Interactions between Hebbian and homeostatic synaptic plasticity in hippocampal circuits

By

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in The University of Michigan
2014

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DEDICATION

To my wife Jill and my son Kurt

ACKNOWLEDGMENTS

I am grateful to those who have contributed to my professional development and helped me complete my graduate studies. I would like to thank the members of my thesis committee, Dr. Michael Sutton, Dr. Geoff Murphy, Dr. Gina Poe, Dr. Rich Hume and Dr. Peter Todd, for generously donating their time for the development of my projects and career goals. I can't imagine assembling a better group of mentors on this journey.

I would especially like to acknowledge my advisor, Dr. Michael Sutton. Thank you for accepting me into your lab and for supporting me throughout my thesis research in myriad ways. I am forever indebted to him for his guidance which made possible this dissertation. Thank you to all of the past and present Sutton Lab members for stimulating discussions and thoughtful advice. In particular, I want to acknowledge postdoctoral fellows Dr. Javier Munoz-Cuevas and Dr. Erin Johnson-Venkatesh for collaborating with me on the projects that account for the data in Chapter 2 and Chapter 3 of this dissertation. In addition, I would like to express my deepest appreciation for my wonderful collaborators, Dr. Peter Todd and Abbie Renoux, without whom Chapter 4 would not have been possible.

I would also like to express my gratitude to Dr. Hisashi Umemori and Dr. Michal Zochowski and their respective lab members for guidance, advice and stimulating discussions over the years. Dr. Shannon Moore provided helpful guidance over the years and I thank her especially for her assistance in the editing of this dissertation. Dr. Gary

Van Hoesen and Dr. Henry Paulson deserve my eternal gratitude for introducing me to the world of academic research when I was just an undergraduate at the University of Iowa, naïve to the possibility that one could make his living pursuing purely academic interests.

I would like to express my appreciation to the Neuroscience Graduate Program, in particular Dr. Edward Stuenkel, Dr. Steve Maren, and Valerie Smith for providing administrative support during my graduate tenure. I would also like to thank the support staff in MBNI for their frequent assistance and grant management.

I am forever grateful to my parents, Jim and Evelynn for cultivating my curiosity of the natural world. I am indebted to my wife, Jill, for her unwavering love, encouragement and support. Your support outside of the lab was critical to the completion of my graduate work. Most importantly, I thank you for bringing our son into this world. Finally, I thank Kurt for providing much relief and great joy to my life during this challenging period.

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ABSTRACT

Hebbian forms of synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD), are thought to underlie learning and memory, but these processes may have a destabilizing effect on neural activity. Homeostatic synaptic plasticity (HSP), often studied as compensatory adaptations driven by perturbations of neuronal activity, is thought to counteract the destabilizing influence of Hebbian plasticity in neural circuits. However, it is unclear how these opposing forces on synaptic efficacy co-exist in neuronal circuits, largely because of the differing preparations and time domains over which they are studied. To investigate interactions between these distinct forms of synaptic plasticity, we characterized a rapid form of HSP expressed at CA3-CA1 synapses in acute hippocampal slices. By altering the frequency of Schaffer collateral stimulation, we induced compensatory changes in synaptic strength that are bidirectional, input-specific and mechanistically distinct from LTP and LTD.

These features allowed us to address the manner by which HSP interacts with Hebbian plasticity at the same population of synapses. Our results reveal that input-specific HSP generally offsets the magnitude of subsequent Hebbian plasticity expression in an additive fashion. Strikingly, we found that prior induction of Hebbian plasticity constrained the magnitude of subsequent HSP expression. This interaction only occurs if both plasticities alter synaptic strength in the same direction, as input-specific HSP was otherwise able to compete with previously established Hebbian plasticity. We identify a

scenario in which neither form of plasticity studied is dependent on new protein synthesis, yet the metaplastic interaction between them is mediated by local protein synthesis. Taken together, the magnitude and durability of synaptic efficacy changes are a product of both Hebbian and homeostatic mechanisms, suggesting that HSP may also influence information coding and storage in neural circuits.

Finally, we examine the nature of activity-dependent biosynthesis of FMRP involved in another local translation-dependent process at synapses, mGluR- LTD. We find that mice with the Fragile X premutation exhibit impaired mGluR-dependent translation of dendritic FMRP and enhanced mGluR-LTD. The synaptic plasticity phenotype is shared with Fragile X Syndrome model mice, yet involves a distinct underlying mechanism, suggesting a possible mechanism for cognitive defects in premutation carriers.

Chapter I

Introduction:

Activity-dependent synaptic plasticity in area CA1

1.1 From Aristotle to Hebb

"Memory is the scribe of the soul"

-Aristotle

This succinct statement attributed to the great Greek philosopher who pondered the nature of memory more than 2000 years ago encapsulates the fundamental importance of memory to humanity. How exactly we can remember events and facts for entire lifetimes has been of interest to scientists since the earliest days of neuroscience. Santiago Ramon y Cajal even conjectured on the mechanisms underlying this process in 1894, speculating that increased mental activity causes greater branching of neuronal processes and connections between them (Jones, 1994, 1999). Later, Donald Hebb described in more depth a candidate cellular mechanism, often referred to as Hebb's rule, or Hebb's postulate (Hebb, 1949). Hebb described a rule whereby the connection between a pair of neurons would become stronger if they were simultaneously active. Within a network of neurons, this process would lead to the formation of cell assemblies, which would serve as the physical memory trace, or engram. An important feature of the

cell assembly is that partial activation would lead to full activation of the assembly, so long as the cells involved were recurrently connected. Taken together, Hebb provided a mechanism whereby synaptic changes could lead to memory formation at the scale of neuronal networks.

Loss of the ability to form new memories through disease or injury has devastating consequences on quality of life. In particular, damage to the human hippocampus has profound negative consequences on declarative memory formation, but not memory storage, since patients display severe anterograde amnesia but retrograde amnesia is typically less severe (Scoville & Milner, 1957; Squire & Wixted, 2011; Zola-Morgan et al., 1986). The basic hippocampal circuit is composed of a trisynaptic loop, a feedforward circuit involving three major regions: The dentate gyrus (1) receives input from the surrounding entorhinal cortex via the perforant pathway. Dentate gyrus neurons project via the mossy fibers to area CA3 (2), which then projects to area CA1 (3) via Schaffer collaterals. CA1 axons then project to the subiculum and back to the cortex (Andersen et al., 1971). Although this basic circuit is an oversimplification of the full hippocampal circuitry, it has proved an extremely useful paradigm in animal studies, which have demonstrated that the rodent hippocampus, and the CA1 hippocampal region in particular, is similarly involved in memory formation (Mayford et al., 2012; Morris et al., 1986; Shimizu, 2000; Tsien et al., 1996). For example, lesions to the rodent hippocampus result in deficits in the Morris water maze assessment of spatial learning and memory (Bliss et al., 2003). The hippocampus is not the only structure to have influence on learning and memory, but I will focus on this region for the purposes of this introduction.

Excitingly, experimental activation of cell assemblies underlying a learned memory may be achievable with currently available techniques. Work from Tonegawa's lab in mice has demonstrated a method to label active hippocampal cells during fear conditioning in one context with light-activated channelrhodopsin-2 (ChR2). Stimulating the labeled cells with the appropriate light while the mouse was in a neutral context elicited freezing behavior, a common measure of fear memory recall in rodents (Liu et al., 2012). They extended these findings in an impressive set of experiments, in which they creating a false memory using a similar approach. Investigators initially labeled a population of cells in the mouse hippocampus active during novel context exploration. These mice were later placed in a second, fear conditioning context while driving spiking in the labeled neurons. By activating the initial cell population during the creation of a fear memory, they tested whether the fear memory would map onto the active cell assembly representing the non-fearful context. Indeed, these mice would freeze upon reintroduction to the initial context but not a completely novel context, suggesting the creation of a false memory and not just generalized fear behavior (Ramirez et al., 2013). With the recent advent of light-activated channels with non-overlapping excitation spectrums it will become possible to activate separate cell assemblies within the same experiment, which may allow more complex investigations of engrams underlying learning and memory (Klapoetke et al., 2014).

1.2 Hebbian synaptic plasticity

1.21 Long Term Potentiation (LTP)

Several decades after Hebb published his postulate, a cellular correlate of Hebb's rule was discovered in the rabbit hippocampus (Bliss & Lømo, 1973). In response to a rapid pattern of perforant pathway stimulation, investigators observed a persistent enhancement of neurotransmission. Thus, it is known as long term potentiation (LTP). LTP is an associative process, as it occurs when a synapse experiences presynaptic activity and postsynaptic depolarization coincidently. Since LTP strengthens the connection between simultaneously active neurons, it can be considered a biological implementation of Hebb's rule.

40 years of studies following up on this initial discovery of a Hebbian LTP have revealed that Hebbian processes come in many varieties (Bliss et al., 2003). It should be noted that Hebbian plasticity has been reported in brain regions outside the hippocampus, including the neocortex and amygdala, but I will restrict my discussion primarily to findings from area CA1 of the hippocampus. Different patterns of electrical stimulation delivered to the axons in a slice preparation produce varying degrees and types of plasticity (Cooper & Bear, 2012; Kirkwood et al., 1993). A single burst of 100 pulses at 100 Hz (a 'tetanus') typically produces a weaker and shorter lasting potentiation of postsynaptic responses. The delivery of multiple tetani produces a much longer lasting and stronger potentiation. Beyond the degree of potentiation, there are mechanistic differences between these plastic changes. For instance, the weaker of the two, commonly referred to as early-phase LTP (E-LTP) does not require new transcription or translation for expression whereas the stronger late-phase LTP (L-LTP) requires new protein synthesis. Before revisiting the protein synthesis requirements of Hebbian synaptic plasticity below, I will introduce a second type of plasticity.

1.22 Long Term Depression (LTD)

The counterpart to LTP is long term depression, or LTD. LTD was discovered in the CA1 region using the acute hippocampal slice preparation (Dudek & Bear, 1992; Stanton & Sejnowski, 1989). Prior to discovery, LTD was predicted to occur under conditions when patterns of input activity fail to activate NMDA receptor-dependent signaling strongly enough to trigger LTP (Bienenstock et al., 1982). The modification threshold is the switch point whereby postsynaptic activity lower than this threshold produces LTD and higher than this threshold produces LTP. Consistent with the prediction, hyperpolarizing the postsynaptic neuron while delivering a typically LTP-inducing stimulation (high frequency stimulation, HFS) resulted in depression of synaptic responses (Stanton & Sejnowski, 1989). Induction of NMDA receptor-dependent LTD at CA1 can reliably be induced by delivering current pulses to Schaffer collaterals at a relatively slow frequency (low frequency stimulation, LFS < 10 Hz). By delivering 900 pulses across a wide range of frequencies in separate slices, Dudek and Bear (Dudek & Bear, 1992) were able to produce a plot of the frequency of presynaptic stimulation frequency versus the sign and degree of synaptic changes which resulted in a response curve similar to the curve produced by Bienenstock, Cooper and Munro (the BCM curve) (Bienenstock et al., 1982).

Strictly speaking, LTD is not accounted for by Hebb's original learning rule. However, since his original description, Hebb's rule was extended to account for LTD, resulting in the Hebbian covariance rule (Dayan & Abbott, 2001; Sejnowski et al., 1989). The covariance rule specifies that synaptic strength increase when presynaptic and postsynaptic activity are positively correlated, but decrease if they are negatively

correlated, thus describing a role for LTD as an associative plasticity. From this point on, when I refer to Hebbian plasticity I will be referring to LTP and LTD. Like LTP, LTD can also be subdivided into a weaker early phase (E-LTD) and a longer lasting late phase (L-LTD) which requires de novo protein synthesis (Bear & Abraham, 1996; Collingridge et al., 2010; Manahan-Vaughan et al., 2000; Sajikumar et al., 2005).

1.23 Hebbian Synaptic Plasticity – Protein Synthesis Dependence

L-LTP is perhaps the most attractive candidate memory mechanism because several of its properties parallel those seen in memory formation. It can produce extremely long-lasting changes in synaptic efficacy, in some cases observed for several months in vivo (Abraham et al., 2002). Conversion of E-LTP to L-LTP requires new protein synthesis, and experiments across multiple systems for the last 50 years have revealed that consolidation of stable memories requires protein synthesis (Flexner et al., 1963; Agranoff et al., 1965, 1966; Davis & Squire, 1984; Sutton et al., 2001; Costa-Mattioli et al., 2009). Interestingly, reactivated memories can be sensitive to disruption and ultimately destabilize unless they undergo a process of reconsolidation that also requires new protein synthesis (Nader & Hardt, 2009; Tronson & Taylor, 2007). Like LTP, L-LTD requires new protein synthesis for the changes to persist in an enduring form (Bear & Abraham, 1996; Collingridge et al., 2010; Manahan-Vaughan et al., 2000; Sajikumar et al., 2005).

As already mentioned, LTP and LTD can be sub-classified based on their dependence on new protein synthesis, tested by global application of translation inhibitors. Delivering a single train of high or low frequency stimulation leads to a

translation-independent enhancement or depression of synaptic strength (early-phase LTP/LTD) lasting 1-3 hrs, whereas repeated stimulation trains lead to more long-lasting changes in synaptic transmission (late-phase LTP/LTD) that require protein synthesis (Frey et al., 1988; Huang & Kandel, 1994; Manahan-Vaughan et al., 2000).

Transcription has also been shown to be important for L-LTP in vitro and in vivo (Alberini, 2009; Frey et al., 1996). Consistent with this view, a number of transcription factors are involved in both LTP and memory, including cAMP response element binding protein (CREB), CCAAT enhancer binding protein (C/EBP), and others (for an extensive review of specific transcription factors underlying LTP and memory, see Alberini, 2009).

1.24 in vivo LTP induced by learning

Another point of connection between LTP and memory is in the common molecular mechanisms required for each. There are a number of molecules important for LTP and memory, which includes, but is not limited to, NMDA receptors (NMDARs), CaMKII, BDNF, IGF2 and Arc/Arg3.1 (Chen et al., 2011; Lu et al., 2008; Morris et al., 1986; Plath et al., 2006; Silva et al., 1992; Silva et al., 1992). Of course, demonstration that LTP and memory involve some of the same molecular mechanisms does not necessarily imply that LTP underlies or causes memory consolidation. For instance, in vivo NMDAR blockade disrupts LTP and memory formation (Morris et al. 1986), but Autry et al. (2011) found that NMDAR blockade led to an increase in hippocampal protein synthesis and BDNF production. This finding provides but one potential consequence of NMDAR blockade beyond selectively disrupting LTP induction, and there are almost certainly other unappreciated effects. However, together with the points

I have already discussed, it is generally assumed that Hebbian plasticity plays a dominant role in memory formation. In addition, numerous studies have found strong correlations between LTP expression and memory performance (Malenka & Bear, 2004). For a recent example, applying recombinant insulin-like growth factor 2 (IGF-2) to the rodent hippocampus leads to both enhanced LTP and greater memory performance on an inhibitory avoidance task, whereas disrupting IGF-2 signaling blocked both of these effects (Chen et al., 2011). Notably, LTP-like changes have been recorded in the hippocampus in response to learning in awake, behaving animals, and this LTP occludes subsequent tetanus-induced potentiation suggesting the mechanisms underlying learning-induced potentiation are similar to the LTP typically studied (Neves et al., 2008; Whitlock et al., 2006).

1.3 Molecular mechanisms underlying Hebbian synaptic plasticity

1.31 NMDA receptors and calcium

Hebbian forms of plasticity at CA3-CA1 synapses are largely induced and expressed postsynaptically (Malenka & Bear, 2004). One of the most fundamental proteins in both LTP and LTD is the glutamatergic NMDA receptor. This ionotropic receptor is permeable to Ca^{++} ions, in addition to Na^{++} ions, but is gated by both glutamate and membrane voltage. The voltage gate is achieved by a Mg^{++} ion block in the pore of the channel that is removed upon sufficient depolarization. This property allows the receptor to act as a detector of coincident activation between a presynaptic input and the postsynaptic target, an essential feature of a Hebbian process. Importantly, NMDAR antagonists block LTP induction in vitro and in vivo (Abraham et al., 1987;

Morris et al., 1986). In vivo disruption of NMDAR activation, pharmacologically or genetically, also blocks formation of certain types of memory, consistent with the idea that NMDAR-dependent LTP underlies memory (Morris et al., 1986; Tsien et al., 1996; but see Bannerman et al., 2012). LTD also requires NMDAR activation (Dudek and Bear, 1992), so in vivo blockade of NMDARs will affect both types of Hebbian plasticity. Thus, while this data implicates Hebbian synaptic plasticity in memory formation, it is not clear that LTP alone is required. Furthermore, NMDAR involvement in processes other than LTP/LTD may contribute to the memory deficits observed. A common feature of major synaptic plasticity subtypes is their dependence on calcium signaling. Since NMDA receptors are permeable to Ca^{2+} ions and they are activated during LTP and LTD induction, activated NMDA receptors are a likely source of elevated calcium required for LTP and memory (Wayman et al., 2008).

1.32 CaMKII

Elevation of postsynaptic calcium binds to calmodulin and activates calcium/calmodulin-dependent protein kinase II (CaMKII), a dodecameric holoenzyme strongly implicated in LTP and memory formation (Lisman et al., 2012). Active CaMKII phosphorylates CaMKII *in trans* at T286 which keeps the kinase in a persistently active state. Introduction of constitutively active CaMKII potentiates synaptic responses and preventing T286 phosphorylation blocks LTP and memory (Lledo et al., 1995; Giese et al., 1998). Interestingly, active CaMKII is targeted to the postsynaptic density of active synapses, providing part of the mechanism for the synapse specificity of LTP. At active synapses, CaMKII activity leads to enhanced AMPA receptor (AMPAR) signaling. Potentiated AMPAR-mediated currents can occur via increase single channel

conductance, increased AMPAR surface accumulation, or both (Bredt & Nicoll, 2003; Poncer et al., 2002). Phosphorylation of AMPAR and the auxillary subunit stargazin by CaMKII are important for proper AMPAR delivery to synapses (Opazo et al., 2010; Tomita et al., 2005). Phosphorylation of the GluA1 AMPAR subunit at S831 by CaMKII increases the single channel conductance and is thought to occur in the early stages of LTP expression (Bredt & Nicoll, 2003).

1.4 The problem with purely Hebbian circuits

Although there is strong evidence that Hebbian modifications are a crucial component of learning and memory, they cannot be the only mechanism operating in neural networks. Networks that operate purely by Hebbian plasticity rules have been shown to be inherently unstable due to the positive feedback nature of LTP/LTD (Miller, 1996). If the connection between any two neurons is strengthened by coincident activity, then one can assume after a sufficiently long period of time that the neurons will be coincidently active and strengthened, which will increase the probability of subsequent strengthening. In time, the connections will be strengthened to saturation and the overarching network will be inflexible. The discovery of LTD was initially thought to counteract this problem by weakening the connections when activity of neuron pairs did not coincide (Dudek & Bear, 1992; Stanton & Sejnowski, 1989). However, computational models have revealed that networks expressing both of these processes are still unstable (Dayan & Abbott, 2001; Turrigiano & Nelson, 2000). Thus, a number of solutions have been explored which serve to confer network stability. One solution based primarily on the computational modeling data was to augment the Hebbian learning rule with an artificial renormalization term to constrain saturating values the synaptic weights

(Oja, 1982). However, there is now strong evidence for empirically-based solutions relevant to the stability of neural networks. For the remainder of this introduction, I will discuss two of these research areas: metaplasticity and homeostatic plasticity.

1.5 Metaplasticity

An interesting feature of synaptic plasticity is that the plasticity processes are not fixed, but are altered depending on recent history, a phenomenon known as metaplasticity (Abraham & Bear, 1996). This 'plasticity of plasticity' adds substantial complexity to the understanding of neural circuits. On a technical level, this means that one has to take into account the history of synaptic activation to predict the outcome of a plasticity-inducing protocol. More importantly, it suggests that an understanding of metaplasticity will give insight into how plasticity processes, most often studied in simplified slice preparations, function in dynamic neural networks of living animals.

The stabilizing element of metaplasticity can readily be seen using the BCM curve previously discussed. The modification threshold was defined as the junction point between activity levels that would produce LTD and LTP. A network with a fixed modification threshold is unstable, but if that threshold can vary based on the history of activity, then it may be possible to add stability. To confer stability, the modification threshold must vary in a manner to make it more difficult to achieve the same direction of change (Bienenstock et al., 1982). In Chapter III, I describe a novel form of metaplasticity that is consistent with this requirement.

1.6 Homeostatic Plasticity

1.61 Theoretical need for homeostatic plasticity

LTD was originally thought to contribute to the stability of neural networks by balancing out LTP, but computational models revealed continued instability (Dayan & Abbott, 2001; Dudek & Bear, 1992; Stanton & Sejnowski, 1989). These models show that Hebbian processes will lead to instability in neural circuits due to their positive-feedback nature if not balanced by a negative-feedback mechanism (Renart et al., 2003). Negative-feedback processes which compensate for changes in neural activity have been described in neurons and neuronal networks, and these define so-called "homeostatic" forms of plasticity. The earliest relevant studies were conducted in crustacean stomatogastric ganglion. Isolating a rhythmically firing neuron from this network abolished its rhythmic activity. However, the rhythmic firing returned after several days in single neuron cultures (Turrigiano et al., 1994). This study revealed that an isolated neuron has a preferred activity pattern, or a set point of firing rate. Deviation from this set point can be detected, and compensated for by some mechanism. This and similar studies have led to the notion that neurons homeostatically regulate their own activity through internal mechanisms that achieve homeostasis of activity rates (Marder & Prinz, 2002). Although complete isolation of a neuron from its network may be a rather drastic scenario, neurons face a variety of activity altering situations throughout the life of an organism, including growth, development and extreme sensory events, such as eye opening in the rodent (Turrigiano & Nelson, 2004). Given the apparent stability of neural systems through these challenges, homeostatic plasticity is likely a fundamental component of nervous systems. Although a strict definition of homeostatic plasticity is that some feature of neuronal activity is maintained steadily, the term is often used to

describe any compensatory process that seeks to achieve homeostasis, whether or not it is completely successful.

1.62 Homeostatic Synaptic Plasticity (HSP)

Forms of plasticity involving compensatory changes enacted at synapses are generally referred to as homeostatic synaptic plasticity (HSP). In addition to synaptic forms of homeostatic plasticity, compensatory changes in intrinsic excitability have also been found to promote stable firing rates. The properties and mechanisms of intrinsic homeostatic plasticity will not be explicitly discussed here (for an excellent review, see Turrigiano, 2011).

Homeostatic forms of synaptic plasticity which compensate for changes in activity can be expressed at different levels within a neuron, either on a global scale involving cell-wide changes or on a local level, implementing changes in a spatially restricted manner. Although a spectrum of HSP subtypes may exist, I will distinguish between two broad categories of HSP: global HSP and local HSP. It is currently accepted that both forms exist at excitatory synapses, but initial studies were interpreted to reflect a single global HSP mechanism and this view dominated the literature for several years (Turrigiano & Nelson, 2004). Although compensatory changes at inhibitory synapses have also been observed (Bateup et al., 2013; Echegoyen et al., 2007), I will concentrate my discussion of HSP to excitatory synapses.

1.63 Global HSP

In early studies using dissociated cultures of neocortical neurons, Turrigiano and colleagues (1998) found that chronically perturbing neural activity gave rise to changes in

the efficiency of synaptic transmission, as measured by changes in the size of miniature excitatory postsynaptic potentials (mEPSCs) resulting from spontaneous release of a single synaptic vesicle. Chronic blockade (48 hours) of action potentials in cultured neocortical neurons with the voltage-gated sodium channel antagonist tetrodotoxin (TTX) produced a compensatory increase in mEPSC amplitudes due to a transcription-dependent accumulation of GluA2-containing AMPARs (Gainey et al., 2009). HSP is a bidirectional process, as chronic blockade of $GABA_A$-mediated inhibition with bicuculline, which increases overall activity, resulted in decreased mEPSC amplitudes (Turrigiano et al., 1998).

Early analysis revealed that the size of mEPSCs were adjusted multiplicatively and thus synaptic transmission was thought to scale up or down in response to changes in average activity (Turrigiano & Nelson, 2004). However, since the activity-altering manipulation would likely involve all synapses, the interpretation of these changes as global HSP may have been premature. A subsequent demonstration that blocking postsynaptic firing with microperfusion of TTX at the soma induces AMPAR accumulation throughout dendrites provided stronger evidence that blocking postsynaptic spiking leads to global scaling up of synaptic weights (Ibata et al., 2008). More recently, Goold and Nicoll (2010) showed that chronically driving action potentials in ChR2 expressing neurons using repeated photoactivation causes a cell-autonomous decrease in AMPAR and NMDAR mediated currents. Notably, chronic blockade of action potentials has also been shown to induce HSP in vivo. Surgical implantation of TTX impregnated Elvax polymer near CA1 in rats led to an increase in the amplitude of mEPSCs in acute hippocampal slices made 2 days following implantations (Echegoyen et al., 2007). That

same year, it was reported that dark rearing mice leads to a multiplicative increase in mEPSCs in visual cortical neurons (Goel & Lee, 2007), similar to the effect observed in cultured neurons (Turrigiano et al., 1998).

1.64 Local HSP

Although there is evidence for global HSP at excitatory synapses (Ibata et al., 2008; Turrigiano et al., 1998; Goold & Nicoll, 2010), the existence of local HSP has also been substantiated (Branco et al., 2008; Hou et al., 2011; Hou et al., 2008; Ju et al., 2004; Sutton et al., 2006). Based on early studies, it was generally assumed that HSP operates on a very slow timeframe of hours to days. More recently, several studies have identified rapid forms of homeostatic plasticity that operate from within a few hours to mere minutes (Branco et al., 2008; Frank et al., 2006; Hou et al., 2011; Ibata et al., 2008; Ju et al., 2004; Sutton et al., 2006), suggesting that homeostatic plasticity is not necessarily a slow process sensitive only to chronic activity manipulations. One of the earliest studies demonstrating rapid, local HSP was found by blocking NMDA receptors in addition to action potentials (TTX+APV) in cultured hippocampal neurons, leading to an increase in the amplitude of mEPSCs within 60 minutes (Sutton et al., 2006). Impressively, local microperfusion of APV across a subsection of dendrite induced protein-synthesis dependent increases in AMPAR accumulation only within the perfused region, indicating spatially restricted alterations in synaptic activity drive local compensatory adaptations. Unlike chronic treatment with TTX alone, this rapid compensatory process is mediated by postsynaptic insertion of GluA2-lacking, Ca^{++}-permeable AMPARs (Sutton et al., 2006; Gainey et al., 2009). A similar result was obtained by AMPAR blockade in place of NMDAR blockade, although this treatment has also been shown to involve presynaptic

changes (Henry et al., 2012; Jakawich et al., 2010). Both TTX+APV and AMPAR blockade treatments lead to dendritic protein synthesis via dephosphorylation of eukaryotic elongation factor-2 (Henry et al., 2012; Nosyreva et al., 2013; Sutton et al., 2007), suggesting at least partially overlapping mechanisms.

In another study, reducing glutamate release via chronic hyperpolarization of presynaptic neurons by expressing the inwardly rectifying potassium channel Kir2.1 induced a local increase in AMPAR accumulation at postsynaptic targets (Hou et al., 2008). As neighboring synapses terminated by non-Kir2.1 neurons showed no AMPAR accumulation, this study revealed a synapse-specific form of HSP. On the flip side, increasing activity at presynaptic terminals by expressing and activating light-gated receptors lead to a decrease in AMPAR abundance only at those excited synapses (Hou et al., 2011). In this study, investigators detected compensatory changes after only 30 minutes of light stimulation (delivered every 20 seconds). Interestingly, this input-specific, rapid change depended on calcium and NMDAR activation, all features common with Hebbian synaptic plasticity.

The most rapid compensatory process reported to date was observed at the Drosophila neuromuscular junction. Application of philanthotoxin to block postsynaptic glutamate receptors resulted in a compensatory increase in presynaptic release of glutamate within 5-10 minutes, revealing that some neurons have the potential to rapidly compensate for detected deviations in activity levels (Frank et al., 2006). I will provide evidence for a rapid compensatory process operating at vertebrate central neurons operating over a similar timeframe in Chapter II.

1.65 Hebbian - Homeostatic plasticity interaction

One issue that remains poorly understood is how Hebbian synaptic modifications endure in the face of homeostatic mechanisms that should theoretically reverse them. One appealing solution to this paradox has homeostatic plasticity uniformly scaling all the synapses of a neuron in order to compensate for chronic changes in activity (global HSP), which might preserve relative changes in synaptic strength produced through Hebbian modification (Turrigiano & Nelson, 2000). However, it is unclear whether maintaining relative synaptic weights preserves information storage capabilities or confers stability to neural networks.

Since homeostatic and Hebbian forms of synaptic plasticity have been studied independently of each other, how these seemingly opposed processes function together at the same synapses is unclear. Prior investigations into the interplay between LTP/LTD and homeostatic plasticity mainly come from theoretical studies of model neurons. Analysis of these models revealed that homeostatic plasticity may play a lead role in influencing synaptic transmission in hippocampal CA1 neurons (Rabinowitch & Segev, 2006; Yeung et al., 2004). However, experimental support for proposed interactions is lacking. For example, Bonhoeffer and colleagues found that the decay of LTP to baseline was dependent on test pulse frequency, a finding they postulated as reflecting a homeostatic process (Fonseca et al., 2004). However, it is unclear whether the decay represents an actual homeostatic process or some other mechanism, since studies of homeostatic plasticity have largely been confined to cultured networks of neurons. In contrast, Hebbian forms of plasticity are best studied in hippocampal slice preparations where the intrinsic hippocampal circuitry is preserved. The inability to study Hebbian

and homeostatic plasticity mechanisms over the same time-scale and at the same population of synapses has greatly impeded our ability to evaluate theoretical interactions between homeostatic and Hebbian plasticity. Such issues of metaplasticity have general significance beyond the hippocampus, and it is likely that empirical studies will provide general insights into this overall issue. In Chapters II and III, I will demonstrate methods to overcome this challenge.

1.7 Local protein synthesis and Fragile X Mental Retardation Protein (FMRP)

1.71 Local translation

Since polyribosomes were first detected in dendrites (Steward & Levy, 1982), the functional role of local protein synthesis in dendrites has been under intense investigation (e.g., Costa-Mattioli et al., 2009; Sutton & Schuman, 2006). In line with this, numerous studies have reported mRNA trafficking out to dendrites, resulting in a constantly growing list of dendritically localized mRNAs (Martin & Zukin, 2006; Cajigas et al., 2012). Several forms of synaptic plasticity already discussed are locally expressed and require de novo protein synthesis, suggesting that protein synthesis is occurring locally, near synaptic sites. Indeed, local dendritic translation is required for LTP (Huang & Kandel, 2005; Kang & Schuman, 1996), non-NMDAR mediated LTD (Huber et al., 2000; discussed below), and local HSP (Soden & Chen, 2010; Sutton et al., 2006). Regarding the last point, the finding that miniature release events suppress dendritic translation in their postsynaptic targets led to the initial discovery of local HSP (Sutton et al., 2004; Sutton et al., 2006).

1.72 FMRP is a translation suppressor

An RNA-binding protein that regulates dendritic translation is the Fragile X mental retardation protein (FMRP) which suppresses translation by blocking ribosomal scanning. As the name suggests, mutations in the Fmr1 gene which encodes FMRP cause the neurodevelopmental disorder Fragile X Syndrome (FXS). When FMRP is phosphorylated it binds target transcripts and suppresses translation. FMRP is phosphorylated by p70 S6 kinase (S6K) (Narayanan et al., 2008). Dephosphorylation of FMRP by protein phosphatase 2A (PP2A) causes unbinding from RNA and an upregulation of translation of FMRP targets (Santoro et al., 2012). FMRP is a key molecule associated with local protein synthesis, since it is not only found near synapses, but has also been shown to regulate translation of dendritic transcripts (Bassell & Warren, 2008). The role of FMRP-regulated local translation has been a topic of great interest recently, particularly its role in protein synthesis dependent form of synaptic plasticity I have not yet discussed.

1.73 FMRP in synaptic plasticity

At CA1 synapses, activation of group 1 mGluRs via low frequency stimulation (LFS) in the presence of APV, paired-pulse LFS or direct application of an mGluR agonist leads to a form of long term depression (mGluR-LTD) that is independent of NMDAR activation and calcium (Fitzjohn et al., 2001; Fitzjohn et al., 1999; Kemp & Bashir, 1999; Oliet et al., 1997; Overstreet et al., 1997; Palmer et al., 1997; Schnabel et al., 1999). In synaptoneurosome preparations, mGluR agonists drive local protein synthesis (Weiler et al 1993). Interestingly, mGluR-LTD requires de novo dendritic protein synthesis (Huber et al., 2000). Physically removing the cell body layer from the apical dendrite layer where recordings took place permits mGluR-LTD expression.

Induction of mGluR-LTD was abolished in the presence of translation inhibitors anisomycin, cyclohexaminde or an mRNA cap analogue (introduced postsynaptically), but not the transcription inhibitor actinomycin D (Huber et al., 2000). Thus, activation of mGluRs is thought to induce local translation of LTD-associated proteins.

In Fmr1KO mice, mGluR-LTD is enhanced, presumably due to increased expression of LTD-associated proteins (Hou et al., 2006; Huber et al., 2002). In striking contrast to their WT counterparts, FMR1KO mice exhibit mGluR-LTD that is not dependent on new protein synthesis (Hou et al., 2006; Nosyreva & Huber, 2006; Volk et al., 2007). These findings have led to a proposal whereby a constitutive abundance of LTD-associated proteins in the dendrites leads to exaggerated mGluR-signaling which is normally balanced by FMRP suppression of LTD proteins (Bear Huber Warren REVIEW). Consistent with this proposal, decreasing mGluR signaling via genetic (Dölen et al., 2007) or pharmacological (Michalon et al., 2012) methods in Fmr1KO mice restores mGluR-LTD expression levels.

FMRP has also been shown to be involved in local HSP (Soden & Chen, 2010). Fmr1KO mice don't exhibit the protein-synthesis dependent increases in mEPSC amplitude previously observed after long term treatment with TTX and APV (Aoto et al., 2008; Soden & Chen, 2010). Surface biotinylation experiments show that Fmr1KO neurons don't show an increase in surface GluA1 levels as seen in WT neurons (Soden & Chen, 2010). Despite this recent study and the overwhelming evidence that FMRP is central to proper mGluR signaling and LTD, NMDAR-dependent forms of LTP and LTD are largely unaffected in FMR1KO mice (Sidorov et al., 2013).

1.74 Fragile X Syndrome (FXS) and Fragile X-Associated Tremor Ataxia Syndrome (FXTAS)

FMRP was discovered in the search for the genetic basis of what is now called Fragile X Syndrome (FXS), but was originally described as Martin-Bell Syndrome (Krueger and Bear 2011). As the name suggests, the FMR1 gene is located on the X chromosome. Hyper-methylation of an expanded CGG sequence repeat (>200 repeats) in the 5' UTR of FMR1 silences its expression, and loss of Fmr1 mRNA and FMRP leads to autism and intellectual disability (Kremer et al., 1991; Pieretti et al., 1991). The work discussed above has led to the mGluR theory of FXS, and since decreased mGluR signaling can correct the deficits reported in Fmr1KO mice, clinical trials in FXS patients are underway (Krueger et al., 2011).

Intermediate CGG repeat expansions between ~45 and 200 repeats (a "premutation") are associated with the conditions Fragile X-associated Tremor Ataxia Syndrome (FXTAS) (Berry-Kravis et al., 2007) in elderly males and Fragile X-associated Primary Ovarian Insufficiency (FXPOI) in females (Hagerman & Hagerman, 2004). Male premutation carriers with FXTAS present with gait ataxia, action tremor, dementia and neuropsychiatric symptoms (Berry-Kravis et al., 2007; Jacquemont & Hagerman, 2004). FXS was originally characterized as a neurodevelopmental disorder and FXTAS as a neurodegenerative disorder (Berry-Kravis et al., 2007), but that dichotomy is complicated by the finding that premutation carriers also display higher rates of autism and ADHD-like symptoms at younger ages (Farzin et al., 2006; Hagerman, 2013). Given the partially overlapping symptoms between FXS patients and young premutation carriers, it is reasonable to ask whether they share a common pathophysiology. This

question will be addressed in Chapter IV investigating mGluR-mediated signaling and LTD in a CGG knock-in mouse model carrying a premutation-sized repeat.

1.8 Bibliography

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Chapter II

Rapid, input-specific homeostatic synaptic plasticity at CA3-CA1 synapses compensates for changes in action potential frequency

2.1 Introduction

Homeostatic synaptic plasticity (HSP) has emerged as the negative feedback counterpart to Hebbian processes, such as long-term potentiation (LTP) and long-term depression (LTD), that are thought to underlie learning and memory (Bliss & Lømo, 1973; Hebb, 1949; Turrigiano & Nelson, 2000). Despite this widespread notion, HSP has rarely been studied in the same context as Hebbian plasticity. Three major hurdles exist in addressing the relationship between Hebbian and homeostatic forms of synaptic plasticity: 1) they are typically studied in different preparations, 2) they are studied using distinct induction methods, and 3) they are studied over greatly varied time-scales. For example, LTP and LTD are typically examined in acute brain slice preparations and rapidly induced using electrical stimulation within seconds to minutes, whereas HSP has routinely been studied in cultured neurons in response to pharmacological manipulation of activity over multiple hours to days (Malenka & Bear, 2004; Turrigiano & Nelson, 2004).

