Metabolic Regulation of Synaptic Plasticity in the Healthy and Diseased Brain

By

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Abstract

Changing strength of synaptic connections, termed synaptic plasticity, allows for the continual maintenance and flexibility of the nervous system. Unfortunately, the same biochemical processes underlying normal plasticity mechanisms may ultimately contribute to the development of hyperexcitable circuits and epilepsy. In its many forms, epilepsy involves the dysregulation and over-synchronization of neuronal firing patterns leading to seizures. In many cases, epilepsy is believed to develop and progress via alterations in synaptic structure and strength, which may represent a therapeutic window. We therefore sought to understand the signaling pathways and plasticity changes in the hippocampus that may be common to both synaptic plasticity and epileptogenesis. This thesis describes a signaling pathway that connects brain energy metabolism and synaptic plasticity. We demonstrate that the cellular energy sensor 5'AMP activated protein kinase (AMPK) can signal to reduce protein translation-dependent synaptic plasticity in the mouse hippocampus. This is accomplished by inhibition of the mammalian target of rapamycin (mTOR) pathway and consequent attenuation of long-term potentiation and long-term depression.

In a mouse model of tuberous sclerosis (TSC) where overactivation of mTOR causes plasticity and behavioral abnormalities, it was found that activation of AMPK can serve to restore appropriate signaling and plasticity in this genetic disease model. Furthermore, we illustrate that TSC2 mutants display an increased susceptibility to the development of epileptiform bursting activity in hippocampal slices. This aberrant activity was due to overactive mGluR5-Erk signaling and could be restored to wild-type levels upon reduction of this pathway.

In summary, my thesis research has defined a signaling route that connects cellular energy metabolism to synaptic plasticity by inhibiting a pathway that is positively associated with the progression of epilepsy. Therefore, we hope that our work may provide novel therapeutic targets for the treatment of conditions where inappropriate plasticity is implicated, such as epilepsy.

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Chapter 1

Introduction to brain plasticity and epilepsy

1. Introduction

In order to learn and adapt to a changing environment, the mammalian nervous system has developed a profound capacity to change; a process known as neural plasticity. This ongoing process involves the strengthening and weakening of existing neuronal connections, the formation and retraction of new connections, as well as the birth of neurons. These plasticity mechanisms support the continual modification of neural architecture and signaling networks within the brain. Until the 20th century, however, the central nervous system was perceived to be relatively rigid. During his Croonian lecture in 1894, Ramón y Cajal suggested that neural processes elaborate and grow with time. In 1949, the psychologist Donald Hebb published his famous "*Hebbian Theory"* of synaptic plasticity:

"When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." (Hebb, 1949)

Hebb's simple idea was that neural activity somehow increased the functional connectivity between neurons; a process that could underlie the activity-dependent neural encoding of experience. Activity-dependent plasticity, alternatively referred to as Hebbian plasticity, has since become a central tenet of neuroscience research. Since his initial postulate, considerable work has demonstrated that the cellular

basis underlying Hebb's increased "efficiency" is subserved by enhanced synaptic efficacy.

Changing synaptic strength, and consequent alterations in network activity, have implications for a diverse spectrum of neurological functions, such as learning and memory, behavior, brain development, psychology, and disease. As research progressively elucidates the etiology of disease, it is becoming increasingly evident that aberrations of neural plasticity underlie many neurological disorders. Examples include epilepsy(Sutula and Steward, 1986; Graef and Godwin, 2010), autism(Gipson and Johnston, 2012), fragile X syndrome(Krueger and Bear, 2011), tuberous sclerosis(Kwiatkowski and Manning, 2005), and Alzheimer's disease(Spires-Jones and Knafo, 2012). With this in mind, we sought to understand the process of synaptic plasticity in hopes of finding therapeutic options for treating neurological disease.

Specifically, this thesis work focused on the role of the cellular energy sensor, 5' adenosine monophosphate activated protein kinase (AMPK), in modulating hippocampal synaptic plasticity. Experimental and conceptual emphasis was placed upon signaling pathways that are common to activity-dependent plasticity and potentially epilepsy. Since plasticity mechanisms are likely to contribute to the development of hyperexcitable networks in epilepsy, our work may have implications for the treatment of epilepsy, and possibly could extend to other neurological conditions where aberrant plasticity is implicated. This introduction

will provide a description of the signaling and structural mechanisms inherent to hippocampal synaptic plasticity, and then discuss the potential contributing role of these plasticity mechanisms in the development of epilepsy.

1.1. The hippocampal formation and slice electrophysiology

There are definite region-specific differences in plasticity mechanisms across the central nervous system. Although many aspects of plasticity discussed here overlap across multiple brain regions, this document will be concerned with plasticity in the hippocampus. The hippocampal formation is buried in the temporal lobe of humans and is required for the acquisition of new memories. Considerable progress into understanding long-term synaptic alteration has been made using the transverse hippocampal slice preparation. This cut maintains the primary connections within the hippocampus, the so-called, trisynaptic circuit. Afferent projections from the entorhinal cortex synapse with dentate granule cells in the hippocampal dentate gyrus via the perforant pathway. Granule cells project axons to synapse with CA3 pyramidal neurons via the mossy fiber pathway. CA3 neurons send a prominent bundle of axons, called the Schaeffer collaterals, to synapse with the dendrites of CA1 pyramidal neurons. Long-term synaptic plasticity occurs at each of these sites, though the cellular mechanisms appear to be fundamentally different. For example, long-term plasticity at the mossy fiber-CA3 synapse does not require the N-methyl-D-aspartate (NMDA) type of glutamate receptor(Harris and

Cotman, 1986), a type of coincidence detector that is described in the next section. Conversely, the NMDA receptor is required for the induction of most types of long-term plasticity at CA3-CA1 synapses(Barco et al., 2006). These differences in regional specificity are likely due to structural as well as molecular differences between these two subregions of the hippocampus. Without doubt, the regulation of activity and synaptic alteration across the hippocampus is important for appropriate learning and memory. Furthermore, disease-related plasticity abnormalities have been described in each of these main hippocampal regions. For the sake of simplicity, our work has focused on the CA3-CA1 synapse, and therefore the plasticity and signaling mechanisms discussed here will be primarily concerned with this area.

Following slicing and removal of cortex, hippocampal slices are allowed to equilibrate in buffer for an extended period, usually 2-4 hours. During this time, signaling pathways and protein synthesis can return to steady-state levels(Osterweil et al., 2010). To monitor synaptic transmission at CA3-CA1 synapses, extracellular recording electrodes can be inserted into the CA1 stratum radiatum dendritic region of CA1 pyramidal cells. Stimulation of the output axons from CA3, the Schaeffer-collaterals, causes action potentials to propagate to synaptic terminals in CA1. Release of neurotransmitter causes postsynaptic excitation and dendritic depolarization via influx of Na^+ and Ca^{2+} ions. The flow of positive charges is detected as a downward deflection in the local extracellular field potential

surrounding the recording electrode. The rate of change of the field potential is a measure of the postsynaptic response and corresponds to the level of depolarization of CA1 dendrites. Test pulses are then given until the slice elicits a stable level of postsynaptic response, at which time the experimenter can apply chemicals and/or electrical stimulation to begin the experiment.

1.2. Long-term potentiation

Encoding of information in the mammalian brain involves stable changes in synaptic efficacy; long-term potentiation (LTP) is believed to represent one form of lasting alteration in synaptic strength underlying memory formation(Bliss and Lomo, 1973). LTP is a stable, activity-dependent increase in synaptic efficacy initiated by robust firing of presynaptic neurons. The rapid induction of LTP results from the coincident binding of neurotransmitter to postsynaptic receptors coupled with a postsynaptic depolarization. In the brain, and especially in the hippocampus, excitatory glutamate is released by presynaptic terminals into the synaptic cleft and is bound by a variety of post-synaptic glutamate receptors: ligand-gated ionic channels, which include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and NMDA receptors; and G protein-linked metabotropic glutamate receptors (mGluR). Each receptor has particular roles in the creation of postsynaptic currents and activation of intracellular signaling pathways, however the NMDA receptor is considered to be crucial for licensing LTP (Collingridge et al., 1983;

Collingridge and Bliss, 1995). The NMDA channel detects the coincident binding of glutamate and postsynaptic depolarization, which only occurs when there is robust and repeated presynaptic activity. When the postsynaptic neuron is sufficiently depolarized to allow release of a Mg^{2+} block in the NMDA receptor pore, the glutamate-bound NMDA receptor will flux Na^{+} and Ca^{2+} ions further depolarizing the postsynaptic neuron and initiating a complex cascade of signaling events(Collingridge and Bliss, 1995). This signaling alters ion channel permeability, increases receptor trafficking, and stabilizes cytoskeletal scaffolding proteins related to synapse fortification. These rapid signaing events involve multiple protein phosphorylation cascades that occur in the presence of translation and transcription inhibitors and in the absence of cell bodies(Vickers et al., 2005).

In the hippocampal slice, LTP can last for many hours and this maintenance requires both translation of existing mRNA transcripts and de novo mRNA transcription(Frey et al., 1988; Frey et al., 1996). LTP can be divided into at least two phases: protein synthesis-independent Early LTP (E-LTP) and Late-LTP (L-LTP), which requires protein translation(Casadio et al., 1999). E-LTP is achieved through persistent activation of protein kinases and increased ion channel permeability. E-LTP can be elicited with a single stimulus train and occurs in the presence of translational and transcriptional inhibitors(Barco et al., 2006). In contrast, induction of L-LTP requires multiple stimulus trains and relies on protein synthesis(Frey et al., 1988) and transcriptional activation(Frey et al., 1996). Furthermore, L-LTP requires

the NMDA receptor, whereas E-LTP does not(Collingridge and Bliss, 1995). These phases are not mutually exclusive, and the post-translational modifications that support short-term E-LTP are likely to contribute towards the induction of lasting L-LTP, hereafter referred to as simply LTP.

1.3. Dendritic signaling underlying long-term plasticity

Glutamatergic transmission induces a complex cascade of signaling events in the postsynaptic neuron. This milieu of signaling is beyond the scope of this introduction so I will attempt to narrow it down to a few critical events that are required for LTP induction and/or maintenance; specifically, increased postsynaptic Ca^{2+}, extracellular signal-regulated protein kinase (ERK) activation, calcium/calmodulin-dependent protein kinase (CaMKII) activation, transcriptional activation, and postsynaptic protein translation. Importantly, each of these events is downstream of and is triggered by the influx of postsynaptic Ca^{2+} through NMDA receptors and release from intracellular stores. Rising Ca^{2+} activates CaMKII and Protein Kinase C (PKC), among other targets. Persistently activated CaMKII stimulates transcription and phosphorylates AMPA receptors to trigger their insertion into the postsynaptic membrane(Malinow and Malenka, 2002). PKC also positively regulates AMPAR function, as well as stimulating ERK signaling. ERK targets transcriptional activation and stimulates protein translation, partially through the mTOR pathway(Carriere et al., 2010). The multiple and overlapping

effects of CaMKII, PKC, and ERK, exemplify the complex postsynaptic signaling involved in the induction of LTP.

The postsynaptic translational machinery required for LTP maintenance is regulated by the mammalian Target of Rapamycin (mTOR) pathway and is selectively activated at stimulated synapses(Casadio et al., 1999; Tang et al., 2002). mTOR is a highly conserved serine/threonine kinase that integrates growth factor, nutrient, and energy signals to promote cell growth, proliferation, and survival(Laplante and Sabatini, 2009). mTOR nucleates two signaling complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2). mTORC1 contains the Regulatory Associated Protein of TOR (RAPTOR), Proline-rich Akt substrate of 40kDa (PRAS40), Sec13 protein 8 (mLST8/GβL), and DEP domain-containing interacting protein (DEPTOR)(Laplante and Sabatini, 2009). Importantly, mTORC1 is responsible for activity-dependent dendritic protein translation and is sensitive to inhibition with the specific mTOR inhibitor rapamycin. RAPTOR is an obligate binding partner that is believed to serve as a scaffolding protein whose association with mTORC1 facilitates signaling(Oshiro et al., 2004). The role of mTORC2 in synaptic plasticity is not well characterized, though it responds to p38 Mitogen Activated Protein Kinase (p38 MAPK) signaling, which itself has been linked to plasticity(Moult et al., 2008). mTORC2 does not contain RAPTOR, does not directly impact activity-dependent translation nor does it respond to rapamycin. Therefore,

the remaining discussion of mTOR-dependent plasticity will be limited to considerations of mTORC1 signaling.

Two prominent upstream signaling pathways converge to activate mTORC1; the phosphoinositide-3 kinase (PI3K)-protein kinase B (Akt) pathway, and the mitogen-activated protein kinase (MAPK)-ERK pathway. Multiple postsynaptic receptors couple neurotransmitter binding to phosphoinositide hydrolysis at the intracellular membrane to promote PI3K activity and consequent activation of membrane-bound Akt. Activated Akt translocates to the cytoplasm and derepresses mTORC1 by inhibiting tuberous sclerosis complex 2 (TSC2)(Dan et al., 2002)(Figure 1.1). Canonically, brain-derived neurotrophic factor (BDNF), an important neurotrophin involved in neuroplasticity, functions to stimulate the PI3K-Akt-mTOR pathway to promote LTP and memory(Tang et al., 2002; Slipczuk et al., 2009). The MAPK-ERK pathway can be activated by metabotropic glutamate receptors through phospholipase C-mediated cleaveage of PIP2 into diacylglygerol (DAG) and IP3, which activate PKC-MEK-ERK signaling(Wisniewski and Car, 2002)(Figure 1.1). Activated ERK stimulates translation through multiple and diverse mechanisms; including TSC2 inhibition to activate mTOR(Roux et al., 2004; Ma et al., 2005), direct phosphorylation RAPTOR to maintain mTORC1 formation(Carriere et al., 2010), indirect activation of p70 ribosomal S6 kinase (S6K)(Anjum and Blenis, 2008), and activation of MAPK interacting kinase 1/2 (Mnk1/2) that signals to eIF4E to promote translational initiation(Banko et al., 2006; Shveygert et al., 2010)(Figure 1.1). Thus,

MAPK-ERK signaling stimulates postysynaptic translation via mTOR-dependent as well as mTOR-independent mechanisms.

Upon activation, mTOR phosphorylates and activates key positive regulators of protein translation such as S6K that goes on to activate the ribosomal protein S6 (S6) leading to ribosomal recruitment to mRNA transcripts. mTOR also phosphorylates and inactivates 4E binding protein 1 (4EBP1), resulting in release of eIF4E, which is a vital component of the translational initiation machinery(Hay and Sonenberg, 2004)(Figure 1.1). The translational targets of mTOR are numerous and not fully characterized, however, several reports have demonstrated that mTOR regulates the translation of proteins involved in synaptic plasticity. Schratt *et al.* catalogued BDNF-induced translation products that were blocked with rapamycin; notably, mTOR controls production of CaMKII, AMPA receptor subunit 1 (GluA1), and Homer2(Schratt et al., 2004). Notably, GluA1 is required for LTP(Selcher et al., 2012), and is targeted by CaMKII and PKC to enhance AMPA receptor conductance(Jenkins and Traynelis, 2012). Inhibition of mTOR blocks activity-dependent production of PKMζ(Kelly et al., 2007). In LTP, PKMζ is autonomously active to regulate AMPAR surface expression and is the only protein identified thus far to be indispensible for LTP and memory(Sacktor et al., 1993; Migues et al., 2010). Given the role of synaptic CaMKII, PKMζ, and AMPA receptors in synaptic plasticity, it is clear how mTOR-dependent translation of these proteins is critical for LTP induction and maintenance.

Importantly, mTOR-dependent signaling is regulated by nutrient and energy availability. Under conditions of energy stress, the energy sensor, 5' adenosine-monophosphate activated protein kinase (AMPK), activates TSC2 to shut down the mTOR pathway(Inoki et al., 2003a; Towler and Hardie, 2007). AMPK regulates mTOR-dependent LTP(Potter et al., 2010) and learning and memory(Dash et al., 2006); the implications of which form the basis of this thesis document and are discussed in detail in the following sections. The availability of amino acids is sensed by mTORC1 via the Rag GTPases that bind RAPTOR and cause it to dissociate from mTORC1 when amino acids are limiting(Sancak et al., 2008). The appropriate integration of the variable inputs to mTORC1 is important for normal neuronal function and plasticity; a process that may be dysregulated in diseases such as epilepsy.

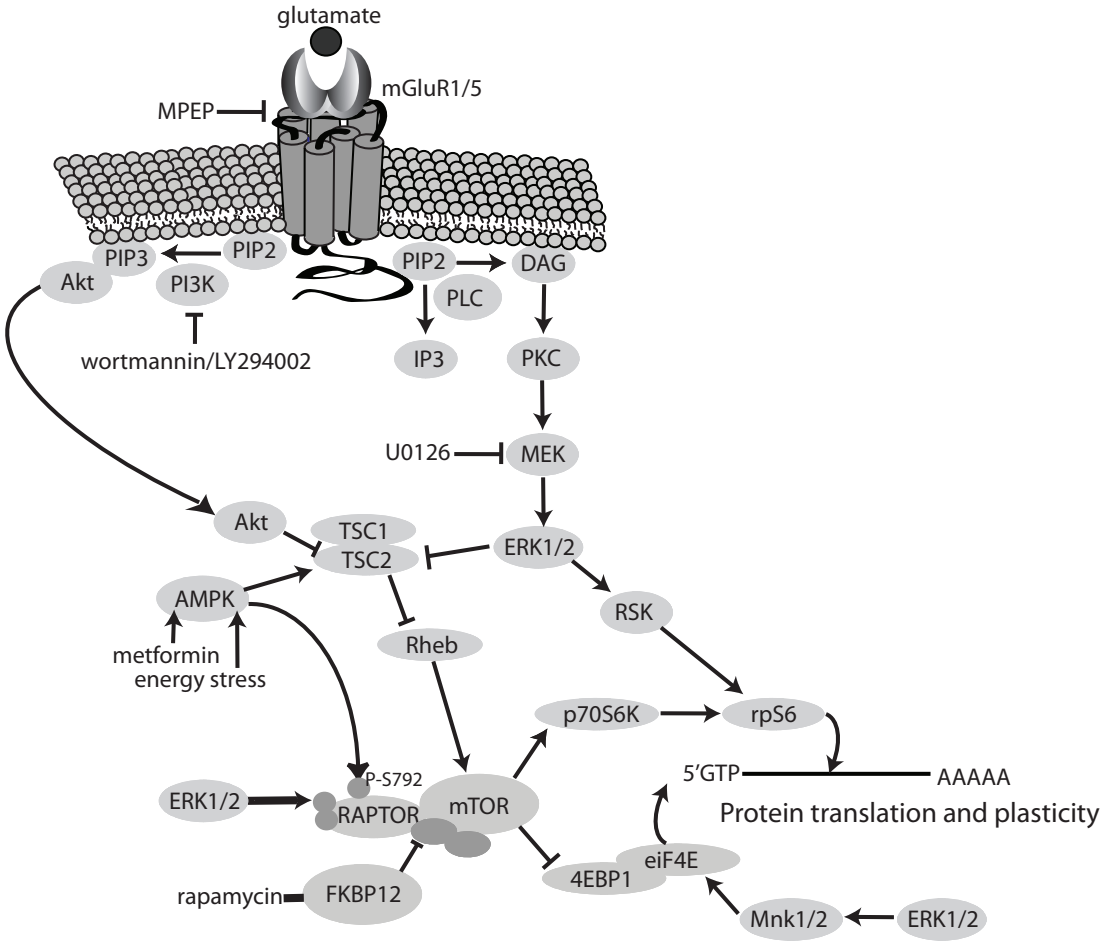

Figure 1.1. Signaling schematic of mGluR signaling.

Hydrolysis of phosphoinositide-2 phosphate (PIP2) by PI3K and PLC respectively activate the Akt and MAPK-ERK pathways. Akt and Erk signal to derepress mTORC1 by inhibiting TSC2, thereby activating mTOR signaling. Additionally, Erk activates RAPTOR to enhance mTORC1 activity, and separately activates RSK and Mnk to enhance protein translation. AMPK serves an opposing function; activation of TSC2 to inhibit mTOR, as well as phosphorylation of RAPTOR-S792 to promote its dissociation from mTORC1. The pharmacological inhibitors used to modulate specific pathways are included above in plain text.

1.4. Long-term depression

LTP can endure for many months if not years in the behaving animal. If activity drives synaptic potentiation, and LTP lasts a very long time, then gradually all the synapses would eventually be driven towards maximal potentiation. In this scenario, synapses would be saturated and there would be no more plasticity, and therefore no learning and memory. Thus, there needs to be a mechanism to reduce synaptic potentiation. Long-term depression (LTD) of synaptic transmission is, in some respects, the physiological opposite of LTP. LTD involves the removal and sequestration of postsynaptic AMPA receptors to produce an enduring reduction in postsynaptic response(Beattie et al., 2000; Waung et al., 2008). Interestingly, most forms of LTP and LTD involve NMDA receptor activity and postsynaptic protein translation. The molecular events that determine whether a synapse is potentiated or depressed rely on a complex relationship of stimulus frequency and duration(Fujii et al., 2010).

LTD at hippocampal CA3-CA1 synapses is commonly induced using several different stimulation protocols; low frequency stimulation (LFS; 1 Hz for 900 seconds), paired-pulse LFS (PP-LFS), or applying pharmacological group 1 metabotropic glutamate receptor (mGluR) agonists. Although they produce similar physiological responses; namely LTD, LFS-LTD and mGluR-LTD have fundamental differences in terms of their induction and dependence on a variety of signaling pathways. For example, LFS-LTD is unaffected by inhibitors of PI3K, ERK, CaMKII, or

mTOR(Peineau et al., 2009), whereas mGluR-LTD is dependent upon all of these kinases(Gallagher et al., 2004; Hou and Klann, 2004; Mockett et al., 2011). Furthermore, LFS-LTD is not protein synthesis-dependent(Huber et al., 2000; Peineau et al., 2009) but requires NMDA receptor activation(Dudek and Bear, 1992; Mulkey and Malenka, 1992).

Activation of group 1 mGluRs (mGluR1 and mGluR5) with (S)-3,5-dihydroxyphenylglycine (DHPG) induces second messenger cascades that impact receptor stability and protein translation. Group 1 mGluRs are G_q-coupled receptors that signal to activate phospholipase C, PI3K-Akt, and MAPK-ERK signaling. Importantly, mGluR activation is a major driver of mTOR-dependent translation that supports mGluR-LTD and is blocked with rapamycin (Hou and Klann, 2004) and other translational inhibitors(Huber et al., 2000). Similarly, PP-LFS-induced LTD activates mGluRs, is blocked by mGluR5 antagonists(Moult et al., 2008) and relies on postsynaptic protein translation for expression of LTD(Huber et al., 2000). Thus PP-LFS-induced LTD has been described as a form of synaptic mGluR-LTD.

Hippocampal LTD facilitates the acquisition of novel memories (Manahan-Vaughan and Braunewell, 1999) and is required for spatial memory(Ge et al., 2010). Furthermore, spatial exploration can involve a reversal of hippocampal LTP(Xu et al., 1998; Abraham et al., 2002). Given the importance of LTD in learning, and the preponderance of evidence supporting a role for LTP in learning and memory(Barco

et al., 2006), it is likely that the process of learning involves a complicated balance of LTP and LTD of synaptic transmission.

1.5. Energy metabolism and AMPK in the brain

The availability of cellular energy is essential for proper brain function. Traditional views held that the brain was 'energetically favored' and that glucose levels were saturating and did not significantly change under normal physiological conditions. Considerable evidence demonstrates that this traditional view is inaccurate and that fluctuations in brain glucose levels dramatically impact neurological functions(Gold, 1986; McNay and Gold, 2002). Extracellular brain glucose is heavily compartmentalized and steeply declines by over 30% in the hippocampus of rats during exploratory behavior(McNay et al., 2000). Accordingly, glucose administration significantly improves learning and memory in rodents and humans and can reverse age-related cognitive decline(McNay et al., 2001; Korol, 2002). Due to the limiting nature of glucose in the brain, appropriate cellular responses to energy stress are crucial for the rapid restoration of energy homeostasis. This is partially achieved through the autoregulatory feedback signaling of energy metabolism pathways; however, it is clear that additional sensors of cellular energy levels are necessary for adequate responses to energy stress.

5' Adenosine Monophosphate-Activated Protein Kinase (AMPK) is a critical sensor of cellular energy status that functions by sensing fluctuations in the

AMP:ATP ratio, which increases with high energy usage. AMPK activation can be elicited through starvation, low-carbohydrate diets, exercise, or a number of small molecules (Figure 1.2). AMPK is also sensitive to a number of cytokines, including leptin, ghrelin, adiponectin, cannabinoids, and interleukin-6(Hardie, 2007). Plant polyphenols such as resveratrol from grapes and epigallocatechin-3-gallate in green tea, are natural AMPK agonists(Baur et al., 2006; Hwang et al., 2007). Glycolytic inhibition with 2-deoxy-D-glucose (2DG) causes energy stress, raises AMP levels, and leads to activation of AMPK(Rubin et al., 2005; Potter et al., 2010). The anti-diabetic drug metformin induces energy stress by inhibiting the mitochondrial complex I(Towler and Hardie, 2007). Importantly, metformin is known to cross the blood-brain barrier(Ma et al., 2007) and is an FDA-approved compound, which means it could be readily adapted for use to treat neurological disease.

