced the same cellular and behavioral phenotype as the commercially available 6N mouse lines, suggesting that Cyfip2 activity has an important role in drug-induced behaviors. However, further studies are needed to determine how the polymorphism found

in 6N animals behavioral differences seen in response to chronic ethanol exposure.

A potential confound in these chronic intermittent ethanol studies was the observation that weight in both groups of 6N mice fluctuated throughout each cycle of CIE vapor and two-bottle choice following the first four weeks of baseline drinking. It is important to note that during the fourth and final chamber exposure, both animals experienced higher than expected blood ethanol levels. Following this vapor exposure cycle, mice in both 6J and 6N ethanol groups lost an average of 4-5 grams in weight. The 6J mice gained all of the lost weight back by the time of sacrifice; 6N mice, however, did not. This may have important implications for actin dynamics and spine morphology. Alterations in body weight are associated with either an increase or decrease in spine density, depending on brain region (Stranahan et al 2008; Stranahan et al 2009; Fan et al 2015). A reduction in spine density was observed in the 6N naive group. However, these data are consistent with previously published studies, showing this same decrease in the nucleus accumbens. 6N ethanol-treated mice showed a slight increase in spine density compared to the 6N naive group. It is difficult to speculate whether this sudden change in body weight contributed to the behavioral and cellular phenotype.

Results presented in this chapter reveal the importance of homeostatic mechanism in dendrites and dendritic spines for regulating protein expression, spine morphology, and drinking behaviors. With the polymorphism in Cyfip2 in 6N animals, failure to induce appropriate mechanisms to maintain the necessary

activity-dependent changes in protein expression and actin polymerization in order to adapt to chronic ethanol exposure produced not only differences in baseline drinking, but also changes in escalation. Although there is no baseline difference in protein expression, as seen with spine density, this indicates that basal state protein expression may be regulated in a different manner. However, activity-dependent mechanisms may require the ability to change both protein expression and actin polymerization to reestablish homeostasis.

Studies in this chapter addressed whether changes in protein expression are also accompanied by changes spine morphology. The naturally occuring polymorphism in the *cyfip2* gene between the -J and -N substrains of C57BL/6 provides the opportunity to address how differences in spine morphology may affect both baseline and ethanol-induced activity-dependent dendritic protein synthesis. Further studies are needed to determine the nature of the connection between protein translation and spine morphology, and how these two processes may alter drinking behaviors.

Chapter 5

Discussion and Future Directions

Translational Changes and Plasticity

Synaptic plasticity is an essential process that allows for homeostatic neuronal adaptations in response to changes in the cellular environment in order to maintain balanced homeostatic function. Plasticity in the hippocampus is necessary for alterations in synaptic activity that have important implications for hippocampal function, including memory processing and consolidation

(Grosshans et al 2001; Fukazawa et al 2003; Hoeffer et al 2013; Bailey et al 2015). Long-term maintenance of these adaptations requires alterations in protein homeostasis and translation of new proteins (Kang et al 1996; Klann and Richter 2007; Im et al 2009; Panja et al 2014). Cellular processes previously investigated that regulate activity-dependent protein synthesis have focused on transcription and somatic translation. However, more recent studies have demonstrated that maintenance of synaptic plasticity also requires alterations in local dendritic translation (Kang et al 1996; Klann and Richter 2007; Liu-Yesucevitz et al 2011). Activity-dependent *de novo* protein synthesis requires a series of highly coordinated synaptic events that are tightly regulated at each step. Pathologies, such as fragile X syndrome, that are characterized by aberrant translational regulation leads to alterations in basal protein levels and deficits in maintaining synaptic plasticity (Li et al 2001; Hagerman and Stafstorm 2009; Darnell et al 2011; De Rubeis and Bagni 2011; Henry 2011). This insufficient regulation of translation is accompanied by cognitive deficits and developmental delays, and the investigation of the how these components are precisely regulated remains an active area of research.