Recently, several studies have identified rapid forms of homeostatic plasticity that operate from within a few hours to mere minutes (Branco et al., 2008; Frank et al., 2006;

Hou et al., 2011; Ju et al., 2004; Sutton et al., 2006), suggesting that homeostatic plasticity is not necessarily a slow process. These observations prompted us to ask whether HSP could be examined in the relatively short-lasting (~10 hours) preparation of the acute hippocampal slice, in which LTP and LTD are typically studied. Rather than using pharmacological manipulators of activity, we postulated that altering the frequency of afferent stimulation to manipulate activity levels impinging upon CA1 synapses would similarly reveal rapid compensatory changes in synaptic strength. The existence of HSP induced in this fashion would allow for investigation of potential interactions between Hebbian and homeostatic forms of synaptic plasticity.

Here, we demonstrate a rapid form of homeostatic synaptic plasticity at CA3-CA1 synapses in acute hippocampal slices. By altering the frequency of Schaffer collateral stimulation, we observe compensatory strengthening or weakening of synaptic inputs depending on whether the frequency is decreased or increased, respectively. We find that NMDA receptor (NMDAR) activation and post-synaptic calcium are necessary for the expression of compensatory changes in synaptic strength. Unlike the mechanisms underlying LTP and LTD, we find that homeostatic synaptic plasticity induced by a shift in input stimulation frequency occurs independently of CaMKII or calcineurin activity. These features allowed us to address the manner by which this HSP co-exists with Hebbian plasticity at the same population of synapses. We find that homeostatic synaptic compensation offsets the magnitude of LTP subsequently induced, but does not alter the relative change in synaptic strength from the "scaled" baseline. Finally, we demonstrate that homeostatic plasticity can enhance the magnitude and durability of previously established LTP, revealing that the net output of this network is the result of intricate

interactions between Hebbian and homeostatic plasticity. These results demonstrate the advantages of inducing input-specific HSP in acute hippocampal slices for studying potential interaction with Hebbian processes.

2.2 Materials and Methods

2.21 Acute hippocampal slice preparation

All procedures involving animals were approved by the University Animal Care and Use Committee. Sprague Dawley rats, aged 2-3 weeks, were decapitated and the hippocampal lobules were rapidly isolated in artificial cerebral spinal fluid (aCSF). Older rats were similarly dissected. aCSF contained (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2P0_4, 26.3 $NaHCO_3$, 11 glucose, 1.3 $MgSO_4$, and 2.5 $CaCl_2$. Transverse slices (400 µm) of the hippocampus were cut using a tissue chopper (Stoelting). Slices were then incubated at room temperature in a humidified interface chamber for at least 2 hours before recording.

2.22 Field electrophysiology

Hippocampal slices were transferred to a recording chamber, maintained at 26-28°C and continuously perfused at 1-2 ml/min with oxygenated aCSF. Area CA1 was visualized with an Olympus SZ51 dissecting microscope, which was also used for electrode placement. Recording electrodes were pulled from borosilicate capillary glass and filled with 3M NaCl (1.7 mm o.d.; VWR International). The recording pipette was placed in the stratum radiatum of CA1. Recordings were made with a MultiClamp 700B amplifier, collected using Clampex 10.2, and analyzed using Clampfit 10.2 (Molecular Devices). Field excitatory postsynaptic potentials (fEPSPs) were evoked using cluster electrodes

also placed in the stratum radiatum of CA1 (FHC). Current between 0.02-0.25 mA for 0.1s was delivered with an ISO-flex stimulus isolator (AMPI). For experiments, current was set at a level to elicit 50% of the maximum response. In experiments where two pathways were stimulated, electrodes were placed on opposite sides of the recording electrode. We verified pathway independence by applying two pulses with a 50 msec interpulse interval to the two pathways and screening for less than 10% paired-pulse facilitation. For all studies, manipulation of frequency followed 30 minutes of stable baseline. If pharmacological agents were included in the experiments, they were applied for 30 minutes after stable baseline was obtained, and then plasticity was induced. The following pharmacological inhibitors were used: D,L-APV (100 µM; Tocris), nifedipine (10 µM; EMD Biosciences), FK506 (50 µM; LC Laboratories), KN-93 (10 µM; Calbiochem), and KN-62 (10 µM; Calbiochem). D,L-APV was re-suspended in water; all other drugs were dissolved in DMSO. When the drugs were dissolved in DMSO, 0.1% DMSO was added to the aCSF in control experiments to account for possible effects of DMSO.

2.23 Whole-cell patch-clamp electrophysiology

CA1 pyramidal neurons were targeted using a blind, whole-cell patch-clamp approach. Preparation and equipment was the same as for the field recordings, except that recording electrodes (with resistances of 4–6 MΩ) contained (in mM) 115 $KMeSO_4$, 15 KCl, 5 NaCl, 0.02 EGTA, 1 $MgCl_2$, 10 Hepes, 10 Na_2-phosphocreatine, 4 Mg-ATP, and 0.3 Na-GTP. For BAPTA experiments, the concentration of $KMeSO_4$ was decreased to 95 mM and 10 mM BAPTA-tetraK+ (Invitrogen) was included in the recording solution. CA1 pyramidal cells were patched and either eEPSCs or eEPSPs were evoked with stimulating

electrodes were placed in stratum radiatum of CA1. Stimulation frequency was altered 30 minutes after patch, and, if possible, returned to the baseline frequency after another 30 minutes. For voltage-clamp experiments, cells were held at -70 mV. To measure NMDAR currents, the holding potential was switched to +40 mV and NMDAR current was measured as the amplitude at 50 ms. NMDAR currents were measured before baseline collection and then again after 30 minutes of altered afferent stimulation frequency.

2.24 Statistics

The results are presented as mean ± SEM and were evaluated using one-way ANOVA, followed by a Tukey post-hoc comparison of groups or Student's t-test where appropriate. The level of significance was set at $p<0.05$.

2.3 Results

2.31 CA3-CA1 synapses exhibit rapid homeostatic compensation to changes in evoked synaptic transmission in acute hippocampal slices.

Instead of using pharmacological blockade or enhancement of activity to evoke homeostatic synaptic plasticity (HSP), we asked whether compensatory changes in synaptic transmission accompany conditions where the frequency of input stimulation is manipulated as a way to alter activity within a network. For this purpose, we used field excitatory postsynaptic potential (fEPSP) recordings in stratum radiatum of CA1 where synaptic responses elicited by stimulating CA3 axons (Schaffer collaterals) can be monitored over an extended time period.

Hebbian forms of synaptic plasticity obey a frequency-response rule, where elevating the frequency of afferent stimulation produces LTD in the 0.5 – 3 Hz range and LTP in the 10 – 200 Hz range (Cooper & Bear, 2012; Dudek & Bear, 1992). Outside of these LTP/LTD induction ranges, CA3-CA1 synaptic strength generally remains stable so long as fEPSPs are continuously elicited at a constant frequency. We thus explored whether compensatory changes in synaptic strength emerge in response to increasing or decreasing net input over time within this 'stable' frequency range. We began by recording stable fEPSPs at a commonly utilized basal stimulation frequency of 0.1 Hz (1 pulse/10 seconds) and examined the consequences of reducing net input over time by shifting the test frequency to $5.6x10^{-4}$ Hz (1 pulse/30 minutes). We found that this high-to-low frequency shift induced a rapid compensatory increase in synaptic strength that emerged within 30 minutes (the first data point after the frequency switch) and stabilized at a new higher level within approximately 60 minutes (Fig. 2.1*A*). 60 minutes following the frequency shift, synaptic efficacy is significantly stronger than during the baseline period ($p=0.036$ by Student's t-test; $n=6$). This increase in synaptic efficacy was readily reversible when test stimulation was reversed back to the original test frequency of 1 pulse/10 seconds (Fig. 2.1*A*). This compensation exhibits a key feature of homeostatic plasticity: synaptic strength is inversely responsive to the levels of synaptic activity over time.

Other features of HSP are less uniform. Some forms of homeostatic plasticity operate cell-wide (Ibata et al., 2008; Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998), while other studies demonstrate local homeostatic mechanisms where activity at a restricted set of synapses is used to locally adjust synaptic function (Branco

& Hausser, 2010; Hou et al., 2011; Hou et al., 2008; Ju et al., 2004; Sutton et al., 2006). To examine if the homeostatic changes we observe are globally or locally implemented, we stimulated two independent input pathways that converge onto a common population of CA1 cells by placing two stimulating electrodes in the stratum radiatum of CA1, on either side of the recording electrode. After ensuring pathway independence, stable fEPSPs were recorded in each pathway stimulated at 0.1 Hz (1 pulse/10 seconds). One path (control) remained at 1 pulse/10 seconds throughout the experiment, whereas the frequency of stimulation in the second (test) pathway was shifted to $5.6x10^{-4}$ Hz (1 pulse/30 minutes). As shown in Figure 2.1*B*, the test pathway demonstrated a clear and reversible increase in synaptic efficacy, while the control pathway remained unchanged. An hour after the frequency change, responses in the test pathway were significantly greater than during the baseline ($p=0.013$ by Student's t-test; $n=5$), but responses in the control pathway remained the same size ($p=0.433$ by Student's t-test; $n=5$). These results demonstrate that these rapid compensatory changes in synaptic strength are implemented locally, in an input-specific fashion.

To better define how local compensatory responses emerge following changes in synaptic activity, we systematically altered the disparity between initial and final input frequency. When we shifted stimulation frequency from 0.05 Hz (1 pulse/20 sec) to lower frequencies, we found a progressive increase in synaptic efficacy proportional to the extent of activity change that plateaued at a final frequency of $3.3x10^{-3}$ Hz (1 pulse/5 min). Shifts from 0.05 Hz (1 pulse/20 sec) to either $3.3x10^{-3}$ Hz (1 pulse/5 min) or $5.6x10^{-4}$ Hz (1 pulse/30 minutes) resulted in significantly stronger synaptic responses one hour after the shift ($p=0.011$ by ANOVA; $n=6$; Fig. 2.1*C-D*). When the stimulation

frequency was not altered, responses remained stable throughout the course of the experiment.

At this point, we asked whether homeostatic weakening could be evoked by increasing the stimulation frequency from a relatively low frequency to a relatively high stimulation frequency. Note, however, that this higher frequency range is outside the frequency range for LTP or LTD. When we shifted from $3.3x10^{-3}$ Hz (1 pulse/5 min) to higher stimulation frequencies we found a progressive decrease in synaptic efficacy correlated with the magnitude of the frequency shift (Fig. 2.1*E-F*), illustrating that homeostatic compensation of synaptic efficacy is bi-directional. Shifts from $3.3x10^{-3}$ Hz (1 pulse/5 min) to either 0.017 Hz (1 pulse/60 sec) or 0.05 Hz (1 pulse/20 sec) resulted in a significantly decreased synaptic response ($p<0.001$ by ANOVA; n=5). In all cases, this compensatory alteration in synaptic efficacy was reversed upon re-establishment of the initial stimulation frequency (data not shown). Homeostatic weakening, like strengthening, occurred locally, in an input specific manner (data not shown). Shifting stimulation frequency to 0.017 Hz (1 pulse/60 sec) had opposite consequences depending on whether such a shift resulted in a net reduction (Fig. 2.1*C*) or increase (Fig. 2.1*E*) in synaptic efficacy over time. This demonstrates that the change in activity, rather than the final frequency itself, is the important determinant of the direction and magnitude of compensatory changes in synaptic strength. Hence, CA1 synapses are subject to rapid, bi-directional, and local homeostatic control. For the remainder of the study, we perform all experiments using frequency shifts from $3.3x10^{-3}$ Hz (1 pulse/5 min) to 0.05 Hz (1 pulse/20 sec) or 0.05 Hz (1 pulse/20 sec) to $3.3x10^{-3}$ Hz (1 pulse/5 min).

2.32 Local homeostatic plasticity is developmentally regulated.

HSP studied in either cultured neurons (Wierenga et al., 2006) or with *in vivo* activity blockade (Echegoyen et al., 2007) is known to be developmentally regulated. Therefore, we next compared homeostatic compensation in slices from young rats (P14-21) and slices from older rats (P42-56). Slices from older animals still exhibited local, bi-directional compensation of synaptic efficacy when the stimulation frequency was increased (1 pulse/5 min → 1 pulse/20 sec) or reduced (1 pulse/20 sec → 1 pulse/5 min) (Fig. 2.2). For synaptic weakening, an hour after the frequency change responses from both young and older rats are significantly decreased, but not significantly different from one another ($p=0.01$ by ANOVA; $n=8$). One hour after the frequency change only responses from younger rats are significantly increased in homeostatic strengthening experiments ($p=0.002$ by ANOVA; $n=9$). However, the magnitude of this compensation in both directions was significantly weaker than that observed in younger slices, indicating that rapid homeostatic control of synaptic function diminishes, but remains present, as hippocampal circuits mature.

2.33 Homeostatic synaptic plasticity is expressed postsynaptically in CA1 pyramidal neurons.

Having established the presence of rapid HSP in acute hippocampal slices, we wanted to study the mechanism underlying this phenomenon. We first examined paired-pulse facilitation (PPF) during homeostatic weakening of synaptic transmission. PPF increases dramatically in the second postnatal week of life and is relatively stable by the age we made slices at for our studies (P14-P21) (Wasling et al., 2004). Changes in PPF are inversely associated with alterations in presynaptic release probability. To examine

PPF throughout the entire time-course of homeostatic plasticity, we used a paired-pulse stimulation protocol (inter-pulse interval of 50 ms). We found that increasing the stimulation frequency induced a clear and sustained decrease in fEPSP slope for both the first and second pulse; however, the magnitude of PPF remained stable (Fig. 2.3*A*; p=1.0 by ANOVA; n=7). Similarly, there was no change in the magnitude of PPF throughout experiments when homeostatic strengthening was induced (Fig. 2.3*B*; p=0.999 by ANOVA; n=9).

These results demonstrate that homeostatic compensation of synaptic efficacy is not accompanied by changes in presynaptic release probability, suggesting a postsynaptic locus for expression. To test this possibility directly, we asked whether buffering postsynaptic Ca^{2+} could prevent the homeostatic changes in synaptic strength. We first confirmed that local homeostatic plasticity is revealed in whole-cell patch-clamp recordings from individual CA1 pyramidal neurons. We stimulated two independent populations of inputs onto a single pyramidal neuron in CA1, and found that after 30 minutes of baseline recording increasing the test frequency induced a clear and reversible decrease in evoked EPSP (eEPSP) amplitude in the test pathway while the control pathway (maintained at $3.3x10^{-3}$ Hz throughout) remained stable (Fig. 2.4*A*), consistent with our previous observations and further demonstrating that homeostatic strengthening is input-specific. Similarly, synaptic strengthening can be seen in an individual pyramidal cell by whole-cell patch-clamp recording by decreasing stimulation frequency after a 30 minute baseline period (Fig. 2.4*B*). Again, an independent pathway, in which stimulation is unaltered, remained stable throughout the course of the experiment. For

both homeostatic synaptic weakening and strengthening, return to baseline is evident after the frequency is returned to the original frequency.

Returning to the question of whether HSP is expressed in the postsynaptic compartment, we included the rapid Ca^{2+} chelator BAPTA (10 mM) in the whole-cell pipette. The inclusion of BAPTA prevented homeostatic synaptic weakening in the test pathway (Fig. 2.4*C*-*D*). During the homeostatic weakening period, there was a significant decrease in eEPSP amplitude in the absence of BAPTA (p=0.029 by Student's t-test; n=8). This decrease was absent when BAPTA was included in the recording pipette (p=0.309 by Student's t-test; n=8). Homeostatic strengthening was similarly blocked by BAPTA (p=0.102 by Student's t-test; n=11) (Fig. 2.4*E*); homeostatic strengthening was clearly observed in experiments without BAPTA (p=0.003 by Student's t-test; n=11). Taken together, these results demonstrate that postsynaptic Ca^{2+} is required for HSP induced by a frequency shift.

Having established that acute HSP is postsynaptically expressed, the involvement of AMPA and NMDA receptors was examined. To do this, AMPAR-mediated currents and NMDAR-mediated currents were measured before the start of baseline stimulation and 30 minutes after homeostatic weakening in whole-cell patch-clamp recording. There is a compensatory decrease in AMPAR responses, but the NMDAR responses stay stable (Fig. 2.4*F*-*G*), suggesting HSP involves a change in AMPAR currents, but no change in the NMDAR currents. Normalized AMPAR eEPSCs were significantly decreased after HSP (p=0.019; n=7); whereas, normalized NMDAR eEPSCs were not altered by HSP

(p=0.191; n=7). Therefore, a presynaptic mechanism is not responsible, since these would alter AMPAR and NMDAR responses equally.

2.34 NMDA receptors, but not L-type calcium channels, are required for homeostatic synaptic plasticity.

To examine a potential synaptic source for observed requirement for postsynaptic Ca^{2+}, we examined HSP in the presence of the NMDAR antagonist APV. Whereas in interleaved control experiments, high-to-low frequency shifts (1 pulse/20 sec → 1 pulse/5 min) induced rapid and reversible homeostatic synaptic strengthening, we found that the application of APV (50 μM) during induction significantly reduced this effect (Fig. 2.5*A*). Homeostatic synaptic strengthening was blocked when measured an hour into the frequency switch by the addition of APV when compared to control conditions (p=0.002; n=10 (Control), 11 (APV)). Similarly, application of APV reduced the magnitude of homeostatic synaptic weakening (Fig. 2.5*B*). Homeostatic synaptic weakening was also blocked by the addition of APV (p=0.033; n=4 (Control), 7 (APV)).

We investigated whether L-type voltage-gated calcium channels could also be a source of the calcium underlying HSP by bath-applying the inhibitor nifedipine (10 μM). We found that neither homeostatic strengthening (Fig. 2.5*C;* p=0.726; n=13 for each condition) nor homeostatic weakening (Fig. 2.5*D;* p=0.426; n=5 for for each condition) was blocked by the presence of nifedipine. Therefore, calcium from NMDA receptors, but not L-type calcium channels likely contributes to HSP in acute hippocampal slices.

2.35 Homeostatic plasticity in acute hippocampal slices is mechanistically distinct from LTP and LTD.

Postsynaptic calcium and NMDA receptor activation are a critical part of the signaling cascade for LTP and LTD, leading to the question of how much the downstream signaling of homeostatic plasticity in acute hippocampal slices overlaps with LTP and LTD. In the hippocampus, blocking calcium-calmodulin dependent kinase II (CaMKII) activity blocks the generation of LTP (Barria et al., 1997; Bortolotto & Collingridge, 1998; Ito et al., 1991). We began by confirming that the CaMK inhibitor KN-93 (10 μM) blocks LTP (Fig. 2.6*A;* Barria et al., 1997; Ito et al., 1991). LTP is significantly weaker in conditions containing KN-93, one hour after LTP induction ($p=0.022$ by Student's t-test; n=8). Unlike with LTP, homeostatic strengthening was not affected by application of either KN-93 or a second CaMK inhibitor, KN-62 (Fig. 2.6*B*; $p=0.868$ by Student's t-test; n=6). Likewise, an inhibitor of calcineurin (FK506, 50 μM) which blocks LTD (Mulkey et al., 1994; Fig. 2.6*C*; $p=0.226$ by Student's t-test; n=8 (control), 10 (FK506)) failed to alter the expression of homeostatic weakening (Fig. 2.6*D*; $p=0.05$ by Student's t-test; n = 5). Taken together, these results demonstrate that homeostatic synaptic plasticity in acute hippocampal slices is mechanistically distinct from LTP and LTD.

2.36 Homeostatic synaptic weakening does not alter the relative magnitude of Hebbian Plasticity.

Thus far we have demonstrated that HSP in acute slices is implemented locally and postsynaptically with some mechanistic similarities to Hebbian plasticity; therefore we asked how these forms of synaptic plasticity interact. We first addressed whether prior induction of HSP alters the magnitude of LTP induced by a single train of high-frequency stimulation (1xHFS; 100 Hz, 1s). We began by inducing homeostatic

weakening in one of two independent inputs onto a common population of CA1 dendrites and then compared the relative magnitude of LTP in the two inputs when LTP was induced in both. Both pathways received baseline stimulation (1 pulse/5 min). Then, stimulation frequency was increased in one pathway to 1 pulse/20 sec to induce homeostatic weakening. 60 minutes later both pathways received a train of 100 Hz for 1 second to induce LTP. We found that induction of homeostatic weakening significantly decreased the magnitude of LTP relative to the homeostatic-naive input 30 minutes after the induction of LTP (Fig. 2.7*A*; p=0.005 by Student's t-test; n=10 control, 8 HSP); however, when we plotted the magnitude of LTP relative to the scaled baseline (i.e., the 20 minute period immediately prior to LTP induction), we found that the decrease in LTP was entirely accounted for by the superimposed homeostatic weakening of synaptic strength (Fig. 2.7*B*; p=0.475 by Student's t-test). These findings thus provide evidence for the theoretical notion that homeostatic plasticity operates in such a way as to preserve the capacity for Hebbian activity-dependent changes in synaptic strength.

2.37 Local cooperation between Hebbian and homeostatic synaptic plasticity

While our data suggest that rapid homeostatic plasticity preserves information coding capabilities in neural circuits, the manner by which homeostatic plasticity interacts with already established Hebbian modifications has been a topic of theoretical debate. To examine whether the two forms of plasticity could cooperate on a local level, we used 2-pathway experiments and induced LTP (1xHFS) in both inputs while stimulating at a frequency of 1 pulse/20 sec, then shifted one input to 1 pulse/5 min to induce homeostatic strengthening 30 minutes following LTP induction. While both pathways exhibited comparable levels of LTP over the first 30 min, induction of local

homeostatic strengthening significantly enhanced the magnitude and durability of LTP over the next 3 hours (Fig. 2.8). The pathway undergoing homeostatic strengthening on top of LTP have a higher level of synaptic efficacy 2 hours after the frequency switch than the control pathway ($p=0.001$ by Student's t-test; $n=11$). These results suggest that homeostatic plasticity can cooperate with Hebbian plasticity in an input-specific manner. These observations further suggest that homeostatic and Hebbian forms of plasticity co-exist at the same synapses and operate together to influence the magnitude and persistence of activity-dependent changes in synaptic efficacy.

2.4 Discussion

Our results show that CA1 synapses rapidly compensate for changes in the activity of their inputs. While the ability of CA1 neurons to undergo homeostatic synaptic plasticity is well established in dissociated cultures (Turrigiano & Nelson, 2004), here we demonstrated input-specific synaptic compensation at CA1 synapses in an intact, acute hippocampal slice preparation. This novel form of HSP is sensitive to NMDA receptor blockade and calcium chelation, but does not require CaMKII or calcineurin activity. We show that prior induction of synaptic compensation offsets the magnitude, but not the relative size, of subsequently induced LTP. Finally, we show that HSP can enhance the durability of LTP-initiated synaptic changes. Synaptic efficacy changes can thus be a product of both Hebbian and homeostatic mechanisms, suggesting that HSP may also influence information coding or storage in neural circuits.

2.41 Rapid synaptic compensation in response to a change in input activity over time

To date, the most common means of inducing HSP has been pharmacological blockade of activity which either increases or decreases network activity levels, depending on the specific target. For the present study, we sought to take advantage of the hippocampal slice preparations highly stereotyped organization by electrically stimulating Schafer collaterals projecting from area CA3 and terminating on CA1 dendrites in stratum radiatum. This recording setup is typically used in LTP and LTD studies. Unlike the induction of LTP or LTD though, we kept the frequency of stimulation below the range that would normally produce Hebbian changes (Cooper & Bear, 2012; Dudek & Bear, 1992). We reasoned that despite the generally low frequencies of afferent stimulation, changes in activity within this frequency band would be sensed by CA1 neurons and compensation of synaptic transmission would result. We found robust compensation for even moderate frequency shifts (see Fig. 2.1*C,E)*. Intact, living brains are highly dynamic and one intriguing possibility is that processes similar to the HSP we demonstrate here *ex vivo* are constantly opposing subtle shifts in activity levels in order to promote stability *in vivo*. Although it remains to be investigated, a dysfunction in such a process would potentially lead to unbalanced activity levels in the brain, perhaps leading to seizure disorders or cognitive impairment.

Since the discovery of homeostatic synaptic plasticity operating at central synapses, work from multiple investigators has demonstrated there a distinct classes of HSP which can be categorized based on various properties. HSP can act in a cell-wide fashion to adjust strengths of all synapses (Goold & Nicoll, 2010; Ibata et al., 2008; O'Brien et al., 1998; Turrigiano et al., 1998) or it can act more locally, even an individual synapse (Beique et al., 2011; Hou et al., 2008, 2011; Lee, Yasuda, & Ehlers, 2010; Sutton

et al., 2006). Here we demonstrate a novel implementation of HSP that is input-specific and therefore acting locally. Although our level of analysis does not have the resolution to answer whether it operates at individual synapses or subsections of the dendrite, is not expressed over the entire cell. If it were, then we would expect to see both pathways respond to the change in evoked activity of a single pathway (Fig. 2.1*B*).

HSP can also be classified by the time scale over which it is expressed. Some forms take at least 24 hours for measurable compensation to occur (O'Brien et al., 1998; Turrigiano et al., 1998) while others can be induced in as little as a few hours or less (Henry et al., 2012; Jakawich et al., 2010; Ju et al., 2004; Sutton et al., 2006). Strikingly, we find that HSP elicited in acute hippocampal slices rapidly begins to compensate for the abrupt change in afferent stimulation frequency and typically approaches asymptotic levels between 30 and 60 minutes (for examples, see Fig.1*C* and Fig.1*E*). While our studies may demonstrate the fastest compensation to a change in activity seen in mammalian neurons, this timescale is in line with that observed in invertebrates. For example, at the Drosophila NMJ, homeostatic compensation of presynaptic release is observed within a few minutes of postsynaptic receptor blockade (Frank et al., 2006).

2.42 Postsynaptic compensation to the change in activity levels

There could have been many potential ways for HSP to be expressed in the hippocampus due to our shift in the frequency of Schaffer collateral stimulation. First, the change in activity in the presynaptic fibers being stimulated could lead to a compensatory change in presynaptic properties such as neurotransmitter release probability or quantal content. Second, CA1 excitability could be altered by the shifts in

input activity. Third, postsynaptic properties such as receptor number, composition or density could undergo modification. Fourth, entirely new synapses could be created or destroyed. 'Silent' synapses are known to exist at CA3-CA1 synapses, so one method of compensating for activity changes would be adjust the silence state of CA1 synapses. Finally, any combination of these possibilities could occur. For any of these changes to be considered compensatory, they must be in the opposite direction as the activity shift. For example, an increase in the afferent stimulation rate could lead to a decrease in the number of AMPARs expressed at postsynaptic membranes. We found evidence that changes occurred in the postsynaptic compartment (Fig. 2.4*A, B)*. Importantly, chelating post-synaptic calcium blocked these changes, revealing that calcium is required for expression of HSP (Fig. 2.4*D, E*). In contrast, we found no evidence that presynaptic release probability is altered by modifying the stimulation rate (Fig. 2.3*A, B*). In addition, we show that post-synaptic AMPAR-mediated, but not NMDAR-mediated, responses are modified following activity manipulation (Fig. 2.4F, *G*). Taken together, our results provide strong evidence that HSP in acute hippocampal slices is a post-synaptic process.

Previous studies have demonstrated that slow test pulse stimulation to naïve CA1 synapses can depress synaptic strength in young rodents (Xiao et al., 2004). Our results indicate that the previously observed test pulse induced depression is likely a specific case of homeostatic synaptic weakening. Similar to previous results, we found that this plasticity is developmentally regulated and requires postsynaptic calcium (Fig. 2.2; Xiao et al., 2004). Some of these studies observed that ceasing activity for a relatively long period of time would allow synaptic responses to recover to baseline levels

(Abrahamsson et al., 2007). Here, we show evidence that the observed recovery can be explained by the induction of homeostatic synaptic strengthening due to the decrease in stimulation rate (see Fig.1*C*,*D*).

2.43 HSP is distinct from LTP or LTD

Rapid HSP in acute hippocampal slices shares some features with Hebbian plasticity. Therefore, we investigated the extent to which the molecular pathways between these plasticity classes overlap. We found that postsynaptic calcium is required for the expression of HSP (Fig. 2.4), as it is for both LTP and LTD (Wayman et al., 2008). Induction of LTP leads to an influx of calcium ions, which activate calcium-calmodulin dependent kinase II (CaMKII). Disruption of CaMKII activity prevents the expression of LTP (Bortolotto & Collingridge, 1998; Ito et al., 1991; Fig. 2.6*A*). Although HSP requires calcium, blockade of CaMKII did not affect the expression of homeostatic strengthening (Fig.6*B*), suggesting that the signaling mediators of HSP and LTP are different. Like LTP, LTD requires the influx of calcium ions. However, LTD is produced when the protein phosphatase calcineurin (also known as PP2B) is activated by calcium. Blocking calcineurin activity abolishes the expression of LTD (Mulkey et al., 1994), but did not affect the expression of homeostatic weakening (Fig. 2.6*D*).

2.44 Interactions between homeostatic and Hebbian synaptic plasticity

One of the main advantages of the approach we took here is that it permits direct studies of potential interactions between Hebbian and homeostatic forms of plasticity. Previous studies have identified that plasticity processes are not static processes, but can undergo activity-dependent modifications in a process known as metaplasticity

(Abraham, 2008). We modeled our experimental design after typical metaplasticity studies which initially apply a priming event prior to the induction of an activity-dependent change, such as LTP or LTD (Abraham, 2008). We have found that prior induction of homeostatic plasticity offsets the magnitude of LTP subsequently induced, but does not alter the relative change in synaptic strength from the "scaled" baseline. These observations provide evidence for the theoretical notion that homeostatic plasticity operates in such a way as to preserve the capacity for Hebbian activity-dependent changes in synaptic strength (Rabinowitch & Segev, 2008).

In a separate set of experiments, we reversed the paradigm for studying metaplasticity (Fig. 2.8). Here, we initially induced a weak form of LTP in two pathways, and then induced homeostatic strengthening in only one of the pathways. We found that HSP enhanced the magnitude and durability of normally weak and short-lived LTP, suggesting that Hebbian and homeostatic plasticity can work in concert to regulate the final output of the network. Furthermore, this data reveals that stable, long-lasting changes in synaptic efficacy can be achieved with only weak associative plasticity processes in neurons that have a multitude of plasticity processes to access. This may have important implications for engram stability.

2.5 Acknowledgements

The work described in Chapter II was done in collaboration with postdoctoral fellows, Dr. Francisco Javier Munoz-Cuevas and Dr. Erin M. Johnson-Venkatesh, in Dr. Michael M. A. Sutton's laboratory. This work was supported by grants F31NS073372 (A.J.I.) from the National Institutes of Neurological Disorders and Stroke and

RO1MH085798 (M.A.S.) from The National Institute of Mental Health and a grant from the Pew Biomedical Scholars Program (M.A.S.). We also thank Hisashi Umemori and members of the Sutton laboratory for many helpful discussions.

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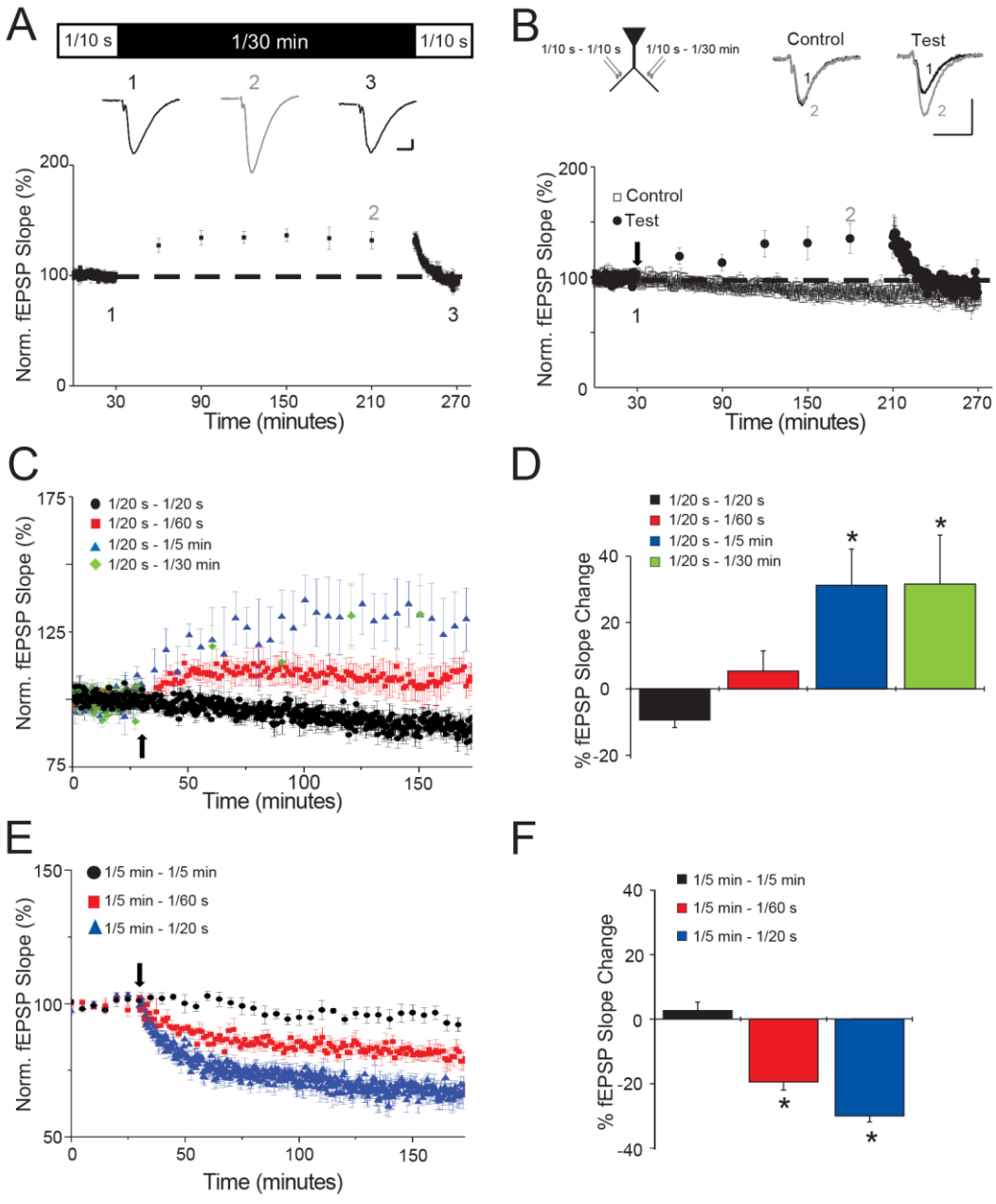
2.7 Figure Legends

Figure 2.1. Homeostatic plasticity in acute hippocampal slices is local, bidirectional, and reversible. A, Recording fEPSPs in CA1 of the hippocampus by stimulating the Shaffer collaterals. When the frequency of stimulation was switched from 1 pulse/10 seconds to 1 pulse/30 minutes, the result is a synaptic strengthening that is reversible upon resumption of 1 pulse/10 seconds stimulation frequency. Example traces are inset. Scale bar: 20 ms (horizontal) and 0.5 mV (vertical). B, When two independent pathways are stimulated at 1 pulse/10 seconds and the stimulation frequency is altered in only one pathway from to 1 pulse/30 minutes, only the pathway with the altered frequency (test) shows homeostatic synaptic strengthening. Simplified, theoretical diagram of experimental paradigm is inset to the left. Example traces are inset to the right. Scale bar: 20 ms (horizontal) and 0.5 mV (vertical). C, Slices were stimulated once every 20 seconds for 30 min, and then the stimulation frequency was either kept the same or shifted to once every 60 seconds, once every 5 min, or once every 30 min. D, After 1 hour of the new stimulation frequency both the pathways shifted to once every 5 minutes and once every 30 minutes had significantly stronger responses than the pathway that was kept constant (*$p=0.011$ by ANOVA; $n=6$). E, Slices were stimulated once every 5 minutes for 30 min, and then the stimulation frequency was either kept the same or shifted to once every 60 seconds or once every 20 seconds. F, After 1 hour of the new stimulation frequency both the pathways shifted to once every 60 seconds and once every 20 seconds had significantly weaker responses than the pathway that was kept constant ($p<.001$ by ANOVA; $n=5$).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Control
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1/20 s - 1/30 min
Norm. fEPSP Slope (%)
Time (minutes)
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Norm. fEPSP Slope (%)
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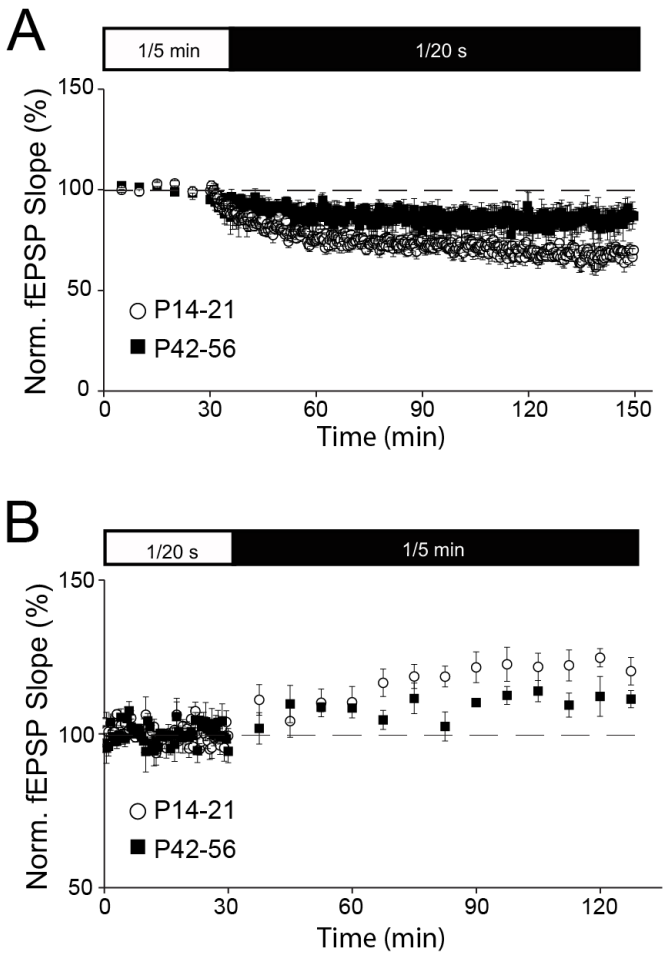
1/5 min - 1/60 s
1/5 min - 1/20 s
% fEPSP Slope Change