AMPK is a heterotrimeric protein consisting of a catalytic α subunit, a functional β subunit, and a structural γ subunit. Binding of AMP and ATP in the Bateman domains within the β subunit induces a conformational shift in AMPK exposing the activation loop in the α subunit. Phosphorylation of Thr172 by upstream kinases, LKB1 and CaMKKβ, induce a greater than 1000 fold increase in kinase activity(Suter et al., 2006). Upon activation, AMPK coordinates an energy-conserving program by activating processes that increase cellular ATP production and reducing bioenergetically expensive processes to limit ATP consumption. ATP production is increased by inducing cell-surface expression of glucose transporters,

activating glycolytic enzymes, increasing mitochondrial biogenesis, and in some cases by inducing autophagy(Hardie, 2007). ATP consumption is suppressed by inhibiting energy-intensive pathways such as fatty acid synthesis, cell division (in cycling cells) and protein translation by inhibiting elongation factor 2 kinase (EF2K), and by shutting down the mTOR pathway(Inoki et al., 2003a; Towler and Hardie, 2007)(Figure 1.2). AMPK inhibits mTOR signaling by activating TSC2 to inhibit Rho Enriched in Brain (Rheb) GTPase activity and silence mTORC1(Inoki et al., 2003a). AMPK can also inhibit mTORC1 activity via phosphorylation of the obligate mTOR complex binding partner, RAPTOR causing its dissociation from and consequent inhibition of mTORC1(Gwinn et al., 2008)(Figure 1.1). This provides yet another pathway by which cellular energy status controls protein translation.

AMPK is a major regulator of energy metabolism in virtually all eukaryotic cells(Hardie, 2011). AMPK is ubiquitous throughout the central nervous system; AMPK impacts nervous system development(Culmsee et al., 2001), neuronal guidance and differentiation(Amato et al., 2011), induces ketogenesis in cortical astrocytes(Blazquez et al., 1999), modulates the hypothalamic response to leptin and ghrelin(Kim et al., 2004), blocks peripheral pain sensitization(Tillu et al., 2012), and regulates hippocampal learning and synaptic plasticity(Dash et al., 2006; Potter et al., 2010), to name a select few. Given the role of AMPK in a variety of nervous system functions and development, it is not surprising that this critical energy sensor exerts its effects through mTOR signaling; a pathway involved in plasticity and growth.

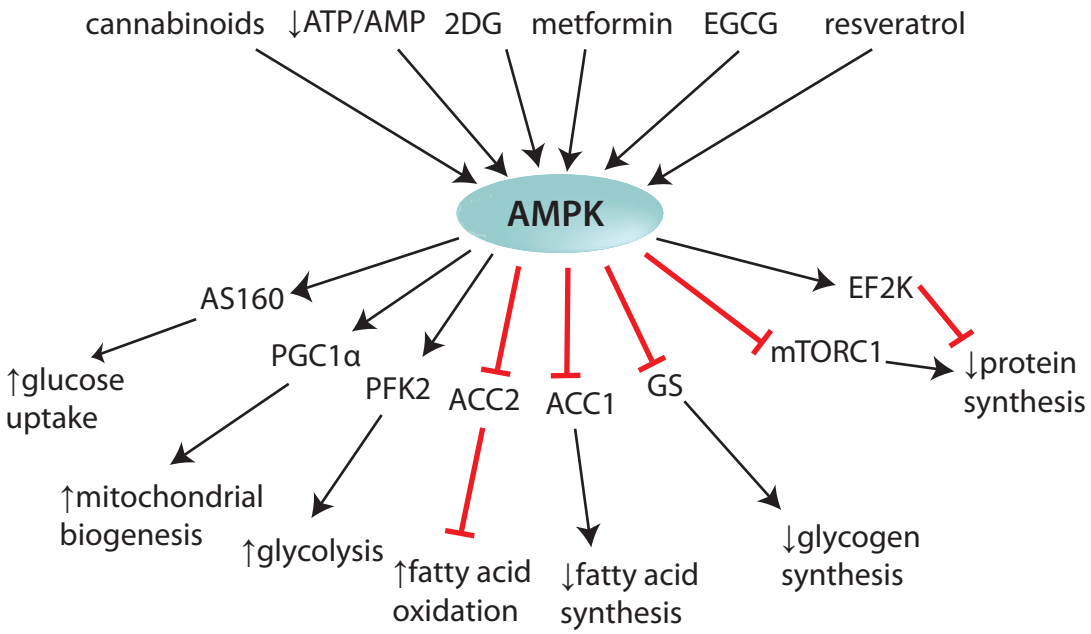

Figure 1.2. AMPK activation and energy-conserving signaling. AMPK is activated by a number of small molecules, including endogenous cannibinoids, and plant polyphenols such as epigallocatechin-3-gallate (EGCG) and resveratrol. The canonical mode of AMPK activation is through a reduced ATP/AMP ratio that occurs during energy stress. Alternatively, energy stress can be initiated through glycolytic inhibition with 2DG or mitochondrial inhibition with metformin. AMPK exerts its energy-conserving program by activating (black arrows) or inhibiting (red bars) various cellular processes. This is not an exhaustive list of AMPK signaling components, however does illustrate the multitude of pathway impacted by AMPK. The final result of AMPK signaling is illustrated at the end of pathway. Abbreviations: 2DG, 2-deoxy-D-glucose; EGCG, epilgallocatechin-3-gallate; AS160, Akt substrate of 160 kDa; PGC1α, PPARγ co-activator 1α; PFK2, 6-phosphofructo-2-kinase; ACC1, acetyl-CoA carboxylase-1; ACC2, acetyl-CoA carboxylase-2; GS, glycogen synthase; mTORC1, mammalian target of rapamycin complex 1; EF2K, elongation factor 2 kinase.

1.6. Epilepsy-related plasticity

Occurring in approximately 1% of the human population, epilepsy is a neurological disorder that involves dysregulated synchronous firing of cortical and/or subcortical neurons that progress to seizure. Epilepsy is a not a single disease, but rather is the common symptomatic manifestation of a variety of neurological complications, such as traumatic brain injury, stroke, infection, genetic predisposition, and structural malformations. Each of these abnormalities impacts network activity in different ways to promote the development of hyperexcitable neural circuitry that is capable of producing seizures.

Epileptogenesis involves a progressive alteration of neuronal network activity that increases recurrent network firing leading to seizures. These neuronal circuits undergo long-term structural and functional synaptic changes including increasing synaptic contacts and synaptic efficacy(Sutula, 2004), which are activity-dependent processes. Therefore, it seems likely that LTP and LTD mechanisms are involved in the pathological progression of epilepsy(McEachern and Shaw, 1999).

Dysregulated and heightened mTOR signaling is implicated in many pathological systems including epilepsy(Buckmaster et al., 2009; Zeng et al., 2009), tuberous sclerosis(Crino and Henske, 1999), autism spectrum disorders, and cancer(de Vries, 2010). In the kainic acid model of temporal lobe epilepsy, rapamycin effectively blocked mossy fiber sprouting, seizure-induced neurogenesis, cellular apoptosis, and epileptogenesis(Zeng et al., 2009). In this study, Zeng *et al*

(2009) noted no difference in seizure severity or duration of status epilepticus between control and rapamycin-treated mice. Rapamycin treatment before or after status epilepticus was capable of preventing the subsequent development of spontaneous seizures(Zeng et al., 2009). This suggests that mTOR is not involved in seizure-related activity, however may regulate seizure-induced plasticity. Recently, Huang *et al* (2010) illustrated that there is hyperactive mTOR in rats with acquired chronic spontaneous seizures in a pilocarpine model of epilepsy(Huang et al., 2010). Rapamycin delivery after the acquisition of spontaneous seizures reduced seizure severity and mossy fiber (MF) sprouting(Huang et al., 2010). MF sprouting of the hippocampal dentate gyrus is commonly observed in human epilepsy as well several epilepsy models of temporal lobe epilepsy(Sutula et al., 1992). The laboratory of Paul Buckmaster has specifically investigated the role of mTOR signaling in MF sprouting and its contribution to epileptogenesis. Kainic acid-induced status epilepticus potently activates the mTOR pathway and rapamycin post-treatment significantly reduces MF sprouting(Buckmaster et al., 2009). However, in subsequent studies using this model, rats post-treated with rapamycin still acquired epilepsy and suffered spontaneous seizures at rates similar to vehicle-treated controls(Buckmaster and Lew, 2011). This finding suggests that reduction of mTOR signaling during seizure may be required to inhibit epileptogenesis.

LTP induction causes rapid morphological alterations of synaptic structure involving the growth of new dendritic protrusions which mature into functional

synapses(Maletic-Savatic et al., 1999), increased synaptic density(Barco et al., 2006), and a selective stabilization of newly acquired structures(De Roo et al., 2008). In addition to structural similarities, electrical kindling (epileptogenesis model) and LTP share molecular determinants such as dependence on macromolecular synthesis of mRNA and proteins, and are facilitated by BDNF administration(Messaoudi et al., 1998). The numerous similarities in the molecular and structural mechanisms supporting epileptogenesis and LTP, suggest that these two processes are intimately related. Thus, given the role of metabolism in the treatment of epilepsy (via the ketogenic diet), deciphering the role of metabolism in synaptic plasticity may have direct implications for the treatment of epilepsy and tuberous sclerosis.

1.7. Tuberous sclerosis

Tuberous sclerosis complex (TSC) is a multisystem autosomal dominant disorder that is characterized by the development of systemic benign hamartomas and cortical tubers, mental disability, autism, and epilepsy(Curatolo et al., 2002; Crino et al., 2006). Affecting approximately 1 in 6,000 people, TSC is caused by mutations in either of the tumor suppressor genes *TSC1* or *TSC2*, which result in altered signaling through multiple cellular pathways that impact neurological processes such as nervous system development, neuronal migration, and synaptic function(Crino and Henske, 1999; Curatolo et al., 2002; Kwiatkowski and Manning, 2005). Epilepsy occurs in approximately 90% of patients and often during the first

year of life(Shepherd et al., 1995; Curatolo et al., 2002; Crino et al., 2006; Franz et al., 2010). Severe cognitive deficits are observed in 30% of patients, while the remaining 70% have varying degrees of mental ability ranging from moderate intellectual disability to normal intelligence(de Vries, 2010). Infantile autism is seen in about 30% of TSC patients, though autistic-like behaviors are noted in approximately 50% of those with the disease(de Vries, 2010). Until recently, the neuropsychiatric phenotype of TSC was considered to be a consequence of structural abnormalities within the central nervous system and the associated seizure activity. However, now TSC is considered to be a disease of overactive signaling through the mammalian target of rapamycin (mTOR) pathway(Kwiatkowski and Manning, 2005).

TSC1 and TSC2 form a heterodimeric complex (TSC1/2) that receives signals from PI3K-Akt(Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002), MAPK-ERK(Roux et al., 2004; Ma et al., 2005), AMPK(Inoki et al., 2003a), TNFα-IκB kinase β signaling(Lee et al., 2008), and glycogen synthase kinase-3β(GSK3β)(Inoki et al., 2006). In this manner, TSC1/2 functions as a signaling node that modulates the activity of the mTOR complex-1(mTORC1)(Inoki et al., 2003b; Tee et al., 2003).

Loss of TSC1/2 function results in disinhibition and consequent hyperactive mTORC1 signaling(Kwiatkowski and Manning, 2005). Since mTOR controls cellular growth and survival, overactive mTOR signaling can explain many of the pathological characteristics of TSC; namely, cellular hypertrophy, tumor formation, developmental malformations, and cognitive deficits. Accordingly, mTOR inhibition

with rapamycin has shown promising clinical effectiveness in reducing tumor burden in TSC patients(Franz et al., 2006; Bissler et al., 2008). In mouse models of TSC, rapamycin treatment improved median survival and ameliorated neurofilament and myelination abnormalites(Meikle et al., 2008). In TSC1-GFAP conditional knockout mice (astrocytic knockout), rapamycin prevented epileptogenesis and structural histopathologies(Zeng et al., 2008). These data suggest that modulation of mTOR may be therapeutic for the treatment of TSC. TSC is diagnosed early in life, however the development of cortical tubers that lead to epilepsy can occur later life. Attenuation of mTOR signaling within this early neurodevelopmental period could have beneficial effects for reducing the development of many aspects of this disease. The contribution of overactive mTOR to tumorigenesis in TSC is well established, however the role of mTOR in epileptogenesis is less clear. Epileptogenic foci are commonly associated with cortical tubers(Curatolo et al., 2002), though it is unknown whether it's the structural abnormalities or altered signaling surrounding the tuber that is epileptogenic(Holmes and Stafstrom, 2007). Experimental models of TSC are perhaps the best models for investigating the role of mTOR signaling in epilepsy.

In the hippocampal slice, overactive mTOR activity produces abnormal LTP and LTD; two well-characterized mTOR-dependent processes. Ehninger *et al* demonstrated that learning and memory deficits in mice with inactivating heterozygous mutations in TSC2 ($TSC2^{+/-}$) could be ameliorated with rapamycin.

These authors demonstrated that $TSC2^{+/-}$ mice had 'aberrant induction' of LTP due to hyperactive mTOR signaling, which was also reduced with rapamycin (Ehninger et al., 2008). Bateup *et al* observed a loss of mGluR-LTD in mice with postnatal deletion of hippocampal TSC1(Bateup et al., 2011). Elucidation of the altered signaling pathways underlying the expression of aberrant hippocampal plasticity may provide insights into possible therapeutic targets to treat TSC.

In the following data chapters, evidence will be presented illustrating the role of AMPK in regulating synaptic plasticity in wild-type hippocampus. This is likely due to reduction of mTOR signaling (Chapter 2). This work was extended to a mouse model of TSC ($TSC2^{+/-}$ heterozygous mice) where overactive mTOR signaling is implicated in the plasticity abnormalities and disease etiology. Chapter 3 addresses a role for AMPK dependent modulation of LTP in $TSC2^{+/-}$ hippocampus. While investigating a role for AMPK in LTD in $TSC2^{+/-}$ mice, we uncovered a hyperactive mGluR5-Erk signaling axis in these mutants. Chapter 4 is devoted to the investigation of the nature and implications of these overactive pathways in LTD, epileptiform bursting, and behavior.

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Chapter 2

Metabolic regulation of neuronal plasticity by the energy sensor AMPK

Notes on collaborations and publications

This chapter is an adaptation of the following publication:

Potter WB, O'Riordan KJ, Barnett D, Osting SM, Wagoner M, Burger C, Roopra A (2010) Metabolic regulation of neuronal plasticity by the energy sensor AMPK. PLoS One 5:e8996.

Electrophysiology was conducted in collaboration with Ken O'Riordan.

Immunohistochemistry was conducted in collaboration with Sue Osting and the lab of Corinna Burger.

Data for Figure 2.1c was acquired in collaboration with Matt Wagoner.

All other work was completed by Wyatt Potter.

Abstract.

Long Term Potentiation (LTP) is a leading candidate mechanism for learning and memory and is also thought to play a role in the progression of seizures to intractable epilepsy. Maintenance of LTP requires RNA transcription, protein translation and signaling through the mammalian Target of Rapamycin (mTOR) pathway. In peripheral tissue, the energy sensor AMP-activated Protein Kinase (AMPK) negatively regulates the mTOR cascade upon gycolytic inhibition and cellular energy stress. We previously demonstrated that the glycolytic inhibitor 2-deoxy-D-glucose (2DG) alters plasticity to retard epileptogenesis in the kindling model of epilepsy. Reduced kindling progression was associated with increased recruitment of the nuclear metabolic sensor CtBP to NRSF at the BDNF promoter. Given that energy metabolism controls mTOR through AMPK in peripheral tissue and the role of mTOR in LTP in neurons, we asked whether energy metabolism and AMPK control LTP. Using a combination of biochemical approaches and field-recordings in mouse hippocampal slices, we show that the master regulator of energy homeostasis, AMPK couples energy metabolism to LTP expression. Administration of the glycolytic inhibitor 2-deoxy-D-glucose (2DG) or the mitochondrial toxin and anti-Type II Diabetes drug, metformin, or AMP mimetic AICAR results in activation of AMPK, repression of the mTOR pathway and prevents maintenance of Late-Phase LTP (L-LTP). Inhibition of AMPK by either compound-C or the ATP mimetic ara-A rescues the suppression of L-LTP by energy stress. We also show that enhanced LTP via AMPK inhibition requires mTOR signaling. These

results directly link energy metabolism to plasticity in the mammalian brain and demonstrate that AMPK is a modulator of LTP. Our work opens up the possibility of using modulators of energy metabolism to control neuronal plasticity in diseases and conditions of aberrant plasticity such as epilepsy.

2.1. Introduction

Long Term Potentiation (LTP) is thought to represent one form of durable alteration in synaptic strength underlying memory formation(Bliss and Lomo, 1973). Despite this potential role in learning and memory, the ability to control aberrant LTP formation under pathological conditions may be of therapeutic value. The initiating events in LTP expression are rapid and require neither de-novo protein synthesis nor transcription (termed Early LTP – E-LTP), however LTP maintenance requires both protein translation and mRNA transcription (Late LTP – L-LTP) (Nguyen et al., 1994; Frey and Morris, 1997; Casadio et al., 1999; Vickers et al., 2005). This protein synthesis appears to be dependent on the mammalian Target of Rapamycin (mTOR), a kinase complex that phosphorylates and activates key positive regulators of protein translation including p70S6 kinase (p70S6K), which then further phosphorylates the downstream target, ribosomal protein S6 (rpS6)(Tang et al., 2002; Tsokas et al., 2007). These downstream effectors act to increase translation of select mRNAs that enhance overall translational capacity (Tang et al., 2002; Hay and Sonenberg, 2004; Tsokas et al., 2007).

In non-neuronal systems the mTOR complex can be controlled by cellular energy levels via the metabolic sensor AMP-activated Protein Kinase (AMPK) (Inoki et al., 2003). A reduced cellular ATP concentration results in elevation of AMP levels (Hardie and Hawley, 2001) that, in concert with upstream kinases leads to full activation of AMPK (Hawley et al., 1996; Stein et al., 2000; Shaw et al., 2005). Activated AMPK coordinates an energy-conserving program by increasing cellular

ATP production and reducing ATP consumption by shutting down energy intensive processes such as mTOR-dependent protein translation (Inoki et al., 2003; Towler and Hardie, 2007). AMPK inhibits mTOR via phosphorylation and activation of the Tuberous Sclerosis Complex heterodimer (TSC1/2) as well as directly phosphorylating the RAPTOR subunit of mTOR complex-1 (Gwinn et al., 2008) (Fig. 1A).

The anti-diabetic drug and mitochondrial complex-1 toxin metformin is a potent activator of AMPK and both AMP dependent and independent mechanisms such as reactive nitrogen species generation have been implicated in AMPK activation by metformin (Zhou et al., 2001; Hawley et al., 2002; Zou et al., 2004). The glucose analogue 2-deoxy-D-glucose (2DG) is also able to activate AMPK through glycolytic inhibition(Rubin et al., 2005) and we recently showed that 2DG administration suppresses epileptogenesis in the kindling model(Garriga-Canut et al., 2006).

It is not known how the mTOR pathway in neurons, and thus L-LTP expression, is controlled by neuronal metabolism. Given that sustained LTP requires mTOR signaling, that mTOR is under metabolic control via AMPK, and previous reports suggest a link between energy metabolism and LTP (Kamal et al., 1999; Ai and Baker, 2006; Sadgrove et al., 2007), we reasoned that energy metabolism could regulate L-LTP via AMPK. Here we demonstrate that the energy sensor AMPK controls hippocampal L-LTP and provide evidence that this control is exerted through mTOR signaling.

2.2. Methods

All procedures were performed with the approval of the University of Wisconsin-Madison School of Medicine and Public Health Institutional Animal Care and Use Committee and according to national guidelines and policies.

Electrophysiology

All electrophysiology was performed on 4-6wk old C57BL/*6* mice. Immediately after euthanasia the brain was removed from the skull and submerged in ice cold cutting solution (CS) [in mM]: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 5 glucose, 0.6 ascorbate). The hippocampi were sectioned transversely in a Vibratome (St. Louis, MO) into 400 μM slices immersed in ice-cold CS. Slices were allowed to recover for 45 min at room temperature (RT) in 50:50 CS: artificial cerebrospinal fluid (ACSF) [in mM]: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 CaCl, 1 $MgCl_2$, 25 glucose), and a further 45 min at RT in 100% ACSF before being transferred to an interface chamber (Fine Science Tools, Foster City, CA) bathed in 100% ACSF (1 mL/min) at 32°C (TC-324B, Warner Instrument Corporation, Hamden, CT) for 2 hours prior to treatment. All solutions were carb-oxygenated (95/5, O_2/CO_2). Enameled bipolar platinum-tungsten (92:8 Pt:Y) stimulating electrodes were placed along the Schaeffer-Collateral pathway. Field EPSPs were recorded from CA1 *stratum radiatum*, with ACSF-filled recording electrodes (5 MΩ). Baseline synaptic transmission was assessed for each individual slice by applying gradually increasing stimuli (0.5V – 15V, 25nA – 1.5μA, A-M

Systems model 2200 stimulus isolator, Carlsborg, WA) to determine the input:output relationship. All subsequent experimental stimuli were 50% of the intensity of the maximum evoked fEPSP slope (i.e. PPF, HFS, TBS). Paired-pulse facilitation was performed prior to the induction of LTP. PPF consisted of an initial single stimulus to the Schaeffer Collateral bundle followed by a second stimulus of equal magnitude. This paradigm was repeated with increasing time intervals between the two pulses. fEPSP slope measurements from the second pulse were plotted as a percentage of initial slope. Field EPSP waveforms were generated with a sampling rate of 100 kHz using Clampex software. LTP was induced with either high frequency stimulation (4 stimulations of 100 Hz each lasting for 1 second) or theta burst stimulation applied to the Schaeffer-collaterals and fEPSPs were measured in *stratum radiatum*. Theta burst stimulation consisted of 10 bursts/train, and 3 trains/stimulus with a 20 second intertrain interval. Each burst contained 4 stimulations at 100 Hz with an interburst interval of 200 msec. Synaptic efficacy was continually monitored (0.05 Hz). Every 2 min sweeps were averaged; the fEPSP's were amplified (A-M Systems model 1800), digitized (Digidata 1322B, Molecular Devices, Sunnyvale, CA) and then analyzed (pClamp, Molecular Devices). Two-way ANOVA (drug and time) with repeated measures (mixed model) and Bonferroni posttests were used for statistical analysis for drug effect over all points.

Tissue homogenization for biochemistry

Following drug application and/or stimulation, slices were flash frozen in eppendorf tubes on dry ice. Slices were subsequently lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) + mammalian protease inhibitor (1:100, Sigma, St. Louis, MO) + phosphatase inhibitors [in mM] (10 NaF, 2 Na Vanadate, 4 Na pyrophosphate, 10 β-glycerophosphate). Slices were then triturated with a 28.5 gauge insulin syringe to shear up DNA. Lysates were spun down at 12 krpm for 30 min and supernatants kept and quantified using the DC Protein Assay (Bio-Rad, Hercules, CA). Appropriate amounts of 5X loading buffer (0.5 M Tris, 10% SDS, 50% glycerol, 10 mM EDTA,1% β-mercaptoethanol) were added to protein extracts and boiled at 95°C for 3 min. All solutions were diluted with Milli-Q water (Milli-Q UF Plus, Millipore, Bedford, MA).

Western blotting

Protein extracts were loaded at 30 μg/lane in gradient (4-20%) tricine gels (Pierce Biochem, Rockford, IL) and resolved with standard electrophoresis in HEPES buffer (100 mM Tris, 100 mM HEPES, 0.1% SDS) and transferred overnight at 4°C onto PVDF membranes (Millipore, Bedford, MA) with tris-glycine buffer (20 mM Tris, 1.5 M glycine). Membranes were blocked with Tris-buffered salt solution with Tween-20 (TBST; 20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) and 5% milk fat for 1 hr prior to addition of primary antibody. To optimize binding, primary antibodies were either diluted in 5% milk fat or TBST. p-AMPK (Thr172) (1:1000

overnight at 4°C in TBST, Cell Signaling, Danvers, MA), p-p70S6K (Thr389) (1:1000 overnight at 4°C in TBST, Cell Signaling), p-rpS6 (Ser235/236) (1:1000 overnight at 4°C in TBST, Cell Signaling), total-p70S6K (1:1000 overnight at 4°C in TBST, Cell Signaling), total-rpS6 (1:1000 overnight at 4°C in TBST, Cell Signaling), βIII tubulin (1:10,000 for 1 hr at RT in milk, Promega, Madison, WI), actin (1:10,000 for 1 hr at RT in milk, Millipore). Membranes were incubated for 1 hr in horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (1:10,000) (Santa Cruz Biotech All solutions were diluted with Milli-Q water (Milli-Q UF Plus, Millipore, Bedford, MA). Protein bands were detected using SuperSignal West Femto ECL reagent (Pierce Biochem) and visualized using Kodak Image Station 2000R and Kodak 1D Image Analysis software, which was also used to quantify protein bands and the two-tailed Student's T-test was employed for statistical analysis.

Immunofluorescence and microscopy.

Tissue analysis was performed on 400μm hippocampal slices following incubation in either ACSF or ACSF plus 10 mM 2DG. Slices were incubated overnight in a fixative solution containing 4% formaldehyde (freshly depolymerized from paraformaldehyde; Sigma, St. Louis, MO) in 0.1 M phosphate buffer, pH 7.4 (PB). Slices were removed from fixative, rinsed in PB and cryoprotected in 50% PB/50% cryoprotectant with 20% sucrose and 5% glycerol for 1 hour, followed by 100% cryprotectant with 20% sucrose and 5% glycerol for 1 hour. Slices were frozen with

dry ice and sectioned on a sliding microtome in the coronal plane at 30μm thickness. Sections were transferred to PB at 4ºC (with 0.01% sodium azide if stored for more than 2 days). Anti-phospho-AMPKα (Thr172) was purchased from Santa Cruz (sc-33524, lot F0209). Anti-NeuN was purchased from Millipore (MAB377, lot LV1573084). Sections from both septal and temporal hippocampus were prepared for light microscopy.