Previous studies have identified the mTORC1 translational pathway as one of the main cellular mechanism regulating activity-dependent translation in dendrites. The sensitivity of synaptic plasticity and memory consolidation to inhibition of mTORC1 implies that coordinated activity of mTORC1 substrates is likely necessary for maintenance of homeostasis (Gong et al 2006; Bekinschtein et al 2007; Meng et al 2013; Russo et al 2013; Takei and Hiroyki 2014; Dibble

and Cantley 2015). In response to chronic ethanol exposure, studies with *in vivo* rat models revealed an increase in several downstream components as well as alterations in protein expression at glutamatergic synapses and in dendritic spines (Nesta et al 2010; Barack et al 2013; Nesta et al 2014). Components of this pathway include kinases, mRNA-binding proteins, and ribosomal subunits that control mRNA trafficking and stability, assembly of the active ribosome, and the rate of translation. Different substrates in this pathway are discretely activated to fine-tune protein synthesis that is necessary for alterations in synaptic activity (Ma and Blenis 2009; Urbanska et al 2012; Meng et al 2013; Takei and Hiroyki 2014). However, how the different proteins in the mTORC1 pathway are tightly regulated is not known.

Studies examining mTORC1 signaling following chronic ethanol exposure have demonstrated that this signaling pathway and its substrates have an important role in maintaining ethanol-induced alterations in protein expression (Nesta et al 2010; Barak et al 2013; Sabino et al 2013). Studies in this dissertation revealed that ethanol-induced alterations in expression of proteins that are key mediators of neuronal excitability in dendrites and dendritic spines may also be regulated by mTORC1 pathway. Results in these experiments from both *in vivo* and *in vitro* studies show that chronic exposure to ethanol causes an increase in GluN1 and GluN2B subunit expression in the hippocampus but a reduction in Kv4.2 and KChIP3 protein levels. Together, these changes in protein expression may tip the equilibrium in cellular signaling toward excitability to reestablish neuronal homeostasis during chronic ethanol exposure. These

studies also provide evidence that FMRP, a downstream substrate in the mTORC1 pathway, may be an important mediator of alterations in NMDA receptor, Kv4.2, and KChIP3 protein expression. As an mRNA-binding protein, FMRP interacts with a variety of different mRNAs to inhibit their translation (Laggerbauer et al 2001; Jones 2003). Although FMRP is a translational repressor, its activity is necessary for maintenance of basal protein synthesis and activity-dependent translation in dendrites and dendritic spines (Zalfa et al 2006; Henry 2011; Schaeffer et al 2012). In the absence of FMRP, local dendritic translation is uncoordinated, leading to disorganization of translation with no *de novo* synthesis of the specific proteins that are required to maintained synaptic plasticity.

FMRP and Ethanol Exposure

Ethanol-induced changes in FMRP and its binding partners

Studies investigating alterations in synaptic plasticity in response to ethanol exposure have identified a role for protein translation in maintaining neuronal homeostasis (Nesta et al 2011; Barak et al 2013). Studies presented here revealed that not only does chronic ethanol exposure induce alterations in proteins that modulate synaptic activity, but they also provide insight into how ethanol may induce these changes in protein levels. Results from Chapter 2 demonstrated an increase in FMRP phosphorylation following exposure to acute

and chronic ethanol with no change in expression. Additional experiments also showed alterations in FMRP-mRNA interactions in ethanol-treated slice cultures compared to control cultures. Studies in Chapter 3 then demonstrate that blockade of ethanol-induced increases in FMRP phosphorylation through inhibition of S6K1 activity prevent alterations in GluN2B, Kv4.2, and KChIP3 in response to chronic ethanol exposure. These observations suggest that ethanol exposure not only increases FMRP activity, but also alters FMRP-mRNA interactions.

Findings presented in Chapters 2 and 3 provide support to the suggestion that FMRP is an essential component of synaptic homeostasis during ethanol exposure, and that FMRP is necessary for coordinated activity-dependent translation in response to ethanol exposure. Previous studies have focused on mTORC1 as a global regulator of activity-dependent translation (Gong et al 2006; Nesta et al 2010; Barak et al 2013; Brewster et al 2013). In contrast, this dissertation provides insight into the role of a specific mTORC1 substrate in discrete regulation of proteins altered by chronic ethanol exposure.

It is important to note that inhibition of FMRP activity prevented ethanol-induced increases in GluN2B, but not GluN1 protein expression. This suggests that FMRP activity does not have a critical role in regulating overall increases in NMDA receptor protein expression. Rather FMRP may indirectly influence NMDA receptor function or localization through regulation of receptor subunit composition.

Another implication of these FMRP experiments is that these studies focused on FMRP in a typically developing brain. Research examining the role of FMRP in synaptic plasticity often utilize clinical populations with aberrant FMRP function, as seen in fragile X syndrome, or mouse models that lack the FMRP protein (Gross et al 2011; Lee et al 2011; Jeon et al 2012; Hoeffer et al 2013. Although these types of studies have provide important insight into the irregularities in cellular structure and function related to the loss of FMRP, there is little research in model systems with typical FMRP protein expression. Experiments in this dissertation focused on the role of FMRP in activity-dependent protein synthesis when FMRP expression is regulated via normal cellular processes. Results from these studies may contribute to the understanding of basic FMRP function in dendritic translation.