Figure 2.2. Homeostatic synaptic plasticity is stronger in younger rats. A, In rats at either P14-21 or P42-56, the Shaffer collaterals were stimulated at 1 pulse/5 min, and then the stimulation frequency was shifted to 1 pulse/20 sec. The induced homeostatic weakening was weaker in the slices from older rats, but still present. B, In rats at either P14-21 or P42-56, fEPSPs were evoked at 1 pulse/20 sec, and then, the stimulation frequency was shifted to 1 pulse/5 min. The induced homeostatic strengthening was weaker in the slices from older rats, but, again, still present.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Time (min)
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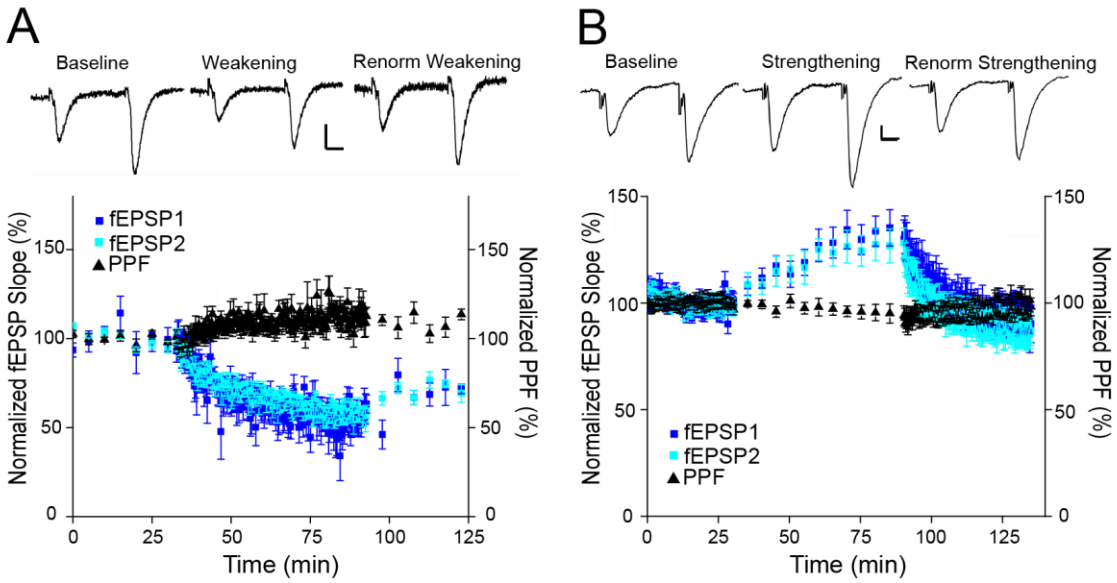
1/5 min
Norm. fEPSP Slope (%)
150
100
50
P14-21
P42-56
0
30
60
90
120
Time (min)

Figure 2.3. HSP does not involve a change in presynaptic release probability. A, Stimuli pairs (given 50 ms apart) were delivered at a rate of once every 5 min for 30 min and then the rate was switched to once every 20 seconds for 30 min and then returned to the original frequency; the normalized fEPSP slope of both the first and the second response is shown in blue. Paired-pulse facilitation (PPF), shown in black, remained unchanged throughout the experiment. Example traces are shown above during the original baseline, the weakening period, and then the weakening trace is renormalized to the amplitude of the baseline period to demonstrate that PPF stayed constant. Scale bars: 0.5 mV (vertical), 20 ms (horizontal). B, Stimuli pairs (given 50 ms apart) were delivered at a rate of once every 20 seconds for 30 min and then the rate was switched to once every 5 min for 30 min and then returned to the original frequency; the normalized fEPSP slope of both the first and the second response is shown in blue. PPF, shown in black, remained unchanged throughout the experiment. Example traces are shown above during the original baseline, the strengthening period, and then the strengthening trace is renormalized to the amplitude of the baseline period to demonstrate that PPF stayed constant. Scale bars: 0.5 mV (vertical), 20 ms (horizontal).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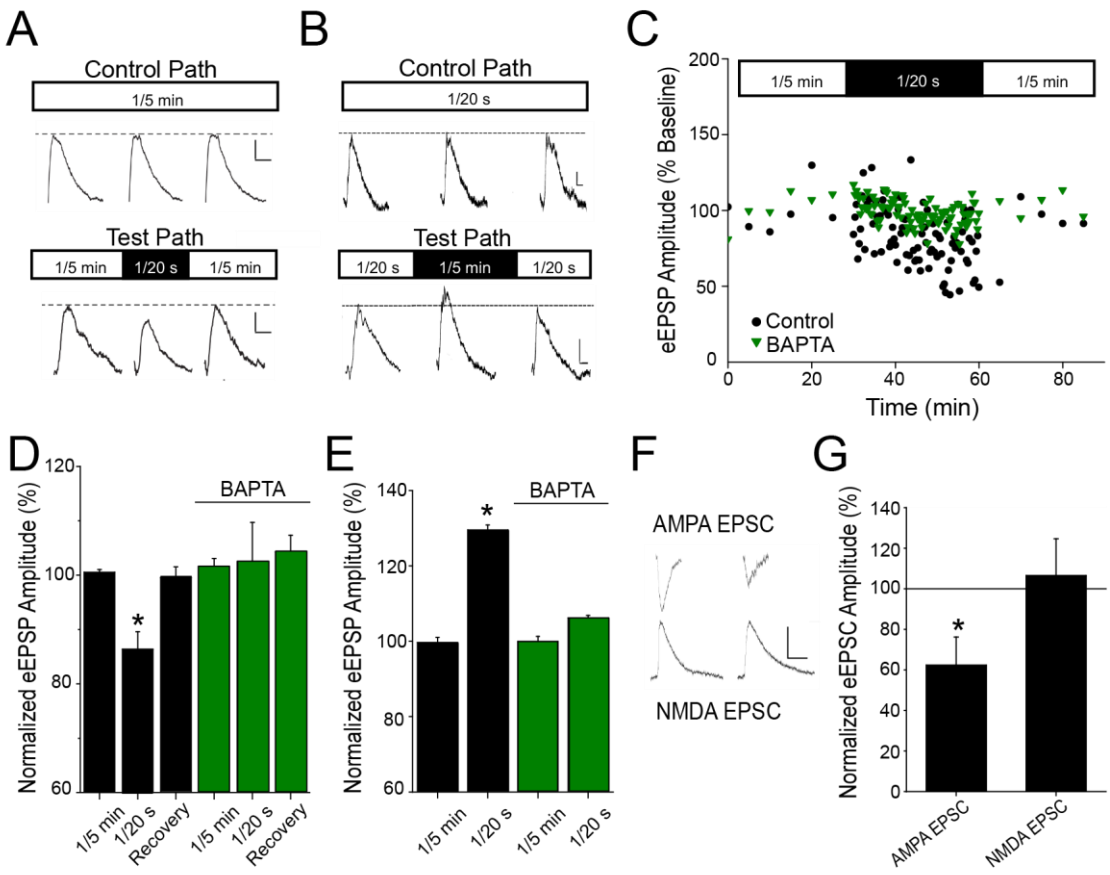
Baseline
Strengthening
Renorm Strengthening
fEPSP1
fEPSP2
PPF
Normalized fEPSP Slope (%)
Normalized PPF (%)
Time (min)

Figure 2.4. HSP in acute hippocampal slices is driven by postsynaptic calcium influx. A, CA1 pyramidal neurons were recorded in whole-cell current clamp mode while eEPSPs from non-overlapping inputs were evoked with stimulating cluster electrodes. eEPSPs were obtained via whole-cell patch-clamp recording of pyramidal cells in CA1 of the hippocampus. Example traces demonstrating when two independent pathways were stimulated at 1 pulse/20 sec and then one pathway had the stimulation frequency shifted to 1 pulse/5 min (test pathway) while the other pathway remained at 1 pulse/20 sec (control pathway), only the pathway with the frequency change demonstrated a decreased eEPSP amplitude. Increasing the frequency of stimulation induced a selective decrease in eEPSP amplitude in the test pathway, demonstrating that homeostatic weakening is input-specific. B, Example eEPSPs that were recorded in two independent pathways stimulated at a baseline frequency of 1 pulse/5 min followed by a shift in the test path to 1 pulse/20 sec while the other pathway remained at 1 pulse/5 min. Decreasing the frequency of stimulation induced a selective increase in eEPSP amplitude in the test pathway, demonstrating that homeostatic strengthening is also input-specific. C, Example recording of normalized eEPSP amplitude across time for a cell with (black) and a cell without (green) BAPTA in the recording electrode. Homeostatic synaptic weakening was not seen in the presence of BAPTA, suggesting that postsynaptic calcium is required for negative homeostatic plasticity. D, Homeostatic weakening is blocked by the inclusion of BAPTA in the recording electrode. There was a significant depression in the amplitude during the homeostatic weakening period in the absence of BAPTA (*p=0.029; n=8). This depression was abolished when BAPTA was included in the recording pipette (p=0.309; n=8). E, Summary data, averaging the eEPSP amplitude

during the first 30 min (1 pulse/5 min) and the second 30 min (1 pulse/20 sec) for pyramidal neurons recorded with normal internal solution and neurons recording with BAPTA in the internal solution. There was a significant increase in amplitude when the frequency was switched to 1 pulse/20 sec in the absence of BAPTA (*p=0.003; n=11). This enhancement was abolished when BAPTA was included in the recording pipette (p=0.102; n=11). F, Example NMDAR and AMPAR traces obtained at +40 and -70, respectively both during a baseline period and after 30 min of HSP induction. NMDAR responses were measured at a 50 ms time point. Scale bars represent 100 pA (NMDAR, vertical), 50 pA (AMPAR, vertical), and 50 ms (horizontal). G, Normalized AMPAR eEPSCs were significantly decreased after HSP (*p=0.019; n=7); whereas, normalized NMDAR eEPSCs were not altered by HSP (p=0.191; n=7). Homeostatic weakening involves a decrease in AMPAR currents, but no change in the NMDAR current.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Test Path
1/20 s
1/5 min
1/20 s
C
1/5 min
1/20 s
1/5 min
eEPSP Amplitude (% Baseline)
200
150
100
50
0
Control
BAPTA
0
20
40
60
80
Time (min)
D
Normalized eEPSP Amplitude (%)
120
100
80
60
BAPTA
*
1/5 min
1/20 s
Recovery
1/5 min
1/20 s
Recovery
E
Normalized eEPSP Amplitude (%)
140
120
100
80
60
BAPTA
*
1/5 min
1/20 s
1/5 min
1/20 s
F
AMPA EPSC
NMDA EPSC
G
Normalized eEPSC Amplitude (%)
140
120
100
80
60
40
20
0
*
AMPA EPSC
NMDA EPSC

Figure 2.5. NMDA receptors, but not L-type calcium channels, are a source of calcium required for homeostatic synaptic plasticity. A-B, Field recordings from CA1 in the hippocampus were used to investigate the source of calcium involved in homeostatic synaptic plasticity. To block NMDA receptors, APV (100 μM) was added after a 30 minute stable baseline was obtained and was included for the remainder of the experiment. Homeostatic synaptic strengthening was blocked by the addition of APV (A, *p=0.002; n = 10 (control), 11 (APV)). Homeostatic synaptic weakening was blocked by the addition of APV (B, *p = .033; n = 4 (control, black), 7 (APV, red)). C-D, The role of L-type calcium channels was investigated using nifedipine (10 μM). After 30 min of stable baseline, nifedipine was included in the perfusion aCSF to block L-type calcium channels, control experiments included DMSO (0.1%). Homeostatic synaptic strengthening was unaffected by the addition of nifedipine (C, p=0.726; n=13 for both control and nifedipine). Homeostatic synaptic weakening was unaffected by the addition of nifedipine (D, p=0.426; n=5 for both control and nifedipine pathways).

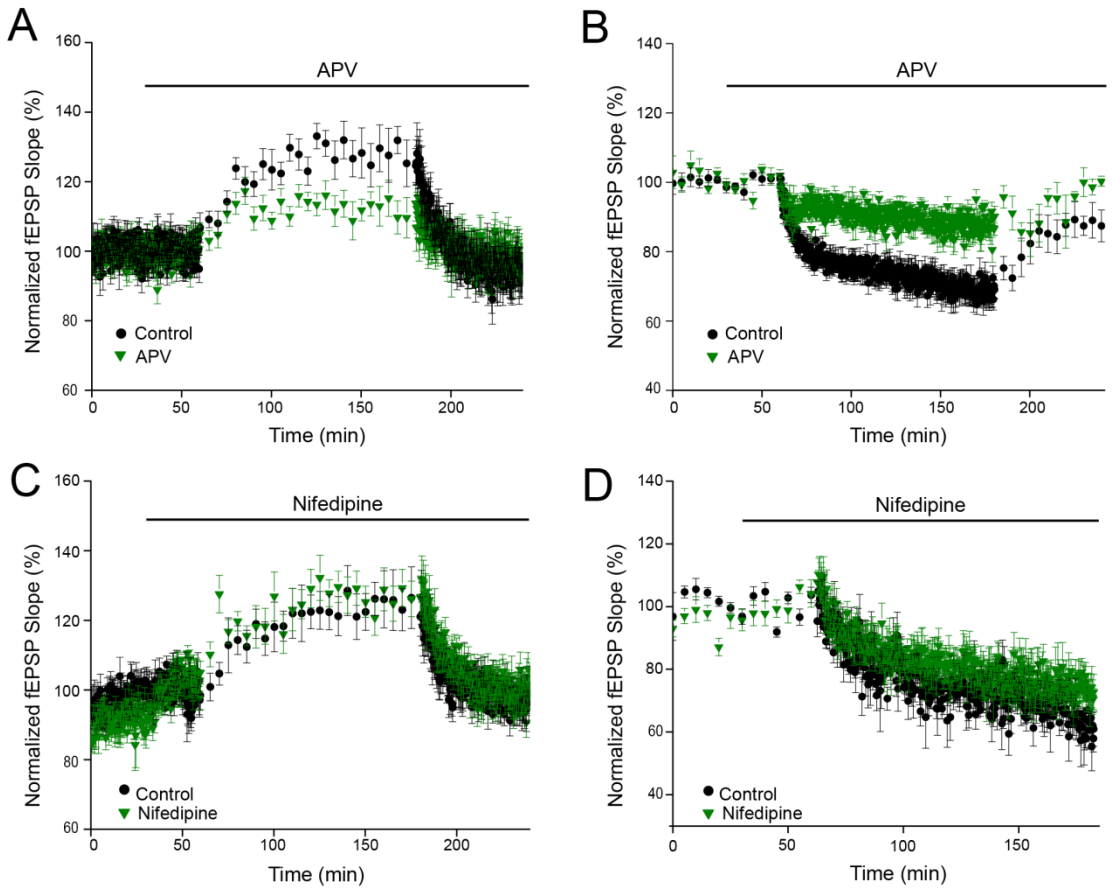

A
APV
Normalized fEPSP Slope (%)
Control
APV
Time (min)
B
APV
Normalized fEPSP Slope (%)
Control
APV
Time (min)
C
Nifedipine
Normalized fEPSP Slope (%)
Control
Nifedipine
Time (min)
D
Nifedipine
Normalized fEPSP Slope (%)
Control
Nifedipine
Time (min)

Figure 2.6. Homeostatic synaptic plasticity in acute hippocampal slices is mechanistically distinct from LTP and LTD. A-B, After a 30 min period of stable baseline, KN-93 (10 μm) or 0.1% DMSO (control) was included in the aCSF for another 30 min, then a single train of 100 Hz was delivered to slices (A) or the frequency of stimulation was switched to 1 pulse/20 sec (B). In the HSP experiments, no difference in the outcome was seen between KN-93 or KN-62 was used so data here are pooled. KN-93 and KN-62 were used to block CaMKII activity. Inhibition of CamKII activity blocked the LTP, but not the HSP. C-D, After a 30 min period of stable baseline, FK506 (50 μm) or 0.1% DMSO (control) was included in the aCSF for another 30 min, then (C) a single train of 1 Hz was delivered to slices or (D) frequency of stimulation was switched to 1 pulse/5 min. FK506, a calcineurin inhibitor, blocked LTD, but not homeostatic weakening.

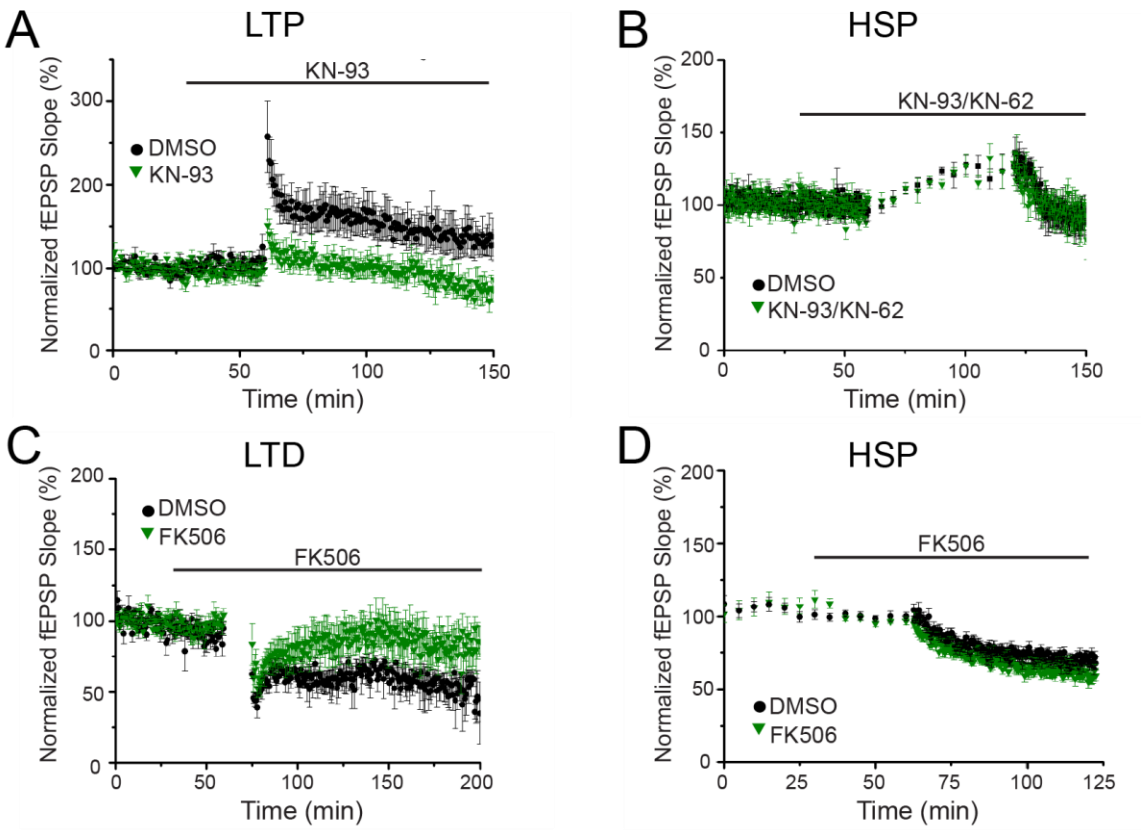

A
LTP
KN-93
DMSO
KN-93
Normalized fEPSP Slope (%)
Time (min)
B
HSP
KN-93/KN-62
DMSO
KN-93/KN-62
Normalized fEPSP Slope (%)
Time (min)
C
LTD
DMSO
FK506
FK506
Normalized fEPSP Slope (%)
Time (min)
D
HSP
FK506
DMSO
FK506
Normalized fEPSP Slope (%)
Time (min)

Figure 2.7. Homeostatic synaptic weakening does not alter the relative magnitude of Hebbian Plasticity. A, In two pathway experiments, homeostatic weakening was induced in one pathway by shifting the frequency of stimulation from once every 5 minutes to once every 20 seconds. An hour after homeostatic weakening, a single train of HFS was delivered to both pathways. B, Renormalization of the previous panel. Data were renormalized to the level before 1 X HFS LTP induction demonstrating that prior induction of homeostatic synaptic weakening offsets the magnitude of 1x HFS LTP, but does not alter the relative change in synaptic strength from the "scaled" baseline (n=8, 10).

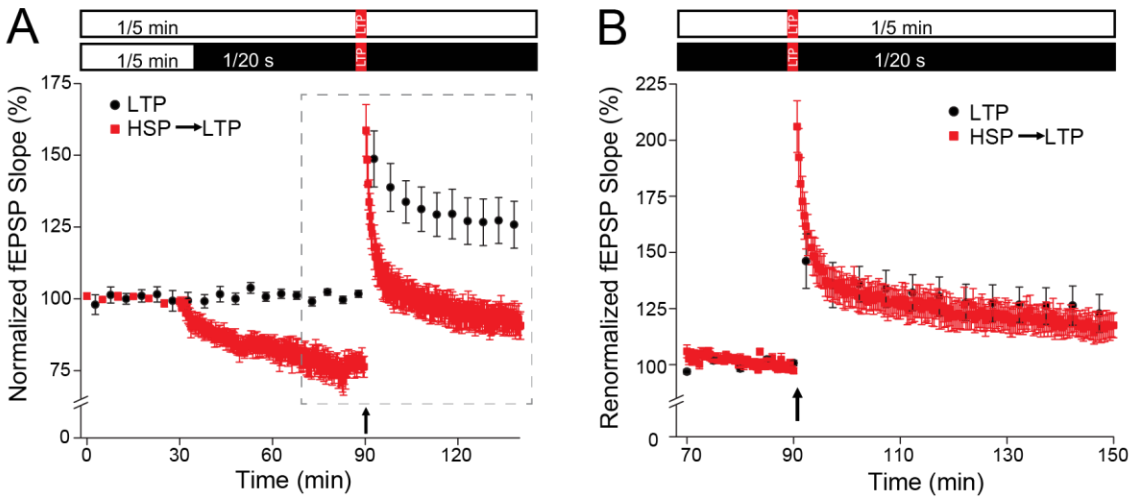
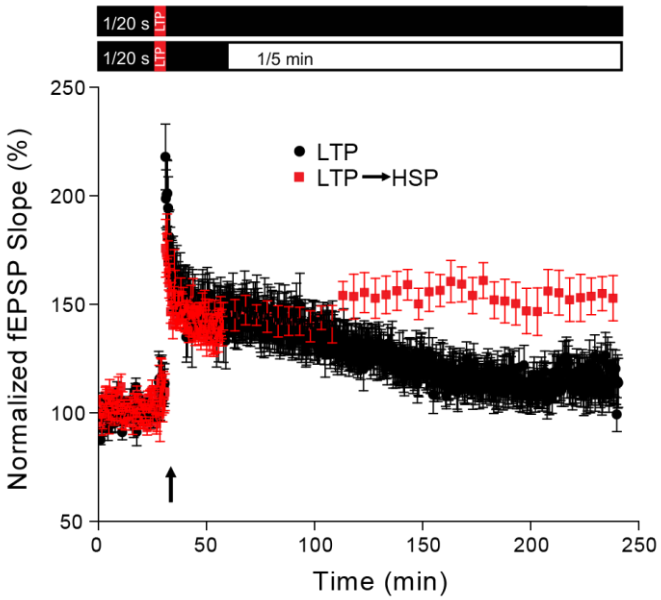

A
1/5 min
1/5 min
1/20 s
LTP
LTP
HSP→LTP
Normalized fEPSP Slope (%)
175
150
125
100
75
0
0
30
60
90
120
Time (min)
B
1/5 min
1/20 s
LTP
LTP
HSP→LTP
Renormalized fEPSP Slope (%)
225
200
175
150
125
100
0
70
90
110
130
150
Time (min)

Figure 2.8. Homeostatic synaptic plasticity can enhance the magnitude and durability of weak LTP in an input-specific manner. In two pathway experiments, 1 X HFS LTP was induced in both pathways, then homeostatic strengthening was induced in only one of the pathways by switching the stimulation frequency from 1 pulse/20 sec to 1 pulse/5 min. Here, HSP may be operating in such a way as to preserve Hebbian changes in this circuit.


1/20 s
LTP
1/20 s
LTP
1/5 min
LTP
LTP→HSP
Normalized fEPSP Slope (%)
250
200
150
100
50
0
50
100
150
200
250
Time (min)

Chapter III

Hebbian and homeostatic plasticity interact at the same synaptic inputs: Metaplasticity mediated by local protein synthesis

3.1 Introduction

The mammalian hippocampus is known to be critical for the formation of long-term memories. Studies in both humans (Scoville & Milner, 1957; Zola-Morgan et al., 1986) and animal models (Mayford et al., 2012; Tsien et al., 1996) have clearly shown the importance of the hippocampus, and the CA1 hippocampal region in particular, in long-term memory formation. Long-lasting activity-dependent modifications to synaptic strength, such as long-term potentiation (LTP) and long-term depression (LTD), are widely thought to form the cellular basis of information storage in hippocampal circuits (Bliss & Lømo, 1973; Dudek & Bear, 1992; Malenka & Bear, 2004). LTP and LTD are often referred to as "Hebbian" forms of synaptic plasticity, because their induction at individual synapses is linked with the correlation between that synapse's activation and postsynaptic firing, a learning rule postulated by Donald Hebb (Hebb, 1949). Although there is strong evidence that Hebbian modifications are a crucial component of learning and memory, they cannot be the only mechanism operating in neural networks. Computational models show that Hebbian processes will lead to instability in neural

circuits due to their positive-feedback nature if not balanced by a negative-feedback mechanism (Dayan & Abbott, 2001; Renart et al., 2003). Negative-feedback processes which compensate for changes in neural activity also exist at synapses in the brain and are generally referred to as homeostatic synaptic plasticity (HSP; Turrigiano & Nelson, 2004)). For example, blocking neuronal activity in dissociated neuronal networks with the voltage-gated sodium channel blocker tetrodotoxin leads to a compensatory increase in synaptic strength whereas raising activity with $GABA_A$ receptor antagonist bicuculline causes a decrease in synaptic strength (Turrigiano et al., 1998). Homeostatic plasticity can be implemented on a cell-wide scale (global HSP; Ibata et al., 2008; Turrigiano et al., 1998) as well as a non-global scale, even at individual synapses (Branco et al., 2008; Hou et al., 2011; Hou et al., 2008; Ju et al., 2004; Sutton et al., 2006).

Since homeostatic and Hebbian forms of synaptic plasticity have largely been studied independently of each other, how they function together at the same synapses is largely unknown. Hypothetical scenarios regarding how these ostensibly conflicting plasticity processes could be interacting have been generated (Rabinowitch & Segev, 2008; Turrigiano & Nelson, 2000), but experimental support for these ideas is scarce. For example, global homeostatic synaptic plasticity, which uniformly scales all the synapses of a neuron in order to compensate for chronic changes in activity, is hypothesized to preserve relative changes in synaptic strength caused by Hebbian processes while promoting stability (Turrigiano & Nelson, 2000). However, it is unclear how locally acting HSP might interact with Hebbian synaptic plasticity at the same inputs, potentially in direct competition over control of synaptic efficacy. In the previous chapter, I demonstrated a rapid form of HSP expressed at CA3-CA1 synapses in acute

hippocampal slices. By altering the frequency of Schaffer collateral stimulation, we induced compensatory changes in synaptic strength that are bi-directional and input-specific. I also showed that this model system can be used to investigate potential interactions between Hebbian and homeostatic synaptic plasticity.

In this chapter, we continue to investigate how HSP operates in conjunction with Hebbian plasticity to influence the net efficacy of synaptic transmission at CA3-CA1 synapses in hippocampal slices. We found that prior induction of Hebbian plasticity constrained the magnitude of HSP subsequently induced. Interestingly, this metaplastic interaction was strictly dependent on the order of induction: prior induction of HSP did not alter the relative change in synaptic efficacy induced with LTP/LTD induction. Although neither form of plasticity studied is dependent on protein synthesis, the metaplastic interaction was abolished by blocking local protein synthesis near synaptic sites.

3.2 Materials and Methods

3.21 Acute hippocampal slice preparation

All procedures involving animals were approved by the University Animal Care and Use Committee. Acute hippocampal slices were prepared as previously described (Chapter II). P14-21 Sprague Dawley rats were decapitated and the hippocampal lobules were isolated. Transverse slices (400μm) of the hippocampus were cut using a tissue chopper (Stoelting, Wood Dale, IL). Slices were then incubated at room temperature in a humidified chamber for at least 2 hours before recording.

3.22 Electrophysiology

Hippocampal slices were transferred to a recording chamber and continuously perfused at a rate of ~1.5 ml/min with artificial cerebral spinal fluid (aCSF) heated to 27-28°C with an in-line solution heater and equilibrated with 95% oxygen/ 5% carbon dioxide. aCSF contained (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2P0_4, 26.3 $NaHCO_3$, 11 glucose, 1.3 $MgSO_4$, and 2.5 $CaCl_2$, pH 7.4. Area CA1 was visualized with an Olympus SZ51 dissecting microscope. Recording electrodes were pulled from borosilicate capillary glass (1.7 mm o.d.; VWR International, Radnor, PA) and filled with 3M NaCl or aCSF. The recording pipette was placed in the center of CA1 stratum radiatum. Extracellular recordings were made with a MultiClamp 700B amplifier, collected using Clampex 10.2, and analyzed using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA). Field excitatory postsynaptic potentials (fEPSPs) were recorded in response to Schaffer collateral stimulation using bipolar stainless steel electrodes also placed in the stratum radiatum of CA1 (FHC, Bowdoin, ME). Current was delivered with an ISO-Flex stimulus isolator (A.M.P.I., Jerusalem, Israel) and set between 0.02 - 0.25 mA (0.1 msec pulse width). Current was set to ~50% of the maximum determined by generating input/output (I/O) curves before each experiment. In 2-pathway experiments, stimulating electrodes were placed on opposite sides of the recording electrode. Pathway specificity in every experiment was confirmed by less than 10% paired-pulse facilitation between inputs (ISI = 50 msec). Baseline recordings were considered stable when the fEPSP slope changed less than ±5% over a continuous 30 min period. For interaction studies, experiments which failed to elicit LTP of at least 20% were omitted from analysis. Long-term potentiation was induced by using either a single train of 100 Hz (1 second) or 4x100 Hz (1s duration each) separated by 5 minutes. Long-term depression were induced

by using either a 1Hz (900 pulses, 15 minutes duration) or 4x 1Hz (900 pulses, 15 minute duration separated by 15 minutes). Data were analyzed by applying a Student's t-test (paired or unpaired), considering $p<0.05$ as statistically significant.

3.23 Local diffusion of protein synthesis inhibitor

Drug diffusing extracellular electrodes were fabricated from capillary glass tubing (G150-4, Warner) using a P-97 Flaming-Brown pipette puller (Sutter Instruments) pulled to resistances between 0.8 and 1.0 MΩ. To test the puller conditions needed to locally diffuse drug from the recording electrode, CNQX was initially included in the recording pipette and was shown to block the bulk of fEPSP signal in response to afferent stimulation, while a nearby recording electrode filled with aCSF showed no decrement in fEPSP signal (data not shown). Subsequent testing confirmed emetine in the recording electrode failed to display the late-phase LTP recorded via the nearby vehicle-containing recording electrode (data not shown). The fEPSP signal was amplified 1000 times with a DAM-50 DC differential amplifier (WPI). For experiments, recording electrodes were filled with emetine diluted in aCSF or aCSF alone and placed in stratum radiatum in the same plane as the apical dendrites, with one electrode site more proximal to the soma than a distally placed electrode (see Fig. 3.7). A single bipolar stimulating electrode was used to deliver an identical stimulation pattern to Schaffer collaterals.

3.3 Results

3.31 Local HSP can compete with previously established Hebbian plasticity

Having previously established that rapid, input-specific HSP can be expressed at CA3-CA1 synapses in response to subtle shifts in the rate of afferent stimulation, we set out to test how HSP and Hebbian plasticity interact within the same circuit. While our previous study demonstrated that rapid HSP preserves the capacity for LTP in neural circuits (Chapter II), the manner by which homeostatic plasticity interacts with already established Hebbian modifications has been a topic of theoretical debate (Rabinowitch & Segev, 2008; Turrigiano & Nelson, 2000). Hence, we next examined whether prior induction of Hebbian plasticity alters rapid homeostatic compensation of synaptic strength. In these experiments, we first induced Hebbian plasticity (LTP or LTD) in one of two independent inputs onto a common population of CA1 dendrites and then compared the relative magnitude of homeostatic compensation between the two inputs in response to the same frequency shift delivered to both pathways.

We first examined conditions where homeostatic plasticity should compete with established Hebbian modifications – that is, when the homeostatic changes in synaptic strength oppose the established Hebbian changes. After an initial collection of stable baseline responses for 30 min, LTP (1x HFS) was induced in one pathway, resulting in a significant potentiation of synaptic responses. When we shifted the test frequency from 1 pulse every 5 minutes to 1 pulse every 20 seconds 60 minutes after the induction of LTP, we found that the magnitude of homeostatic weakening was offset from the naïve (non-LTP) input (Fig. 3.1A, left). However, renormalizing the data to the potentiated baseline to examine the relative homeostatic weakening revealed that the magnitude and time-course of homeostatic synaptic weakening in this pathway was indistinguishable from the naïve pathway. Likewise, when we shifted the test frequency from 1 pulse/20 seconds to

1 pulse/5 minutes to induce homeostatic strengthening 60 min after the induction of LTD (1xLFS) in one pathway, the compensatory increase in synaptic strength in the LTD path was offset (Fig. 3.1B, left), but otherwise identical to that of the naïve pathway (Fig. 3.1B, right). Taken together, these results suggest that homeostatic forms of synaptic plasticity can compete with established Hebbian forms of synaptic plasticity.

3.32 Hebbian plasticity exerts a pathway-specific constraint on subsequent synaptic compensation

Next, we designed a protocol that would fully assess the ability of HSP to interact with a strong form of LTP (Fig. 3.2A). In these 2-pathway experiments, fEPSPs were measured over a 30 minute baseline period at 1 pulse every 40 seconds to ensure stability in both pathways. In order to address the possibility that the initial signaling frequency controls the interaction, the baseline frequency was the same in each condition. LTP was then induced in one (test) pathway by delivering 4 spaced (ITI = 5 min) trains of 100Hz stimulation (4xHFS, 1 sec), while the control pathway continued to receive stimulation at 1 pulse/40 seconds but received no LTP induction. fEPSPs were monitored for an additional 30 min following LTP induction, then HSP was induced in both pathways by shifting the frequency of stimulation to a new frequency that induced either homeostatic weakening, homeostatic strengthening or no compensation (Fig. 3.2A). The relative magnitude of compensation was assessed by renormalizing the fEPSPs to the 20 min period immediately prior to HSP induction. We found that prior induction of saturating LTP does not alter the relative magnitude of homeostatic synaptic weakening under competing conditions, but significantly reduces the relative magnitude of homeostatic synaptic strengthening under cooperating conditions 90 min following HSP induction

(paired t-test, $p < 0.05$) (Fig. 3.2). Thus, Hebbian and homeostatic plasticity interact asymmetrically at hippocampal CA3-CA1 synapses to influence synaptic efficacy changes in response to activity.

The magnitude of homeostatic strengthening expressed in the previous experiment's naïve control path was not particularly strong, raising a potential concern with the parameters of HSP induction in that experiment. Since homeostatic plasticity is sensitive to degree of activity disparity (Chapter II), the experiment was run again utilizing a greater frequency disparity to induce stronger compensatory strengthening. In both pathways, a 30 minute stable baseline was obtained at a stimulation rate of 1 pulse/40 seconds before inducing a strong form of LTP induced by four trains of high-frequency stimulation (4xHFS; 100 Hz for 1 sec, 5 minutes apart) in one pathway (Fig. 3.3A), similar to the previous experiment (Fig. 3.2). In this case, stronger homeostatic strengthening was induced in both pathways to directly compare the naïve pathway to the pathway with a history of Hebbian modification by decreasing the frequency of afferent stimulation to 1 pulse every 15 minutes (Fig. 3.3A). Plotting the magnitude of homeostatic strengthening relative to the potentiated baseline (i.e., the 15 min period immediately prior to homeostatic strengthening), revealed that HSP expression was constrained by prior LTP induction (Fig. 3.3B), as observed with a weaker homeostatic strengthening protocol (Fig. 3.2).

We next examined whether a strong form of LTD exerts a similar influence over HSP. In this case, baseline was collected at 1 pulse/5 minutes before inducing LTD (4xLFS; 900 pulses at 1 Hz, 15 minutes apart) in one pathway (Fig. 3.3B, left). To test whether LTD interacts with an HSP that would alter synaptic strength in the same

direction, we induced homeostatic weakening by increasing the rate of afferent stimulation to 1 pulse/20 seconds in both pathways after 60 min of LTD expression (Fig. 3.3B, left). As with LTP, prior LTD alters the absolute and relative magnitude of subsequent homeostatic weakening, revealing a similar pathway-specific constraint on HSP (Fig. 3.3B).