Frozen sections were rinsed in 0.01M phosphate buffered saline (PBS) with 0.1% saponin (product S7900; Sigma Aldrich) and 2% bovine serum albumin (BSA; Calbiochem, La Jolla, CA), blocked in the same buffer with 20% normal goat serum for 45 minutes, incubated overnight in primary antiserum to p-AMPKα (1:500) and primary antiserum to NeuN (1:1000) with 0.1% normal goat serum, washed in buffer, incubated for 2 hours in 1:500 AlexaFluor 488 goat anti-rabbit IgG (product A11034, lot 461250 from Molecular Probes, Eugene, OR) and 1:500 AlexaFluor 594 goat anti-mouse IgG (product A11032, lot 419361 from Molecular Probes, Eugene, OR) in PBS with 0.1% saponin and 2% BSA, rinsed with PB and mounted with SlowFade Light Antifade Kit (product S2828, lot 54616A from Molecular Probes, Eugene, OR).

Three "positive" controls were performed to minimize the possibility of artifactual staining. 1) p-AMPKα and NeuN were visualized with both immunofluorescence and immunoperoxidase reaction methods. 2) Dilution series were carried out to obtain optimal staining dilutions. 3) p-AMPKα and NeuN antisera were visualized on transcardial perfused tissue fixed with 4%

formaldehyde. No discrepancies were observed in the pattern of label in these comparisons. For a "negative" control, immunoreactions were run without primary antisera. No label was observed with this control.

Light microscopic imaging was performed with a digital camera (Spot II; Diagnostic Instruments, Sterling Heights, MI) on a Nikon E600 Eclipse epifluorescent microscope with x2-60 planapochromatic objectives and a standard FITC filter cube (FITC; EX 465-495 nm; DM 505 nm; BA 515-555 nm) and TRITC filter cube (TRITC; EX 528-553 nm; DM 565 nm; BA 600-660 nm). Images were obtained with the x40 objective. Fluorescent images were acquired at an initial 36-bit tone scale and saved as 16-bit files. Light microscopic images were prepared for reproduction in Adobe Photoshop 7.0 with minimal adjustments in the tone scale, contrast, hue and subsequent sharpening with the unsharp mask algorithm.

2.3. Results

AMPK activation in the hippocampus represses mTOR signaling.

To test the hypothesis that metabolism can control L-LTP via the action of AMPK on mTOR signaling, we first tested whether energy stress could activate AMPK in the hippocampus. Hippocampal slices were incubated in either ACSF or ACSF containing the glycolytic inhibitor 2-deoxy-D-glucose (2DG) for 30 minutes, and AMPK activation was assessed by measuring phosphorylated AMPKα-T172 (p-AMPK). Exposure to 10mM 2DG (a concentration that allows for competitive inhibition of transporters and kinases of 25 mM glucose) resulted in a 2 to 3 fold induction of p-AMPK compared to control slices (Fig. 2.1A and 2.1B). This AMPK activation correlated with a 2 fold reduction in ATP levels (Fig. 2.1C). The anti-diabetic drug metformin is a potent activator of AMPK in other tissues: Figures 2.1A and 2.1B show that 5μM metformin and the related molecule phenformin are also able to activate AMPK 2 to 3 fold in the hippocampus. To determine whether AMPK activation occurs in CA1 dendrites (the site of mTOR activation upon LTP induction(Tsokas et al., 2007)) we visualized p-AMPK using immunofluorescence on mouse hippocampal slices. Figure 2.2 shows that p-AMPK immunoreactivity is present both in the dendrites of the stratum radiatum (SR) and cell bodies of the pyramidal layer in CA1. Addition of 2DG results in a transition from a punctate pattern to a smoother and brighter pattern in the dendrites (compare panels 1 and 4). There is also a marked increase in cell body staining in the presence of the AMPK

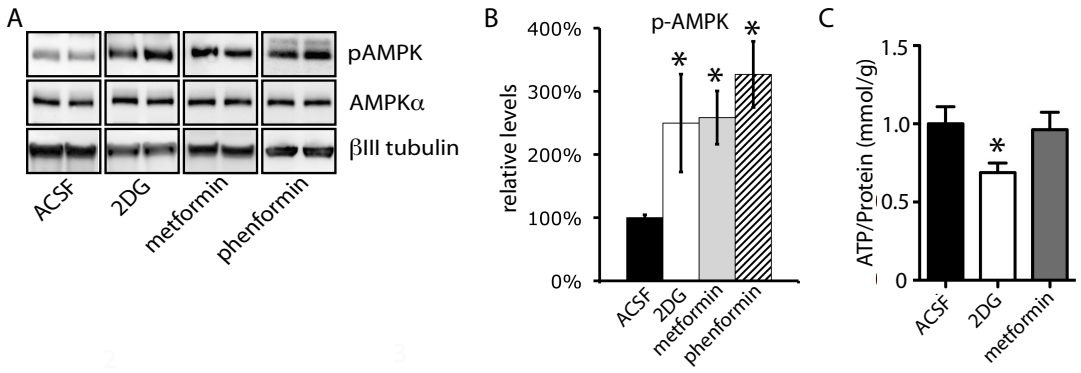

Figure 2.1. Metformin and 2DG activate AMPK in mouse hippocampus

A) AMPK is activated 30 min after exposure to 2DG (10mM, p=0.019, n=9), metformin (5μM, p=0.005, n=6), or phenformin (10μM, p=0.018, n=6). Hippocampal slices were incubated in ACSF and drug for 30 minutes and subjected to western blot with anti-phospho-Thr172-AMPK antibody followed by βIII-tubulin as a loading control. Representative western blots of duplicate lanes are shown, together with their quantification from at least 10 samples per condition (**B**). **C**) ATP levels are reduced in the presence of 10mM 2DG (p=0.032, n=7), but not 5μM metformin (p=0.825). Tissue was lysed and subjected to a CellTiter-Glo ATP assay (Promega).

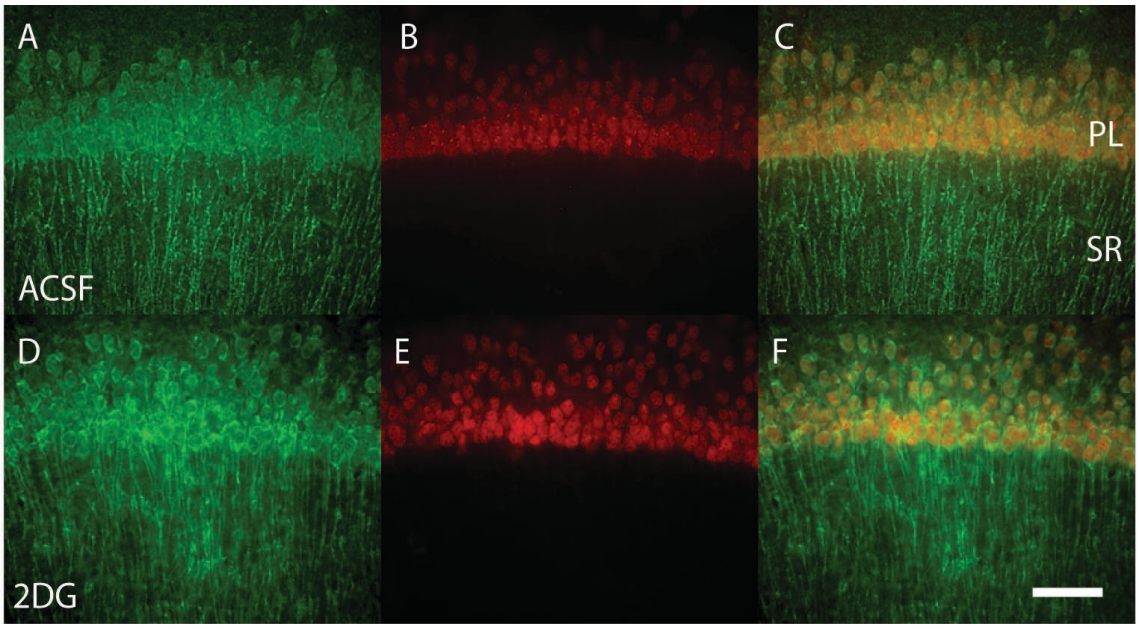

Figure 2.2. 2DG activates AMPK in CA1 hippocampus

A) Basal levels of p-AMPK in CA1 hippocampus. **B**) Neu-N labeling of CA1 stratum pyramidale (PL). **C**) Overlap of panels A and B. **D**) 2DG activates AMPK in cell bodies of the pyramidal layer (PL) and dendrites of the stratum radiatum (SR). **E**) Neu-N labeling in 2DG-treates slices. **F**) Overlap of panels D and E. Anti-phospho-Thr172-AMPK immunoreactivity is displayed in green. Neu-N is displayed in red. Scale bar: 10 μm.

activator 2DG. The increased diffuse staining throughout the SR in the presence of 2DG may be due to activation of AMPK in the glial compartment. However, the observed activation of AMPK in the dendrites demonstrates that hippocampal neurons contain the necessary components to activate AMPK upon administration of known AMPK inducers.

The mTOR pathway is activated in CA1 within 5 minutes of High Frequency Stimulation (HFS) and is necessary for expression of L-LTP(Cammalleri et al., 2003; Tsokas et al., 2007). Activation of mTOR can be monitored by phosphorylation of its downstream targets and so we looked at the mTOR substrate p70-S6Kinase (p70S6K) and its substrate ribosomal protein S6 (rpS6) due to their established role directly downstream of the mTOR kinase. We confirmed the observations of Tsokas *et al.* that HFS of the Schaeffer Collaterals induces phosphorylation of the mTOR cascade components p70S6K and rpS6(Tsokas et al., 2007) as judged by western blotting of protein from stimulated or un-stimulated slices (Fig. 2.3). Consistent with the hypothesis that AMPK activation suppresses mTOR signaling in the hippocampus, high frequency stimulation failed to induce rpS6 or p70S6K phosphorylation in the presence of 2DG.

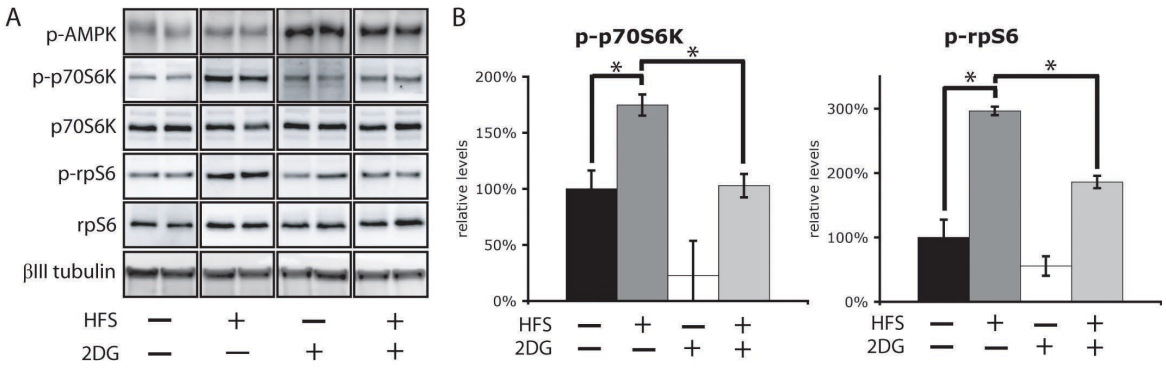

Figure 2.3. AMPK activation represses the mTOR pathway

A) HFS was delivered to the Schaeffer-collateral pathway of slices in the presence or absence of 10mM 2DG for 30 minutes. Slices were then subjected to western blot analysis using anti-phospho-Thr389-p70S6K, anti-p70S6K, anti-phospho-Ser235/236-rpS6 or anti-rps6 antibody. Representative western blots of duplicate lanes and quantification (**B**) of 12 samples per condition are shown. Error bars show standard error of the mean (s.e.m). ∗$p<0.05$

AMPK activation prevents L-LTP expression.

Given the necessity of mTOR signaling for L-LTP expression, we predicted that AMPK activation should prevent L-LTP expression. To test this hypothesis, we initially tested the effects of the AMPK activator 2DG on LTP that was induced using 2 different mTOR dependent paradigms: HFS and Theta Burst Stimulation (TBS)(Alarcon et al., 2004). HFS or TBS was delivered between CA1 and CA3 in the Schaeffer-collateral bundle in the presence or absence of 2DG and field excitatory post-synaptic field potentials (fEPSPs) were recorded in the stratum radiatum of CA1. Following either HFS or TBS, the induction step of LTP was indistinguishable between 2DG treated and control slices (Fig. 2.4A and 2.4B, respectively), consistent with this step being mTOR-independent(Tang et al., 2002). However 60 minutes post-stimulation the 2DG-treated slices failed to maintain L-LTP, which falls to 10% of untreated over the course of 3 hours in the HFS paradigm and 30% in the TBS paradigm (Figure 2.4).

We reasoned that if 2DG inhibits L-LTP via AMPK activation, then other AMPK activators should also inhibit L-LTP. Therefore we tested whether the AMPK activators metformin(Zhou et al., 2001) or 5-aminoImidazole-4-carboxamide ribonucleoside (AICAR)(Corton et al., 1995) could also inhibit L-LTP induced by TBS or HFS. Figure 2.4A and 2.4B show that similar to 2DG, 5μM metformin suppressed L-LTP but did not impact LTP induction. Figure 2.4B also shows that AICAR inhibits L-LTP induced by TBS. Therefore, three independent and structurally unrelated

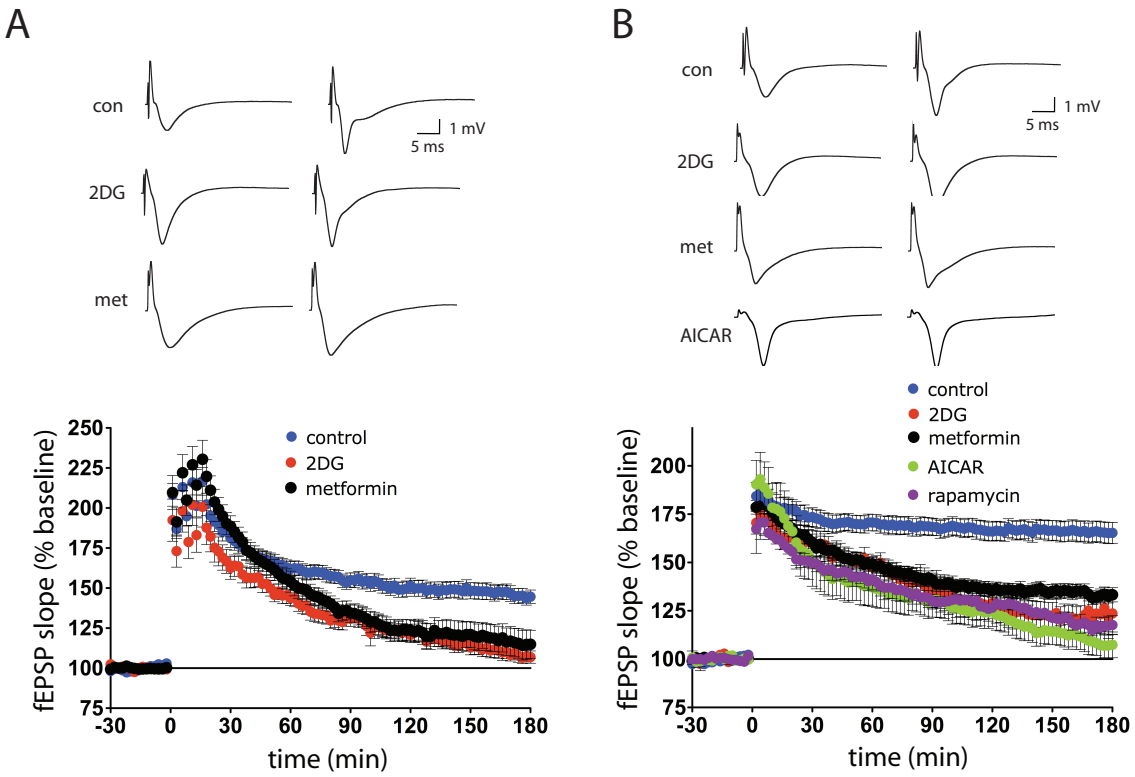

Figure 2.4. AMPK activation inhibits L-LTP expression. **A**) AMPK activation inhibits LTP induced by HFS. 10mM 2DG (n=10) reduces L-LTP to 10% of control (n=20) (p=0.034). 5μM metformin (n=8) reduces L-LTP to 40% of control (p=0.028). **B**) AMPK activation inhibits LTP induced by TBS. 10mM 2DG reduces L-LTP (p=0.022, n=7) to 30% of control (n=13), 5μM metformin reduces L-LTP (p=0.042, n=12) to 51% of control. 1mM AICAR reduces L-LTP (p=0.0025, n=8) to 11% of control. A and B) Inset: representative fEPSP traces shown were taken 4 minutes prior and 180 minutes after stimulation. Error bars show s.e.m.

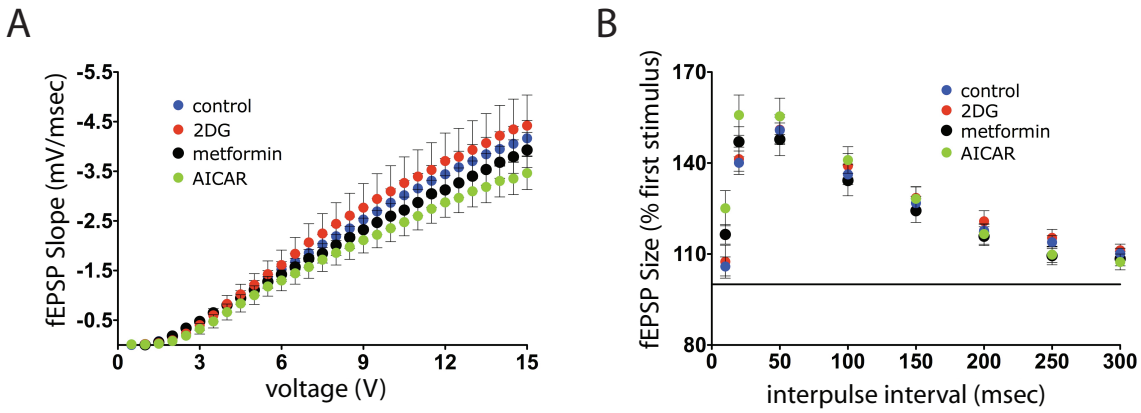

Figure 2.5. AMPK agonists do not affect basic synaptic transmission

A) Input-output relationships for Schaeffer-collateral stimulation were not significantly different between ACSF (n=27), 10mM 2DG (p=0.702)(n=14), 5μM metformin (p=0.573)(n=17) or 1mM AICAR (p=0.223)(n=8). **B)** Paired Pulse Facilitation is not affected by 2DG (p=0.6006; n=11), metformin (p=0.9076)(n=17), or AICAR (p=0.2061)(n=8) compared to ACSF alone (n=14). Results are plotted as the ratio of fEPSP slopes (2^{nd} stimulus/1^{st} stimulus X100) as a function of interpulse interval (0-300msec). Error bars show s.e.m.

activators of AMPK eliminated L-LTP that was elicited by two different stimulation paradigms. Importantly, 2DG, AICAR and metformin did not affect synaptic transmission *per se* because there was no significant difference in the input/output relationship of the fEPSP slope magnitude as a function of stimulus voltage when compared to ACSF alone (Fig. 2.5A).

If AMPK activation works to suppress mTOR signaling to inhibit L-LTP, then the effects of AMPK activation would be predicted to be postsynaptic given that LTP induction results in dendritic rather than axonic mTOR activation(Tsokas et al., 2007). To test this we assessed whether 2DG, metformin or AICAR affected Paired-Pulse Facilitation (PPF) (a protocol widely used to assess a presynaptic component of a response to a stimulation or compound) (Dobrunz and Stevens, 1997). The PPF protocol consists of an initial stimulation followed by a second stimulation after a given interval. The second stimulus evokes a greater response (measured as fEPSP slope) than the initial stimulus due to residual Ca^{2+} in the presynaptic terminal which facilitates an increased amount of neurotransmitter release. If a compound inhibits release, there will be less neurotransmitter released initially, and therefore the second stimulus elicits a greater release of neurotransmitter and produces an increased postsynaptic response. Figure 2.5B shows that there was little difference in PPF between control slices and the AMPK agonists. This result is consistent with the hypothesis that AMPK activation acts to inhibit mTOR signaling to suppress L-LTP.

If AMPK activation is necessary for 2DG, metformin or AICAR to inhibit L-LTP, then addition of an AMPK inhibitor should prevent 2DG, metformin or AICAR from repressing LTP. Figure 2.6 shows that in the presence of the potent AMPK inhibitor compound-C (Zhou et al., 2001), 2DG treatment failed to activate AMPK (Fig. 2.6D). Accordingly, in the presence of compound-C, 2DG had no effect on TBS-induced LTP expression (Fig. 2.6A). AMPK inhibition using a structurally unrelated molecule, ara-A (Henin et al., 1996) also prevented 2DG from repressing LTP expression (Fig. 2.6A). LTP suppression by metformin was similarly blocked by either compound-C or ara-A (Fig. 2.6B). Finally, LTP suppression by the AMPK activator ACIAR was prevented by compound-C (Fig. 2.6C). These data with three AMPK activators and two inhibitors support the hypothesis that LTP expression in the hippocampus is under metabolic control via the metabolic sensor AMPK.

LTP inducing stimuli (e.g. TBS and HFS) rapidly activate postsynaptic translational machinery in order to produce key proteins involved in the induction and maintenance of LTP (Casadio et al., 1999; Guzowski et al., 2000). In the presence of protein synthesis inhibitors, LTP-inducing stimuli produce short-term potentiation, yet fail to produce lasting LTP (Krug et al., 1984; Frey et al., 1988). However, LTP is unaffected if protein synthesis inhibitors are added just after the tetanus (Cammalleri et al., 2003). Late-Phase LTP elicited by either HFS or TBS is eliminated by the mTOR inhibitor rapamycin. Further, Cammalleri *et al.* demonstrated a critical time window of rapamycin sensitivity in inhibition of LTP: exposure to rapamycin during tetanic stimulation is sufficient to prevent expression

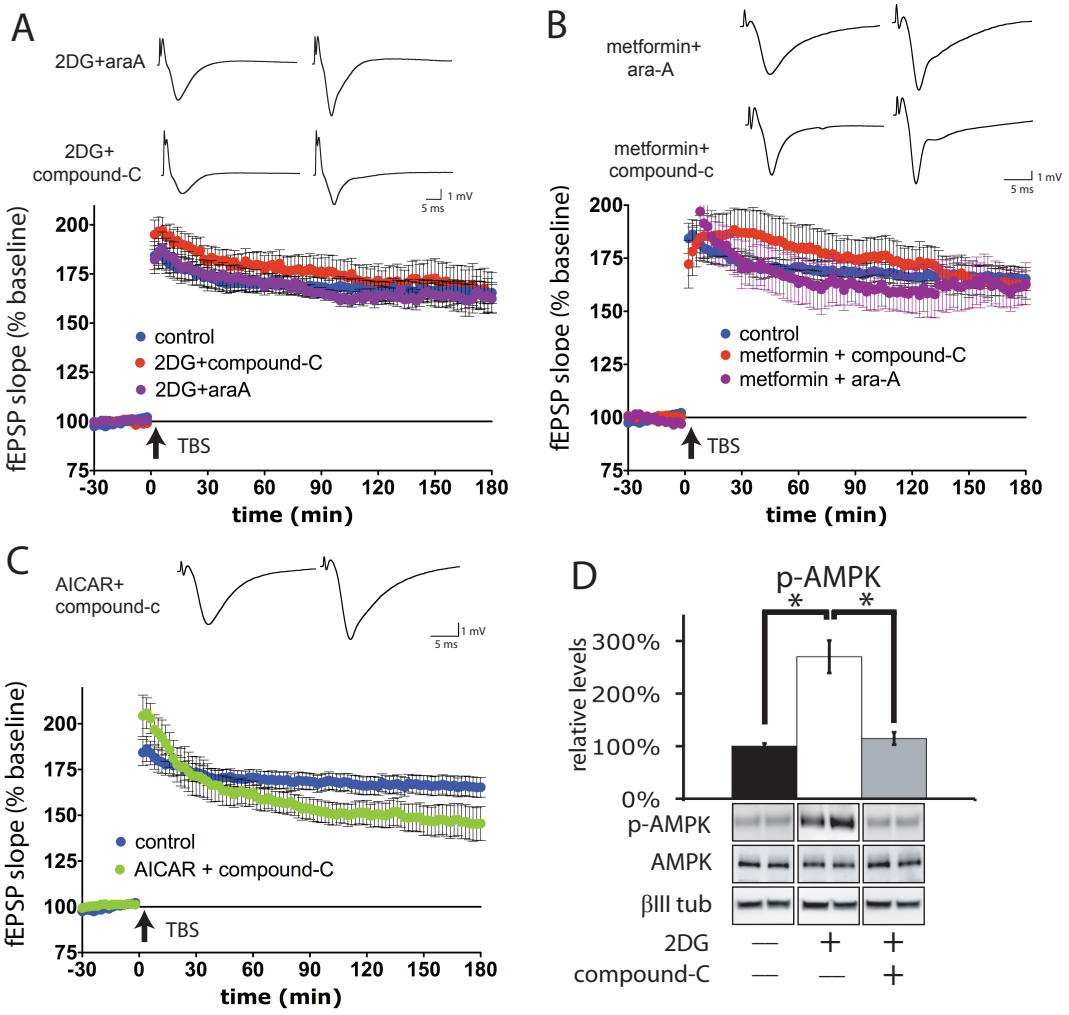

Figure 2.6. AMPK antagonists rescue LTP expression. A) 1μM compound-C (n=9) or 100μM araA (n=6) prevent 2DG-mediated loss of LTP(A) and metformin-mediated loss of LTP (**B**). **C**) 1μM compound-C prevents AICAR-mediated loss of LTP (n=6). A-**C**) Inset: representative fEPSP traces shown were taken 4 minutes prior and 180 minutes after stimulation. **D**) 1μM compound-C abolishes 2DG-mediated AMPK activation. Slices were incubated in ACSF (n=8), 10mM 2DG (n=5) or both 10 mM 2DG and 1μM compound-C (n=6) for 30 minutes. *p=0.0002. Error bars show s.e.m.

of LTP two hours later(Cammalleri et al., 2003). If AMPK activation represses mTOR signaling to inhibit LTP, then metformin exposure solely during the stimulation protocol should be sufficient to prevent LTP expression. Figure 2.7B shows that metformin administration during TBS followed by wash-out immediately after stimulation generates a loss of LTP similar to that seen in the continued presence of metformin (compare to Fig. 2.4). To show that this was not due to persistence of metformin after wash out, metformin was added and washed out prior to stimulation, with no effect on LTP (Fig. 2.7A). Also, addition of metformin after stimulation for the remaining three hours had no significant effect on LTP magnitude (Fig. 2.7C). This result suggests that a critical period of metformin/AMPK sensitivity exists during the induction phase of LTP, which overlaps with the mTOR dependent period defined by Cammalleri *et al*.