S6K1-mediated FMRP activity during chronic ethanol exposure

FMRP is part of a larger mTORC1 signaling pathway that mediates activity-dependent translation (Holz et al 2005; Ma and Blenis 2009; Russo et al 2013). This pathway includes numerous downstream kinases that activate different components for proper coordination of translation. *In vitro* studies in Chapter 3 demonstrated that both increased FMRP phosphorylation at S499 and alterations in FMRP-mRNA binding are regulated by S6K1 activity. Experiments in a rat model of chronic exposure also demonstrated an increase in S6K1 phosphorylation as well as an increase in protein expression for other mRNAs

that are downstream substrates of FMRP (Nesta et al 2010; Barak et al 2013; Nesta et al 2014). Importantly, decreased S6K1 activity *in vivo* has been shown to prevent ethanol-seeking behaviors and ethanol-induced increases in protein expression (Barak et al 2013). Taken together, these results suggest that FMRP phosphorylation and activity are likely mediated by S6K1 during ethanol exposure.

FMRP contains multiple binding sites for mRNAs and regulatory proteins, and its activity can be regulated through different cellular pathways. S6K1-mediated phosphorylation of S499 on FMRP provides important insight into how FMRP activity is regulated during ethanol exposure (Bardoni et al 1997; Brown et al 1995; Jones 2003; Bartley et al 2014). Phosphorylation at each of its three serine residues differentially regulates activity to induce binding to 4E-BPs and the ribosome to suppress translation, interaction with mRNAs, or interaction with miRNAs that inhibit FMRP function (Bardoni et al 1997; Li et al 2001; Jones 2003; Chen and Joseph 2015). Regulation of FMRP through S6K1 suggests a potential mechanism by which neurons maintain protein homeostasis during chronic ethanol exposure.

Spine morphology and activity-dependent translation

Regulation of WAVE through Cyfip2 activity

Long-term maintenance of protein homeostasis in dendrites is often accompanied by alterations in actin polymerization and spine morphology. As with protein translation, actin polymerization is a dynamic process that involves several discretely coordinated components. Regulation of F-actin formation through the WAVE complex is mediated through Cyfip2. Interaction between Cyfip2 and WAVE prevents actin polymerization, while Rac1-dependent activation of Cyfip2 induces disassociation from the complex (Miki et al 1998; Zhao et al 2003; Bongmba et al 2011; Bellot et al 2014). This disassociation of Cyfip2 from WAVE allows for disinhibition of the complex and active actin polymerization (Smith and Rong 2004; Pilpel et al 2005; Kim et al 2006). Decreased expression of Cyfip2 leads to dysregulated actin dynamics and an increase in immature dendritic spines (Hoeffer et al 2013; Bellot et al 2014). Additionally, this decrease in Cyfip2 is also correlated with an alteration in basal protein levels (Napoli et al 2008; Panthania et al 2014; Han et al 2015). Likewise, individuals with fragile X syndrome who lack FMRP and exhibit aberrant basal and activity-dependent protein expression, also have increases in Cyfip2 (Hoeffer et al 2013). This presents the possibility that chronic ethanol-induced alterations in protein expression may be connected to ethanol-induced differences in spine

morphology, and that FMRP and Cyfip2 both have an essential role in this process.

As shown in Chapter 4, C57BL/6N mice (6N) with a polymorphism substituting a phenylalanine in place of a serine residue in the *cyfip2* gene exhibited a decrease in spine density characterized by a decrease in long/thin spines compared to C57BL/6J mice (6J). Exposure to chronic ethanol resulted in an increase in spine density in 6J mice but not 6N mice. Of note, this SNP does not affect the levels of Cyfip2 protein, but rather it decreases the half-life of the protein and likely an increase in Cyfip2 turnover (Kumar et al 2013). This increase in Cyfip2 turnover may interfere with discrete regulation of the WAVE complex. While Cyfip2 is an inhibitor of WAVE activity, this inhibition may be necessary to properly coordinate the different components needed for adaptive cytoskeletal remodeling. Without this coordination, actin dynamics may remain unregulated and unresponsive to alterations in synaptic activity.