Although we have interpreted the differences in HSP expression levels in pathways with a history of Hebbian plasticity as due to a homosynaptic constraint, we could not exclude the possibility of that the observed constraint wasn't a result of ceiling/floor effects. This explanation is especially salient since the strong induction protocols used produce saturating forms of LTP and LTD (Abraham & Tate, 1997; Heynen et al., 1996; data not shown). To circumvent this potential confound, we performed the experiment as before but with a weak LTD induction protocol (1xLFS; 900 pulses at 1 Hz). Even expression of weak, non-saturating LTD constrains the relative magnitude of subsequent homeostatic strengthening (Fig. 3.3C). Thus, Hebbian plasticity exerts a pathway-specific constraint on subsequent synaptic compensation expressed in the same direction.

3.33 Homeostatic plasticity preserves the capacity for Hebbian plasticity

We next addressed whether prior induction of homeostatic plasticity alters the magnitude of LTD induced by a single epoch of low frequency stimulation (1xLFS; 900 pulses at 1 Hz). In this experiment, we scaled one of two independent inputs onto a common population of CA1 dendrites and then compared the relative magnitude of LTD induced in the two inputs. We found that homeostatic weakening by shifting the test

frequency from 1 pulse/5 minutes to 1 pulse/20 seconds decreased the absolute magnitude of LTD relative to the non-scaled input (Fig. 3.4A, left); however, when we plotted the magnitude of LTD relative to the scaled baseline (i.e., the 20 min period immediately prior to LTD induction), we found that the decrease in LTD was entirely accounted for by the superimposed homeostatic weakening of synaptic strength (Fig. 3.4A, right). This reversal of the paradigm in Fig. 3.3C reveals that the interaction between LTD and HSP is order-dependent and unlikely a result of a floor effect. Thus, LTD exerts an asymmetric constraining influence on subsequent homeostatic weakening.

To examine whether such offset of Hebbian plasticity was a general feature of homeostatic compensation, we examined if prior homeostatic weakening had a similar effect on the saturation of LTP induced by 4 spaced trains of HFS (4xHFS; 100Hz for 1 s, 5 min apart). Again, we found that the magnitude of saturated LTP was offset by prior homeostatic weakening, diminishing the total increase in synaptic strength (Fig. 3.4B, left), but not the relative increase in synaptic efficacy from the scaled baseline (Fig. 3.4B, right). These findings provide further evidence for the theoretical notion that homeostatic plasticity operates in such a way as to preserve the capacity for Hebbian activity-dependent changes in synaptic strength.

3.34 Metaplastic interaction is mediated by local protein synthesis

We hypothesized that the metaplastic influence of LTD on homeostatic weakening depends on the synthesis of new proteins. We first tested whether rapid, local HSP in acute hippocampal slices depends on new protein synthesis. Bath application of the protein synthesis inhibitor emetine 30 minutes prior to frequency shifts did not alter

the subsequent expression of either homeostatic strengthening (Fig. 3.5, left) or homeostatic weakening (Fig. 3.5, right). Unlike previously reported forms of rapid, local homeostatic plasticity (Sutton et al 2006; Jakawich et al. 2010), frequency shift-induced compensation does not depend on new protein synthesis for its expression in either direction.

In order to address whether the constraining influence of LTD on homeostatic weakening depends on the synthesis of new proteins, we took advantage of the facts that neither the weak LTD induction protocol (1xLFS; 900 pulses at 1 Hz) nor homeostatic weakening requires protein synthesis (Huber et al., 2000; Fig. 3.5). We induced non-saturating LTD in one pathway, followed by homeostatic weakening in both pathways as in Figure 3.3C, except this time we bathed slices in the protein synthesis inhibitor emetine or cyclohexamide (Fig. 3.6). Unlike slices bathed in vehicle alone, slices bathed with either protein synthesis inhibitor did not display the pathway-specific constraint. This result supports the hypothesis that the metaplastic influence of LTD on homeostatic weakening depends on synthesis of new proteins.

Given that both forms of plasticity are expressed locally, and that the metaplastic interaction occurred in an input-specific manner, we wondered if the protein synthesis mediating the interaction was also occurring in a spatially restricted manner. To investigate this possibility, two recording electrodes were placed in the same column of CA1 with a single stimulating electrode (fig. 7). One of the recording electrodes contained aCSF vehicle while the other contained emetine, with electrodes designed to release contents only over a limited area (data not shown). In this case, the suppression of homeostatic weakening by previous LTD was blocked locally by the focal application

of emetine (fig. 8). Selective blockade of protein synthesis at either proximal or distal apical dendrites reveals that metaplastic interactions are not isolated to one dendritic compartment (Fig. 3.9). Taken together, these data reveal that local protein synthesis mediates the interaction between Hebbian and homeostatic synaptic plasticities.

3.35 Protein synthesis dependent cooperation between Hebbian and homeostatic synaptic plasticity

Given that weak, non-saturating LTD exerts a protein synthesis dependent effect on homeostatic weakening, we reasoned that non-saturating forms of LTP would exert the same effect on homeostatic strengthening. To test this, we began by inducing weak LTP (1xHFS, 100Hz for 1 s) in one pathway before homeostatic strengthening in both pathways (Fig. 3.10A). The overall magnitude of fEPSP slopes was increased due to LTP expression, but unlike with other tested forms of LTP and LTD (Fig. 3.3), weak LTP did not alter the relative magnitude of homeostatic strengthening. Induction of slightly stronger forms of LTP with 2 trains of HFS (Fig. 3.10B) or theta-burst stimulation (25 bursts of four pulses at 100 Hz with 200 msec interburst intervals; Fig. 3.10C) similarly failed to elicit the pathway-specific constraint.

We previously found that HSP can prolong the potentiation of normally weak, short-lived LTP (see Chapter II), suggesting that Hebbian and homeostatic plasticity can cooperate to regulate the final output of the network. Intriguingly, homeostatic strengthening after weak LTP, which is itself protein synthesis independent, resembles late phase LTP, which is protein synthesis dependent (Chapter II; Fig. 3.11, left). Neither this form of weak LTP nor HSP are dependent on protein synthesis (Fig. 3.5). Under

some circumstances, Hebbian plasticity can constrain the magnitude of subsequent HSP in a protein synthesis dependent manner. Hence, we examined whether a similar protein synthesis dependent constraint occurs alongside the cooperation between LTP and HSP. To examine this possibility, we again used 2-pathway experiments and induced LTP (1xHFS) in both inputs while stimulating at a frequency of 1 pulse/20 seconds, then shifted one input to 1 pulse/5 minutes to induce homeostatic strengthening 30 min following LTP induction in the presence of the protein synthesis inhibitor emetine. Inhibition of protein synthesis results in significantly stronger homeostatic strengthening compared to experiments excluding inhibitor (Fig. 3.11). Despite evidence using a different protocol that weak LTP does not constrain subsequent HSP (Fig. 3.10), these data suggest there is an interaction between weak LTP and homeostatic strengthening which is regulated by protein synthesis.

3.4 Discussion

The present study probed several potential interactions between Hebbian plasticity and a rapid, input-specific form of HSP. Our results reveal that this form of HSP generally offsets the magnitude of subsequent Hebbian plasticity expression in an additive fashion. Strikingly, we found that prior induction of Hebbian plasticity constrained the magnitude of HSP subsequently induced, but only in cases where both plasticities altered synaptic strength in the same direction. Using induction protocols in which neither Hebbian plasticity nor HSP depends on new protein synthesis, we show that the metaplastic interaction between them is mediated by local protein synthesis. Finally, we follow up on a previous result demonstrating an unexpected form of

cooperation between LTP and HSP by unmasking a concurrent metaplastic interaction mediated by new protein synthesis.

3.41 Local HSP can compete with previously established Hebbian plasticity

Given that Hebbian forms of synaptic plasticity can endure for months in vivo (Abraham et al., 2002), one might predict that prior induction of Hebbian plasticity would suppress or constrain the magnitude of local homeostatic plasticity that would oppose the Hebbian changes, since the latter would limit the durability of the former. However, we found that homeostatic compensation was similar between inputs, regardless of whether Hebbian plasticity had been induced or not (Fig. 3.1). This is perhaps not too surprising, given that compensation would do little good if it were being disabled in the exact inputs that were deviating from baseline. As paradoxical as it may seem, we cannot rule out the possibility that Hebbian plasticity exerts a non-local constraint on the magnitude of opposing HSP since our comparisons were between pathways. Of course, since LTP and LTD do eventually decay back to baseline, perhaps finding that the competing HSP is intact is not only unsurprising, but may indicate that decay of LTP and LTD can be accounted for by a compensatory process. One prediction of this idea is that HSP would not compete as efficiently with longer lasting forms of LTP, such as late-phase LTP (L-LTP) which has a much slower decay time. However, we found no evidence of diminished homeostatic weakening following L-LTP (Fig. 3.2).

3.42 Hebbian plasticity exerts a pathway-specific constraint on subsequent synaptic compensation

Perhaps the most striking result of the present work is that LTP and LTD exert an input-specific suppressive influence over HSP. HSP can be induced in either direction, yet the influence of Hebbian plasticity is limited to the HSP that adjusts synapses in the same direction, not just on homeostatic strengthening or weakening (Fig. 3.2, 3. 3). This indicates that LTP induction results in a signal that constrains the relative magnitude of homeostatic strengthening, while LTD induction results in a signal that constrains the relative magnitude of homeostatic weakening. Another interpretation of these data is that Hebbian synaptic plasticity saturates the changes in synaptic efficacy such that further changes by HSP are impeded, a so called ceiling, or floor, effect. Another possibility is that Hebbian plasticity is occluding subsequent HSP, indicating a large degree of overlap in underlying mechanisms. However, we do not believe either of these alternative explanations to be correct based on follow up experiments.

First, when we utilized a non-saturating form of LTD, we still found a significant decrement in the magnitude of subsequent homeostatic weakening (Fig. 3.3C). Second, when we reversed the contingency such that homeostatic weakening preceded non-saturating LTD, there was no observable interaction (Fig. 3.4A). Similarly, HSP does not interfere with the subsequent expression of saturating LTP (Fig. 3.4B) or non-saturating LTP (Chapter II). Some forms of metaplasticity which do alter the expression of Hebbian plasticity have been shown to work via changes in NMDA receptor (NMDAR) signaling (Abraham, 2008; Gambrill et al., 2011). Thus, our finding that HSP does not influence subsequent Hebbian plasticity is in line with previous evidence that rapid HSP does not alter NMDAR-mediated currents (Chapter II). Finally, the most convincing evidence that the homosynaptic constraint is not an epiphenomenon of a floor effect

comes from experiments which probed the protein synthesis dependence of the metaplastic interaction**.** Since blocking new protein synthesis abolished the interaction, it is unlikely that the interaction is a result of floor effect. This result is particularly striking, since neither weak LTD nor HSP are dependent on protein synthesis for their own expression (Fig. 3.5), but the interaction between them requires intact protein synthesis.

The experiments presented here raise the possibility that other forms of synaptic plasticity may interact with HSP. One particularly interesting hypothesis is that metabotropic glutamate receptor-dependent LTD (mGluR-LTD) interacts with homeostatic weakening. mGluR-LTD persists in the presence of NMDAR antagonists (Oliet et al., 1997). The types of LTP and LTD examined in the present studies depend on NMDAR activation (Malenka & Bear, 2004), as does homeostatic strengthening and weakening (Chapter II). An interaction between mGluR-LTD and HSP would suggest a separate underlying signaling cascade. We found strong interactions of HSP with NMDAR forms of LTD (Fig. 3.3B,C) so it is conceivable that any similar interaction with mGluR-LTD would involve synthesis of the same HSP-disrupting protein(s).

3.43 Metaplastic interaction is mediated by local protein synthesis

Another striking finding is that the metaplastic interaction requires protein synthesis. We were able to ascertain this protein synthesis dependency by examining interactions between protein synthesis-independent forms of synaptic plasticity (Fig. 3.6). Because of this advantage, we were able to bath apply protein synthesis inhibitors throughout the experiment. Unfortunately, we could not use this approach to test whether

the interaction between HSP and late-phase LTP/LTD since these depend on new protein synthesis (Malenka & Bear, 2004).

Following up with the interaction we could examine, we spatially localized a protein synthesis inhibitor to the apical dendrite layer stratum radiatum of area CA1 and discovered a novel role for local protein synthesis in mediating metaplastic interaction. De novo translation mediating synaptic plasticity can occur within dendrites near synapses (Sutton & Schuman, 2006), and this mechanism fits with our results. To confirm dendritic protein synthesis, future experiments could physically isolate the dendritic compartment from the soma, the principal site of protein synthesis (Huber et al., 2000; Kang & Schuman, 1996). In addition, future studies could examine whether transcription, which takes place in the soma, is required for the interaction by bath application of the drug actinomycin-D.

The experiments performed do not reveal when the translation underlying the interaction occurs. One possibility is that LTP and LTD induction concurrently drive the creation of proteins that are involved with the interaction, presumably initiating a signaling cascade that remains active for the time course of the experiments. A second possibility is that the translation machinery is somehow altered by LTP and LTD induction, such that when HSP is induced, new protein synthesis is required. This possibility could be tested by applying protein synthesis inhibitors after LTP and LTD induction but before HSP induction.

What remains to be identified is the relevant translation regulatory signaling pathways involved and which transcripts are being translated. Both mTor and

ERK/MAPK signaling pathways are involved in the regulation of protein-synthesis dependent forms of plasticity (Gallagher et al., 2004; Hoeffer & Klann, 2010; Lynch, 2004). The proteins being synthesized could directly underlie HSP, with new protein synthesis altering the balance of relevant players and preventing normal HSP expression. Another possibility is that a molecule unnecessary for normal HSP expression is synthesized that can functionally interact with the HSP machinery. To best address these possibilities, it would be useful to know the exact molecular mechanisms underlying rapid HSP in acute hippocampal slices, which would provide a candidate list of transcripts. Since this compensatory plasticity was only recently discovered, the molecular mechanisms are still being investigated.

3.44 Protein synthesis dependent interaction concurrent with cooperation between Hebbian and homeostatic synaptic plasticity

In the present study, we also followed up on an earlier finding that HSP could cooperate with LTP to increase the endurance of the synapse potentiation (Chapter II, Fig. 3.11). Although this type of scenario seems counterintuitive at first glance, it is not unlikely that a potentiated input in a behaving organism's brain would subsequently enter a relatively inactive state. In this case, the potentiation may decay too rapidly in the absence of a protective, input-specific compensatory response. Here, we found that in such a scenario, homeostatic strengthening following LTP is likely being constrained in a protein synthesis dependent manner (Fig. 3.11). This result may suggest that although HSP can serve to prevent decay of Hebbian changes, it does so with limited capabilities, perhaps to prevent the complete salvation of inactive inputs.

To move beyond mere speculation of the functional effects of these interactions, one could take advantage of current computation models of neural networks and implement the reported interactions as modification rules (Dayan & Abbott, 2001). We hypothesize that these rules would have a stabilizing effect on the neural networks, since all discovered constraints prevent further deviations in synaptic strength away from the original baseline. Perhaps the most puzzling question is in regard to the nature of the two classes of synaptic plasticity studied: How can rapid, local HSP co-exist with LTP/LTD and not erase their changes? Given that the actual dynamics of these processes are likely different in vivo, it will be important to determine whether the reported interactions occur in an intact organism.

3.5 Acknowledgements

The work described in Chapter III was done in collaboration with postdoctoral fellows, Dr. Erin M. Johnson-Venkatesh and Dr. Francisco Javier Munoz-Cuevas, in Dr. Michael M. A. Sutton's laboratory. This work was supported by grants F31NS073372 (A.J.I.) from the National Institutes of Neurological Disorders and Stroke and RO1MH085798 (M.A.S.) from The National Institute of Mental Health and a grant from the Pew Biomedical Scholars Program (M.A.S.). We also thank Hisashi Umemori and members of the Sutton laboratory for many helpful discussions.

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3.7 Figure Legends

Figure 3.1- Homeostatic synaptic weakening does not alter the relative magnitude of Hebbian Plasticity. Prior induction of either (A) 1x HFS LTP or (B) 1x LFS LTD offsets the magnitude of homeostatic plasticity subsequently induced in the opposing direction, but does not alter the relative change in synaptic strength (n=12 in (A), n=8 in (B)). Thus, both forms of plasticity are able to compete with each other at the same synapses.

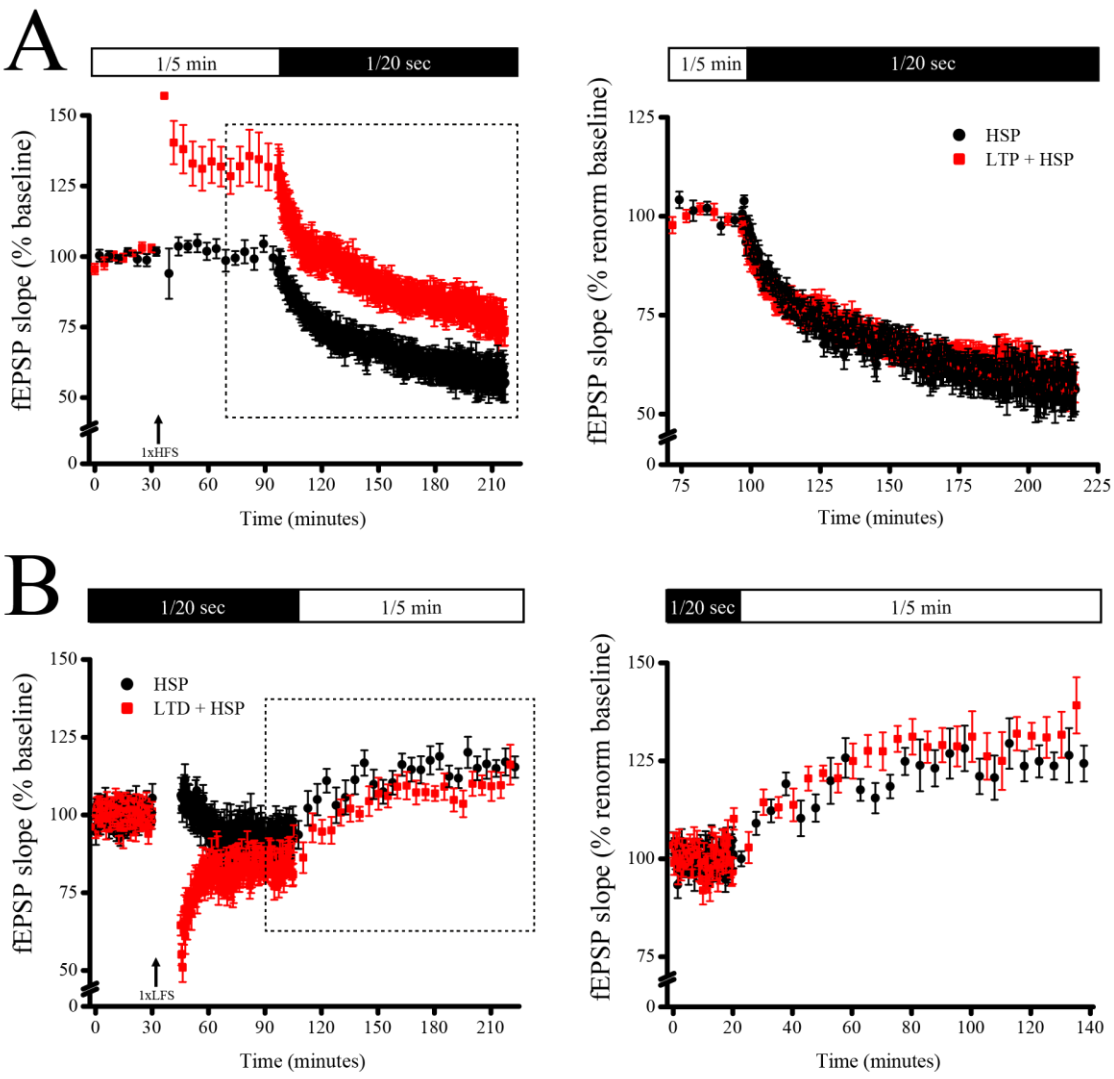

A
1/5 min
1/20 sec
fEPSP slope (% baseline)
150
125
100
75
50
0
1xHFS
0
30
60
90
120
150
180
210
Time (minutes)
1/5 min
1/20 sec
HSP
LTP + HSP
fEPSP slope (% renorm baseline)
125
100
75
50
0
75
100
125
150
175
200
225
Time (minutes)
B
1/20 sec
1/5 min
HSP
LTD + HSP
fEPSP slope (% baseline)
150
125
100
75
50
0
1xLFS
0
30
60
90
120
150
180
210
Time (minutes)
1/20 sec
1/5 min
fEPSP slope (% renorm baseline)
150
125
100
75
0
0
20
40
60
80
100
120
140
Time (minutes)

Figure 3.2- Prior induction of 4xHFS LTP does not alter the relative magnitude of homeostatic synaptic weakening, but significantly reduces the relative magnitude of homeostatic synaptic strengthening. (A) Schematic of experimental setup, illustrating 2-pathway experiments initially stimulated at 1 pulse every 40 seconds. During this period, saturating LTP (4x HFS) is induced in the test pathway. Subsequently, both pathways are switched to the same new stimulating frequency to induce homeostatic strengthening, no homeostatic compensation or homeostatic weakening. (B) Mean +/- SEM from pooled experiments in which no homeostatic compensation is induced and the frequency is of maintained at 1 pulse every 40 seconds. (C) Mean +/- SEM from pooled experiments in which homeostatic weakening is induced at a final frequency of 1 pulse every 10 seconds. (D) Mean +/- SEM from pooled experiments in which homeostatic strengthening is induced at a final frequency of 1 pulse every 5 minutes. (E) Partial log-plot of the renormalized mean +/- SEM renormalized fEPSP slope values (*$p<0.05$, n=9, ISI 10sec, n=7, ISI 20sec, n=3, ISI 40sec, n=6, ISI 5min).

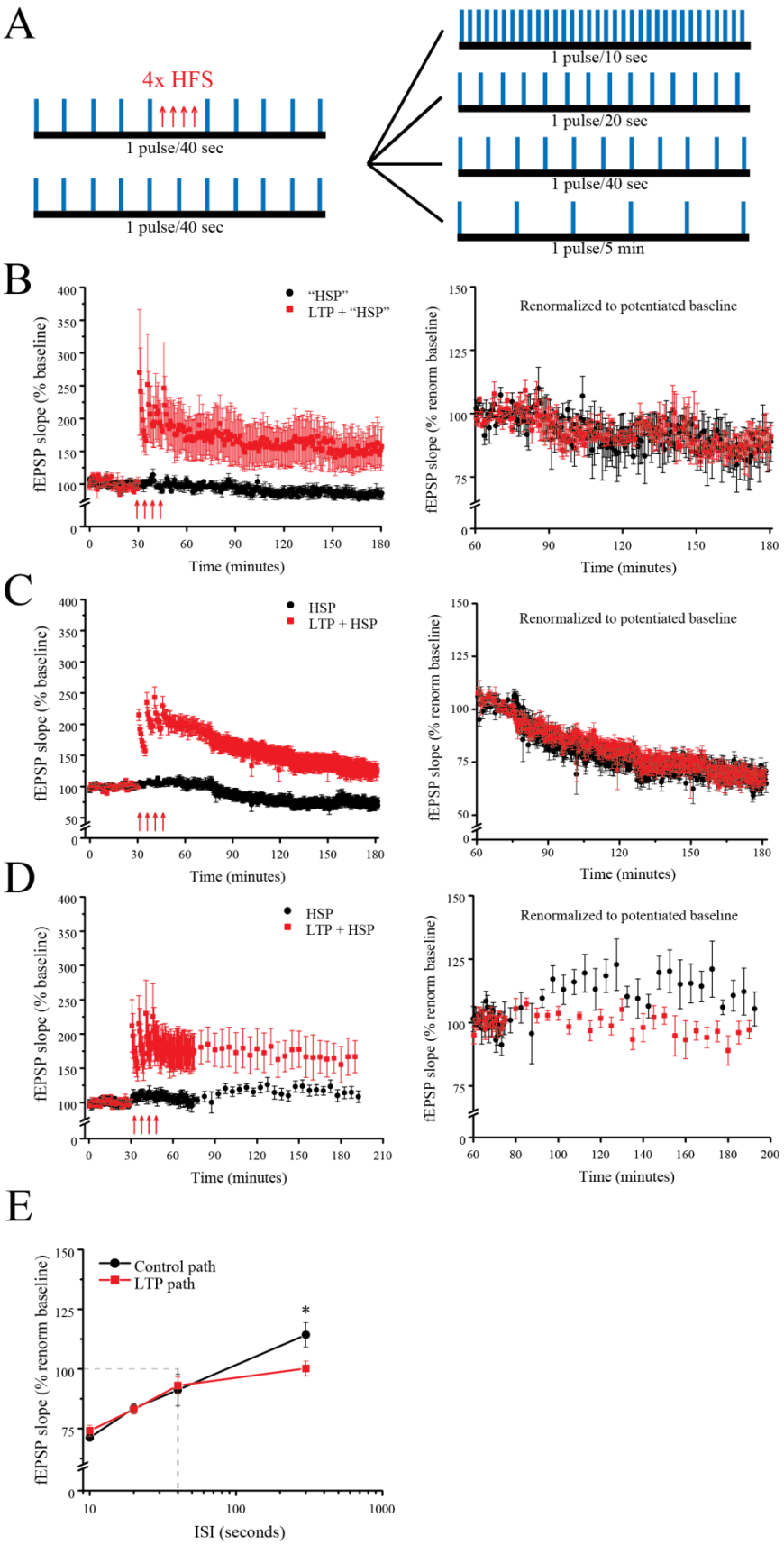

A
4x HFS
1 pulse/40 sec
1 pulse/40 sec
1 pulse/10 sec
1 pulse/20 sec
1 pulse/40 sec
1 pulse/5 min
B
"HSP"
LTP + "HSP"
fEPSP slope (% baseline)
Time (minutes)
Renormalized to potentiated baseline
fEPSP slope (% renorm baseline)
Time (minutes)
C
HSP
LTP + HSP
fEPSP slope (% baseline)
Time (minutes)
Renormalized to potentiated baseline
fEPSP slope (% renorm baseline)
Time (minutes)
D
HSP
LTP + HSP
fEPSP slope (% baseline)
Time (minutes)
Renormalized to potentiated baseline
fEPSP slope (% renorm baseline)
Time (minutes)
E
Control path
LTP path
*
fEPSP slope (% renorm baseline)
ISI (seconds)

Figure 3.3- Hebbian plasticity exerts a pathway-specific constraint on subsequent homeostatic compensation. (A) In 2-pathway experiments, strong LTP (arrows indicate 4 trains of HFS) was induced in the test pathway followed by a frequency shift from 1 pulse/40sec to 1 pulse/15min in both pathways to induce homeostatic strengthening. Renormalizing the fEPSP responses to the 15 minute period prior to the frequency shift reveals homeostatic compensation is weaker in the pathway which expressed LTP than the naïve pathway (*$p<0.05$, $n=5$). (B) Similar to LTP, prior induction of strong LTD (4 trains LFS) significantly reduces the relative magnitude of homeostatic compensation in the opposing direction (*$p<0.05$, $n=4$). (C) Induction of weak LTD (1 train LFS) reduces the relative magnitude of subsequent homeostatic strengthening (*$p<0.05$, $n=14$).

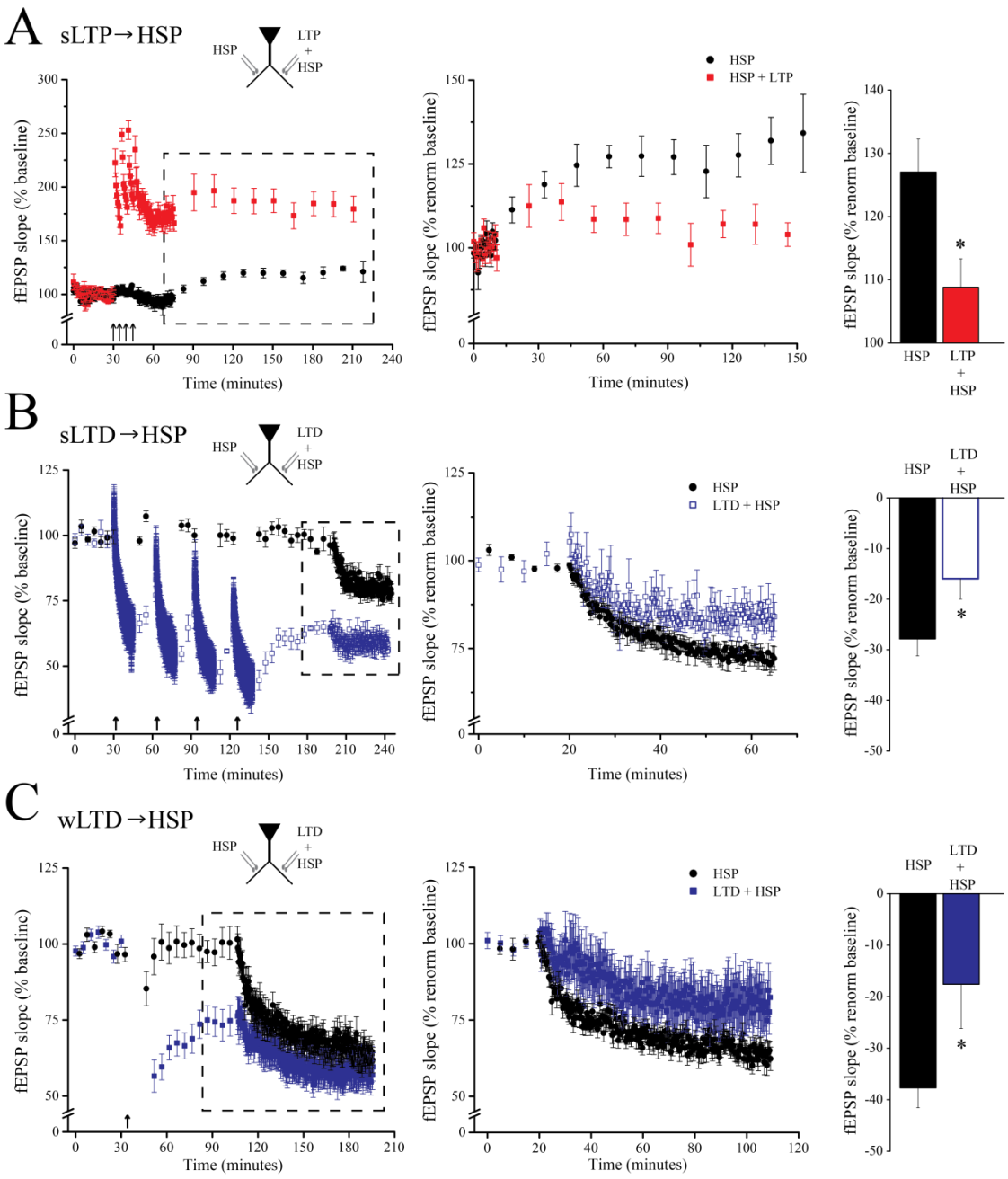

A
sLTP→HSP
HSP
LTP + HSP
fEPSP slope (% baseline)
Time (minutes)
HSP
HSP + LTP
fEPSP slope (% renorm baseline)
Time (minutes)
fEPSP slope (% renorm baseline)
HSP
LTP + HSP
*
B
sLTD→HSP
HSP
LTD + HSP
fEPSP slope (% baseline)
Time (minutes)
HSP
LTD + HSP
fEPSP slope (% renorm baseline)
Time (minutes)
fEPSP slope (% renorm baseline)
HSP
LTD + HSP
*
C
wLTD→HSP
HSP
LTD + HSP
fEPSP slope (% baseline)
Time (minutes)
HSP
LTD + HSP
fEPSP slope (% renorm baseline)
Time (minutes)
fEPSP slope (% renorm baseline)
HSP
LTD + HSP
*

Figure 3.4- Homeostatic plasticity preserves the capacity for Hebbian plasticity. (A) Prior induction of homeostatic synaptic weakening offsets the overall magnitude of weak LTD subsequently induced, but does not alter the relative change in synaptic strength from the scaled baseline (n=11). (B) Similarly, prior induction of homeostatic synaptic weakening offsets the overall magnitude of strong LTP subsequently induced, but does not alter the relative change in synaptic strength from the scaled baseline.

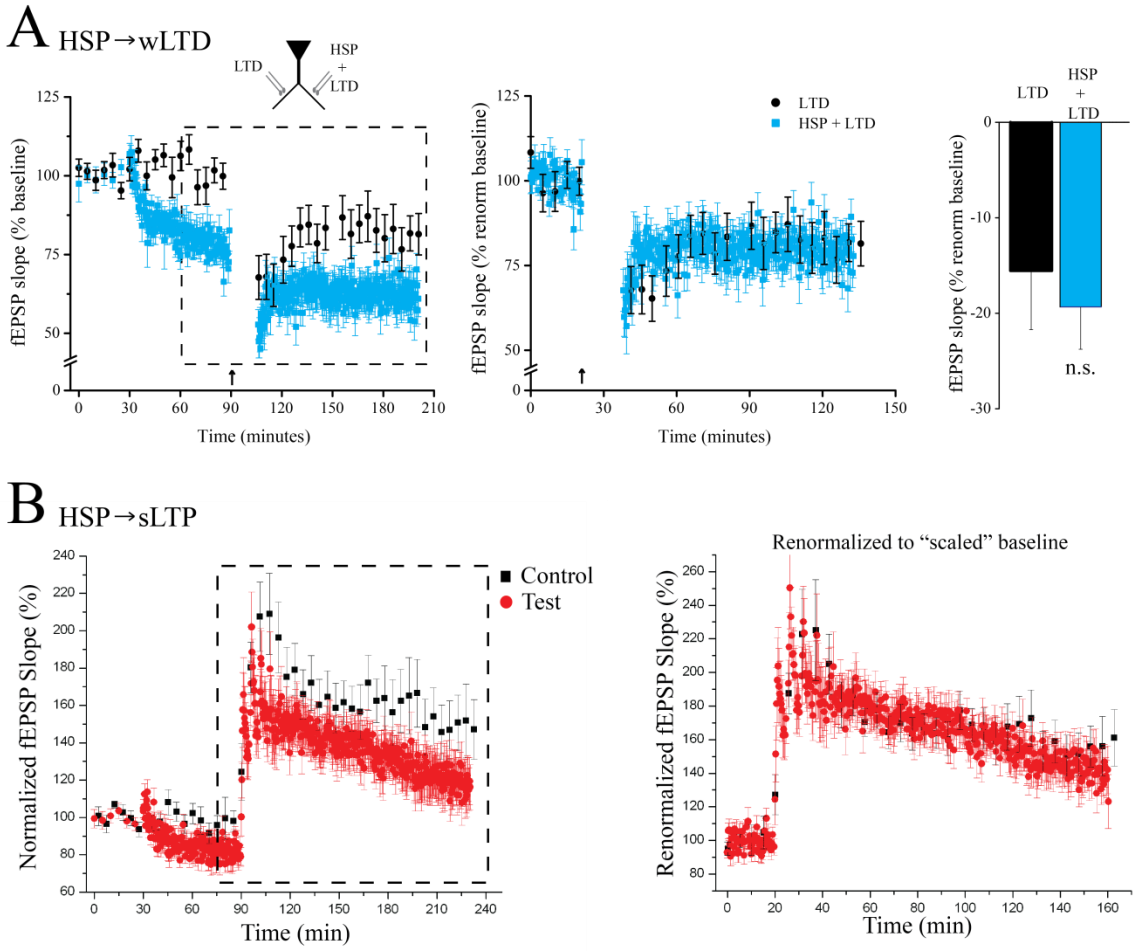

A HSP→wLTD
LTD
HSP + LTD
fEPSP slope (% baseline)
Time (minutes)
fEPSP slope (% renorm baseline)
LTD
HSP + LTD
Time (minutes)
LTD
HSP + LTD
fEPSP slope (% renorm baseline)
n.s.
B HSP→sLTP
Normalized fEPSP Slope (%)
Control
Test
Time (min)
Renormalized to "scaled" baseline
Renormalized fEPSP Slope (%)
Time (min)

Figure 3.5-HSP is not blocked by protein synthesis inhibitors. Unlike other forms of homeostatic plasticity, frequency shift-dependent compensation does not depend on new protein synthesis for its expression in either direction. Bath application of emetine 30 minutes prior to frequency shifts did not alter the subsequent expression of either homeostatic strengthening (left) or homeostatic weakening (right). Histograms represent magnitude of homeostatic strengthening or weakening 90 minutes following frequency shift (n=4 control, 5 emetine for strengthening; n=13 control, 7 emetine for weakening).