To explore the role of mTOR in AMPK modulation of LTP we took advantage of the observation that treating slices with the AMPK inhibitors compound-C or ara-A, in the absence of 2DG or metformin, results in hyper-potentiation (Fig. 2.8A). If AMPK inhibition results in heightened LTP due to de-repression of mTOR signaling then in the presence of the mTOR inhibitor rapamycin, compound-C should have no effect. Figure 2.8B shows that as reported by others, rapamycin eliminates LTP induced by TBS. Compound-C fails to elevate LTP in the presence of rapamycin suggesting that AMPK modulation of LTP requires mTOR signaling and that mTOR is downstream of AMPK. In aggregate, these physiological measurements, together

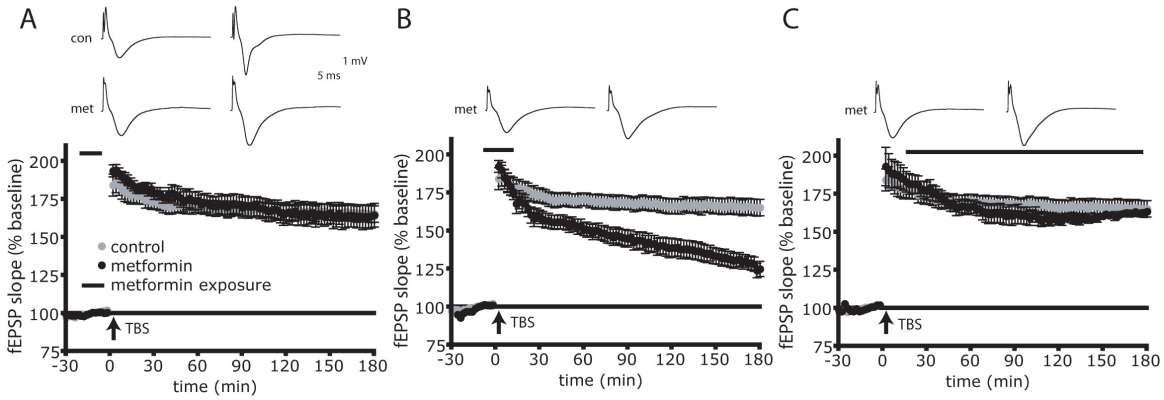

Figure 2.7. AMPK activation suppresses LTP within a time-restricted window. **A**) 5μM metformin was added 20 minutes prior to TBS and washed out immediately prior to TBS (n=8). LTP at 180 minutes post TBS was equal to control (n=17), **B**) metformin was added immediately prior to TBS and washed out immediately after TBS (n=8). LTP was reduced to 30% of control (p=0.0172). **C**) metformin was added 5 min after stimulation for the duration of the experiment (n=5). LTP at 180 minutes was similar to control. Control data in Fig. 5A is reproduced in Fig. 5B and Fig. 5C for comparison. Inset: Representative fEPSP traces shown were taken 4 minutes prior to and 180 minutes after TBS. Error bars show s.e.m.

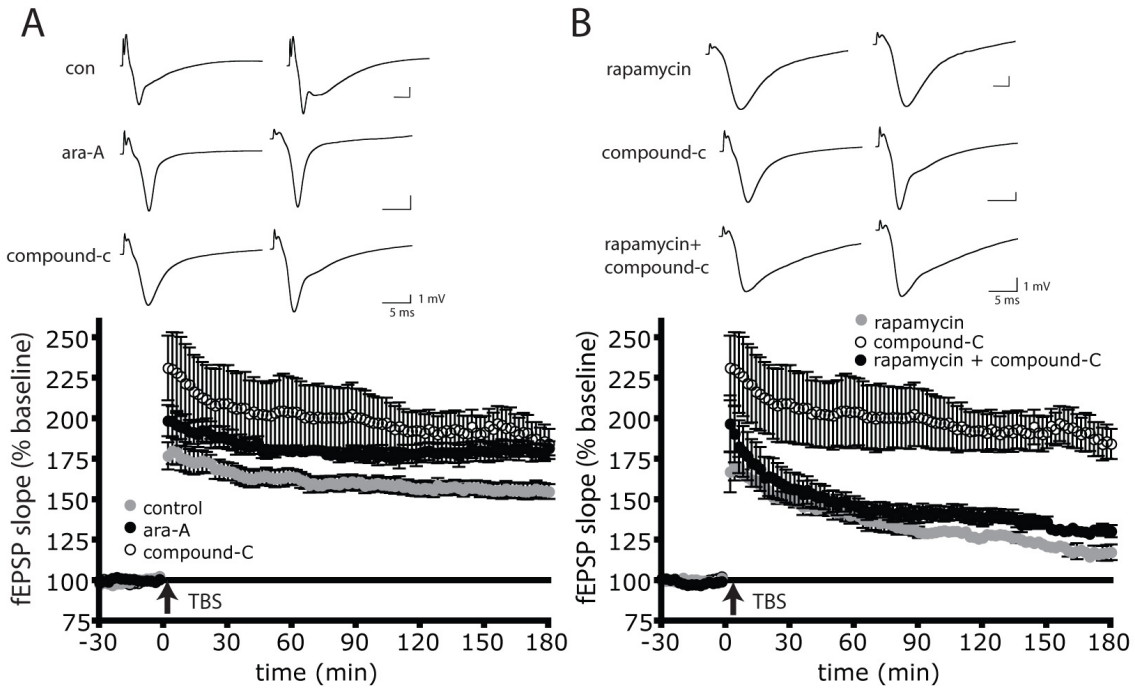

Figure 2.8. AMPK regulation of LTP is rapamycin sensitive. **A**) 1μM compound-C (n=8, p=0.0069) or 100μM ara-A (n=8, p=0.0045) results in heightened potentiation after TBS compared to ACSF alone (n=14). **B**) 1μM rapamycin results in suppression of LTP to 33% of control (n=4, p=0.0097). 1μM compound-C in the presence of rapamycin fails to significantly enhance LTP above rapamycin alone (n=9, p=0.2706). Control data in Fig. 6A is reproduced in Fig. 6B for comparison. Inset: Representative fEPSP traces shown were taken 4 minutes prior to and 180 minutes after TBS. Error bars show s.e.m.

with the molecular analyses suggest a model whereby AMPK activation inhibits maintenance of LTP at least in part, through suppression of the mTOR pathway.

2.4. Discussion

The work presented here suggests that LTP is under metabolic control via the energy sensor AMPK. This is supported by the observation that three structurally distinct activators of AMPK (2DG, metformin and AICAR) suppress LTP expression in two different LTP paradigms (HFS and TBS) (Fig. 2.4). Secondly, LTP suppression by 2DG, metformin and AICAR can be overcome by two inhibitors of AMPK that are structurally and mechanistically distinct (compound-C and ara-A) (Fig. 2.6). It is likely that the suppressive effects of AMPK activation work at least in part through the mTOR pathway. This hypothesis is supported by the observation that the AMPK activator 2DG suppresses phosphorylation and activation of the downstream mTOR pathway components, p70S6K and rpS6 upon High Frequency Stimulation (Fig. 2.3). Secondly, rapamycin prevents the enhancement of LTP by the AMPK inhibitor compound-C suggesting that AMPK is upstream of mTOR in regulating LTP (Fig. 2.8). Thirdly, AMPK activators fail to alter PPF, which suggests a post-synaptic mode of action, consistent with the site of mTOR activity(Tang et al., 2002; Tsokas et al., 2005). Fourthly, AMPK activation restricted to the stimulation period is sufficient to prevent LTP expression three hours later, an observation consistent with the time period required for mTOR activity to maintain LTP(Cammalleri et al., 2003). Data presented here connects AMPK to neuronal mTOR signaling thereby linking energy

and metabolic status to LTP. In conjunction with published work describing the role of mTOR in LTD(Hou and Klann, 2004), our work suggests that neuronal plasticity in general is under the direct control of cellular energy metabolism through AMPK.

Late-Phase LTP requires MAPK/ERK and PI3/AKT signaling that activates the mTOR pathway and dendritic protein translation(Tsokas et al., 2007). LTP also has a requirement for gene transcription including a critical role for the cAMP Response Element Binding protein (CREB) activity and BDNF expression(Casadio et al., 1999; Guan et al., 2002; Barco et al., 2005). Interestingly, CREB and mTORC1 signaling is known to be modulated by AMPK in peripheral tissue(Shaw et al., 2005; Horike et al., 2008; Thomson et al., 2008), suggesting that AMPK may also signal to CREB to impact plasticity.

In addition to positively regulating protein synthesis, mTOR contributes to a variety of other cellular processes, some of which have been linked to neuronal function. mTOR activity impacts mitochondrial function by increasing oxidative capacity and promoting the transcription of key components of the electron transport chain (Schieke et al., 2006; Cunningham et al., 2007). Consequently, hyperactive mTOR signaling increases the generation of reactive oxygen species (ROS), which are required for LTP (Knapp and Klann, 2002). Hence it is possible that inhibition of mTOR signaling by AMPK activation could suppress LTP via a reduction in ROS production.

Additionally, mTOR acts to inhibit glycogen synthase kinase 3β (GSK3β) signaling (Meske et al., 2008). Given the potential role of GSK3β in enhancing LTP

(Peineau et al., 2008), activation of GSK3β via mTOR inhibition may also contribute to loss of LTP in our system. Nevertheless, our results suggest that the negative regulation of mTOR signaling by AMPK links energy sensing to plasticity.

Several reports have demonstrated an increased fEPSP when glucose is replaced by 2DG for some time period (Tekkok and Krnjevic, 1996; Xu and Krnjevic, 2001; Godfraind and Xu, 2006). In these studies, 2DG application results in a decreased fEPSP slope, which is reversed upon switching back to normal ACSF leading to potentiation. Zhao *et al*. reported that 2DG suppresses synaptic transmission through release of adenosine and activation of pre-synaptic adenosine receptors (Zhao et al., 1997). This would imply a pre-synaptic mode of action for 2DG and would result in an altered PPF (compared to control) at CA1. In our hands we do not see an effect on PPF by 2DG or metformin, which is consistent with the hypothesis that the effects of AMPK are primarily post-synaptic. It is therefore unlikely that the negative regulation of LTP by AMPK works through adenosine release. The discrepancy between the results reported by Zhao *et al*. and our work is most likely due to the fact that Zhao *et al*. replace glucose with 2DG in their ACSF. We administer 10mM 2DG in the presence of 25mM glucose in ACSF and this results in a tempering of ATP production to 69% of ACSF alone (Fig. 2.1C), yet does not alter baseline synaptic responses out to four hours of recording (data not shown). This major difference in paradigm most likely explains why Zhao *et al*. observe a pre-synaptic effect by 2DG via adenosine release whereas our 2DG effects are post-synaptic, as judged by the lack of change in PPF.

Our findings that AMPK controls LTP may provide a cellular and molecular basis for the observations that increased glucose availability enhances learning and memory(Gold, 1986; Azari, 1991; Kopf and Baratti, 1996; McNay et al., 2001). During exploratory behavior, plasma brain glucose levels drop. When glucose levels are maintained with glucose injections, rats perform better in a passive avoidance test compared to controls. Rats injected with glucose immediately after training in this same avoidance test showed enhanced retention of memory 24 hours later compared to controls. However, injection 1 hour after training had no effect on retention(Gold, 1986). Similarly, intra-amygdala glucose injection helped the extinction of conditional place preference only when glucose administration occurred immediately after training but not two hours later(Schroeder and Packard, 2003). The ability of glucose to impact learning and memory tasks implies that learning and memory mechanisms are under metabolic control.

The inability of glucose to improve behavioral outcomes when administered 1-2 hours after training as compared to directly after is consistent with our findings that AMPK activation must occur during the stimulation period to inhibit LTP. mTOR activity is not continuously required after LTP induction in order to sustain LTP but instead is necessary only during the stimulus period(Cammalleri et al., 2003). This suggests that *de novo* protein synthesis upon induction generates products required to maintain LTP in the absence of continued stimulation. In keeping with this model, our data shows that a narrow time window exists during which AMPK activation is sufficient to suppress LTP: the presence of metformin just during the stimulus

period followed by wash-out prevents LTP maintenance whereas addition of metformin immediately after stimulation for the duration of the experiment has no effect on LTP (Fig. 2.7). This result suggests that acute suppression of mTOR via AMPK activation during the short induction phase of LTP prevents expression of chronic LTP.

It is tempting to consider that AMPK modulation of LTP *in vitro* reflects AMPK modulation of learning and memory *in vivo*. Indeed Dash *et al.* demonstrated that activation of neuronal AMPK with hippocampal injections of AICAR reduced long-term memory and this was associated with reduced phosphorylation of mTOR cascade components. Importantly, glucose injection directly into the rat hippocampus improved long-term spatial memory in the Morris Water Maze and this was associated with decreased AMPK phosphorylation/activation and increased activity of the mTOR cascade(Dash et al., 2006). The fact that AMPK activity can be reduced in the hippocampus and result in heightened long-term memory demonstrates that under physiological conditions, latent AMPK activity tempers long-term memory mechanisms. This notion is consistent with the observation that application of AMPK inhibitors (compound-C and ara-A) in the absence of AMPK agonists result in heightened LTP (Fig. 2.8A). Therefore we argue that metabolic regulation of LTP and long-term memory is not only important under pathological conditions where energy stress may be present but is also important under physiological conditions. The results presented here along with published work

supports a model whereby neuronal energy status controls LTP maintenance through the action of AMPK on mTOR dependent dendritic protein translation.

The possibility of controlling plasticity through AMPK may offer a route to therapeutic intervention in certain neurological disorders. Tuberous Sclerosis is caused by mutations in the genes encoding either TSC1 or TSC2 and is often associated with autism, mental retardation and epilepsy(1993; van Slegtenhorst et al., 1997). Loss of TSC1/2 function results in heightened mTOR activity and inappropriate LTP induction upon a single high frequency stimulation in slice experiments(Ehninger et al., 2008). Importantly, addition of the mTOR inhibitor rapamycin reverses many of the behavioral deficits in TSC2 mutant mice and prevents inadvertent maintenance of LTP. These intriguing results indicate that pharmacological suppression of heightened mTOR signaling in Tuberous Sclerosis patients might be of therapeutic value. Our work suggests that the widely used anti-type II diabetes drug metformin can suppress mTOR signaling through activation of AMPK in the hippocampus. Metformin is used by around 35 million people in the U.S alone, is able to cross the blood-brain barrier and has few contra-indications when prescribed appropriately(Zhou et al., 2001; McCullough et al., 2005; Ma et al., 2007). The absence of a functional TSC1/2 complex prevents AMPK from inhibiting mTOR signaling through phosphorylation of TSC2, however work by Gwinn *et al.* shows that AMPK can also suppress mTOR through the targeting of RAPTOR (Gwinn et al., 2008). Therefore, heightened mTOR signaling in TSC patients could feasibly be attenuated therapeutically through the action of metformin on AMPK and RAPTOR.

We show that the glycolytic inhibitor 2DG is a potent activator of AMPK, prevents stimulation-induced mTOR activation, and suppresses LTP in an AMPK-dependent manner. These properties are of particular interest because we recently showed that 2DG retards epileptogenesis in the rat electrical kindling model of temporal lobe epilepsy(Garriga-Canut et al., 2006). This modulation of activity dependent plasticity was associated with reduced BDNF and TrkB expression and increased recruitment of the metabolic sensor CtBP to the transcriptional repressor NRSF/REST(Roopra et al., 2001; Chinnadurai, 2002). BDNF is an upstream component of the mTOR cascade and is sufficient to enhance potentiation at CA1 synapses in a rapamycin sensitive manner(Tang et al., 2002). Therefore mTOR dependent LTP may be regulated by metabolism via a transcription dependent mechanism through CtBP and via a post-translational mechanism through AMPK. It will be interesting to test whether metformin, working through AMPK has the same effects as 2DG working through CtBP in retarding kindling and epileptogenesis. In summary, we show that plasticity is under metabolic control through the activity of the master energy regulator, AMPK.

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Chapter 3

AMPK eliminates aberrant LTP in $TSC2^{+/-}$ mice

Notes on Collaborations

Electrophysiology was conducted in collaboration with Ken O'Riordan.

Immunoprecipitations and western blotting were done in collaboration with Allison Kirchner.

Abstract

The mammalian target of rapamycin (mTOR) signaling pathway regulates a variety of cellular processes involved in growth, survival, proliferation, and neural plasticity. In tuberous sclerosis complex (TSC), mutations in the tumor suppressor genes encoding tuberin (TSC1) or hamartin (TSC2), result in overactivation of mTOR signaling which manifests with systemic tumor formation, mental retardation, and epilepsy. The activity of mTOR is positively regulated by the presence of its obligate binding partner, the Regulatory Associated Protein of TOR (RAPTOR), a scaffolding protein that integrates multiple intracellular and extracellular signals. The energy sensor, 5' AMP-activated protein kinase (AMPK) inhibits mTOR-dependent hippocampal plasticity in wild-type mice, but it is unknown whether AMPK can communicate to mTOR in $TSC2^{+/-}$ mutant hippocampus. Here we show that AMPK activation eliminates aberrant LTP in $TSC2^{+/-}$ mutant hippocampus. AMPK activation results in a significant increase in phosphorylated RAPTOR selectively in $TSC2^{+/-}$ mutant but not wild-type hippocampus. Accordingly, AMPK activation in $TSC2^{+/-}$ hippocampus caused dissociation of the mTORC1 signaling complex in $TSC2^{+/-}$ mutant but not wild-types. These data suggest that AMPK primarily signals through TSC1/2 in wild-type hippocampus, however in $TSC2^{+/-}$ mutants, AMPK is capable of impacting mTOR signaling via RAPTOR inactivation. Thus, AMPK could represent a potential therapeutic target for neurological disorders associated with hyperactive mTOR signaling, such as TSC.

3.1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder characterized by the formation of benign tumors, mental retardation, autism, and epilepsy(Curatolo et al., 2002; Crino et al., 2006). With a prevalence of approximately 1 in 6,000 births, TSC is caused by mutations in either of the tumor suppressor genes encoding hamartin (TSC1) or tuberin (TSC2). Reduced TSC1/2 function results in derepression and hyperactivation of mTOR signaling. Since mTOR activity positively controls cellular survival and growth, overactive mTOR signaling results in dysregulated cell growth, tumor formation, and neurological dysfunction.

TSC1/2 is a GTPase activating protein complex that receives input from at least three major pathways: the PI3K-Akt pathway, the ERK-MAPK pathway, and the LKB1-AMPK pathway(Kwiatkowski and Manning, 2005). The PI3K and ERK pathways inactivate TSC1/2 activity, leaving the TSC1/2 effector protein, Rheb, in an active GTP-bound state that sustains mTOR activity. The LKB1-AMPK pathway, however, is the primary activating input to TSC1/2 leading to mTOR inhibition(Inoki et al., 2003). In addition to signaling through TSC1/2, AMPK can also inhibit mTOR by phosphorylating Regulatory Associated Protein of TOR (RAPTOR) *in vitro*(Gwinn et al., 2008). Phosphorylation of RAPTOR induces 14-3-3 binding, which leads to its dissociation from mTORC1 and consequent inhibition of the mTOR complex(Gwinn et al., 2008). This alternate pathway is heightened in cells lacking TSC1/2, suggesting that inactivating mutations in TSC1/2 would lead to increased signaling through AMPK-RAPTOR.

In the hippocampus, the mTOR pathway regulates the activity-dependent translation of numerous proteins involved in the induction and expression of long-lasting forms of synaptic plasticity. Dysregulated protein translation is implicated in a growing number of neurological disorders including autism, fragile X syndrome, Alzheimer's disease, and epilepsy. In mouse models of tuberous sclerosis, rapamycin treatment improved median survival and ameliorated neurofilament and myelination abnormalites(Meikle et al., 2008). Ehninger *et al*. demonstrated that learning and memory deficits in mice with inactivating heterozygous mutations in TSC2 ($TSC2^{+/-}$) could be ameliorated with rapamycin. These authors demonstrated that $TSC2^{+/-}$ mice had 'aberrant induction' of LTP due to hyperactive mTOR signaling, and that the aberrant LTP was reduced with rapamycin. These data suggest that modulation of mTOR may be therapeutic for the treatment of tuberous sclerosis.

We show that AMPK activation with metformin eliminates aberrant LTP in $TSC2^{+/-}$ hippocampus. Metformin treatment resulted in significantly higher phosphorylation of RAPTOR in $TSC2^{+/-}$ but not wild-type hippocampal slices. This increased P-RAPTOR resulted in a consequent dissociation of the RAPTOR-mTORC1 complex. These results indicate that AMPK activation could potentially serve to reduce mTOR activity in TSC.

3.2. Methods

Electrophysiology

Methods were previously described in detail (Potter et al., 2010). All electrophysiology was performed on male 5-10wk old $TSC2^{+/-}$ and wild-type littermate mice (C57BL/6 background). Field EPSPs were recorded from CA1 *stratum radiatum*, with ACSF-filled recording electrodes (1.4-2 MΩ). Baseline synaptic transmission was assessed for each individual slice by applying gradually increasing stimuli (0.5V – 20V, 25nA – 2.0μA, A-M Systems model 2200 stimulus isolator, Carlsborg, WA) to determine the input:output relationship. All subsequent experimental stimuli were set to an intensity that evoked 50% of maximum fEPSP slope. ***LTP induction -*** LTP was induced with theta burst stimulation applied to the Schaeffer-collaterals and field EPSPs were measured in *stratum radiatum*. Theta burst stimulation consisted of a single stimulation train consisting of 10 bursts (referred to as 1XTBS in text). Each burst contained 4 stimulations at 100 Hz with an interburst interval of 200 msec.

Immunoprecipitation from hippocampal tissue

Hippocampal tissue was homogenized in ice-cold CHAPS lysis buffer (in mM) (150 NaCl, 40 HEPES, 2 EDTA, 10 pyrophosphate, 10 glycerophosphate, 4 orthovanadate, 0.3% CHAPS). Following quantification, equal amounts of cell extract were used for immunoprecipitation with 0.5 μg anti-mTOR antibody. For sham, an antibody (0.5μg) specific to the unrelated protein LIN28 (Cell Signaling) was used. Samples

were incubated on a rotator at 4°C for 2 hours. 20μg of whole cell extract was used for quantitative comparisons. 20 μg of washed Sepharose G beads were added to each sample and spun on the rotator for an additional hour at 4°C. Samples were spun down briefly, washed, and combined with SDS gel loading buffer.

3.3. Results

AMPK activators block aberrant LTP in TSC2$^{+/-}$ CA1 hippocampus.

At Schaeffer collateral-CA1 synapses, TSC2$^{+/-}$ mouse hippocampal slices express LTP from a single theta burst stimulation (1XTBS); a stimulus which fails to elicit lasting potentiation in hippocampal slices from wild-type littermates (WT) (Ehninger et al., 2008)(Figure 3.1a). Our previous work showed that AMPK can suppress LTP in wild-type hippocampus(Potter et al., 2010). We therefore sought to assess whether AMPK activation could suppress the 1XTBS-induced LTP in TSC2$^{+/-}$ hippocampus. TSC2$^{+/-}$ slices stimulated in the presence of the AMPK activator, metformin, failed to express 1XTBS LTP (Figure 3.1a). Furthermore, addition of the AMPK antagonists, ara-A or compound C, prevented metformin from suppressing aberrant LTP (Figure 3.1b and 3.1c, respectively), indicating that metformin impacted LTP through AMPK activation.