Results from experiments in Chapter 4 suggest a connection between the regulation of spine morphology and activity-dependent protein translation. Although 6N mice had a reduced spine density, there was no difference in basal protein levels compared to the 6J mice. However, unlike 6J mice, the 6N group did not display ethanol-induced alterations in protein expression or FMRP phosphorylation. One potential interpretation of these data are that protein expression and spine morphology may be differentially regulated under basal conditions, but both processes must occur in tandem to develop sustained homeostatic adaptations to alterations in neuronal activity.

Importance of Cyfip2 and FMRP during ethanol consumption

Alterations in both protein expression and spine morphology in response to chronic ethanol exposure suggest that both of these processes are important for maintaining neuronal homeostasis in response to changes in synaptic plasticity. Data presented in Chapter 4 indicates that for activity-dependent adaptations to occur, both changes in spine morphology and protein synthesis are necessary. Results from these studies and others indicate that inhibition of either of these two mechanisms prevents ethanol-induced cellular changes. Additionally, these cellular changes may also influence behavioral phenotypes. As shown in Chapter 4, 6N mice consumed less ethanol compared to 6J mice in a long-term CIE/two-bottle choice drinking paradigm. Although other factors may contribute to this difference in consumption, when these drinking data are considered with activity-dependent differences in protein expression and spine morphology, these data suggest that Cyfip2 function may have an important role in the development of drinking behaviors. These results also present the idea that the ability of neurons to induce specific homeostatic mechanisms is directly connected to the development of ethanol drinking and dependence. If 6N mice cannot initiate the appropriate mechanism to adapt to ethanol-induced alterations in homeostasis, does this contribute to the decreased ethanol intake? Conversely, are 6J mice more susceptible to acquiring high ethanol drinking behaviors because they can induce this specific homeostatic mechanism?

Future Directions

This project outlines some important findings regarding how ethanol may induce alterations in protein expression and spine morphology. Future studies to further investigate how homeostatic protein translation is mediated in ethanol exposure are needed. Results in Chapter 2 indicate that although protein expression in dendrites did not change after acute ethanol exposure, FMRP phosphorylation was increased after only 24 hours of ethanol treatment. Follow-up experiments could focus on FMRP activity during this period. FMRP can regulate translation through several different means including interaction with the active ribosome or other mRNAs that encode proteins needed for short-term adaptations to ethanol. As a key mediator of 5' TOP mRNAs, alterations in FMRP activity may also be important for translation of proteins that make up the active ribosome along with other mRNA binding proteins and key translational regulators (Jefferies et al 1997; Darnell et al 2013). As a result of this increase in phosphorylation, FMRP may have a significant role in mediating the transition in neuronal homeostasis that occurs during the shift from acute to chronic ethanol exposure.

An important direction for these future studies could focus on the questions presented above. For example, are specific cellular homeostatic mechanisms directly connected to drinking behavior, and how might activity-dependent protein translation and actin dynamics be regulated? Cyfip2 is also mediated by

upstream of Rac1 by S6K1 (Miki et al 1998; Castets et al 2005; Tolias et al 2005). Therefore, S6K1 may serve as the common element of each mechanism regulating both spine morphology and protein translation **(Figure 5-1)**. Research in non-neuronal cell types indicate that inhibition of S6K1 decreases interactions between GTP-bound Rac1 and Cyfip, as well as preventing Cyfip2-mediated cytoskeletal remodeling (Castets et al 2005). These studies also demonstrate that Cyfip2-mediated actin dynamics are dependent on another downstream substrate of S6K1, specifically, the Rac1 GEF Tiam1 (Castets et al 2005; Tolias et al 2005). As with S6K1 inhibition, blockade of Tiam1 activity prevented cytoskeletal alterations due to Cyfip2 activity (Castets et al 2005). Future experiments may also investigate these mechanisms in other brain regions implicated in alcohol addiction and ethanol-induced cognitive deficits. Both the S6K1 inhibitor used in these studies as well as a Tiam1-specific inhibitor are commercially available.

Studies comparing the behavioral and cellular phenotype of C57BL/6J to -6N mice would build upon the body of work presented here. 6N mice consumed less ethanol compared to 6J mice, but like the 6J group, the 6N group demonstrated an escalation in ethanol intake following vapor chamber exposure. Investigation of ethanol-induced behavioral or cognitive deficits will further characterize the phenotypic differences that may exist as a result of this *cyfip2* polymorphism. Although these are two distinct substrains of mice, a group has created a knock-in of the 6N SNP on a 6J background, making the only

difference between the two strains the *cyfip2* gene, eliminating one of the variables in these studies (Kumar et al 2013).