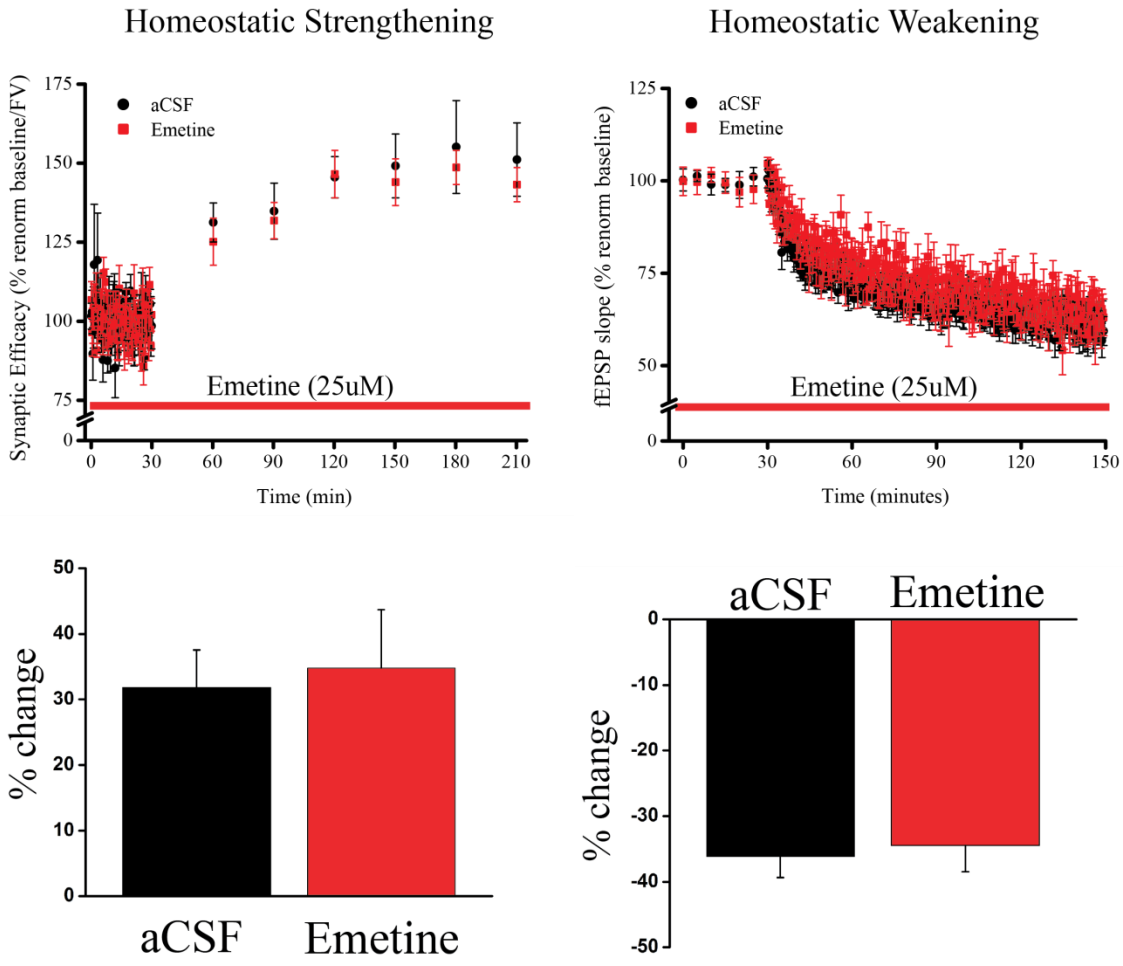

Homeostatic Strengthening
Homeostatic Weakening
Synaptic Efficacy (% renorm baseline/FV)
aCSF
Emetine
Emetine (25uM)
Time (min)
fEPSP slope (% renorm baseline)
Time (minutes)
% change
aCSF
Emetine

Figure 3.6- Interaction between LTD and homeostatic weakening requires translation. Bath application of emetine or cyclohexamide rescues full expression of homeostatic weakening following LTD in the test pathway. Renormalizing the fEPSP slope to the post-LTD baseline reveals that HSP is significantly reduced in comparison to the pathway that is naive to LTD, but protein synthesis inhibitors abolish the difference between pathways by enhancing the strength of HSP in the pathway with a history of LTD. Histograms depict magnitude of HSP 90 minutes following induction of homeostatic weakening. (*$p<0.05$, n=14 aCSF group, 7 emetine, 10 cyclohexamide group).

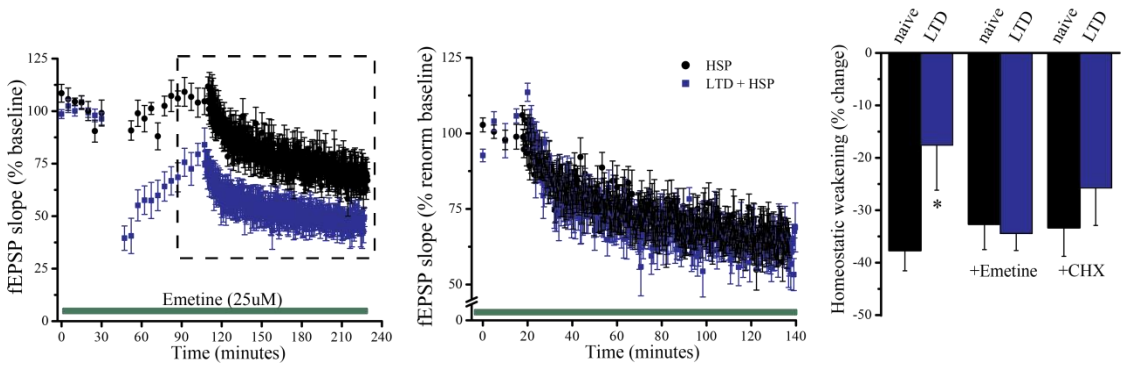

fEPSP slope (% baseline)
Emetine (25uM)
Time (minutes)
fEPSP slope (% renorm baseline)
HSP
LTD + HSP
Time (minutes)
Homeostatic weakening (% change)
naive
LTD
naive
LTD
naive
LTD
*
+Emetine
+CHX

Figure 3.7-Positioning of electrodes for focal diffusion experiments. In order to inhibit protein synthesis locally, emetine is applied via one recording electrode to a set of distal synapses while vehicle (aCSF) is applied via a second recording electrode to synapses more proximal to the cell body layer. A captured image of the recording setup illustrates that the synapses being recorded from originate from the same neurons. GR=Granular layer of DG, MOL=molecular layer of DG, SLM=stratum lacunosum-moleculare, RAD=stratum radiatum, PY=stratum pyramidale, OR=stratum oriens.

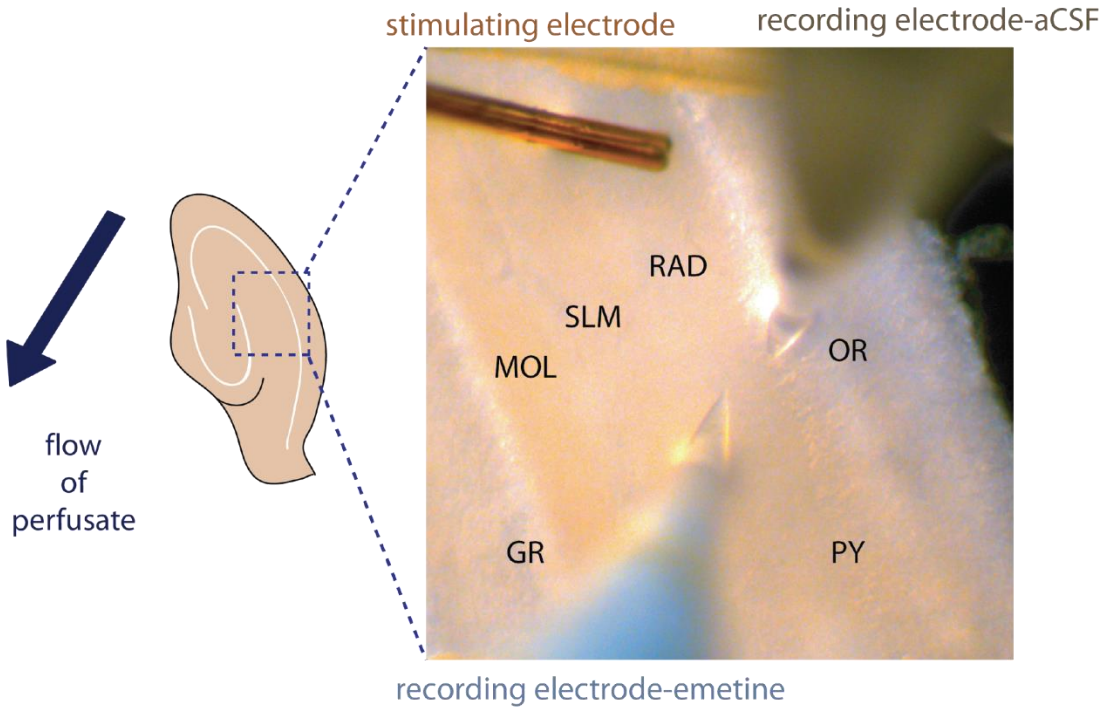

stimulating electrode
recording electrode-aCSF
RAD
SLM
MOL
OR
GR
PY
flow
of
perfusate
recording electrode-emetine

Figure 3.8-Local protein synthesis mediates the interaction between Hebbian and homeostatic synaptic plasticities. Focal application of emetine to dendrites in stratum radiatum via a recording electrode did not alter the magnitude of LTD, but did enhance the magnitude of homeostatic weakening following LTD expression compared to a second recording electrode diffusing aCSF alone (*$p<0.05$, $n=10$).

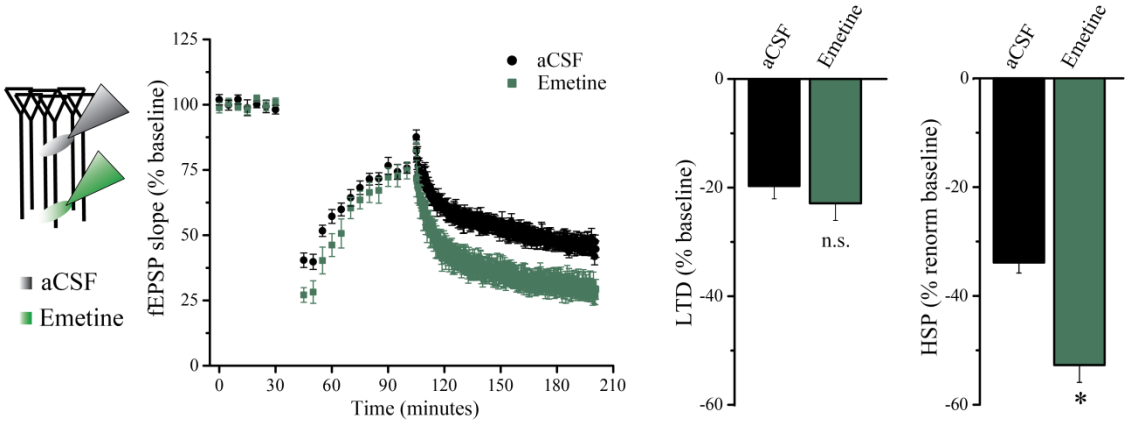

aCSF
Emetine
fEPSP slope (% baseline)
125
100
75
50
25
0
0
30
60
90
120
150
180
210
Time (minutes)
aCSF
Emetine
LTD (% baseline)
0
-20
-40
-60
n.s.
HSP (% renorm baseline)
*

Figure 3.9-Selective blockade of protein synthesis at either proximal or distal apical dendrites reveals that metaplastic interactions are not isolated to one dendritic compartment. (A) Focal application of emetine to dendrites in stratum radiatum to a region distal to stratum pyramidale via a second recording electrode did not alter the magnitude of LTD, but did enhance the magnitude of homeostatic weakening following LTD expression (*$p<0.05$, n=5). (B) In separate experiments, emetine applied to proximal dendrites revealed the same rescue of homeostatic weakening seen with distal application (* $p< 0.05$, n=5).

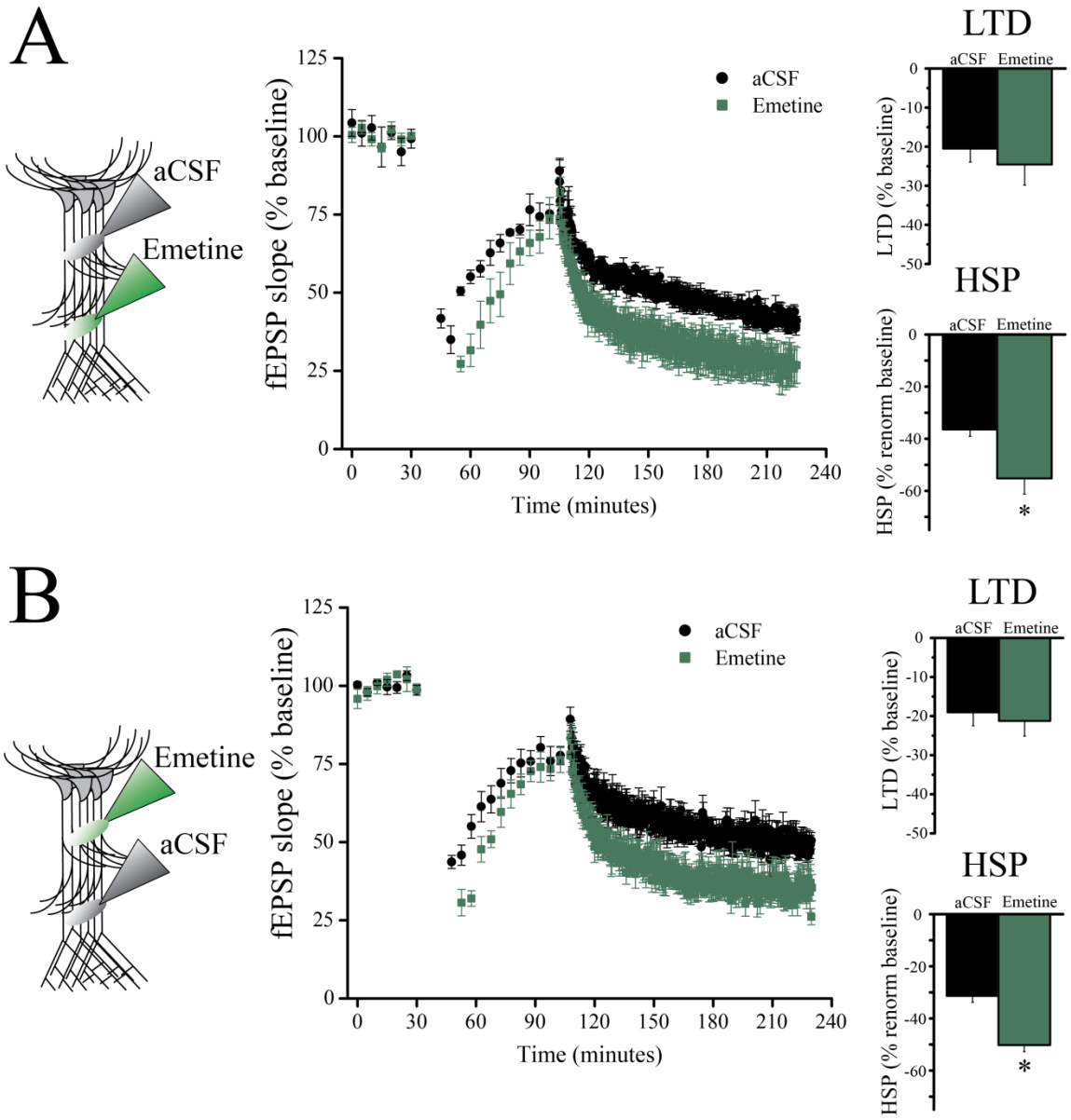

A
aCSF
Emetine
fEPSP slope (% baseline)
Time (minutes)
aCSF
Emetine
LTD
LTD (% baseline)
HSP
HSP (% renorm baseline)
*
B
Emetine
aCSF
fEPSP slope (% baseline)
Time (minutes)
aCSF
Emetine
LTD
LTD (% baseline)
HSP
HSP (% renorm baseline)
*

Figure 3.10-Weak LTP preserves the capacity for homeostatic strengthening. Prior induction of either 1x HFS LTP (A), 2x HFS LTP (B) or TBS LTP (C) offsets the magnitude of homeostatic plasticity subsequently induced, but does not alter the relative change in synaptic strength. In each experiment, homeostatic strengthening was induced using a frequency shift from 1 pulse/20 seconds to 1 pulse/5 minutes.

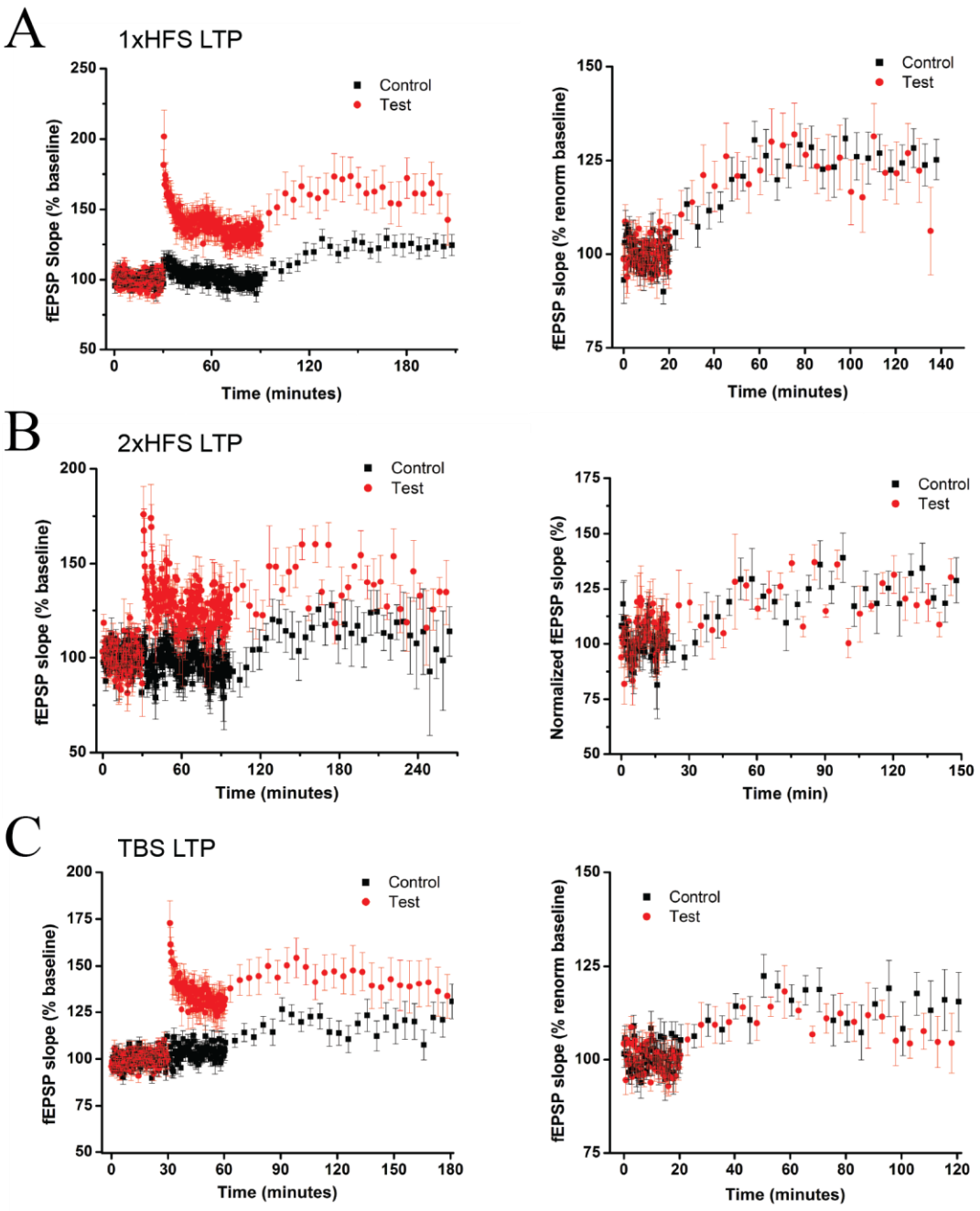
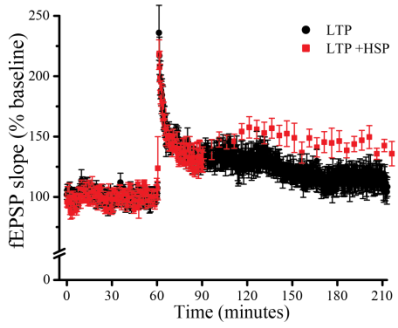
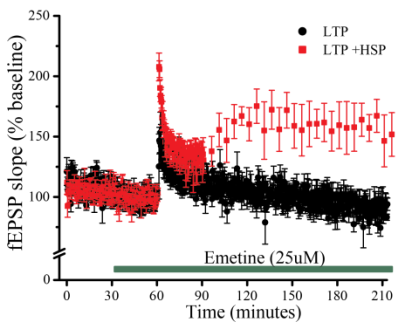
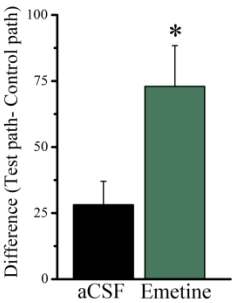

A
1xHFS LTP
Control
Test
fEPSP Slope (% baseline)
Time (minutes)
fEPSP slope (% renorm baseline)
B
2xHFS LTP
fEPSP slope (% baseline)
Normalized fEPSP slope (%)
Time (min)
C
TBS LTP

Figure 3.11-Protein synthesis dependent interaction concurrent with cooperation between Hebbian and homeostatic synaptic plasticity. (A) In 2-pathway experiments, homeostatic synaptic strengthening increases the magnitude of synaptic strength at synapses with previously established 1x HFS LTP. Both pathways initially undergo induction of weak LTP (1 train HFS), followed by induction of homeostatic strengthening in only one pathway. This long-lasting enhancement was not abolished by application of protein synthesis inhibitors, but was in fact significantly increased (*$p<0.05$, n=6 control group, 4 emetine group).


LTP
LTP +HSP
fEPSP slope (% baseline)
Time (minutes)


LTP
LTP +HSP
Emetine (25uM)
fEPSP slope (% baseline)
Time (minutes)


*
Difference (Test path- Control path)
aCSF
Emetine

Chapter IV

Impaired activity-dependent FMRP translation and enhanced mGluR-dependent LTD in Fragile X premutation mice

This chapter has been published:

Adam J. Iliff, Abigail J. Renoux*, Amy Krans, Karen Usdin, Michael A. Sutton, and Peter K. Todd. Hum. Mol. Genet. (2013) 22 (6): 1180-1192.*
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4.1 Introduction

Fragile X Syndrome (FXS) is the most common known monogenic cause of autism and intellectual disability, affecting upwards of 1 in 4000 boys and 1 in 8000 girls (Hernandez et al., 2009; Rogers et al., 2001). FXS results from the expansion of a CGG microsatellite repeat in the 5′ untranslated region (UTR) of the FMR1 gene on the X chromosome. In humans, this sequence is normally <45 CGG repeats. Expansions to >200 repeats trigger hypermethylation of the repeat and FMR1 promoter, resulting in transcriptional silencing of the FMR1 gene and the absence of the Fragile X mental retardation protein (FMRP), (Bell et al., 1991; Kremer et al., 1991; Oberle et al., 1991; Pieretti et al., 1991; Verkerk et al., 1991).

FMRP is an RNA-binding protein that regulates activity-dependent translation of associated transcripts at the synapse (Bassell & Warren, 2008). Mice lacking FMRP (Fmr1 KO mice) exhibit specific defects in synaptic signaling mediated through group I

metabotropic glutamate receptors (mGluRs; Huber et al., 2002). At CA3–CA1 synapses in the hippocampus, mGluR activation normally leads to a long-term depression (LTD) of synaptic efficacy that requires new dendritic protein synthesis (Huber et al., 2000; Nosyreva & Huber, 2006; Park et al., 2008; Shepherd et al., 2006). mGluR agonists trigger rapid FMRP dephosphorylation and degradation, which allows synaptic translation of FMRP-associated transcripts (Hou et al., 2006; Nalavadi et al., 2012; Niere et al., 2012). In mice lacking FMRP, mGluR-LTD is enhanced and no longer requires new protein synthesis, and mGluR agonists fail to trigger the translation of FMRP target mRNAs (Huber, et al., 2002; Muddashetty et al., 2007; Niere, et al., 2012; Nosyreva & Huber, 2006; Todd et al., 2003). The absence of FMRP is thought to decouple mGluR 1/5 activity from protein translation, such that basal dendritic translation of these target mRNAs is increased, but mGluR-coupled dendritic translation is lost (Bear et al., 2004).

One of the dendritically localized transcripts whose translation is regulated by mGluR signaling is FMRP itself (Antar et al., 2004; Hou, et al., 2006; Todd et al., 2003; Weiler et al., 1997). Although the function of this newly synthesized FMRP is unknown, it has been proposed to act as a brake on local protein production, hence constraining LTD by limiting the new translation of LTD effector proteins (Bear, et al., 2004; Todd & Malter, 2002). A critical prediction of this model is that the magnitude of LTD should be enhanced by diminished mGluR-dependent translation of FMRP. Despite its appeal, and its consistency with studies using the Fmr1 knockout mouse as an experimental model, this idea has never been directly tested.

‘Premutation’ expansions at the FMR1 locus to between 55 and 200 CGG repeats are associated with the age-related neurodegenerative condition Fragile X-associated

Tremor Ataxia Syndrome (FXTAS) (Berry-Kravis et al., 2007; Bourgeois et al., 2009; Greco et al., 2006; Sullivan et al., 2005). This disorder, characterized clinically by gait ataxia, action tremor, dementia and neuropsychiatric symptoms, occurs in ~40% of male premutation carriers over the age of 50 (Jacquemont et al., 2004). However, premutation range repeats are relatively common in the population [estimates upwards of 1:813 males and 1:259 females (Jacquemont et al., 2007; Seltzer et al., 2012)], and have the potential to significantly influence the risk of other human diseases. Recent studies in young premutation carriers demonstrate higher rates of autism and attention deficit hyperactivity disorder (ADHD)-like symptoms in the absence of FXTAS symptoms (Clifford et al., 2007; Farzin et al., 2006; Grigsby et al., 2006; Loesch et al., 2002; Loesch et al., 2003; Loesch et al., 2004; Loesch et al., 2007) and FXS phenotypes have been reported in larger premutation and unmethylated full mutation carriers who produce FMR1 mRNA but inefficiently translate FMRP (Allen et al., 2005; Chonchaiya et al., 2009; Feng et al., 1995; Hagerman et al., 1996; Jacquemont et al., 2011; Tassone et al., 2000; Tassone et al., 2000).

Unlike full mutation expansions, premutation-sized repeats are unmethylated and over-transcribed, leading to a 2–8-fold elevation in the production of FMR1 mRNA (Tassone et al., 2007; Tassone et al., 2000; Todd et al., 2010). However, the CGG repeat expansion forms a hairpin loop in the 5′ UTR of the RNA transcript that impairs ribosomal scanning and induces significant translational inefficiency (Kaufmann et al., 1999; Ludwig et al., 2011; Primerano et al., 2002; Zumwalt et al., 2007). This leads to low-normal or decreased basal FMRP expression in Fragile X-premutation carriers, depending on the repeat size (Kaufmann, et al., 1999; Tassone et al., 2004). The

neurodegeneration seen in FXTAS and other age-related premutation phenotypes are thought to result primarily from an RNA gain-of-function mechanism (Hagerman, 2012; Li & Jin, 2012; Renoux & Todd, 2012). In contrast, work in two independently generated FMR1 premutation mouse models suggests an additional role for FMRP insufficiency in aspects of the premutation phenotype, especially in younger animals that do not yet demonstrate neurodegenerative sequelae (Berman & Willemsen, 2009; Chen et al., 2010; Cunningham et al., 2011; Qin et al., 2011). Defects in these mice include alterations in neuronal migration, dendritic branching, synaptic activity in cultured neurons, and behavioral defects including altered performance on measures of anxiety and social interaction (Cao et al., 2012; Entezam et al., 2007; Qin, et al., 2011).

Given the known critical roles for FMRP in synaptic function and the translational inefficiency induced by CGG repeat expansions, we hypothesized that mice with large unmethylated CGG repeat expansions would exhibit a specific defect in their ability to rapidly translate FMRP at synapses. A defect in activity-dependent synthesis of FMRP would allow for the analysis of the function of newly produced synaptic FMRP, including its role in long-lasting forms of synaptic plasticity. We therefore evaluated dendritic FMRP synthesis and synaptic function in a premutation mouse model where a CGG repeat expansion has been knocked into the mouse Fmr1 locus (Entezam, et al., 2007; Qin, et al., 2011).

Here, we show that mice with 120–150 CGG repeats in the mouse Fmr1 5′ UTR have modestly reduced basal FMRP expression despite elevated Fmr1 mRNA levels, consistent with a robust impairment in translational efficiency. Strikingly, these animals exhibit impaired mGluR-dependent translation of dendritic FMRP. Young CGG KI mice

exhibit normal basal synaptic properties, but enhanced mGluR-LTD, as in Fmr1 KO mice (Huber, et al., 2002). However, the mechanism underlying this functional alteration is distinct from that in Fmr1 KO animals, as mGluR-LTD in CGG KI mice remains dependent on new protein synthesis. Our results provide a link between local FMRP synthesis and mGluR-dependent synaptic plasticity, and raise the possibility that some aspects of the cognitive defects observed in premutation carriers and unmethylated FXS patients may result from altered activity-dependent translation of FMRP.

4.2 Materials and Methods

4.21 Mice and cell culture

Animal use followed NIH guidelines and was in compliance with the University of Michigan Committee on Use and Care of Animals. DNA was extracted from tail biopsies and isolated with DirectPCR lysis reagent (Viagen) and proteinase K (0.2 µg/µl, Roche), incubated overnight at 55°C. Proteinase K was heat inactivated and DNA samples were genotyped first with primers against the Y chromosome (5′GTGAGAGGCACAAGTTGGC, 5′GTCTTGCCTGTATGTGATGG) to determine the sex of each animal using Platinum® PCR Supermix (Invitrogen). To amplify the knocked-in CGG repeat expansion, we targeted mouse specific Fmr1 allele (5′AGCCCCGCACTTCCACCACCAGCTCCTCCA, 5′GCTCAGCTCCGTTTCGGTTTCACTTCCGGT) in male hemizygous animals using the Expand High Fidelity PCR System (Roche) supplemented with 2 m Betaine (Sigma) and 5% dimethyl sulfoxide (DMSO; Fisher Scientific) as described previously (Tassone et al., 2008). As genotyping was performed on tail samples early in life, small expansions

in repeat length may have occurred due to somatic instability in older animals (Lokanga et al., 2012). Dissociated hippocampal neuron cultures were prepared from postnatal (P1–3) mice as previously described (Jakawich et al., 2010). Experiments were performed at 14–17 days in vitro (DIV).

4.22 Drugs

(RS)-3,5-DHPG (Tocris) was prepared fresh each day in sterile water, or artificial cerebrospinal fluid (aCSF, in mm: 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$ and 10 dextrose). Anisomycin (Sigma) was prepared as a 1000× stock in DMSO, stored at −20°C, and diluted to final concentration in aCSF or conditioned media.

4.23 Western blotting

Brain lysate samples were homogenized in RIPA buffer (50 mm Tris–HCl, 150 mm NaCl, 0.1% SDS, 1% NP−40, 0.5% deoxycholic acid-sodium salt, pH 7.4) containing Complete Mini protease inhibitor cocktail (Roche). Samples were sonicated and centrifuged, and total protein content of the supernatant measured using a DC Protein assay (Bio-Rad). Equal amounts of protein were mixed with 4× Laemmli buffer and boiled for 5 min before separation on 10 or 12% polyacrylamide gels. Gels were transferred to PVDF membranes and blocked with Tris-buffered saline containing 0.1% Triton-X (TBST) and 5% non-fat milk for 60 min at RT, and incubated with an antibody against FMRP (Millipore mouse monoclonal 1C3 1:1000 or Abcam rabbit polyclonal 17 722, 1:1000) or PSD-95 (Abcam, 6G6–1C9, 1:2000) overnight at 4°C. After washing with TBST, blots were incubated with a corresponding HRP-conjugated secondary antibody (anti-rabbit or anti-mouse 1:5000; Jackson Immunoresearch); this was followed

by chemiluminescent detection (Western Lightning Plus-ECL, PerkinElmer). The same blots were reprobed with a mouse monoclonal antibody against β-tubulin (University of Iowa's Developmental Studies Hybridoma Bank E7, 1:5000) or β-actin (Sigma 1:5000) to confirm equal loading. Band intensity was quantified in the linear range with densitometry using NIH ImageJ.

4.24 Quantitative polymerase chain reaction

Dissected cortex or hippocampi from P28–60 male mice were flash-frozen and stored at −80°C. RNA was extracted using TRIzol Reagent (Invitrogen), following the manufacturer's guidelines. Equal amounts of extracted RNA (1 μg) were used to generate cDNA (iScript™ cDNA synthesis kit, Bio-Rad). Quantitative polymerase chain reaction (QPCR) was performed using iQ™ SYBR© Green Supermix (Bio-Rad) and primers against the 2/4 (5′CATGAAGATTCAATAACAGTTGC, 5′CACTTTAGCTAACCACCAACAG) or 16/17 (5′CCGAACAGATAATCGTCCACG, 5′ACGCTGTCTGGCTTTTCCTTC) exons of mouse Fmr1, and actin (5′GGCATCCTCACCCTGAAGTA, 5′AGAGGCGTACAGGGATAGCA). Samples were run in triplicate, and Fmr1 expression data normalized to actin expression for each sample.

4.25 Translational efficiency calculation

The translational efficiency ratio was calculated by deriving FMR1 mRNA expression levels determined by qRT–PCR from one cortex while total protein lysates were prepared from the contralateral cortex from the same animal. For each animal, cortical FMR1 mRNA expression (relative to actin) was normalized to the mean FMR1

mRNA expression in control cortices. Similarly, cortical FMRP levels were expressed as a ratio to actin expression and then normalized to the mean FMRP expression in control cortices. These numbers were then expressed as a ratio of normalized FMRP expression/normalized FMR1 mRNA expression. Finally, the mean value of this ratio in WT animals was set at 100 and all individual animal values were expressed as a percentage of this number.

4.26 Synaptoneurosomes

SNs were prepared from male P14–21 WT and CGG KI mice as described previously (Hollingsworth et al., 1985; Muddashetty, et al., 2007). Briefly, cortices were homogenized in 3 ml of homogenization buffer [containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.53 KH_2PO_4, 212.7 glucose and 1 DTT, pH 7.4], supplemented with Complete Mini protease inhibitor cocktail (Roche) on ice. Samples were passed through a 100 μm nylon mesh filter, followed by two 10 μm nylon mesh filters (Millipore), followed by centrifugation at 1000 g for 15 min at 4°C. The pellets were suspended in 1.1 ml homogenization buffer per cortex. SN preparations were divided into 10 × 100 μl aliquots for technical duplicates, and pre-warmed for 10 min at 37°C before stimulation with (RS)-3,5-DHPG (Tocris, 100 μM). After incubation with DHPG at 37°C, samples were passed through a 28 gauge needle, and processed for western blotting as above. Expression of all samples was normalized to unstimulated samples maintained at 37°C for 60 min and statistical significance was determined using a Kruskal–Wallis one-way analysis of variance. Similar results were observed when comparisons were done with pre-stimulated samples (i.e. samples from the same SN prep that were never warmed to 37°C, data not shown).

4.27 Immunohistochemistry

Animals were anesthetized with 0.2 mg Ketamine/20 µg Xylazine per kilogram prior to transcardial perfusion (2 ml per min) with 5–10 ml of ice-cold sterile phosphate-buffered saline (PBS) and 5–10 ml of 4% paraformaldehyde (PFA) followed by brain dissection. Brains were sunk in 30% sucrose in PBS at 4°C prior to sectioning with a vibratome at 30 µm. Free-floating sections were stored in cryostorage (30% sucrose, 33.33% ethylene glycol, 0.05 m PB pH 7.4) at −20°C. Sections were removed from cryostorage by rotating in PBS at 4°C overnight. Sections were permeabilized in 0.1% Triton X in PBS for 5 min, followed by staining with DAPI (1:10 000) for 15 min at room temperature. Sections were washed 2× with PBS, and mounted on slides in ProLong® Gold Antifade Reagent with DAPI (Invitrogen).

4.28 Immunocytochemistry and microscopy

All experiments were conducted at 37°C. Neurons were treated with anisomycin (40 µM) or vehicle (DMSO 1:1000) for 30 min in conditioned media. Cultures were then stimulated with DHPG (100 µM) for 20 min in the presence of anisomycin, or left as controls with vehicle, or with anisomycin alone. After treatment, neurons were fixed with warmed 4% PFA/4% sucrose in PBS with 1 mm $MgCl_2$ and 0.1 mm $CaCl_2$ (PBS-MC), permeabilized (0.1% Triton X in PBS-MC, 5 min), blocked with 5% normal goat serum in PBS-MC for 1 h and labeled with an antibody against FMRP (Millipore 1C3 1:200 or Abcam 17722 1:500). For co-labeling of dendrites, we used antibodies against Map2 (Sigma M4403 1:1000, Millipore AB5622 1:1000) for 60 min at RT, or overnight at 4°C.

Secondary detection was achieved with Alexa 488-, 555- or 635-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:500 or 1:1000) for 60 min at RT.

All imaging was performed on an inverted Olympus FV1000 laser scanning confocal microscope with identical acquisition parameters for each treatment condition. Image analysis was performed on maximal intensity z-projected images using custom-written analysis routines for ImageJ. Statistical analysis utilized a one-way analysis of variance (ANOVA) to detect differences across conditions within genotype. N≈ 20–40/condition across multiple individual experiments for each genotype.

4.29 Electrophysiology

Hippocampal slices were prepared from P35–42 male CGG KI mice and their male WT littermates. Mice were lightly anesthetized with isoflurane before decapitation. Then, the brain and hippocampal lobules were rapidly removed and placed in ice cold artificial cerebrospinal fluid [aCSF, containing in mm: 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 10 dextrose (pH 7.4) saturated with 95% O2, 5% CO2]. Transverse slices (400 μm) of the hippocampus were cut using a tissue chopper (Stoelting, Wood Dale, IL, USA) and CA3 was surgically isolated from CA1 with a scalpel. Slices recovered for 2–5 h at room temperature in a submersion chamber containing aCSF prior to recording. For recording, hippocampal slices were transferred to a recording chamber and continuously perfused at 32°C with aCSF at a rate of 1–2 ml/min.

