Homeostatic Metaplasticity in Hippocampal Neuronal Networks

by

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DEDICATION

I dedicate my dissertation work to my family and friends. Thank you for your unwavering support throughout this process.

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ABSTRACT

Synaptic scaling is a form of homeostatic plasticity that initiates compensatory adaptations in synaptic strength to buffer chronic aberrant levels of activity within neural circuits. L-type voltage-gated Ca^{2+}- channels (LTCCs) play a key role in the induction of this process as evident in the regulation of scaling mediated by LTCC signaling blockade with dihydropyridine antagonists. These agents, however, do not distinguish between the two LTCC subtypes $Ca_V1.2$ and $Ca_V1.3$ expressed in the brain. Outlined in Chapter 2, we investigated the unique roles of these LTCC subtypes and found that the deletion of $Ca_V1.2$ in excitatory neurons induced a significant increase in basal synaptic strength and surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) whilst occluding TTX-induced synaptic upscaling. By contrast, TTX-induced upscaling of miniature excitatory postsynaptic currents (mEPSCs) is lost in $Ca_V1.3$ deficient neurons, with no alterations in basal synaptic properties accompanying $Ca_V1.3$ deletion. In addition to mechanisms that induce synaptic scaling, we investigated whether previous homeostatic functional alterations reverse upon activity renormalization and whether a previous history of homeostatic scaling in networks altered subsequent homeostatic responses to chronic activity manipulations. We identified a novel "resetting" phase of synaptic scaling whereby homeostatic changes in synaptic strength revert to basal levels after activity renormalization. Furthermore, future synaptic scaling in response to the same, and even opposite, activity challenges is robustly suppressed by a prior history of scaling in hippocampal neurons. This history-dependent suppression is specific to

homeostatic plasticity as networks with prior scaling history showed no deficits in Hebbian forms of synaptic potentiation (cLTP). We further demonstrated that hippocampal neurons with a prior history of synaptic scaling exhibited widespread alterations in activity-dependent transcriptional regulation despite normal engagement of activity-dependent signaling through the ERK/MAPK signaling pathway. Taken together, our data suggests that LTCC subtypes, Cav1.2 and Cav1.3, play nonredundant roles in the induction of synaptic scaling and that the history of homeostatic signaling in neural circuits plays a key role in shaping future compensatory adaptations to chronic changes in network activity.

CHAPTER I

Introduction

Significance of synaptic plasticity within the brain

Our experiences to an external environment, whether they are the warmth of a summer day, a stressful grizzly bear encounter, or a delightful Omakase lunch, all emerge from neural representations encoded by specific circuits in the brain. While stereotyped connections of specific neuron types are defining features of neural circuits, the ability of those connections to become stronger or weaker, or remodeled after different experiences allows us the ability to adapt successfully in a changing environment. This remodeling of neural connections is referred to as "synaptic plasticity," and is one of the most fascinating characteristics of the mammalian brain because it allows neural activity generated by experience to shape neural circuit architecture and thereby neural representations of thoughts, feelings, and behavior. Indeed, intense research over the last 50 years has revealed a central role for synaptic plasticity in neural development (Chen & Tonegawa, 1997), learning and memory (Neves et al., 2008; Maren, 2003), and the etiology of several neuropsychiatric disorders (Lau & Zukin, 2007; Nanou & Catterall, 2018). Thus, elucidating the underlying molecular mechanisms of synaptic plasticity in a variety of model organisms and brain regions is critical for understanding the neural basis of normal healthy and pathological brain function.

Given the diverse roles for synaptic plasticity, it is not surprising that many distinct forms have been formally described that differ in their functional role, temporal dynamics, and underlying molecular mechanism. Emerging from this heterogeneity of synaptic remodeling mechanisms is one of the major conundrums in Neuroscience – How do all these distinct forms of synaptic plasticity work in concert to maintain proper brain function? This question becomes exceedingly complex because the potentiation or depression of synaptic neurotransmission by activity can happen within very wide temporal domains, ranging from milliseconds to days and presumably years for long-term memory (Zenke et al., 2017; Zenke & Gerstner, 2017). As such, a major factor distinguishing between different forms of synaptic plasticity is whether functional changes are relatively transient (short-term plasticity; milliseconds to minutes) or durable (long-term plasticity; hours to years).

Short-term synaptic plasticity

At chemical synapses, an action potential propagates down the axon and activates specific voltage-gated Ca^{2+} channels (VGCCs) at the axon terminal, the presynaptic region of the synaptic connection between neurons. The ensuing influx of Ca^{2+} ions through VGCCs trigger the rapid release of synaptic vesicles containing neurotransmitter, which diffuse a short distance before being detected by receptors on the postsynaptic cell. Unbound neurotransmitters in the synaptic cleft are either degraded by specific enzymes (Fon & Edwards, 2001) or recycled by specific neurotransmitter transporters (Murphy-Royal et al., 2017), preventing sustained postsynaptic stimulation after neurotransmitter release. Ultimately, presynaptic signaling elicits changes in ion permeability in the postsynaptic neuron leading to postsynaptic currents (PSCs)

and postsynaptic potentials (PSPs) that are integrated with other signaling to alter postsynaptic output.