Interestingly, in 6N mice, both the water and CIE-exposed groups gained more weight by the end of the baseline-drinking period compared to 6J mice. Research in Prader-Willi phenotype, a form of obesity connected with fragile X syndrome, has implicated Cyfip2 in dysregulation of leptin signaling (Hoeffer et al 2013). Additionally in preclinical obesity studies, 6N mice are prone to develop diet-induced obesity when given free access to food (Fan et al 2015). Future studies may investigate whether the genotypic differences in 6J and 6N mice affect reward choice. Results may reveal an interesting difference in 6N mice between natural rewards (food) and unnatural rewards (ethanol).

In conclusion, the work presented in this dissertation revealed that chronic ethanol exposure induces homeostatic alterations in protein translation that likely occur through modulation of FMRP activity. These alterations in translation were also accompanied by changes in dendritic spine density and spine morphology. The aim of these experiments was to examine the role of FMRP in activity-dependent, homeostatic protein synthesis in dendrites and dendritic spines in response to chronic ethanol exposure. Additionally, these studies also investigated whether spine morphology is associated with these FMRP-mediated changes in translation and if these two processes have a potential role in mediating ethanol drinking behaviors. The mechanism driving these neuronal adaptations during ethanol exposure, and how this may affect behavior is not well understood. Results from these studies provide evidence of a specific

mechanism that may be essential for chronic ethanol-induced cellular adaptations.

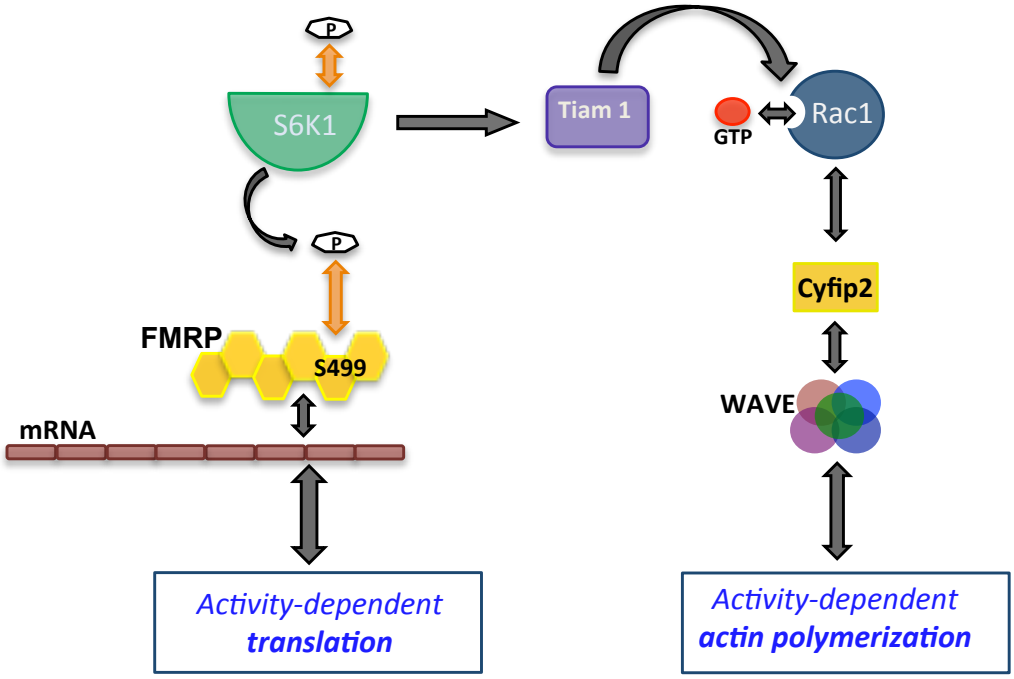

Figure 5-1 Potential S6K1-mediated mechanism driving activity-dependent alterations in protein translation and spine morphology.

Increases in protein translation through FMRP-mRNA binding induced by S6K1 activity leads to activity-dependent translation of specific proteins. S6K1 may also activate the GEF Tiam1, and Rac1 activation through GTP binding. Cyfip2 interactions with GTP-bound Rac1 causes dissociation with the WAVE complex, and allows for actin polymerization and spine development. S6K1 may be the common mediator that pairs ethanol-induced translational changes with activity-dependent alterations in dendritic spines.

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