Recording electrodes were pulled from borosilicate capillary glass (G150-4, Warner) and filled with aCSF. The recording pipette was placed in the middle of stratum

radiatum of CA1. Synaptic responses were elicited using cluster stimulation electrodes (FHC, Bowdoin, ME, USA) placed in CA1stratum radiatum, lateral to the recording electrode. Current was delivered for 100 μs with an ISO-flex stimulator (AMPI, Jerusalem, Israel). Stable baseline responses were collected every 30 s (0.033 Hz) by using a stimulation intensity (20–140 μA) yielding ~50% of the maximal synaptic response. The fEPSP signal was amplified 1000 times with a DAM-50 DC differential amplifier (WPI) and filtered at 3 kHz. Recordings were collected at 10 kHz using Clampex 10.2 and analyzed using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA, USA). For all experiments, the initial slope of each fEPSP was expressed as the percentage of the baseline average. Pooled data represent the mean fEPSP slope (±SEM). Statistical significance was determined using an independent t-test, $P < 0.05$.

4.3 Results

4.31 Reduced FMRP translational efficiency in premutation model mice

To evaluate the neurobiological effects of 'premutation' range CGG repeats in the Fmr1 gene, we utilized a mouse model of the Fragile X premutation which contains ~120–150 CGG repeats knocked-in to the endogenous mouse Fmr1 5′ UTR [CGG KI, Entezam, et al., 2007; Fig. 4.1A]. Similar to human premutation patients, the expression of Fmr1 mRNA is significantly increased in cortical tissue [Fmr1 2/4 exon junction wild-type (WT) 1 ± 0.27, KI 5.24 ± 0.98 $P < 0.05$; Fmr1 16/17 exon junction WT 1 ± 0.24, KI 4.46 ± 0.74; $P < 0.05$, $n = 5$; Fig. 4.1B], as well as hippocampus (Fmr1 2/4 exon junction WT 1 ± 0.07, KI 4.04 ± 1.11 $P < 0.05$; Fmr1 16/17 exon junction WT 1 ± 0.07, KI 4.40 ± 1.99, data not shown) in CGG KI mice at 1 month of age (P28–37) compared with

littermate controls (Fig. 4.1B and data not shown). Despite this increase in mRNA, FMRP expression is significantly reduced in both CGG KI cortex (P28–37, WT 100 ± 10.09%, KI 37.50 ± 4.37%, $P < 0.05$, n = 5; Fig. 4.1C) and hippocampus (P35–60, WT 100 ± 17.00%, KI 44.93 ± 14.71%, $P < 0.05$, n = 5; Fig. 4.1D) from young animals compared with littermate controls. To determine the relative translational efficiency of Fmr1 mRNA in cortical tissues, we created a ratio of total FMRP/relative Fmr1 mRNA from the same animals. Using this analysis, we find that the efficiency of Fmr1 mRNA translation is dramatically reduced in young CGG KI mice compared with littermate controls (FMRP CTX/Fmr1 mRNA; WT 100 ± 21.26%, KI 7.60 ± 0.99%, $P < 0.05$, n = 5; Fig. 4.1E).

Consistent with previous reports (Entezam, et al., 2007; Qin, et al., 2011), FMRP is also reduced in the cortex of older (6-month-old) CGG KI mice (WT 100 ± 17.57%, KI 18.57 ± 2.68%, $P < 0.05$, n = 3; Fig. 4.1C) and, interestingly, when compared with WT littermates, the reduction in FMRP expression is greater in older CGG KI animals than in younger animals (1 month: KI 37.50 ± 4.37%, n = 5; 6 month: KI 18.57 ± 2.68%, n = 3; $P < 0.05$). This may reflect either a relatively greater decrease in FMR1 transcription in CGG KI versus WT mice with age or could result from somatic instability that is known to occur in these mice (Lokanga, et al., 2012; Singh et al., 2007).

4.32 Activity-dependent synaptic translation of FMRP is impaired in CGG KI mice

To examine the sub-cellular distribution of FMRP in CGG KI neurons, we generated dissociated hippocampal neurons from CGG KI and WT littermate controls (P1–3). Neurons were probed with antibodies to FMRP on day in vitro (DIV) 14–17 (Fig.

4.2A), and FMRP expression in somatic and dendritic regions was assessed. FMRP expression was reduced in both the cell soma and proximal dendrite by similar amounts (soma: WT 100 ± 5.74%, KI 49.82 ± 2.69%, $P < 0.05$; dendrite: WT 100 ± 4.63%, KI 66.87 ± 2.94%, $P < 0.05$, n = 23–24 neurons from 2 animals; Fig. 4.2B–D), suggesting that, while FMRP expression is lower, what FMRP is expressed in CGG KI neurons is appropriately distributed.

The reduced efficiency of Fmr1 mRNA translation in CGG KI mice suggests that rapid, mGluR-dependent synthesis of FMRP might also be disrupted in the CGG KI mice. To address this question, we first examined changes in FMRP expression upon mGluR1/5 stimulation in synaptoneurosomes (SNs), a biochemical preparation enriched for synaptic components and often used as a means to examine protein synthesis at isolated synapses (Muddashetty, et al., 2007; Weiler, et al., 1997). SNs were prepared from the neocortex of P14–21 CGG KI mice and their WT littermates. In all experiments, we verified the appropriate enrichment of the synaptic scaffolding protein PSD-95 at different stages of SN preparation, and found that the enrichment of PSD-95 was similar between WT and CGG KI mice (Fig. 4.3A). PSD-95 expression in SNs were similar in WT and CGG KI mice (WT 100 ± 23.8%, CGG KI 99.2 ± 24.8%, n = 6). Consistent with our immunocytochemical results (Fig. 4.2), the expression of FMRP in unstimulated SNs was reduced in CGG KI, relative to WT mice (% WT, 42.92 ± 21.51%, $P < 0.05$, n = 5; Fig. 4.3B). We next examined changes in FMRP expression in response to mGluR stimulation: SNs were stimulated with the group 1 mGluR agonist, (RS)-3,5-dihydroxyphenylglycine (DHPG, 100 μM) for either 10 or 30 min at 37°C. Similar to effects seen previously in WT SNs (Weiler, et al., 1997), DHPG induced significant

increases in FMRP at both 10 and 30 min time points relative to controls (10 min: WT 199.77 ± 56.97%; 30 min: WT 202.13 ± 54.83%, $P < 0.05$, n = 15; Fig. 4.3C and D). This increase was dependent on new protein synthesis (% 30 min untreated samples: 30 min DHPG: 162.5 ± 32.6%; 30 min DHPG + Anisomycin: 124.5 DHPG + Anisomycin 15.1%, expressed as % untreated n = 6). In contrast, SNs prepared from CGG KI mice did not show changes in FMRP expression in response to DHPG stimulation, consistent with impaired mGluR-dependent translation (Control: KI 42.92 ± 21.51%; 10 min: KI 31.16 ± 9.35%; 30 min: KI 40.84 ± 20.12%; NS, n = 5; Fig. 4.3C and D).

To further assess mGluR-dependent FMRP translation in CGG KI neurons, we took advantage of mice expressing green fluorescent protein (GFP) on the X chromosome to generate hippocampal cultures where neurons harboring the premutation are intermingled with normal length CGG repeat WT neurons (Fig. 4.4A–C). This approach allows us to evaluate cell-autonomous roles of the premutation by comparing CGG KI neurons with neighboring WT neurons in the same culture, a strategy similar to that used previously for other X-linked mutations (Hadjantonakis et al., 1998; Hanson & Madison, 2007; Kalantry et al., 2009; Niere, et al., 2012). Mice expressing GFP on the X chromosome were crossed with CGG KI mice to generate heterozygous XGFP/CGG KI females (Fig. 4.4A). This cross generates females possessing one WT X chromosome with a normal copy of Fmr1 and GFP and one X chromosome with a premutation range CGG repeat knocked-in to the Fmr1 allele, but no GFP. Owing to X-inactivation, roughly half the neurons will inactivate the CGG KI X chromosome and express normal Fmr1 mRNA along with GFP. The remaining neurons will inactivate the GFP-expressing chromosome and instead express the CGG KI Fmr1 allele. Analysis of dissociated

neuronal cultures and histological staining of hippocampi shows roughly equal proportions of GFP+ and GFP− cells in both XGFP/WT and XGFP/KI female mice (Fig. 4.4B and data not shown).

We first confirmed the effects of CGG repeat expansions on basal FMRP expression in XGFP/CGG KI cultures. GFP(−)/CGG KI(+) neurons exhibit reduced FMRP immunoreactivity in mixed XGFP/CGG KI cultured networks at DIV 14–17 compared with neighboring GFP(+)/FMR1 WT neurons (Fig. 4.4C–E). Consistent with studies in non-mosaic neuronal cultures (Fig. 4.2), these effects were seen both in the soma (WT 100 ± 4.79%, KI 30.07 ± 1.70%, $P < 0.05$, n = 14–24 neurons; Fig. 4.4D and E) and in both proximal and distal dendritic segments of CGG KI GFP neurons (0–40 μm: WT 100 ± 8.65%, KI 46.46 ± 7.03%; 40–80 μm: WT 100 ± 17.22, KI 51.47 ± 5.92%; 80–120 μm: WT 100 ± 15.05%, KI 55.05 ± 5.36%, $P < 0.05$, n = 13–23 neurons; Fig. 4.4D). The total amount of FMRP detected decreases with distance from the cell soma in both control and CGG KI neurons. However, the relative difference in expression of basal FMRP between WT and CGG KI neurons is smaller in proximal and distal dendritic compartments than in the cell soma, suggesting that decreases in FMRP reflect a primary failure in translational efficiency rather than a breakdown in FMRP transport into dendrites. We next examined whether the premutation had a cell-autonomous effect on mGluR-initiated translation of new FMRP. XGFP/CGG KI cultures were stimulated with DHPG (100 μM, 20 min) prior to FMRP and Map2 immunostaining. After mGluR activation, WT neurons showed a significant increase in dendritic FMRP immunoreactivity (Control: WT 100 ± 6.85%; DHPG: WT 133.74 ± 11.46%, $P < 0.05$; Fig. 4.4F–H) and this effect was blocked by pretreatment with the

protein synthesis inhibitor anisomycin (40 μM, 30 min prior to and throughout DHPG application; Anisomycin + DHPG: WT 96.81 ± 7.75%; Anisomycin: WT 102.53 ± 7.50%; Fig. 4.4H). In contrast, DHPG did not alter FMRP expression in CGG KI neurons in the presence or absence of anisomycin (Control: KI 49.70 ± 4.33%; DHPG: KI 50.42 ± 6.20%; Anisomycin + DHPG: KI 38.73 ± 2.74%; Anisomycin: KI 42.04 ± 3.20%; NS; Fig. 4.4F–H). These data support the hypothesis that premutation range expanded CGG repeats impair mGluR-dependent synthesis of FMRP in a cell-autonomous fashion.

4.33 Enhanced mGluR-LTD in hippocampal slices prepared from CGG knock-in mice

Since mGluR-dependent translation is critical for certain forms of synaptic plasticity that are altered in FXS model mice, we next tested whether there was any overlap between the synaptic plasticity phenotypes in Fmr1 KO mice and CGG KI mice. We first examined basal synaptic properties at CA3–CA1 synapses in acute hippocampal slice preparations from young CGG KI mice with their WT littermates (P31–35). Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating Schaffer collaterals and recording in stratum radiatum of area CA1. In response to a series of stimulation pulses of increasing intensity, we found that the corresponding increase in fEPSP slope was nearly identical in WT and CGG KI mice (Fig. 4.5A). These largely overlapping input/output curves show that CGG KI mice do not exhibit alterations in basal synaptic efficacy relative to WT mice. In addition, we tested whether paired pulse facilitation, a measure of short-term synaptic plasticity and presynaptic function, was altered in CGG KI mice. In response to pairs of stimulation pulses with varying inter-pulse intervals, WT and CGG KI mice exhibited similar robust facilitation of the second synaptic response at all intervals (Fig. 4.5B), suggesting that the neurotransmitter release

probability is largely similar between the two genotypes. Hence, basal synaptic function is similar between CGG KI mice and their WT littermates.

Our results suggest that premutation range repeats impair FMRP translation even in young mice, raising the question of whether this loss of new FMRP synthesis might mimic aspects of the FXS phenotype. To test this idea, we next examined mGluR-dependent LTD at these CA3–CA1 synapses. After confirming that evoked fEPSPs were stable over time, LTD was induced by brief application of DHPG (100 μM, 10 min; Fig. 4.6). As previously described, DHPG treatment induced a sustained depression of fEPSPs in WT slices that persisted well beyond drug application (Fig. 4.6). Interestingly, we found that this mGluR-dependent LTD was significantly exaggerated in slices from CGG KI mice (Fig. 4.6A), a synaptic phenotype that is similar to Fmr1 KO mice (Huber, et al., 2002). These results demonstrate that, even during early life, the expanded premutation CGG repeat in the Fmr1 gene leads to altered hippocampal synaptic plasticity.

4.34 Enhancement of mGluR-LTD in premutation and FXS model mice are mechanistically distinct

Like young CGG KI mice, Fmr1 KO mice also exhibit enhanced mGluR-LTD (Huber, et al., 2002). Since this exaggerated mGluR-LTD in FXS model mice is thought to contribute to intellectual disability and/or autistic features in FXS, it was of interest to determine to what extent the exaggerated LTD in each case was due to similar or distinct mechanisms. To explore this issue, we examined whether protein synthesis inhibitors would impair the induction of mGluR-LTD in CGG KI and Fmr1 KO mice. In WT mice, mGluR-LTD requires rapid dendritic protein synthesis for its induction (Huber et al.,

2000), whereas mGluR-LTD in Fmr1 KO mice is completely resistant to protein synthesis inhibitors (Hou, et al., 2006; Nosyreva & Huber, 2006). Consistent with these findings, we found that the magnitude of mGluR-LTD in Fmr1 KO mice was not affected by blocking protein synthesis with anisomycin (Fig. 4.6B). In contrast, the enhanced mGluR-LTD seen in young CGG KI mice was significantly diminished with anisomycin (Fig. 4.6C), indicating that mGluR-LTD remains dependent on new protein synthesis in these mice, as in WT mice. Taken together, these results suggest that while young FXS and premutation model mice share the same exaggerated mGluR-LTD phenotype, the mechanism underlying this plasticity is distinct in the two mouse models.

4.4 Discussion

The roles of FMRP in both normal and aberrant control of synaptic function have received considerable attention in the past two decades. This effort has been greatly facilitated by work in the Fmr1 KO mouse, which recapitulates several important features of FXS, and has been instrumental in the rapid development of novel therapeutic approaches (Bhakar et al., 2012). In addition, significant advances have been made in our understanding of the molecular consequences of premutation CGG repeat expansions, which enhance FMR1 transcription but impair FMRP translation and elicit toxicity directly as RNA (Renoux & Todd, 2012). In contrast, considerably less is known about the impact of premutation range CGG repeat expansions on neuronal function. Premutation expansions do not typically lead to overt intellectual disability, but they are increasingly linked to a broad range of important clinical phenotypes in patients, including neuropsychiatric symptoms and autistic features earlier in life (Berry-Kravis, et al., 2007; Bourgeois, et al., 2009; Clifford, et al., 2007; Farzin, et al., 2006; Grigsby, et

al., 2006; Loesch, et al., 2004). These clinical features are recapitulated in Fragile X premutation model mice that exhibit altered social interactions and anxiety behaviors compared with littermate controls (Qin, et al., 2011). We therefore examined neuronal function in young Fragile X premutation model mice, with a specific focus on the impact of the CGG repeat on activity-dependent FMRP translation.

Our results demonstrate that premutation model mice exhibit a dramatic decrease in the translational efficiency of Fmr1 mRNA that impairs rapid, activity-dependent synthesis of FMRP in dendrites. This defect in local FMRP synthesis is associated with exaggerated mGluR-dependent LTD, a phenotype first reported in Fmr1 KO mice. This shared synaptic phenotype, however, is mechanistically distinct between Fmr1 KO and premutation model mice, as mGluR-dependent LTD in CGG KI mice remains dependent on new protein synthesis (Fig. 4.6B and C). Coupled with data demonstrating altered dendritic spine morphology and development in CGG KI mice (Chen, et al., 2010; Qin, et al., 2011), our results reveal a shared defect in synaptic plasticity in FXS and premutation model mice and suggest an important role for activity-dependent FMRP synthesis at synapses in regulating the magnitude of synaptic strength.

FMRP is an RNA-binding protein found associated with stalled ribosomes (Laggerbauer et al., 2001), where it acts primarily as a translational suppressor (Darnell et al., 2011; Laggerbauer, et al., 2001; Li et al., 2001). mGluR signaling induces dephosphorylation of FMRP, which then dissociates from polysome–transcript complexes and is rapidly degraded, leading to an activity-dependent burst of translation of FMRP target mRNAs (Fig. 4.7A) (Nalavadi, et al., 2012; Narayanan et al., 2007; Narayanan et al., 2008). Intriguingly, FMRP also binds and regulates the translation of its

own mRNA in vitro and FMRP is rapidly synthesized at synapses in response to mGluR activation in vivo (Hou, et al., 2006; Li, et al., 2001; Siomi et al., 1994; Todd, et al., 2003; Todd et al., 2003; Weiler, et al., 1997). The role of FMRP as a translation repressor, and the clear role of certain FMRP targets (e.g. activity-regulated cytoskeletal-associated protein; Arc) as mediators of mGluR-LTD (Park, et al., 2008; Waung et al., 2008), has bolstered the hypothesis that newly synthesized FMRP functions to provide negative feedback on further local translation, thus constraining the magnitude of LTD after mGluR activation (Fig. 4.7A) (Bassell & Warren, 2008; Bear, et al., 2004; Todd & Malter, 2002). This notion of newly synthesized FMRP as a 'brake' on local translation is consistent with observations that mGluR-LTD and other forms of mGluR-mediated plasticity require local protein synthesis in only a brief time window after induction (Huber, et al., 2000; Merlin et al., 1998). In the complete absence of FMRP, mGluR-LTD is enhanced but no longer requires new protein synthesis (Huber, et al., 2002; Nosyreva & Huber, 2006). This has been interpreted as resulting from an uncoupling of mGluR activation and synthesis of critical mGluR-LTD effector proteins (Fig. 4.7B) (Bassell & Warren, 2008; Bear, et al., 2004). Thus, whereas synaptic levels of Arc and other LTD mediator proteins are low basally in WT neurons and increase as a result of mGluR-dependent synthesis, Arc in FMR1 KO neurons is basally elevated, but is no longer synthesized in response to mGluR activation (Fig. 4.7B) (Niere, et al., 2012).

In CGG KI mice, our results demonstrate that mGluR-LTD is exaggerated as in Fmr1 KO mice, but that this enhanced mGluR-LTD remains dependent on new protein synthesis, as occurs typically in WT animals (Fig. 4.6C). We suggest that this protein synthesis-dependent enhancement of mGluR-LTD occurs because of a specific failure in

activity-dependent FMRP production (Fig. 4.7C). Although basal FMRP levels are lower in CGG KI mice, FMRP is maintained in both proximal and distal dendritic compartments at levels that are 40–60% of normal, which is above the threshold at which alterations in mGluR-triggered AMPA receptor (AMPAR) recycling occurs (Nakamoto et al., 2007). This suggests that basal synthesis of FMRP, although inefficient, is adequate to achieve the suppression of translation of LTD effector proteins in the absence of mGluR activity (Fig. 4.7C). However, with mGluR activation, the rapid synthesis of dendritic FMRP is significantly impaired by the CGG repeat expansion. This means that there is inadequate new FMRP produced to halt the ongoing translation of FMRP target mRNAs, leading to an overproduction of these LTD effector proteins. This overproduction of LTD effector proteins presumably drives the enhanced LTD phenotype, but unlike FMR1 KO cultures, production of these proteins remains coupled to mGluR activity, as the release of FMRP cargo transcripts is still required to initiate the LTD (Fig. 4.7C). Within this framework, we propose that new translation of FMRP at synapses is critical for constraining mGluR-LTD, likely through limiting the sustained expression of LTD effectors by repressing their continued synaptic translation. However, some aspects of the effects observed here may also derive from either basal insufficiency of FMRP or from CGG repeat RNA-mediated toxic effects. Future experiments will be required to demonstrate altered synthesis of LTD effector proteins in CGG KI mice and to formally exclude contributions from these additional factors on synaptic function in CGG KI mice.

In humans, the consequences of premutation range CGG repeats are age-dependent. Of relevance, a recent study examined mGluR-dependent synaptic plasticity

in aged animals (10–13-month-old), comparing WT animals and a different mouse model of the fragile X premutation (Hunsaker et al., 2012). They found that aged premutation model mice exhibited weaker immediate synaptic depression following mGluR activation relative to their WT counterparts, but the level of sustained synaptic depression was similar across genotypes. In contrast, in younger animals, we find no difference in acute synaptic depression driven by mGluR activation, but a significant increase in the magnitude of enduring synaptic depression following mGluR stimulation. Although Hunsaker et al. (Hunsaker, et al., 2012) used a different Fmr1 premutation mouse model than the one employed here, these results raise the interesting possibility that the impact of the Fmr1 premutation may evolve as a function of age. One possibility is that the effects of enhanced mGluR-LTD on the development of childhood and early-adult-onset phenotypes in premutation carriers may be dissociable from the development of late-adult-onset FXTAS in premutation carriers, where RNA-mediated toxicity and neurodegeneration might be expected to have a greater impact.

In this work, we focused on the features of mGluR-LTD in young premutation model mice, given that exaggerated hippocampal mGluR-LTD in Fmr1 KO mice is widely considered relevant to the intellectual disability and autistic symptoms seen in FXS. However, it is likely that Fmr1 premutation repeats may have a broader impact on neural excitability. A recent series of in vitro studies demonstrated that neurons cultured from premutation mice develop abnormal firing properties (Cao, et al., 2012). These neuronal networks exhibit clustered firing and increased Ca^{2+} oscillations, as well as disruptions in neurotransmitter transport machinery (Cao, et al., 2012). Neurons derived from induced pluripotent stem cells generated from premutation carrier fibroblasts exhibit

a similar increase in Ca^{2+} dynamics (Liu et al., 2012). The authors speculated that the functional deficits arise from an improper excitation/inhibition ratio created by the altered transport of glutamate and GABA. While changes in the ratio of excitation to inhibition would influence Ca^{2+} dynamics and thus the firing properties of neurons, we did not find evidence of altered basal synaptic transmission in our ex vivo experiments (Fig. 4.5).

Recent clinical evidence highlights potential points of confluence in symptoms found in young premutation carriers with FXS, suggesting that comparisons between FXS and premutation model mice may help to better identify specific behavioral and neurophysiological correlates of disease features. Specifically, work by a number of groups has demonstrated increased rates of autism and ADHD in premutation carriers, as well as neuropsychiatric symptoms, and executive and amygdala dysfunction (Cornish et al., 2005; Farzin, et al., 2006; Hessl et al., 2007; Hessl et al., 2005; Hessl et al., 2011; Hocking et al., 2012; Hunter et al., 2008; Kogan et al., 2008; Loesch, et al., 2003). This amygdala dysfunction and structural changes in premutation carriers without FXTAS correlate with lower blood FMRP expression (Hessl, et al., 2011). Consistent with this, two CGG KI mouse models exhibit numerous behavioral defects that mirror those observed in Fmr1 KO animals (Hunsaker, et al., 2012; Hunsaker et al., 2009; Qin, et al., 2011). We find that FXS model mice and Fmr1 premutation model mice of similar ages share an important synaptic plasticity phenotype. Our data raise the intriguing possibility that neuropsychiatric abnormalities, autism and ADHD-like symptoms in young premutation patients may be linked to the mGluR-dependent plasticity deficits examined in mouse models of these disorders. However, it should be noted that the repeat sizes studied in CGG KI mice here and elsewhere are significantly larger than that seen in the

average premutation carrier, as repeats become progressively less stable with expansions above 55 repeats. These findings are therefore more relevant to those rare patients who have >100 CGG repeats or who have an unmethylated full mutation. This model may be particularly relevant to this latter category, as recent data suggests that a significant (>30%) portion of FXS patients exhibit incomplete FMR1 DNA methylation and some FMR1 RNA transcription (Jacquemont, et al., 2011). Importantly, this epigenetic alteration correlates with clinical severity and response to some experimental therapies (Jacquemont, et al., 2011). As clinical trials proceed in this patient population with agents that either directly or indirectly target the mGluR pathway (Bhakar, et al., 2012; Hagerman et al., 2012; Jacquemont, et al., 2011), it will be important to understand how mechanistic differences in different mutation states elicit altered mGluR-LTD, and incorporate this knowledge into better practice and drug development.

4.5 Acknowledgements

We thank Sundeep Kalantry for his kind gift of XGFP mice and Cara Westmark for providing us with FMR1 KO mice. We are grateful to Cynthia Carruthers and Christian Althaus for their assistance with culture maintenance and preparation and Grace Van Hyfte for assistance with imaging analysis. We also thank Hank Paulson for his insights and comments during the preparation of the manuscript.

This work was supported by the National Institutes of Mental Health (grant number RO1MH085798 to M.A.S.); the National Institutes of Neurological Disorders and Stroke (grant number F31NS073372 to A.J.I.); the National Institutes of Health

(grant number T32GM008322 to A.J.R., K08NS069809 to P.K.T.); the PEW Biomedical Scholars Program to M.A.S.; and the Harris Professorship to P.K.T.

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4.7 Figure Legends

Figure 4.1-Elevated cortical Fmr1 mRNA and decreased Fragile X mental retardation protein (FMRP) in the fragile X premutation mouse. (A) PCR genotyping of CGG KI male mice and WT littermates showing the expanded CGG repeat. KI band corresponds to ~120 repeats; WT band corresponds to 8 CGG repeats. (B) Fmr1 mRNA levels in the cortex of p28–37 fragile X premutation male mice by qPCR using two different sets of primers against Fmr1. The bar graph summarizes three experiments, n = 5. (C) Representative immunoblot to FMRP (1C3 1:1000) in p28–37 male mouse cortices from the indicated genotypes. Below: Summary of three experiments. Mean (±SEM) cortical FMRP in 1-month-old (p28–38; n = 5) and 6-month-old (p177–181; n = 3) CGG KI mice is decreased compared with littermate controls. The relative decrease between genotypes is greater in older animals. (D) Representative immunoblot against FMRP (17722 1:1000) in hippocampi of p35 CGG KI animals compared with WT littermate controls. Below: Mean (±SEM) hippocampal FMRP in p35–p60 male CGG KI mice compared with WT littermate controls. n = 5. (E) Translational efficiency of cortical Fmr1 RNA expressed as the ratio of FMRP to Fmr1 RNA levels in each individual animal, plotted on log10 scale; n = 5. *P < 0.05, Student's t-test.

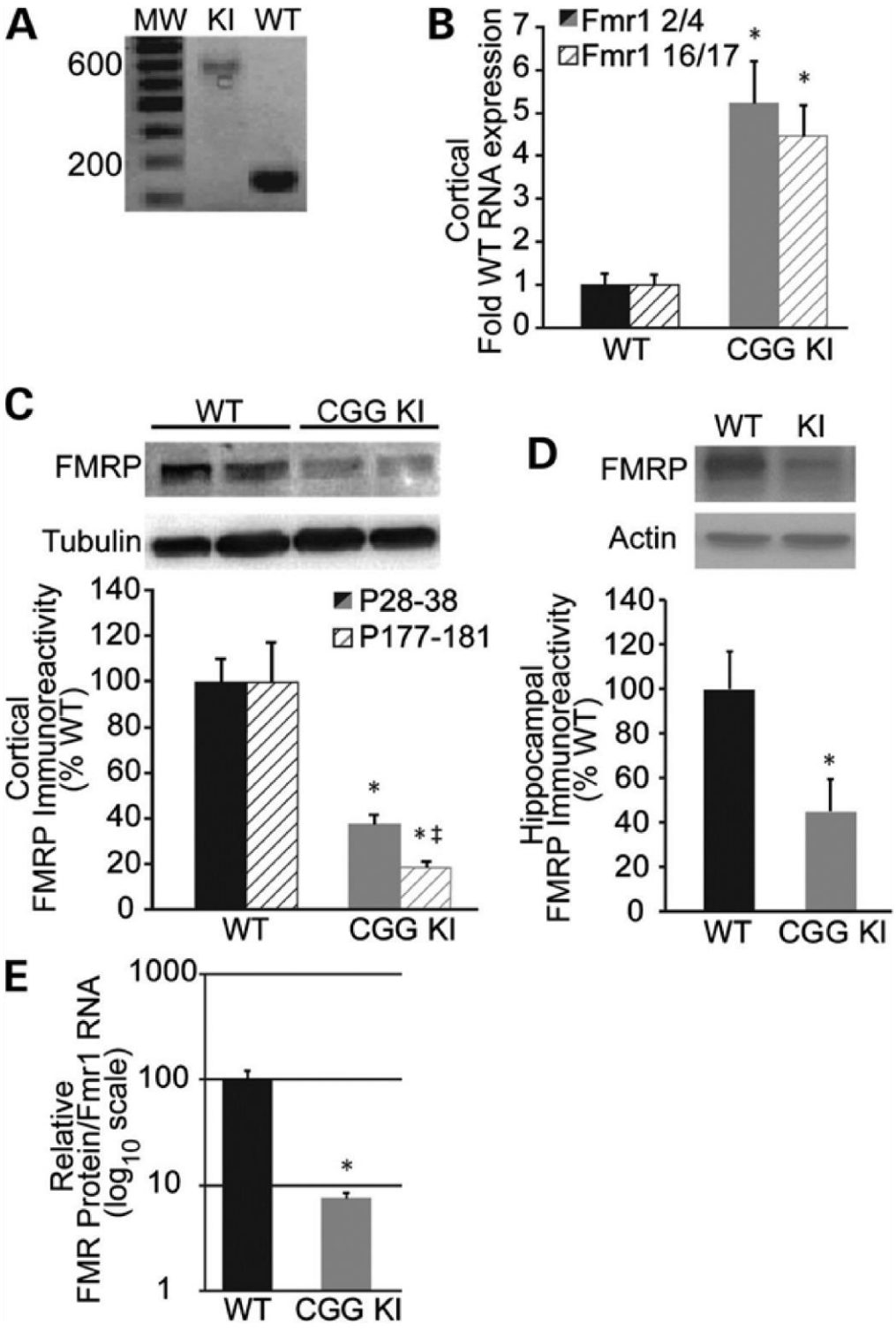

A
MW KI WT
600
200
B
Fmr1 2/4
Fmr1 16/17
Cortical
Fold WT RNA expression
WT
CGG KI
C
WT
CGG KI
FMRP
Tubulin
P28-38
P177-181
Cortical
FMRP Immunoreactivity
(% WT)
WT
CGG KI
D
WT KI
FMRP
Actin
Hippocampal
FMRP Immunoreactivity
(% WT)
WT
CGG KI
E
Relative
FMR Protein/Fmr1 RNA
(log10 scale)
WT
CGG KI

Figure 4.2-Reduced FMRP is distributed throughout dendrites in cultured CGG KI neurons. (A) DIV 14–17 cultured hippocampal neurons from male P1–3 CGG KI and littermate WT animals stained for FMRP (1C3 1:500). (B) 3D surface plot of the relative pixel intensity for the linearized images shown in A demonstrating reduced FMRP expression throughout the soma and dendrite. (C) Total non-zero FMRP fluorescence intensity was quantified in soma, revealing CGG KI neurons have 50% of WT FMRP levels. (D) Summary of fluorescence intensity studies in dendrites (0–40 μm), showing reduced FMRP in CGG KI neurons compared with WT neurons; n = 23–24 neurons from two animals in each group. *P < 0.05, Student's t-test.

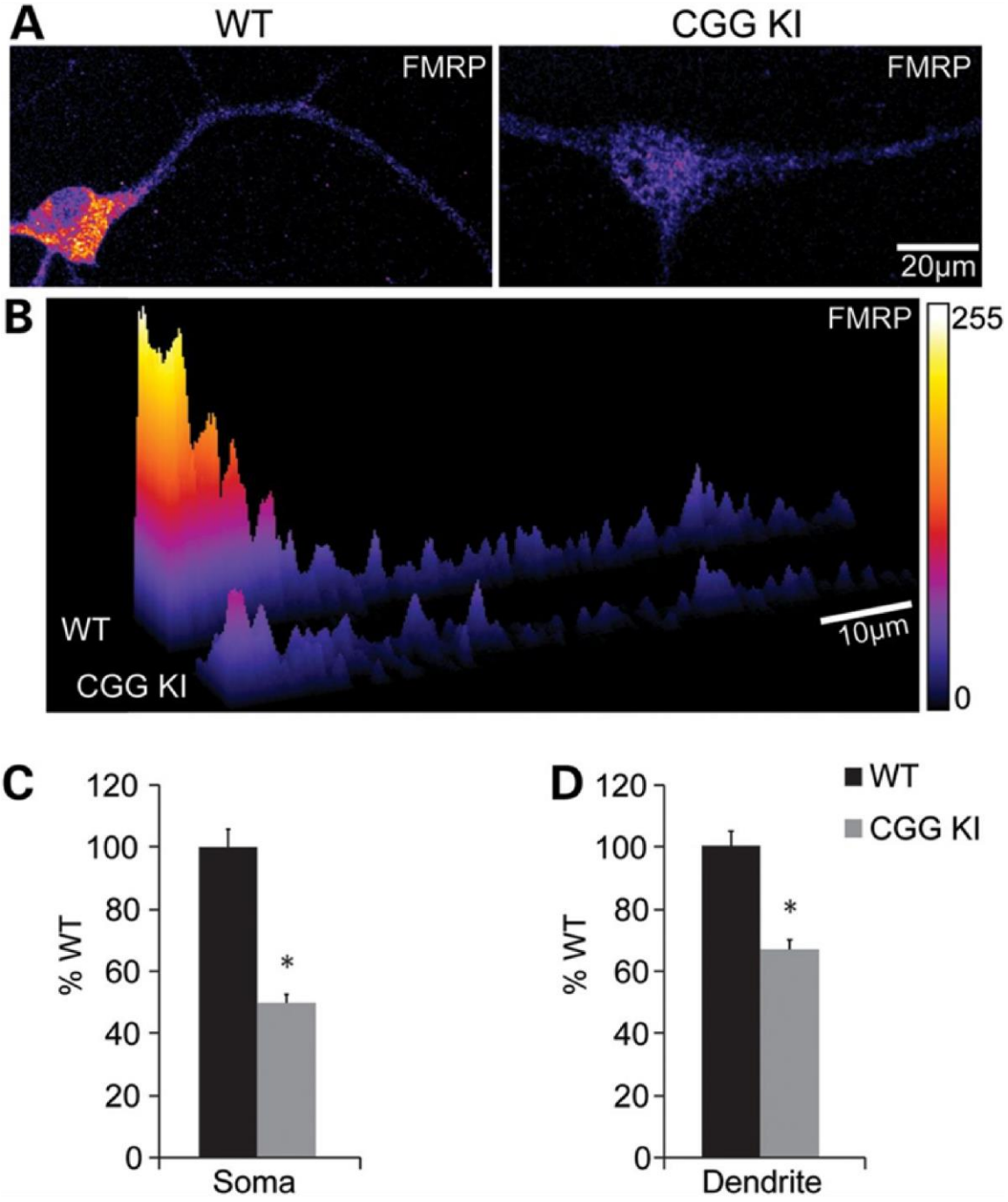

A
WT
CGG KI
FMRP
FMRP
20μm
B
FMRP
255
0
WT
CGG KI
10μm
C
120
100
80
60
40
20
0
% WT
*
Soma
D
WT
CGG KI
120
100
80
60
40
20
0
% WT
*
Dendrite

Figure 4.3-CGG KI SNs do not respond to mGluR stimulation. SNs were prepared from WT and CGG KI cortical homogenates. (A) Verification of SN preparation was confirmed by PSD-95 enrichment between the initial homogenate (H), filtered sample (F), post-centrifugation supernatant (S) and final synaptoneurosome fraction (SN) in each WT and CGG KI preparation. (B) Representative immunoblot against FMRP (17 722 1:1000) in CGG KI SNs compared with littermate WT control. (C) SNs treated with 100 μM 3,5-dihydroxyphenylglycine (DHPG) for 10 or 30 min. Samples were immunoblotted for FMRP (17 722 1:1000) and actin (1:5000). (D) Quantification of FMRP immunoreactivity normalized to untreated samples. WT n = 15, CGG KI n = 5, *P < 0.05, Kruskal–Wallis one-way ANOVA.