AMPK can inhibit mTORC1 either through TSC1/2 or directly by altering binding of the obligate partner protein, Regulatory Associated Partner of TOR (RAPTOR)(Gwinn et al., 2008; Carriere et al., 2010). Given the loss of TSC2 in these mice, we hypothesized that in TSC2$^{+/-}$ hippocampus, AMPK may selectively signal through RAPTOR to reduce mTORC1 activity and block the aberrant LTP observed in TSC2$^{+/-}$ hippocampal slices. Western blot analysis shows that whereas metformin significantly increased phosphorylation of AMPK in both WT and TSC2$^{+/-}$ hippocampus, it increased RAPTOR-S792 phosphorylation in TSC2$^{+/-}$ but not WT hippocampus (Figure 3.2a). In cultured cells, phosphorylation of RAPTOR-S792 by

AMPK causes its dissociation from mTORC1 and consequent reduction in mTORC1 activity (Gwinn et al., 2008). To test whether metformin treatment caused dissociation of RAPTOR from mTORC1 in WT and/or $TSC2^{+/-}$ hippocampi, mTORC1 was immunoprecipitated from hippocampal homogenates and the precipitate probed for RAPTOR. Figure 3.2b shows that $TSC2^{+/-}$ hippocampus exhibits a robust reduction of RAPTOR/mTOR interaction in response to metformin treatment. Unlike in cultured non-neuronal cells and myotubule cells(Gwinn et al., 2008), wild type hippocampus does not display an AMPK-dependent loss of RAPTOR/mTOR interaction (Figure 3.2b). Together, these data support the hypothesis that AMPK blocks aberrant LTP in $TSC2^{+/-}$ slices, at least in part, via phosphorylation of RAPTOR causing dissociation from mTORC1.

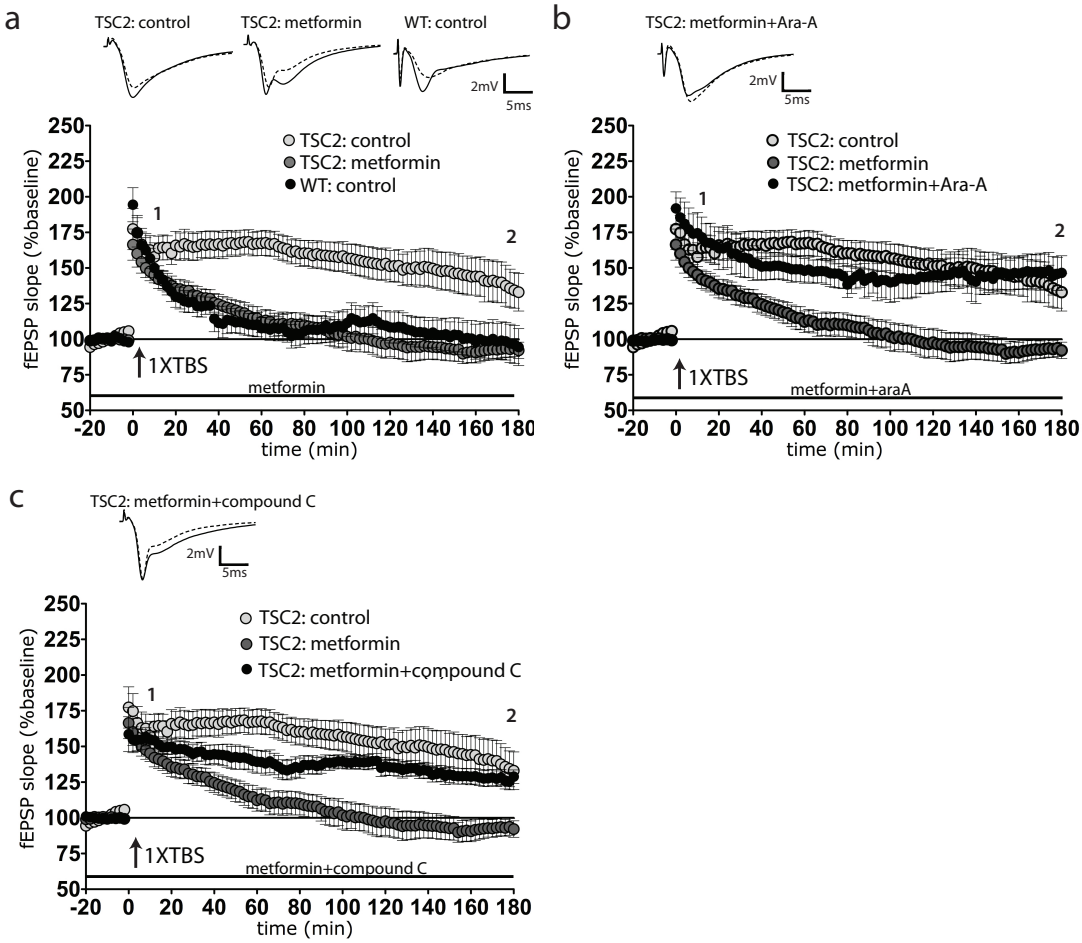

Figure 3.1. AMPK activation blocks 1XTBS-LTP *a*. 1XTBS applied to the Shaffer collateral fiber bundle elicits lasting LTP in $TSC2^{+/-}$ slices [133.0±15.0%, n=9(5)]; whereas 1XTBS produces only short-term potentiation in WT slices [94.4±12.9%, n=7(5)]. This aberrant LTP is eliminated with metformin (5μM, present throughout recording)[92.1±5.8%, n=7(5)]. ***b***. The effect of metformin is blocked with the AMPK inhibitor, Ara-A (100μM)[146.6±12.0%, n=8(5)]. ***c***. The effect of metformin is blocked with the AMPK inhibitor, compound C (1μM)[128.9±4.7%, n=7(4)]. Representative traces: solid line is 4 minutes after TBS '1', dashed line is at the end of the recording '2'.

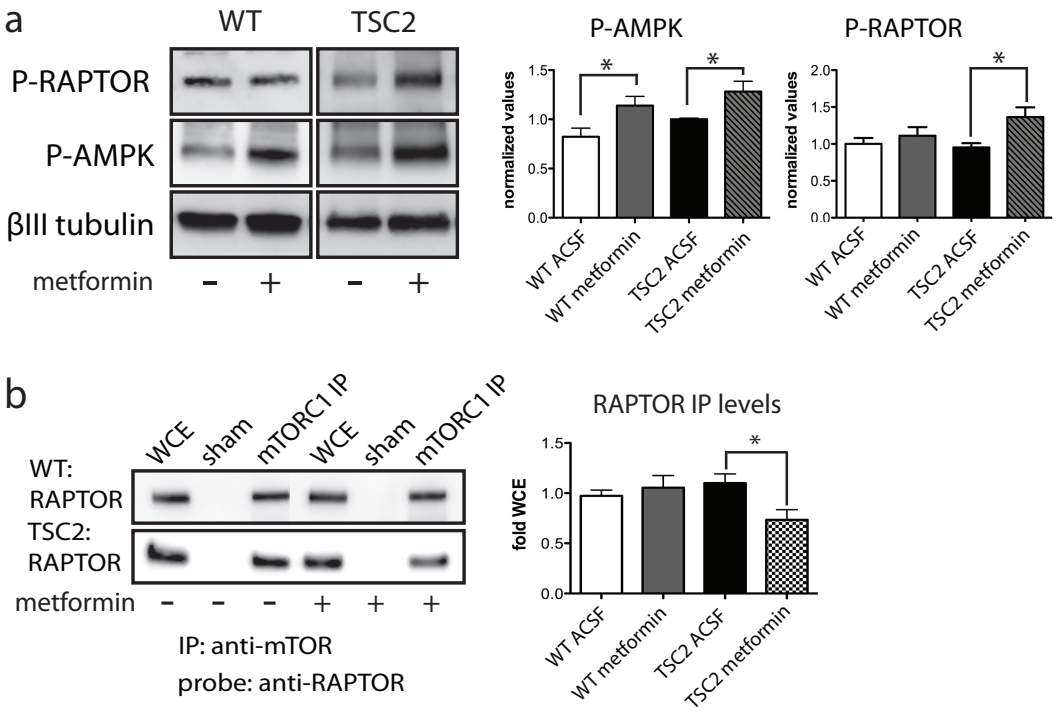

Figure 3.2. Metformin selectively reduces RAPTOR-mTORC1 association in TSC2$^{+/-}$ versus WT hippocampus. ***a***. Metformin activates AMPK in WT (n=14, p=0.0198) and TSC2$^{+/-}$ slices (n=14, p=0.0145), yet selectively phosphorylates RAPTOR in TSC2$^{+/-}$ hippocampal slices (n=11, p=0.0082). ***b.*** Metformin treatment significantly reduces RAPTOR-mTORC1 complexing selectively in TSC2$^{+/-}$ hippocampus (n=5, p=0.0282). WCE-whole cell extract. *p<0.05

3.4. Discussion

We found that the AMPK activator and anti-diabetic drug metformin potently reduces aberrant LTP in $TSC2^{+/-}$ hippocampus, and does so in an AMPK-dependent fashion (Figure 3.1). We provide the first evidence, albeit indirectly, that AMPK can signal to RAPTOR in the brain. Interestingly, our data suggests that AMPK-mediated phosphorylation of RAPTOR and its consequent dissociation from mTORC1 occurs in $TSC2^{+/-}$ but not in WT hippocampus. The reasons for these differences in response are not clear. These data might indicate that in WT hippocampus, AMPK prefers to signal through TSC1/2, however with reduced TSC2, the alternative AMPK-RAPTOR signaling route is enhanced. In the $TSC2^{+/-}$ hippocampus, TSC2 protein levels are reduced to about 75% relative to their WT littermates (Figure 3.3). Therefore, it is likely that AMPK continues to signal through this substantial remaining pool of TSC2. It will be important to determine the level of interaction between AMPK-TSC2, and AMPK-RAPTOR in both WT and $TSC2^{+/-}$. These two interactions are likely to be very brief, which might explain why we have been unable to detect either of these interactions in hippocampal tissue (data not shown). Furthermore, the AMPK and RAPTOR antibodies used thus far have demonstrated low pulldown efficiency. In order to capture the brief interaction, we recently modified our approach to include an *in vivo* crosslinking agent during incubation with metformin (in progress). Though this may maintain the binding of AMPK to its substrates, our detection will still be limited by the precipitation efficiency of our antibodies.

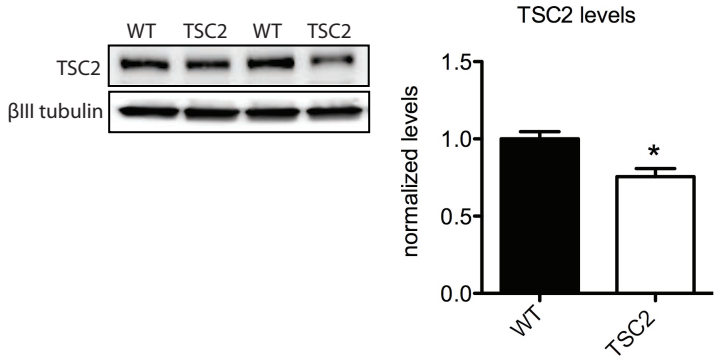

Figure 3.3. TSC2 levels are reduced in TSC2$^{+/-}$ mutant hippocampus.

Homogenates from whole hippocampus show that TSC2$^{+/-}$ mutants express approximately 75% as much TSC2 protein as their WT littermates (Student t-test, n=12). * $p<0.05$

The current interpretation of AMPK-dependent loss of aberrant LTP is limited by the usage of a single AMPK activator. Therefore, we will test whether the AMPK activator, AICAR, produces the same effect of metformin, and importantly, is rendered ineffective in the presence of AMPK antagonists. In cultured fibroblasts, metformin has been reported to be capable of AMPK-independent inhibition of mTOR. Although this alternative signaling may exist in the brain, the data obtained thus far reliably demonstrates a necessary role for AMPK in the effects of metformin on synaptic plasticity in the hippocampus.

There are no reports of altered RAPTOR levels or function in epilepsy-related diseases. However, reduction of several 14-3-3 isoforms is correlated with malformation and epilepsy(Schindler et al., 2006; Cheah et al., 2012). Given that 14-3-3 mediates the degradation of RAPTOR, it is likely that reduced 14-3-3 function could result in increased mTOR signaling. The role of 14-3-3 proteins in plasticity is not clear though reduced 14-3-3 is implicated in neuropsychiatric defects(Cheah et al., 2012) and cortical malformation(Pramparo et al., 2011). Moreover, 14-3-3 isoforms are involved in the degradation of many proteins, and are especially important for the process of autophagy and apoptosis following seizure.

Though the current story is incomplete, the potential implications for the treatment of TSC with AMPK antagonists are attractive. We have preliminary data showing that rapamycin reduces the development of epileptiform bursts in $TSC2^{+/-}$ mutant hippocampus. This suggests that metformin would be similarly effective, possibly by inhibiting RAPTOR-mTOR association. Owing to the success of the

ketogenic diet in the treatment of TSC, it would be exciting to learn whether modulators of energy metabolism could also serve as antiepileptic compounds. If so, metabolic interventions could supplant the use of difficult diets or harsh epilepsy drugs.

3.5. References

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Chapter 4

Heightened mGluR5-Erk1/2 signaling drives aberrant plasticity and epileptiform activity in a mouse model of Tuberous Sclerosis

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Notes on collaboration and publications

This chapter is an adaptation of the following manuscript:

Potter WB, O'Riordan KJ, Kirchner A, Rutecki P, Burger C, and Roopra A. Heightened mGluR5-Erk signaling drives aberrant plasticity and epileptiform activity in a mouse model of Tuberous Sclerosis. *Submitting*

All electrophysiology was done in collaboration with Ken O'Riordan.

Behavior and analysis was done by Ken O'Riordan and Corinna Burger.

Western blotting and immunoprecipitations were done in collaboration with Allison Kirchner.

All other work was done by Wyatt Potter.

Abstract

Tuberous sclerosis complex (TSC) is a multisystem genetic disease that manifests with mental retardation, tumor formation, autism, and epilepsy. Heightened signaling through the mammalian target of rapamycin (mTOR) pathway is involved in TSC pathology, however it remains unclear how other signaling pathways are perturbed and contribute to disease symptoms. We report that a mouse model of TSC ($TSC2^{+/-}$) displays heightened expression of metabotropic glutamate receptor 5 and constitutively activated Erk signaling in the hippocampus. This mGluR5-Erk pathway results in a novel mTOR-independent long-term depression in CA1 hippocampus, and contributes to the development of epileptiform bursting activity in $TSC2^{+/-}$ CA3 hippocampus. Inhibition of mGluR5 or Erk signaling restores appropriate mTOR-dependence to long-term depression, and significantly reduces epileptiform bursting in $TSC2^{+/-}$ slices. We also report that $TSC2^{+/-}$ mice exhibit a subtle perseverative behavioral phenotype that is eliminated by mGluR5 antagonism. This study demonstrates that heightened mGluR5-Erk signaling contributes to the observed plasticity abnormalities, epileptiform activity, and perseverative behavior in $TSC2^{+/-}$ mice.

4.1. Introduction

Tuberous sclerosis complex (TSC) is a multisystem autosomal dominant disorder that is characterized by the development of systemic benign hamartomas and cortical tubers, mental disability, autism, and epilepsy(Curatolo et al., 2002; Crino et al., 2006). Affecting approximately 1 in 6,000 people, TSC is caused by mutations in either of the tumor suppressor genes *TSC1* or *TSC2*, which result in altered signaling through multiple cellular pathways that impact neurological processes such as nervous system development, neuronal migration, and synaptic function(Crino and Henske, 1999; Curatolo et al., 2002; Kwiatkowski and Manning, 2005). Epilepsy occurs in approximately 90% of patients and often during the first year of life(Shepherd et al., 1995; Curatolo et al., 2002; Crino et al., 2006; Franz et al., 2010).

In TSC, reduced TSC1/2 function results in overactivation of the mammalian target of rapamycin (mTOR) pathway. TSC1 and TSC2 form a heterodimeric complex (TSC1/2) that receives signals from protein kinase B(Akt)(Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002), extracellular signal-regulated kinase (Erk1/2)(Roux et al., 2004; Ma et al., 2005), 5' adenosine monophosphate activated protein kinase (AMPK)(Inoki et al., 2003a), TNFα-IκB kinase β signaling(Lee et al., 2008), and glycogen synthase kinase-3β(GSK3β)(Inoki et al., 2006). In this manner, TSC1/2 functions as a signaling node that modulates the activity of the mTOR complex-1(mTORC1)(Inoki et al., 2003b; Tee et al., 2003). mTORC1 regulates post-synaptic protein translation, and thereby controls activity-dependent plasticity; specifically,

long-term potentiation (LTP)(Tang et al., 2002) and a form of long-term depression (LTD) induced by the group 1 metabotropic glutamate receptor (mGluR1 and mGluR5) agonist, (*S*)-3,5-Dihydroxyphenylglycine (DHPG)(Hou and Klann, 2004).

Given its role in synaptic plasticity, work has sought to elucidate a role for mTOR in epileptogenesis and the activity-dependent strengthening of neuronal networks following seizure (Zeng et al., 2008; Buckmaster et al., 2009; Zeng et al., 2009). The specific mTOR inhibitor, rapamycin, blocks the development of spontaneous seizures across multiple models of epileptogenesis (Zeng et al., 2009; Huang et al., 2010; Sunnen et al., 2011)(but see Buckmaster et al., 2011). Since inhibition of mTORC1 may have therapeutic value in treating TSC(Zeng et al., 2008; Krueger et al., 2010), pathways that signal to mTORC1 could represent viable avenues for treating TSC.

Recently, Auerbach *et al.*, showed that when CA3 is removed, wild-type (WT) CA1 displays rapamycin-insensitive mGluR-LTD. In the absence of CA3, mGluR-LTD was decreased in $TSC2^{+/-}$ CA1 and rapamycin treatment increased mGluR-LTD magnitude(Auerbach et al., 2011). Using intact mouse hippocampal slices, we find that the magnitude of mGluR-LTD is similar between $TSC2^{+/-}$ and WT mice. In contrast to WT, mGluR-LTD in the $TSC2^{+/-}$ hippocampus is insensitive to mTOR inhibition. We report that $TSC2^{+/-}$ hippocampus has heightened expression of mGluR5 and constitutively activated Erk. Inhibition of mGluR5-Erk signaling, but not PI3k-Akt signaling, is sufficient to restore mTOR dependence in $TSC2^{+/-}$ hippocampus. Additionally, we report an epileptiform bursting phenotype in $TSC2^{+/-}$

CA3 hippocampus induced by prolonged incubation with DHPG. $TSC2^{+/-}$ slices were more likely to develop long synchronous bursts, compared to WT slices; a phenotype that was eliminated by antagonism of mGluR5-Erk signaling. Finally, we show that $TSC2^{+/-}$ mice exhibit a perseverative behavioral phenotype that is corrected by reducing mGluR5 function.

4.2. Methods

All procedures were performed with the approval of the University of Wisconsin-Madison School of Medicine and Public Health Institutional Animal Care and Use Committee and according to national guidelines and policies.

Drugs

S-3,5-Dihydroxyphenylglycine (DHPG), 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), and anisomycin were purchased from Tocris Bioscience and were solublized in MilliQ water. 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), and rapamycin were purchased from Sigma-Aldrich and dissolved in DMSO. Metformin, and adenine 9-β-D-arabinofuranoside (ara-A) were purchased from Sigma-Aldrich and dissolved in MilliQ water.

Electrophysiology

Methods were previously described in detail (Potter et al., 2010). All electrophysiology was performed on male 5-10wk old $TSC2^{+/-}$ and wild-type littermate mice (C57BL/6 background). Field EPSPs were recorded from CA1 *stratum radiatum*, with ACSF-filled recording electrodes (1.4-2 MΩ). Baseline

synaptic transmission was assessed for each individual slice by applying gradually increasing stimuli (0.5V – 20V, 25nA – 2.0μA, A-M Systems model 2200 stimulus isolator, Carlsborg, WA) to determine the input:output relationship. All subsequent experimental stimuli were set to an intensity that evoked 50% of maximum fEPSP slope. ***LTD induction*** – LTD was induced with bath application of 50μM S-DHPG for 10 min. To account for differences in recording rigs (flow rate, slice placement, etc.), the moment that slices began to demonstrate synaptic depression was set as t=0 for all graphical representations in the results section. Synaptic efficacy was continually monitored (0.033 Hz). ***Induction of epileptiform activity:*** Slices were prepared as described above and exposed to 50 μM S-DHPG for 30 min or DHPG with 40 μM MPEP or 20μM U0126 (MPEP and U0126 were applied 10 min prior to DHPG addition). Slices were then transferred to the recording chamber, allowed to recover for 1 hour. Following recovery, spontaneously occurring activity was monitored in CA3 stratum pyramidale with an extracellular recording electrode. The frequency and duration of activity was characterized as interictal if less than 2 sec and ictal if greater than 2 sec. The proportion of slices demonstrating spontaneously occurring activity was compared between wild type and $TSC2^{+/-}$ mice and slices exposed to DHPG vs. DHPG and MPEP. ***CA3-resection***: Immediately following slicing, cortex was removed from the hippocampal formation and CA3 was severed and discarded. The slices were then allowed to recover for a total of 4 hours as described previously(Potter et al., 2010).

Tissue homogenization and western blotting

Methods were previously described(Potter et al., 2010). Briefly, following drug application and/or stimulation, slices were flash frozen in eppendorf tubes on dry ice. Acutely harvested slices were flash frozen immediately after slicing. Following purification, protein extracts were loaded at 20-30 μg/lane in gradient (4-20%) SDS-PAGE gels (BioRad) and resolved with standard electrophoresis in tris-glycine running buffer (100 mM Tris, 1.5 M glycine, 0.1% SDS) and transferred at 4°C onto PVDF membranes and blocked in 5% milkfat TBST. All primary antibodies were applied overnight at 4°C and were obtained from Cell Signaling (Danvers, MA), except for βIII tubulin, which was obtained from Promega (Madison, WI). Membranes were the washed and incubated for 1 hr in horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (1:10,000) (Santa Cruz Biotech). Protein bands were detected using SuperSignal West Femto ECL reagent (Pierce Biochem) and visualized using a UVP ChemiDoc-it Imaging system with VisionWorks software, which was also used to quantify protein bands.

Immunoprecipitation from hippocampal tissue

Hippocampal tissue was homogenized in ice-cold CHAPS lysis buffer (in mM) (150 NaCl, 40 HEPES, 2 EDTA, 10 pyrophosphate, 10 glycerophosphate, 4 orthovanadate, 0.3% CHAPS). Following quantification, equal amounts of cell extract were used for immunoprecipitation with 0.5 μg anti-mTOR antibody. For sham, an antibody

(0.5μg) specific to the unrelated protein LIN28 (Cell Signaling) was used. Samples were incubated on a rotator at 4°C for 2 hours. 20μg of whole cell extract was used for quantitative comparisons. 20 μg of washed Sepharose G beads were added to each sample and spun on the rotator for an additional hour at 4°C. Samples were spun down briefly, washed, and combined with SDS gel loading buffer.

Radial water maze (RAWM) for Mice

The protocol has been described in detail (Gerstein et al 2011). Briefly, the RAWM consisted of a 2-day training protocol with 15 trials per day. On Day 1, the animals were trained in the visible platform task first (Trials 1-9), then trained on the hidden platform version of the maze (Trials 10-15). All trials on Day 2 utilized the submerged/hidden platform (Trials 16-30). The number of errors (arm entries that did not result in finding the platform) was recorded. Data were collected with VideoTrack v2.5 (ViewPoint Life Sciences Inc, Montreal CANADA). Two way repeated-measures ANOVA with 3-trial bins as the repeated measure was used to compare the time course for errors in RAWM. In examining individual time points, one-way ANOVA was used. Data were analyzed using Prism 5 (Graphpad Software Inc, La Jolla CA) and all data are expressed as means ± SEM.

Reversal Training. On Day 3, animals received six additional trials using a hidden platform that was moved to a different location to learn (reversal training). The new goal arm was two arms away from the location in days 1 and 2. After the hidden platform training was completed, the radial arms were removed and an open pool

task with the visible platform was performed to confirm that the deficits found in the RAWM were not caused by vision or motor performance deficits in the mice. This task consisted of 5 trials, 60 seconds each.

Statistical Analyses

For electrophysiological experiments, Two-way ANOVA with repeated measures (mixed model) and Bonferroni posttests were used for statistical analysis. For western blot analysis with 2 sets of data, two-tailed Student t-tests were used. Western blot analysis where multiple groups were acquired and analyzed together, 1-way ANOVA with Tukey-Krameŕ post-test correction for multiple analyses were used. Chi Square test for trend was used to analyze the contingency data obtained from the epileptiform bursting experiments. For all tests, $p<0.05$ was considered statistically significant.

4.3. Results

TSC2$^{+/-}$ hippocampus exhibits mTORC1-independent mGluR-LTD

In WT hippocampal slices, LTD induced with the group 1 mGluR agonist, DHPG (50μM, 10 min), requires mTORC1-dependent signaling and protein translation (Huber et al., 2000; Hou and Klann, 2004). To assess the role of mTORC1 activity in TSC2$^{+/-}$ LTD, we applied rapamycin to TSC2$^{+/-}$ and WT slices. Consistent with the work of Hou and Klann (2004) rapamycin reduced mGluR-LTD in WT slices (Figure 4.1a). Surprisingly, rapamycin had no effect on mGluR-LTD in TSC2$^{+/-}$ slices (Figure 4.1b). We recently showed that pharmacological activators of AMPK could mimic rapamycin by inhibiting mTOR. Thus we asked whether the AMPK inducer metformin could inhibit mGluR-LTD in TSC2$^{+/-}$ slices. Figure 4.1c shows that, as with rapamycin, metformin also failed to inhibit mGluR-LTD in TSC2$^{+/-}$ slices, but was able to inhibit LTD in WT slices (Figure 4.1d). This inhibition was AMPK-dependent as it could be blocked by the AMPK antagonist, Ara-A (Figure 4.1d). Since mTORC1 governs post-synaptic protein translation, we tested the possibility that mGluR-LTD in TSC2$^{+/-}$ hippocampus may also be protein synthesis-independent. Treatment with the general translational inhibitor, anisomycin, eliminated LTD (Figure 4.2), demonstrating a continued requirement for protein synthesis in LTD expression.