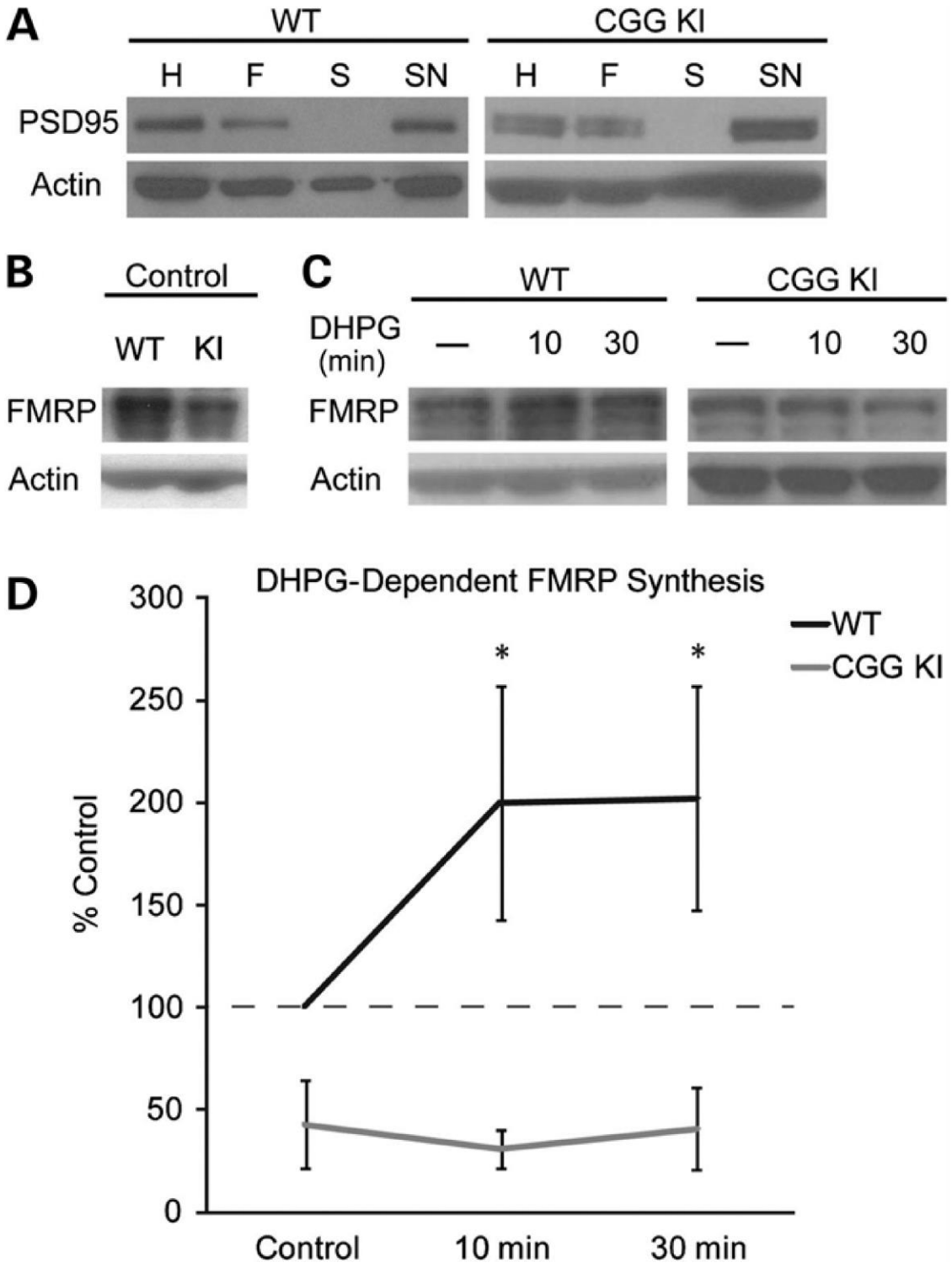

A
WT
CGG KI
H
F
S
SN
H
F
S
SN
PSD95
Actin
B
Control
WT
KI
FMRP
Actin
C
WT
CGG KI
DHPG (min)
—
10
30
—
10
30
FMRP
Actin
D
DHPG-Dependent FMRP Synthesis
WT
CGG KI
% Control
300
250
200
150
100
50
0
*
*
Control
10 min
30 min

Figure 4.4-CGGKI/XGFP heterozygous cultures reveal selective DHPG induction of FMRP in WT neurons. (A) The breeding scheme used to generate mosaic female mice with one WT (GFP+) X chromosome, and one CGG KI (GFP−) X chromosome. (B) Fluorescent nuclei staining (DAPI 1:10 000) in coronal sections from an XGFP/WT female reveal GFP+ and GFP− cells in the hippocampus. (C) Primary hippocampal neurons from mosaic XGFP/CGG KI mice allow both WT (GFP+) and KI (GFP−) neurons in culture. (D) Quantitative analysis on soma from DIV 14–17 XGFP/CGG KI neurons stained for Map2 (Sigma 1:1000) and FMRP (17 722 1:500). CGG KI (GFP−) soma showed reduced basal FMRP fluorescence compared with WT (GFP−) neurons. (E) Basal FMRP expression is maintained in proximal and distal dendrites of CGG KI mice. WT n = 24, CGG KI n = 14, *P < 0.05, Student's t-test. (F) Cultures were treated with DHPG (100 μM for 20 min) prior to FMRP and Map2 staining. (G) Proximal dendrite segments showed selective FMRP immunofluorescence increases in WT (GFP+) neurons, but not in CGG KI (GFP−) neurons. (H) The effects of DHPG are mitigated by pretreatment with anisomycin (40 μM for 30 min) in WT proximal dendrites. There is no effect of DHPG or anisomycin on FMRP expression in the initial segment of CGG KI dendrites. WT n = 15–42 neurons from 1–2 animals, CGG KI n = 7–25 neurons from 1–2 animals. *P < 0.05, one-way ANOVA with Fisher's-LSD.

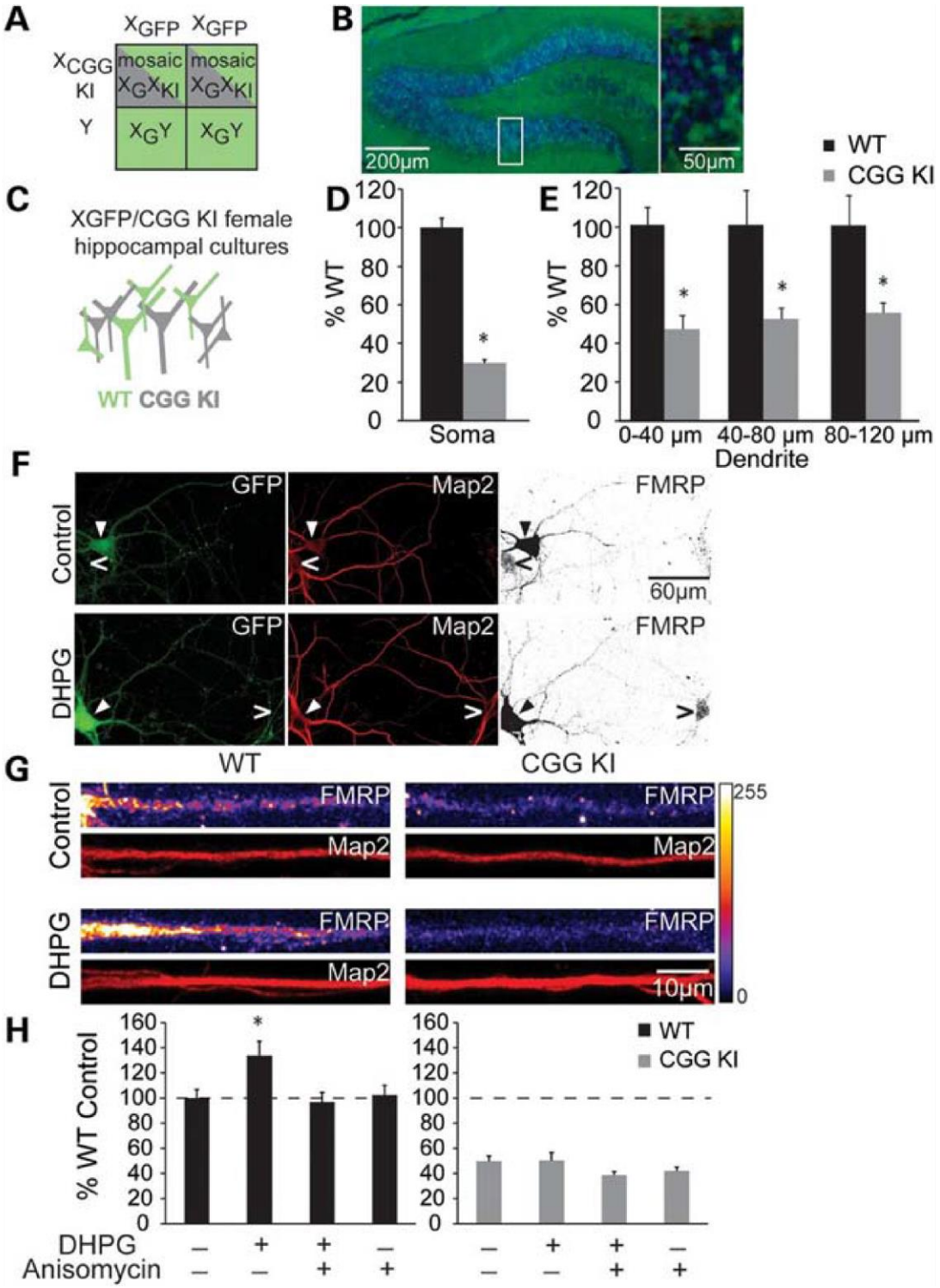

A
XGFP XGFP
XCGG KI
Y
mosaic XGXKI
mosaic XGXKI
XGY
XGY
B
200µm
50µm
WT
CGG KI
C
XGFP/CGG KI female hippocampal cultures
WT CGG KI
D
% WT
Soma
E
% WT
0-40 µm
40-80 µm
80-120 µm
Dendrite
F
Control
DHPG
GFP
Map2
FMRP
60µm
WT
CGG KI
G
Control
DHPG
FMRP
Map2
255
0
10µm
H
% WT Control
DHPG
Anisomycin
WT
CGG KI

Figure 4.5-Basal synaptic function is unchanged in CGG KI mice. (A) Hippocampal field excitatory postsynaptic potentials (fEPSPs) in response to Schaffer collateral stimulation of increasing strength show a similar input/output response curve in CGG KI animals compared with littermate WT controls. n = 19 (WT) and 19 (CGG KI). (B) No difference is detected in paired-pulse facilitation, a measure of basal neurotransmitter release probability, between CGG KI mice and littermate WT mice at any inter-stimulus interval, suggesting that the neurotransmitter release probability at CA3–CA1 synapses is not altered by the premutation. n = 8 (WT) and 8 (CGG KI).

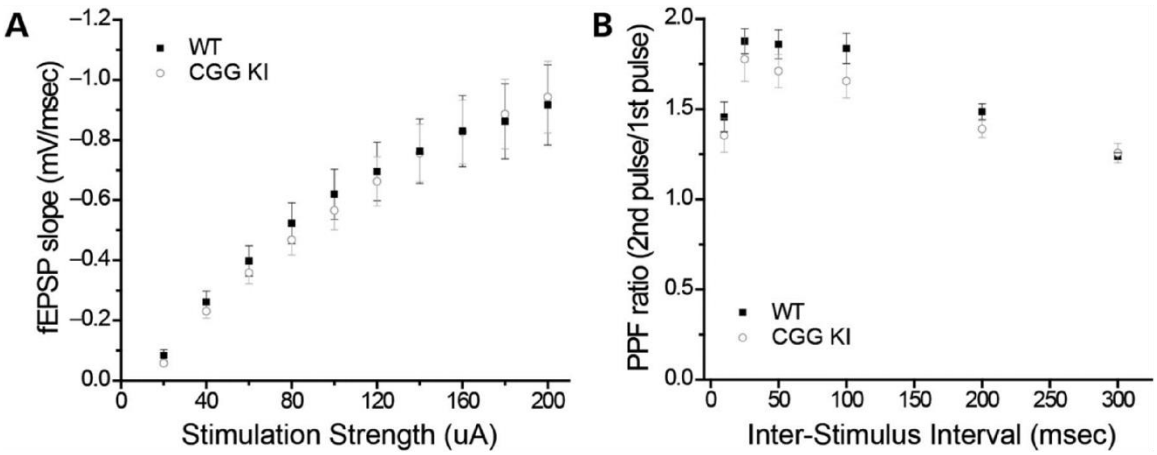

A
WT
CGG KI
fEPSP slope (mV/msec)
−1.2
−1.0
−0.8
−0.6
−0.4
−0.2
0.0
0
40
80
120
160
200
Stimulation Strength (uA)
B
PPF ratio (2nd pulse/1st pulse)
2.0
1.5
1.0
0.5
0.0
WT
CGG KI
0
50
100
150
200
250
300
Inter-Stimulus Interval (msec)

Figure 4.6-Exaggerated mGluR-LTD in CGG KI mice is protein synthesis dependent. (A) Field EPSPs were recorded in CA1 stratum radiatum in response to Schaffer collateral stimulation. Addition of the group 1 mGluR agonist DHPG (100 μM; 10 min) induced LTD at CA3–CA1 synapses; this mGluR-LTD was significantly enhanced in CGG KI mice. n = 9 (WT) and 13 (CGG KI). Inset: Shown are representative averages of four consecutive field potential waveforms from each group during the baseline period and 1 h after LTD induction. (B) mGluR-LTD in FMR1 KO mice persists in the presence of the protein synthesis inhibitor anisomycin (20 μM), as previously reported (Huber, et al., 2002). n = 7 (control) and 8 (aniso). (C) In contrast, the enhanced mGluR-LTD in CGG KI mice remains sensitive to protein synthesis inhibitors. n = 13 (control) and 7 (aniso).

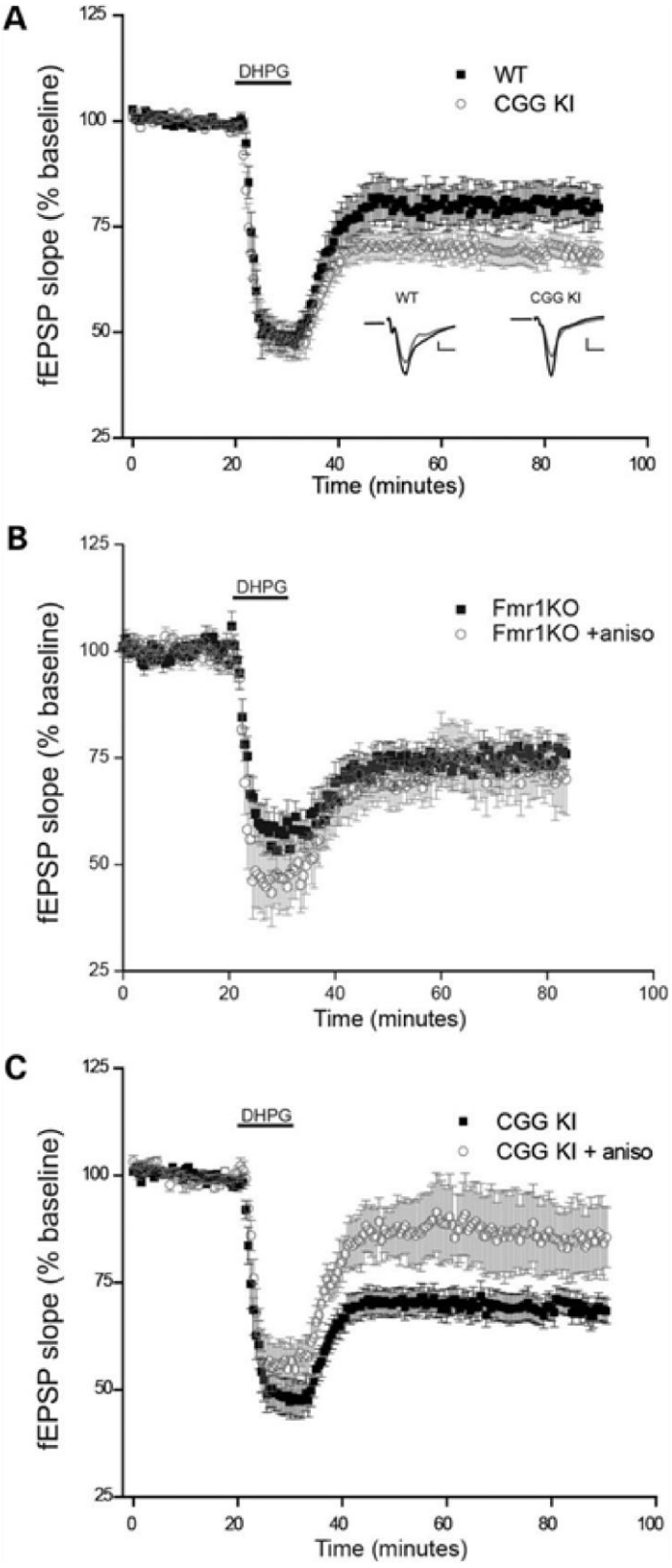
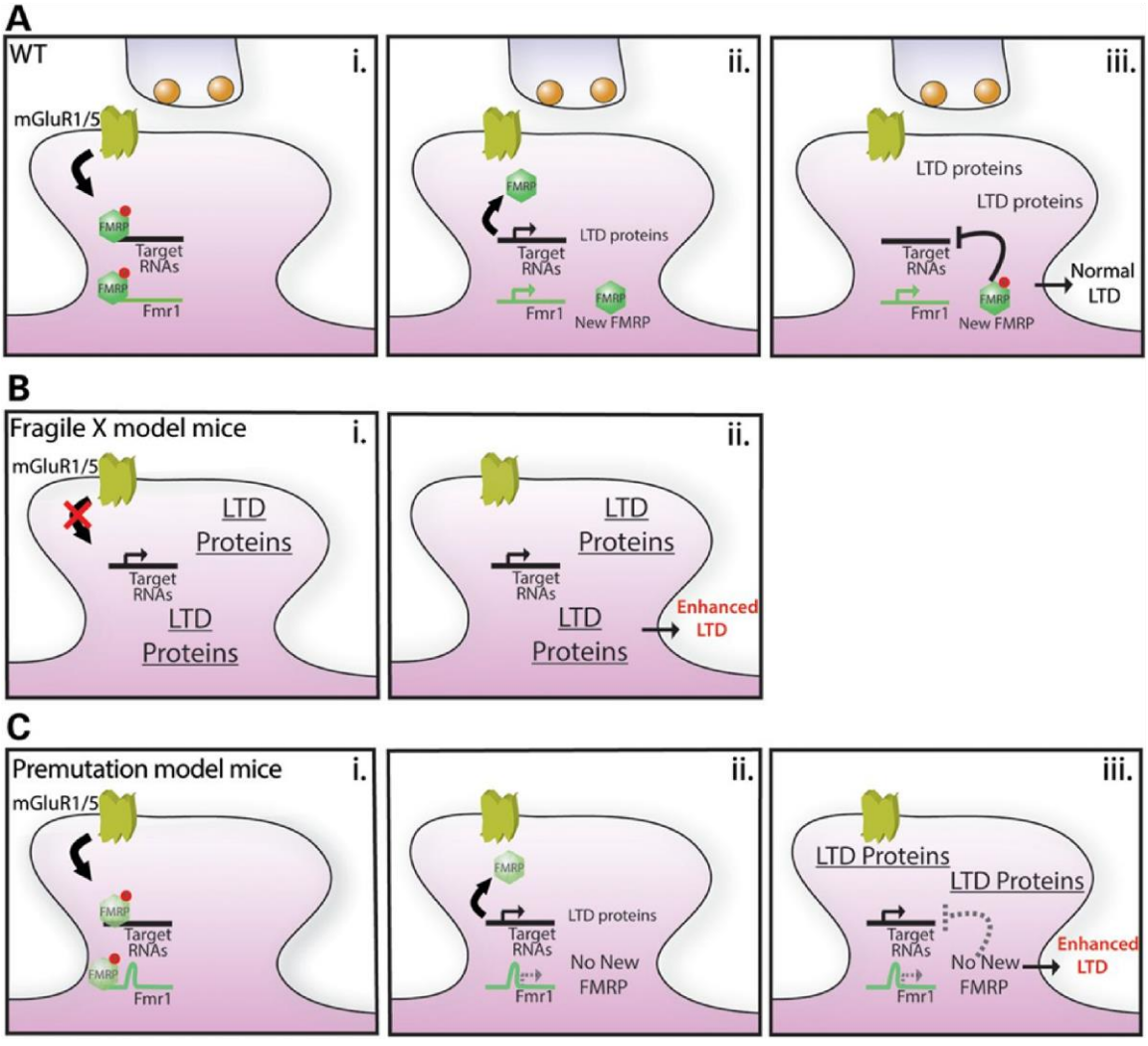

A
125
100
75
50
25
fEPSP slope (% baseline)
DHPG
WT
CGG KI
WT
CGG KI
0
20
40
60
80
100
Time (minutes)
B
125
100
75
50
25
fEPSP slope (% baseline)
DHPG
Fmr1KO
Fmr1KO +aniso
0
20
40
60
80
100
Time (minutes)
C
125
100
75
50
25
fEPSP slope (% baseline)
DHPG
CGG KI
CGG KI + aniso
0
20
40
60
80
100
Time (minutes)

Figure 4.7-A working model of mGluR-LTD in WT, KO and CGG KI mice. Group I mGluR receptors are critical modulators of synaptic overactivity. (A) Normally, FMRP bound transcripts, including Fmr1 mRNA, exist in stalled polyribosomal complexes at synapses. (i) Activation of group I mGluRs triggers the internalization of AMPAR and the dissociation/clearance of FMRP from target mRNAs. (ii) This allows for the rapid translation of proteins required for the maintenance of AMPAR internalization (LTD proteins), leading to long-lasting changes in synaptic strength. In parallel, FMRP is itself synthesized at synapses. (iii) This new FMRP acts as a brake on further translation of mRNA targets. The end result is mGluR-LTD that requires a temporally constrained burst of local protein translation after receptor activation. (B) In FXS model mice, translation of FMRP target transcripts is uncoupled from mGluR signaling. (i) This results in a basal increase in production of LTD proteins. Upon mGluR activation, AMPARs are internalized normally but the presence of excess basal LTD effector proteins leads to the enhancement of mGluR-LTD. As the over-synthesis of LTD effector proteins is not tied to mGluR activation, induction of mGluR-LTD in FXS model mice does not require new protein synthesis. (C) In Fragile X premutation model mice, there is adequate basal expression of FMRP to allow for the localization of FMRP with associated transcripts at synapses. (i) mGluR activation triggers the dissociation of FMRP from these transcripts normally. (ii) However, the CGG repeat expansion blocks rapid FMRP synthesis. Without this new FMRP, there is no brake to prevent the ongoing synthesis of FMRP target transcripts. (iii) The result is overproduction of LTD effector proteins and enhanced mGluR-LTD. In contrast to FXS model mice, synaptic protein translation in premutation model mice remains coupled to mGluR activation and the

mGluR-LTD is thus dependent on new protein synthesis. This working model makes a number of specific predictions which will be tested in future studies.


A
WT
i.
mGluR1/5
FMRP
Target RNAs
FMRP
Fmr1
ii.
FMRP
LTD proteins
Target RNAs
Fmr1
FMRP
New FMRP
iii.
LTD proteins
LTD proteins
Target RNAs
Fmr1
FMRP
New FMRP
Normal LTD
B
Fragile X model mice
i.
mGluR1/5
LTD Proteins
Target RNAs
LTD Proteins
ii.
LTD Proteins
Target RNAs
LTD Proteins
Enhanced LTD
C
Premutation model mice
i.
mGluR1/5
FMRP
Target RNAs
FMRP
Fmr1
ii.
FMRP
LTD proteins
Target RNAs
Fmr1
No New FMRP
iii.
LTD Proteins
LTD Proteins
Target RNAs
Fmr1
No New FMRP
Enhanced LTD

Chapter V

Future Directions and General Discussion

5.1 Summary

The data presented in this dissertation reveal that CA1 synapses can compensate for subtle changes in the activity of their inputs through a rapid, input-specific form of homeostatic synaptic plasticity (HSP). Thus, HSP occurs alongside Hebbian processes at CA3-CA1 synapses in acute hippocampal slices and like Hebbian synaptic plasticity, HSP requires functioning NMDA receptors (NMDARs) and post-synaptic calcium. Unlike the mechanisms underlying LTP and LTD, HSP occurs independently of CaMKII or PP2B activity. Although previously discovered forms of rapid, local forms of HSP require new protein synthesis, this form of HSP does not, suggesting it represents a novel form of synaptic plasticity. Together, these features permitted an investigation into how directly opposed forms of synaptic plasticity can operate at the same population of synapses by proving several potential interactions between Hebbian plasticity and a rapid, input-specific form of HSP. Our results reveal that this form of HSP generally offsets the magnitude of subsequent Hebbian plasticity expression in an additive fashion. We also identified an unforeseen cooperative effect whereby homeostatic plasticity can enhance the durability of established LTP, revealing a condition in which weak LTP stimuli can result in long-lasting changes without de novo protein synthesis. Surprisingly, we

discovered that Hebbian plasticity can constrain the expression of HSP subsequently induced, but only in cases where both plasticities drive synaptic changes in the same direction. We go on to identify a role for local protein synthesis in mediating this metaplastic interaction. Finally, we examine the nature of an activity-dependent biosynthesis of a molecule (FMRP) at synapses involved in the local translation-dependent mGluR-mediated synaptic plasticity. Specifically, we found that a mouse model of the Fragile X syndrome premutation shares a classic synaptic plasticity phenotype with Fragile X syndrome model mice, but involves a distinct underlying mechanism (Chapter IV; Iliff, et al., Hum Mol Gen, 2013). The results from Chapter IV suggest a possible mechanism for the increased vulnerability for autism and ADHD-like symptoms in premutation carriers.

5.2 Molecular mechanisms underlying local HSP in acute hippocampal slices

5.21 Common mechanisms of bidirectional HSP

NMDAR activation and postsynaptic calcium are required for normal HSP expression in acute hippocampal slices (Chapter 2). This was surprising in the sense that Hebbian plasticity also depends on these molecules, yet HSP operates on a very different set of principles. Yet, it may not be surprising that neurons utilize similar activity-dependent signals in order to respond to the degree of evoked activity. Another similarity with LTP/LTD is that HSP is expressed in the postsynaptic compartment via changes in the magnitude of AMPA receptor (AMPAR) mediated currents (Fig. 2.4). Determining exactly how AMPARs are modulated following HSP induction is a process we are only beginning to understand and will require further investigation.

We found that presynaptic release probability is not altered during bidirectional HSP, suggesting that HSP is not expressed in the presynaptic compartment (Chapter 2). Consistent with this view, HSP was both blocked by postsynaptic chelation of Ca^{2+} and expressed as a change in the magnitude of AMPAR (but not NMDAR) mediated currents. This differential regulation of glutamate currents supports a postsynaptic expression locus, since a presynaptic change would alter the currents to a similar degree with no change in AMPA/NMDA ratio. Subsequently, our lab found that GluA1 endocytosis was required for homeostatic weakening using the endocytosis inhibitor D15 (data not shown; 1 mM; Tocris), which has previously been shown to block LTD (Lüscher et al., 1999). We found that GluA1, but not GluA2, trafficking was required for homeostatic weakening in whole cell recordings of CA1 neurons (data not shown). Inclusion of the peptide that binds the C-tail of GluA1 in the recording pipette blocked homeostatic weakening (data not shown; 1mM; glua1-TGL and glua2-SVKI from Tocris).

5.22 S6K activity required for homeostatic strengthening

Since HSP in slices was blocked by introduction of a peptide against the c-terminus of GluA1, we turned our attention towards potential mechanisms involving the C-tail region. The GluA1 C-tail contains a number of phosphorylation sites important for synaptic plasticity (Lu & Roche, 2012). Phosphorylation of S831 increases single channel conductance, yet hippocampal LTP is unaffected in mice lacking S831 phosphorylation, making it a good candidate for mediating homeostatic strengthening (Lu & Roche, 2012). However, S831 is phosphorylated by protein kinase C (PKC) and CaMKII, and our findings suggest that neither of these kinases is required for homeostatic strengthening (Chapter 2; data not shown). S845 phosphorylation leads to

enhanced AMPAR function as well. Once again, hippocampal LTP is unaffected in mice lacking S845 phosphorylation but the activity of the kinase that phosphorylates this residue (PKA) is unnecessary for homeostatic strengthening (data not shown). In contrast, S818 phosphorylation (by PKC) is required for LTP. Finally, T840 phosphorylation is dispensable for LTP and is phosphorylated by PKC and p70 S6 kinase (S6K) (Delgado et al., 2007; Lee et al., 2007). Additionally, treatment of hippocampal slices with NMDA leads to dephosphorylation of this site, a result interpreted as evidence of T840 involvement in NMDAR-LTD (Delgado et al., 2007). In light of our data on the NMDAR dependence of HSP in hippocampal slices (Chapter 2), their result could also be interpreted as evidence of T840 involvement in HSP. Intriguingly, induction of homeostatic increases in synaptic efficacy with AMPAR blockade in dissociated cultures activates S6K activity (Henry et al., 2012). Taken together, regulation of T840 phosphorylation by S6K is an excellent candidate mechanism underlying homeostatic strengthening.

To investigate this possibility, we took advantage of a recently described inhibitor of S6K activity (Pearce et al., 2010). Applying S6K inhibitor PF4708671 (Tocris; 10uM) to hippocampal slices for 30 min blocked the expression of homeostatic strengthening (Fig. 5.1A). S6K activity is regulated by mTOR activity. mTORC1 phosphorylates S6K thereby increasing its activity and thus the phosphorylation of S6K substrates (Caron et al., 2010). We reasoned that inhibiting mTOR activity might similarly disrupt expression of homeostatic strengthening via this pathway. Indeed, application of the mTOR inhibitor rapamycin (100nM ; LC Labs) blocked expression of homeostatic strengthening in hippocampal slices (Fig. 5.1B). A dependence of S6K activity is a shared mechanism

with a rapid, local form of HSP investigated in dissociated hippocampal cultures (Henry et al., 2012). However, S6K activity implicated in HSP studied in culture is involved with mTOR mediated-protein synthesis (Henry et al., 2012), but HSP in slices is independent of new protein synthesis (Chapter 3), suggesting unique compensatory mechanisms. Our working hypothesis is that S6K influences HSP expression via direct phosphorylation of the GluA1 subunit at T840.

5.23 Regulation of GluA1 T840 phosphorylation status may underlie bidirectional HSP

In an attempt to address whether regulation of GluA1 T840 phosphorylation status underlies HSP in slices, we first confirmed that blocking S6K activity led to a reduction in T840 phosphorylation using an antibody against phosphorylated T840 (p-T840). We found in preliminary experiments that one hour treatments with the drug greatly reduced p-T840 signal compared to vehicle treated slices (Fig. 5.2A). Previously it was reported that NMDAR activation by application of NMDA to slices rapidly dephosphorylates T840 (Delgado et al., 2007). We confirmed this result under the same conditions (Fig. 5.2B). We also confirmed another result from the same study (Delgado et al., 2007) that p-T840 signal was enhanced by treatment with the phosphatase inhibitor cantharidin (Fig. 5.2C). These results demonstrate bidirectional regulation of p-T840 state and suggest that a balance of kinase and phosphatase activity maintain p-T840 levels. In a preliminary experiment, we found that induction of homeostatic weakening in slices using one-pathway stimulation decreased p-T840 signal (Fig. 5.2D), but this result requires further validation. Whether p-T840 increases in response to homeostatic strengthening protocols remains to be seen. A parsimonious explanation of the above results is that bidirectional regulation of this site mediates homeostatic strengthening and

weakening. Furthermore, the homeostatic set point of synaptic strength could be the dictated by the interplay between kinase and phosphatase activity acting on the T840 residue, but much more work is needed to validate this potential scenario.

The previous sets of experiments suggest several follow-up experiments. For instance, would driving S6K activity promote homeostatic strengthening? Henry et al (2012) used exogenous expression of the mTOR activating GTPase Rheb and a hyperactive mutant to drive mTOR activity in neurons. Injection of viral vector containing this construct to rat hippocampus could be used to drive mTOR activity in a subset of neurons. Co-expression of a reporter would allow identification of Rheb-expressing neurons and visually-guided patch clamping experiments to test for enhanced homeostatic strengthening. An important caveat if the hypothesis is correct is that driving mTOR activity may occlude subsequent HSP expression.

All the work presented thus far has taken place in acute hippocampal slices for reasons already mentioned. Although this preparation has proved beneficial for the questions being addresses, the use of dissociated neuronal cultures greatly simplifies the investigation into molecular mechanisms. Therefore, we sought a correlate of the HSP characterized in slices in dissociated cultures of hippocampal neurons. Towards this end, we designed a field stimulator that would be able to create a brief voltage field across the network to drive activity in a similar manner to that performed in slices, utilizing that stimulation rates to induce bidirectional HSP. In neurons loaded with the calcium indicator Fluo5F (5 uM; Invitrogen), stimulating with the field stimulator produces brief rises in intracellular calcium (data not shown). Spontaneous calcium spikes were

detected in normal Hepes-buffered saline, but the addition of 50 uM muscimol subdues spontaneous activity while permitting stimulated calcium responses (data not shown).

Consistent with our preliminary results in slices, increasing the frequency of stimulation of stimulation from one pulse every 5 min to one pulse every 20 sec caused a decrease in p-T840 signal. One advantage of using a culture model is the ease with which one can resolve individual synapses. By co-staining for PSD95, we were able to restrict our examination of p-T840 signal to PSD-95+ puncta. This type of analysis revealed that a homeostatic weakening protocol correlates with a significant decrease in p-T840 signal at synapses as compared to a constant stimulation protocol (Fig. 5.3A). Similarly, a homeostatic strengthening protocol (one pulse every 20 sec to one pulse every 5 min) produced an increase in p-T840 signal at PSD95+ puncta (Fig. 5.3B). Future experiments could use this approach combined with inhibitors of S6K to address whether S6K activity is responsible for the increased phosphorylation of T840.

Although we can elicit bidirectional changes in p-T840 levels in dissociated cultures using electrical stimulation, it remains unknown whether this protocol leads to a functional change in synapses. To address this question, future experiments could measure changes in surface AMPAR accumulation. Our lab has previously published studies using methods that would accomplish this goal, using both a surface biotinylation assay (Zhang et al., 2012) and a surface GluA1 immunofluorescence assay (Henry et al., 2012; Jakawich et al., 2010). Additionally, electrophysiological recordings could measure changes in the amplitude of evoked or miniature EPSCs, either of which indicate altered synaptic function. If HSP is produced in culture using our protocol, then the underlying mechanism could be probed. For instance, the role of NMDARs and calcium,

shown to be important for HSP in slices (Chapter 2) could be investigated using the same strategies.

Despite all the potential advantages of using the approaches outlined above, a difficulty remains in testing our working model. Outside of a couple studies, not much is known about the T840 site on GluA1. The creation of a T840A mouse line would be invaluable for studies into the role of T840 phosphorylation in synaptic plasticity. A mouse line containing this mutation along with 4 other mutated GluA1 phosphorylation sites (S831A, T838A, S839A, T840A, S845A) has been created (Lee et al., 2007), but as already mentioned, several of these sites play established roles in synaptic plasticity which may confound interpretations.

5.24 What is the mechanism of homeostatic weakening?

In addition to de novo protein synthesis, degradation has also been shown to play a pivotal role in both L-LTP (Fonseca et al., 2006) and mGluR-LTD (Hou et al., 2006), indicating that a proper balance of protein synthesis and degradation is needed for proper responses in activity-dependent processes (Ehlers, 2003). We found that protein synthesis is dispensable for bidirectional changes in synaptic transmission in response to subtle changes in the frequency of afferent stimulation (Chapter 3). In subsequent experiments, the role of protein degradation in mediating rapid, local HSP in hippocampal slices was explored using the proteasome inhibitor lactacystin (10uM; Sigma) and the ubiquitin-activating enzyme (E1) inhibitor Ube1-41(50uM; Biogenova). Neither of these inhibitors altered the magnitude or time course of homeostatic

weakening, suggesting that neither de novo protein synthesis nor degradation via the ubiquitin-proteasome system mediates homeostatic weakening (data not shown).

In chapter 2, we used treatment of slices with the compound FK506 to inhibit protein phosphatase 2B (PP2B) to demonstrate a mechanistic difference between homeostatic weakening and LTD. Unlike NMDAR-dependent LTD induced by delivery of 900 pulses at 1Hz, FK506 did not alter the expression of homeostatic weakening (Fig 2.5; Mulkey et al., 1994). A pair of phosphatases required for NMDAR-dependent LTD (Mulkey et al., 1993) and known to dephosphorylate T840 GluA1 (Delgado et al., 2007) is protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). In fact, most inhibitors of PP1 also inhibit PP2A with similar potencies (Swingle et al., 2007), so resolving which phosphatase is being inhibited typically involves a complex interplay of multiple inhibitors at non-maximal activity concentrations. Both okadaic acid (Mulkey et al., 1993; Niere et al., 2012; Woo et al., 2002; Young et al., 2006) and calyculin A (Mulkey et al., 1993; Woo et al., 2002) have been used in hippocampal slices previously and could be used to test whether PP1/PP2A phosphatase activity underlies homeostatic weakening. Another PP1/PP2A inhibitor, cantharidin, has been shown to block the dephosphorylation of T840 that occurs in response to NMDAR activation (Delgado et al., 2007), and could potentially be used to investigate both the PP1/PP2A-dependence of HSP and the regulation of GluA1 phosphorylation at T840. Although cantharidin has not been used as extensively as okadaic acid, we and others have found that cantharidin increases basal levels of GluA1 phosphorylation at T840 in hippocampal slices (Delgado et al., 2007; Fig. 5.2C).

We found that NMDAR antagonism blocked the bulk of change in evoked responses during homeostatic strengthening and weakening (Chapter 2). However, some detectable change persisted (Fig. 2.6), suggesting either incomplete NMDAR blockade or the presence of a non-NMDAR mediated component to HSP. A simple hypothesis is that HSP is mediated in part by mGluR activation, as there are both NMDAR- and mGluR-dependent forms of LTP/LTD in CA1. A number of mGluR antagonists have been used successfully in hippocampal slices (Gladding et al., 2009) and could be used to test this hypothesis.

5.3 The complex interplay between Hebbian synaptic plasticity and HSP at CA1 synapses

5.31 Local HSP competes with established Hebbian plasticity

We found that synapses could compensate for changes in activity in a direction that would reverse the changes of a previously established Hebbian process (Chapter 3). For instance, the expression of homeostatic weakening was unaltered at synapses that had a prior history of LTP induction. On the one hand, this result is surprising, because it suggests that these diametrically opposed processes can interfere with each other, reducing the potential for extremely long-lasting changes in synaptic efficacy which is known to occur in vivo (Abraham et al., 2002). On the other hand, the competition between these processes may be a necessary and perhaps stabilizing property of neuronal networks. To produce long lasting changes in LTP, other processes are required, perhaps in part to overcome this competition. It is well established that LTP requires de novo protein synthesis for longer lasting forms of LTP, such as L-LTP. Since we found that

homeostatic weakening could compete with L-LTP too (Chapter 3), the protein synthesis requirement might have more to do with events like spine growth rather than outright disabling of competing local HSP.