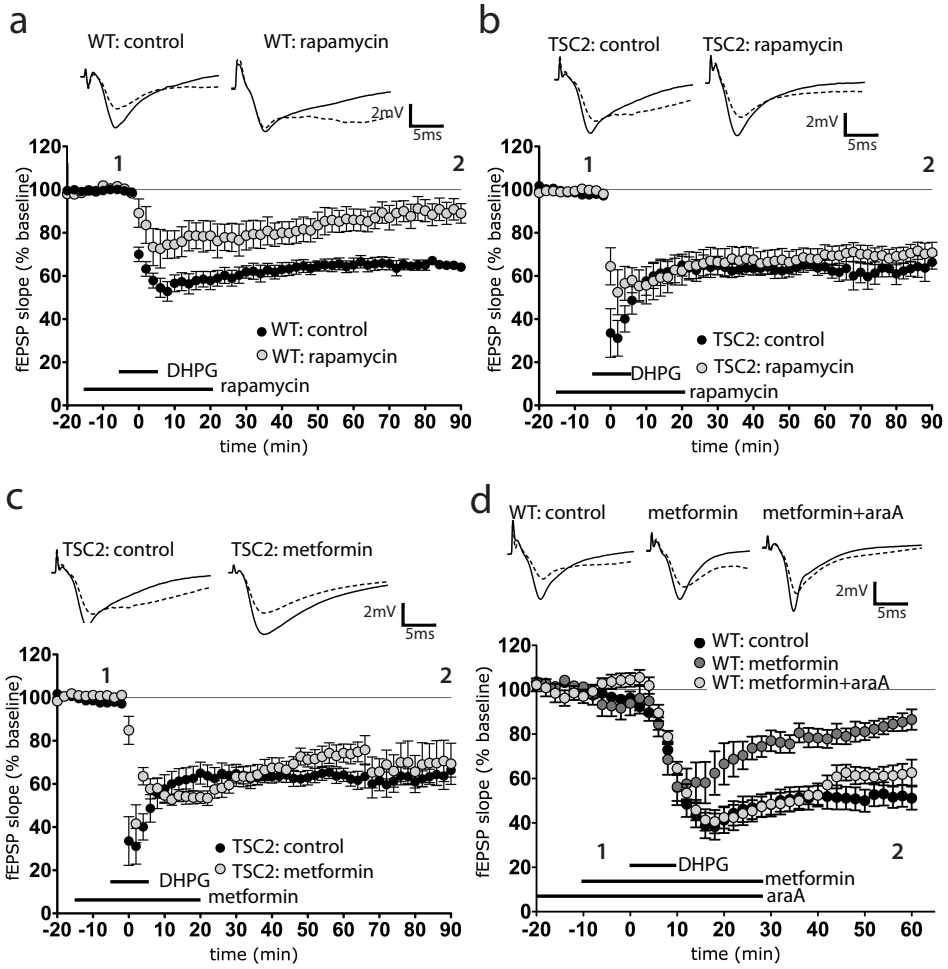

Figure 4.1. TSC2$^{+/-}$ hippocampal slices are immune to mTORC1 inhibition. ***a***. Rapamycin (20nM) reduces mGluR-LTD in WT littermates [84.4±5.4%, n=8(4)] [2-way ANOVA; *F*(1, 18)=17.4, p=0.0006]. ***b***. Rapamycin (20nM) does not affect mGluR-LTD in TSC2$^{+/-}$ hippocampus [70.8±5.4%, n=9(5)]. ***c***. Metformin has no effect on mGluR-LTD in TSC2$^{+/-}$ hippocampus [63.4±8.0%, n=7(3)]. ***d***. Metformin (5μM) significantly reduces mTOR-dependent mGluR-LTD in WT slices [metformin-86.6±4.6%; control-51.2±5.2%][2-way ANOVA; *F*(1, 13)=15.65, p=0.0016], an effect that is reversed with the AMPK inhibitor Ara-A (100μM)[62.7±6.6%, n=9(5)].

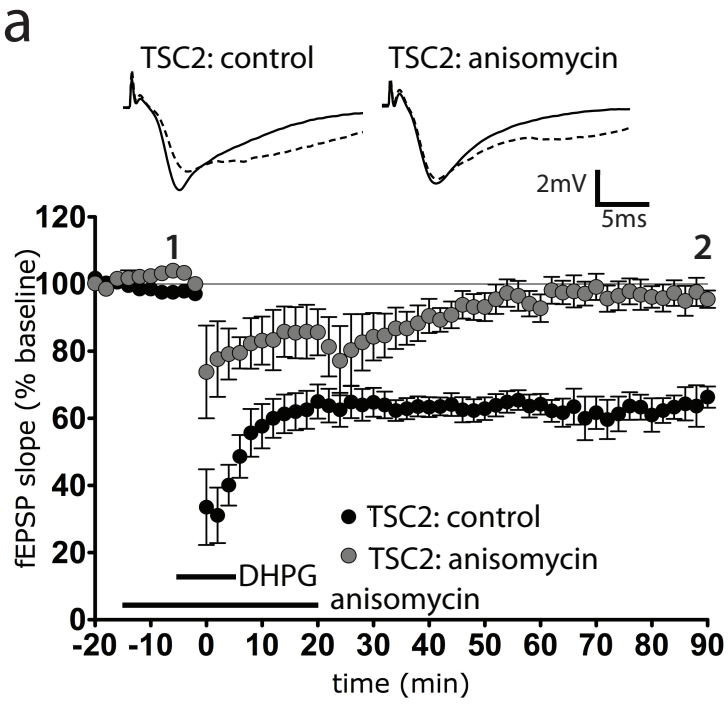

Figure 4.2. mGluR-LTD in TSC2$^{+/-}$ requires protein translation. Inhibition of protein synthesis with anisomycin eliminates mGluR-LTD in TSC2$^{+/-}$ hippocampus [96.5±2.5%, n=6(3)][2-way ANOVA; $F(1, 13)=37.62$, $p<0.0001$].

Rapamycin works, in part, by reducing the association of mTOR with its obligate partner protein, Regulatory Associated Partner of TOR (RAPTOR) to reduce activation of downstream targets, such as rpS6(Hara et al., 2002). Since rapamycin failed to impact mGluR-LTD in $TSC2^{+/-}$ slices, we tested whether it was still capable of reducing RAPTOR-mTOR association. We immunoprecipitated mTORC1 from slices incubated with DHPG for 10 minutes in the presence and absence of rapamycin. In the presence of DHPG, rapamycin treatment significantly reduced RAPTOR-mTORC1 binding equally well in WT and $TSC2^{+/-}$ slices (Figure 4.3a and 4.3b). Additionally, rapamycin significantly reduced p-S6 levels in WT and $TSC2^{+/-}$ slices in the presence of DHPG (Figure 4.3c and 4.3d). These data show that in $TSC2^{+/-}$ slices rapamycin is able to disrupt the mTORC1 complex and inhibit downstream signaling to S6. Together, these data demonstrate that the $TSC2^{+/-}$ hippocampus maintains a requirement for protein translation for the expression of mGluR-LTD, yet appears to have altered signaling that circumvents mTORC1 activity.

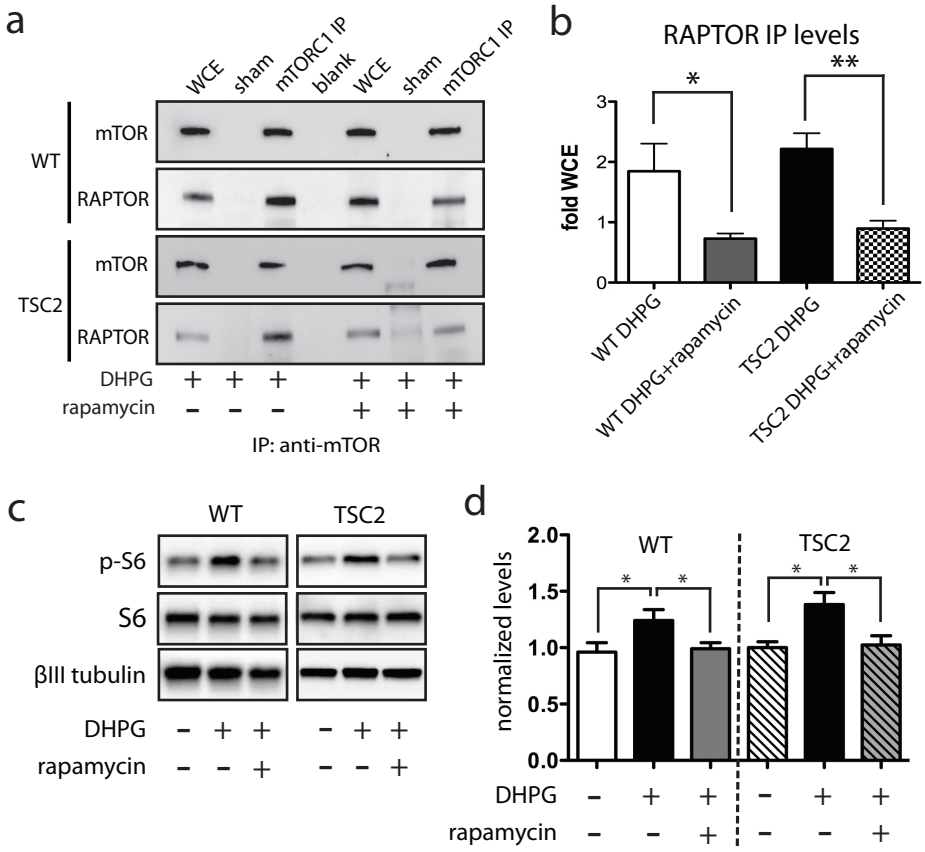

Figure 4.3. Rapamycin inhibits mTOR signaling equally well in WT and TSC2$^{+/-}$ hippocampus. a. Rapamycin (20nM) treatment significantly reduces RAPTOR-mTORC1 association in DHPG-treated WT and TSC2$^{+/-}$ slices; quantified in (**b**)(Student t-test; WT n=4, p=0.030; TSC2$^{+/-}$ n=4, p=0.0043). **c**. Rapamycin significantly reduces p-S6 levels in WT and TSC2$^{+/-}$ hippocampal slices; quantified in (**d**)(Student t-test; WT n=22, p=0.031; TSC2$^{+/-}$ n= 18, p=0.016). Representative traces: solid line is 4 minutes before DHPG '1', dashed line is at the end of the recording '2'. WCE-whole cell extract. *p<0.05, **p<0.005

TSC2$^{+/-}$ mice have constitutively elevated mGluR5-Erk signaling

In Fragile X Syndrome, LTD is rendered mTOR-independent by hyperactive mGluR signaling (Sharma et al., 2010). This observation along with a report of heightened mGluR5 expression in cortical tubers resected from TSC patients(Boer et al., 2008) prompted us to investigate this receptor in the TSC2$^{+/-}$ mouse. Western blot analysis of acutely harvested TSC2$^{+/-}$ hippocampal lysates shows significantly elevated mGluR5 expression levels compared to WT (Figure 4.4a). Group 1 mGluR receptors (mGluR1 and mGluR5) activate G_q-coupled second messenger cascades, which increase PI3K-Akt and Mek-Erk signaling(Ferraguti et al., 1999; Hou and Klann, 2004). We predicted that heightened mGluR5 expression would cause increased activation of these cascades to drive the observed aberrant plasticity in TSC2$^{+/-}$ mice. We therefore compared the activation status of Akt and Erk in acutely harvested WT and TSC2$^{+/-}$ hippocampi. Figure 4.4b shows that TSC2$^{+/-}$ slices exhibit a constitutively heightened level of phosphorylated Erk1/2 (T202/Y204), but no observable difference in phosphorylated Akt (T308), compared to WT slices. To determine whether heightened mGluR5 signaling contributes to the observed rapamycin-insensitive LTD in TSC2$^{+/-}$ hippocampus, we preincubated TSC2$^{+/-}$ hippocampal slices with the non-competitive mGluR5 antagonist, 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP). Despite heightened mGluR5-Erk signaling, preincubation with MPEP (40μM for 20 min, followed by a 20 min washout period) had no effect on mGluR-LTD expression (Figure 4.4c). However, when rapamycin was added during the MPEP washout period, we observed a striking

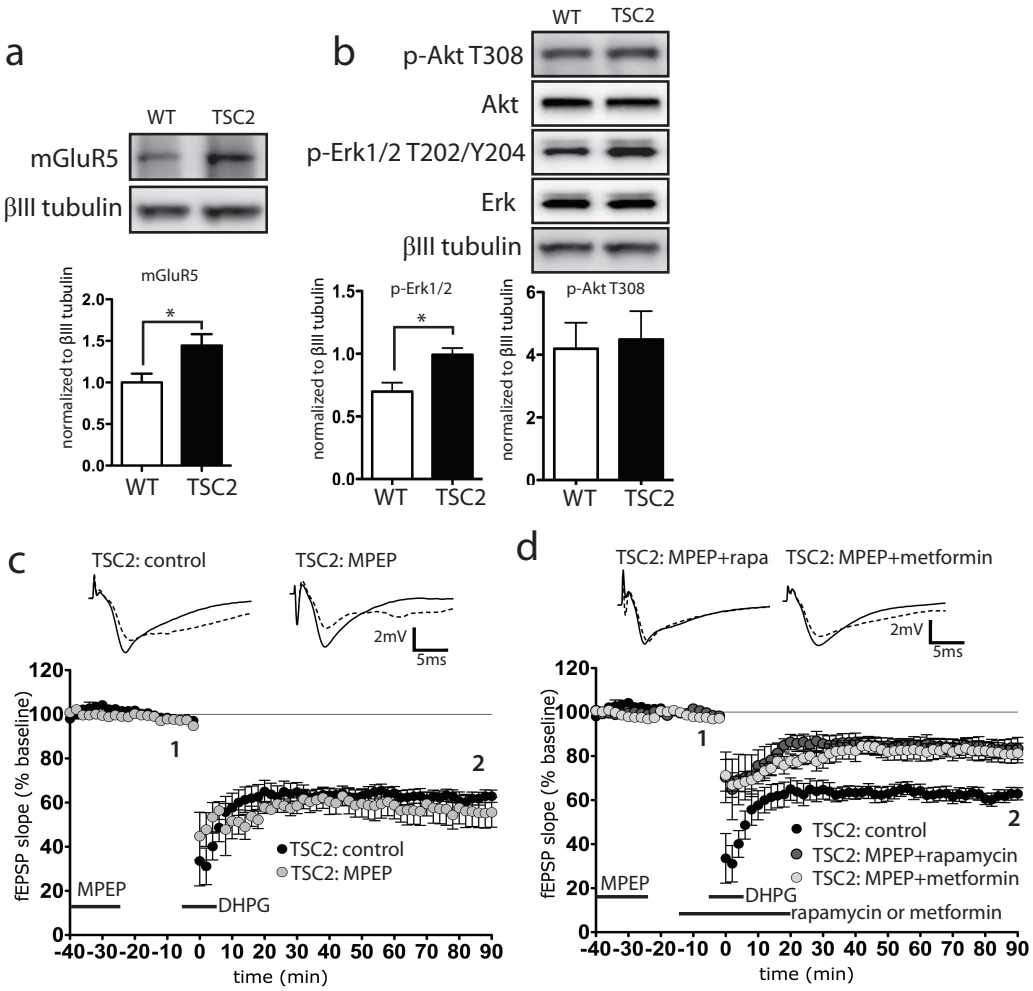

Figure 4.4. mGluR5-Erk1/2 signaling is heightened in TSC2$^{+/-}$ mice. ***a***. Acutely harvested hippocampal tissue displays increased mGluR5 expression in TSC2$^{+/-}$ mice (Student t-test; n=14, p=0.0155). ***b***. Erk1/2 is constitutively phosphorylated in TSC2$^{+/-}$ hippocampus compared to WT (Student t-test; n=9, p=0.0093). There is no difference in phosphorylated Akt between genotypes (Student t-test; n=12, p=0.81). ***c***. MPEP preincubation (40μM, present -40 to -20 min), followed by washout does not impact mGluR-LTD magnitude in TSC2$^{+/-}$ hippocampus [MPEP-55.6±5.7%, n=6(3)]. ***d***. MPEP preincubation restores rapamycin sensitivity [MPEP+rapamycin-83.5±5.4%] and metformin sensitivity of mGluR-LTD [metformin+rapamycin-81.4±4.5%].

restoration of rapamycin-sensitivity and attenuation of LTD magnitude (Figure 4.4d). Metformin showed the same restorative properties (Figure 4.4d). Importantly, the level of reduction of mGluR-LTD with MPEP and rapamycin closely resembled the effect of rapamycin alone in WT mGluR-LTD (compare Figure 4.4d and 4.1a). Consistent with the work of Hou & Klann (2004) in WT slices, MPEP treatment without washout completely eliminated mGluR-LTD in $TSC2^{+/-}$ slices, demonstrating that mGluR5 signaling is required for the induction of LTD by DHPG (Figure 4.5a). Western blot analysis of DHPG-treated $TSC2^{+/-}$ slices showed that pre-incubation with MPEP caused a significant reduction in p-Erk (Figure 4.6, compare lanes 11 and 12) and p-Akt (Figure 4.6, compare lanes 5 and 6). Notably, the untreated control $TSC2^{+/-}$ slices had constitutively elevated phospho-Erk levels compared to WT controls (Figure 4.6, compare lanes 7 and 10).

Given the established role of mGluR receptors in LTP(Bikbaev et al., 2008; Neyman and Manahan-Vaughan, 2008), we postulated that the heightened mGluR5 signaling we observed in $TSC2^{+/-}$ hippocampi may also contribute to the aberrant LTP induced in these slices by a single TBS train(Ehninger et al., 2008). We therefore stimulated $TSC2^{+/-}$ slices with 1XTBS in the presence and absence of MPEP. As predicted, MPEP treatment significantly reduced 1XTBS-induced LTP expression in $TSC2^{+/-}$ compared to control slices (Figure 4.5b). These findings suggest that the aberrant LTD and LTP in $TSC2^{+/-}$ mice are due at least in part to heightened mGluR5 signaling.

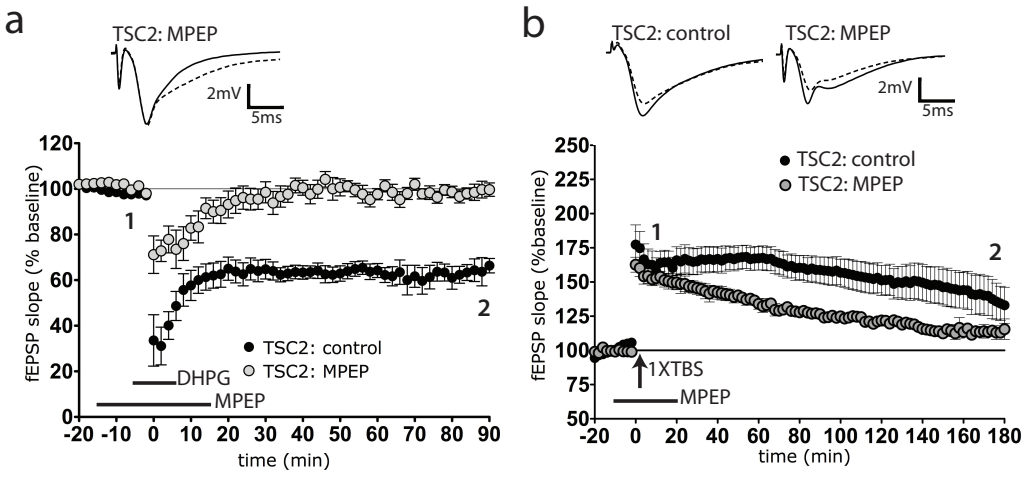

Figure 4.5. mGluR5 inhibition blocks mGluR-LTD and reduces aberrant LTP.

a. MPEP (40μM) incubation during LTD induction (present -20 to 20 min), eliminates mGluR-LTD in $TSC2^{+/-}$ hippocampus [99.7±2.7%, n=5(3)]. ***b***. MPEP (40μM) during 1XTBS significantly reduces LTP [119.5±2.1%, n=6(3)] [2 way ANOVA; n=6, p=0.04]. Representative traces: solid line is 4 minutes before DHPG '1', dashed line is at the end of the recording '2'. *p<0.05

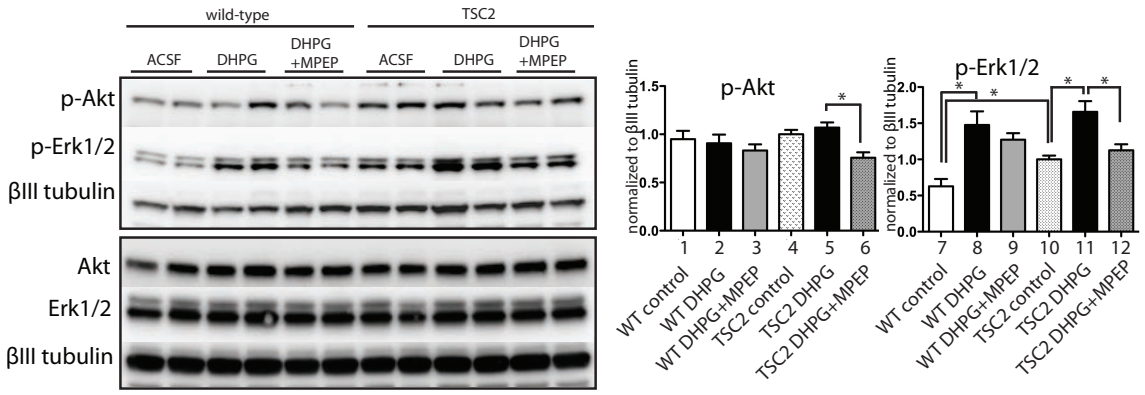

Figure 4.6. MPEP preincubation impacts Akt and Erk signaling in TSC2$^{+/-}$. In WT and TSC2$^{+/-}$ slices, DHPG (50μM, 10 min) significantly increases phospho-Erk1/2 (1 way ANOVA, Tukey's post-test; n=17, p=0.0001). In WT slices, DHPG increased phospho-Erk1/2 levels (1 way ANOVA, Tukey's post-test; n=14, p=0.0004). MPEP preincubation, followed by DHPG treatment causes a significant reduction in p-Akt ($p<0.005$), and p-Erk1/2 ($p<0.0001$) levels in TSC2$^{+/-}$ hippocampus. Total Akt and Erk were run on a separate gels and were not used to normalize.

Reduction of Mek-Erk signaling restores mTORC1 sensitivity in TSC2$^{+/-}$ hippocampus

To determine how PI3K-Akt and Mek-Erk signaling might contribute to the mTOR-independent LTD observed in TSC2$^{+/-}$ slices, we applied pharmacological antagonists of PI3K-Akt signaling (wortmannin and LY294002), and Mek-Erk signaling (U0126) during LTD induction with DHPG. In accordance with previous studies(Hou and Klann, 2004), the expression of LTD was significantly reduced with LY294002 and wortmannin in WT slices (Figure 4.7a). In contrast to WT, antagonism of PI3K-Akt signaling in TSC2$^{+/-}$ slices had no effect on the expression of LTD, nor did it restore rapamycin sensitivity (Figure 4.7b, 4.7c). This demonstrates that PI3K inhibition cannot recapitulate the effects of mGluR5 inhibition in TSC2$^{+/-}$. Interestingly, the Mek-Erk inhibitor U0126 significantly reduced the magnitude of LTD in TSC2$^{+/-}$ slices, an effect that was not enhanced by rapamycin (Figure 4.7d). Importantly, the magnitude of LTD reduction in TSC2$^{+/-}$ slices with U0126 alone mirrored that observed in WT slices exposed to rapamycin (compare Figure 4.7d and Figure 4.1a).

We hypothesized that the hyperactive Erk signaling was bypassing or short-circuiting the mTOR pathway to drive LTD and render it mTOR-independent and rapamycin insensitive. To test whether we could use U0126 to effectively dial down Erk signaling to WT levels and thus restore rapamycin sensitivity in TSC2$^{+/-}$ slices, we bath applied a concentration series of U0126 to TSC2$^{+/-}$ slices and quantified p-Erk levels via western blot (Figure 4.8a). From this we determined that 200nM

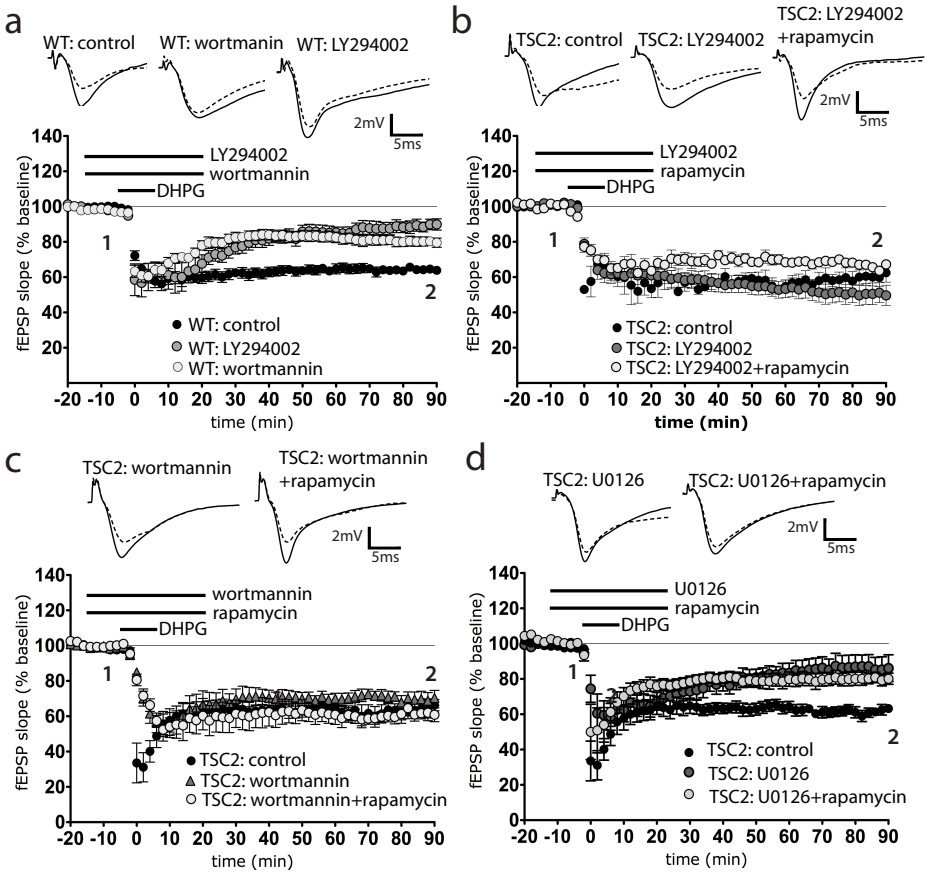

Figure 4.7. Inhibition of Mek-Erk signaling, but not PI3K-Akt, reduces mGluR-LTD in TSC2$^{+/-}$ hippocampus. ***a***. Incubation with the PI3K antagonists LY294002 (10μM) or wortmannin (500nM) reduce mGluR-LTD in WT slices [LY294002-89.9±2.4%, n=9(3); wortmannin-79.5±3.6%, n=13(3); control, n=16(4)]. ***b***. LY294002 has no effect in TSC2$^{+/-}$ hippocampus [49.6±5.3%, n=6(3)], nor does it restore rapamycin sensitivity [67.2±1.4%, n=6(3)]. ***c***. Likewise, wortmannin (500nM) does not impact mGluR-LTD in TSC2$^{+/-}$ hippocampus [70.4±4.3%, n=7(3)], nor does it restore rapamycin sensitivity [57.4±3.5%, n=7(3)]. ***d***. Incubation with the Mek-Erk inhibitor, U0126 (20μM), reduces mGluR-LTD in TSC2$^{+/-}$ slices [86.1± 6.3%, n=9(4)] an effect that is not enhanced with rapamycin [80.1±3.4%, n=8(3)].

U0126 reduced p-Erk levels in $TSC2^{+/-}$ slices to WT levels (corresponding to 62% of $TSC2^{+/-}$, Figure 4.4b). If reduced Erk signaling is sufficient to restore rapamycin sensitivity in $TSC2^{+/-}$ slices, then reduction of p-Erk to WT levels would be expected to leave mGluR-LTD unaffected, yet render slices responsive to rapamycin. Figure 4.8b shows that application of 200nM U0126 did not affect mGluR-LTD magnitude in $TSC2^{+/-}$ slices. However, addition of rapamycin was now capable of reducing mGluR-LTD (Figure 4.8b). These data indicate that the subtle reduction of Mek-Erk signaling was sufficient to restore a WT-like response to rapamycin and recapitulate the effects of preincubated MPEP on mGluR-LTD in $TSC2^{+/-}$ slices (compare Figure 4.8b and Figure 4.4d). There was a trend for a greater reduction in p-S6 levels in slices treated with U0126 plus rapamycin, than rapamycin alone ($p=0.077$, Figure 4.9). These observations, along with the elevated levels of phospho-Erk in $TSC2^{+/-}$ slices, supports the hypothesis that the aberrant plasticity in $TSC2^{+/-}$ slices arises from heightened mGluR5-Erk signaling.

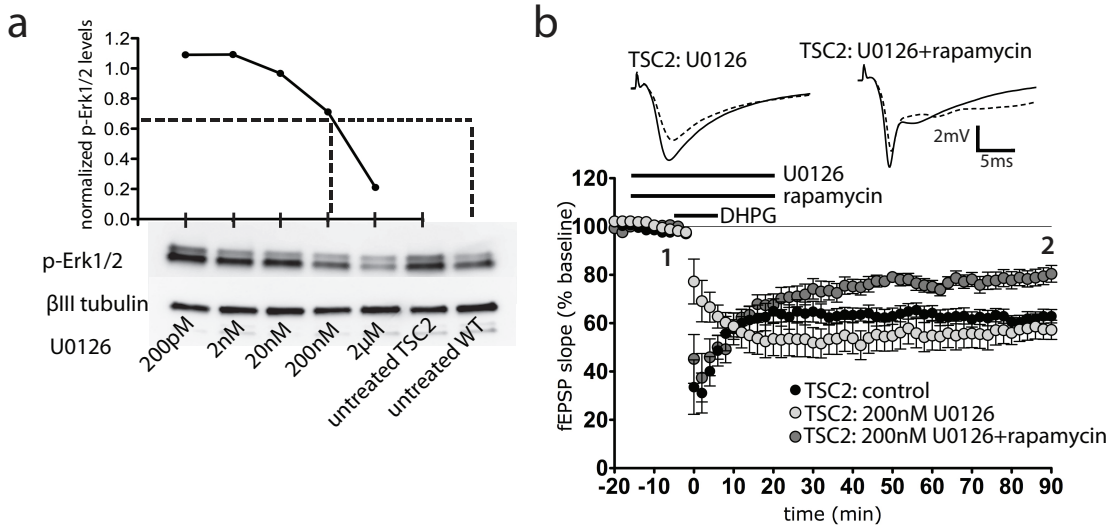

Figure 4.8. Subtle reduction of Erk signaling restores mTORC1 sensitivity.

a. Analysis of p-Erk1/2 levels in response to a U0126 concentration series indicates that 200nM U0126 reduces p-Erk1/2 to untreated WT levels. ***b***. Subtle reduction of Mek-Erk1/2 signaling with 200nM U0126 (-20 to 20 min), does not impact mGluR-LTD magnitude [57.3±4.3%, n=8(3)], yet does restore rapamycin sensitivity [80.4±3.9%, n=7(3), p=0.0054).

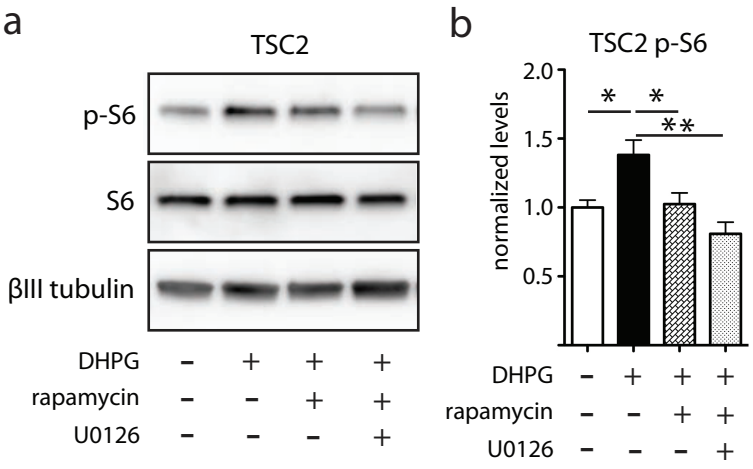

Figure 4.9. Rapamycin reduces p-S6 in both WT and TSC2$^{+/-}$ hippocampus.

a. Rapamycin plus U0126 produced a significant reduction in p-S6 (Student T-test; n=15, p=0.0005) (untreated and DHPG-treated data is reproduced from Figure 1). ***b.*** Quantification of p-S6, normalized to beta-3 tubulin. Representative traces, solid line is 4 minutes before DHPG '1', dashed line is at the end of the recording '2'.

mGluR5 antagonism reduces epileptiform activity in TSC2$^{+/-}$ hippocampus

Epilepsy is very prevalent in TSC patients and occurs in approximately 80-90% of affected individuals and often by the first year of life (Kwiatkowski and Manning, 2005; Kassiri et al., 2011). Epileptiform bursting activity can be induced in hippocampal slices with prolonged activation of group 1 mGluRs with DHPG. This alteration in excitability persists for hours after the removal of DHPG, suggesting that the synchronous bursting activity is due to enduring changes in network plasticity(Merlin and Wong, 1997; Sayin and Rutecki, 2003; Chuang et al., 2005). Due to our observation that mGluR5 expression is heightened in the TSC2$^{+/-}$ hippocampus, we reasoned that TSC2$^{+/-}$ slices should be more susceptible to the development of DHPG-induced epileptiform bursting. To test this, we measured field activity in CA3 stratum pyramidale in TSC2$^{+/-}$ and WT slices following DHPG treatment (50μM, 30 min) and quantified burst number and duration in slices that showed spontaneously occurring synchronous activity. Ictal epileptiform activity was defined as synchronous activity of greater than two seconds with intraburst frequencies of greater than 2 Hz. Interictal epileptiform activity was defined as spontaneously occurring synchronous activity with a duration of less than 2 seconds (examples shown in Figure 4.10a). 1 hour after removal of DHPG, TSC2$^{+/-}$ slices were more likely to exhibit epileptiform activity (Figure 4.10b), produced significantly more long-duration ictal bursts compared to WT (Figure 4.11a), and had significantly longer burst durations (Figure 4.10c). When WT slices were exposed to

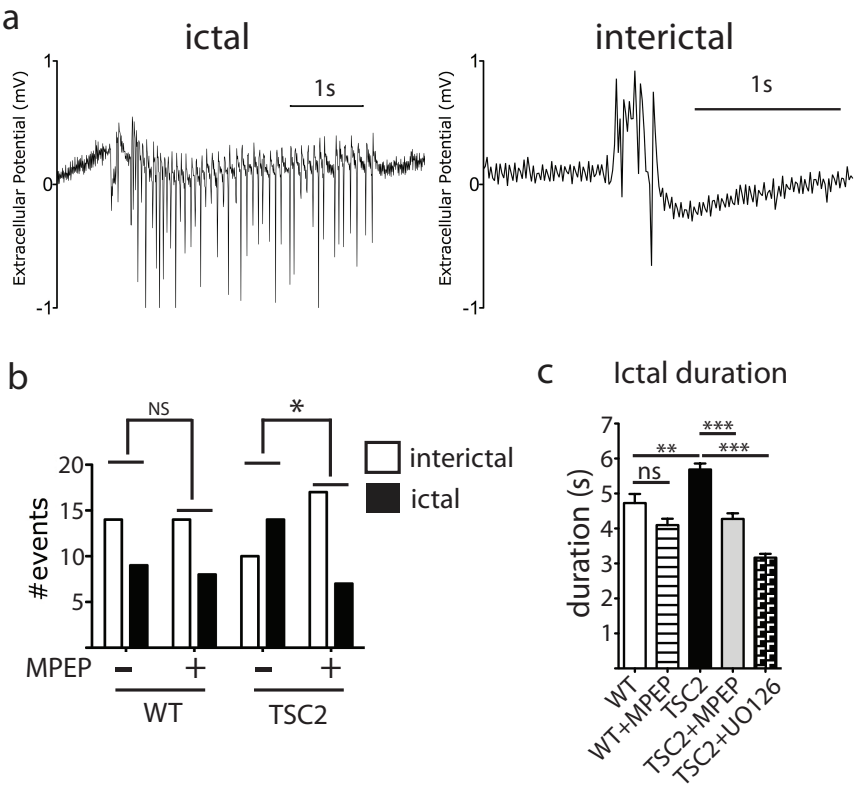

Figure 4.10. Antagonism of mGluR5-Erk signaling reduces epileptiform activity in TSC2$^{+/-}$ CA3 hippocampus. ***a***. DHPG (50μM, 30 min) produces long-duration ictal (>2 sec) and short duration interictal (<2 sec) burst activity in CA3 stratum pyramidale (examples shown). ***b***. Compared to WT slices, TSC2$^{+/-}$ slices are more susceptible to the development of ictal epileptiform activity (WT: 9/23 = 39.1% ictal; TSC2$^{+/-}$: 14/24 = 58.3% ictal). Antagonism of mGluR5 with 40μM MPEP during DHPG treatment, significantly reduces epileptiform bursting in TSC2$^{+/-}$ (Chi square, p=0.0188). WT bursting was not significantly different with MPEP (Chi square, p=0.362). ***c***. Ictal burst duration is greater in TSC2$^{+/-}$ hippocampus compared to WT (Student t-test, p=0.0020), and is significantly reduced with MPEP (p<0.005) and U0126 (p<0.0005).

MPEP during the DHPG incubation, the proportion of slices with epileptiform activity did not change (WT: 39.1% DHPG vs. 36.4% DHPG+MPEP)(Figure 4.10b). However, MPEP caused a significant reduction in the proportion of $TSC2^{+/-}$ slices that developed ictal activity ($TSC2^{+/-}$: 58.3% DHPG vs. 29.2% DHPG+MPEP) (Figure 4.10b). This pronounced effect of MPEP in suppressing the induction of ictal epileptiform activity in $TSC2^{+/-}$ is further demonstrated in Figure 4.11b (compare to WT+/- MPEP in Figure 4.11c). MPEP significantly reduced ictal burst duration in $TSC2^{+/-}$ slices but did not have a significant impact on WT slices (Figure 4.10c). Incubation of $TSC2^{+/-}$ slices with U0126 produced an even greater reduction in bursting duration than MPEP (Figure 4.10c and 4.11d). The enhanced effect of U0126 is likely due to Erk being situated downstream of mGluR1 and mGluR5, which are both activated by DHPG. The CA3 bursting data demonstrates that mGluR5-Erk signaling is involved in the development of epileptiform activity in $TSC2^{+/-}$ CA3 neurons in response to DHPG, and that the enhanced epileptogenic potential of $TSC2^{+/-}$ slices can be blocked with mGluR5-Erk antagonism.

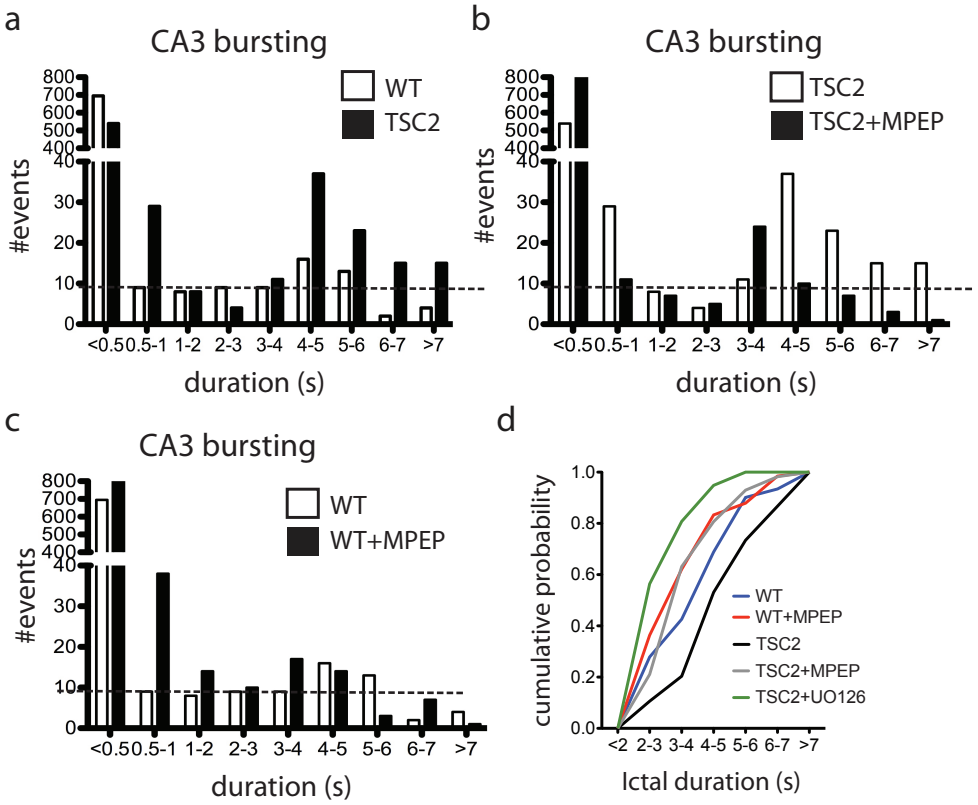

Figure 4.11. Antagonism of mGluR5-Erk signaling reduces epileptiform activity in TSC2$^{+/-}$ CA3 hippocampus. ***a***. TSC2$^{+/-}$ slices develop significantly more long-duration bursts in CA3 neurons than WT slices (Chi square test for trend, p<0.0001). ***b.*** MPEP (40μM) treatment during DHPG incubation significantly reduces CA3 bursting in TSC2$^{+/-}$ slices (Chi square for trend, p<0.0001). ***c.*** Co-incubation of MPEP and DHPG in WT slices produced an insignificant trend towards less bursting in WT slices (Chi square for trend, p=0.182). ***d***. Cumulative probability of developing long duration ictal activity is greatest in TSC2$^{+/-}$ slices and decreases with MPEP and even more so with U0126. NS- not significant, *p<0.05, **p<0.005, ***p<0.0005.

TSC2$^{+/-}$ mice display a perseverative behavioral phenotype that can be corrected by MPEP

In a previous report, Ehninger *et al* (2008) analyzed cognitive and stress-related behaviors associated with tuberous sclerosis in TSC2$^{+/-}$ mice. They reported that TSC2$^{+/-}$ mice display cognitive and stress-related deficits that were corrected by rapamycin (Ehninger et al., 2008). We sought to extend the behavioral analysis to other characteristic behaviors found in tuberous sclerosis, namely autistic perseverative behavior, and to determine if these behaviors in TSC2$^{+/-}$ mice could be due to heightened mGluR5 signaling. We used the radial arm water maze (RAWM) followed by a reversal training protocol to analyze the behavioral phenotype of these mice. TSC2$^{+/-}$ mice performed as well as wildtype littermate controls (Figure 4.12a; Trials 1-30: $F_{(3,27)}$ = 1.44, p=0.1429). On trial 31, the hidden platform was moved to test reversal learning. TSC2$^{+/-}$ mice displayed perseverative phenotype during the 6 trials of reversal training relative to wild type littermates (Figure 4.12b; main effect of genotype $F_{(1,31)}$=5.882, p=0.0034). Specifically, TSC2$^{+/-}$ mice continually visited the target arm where the hidden platform had been previously placed in trials 1-30. This perseverative behavior was corrected in TSC2$^{+/-}$ mice injected with MPEP in that MPEP treated TSC2$^{+/-}$ mice showed no significant difference on the 6 reversal trials relative to wildtype littermates, (Figure 4.12b; main effect of group: $F_{(1,21)}$ = 1.98, p = 0.1747). This suggests that mGluR5 contributes to the behavioral phenotype in TSC2$^{+/-}$ mice. Additional behavioral testing revealed no deficits in sensory, exploratory or motor performance. There were no differences in the performance in

the open pool task between any of the experimental groups (Figure 4.13c; $F_{(3,27)}$ = 0.7313, p > 0.05). Locomotor activity was unaffected by both genotype and treatment as measured in the Open Field task. There were no differences in anxiety and exploration between the experimental groups, as measured by the percentage of time spent in the center (Figure 4.12c (inset); one way ANOVA $F_{(3,28)}$ = 0.47, p > 0.05).

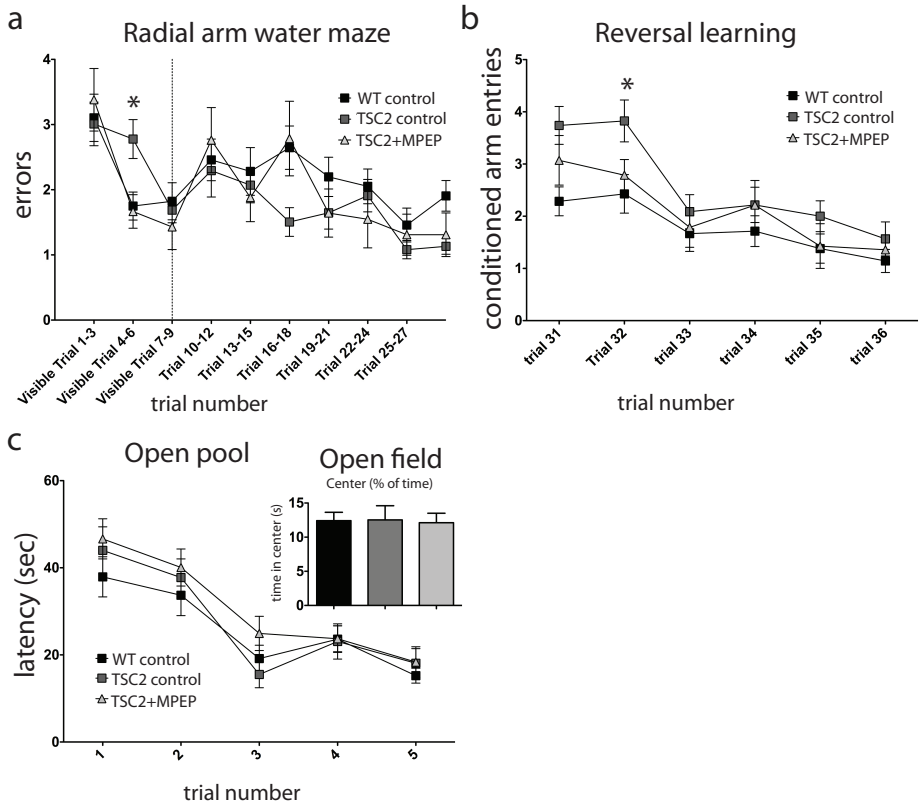

Figure 4.12. **MPEP eliminates a perseverative behavior in TSC2$^{+/-}$ mice.** ***a***. There was no significant difference in learning between groups during RAWM training (2-way ANOVA; WT n=32; TSC n=33; TSC+MPEP n=14, interaction $F(3, 78)=1.299$) (Learning trials 4-6, Mann Whitney t-test; p=0.0257 .TSC n=33;TSC+MPEP n=14) ***b***. TSC2$^{+/-}$ mice make significantly more errors towards the conditioned arm in the RAWM (2-way ANOVA; WT n=21; TSC n=23; $F(3, 43)=9.160$, p=0.0034. For TSC v TSC+MPEP, trial 32 only, Mann Whitney t-test; p=0.0466 .TSC n=23;TSC+MPEP n=14 ***c***. There were no differences for open field performance between genotypes. (WT n=14; TSC n=12; TSC+MPEP n=14). There were no differences for open pool performance between genotypes. (WT n=13; TSC n=12; TSC+MPEP n=14). NS-not significant, *p<0.05, **p<0.005.

4.4. Discussion

While the role of dysregulated mTORC1 signaling in the pathophysiology of TSC has been demonstrated previously(Ehninger et al., 2008; Meikle et al., 2008; Muncy et al., 2009; Auerbach et al., 2011), our work is the first to implicate heightened mGluR5-Erk signaling as a key component of the irregular plasticity and pathological phenotypes in a model of TSC. We show that $TSC2^{+/-}$ mice have increased levels of hippocampal mGluR5 expression, which drives constitutive over-activation of Mek-Erk signaling. Inhibition of mGluR5 signaling with MPEP blocked a novel form of mTORC1-independent mGluR-LTD in $TSC2^{+/-}$ CA1 hippocampus. We illustrate, for the first time, that the $TSC2^{+/-}$ hippocampus displays an increased susceptibility to the development of epileptiform activity following prolonged group I mGluR activation, and that this bursting activity can be suppressed via mGluR5 inhibition with MPEP or inhibition of Erk signaling with U0126. Finally, MPEP corrected a perseverative behavioral phenotype in $TSC2^{+/-}$ mice, which may be a correlate of an autistic-like behavioral aspect in $TSC2^{+/-}$ mice. These findings suggest that modulation of mGluR5 signaling can correct two major pathological aspects of TSC; namely epilepsy and cognitive dysfunction.