Interestingly, the competition is one-sided. Induction of local HSP in either direction caused a change in the magnitude of synaptic responses, but did not alter the relative expression of LTP or LTD induced on top of those changes (Chapter 3). We argued in Chapter 3 that this result is internally consistent, since we found no previous evidence that NMDARs were altered as a consequence of HSP induction (Chapter 2). The strictly additive effect of prior homeostatic plasticity induction may be specific to local, input-specific forms of HSP. Arendt et al (2013) found that 60 hour treatment of organotypic slice cultures with TTX produced a dramatic increase in the expression of subsequent LTP (Arendt et al., 2013). Prolonged TTX treatment caused an increase in both AMPAR and NMDAR-mediated currents, another key difference between our findings (Chapter 2; Arendt et al., 2013). The authors go on to show that the enhanced LTP is not due to the increase in NMDARs, but rather the unsilencing of TTX treatment-created silent synapses. This study uses a different preparation than we use in chapter 2, in addition to different protocols for HSP and LTP induction, all of which may contribute to the differences observed in both studies. An advantage to investigating rapid, local forms of HSP in organotypical slices cultures is that more complex interactions than we examined in Chapter 3 could be probed. For instance, it would be interesting to see if repeated bouts of LTP followed by homeostatic weakening would still lead to LTP saturation.

Another study found that decreasing the rate of stimulation of Schaffer collaterals (similar to our own protocol) or ceasing stimulation altogether increased synaptic transmission and NMDAR-mediated transmission via incorporation of GluN2B subunits (Gambrill et al., 2011). We found increased synaptic transmission using similar protocols but no change in NMDAR-mediated transmission (Chapter 2). It is unclear why these two studies have conflicting results, but a potentially important difference between them is the use of $GABA_A$ receptor antagonist in Gambrill et al (2011), which may alter the network dynamics of CA1 in a way that produces NMDA receptor changes. The authors also argue that LTP is enhanced following upregulation of NMDA receptors, which is consistent with a change in LTP threshold (Abraham, 2008). However, one caveat of that interpretation is that LTP was induced on top of stimulation cessation, which they showed increases synaptic responses. Thus, the reported increase in LTP could be an additive effect of homeostatic strengthening with the LTP (i.e., HSP+LTP) rather than a specific increase in the magnitude of LTP. Future experiments could use decreased stimulation rather than lack of stimulation and wait until responses have stabilized to probe for a specific enhancement in LTP induction.

5.32 Translation regulatory mechanisms underlying metaplastic interaction

Although the molecular mechanisms responsible for homeostatic weakening aren't fully known yet, we found that a protein synthesis-independent form of LTD constrained the magnitude of HSP subsequently induced. This instance of metaplasticity, in which one type of plasticity alters the expression of another, is mediated by local protein synthesis (Chapter 3). A number of questions are generated by these findings and will be discussed here.

5.33 What are the relevant translation regulatory signaling pathways involved?

Both mitogen-activated protein kinase (MAPK) and mammalian Target of rapamycin (mTor) signaling transduction pathways are involved in the regulation of protein-synthesis dependent forms of plasticity (Gallagher et al., 2004; Hoeffer & Klann, 2010; Lynch, 2004). Inhibition of a MAPK sub-class, extracellular-signal regulated protein kinases (ERK) blocks mGluR-LTD but not NMDAR-dependent LTD (Gallagher et al., 2004). We therefore used an inhibitor of ERK activity (U0126) to probe its involvement in signaling the constraint on HSP following LTD induction. Surprisingly, we found that U0126 treatment blocked the expression of NMDAR-dependent LTD (Fig. 5.4A), even though our conditions were highly similar to those previously reported not to disrupt LTD. This result impeded our ability to investigate the interaction. One interpretation of our result is that our induction protocol is invoking an ERK-mediated protein synthesis-dependent form of LTD. However, multiple experiments using translation inhibitors have shown no decrement in the magnitude of LTD (Chapter 3). In our hands, ERKs may be playing a role in LTD expression outside its involvement in protein synthesis. More studies are needed to validate our result, perhaps using other inhibitors of ERK activity.

Another major signaling transduction pathway strongly implicated in translation is the mTOR pathway. mTOR is a serine/threonine kinase with substrates involved in catalyzing translation. One of these substrates is p70 S6 kinase (S6K) which has already been discussed here. We previously found mTOR and S6K to be a molecular players underlying translation-independent homeostatic strengthening. Given that mTOR plays a well-established role in translation, we addressed the possibility that it was involved in

the interaction between Hebbian and homeostatic plasticity. As an initial test of this possibility, we applied rapamycin to slices while performing the LTD-homeostatic weakening experiment described in Chapter 3. We found no difference between rapamycin- and vehicle-treated slices, although there was a slight trend towards greater homeostatic weakening in the presence of rapamycin (data not shown). This non-significant effect occurred regardless of prior LTD induction at the same input. Thus mTOR and S6K activity are likely only required for homeostatic strengthening.

Recent work has implicated another translational regulator, eukaryotic Elongation Factor 2 kinase (eEF2K), in mediating translation dependent synaptic plasticity (Henry et al., 2012; Park et al., 2008; Sutton et al., 2007). Homeostatic increases in synaptic efficacy in dissociated cultures correlate with decreases in eEF2 phosphorylation in addition to increases in protein synthesis (Henry et al., 2012; Sutton et al., 2007; Sutton et al., 2004). In mice lacking eEF2K, mGluR-LTD and L-LTP are deficient in area CA1 (Park et al., 2008). eEF2K is a calcium/calmodulin-dependent kinase whose activity can be triggered by synaptic stimulation (Scheetz et al., 2000). Phosphorylation of eEF2 by its kinase inhibits translation via stalled elongation similar to the method of inhibition by the drug cyclohexamide (Ryazanov et al., 1988). Cyclohexamide, as well as emetine, was demonstrated to abolish the interaction between Hebbian and homeostatic plasticity in Chapter 3, raising the possibility that eEF2K activity may be involved. To examine this possibility, we applied the eEF2K inhibitor NH125 to hippocampal slices while performing the LTD-interaction experiment as described. The constraint of homeostatic weakening was similar between slices bathed in NH125 and DMSO vehicle control (Fig.

5.4B). Thus, it is currently unclear which signal transduction pathway mediate crosstalk between different forms of synaptic plasticity.

Turning to a slightly different approach, we focused on the translational suppressor Fragile X mental retardation protein (FMRP) (Todd & Malter, 2002). If the transcript(s) involved with metaplastic interactions is normally suppressed by FMRP but translated in response to LTP/LTD induction, then perhaps Fmr1KO mouse lacking FMRP would exhibit greater degrees of HSP constraint. This hypothesis builds off of findings in Fmr1KO mice that show exaggerated responses to mGluR stimulation, leading to excess mGluR-LTD (Hou et al., 2006; Huber et al., 2002; Iliff et al., 2013; Chapter 4). mGluR-LTD is dependent on dendritic protein synthesis (Huber et al., 2000), which raised the possibility that FMRP was involved in other forms of synaptic plasticity involving local protein synthesis. However, Fmr1KO mice display largely normal L-LTP (Paradee et al., 1999; Zhang et al., 2009), early phase NMDAR-dependent LTP (E-LTP) and LTD (E-LTD) (Godfraind et al., 1996; Huber et al., 2002; but see Lauterborn et al., 2007), and global HSP (Soden & Chen, 2010). The latter study did find evidence that Fmr1KO mice are deficient in their ability to express a protein synthesis dependent form homeostatic plasticity mediated by retinoic acid (Soden & Chen, 2010), suggesting FMRP can modulate the expression of HSP under certain circumstances. To examine this possibility in the context of our metaplastic interaction, we made hippocampal slices from Fmr1KO mice and their WT littermates as previously described (Chapter 4, Iliff et al., 2013) while performing the LTD-interaction experiment as described above and in Chapter 3. However, the constraint of homeostatic weakening by prior LTD was weak in both genotypes (Fig. 5.5), so further experiments are needed to resolve of the role of

FMRP in mediating the metaplastic interaction. Once recording conditions are optimized to detect the interaction in WT littermates, an accompanying approach may be used to explore a role for FMRP in metaplasticity. Previously, a mouse model overexpressing human FMRP was found to abolish mGluR-LTD (Hou et al., 2006). Future studies could use a similar approach to test for the absence of a metaplastic interaction. At least one previous study has demonstrated a role for FMRP in metaplastic enhancements to a heterosynaptic form of LTP (Connor et al., 2011), setting a precedent for FMRP's involvement in metaplasticity. Still, the major electrophysiological phenotype associated with loss of FMRP is enhanced mGluR-LTD with an associated loss of the requirement for protein-synthesis.

5.34 Which transcripts are being locally translated?

Much less is known about the newly created proteins underlying the metaplastic interaction. If the sign and direction of HSP is determined by a balance of kinase and phosphatase activity as suggested above, then the translated transcripts could either be these kinases or phosphatases. In this scenario, the balance would be shifted away from normal HSP expression. Of course, another possibility is that a protein is created which normally does not partake in HSP, yet alters the properties of HSP, perhaps by shifting the balance of kinase and phosphatase activity. A better understanding of the molecular mechanisms underlying rapid HSP in acute hippocampal slices would aid in the creation of candidate proteins.

Recently, a study in dissociated hippocampal neurons provided possible clues into the identity of proteins involved with our metaplastic interaction. Okuno et al (2012)

followed up on the paradoxical findings that expression of the immediate early gene Arc weakens excitatory synapses (Rial Verde et al., 2006) and is required for mGluR-LTD (Park et al., 2008; Waung et al., 2008), NMDAR-LTD (Plath et al., 2006) and homeostatic plasticity (Beique et al., 2011; Chowdhury et al., 2006), yet is strongly expressed following LTP induction (Messaoudi et al., 2007; Steward et al., 1998; Ying et al., 2002). The authors stimulated neurons with BDNF for 2 hours, a treatment that results in potentiation of synapses and drives Arc expression (Okuno et al., 2012; Ying et al., 2002). Following BDNF-LTP, activity in the cultures was blocked using TTX or CNQX+APV for 2 hours. The cultures which underwent glutamate receptor or action potential blockade exhibited greater Arc accumulation at PSD95+ puncta than cultures treated with no drugs. The authors also found that Arc expression correlated with CaMKIIß expression and that Arc preferentially targets CaMKIIß under conditions of reduced Ca^{2+}, a finding they used to suggest Arc targets inactive synapses containing CaMKIIß and weakens them. The function of this event was proposed to prevent synaptic enhancement only at weak synapses (Okuno et al., 2012). Taken together, the investigators have created an experiment in dissociated cultures whereby LTP induction is followed by a potential homeostatic strengthening induction protocol, as CNQX treatment alone can produce homeostatic strengthening in a similar frame used in the Okuno et al (2012) study (2 hour blockade). Interestingly, enhancing activity (which may promote homeostatic weakening) showed no such effect on Arc accumulation. Additionally, Arc upregulation was enhanced in an input-specific manner, as expression of tetanus toxin to inhibit activity at a small number of presynaptic inputs begat greater changes at those inputs than their neighbors. These results indicate that following LTP,

Arc is targeted to inactive inputs where it may promote weakening of synaptic transmission. If this is occurring in our experiments, then it could explain why we found diminished homeostatic strengthening following LTP, as unmasked by application of protein synthesis inhibitors (Chapter 3). This process would be competing with HSP at the same inputs. If this were the case, then blocking Arc induction, which could be done with available Arc KO mice (Park et al., 2008) should also prevent the observed interaction. CaMKIIß would also be a candidate interaction protein since it associates with Arc under these conditions (Okuno et al., 2012). A molecule acting in a similar fashion as Arc, but in the opposite scenario, would make an excellent candidate protein for the LTD-homeostatic weakening interaction, but the identity of such a candidate remains elusive.

The work collected here demonstrates a distinct form of synaptic compensation operating in an intact hippocampal circuit. The rapid, input-specific properties of this process permitted an exploration into the nature of its interactions with Hebbian synaptic plasticity in acute hippocampal slices. I have revealed a novel type of metaplasticity that crosses plasticity domains and requires de novo local protein synthesis. The functional impact of such interactions is unclear. A reasonable hypothesis might be that it serves as an additional layer of protection from saturating synaptic transmission. My investigations addressed a major theoretical problem in the synaptic plasticity field, although my work is by no means exhaustive. Using our approach, other potential interactions could be investigated. Future studies into the molecular underpinnings of the metaplastic interactions uncovered here are also needed. A major conclusion from my thesis work is that HSP, along with Hebbian plasticity, has a profound effect on the net

efficacy of neurotransmission, arguing that HSP is a critical component of the synaptic mechanisms underlying memory in the brain. As important as the actual findings presented here, I have also demonstrated the usefulness of this approach for studying metaplasticity. A combination of multiple approaches and techniques will be needed to fully understand how dynamic neural networks can encode the memories we cherish so much, and perhaps more importantly, how we can protect this capacity from neurological disease.

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5.5 Figure Legends

Figure 5.1— S6K participates in homeostatic strengthening of CA1 synapses. Sprague Dawley rats, aged 2-3 weeks, were decapitated and the hippocampal lobules were rapidly isolated in artificial cerebral spinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2P0_4, 26.3 $NaHCO_3$, 11 glucose, 1.3 $MgSO_4$, and 2.5 $CaCl_2$. Transverse slices (400 µm) of the hippocampus were cut using a tissue chopper (Stoelting). Slices were then incubated at room temperature in a humidified interface chamber for at least 2 hours before recording. Hippocampal slices were transferred to a recording chamber, maintained at 26-28°C and continuously perfused at 1-2 ml/min with oxygenated aCSF. Area CA1 was visualized with an Olympus SZ51 dissecting microscope, which was also used for electrode placement. Recording electrodes were pulled from borosilicate capillary glass and filled with aCSF. The recording pipette was placed in the stratum radiatum of CA1 and bipolar stimulating electrodes (FHC) were placed on either side of the recording site. fEPSP recordings were made with a MultiClamp 700B amplifier, collected using Clampex 10.2, and analyzed using Clampfit 10.2 (Molecular Devices). Current between 0.02-0.25 mA for 0.1s was delivered with an ISO-flex stimulus isolator (AMPI). For experiments, current was set to elicit 50% of the maximum response. We verified pathway independence by applying two pulses with a 50 msec interpulse interval to the two pathways and screening for less than 10% paired-pulse facilitation. Following 30 minutes stable baseline, (A) the S6K inhibitor PF4708671 [10uM; Tocris] or (B) the mTOR inhibitor rapamycin [100nM; LC Labs] was applied for 30 minutes prior to induction of homeostatic strengthening. Column graphs represent normalized fEPSP slopes 90 minutes into HSP.

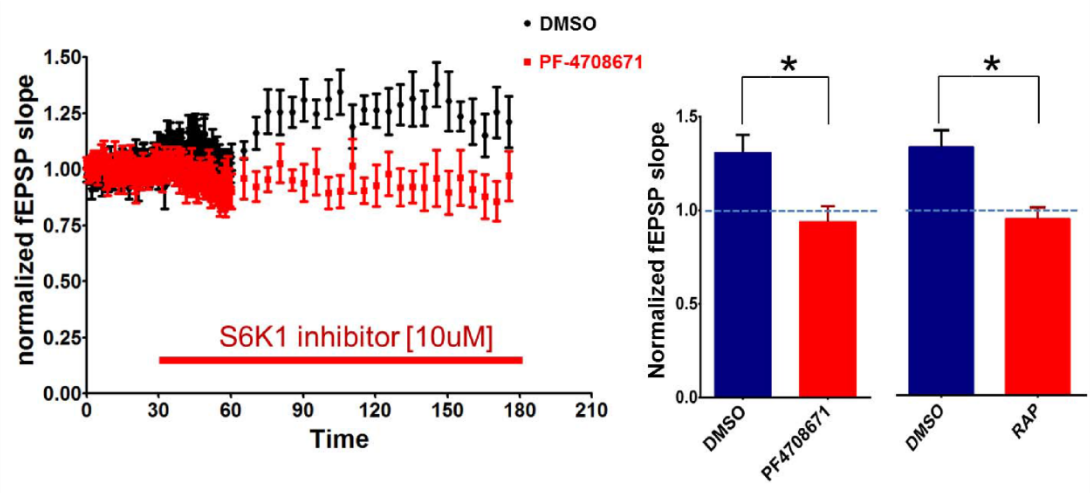

DMSO
PF-4708671
normalized fEPSP slope
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
30
60
90
120
150
180
210
Time
S6K1 inhibitor [10uM]
Normalized fEPSP slope
1.5
1.0
0.5
0.0
*
*
DMSO
PF4708671
DMSO
RAP

Figure 5.2— Phosphorylation of T840 GluA1 regulated by frequency-shifts in slice.

(A) Treatment of hippocampal slices with the PP1/PP2A inhibitor cantharidin (10uM) increases basal T840 GluA1 phosphorylation throughout stratum radiatum. (B) Treatment of slices with the S6K inhibitor PF4708671 (5uM) decreases basal T840 GluA1 phosphorylation. In (A) and (B), acute hippocampal slices were sectioned and incubated as described in Chapter 2. Following a 2 hour incubation period, slices were moved to one of two submersion chambers, containing bubbling aCSF with DMSO vehicle control or drug for 1 hour, and then immediately fixed with 4% PFA. After re-sectioning the slices, they were probed with p-T840 GluA1 and MAP2 primary antibodies. Stained sections were imaged on an epifluorescent microscope with identical setting between groups and stitched together using ImageJ software (NIH). (C) Induction of homeostatic weakening for 30 minutes following a stable 30 minute baseline in one-pathway experiments leads to a decrease in p-T840 immunofluorescence compared to slices constantly stimulated at baseline frequency. (D) We confirmed the finding from Delgado et al. (2007) that treatment of slices with NMDA for 5 minutes produced a significant decrease in p-T840 immunofluorescence compared to matched controls treated with vehicle ($n=3$; $P < 0.05$; white box represents area of quantification).

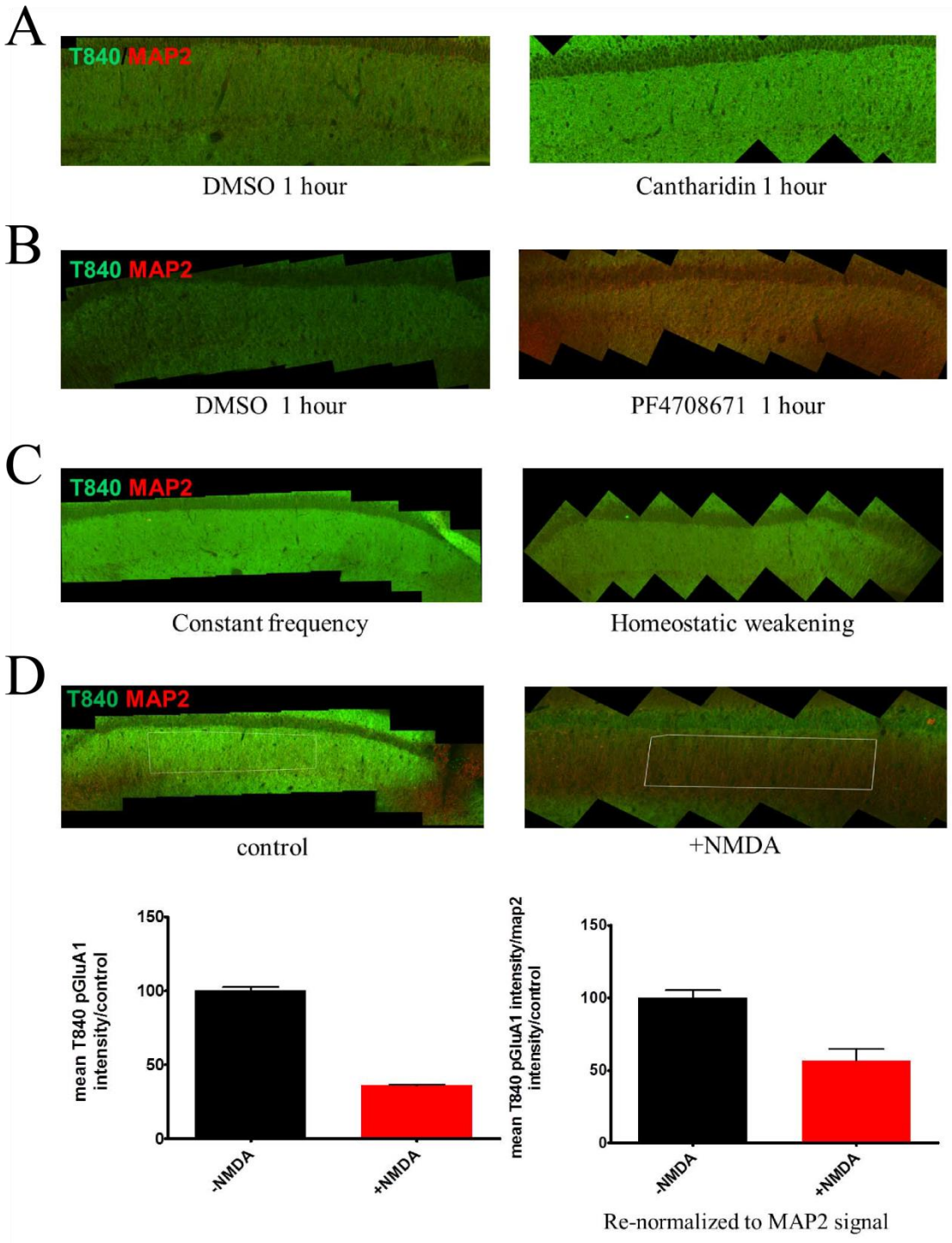
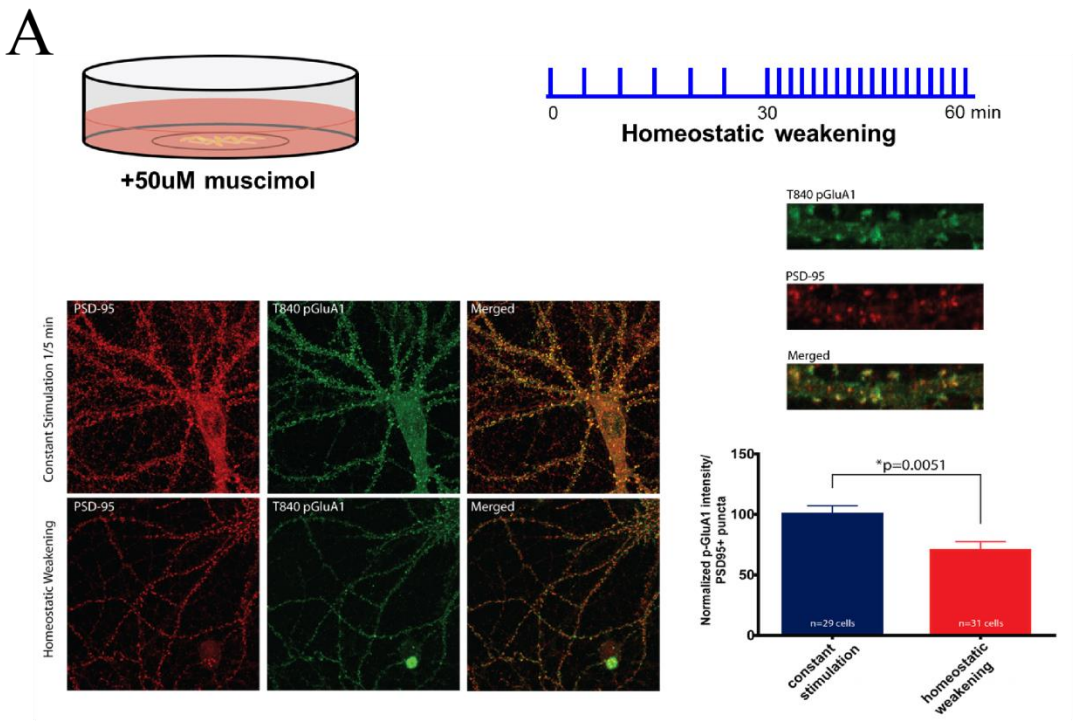

A
T840 MAP2
DMSO 1 hour
Cantharidin 1 hour
B
T840 MAP2
DMSO 1 hour
PF4708671 1 hour
C
T840 MAP2
Constant frequency
Homeostatic weakening
D
T840 MAP2
control
+NMDA
mean T840 pGluA1 intensity/control
150
100
50
0
-NMDA
+NMDA
mean T840 pGluA1 intensity/map2 intensity/control
150
100
50
0
-NMDA
+NMDA
Re-normalized to MAP2 signal

Figure 5.3— Translation regulatory signal transduction pathway involvement with the metaplastic interaction. Dissociated hippocampal cultures (DIV 18-22) were stimulated for 60 minutes with a field stimulator driven by an ISO-Flex stimulus isolator in Hepes-buffered saline containing 50uM muscimol. In (A), cultures were either constantly stimulated at a rate of one pulse every 5 minutes for 60 minutes or switched to a frequency of one pulse every 20 seconds after a 30 minute baseline period to mimic the homeostatic weakening protocol. In (B), cultures were either constantly stimulated at a rate of one pulse every 20 seconds for 60 minutes or switched to a frequency of one pulse every 5 minutes after a 30 minute baseline period to mimic the homeostatic strengthening protocol. Following stimulation, neurons were fixed with 2% PFA and probed with p-T840 GluA1 and PSD95 primary antibodies. A redirected particle analysis (custom ImageJ macro) was performed to quantify the intensity of p-T840 GluA1 signal only at PSD95+ puncta.


A
+50uM muscimol
0
30
60 min
Homeostatic weakening
T840 pGluA1
PSD-95
Merged
Constant Stimulation 1/5 min
Homeostatic Weakening
Normalized p-GluA1 intensity/ PSD95+ puncta
*p=0.0051
n=29 cells
n=31 cells
constant stimulation
homeostatic weakening

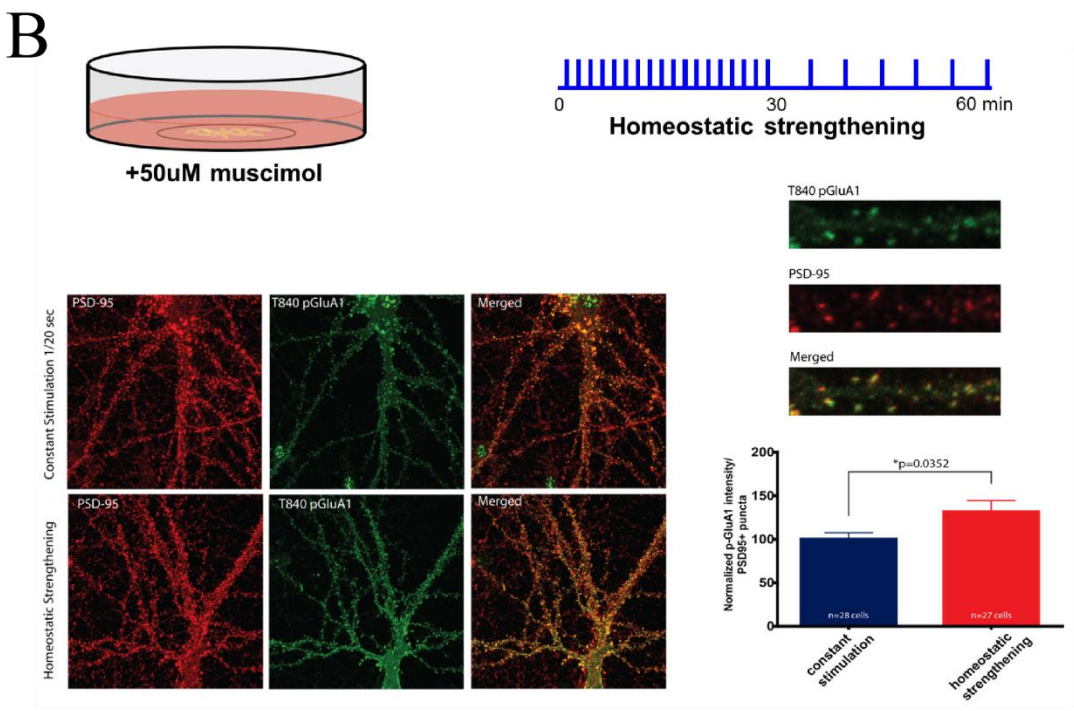
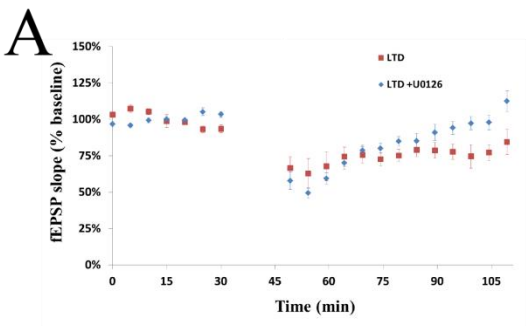

B
+50uM muscimol
0
30
60 min
Homeostatic strengthening
T840 pGluA1
PSD-95
Merged
Constant Stimulation 1/20 sec
Homeostatic Strengthening
Normalized p-GluA1 intensity/ PSD95+ puncta
*p=0.0352
n=28 cells
n=27 cells
constant stimulation
homeostatic strengthening

Figure 5.4— Translation regulatory mechanisms underlying the metaplastic interaction. (A) Treatment of hippocampal slices with the ERK inhibitor U0126 [20uM; Tocris] blocks NMDAR-dependent LTD induced with 900 pulses at 1Hz; n=6 (LTD), 10 (LTD+U0126). (B) In 2-pathway experiments, LTD was induced in one path only, followed by induction of homeostatic weakening in both pathways. Prior LTD constrains the expression of homeostatic weakening. Treatment of hippocampal slices with the eEF2K inhibitor NH125 [10uM; Tocris] does not alter the relative magnitude of homeostatic weakening following LTD induction compared to slices treated with DMSO vehicle.


A
150%
125%
100%
75%
50%
25%
0%
fEPSP slope (% baseline)
LTD
LTD +U0126
0
15
30
45
60
75
90
105
Time (min)

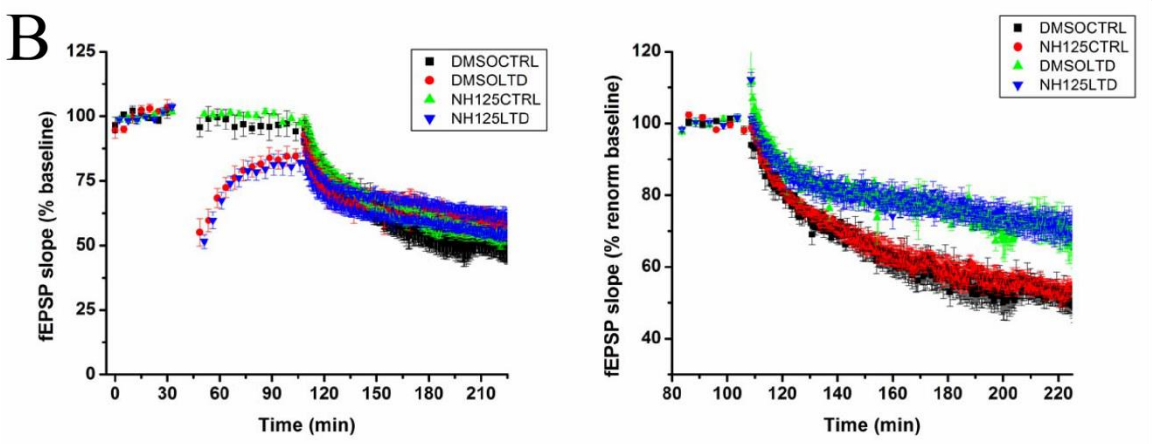
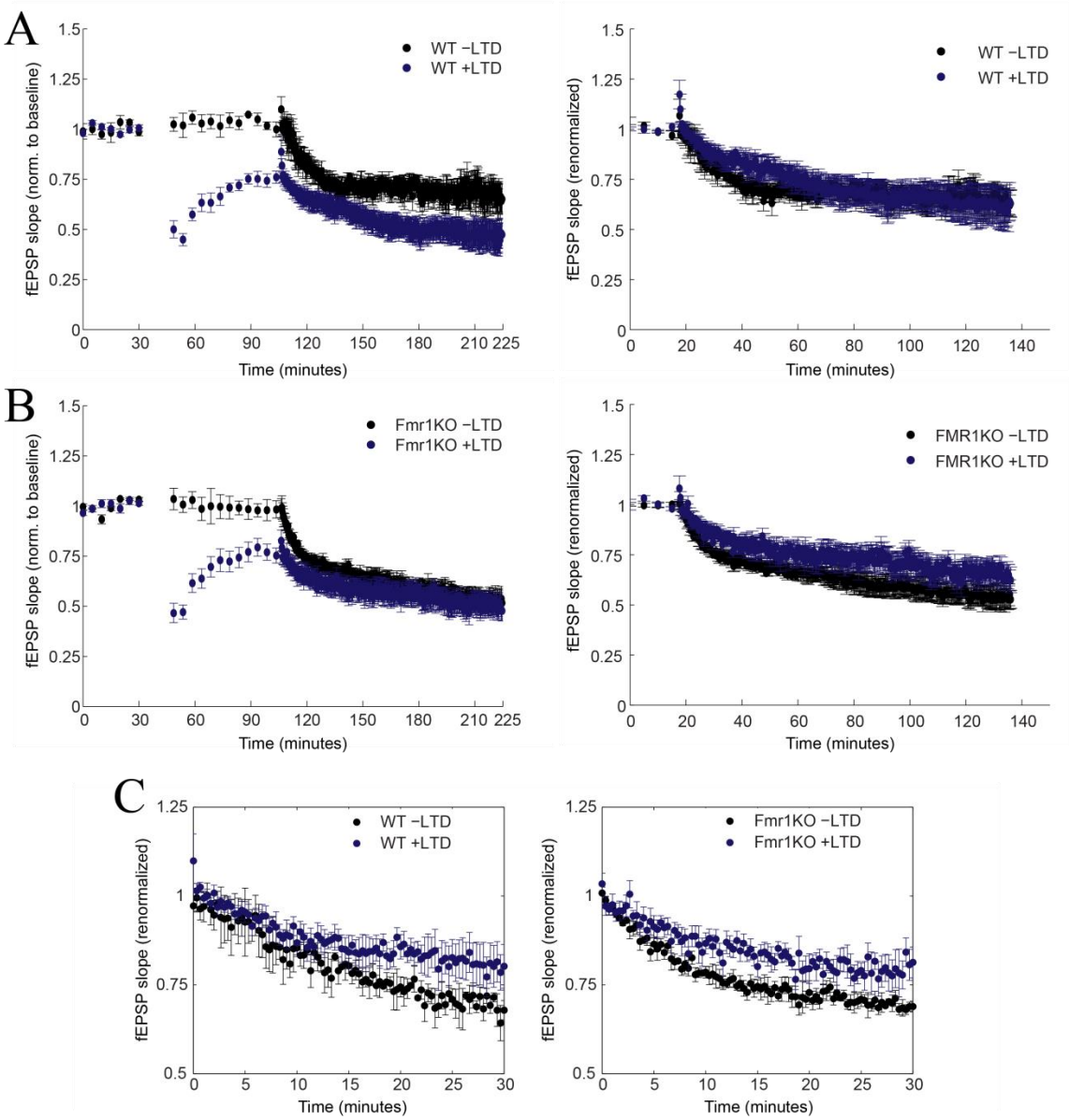

B
125
100
75
50
25
0
fEPSP slope (% baseline)
DMSOCTRL
DMSOLTD
NH125CTRL
NH125LTD
0
30
60
90
120
150
180
210
Time (min)
120
100
80
60
40
fEPSP slope (% renorm baseline)
DMSOCTRL
NH125CTRL
DMSOLTD
NH125LTD
80
100
120
140
160
180
200
220
Time (min)

Figure 5.5— Fmr1KO mice and WT littermates fail to exhibit a pronounced metaplastic interaction. Acute hippocampal slices were prepared from Fmr1KO mice and their WT littermates, as described previously (Iliff et al., 2013). These slices underwent the protocol used in Chapter 3 (see Fig. 3.3C) to demonstrate a metaplastic interaction between NMDAR-LTD and homeostatic weakening. Only experiments exhibiting LTD > 20% were used to probe for a potential interaction. (A) CA1 synapses from WT littermates fail to exhibit a pronounced metaplastic interaction detected under different conditions (see Chapter 3) and in unrelated mouse lines (data not shown). Left, data normalized to the initial baseline. Right, data renormalized to the 20 minute period prior to the induction of homeostatic weakening. (B) CA1 synapses from Fmr1KO mice also fail to exhibit a pronounced metaplastic interaction, similar to WT mice. Left, data normalized to the initial baseline. Right, data renormalized to the 20 minute period prior to induction of homeostatic weakening. (C) An exploded view of the renormalized data projecting the initial 30 minutes of homeostatic weakening. This comparison suggests slightly weaker, but not significant, HSP following LTD in the Fmr1KO mice compared with WT littermates. The weak interaction at WT synapses suggests optimization of recording conditions may be required before satisfactory analysis of FMRP involvement. In addition, the number of slices used in the analysis was fewer than typically used so further experiments are needed to form any strong conclusion (n = 3-6 slices per condition).


A
WT −LTD
WT +LTD
fEPSP slope (norm. to baseline)
Time (minutes)
fEPSP slope (renormalized)
B
Fmr1KO −LTD
Fmr1KO +LTD
FMR1KO −LTD
FMR1KO +LTD
C