In a recent study, Auerbach *et al.* reported reduced mGluR-LTD in CA3-resected hippocampal slices from $TSC2^{+/-}$ mice; a defect that was corrected with rapamycin treatment. Surprisingly, the CA3-resected WT slices did not respond to rapamycin(Auerbach et al., 2011); a finding that disagrees with previous whole slice experiments(Zho et al., 2002; Hou and Klann, 2004)(and Figure 4.1a). To address

the apparent discrepancies between the present study and that of Auerbach *et al*., we performed LTD experiments in CA3-resected slices from WT and $TSC2^{+/-}$ mice. Similar to Auerbach *et al*., we observed that rapamycin did not impact mGluR-LTD in WT CA3-resected slices (Figure 4.12a). However, contrary to Auerbach *et al.*, there was no difference in the magnitude of mGluR-LTD between WT and $TSC2^{+/-}$ (Figure 4.12b) and rapamycin did not impact mGluR-LTD (Figure 4.12c) in $TSC2^{+/-}$ CA3-resected slices. Similar results were obtained when DHPG exposure time was reduced to 5 min (data not shown). In the CA3-resected slices, Auerbach *et al.* did not observe a difference in phosphorylated Erk1/2. To address this discrepancy, we measured p-Erk levels in hippocampal sub-regions (Figure 4.12d). Though there was a trend for increased p-Erk in $TSC2^{+/-}$ CA3 hippocampus ($p=0.29$, Student t-Test), there were no significant differences in p-Erk levels between WT and $TSC2^{+/-}$ hippocampal sub-regions (Figure 4.12d). This indicates that differences in p-Erk are only apparent in whole slice homogenates and explains why Auerbach *et al.* failed to detect these differences in homogenates that lacked the CA3 sub-region. Since severing CA3 render's mGluR-LTD in CA1 rapamycin insensitive (Auerbach et al., 2011)(and Figure 4.12a), it is evident that this manipulation induces dramatic changes in the physiology of CA3-CA1 synapses. Axotomy-induced signaling alters a number of cellular pathways, including mTOR(Park et al., 2008; Leibinger et al., 2012), Erk(Park et al., 2004; Luo et al., 2007) and cellular stress responses(Vinit et al., 2011). Therefore, it is likely that severing of the Schaeffer collaterals produces a myriad of signaling responses that dramatically alter CA1 physiology and makes it

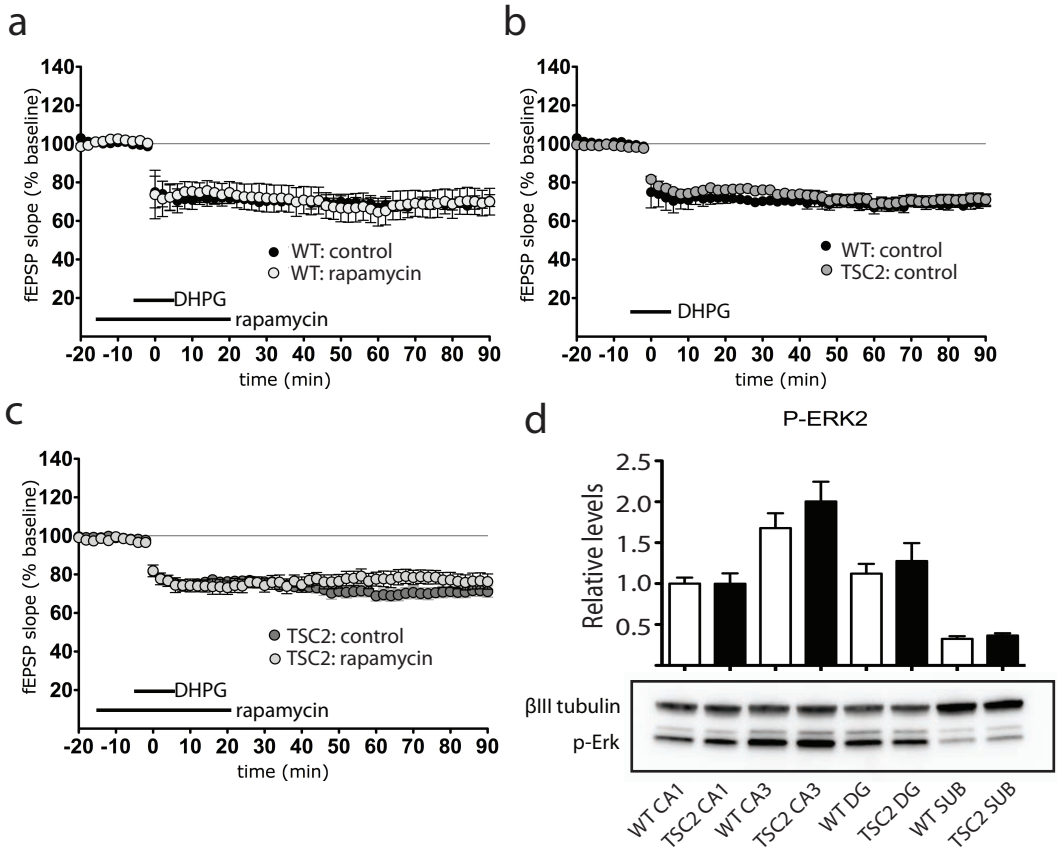

Figure 4.13. CA3-resected WT and TSC2$^{+/-}$ slices display rapamycin-insensitive mGluR-LTD. ***a.*** Rapamycin treatment (20nM) fails to impact mGluR-LTD in CA1 stratum radiatum of WT CA3-resected slices. ***b***. mGluR-LTD magnitude appears identical between WT and TSC2$^{+/-}$ CA1 hippocampus in CA3-resected slices. ***c.*** Rapamycin has no effect on mGluR-LTD in TSC2$^{+/-}$ CA1 hippocampus in CA3-resected slices. ***d.*** Western blot for p-Erk in subregions of WT and TSC2$^{+/-}$ hippocampus. Analysis of p-Erk levels in hippocampal subregions fails to detect a statistical difference.

difficult to draw conclusions regarding results in this reduced preparation. In contrast, whole slice experiments maintain important signaling networks (i.e. mTOR pathway), respond to rapamycin (Zho et al., 2002; Hou and Klann, 2004), are less obscured by damage responses(Park et al., 2004), and are likely to be more physiologically relevant than reduced preparations.

We hypothesize that heightened mGluR5-Erk signaling bypasses the need for mTOR and so renders LTD insensitive to signals that converge on mTOR. Brief preincubation of MPEP, followed by washout, did not by itself inhibit mGluR-LTD magnitude in $TSC2^{+/-}$ hippocampus, but instead restored rapamycin sensitivity to mGluR-LTD (Figure 4.4). This suggests that the $TSC2^{+/-}$ hippocampus is capable of "wild-type" like responses if signaling downstream of mGluR5 can be attenuated, and thus opens up a novel therapeutic avenue. The mechanisms driving upregulation of mGluR5 are not known and we did not detect a difference in mGluR5 transcript between WT and $TSC2^{+/-}$ hippocampus (data not shown). This suggests a post-transcriptional mechanism for mGluR5 upregulation in $TSC2^{+/-}$. We find that inhibition of Mek-Erk signaling, but not PI3K-Akt signaling, recapitulates the effects of MPEP in restoring rapamycin sensitivity to mGluR-LTD, and does so in a dose-dependent fashion (Figure 4.8). Independently of the mTOR pathway, Erk can promote protein synthesis through phosphorylation of Mnk(Banko et al., 2006; Shveygert et al., 2010) as well as through signaling to p90 ribosomal S6 protein kinase (RSK) to increase S6 phosphorylation (Anjum and Blenis, 2008). Reduction of these pathways could explain the effect of U0126 in restoring rapamycin sensitivity.

It is interesting to note that Fragile X Syndrome (FXS), another condition associated with overactive mGluR5 function, shares many aspects of physiology and behavior with TSC. *FMR1* knockout hippocampal slices that were disinhibited with bicuculline to induce synchronous discharges had more prolonged bursting activity and this phenotype was counteracted by MPEP (Chuang et al., 2005). Additional studies in *FMR1* knockout hippocampus demonstrate that basal levels of mGluR5 are unchanged; however, Erk1 is hyperactivated in response to DHPG(Osterweil et al., 2010). The Erk hyperactivation promotes over-translation of mRNA transcripts and can be reduced via antagonism of mGluR5 or Erk signaling, yet is immune to mTORC1 inhibition with rapamycin(Osterweil et al., 2010). It has been reported that *FMR1* knockout mice exhibit rapamycin-insensitive and protein synthesis-independent mGluR-LTD (Nosyreva and Huber, 2006). We observed a similar rapamycin-insensitivity after DHPG treatment, though mGluR-LTD in $TSC2^{+/-}$ remains dependent upon protein synthesis (Fig 4.2). Though subtle differences exist, the similarities suggest that TSC and fragile X mental retardation share some common underlying mechanisms. Moreover, our results, along with the *FMR1* studies mentioned, establish a prominent and perhaps general role for mGluR5 signaling in diseases where dysregulated post-synaptic protein translation is implicated.

Due to the established role of mTORC1 in the pathophysiology of TSC(Kwiatkowski and Manning, 2005) and our recent finding that AMPK modulates hippocampal mTOR(Potter et al., 2010), our initial focus was to determine whether

AMPK activators could reduce mTORC1 activity in the $TSC2^{+/-}$ brain. We find that although AMPK can attenuate mGluR-LTD in WT hippocampus, it fails to reduce LTD in $TSC2^{+/-}$ mice (Figure 4.1). In keeping with our model of hyperactive mGluR5-Erk signaling, preincubation of MPEP restores metformin sensitivity.

The current study implicates heightened mGluR5 function in TSC pathology, thereby suggesting that available mGluR5 antagonists or Erk inhibitors may serve as therapeutic agents for treating people with TSC. It should be noted that Mek/Erk inhibitors are currently in clinical trials for the treatment of cancer(LoRusso et al., 2010) and fenobam is a clinically-available mGluR5 antagonist currently used clinically as an anti-anxiolytic(Jacob et al., 2009). Here we show that modulation of mGluR5-Erk signaling can restore appropriate signaling in a disease model originating from a congenital defect, which implies that symptomatic alleviation in human TSC is possible with anti-mGlur5 and anti-MAPK targeting drugs.

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Chapter 5

Discussion and future directions

Our results define AMPK as a critical modulator of hippocampal LTP and LTD and thereby links energy metabolism to synaptic plasticity. Given the role of energy metabolism in the antiepileptic actions of the ketogenic diet, these results may provide mechanistic insights into the ketogenic diet as well as new potential therapeutic avenues for the treatment of epilepsy. By attenuating activity-dependent potentiation, metabolic control of plasticity may be an important mechanism to prevent the development of hyperexcitable and unstable neural circuitry.

Brief summary

Our initial experiments tested the hypothesis that AMPK activation could inhibit hippocampal LTP at the CA3-CA1 synapse. This hypothesis was confirmed with three mechanistically distinct AMPK activators, across two common LTP induction paradigms. Importantly, the effects of these AMPK activators on LTP were eliminated when combined with either of two structurally distinct AMPK antagonists. Several lines of evidence indicate that AMPK exerts its effects through inhibition of mTOR signaling. Biochemical analysis demonstrated that the AMPK activator, 2DG, significantly reduced the phosphorylation of downstream components of the mTOR signaling cascade. Inhibition of mTOR signaling solely during the high frequency stimulus epoch is sufficient to block LTP expression(Cammalleri et al., 2003). When metformin treatment was restricted to the theta burst stimulus period, followed by washout, LTP was potently blocked.

AMPK activators, 2DG and metformin also inhibited mTOR-dependent mGluR-LTD; an effect that was eliminated by two AMPK antagonists.

Given the role of hyperactive mTOR signaling in the pathology of TSC, we tested whether AMPK activation could impact mTOR-dependent plasticity in a mouse model of TSC. Metformin potently eliminated aberrant LTP in $TSC2^{+/-}$ hippocampus. Where TSC1/2 function is abrogated, AMPK can inhibit mTOR signaling via phosphorylation of RAPTOR, which promotes its dissociation from mTORC1(Gwinn et al., 2008). Accordingly, we observed that metformin led to RAPTOR phosphorylation and reduced RAPTOR-mTOR binding selectively in $TSC2^{+/-}$ hippocampus.

Our next prediction was that, similar to WT hippocampus, metformin should inhibit mGluR-LTD in $TSC2^{+/-}$. To our surprise, treatment with either metformin or rapamycin had no effect on mGluR-LTD in $TSC2^{+/-}$ hippocampal slices. To identify the potential molecular underpinnings contributing to this novel mTOR-independent mGluR-LTD, we investigated signaling components that are altered in TSC. We discovered that these $TSC2^{+/-}$ mice display heightened expression of mGluR5 and constitutively activated Erk signaling. Reduction of mGluR5 or Erk signaling was capable of restoring sensitivity of mGluR-LTD to metformin and rapamycin.

The enhanced mGluR5-Erk signaling in $TSC2^{+/-}$ mice appears to have important physiological ramifications; $TSC2^{+/-}$ mice displayed an increased susceptibility to the development of epileptiform activity in CA3 hippocampus. Reduction of mGluR5 or Erk signaling significantly reduced the development and

severity of epileptiform bursting. Finally, inhibition of mGluR5 corrected a perseverative behavioral phenotype in $TSC2^{+/-}$ mice.

AMPK and the "*Metabolic Theory of Plasticity*"

AMPK is a ubiquitous, metabolically-sensitive signaling hub that modulates a variety of cellular processes in order to maintain appropriate cellular energy levels. The extensive and rapidly expanding literature describing the role of AMPK in health and disease illustrates that AMPK signaling is an integral component of cellular function. The finding that AMPK controls synaptic plasticity has broad implications for the development of neural circuitry. It is generally accepted that learning and memory are encoded through modifications of synaptic contacts; a process that appears to be modulated by AMPK. In the brain, and especially during neural development, synaptic strength can dictate whether a particular connection is preserved or eliminated. Given that synaptic strength directs a changing network structure and AMPK activity regulates LTP and LTD, it is likely that AMPK contributes to the refinement of neural network architecture. Additionally, the mTOR pathway regulates growth cone stability, suggesting that AMPK-dependent modulation of mTOR may impact cortical growth and development. Thus, AMPK signaling may impact synaptic as well as structural plasticity in the developing and adult brain.

As mentioned in the introduction, brain glucose is heavily compartmentalized and significantly decreases in rat hippocampus during explorative learning. Glucose

administration enhances memory formation in rats and humans across multiple cognitive tests(McNay et al., 2000; Scholey et al., 2001; McNay and Gold, 2002). The additional glucose needs to be present during, or immediately following the acquisition phase of the behavioral test. This temporal limitation coincides with the window of mTOR-dependent plasticity. Hippocampal AMPK activation reduces mTOR signaling and inhibits memory formation; whereas glucose injections activate mTOR signaling and facilitate learning and memory(Dash et al., 2006). This demonstrates the physiological relevance of AMPK in plasticity and behavior.

The observation that extracellular glucose is limiting for learning suggests that the brain could actually be energy stressed during learning, which is counter to the notion that the brain is somehow energy privileged. A central tenet of neuroscience is that neural excitation needs to be balanced with inhibition. It is plausible that energy limitation is a physiological adaptation to limit plasticity and that AMPK may be the mediator of this process. The glucose transporter at the blood brain barrier, GLUT1, has an effective K_m of 1.6mM for influx of glucose into the brain parenchyma. In fact, GLUT1 has an asymmetric structure such that its function is inhibited by intracellular ATP. Thus glucose transporters have K_m values close to the free glucose concentration in the hippocampus (1.2mM) to, in effect, throttle glucose uptake. Why would such a system evolve when increased abilities to learn would seem to be evolutionarily advantageous? The answer may be that the same cellular pathways involved in activity-dependent plasticity and learning, could contribute to the development of unstable hyperexcitable circuits. Thus, AMPK could represent an

evolutionarily conserved mechanism to limit mTOR signaling during activity using energy availability as a proxy for neuronal activity. Without the AMPK break, the system would be rendered unstable – a condition analogous to TSC.

Future experiments. There is preliminary data suggesting that under low glucose conditions (2.5mM glucose), hippocampal slices fail to express LTP at CA3-CA1 synapses. Is this due to AMPK activation? If so, we should be able to inhibit AMPK under low glucose conditions and allow the capture of LTP. This signaling should be reflected biochemically in terms of mTOR signaling components. If AMPK antagonists enhance mTOR signaling and LTP under energy-limiting conditions, then administration of these compounds should also enhance learning and memory in behavioral assays. This could be accomplished with cannulated mice that undergo behavioral testing. Dash *et al*. showed that AMPK activation *in vivo* could inhibit mTOR to reduce learning in the morris water maze(Dash et al., 2006). Our previous work was done with 25mM glucose; the experiments outlined above would address the role of AMPK in plasticity and learning *under physiological glucose* conditions. There are mice with floxed AMPK that could be mated with the appropriate Cre-driven mice to produce AMPK conditional knockout mice. If AMPK mediates the loss of LTP under low glucose conditions, then the AMPK knockout mice should be unaffected by low glucose.

Metabolism and epilepsy

Cellular metabolism influences many neurological processes and neurological disease is often associated with metabolic dysfunction. Historically, fasting has been a potent therapeutic for epilepsy. In 1921, the ketogenic diet was described as a carbohydrate-restricted dietary treatment to reduce seizure frequency (Wilder, 1921). In the 90 years since it's inception, the ketogenic diet has proven to be an effective antiepileptic therapy for a wide range of intractable epilepsies, especially in children(Stafstrom, 2004). This high fat, adequate protein, low carbohydrate diet results in drastically lowered plasma glucose levels. The liver responds by producing ketone bodies to serve as an alternative energy source for the body and causes a shift from glycolytic to ketolytic metabolism. The mechanism of action for the anticonvulsant effects of the ketogenic diet remains controversial. There is ample evidence for the anticonvulsant effects of acetone, a prevalent ketone body(Likhodii et al., 2003). Several studies have demonstrated that the ketogenic diet increases adenosine release, which acts as a retrograde signal to inhibit presynaptic release. Accordingly, mutant mice deficient in either adenosine or adenosine receptors are prone to the development of epilepsy(Masino et al., 2011).

In addition to increasing adenosine and ketone body production, the ketogenic diet reduces plasma glucose concentration and glycolysis. A beneficial role for reduced glycolytic rate may be reflected in the observance that the level of ketosis does not often correlate with seizure protection in the ketogenic diet(Greene et al., 2003). Work from our lab demonstrated that glycolytic inhibition with 2DG

significantly retarded the progression of epileptogenesis in the kindling model (Garriga-Canut et al., 2006). This protective effect relied on the reduction of glycolytic NADH that when sensed by CtBP, augmented REST-mediated transcriptional repression of BDNF and TrkB. This exciting antiepileptic action of 2DG prompted us to examine the potential acute effects of glycolytic inhibition. This led to our discovery that AMPK served to regulate mTOR-dependent plasticity in the hippocampus. It is tempting to consider that AMPK is involved in the antiepileptic actions of the ketogenic diet. McDaniel *et al.* reported a potent activation of AMPK in the liver of ketogenic diet-fed rats, yet only observed a trend towards activation of hippocampal AMPK. Reduced mTOR signaling was observed in both tissues (McDaniel et al., 2011). Inhibition of mTOR is essential for liver ketogenesis (Sengupta et al., 2011) which provides an abundance of high energy molecules for the brain(Nakazawa et al., 1983; Pan et al., 1999). This would presumably prevent full activation of AMPK in the hippocampus. Nevertheless, since the ketogenic reduces mTOR signaling in the brain, it is possible that AMPK activators could partially reproduce the effects of the ketogenic diet and be used therapeutically to treat epilepsy.

Epilepsy–related plasticity is often associated with altered ion channel expression and/or function. Two established alterations in channel function implicated in epilepsy are decreased HCN and voltage-gated potassium channels. In addition to reducing activity-dependent translation, AMPK also signals to enhance inhibitory $GABA_B$ receptor activity. Increased $GABA_B$ signaling resulted in enhanced

activity of inward rectifying potassium channels(Kuramoto et al., 2007); a process that increases afterhyperpolarization potentials and decreases the likelihood for neuronal bursting activity. Therefore, AMPK may impact neuronal excitability through multiple beneficial mechanisms.

Recently, anecdotal evidence for the therapeutic potential of metformin came to us by way of a telephone call from a mother whose daughter suffers from severe epilepsy. The mother told us that her 25 year-old daughter experienced many seizures per day, which were medically intractable and often required hospitalization. Through online research and upon reading our results with metformin, this woman convinced the doctor to prescribe metformin to treat her daughter's epilepsy. The seizure frequency dropped dramatically from numerous daily seizures to only 1-2 seizures per month that did not require hospitalization! This woman contacted our lab to inform us and thank us for our contributions to epilepsy research. The impact of metformin on seizure frequency may be specific to this particular epilepsy; catamenial epilepsy, a chronic condition where seizure intensity and duration are exacerbated by estrogen and therefore fluctuate during a woman's menstrual cycle.

Future experiments: Testing the role of AMPK in the antiepileptic effect of the ketogenic diet is not easily addressed with slice electrophysiology. This is due to the difficulty of recapitulating the plethora of metabolic changes that occur in the behaving animal, or human, that receives the ketogenic diet. Indeed, researchers

have assessed the impact on excitability of many compounds that change during the ketogenic diet, and have unfortunately failed to identify the key antiepileptic mode of action of this diet. In order test the role of hippocampal AMPK in the ketogenic diet, AMPK could be deleted selectively in the hippocampus using CRE-mediated recombination. Control and knockdown mice could be fed the ketogenic diet and then subjected to a variety of epileptogenic models (i.e. kainic acid, pilocarpine, kindling, etc.), followed by monitoring for the development of recurrent seizures. If hippocampal AMPK is involved in the protective effects of the ketogenic diet, then AMPK knockdown mice should be unaffected by the diet, whereas control mice will be less likely to subsequently develop epilepsy.

Our data supports a model whereby AMPK reduces LTP through inhibition of the mTOR pathway. However, it is likely that AMPK activation impacts separate processes, such as $GABA_BR$ signaling and consequent alteration in K^+ channel activity. Does chronic AMPK activation, perhaps with metformin, impact $GABA_BR$ function *in vivo*? If so, is this AMPK-$GABA_BR$ signaling antiepileptic? The answer would provide additional support for the potential use of metformin as an antiepileptic compound.

The anecdotal story from the parent begs the question of how could metformin impact chronic epilepsy? Is it through AMPK, or some other mechanism? Fortunately, there is ample work demonstrating a role for estrogen signaling in the facilitation of hippocampal LTP. Therefore, we could test whether metformin inhibits estrogen-induced LTP and then determine if this is dependent upon AMPK

activation. It is then possible that adding exogenous estrogen could exacerbate CA3 bursting. Does metformin reduce the severity of bursting when added to estrogen-treated slices? Since estrogen signaling impacts a number of cellular pathways, including mTOR, we would next test whether rapamycin impacts estrogen-induced LTP and bursting. All of our previous experience suggests that metformin and rapamycin should produce nearly identical results. Any observed difference in the effects of metformin and rapamycin could reflect divergent modes of action for these two compounds.

mGluR-Erk signaling in epilepsy

The mGluR-Erk pathway couples glutamatergic transmission to intracellular responses; a process that is hyperactivated during seizure activity. Therefore, it is not surprising that heightened mGluR and Erk signaling have been linked to the pathology of epilepsy. There is overactivation of Erk in the mesial temporal lobe of epileptic patients(Park et al., 2003) and mice engineered to express constitutively active MEK1 develop epilepsy(Nateri et al., 2007). In the pilocarpine model of epileptogenesis, there is distinct Erk activation in neural precursor cells of the subgranular zone of the hippocampal dentate gyrus(Li et al., 2010). Given that Erk signals to mTOR to promote growth and plasticity, it is likely that heightened Erk signaling contributes to pathological plasticity changes that occur in the dentate gyrus following status epilepticus. Paradoxically, in this same model, Erk activation across the hippocampal formation was significantly lower than controls during the

"chronic" period between status epilepticus and the development of spontaneous seizures. However, Erk activation increased sharply during seizure and then quickly decreased back to baseline over the 15 minutes following seizure cessation(Houser et al., 2008).

Activation of mGluR signaling with DHPG induces epileptiform bursting in CA3 neurons. The development of this bursting is blocked with Mek-Erk antagonists, as well as inhibitors of ionotropic glutamate receptors(Zhao et al., 2004). Similarly, reduction of mGluR5 activity with MPEP reduces seizure development in several epilepsy models: FMR1 mutants over-expressing amyloid beta(Westmark et al., 2009), dis-inhibition of $GABA_AR$ with bicuculline(Chuang et al., 2005), and PTZ-induced epileptogenesis(Mares et al., 2010). Thus, there exists abundant evidence demonstrating a general role for mGluR and Erk signaling contributing to, or exacerbating the development of hyperexcitability. This detrimental signaling axis appears to be overactive in $TSC2^{+/-}$ hippocampus and underlies the aberrant LTP and LTD in these mice (chapters 2 & 3).

Future experiments: Chapter 3 demonstrates that Erk signaling underlies the increased epileptiform bursting in $TSC2^{+/-}$ slices. It would be interesting to investigate which downstream aspects of Erk signaling are causative for the bursting phenotype in $TSC2^{+/-}$ mice. There are specific inhibitors of Rsk, Mnk, and S6K, all of which are downstream of Erk and impact protein translation. Which one, or combination of, is capable of recapitulating the protective effects of U0126?

Inhibition of Mnk significantly reduced phosphorylation eiF4E, yet did not restore rapamycin sensitivity to mGluR-LTD (data not shown). It would be very informative to assess the impact of Rsk inhibition on mGluR-LTD and bursting in $TSC2^{+/-}$ mice.

There is also the question of how mGluR5 levels are upregulated in the $TSC2^{+/-}$ hippocampus. Is there increased translation, transcription, or stabilization involved? We have preliminary data suggesting that mGluR5 levels increase upon DHPG stimulation; an upregulation that appears to be immune to mTOR inhibition with rapamycin. This could reflect rapid translation or stabilization. In basal, unstimulated hippocampus, there are no observable differences in mGluR5 transcript levels between WT and $TSC2^{+/-}$. These data suggest a post-transcriptional role, perhaps due to enhanced synaptic activity, for increased mGluR5 levels in $TSC2^{+/-}$. The elucidation of how mGluR5 levels change in $TSC2^{+/-}$ mutants could uncover potential therapeutic approaches for limiting the pathological progression of TSC.

Final Remarks

Cellular metabolism exerts potent effects on transcriptional and translational processes to modulate the functioning of all cells. This impact is readily observed in the central nervous system and neurological disease is often associated with metabolic dysfunction. Currently available pharmacological agents produce a host of unacceptable side-effects, whereas metabolic interventions may provide alternative options with decidedly less contra-indications. Rapamycin and its analogues, are

currently under intense clinical research for use as anticancer and antiepileptic treatments. Unfortunately, rapamycin is an immunosuppressant that can dispose the patient to opportunistic infections, hyperlipodemia, leukopenia, and thrombocytopenia, which occur frequently (>35%) (Sofroniadou and Goldsmith, 2011). In contrast, the anti-diabetic drug metformin is used by millions of people worldwide with relatively uncommon (6-9%) and minor side-effects when prescribed correctly. It will be important to assess the role of AMPK in the antiepileptic effects of metformin. Given the success of the ketogenic diet for the treatment of epilepsy, it is compelling that other metabolic modulators, such as metformin and 2DG, impact activity-dependent plasticity through pathways that are implicated in the progression of epilepsy. These observations argue for the continued research into the use of metformin and other AMPK agonists in the treatment of epilepsy.

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