The process described above is subject to several forms of dynamic modulation on the order of milliseconds to minutes. Strengthening of the postsynaptic response following presynaptic tetanic stimulation, or short-term synaptic enhancement (STE) comes in many different flavors and its four components are categorized based on the synaptic efficacy decay time constants: fast-decaying facilitation (F1; tens of milliseconds), slow-decaying facilitation (F2; hundreds of milliseconds), augmentation (AUG; thousands of milliseconds), and post-tetanic potentiation (PTP; tens of seconds) (Fisher et al., 1997). Genetic, pharmacological, and behavioral evidence from multiple model systems including intracellular recordings in Aplysia (Sánchez & Kirk, 2000), miniature endplate potential (MEPP) recordings in rat (Nussinovitch & Rahamimoff, 1988) and frog (Zengel & Magelby, 1980) neuromuscular junction (NMJ), and paired whole-cell patch recordings in central synapses of hippocampal slices (Stevens et al., 1994) indicate that these forms of STE are highly conserved. In parallel to enhancement, repetitive activation can also lead to short-term synaptic depression which is largely thought to be due to the depletion of vesicles within the readily releasable pool (Betz, 1970). Given that presynaptic stimulation can result in either short-term enhancement or depression, an obvious question arises – what are the underlying deterministic molecular mechanisms? Since these seminal studies, three quantal parameters have been characterized and are now commonly used to define key properties of synaptic transmission: 1) the maximum number of vesicles released by an AP or the number of functional release sites (n), 2) the probability of vesicular release (p), and the amplitude of the postsynaptic response following the release of a single neurotransmitter vesicle (q).

The role of calcium in short-term plasticity

The crucial role of calcium in STE and the residual Ca^{2+} hypothesis was first proposed in the landmark paper of Katz and Miledi (Katz & Miledi, 1968). They proposed that short-term facilitation at the NMJ is induced by residual calcium after nerve stimulation which they tested using a paired-pulse stimulation paradigm and a Ca^{2+}-free ringer solution. Upon removal of external Ca^{2+} during the initial conditioning pulse, Katz & Miledi observed no significant short-term facilitation at NMJ synapses despite Ca^{2+} being present during the secondary test pulse. This result suggested that there exists some amount of calcium at the nerve terminal after the initial conditioning pulse that persists and interacts with Ca^{2+} influx from the second test pulse and that the increased Ca^{2+} levels is responsible for the facilitation of synaptic transmission. Subsequent studies confirmed the role of residual Ca^{2+} in F1, F2 (Kamiya & Zucker, 1994), AUG (Magelby & Zengel, 1976; Delaney et al., 1994), and PTP (Kretz et al., 1982; Swandulla et al., 1991).

Upon further investigation of the original residual Ca^{2+} model, novel studies confirmed the necessity of expanding it to incorporate both spatial and temporal characteristics of Ca^{2+} ion concentrations; dubbed the spatiotemporal model. Numerous studies have implicated the existence of transient microdomains of elevated Ca^{2+} upon AP arrival at the presynaptic terminal (Llinás et al., 1992; Sugimori et al., 1994; Berridge, 2006), and that there is a dramatic drop off in concentration of the surrounding area within the nerve terminal. Additionally, even a single open VGCC generates a discrete influx of Ca^{2+} centered on its pore (Chad & Eckert, 1984). Collectively, these two models indicate that short-term synaptic plasticity is reliant on the timing and location of Ca^{2+} ions as well as the activity-dependent history within the nerve terminal.

Long-term synaptic plasticity

In contrast to more transient modifications of synaptic neurotransmission, it is widely believed that experience can also profoundly modify subsequent behaviors through long-lasting alterations of synaptic strength. Experience is encoded within the brain as complex events of spatiotemporal patterns of activity in a large ensemble of neurons, or neural circuits. Information is stored in the brain when activity within a circuit results in long-lasting changes in the pattern of synaptic weights. This idea was further refined by Donald Hebb who postulated that associative memories are formed in the brain through the process of strengthening synaptic connections when presynaptic activity correlates with postsynaptic firing (Hebb, 1949). Moreover, the anticorrelation is thought to weaken synaptic connections and result in failure to form the specific associative memory. This form of synaptic plasticity, predicated on the detection of two coincident events, has since been termed "Hebbian" synaptic plasticity and has been intensely studied for its role in the formation of associative memories such as those established by Pavlovian classical conditioning (Pavlov, 1927).

The first experimental evidence for long-lasting activity-dependent changes in synaptic strength emerged in the 1970s from the landmark studies by Bliss, Lomo and their colleagues in Per Anderson's laboratory (Bliss & Lomo, 1973; Bliss & Gardner-Medwin, 1973). These studies, conducted in anesthetized rabbits, demonstrated robust potentiation of postsynaptic responses recorded from granule cells in the dentate gyrus after repetitive stimulation of the afferent perforant pathway. This potentiation was determined by the increases in amplitude of the population excitatory post-synaptic potential (EPSP) and increases in the amplitude and latency of the population spike. In contrast to the fast-decaying kinetics of STE, the synaptic potentiation reported by Bliss and Lomo was far more durable, lasting as long as 10hrs (Bliss & Lomo, 1973)

to 3 days (Bliss & Gardner-Medwin, 1973) after induction. This phenomenon, now termed long-term potentiation (LTP), has been the subject of intense investigation because it is believed to capture important aspects of the cellular mechanism of long-term memory formation. Importantly, LTP is complemented by several forms of long-term depression (LTD), enabling bidirectional malleability within neural circuits. Heterosynaptic LTD was first observed in 1977 where Lynch et al. reported a generalized depression of synaptic efficacy after LTP induction in one set of synapses (Lynch et al., 1977) and has been reliably observed in the dentate gyrus (Abraham & Goddard, 1983) and CA1 (Abraham & Wickens, 1991) regions of the hippocampus. Furthermore, it has become increasingly clear that LTP and LTD are only terms that describe classes of synaptic plasticity; the underlying molecular mechanism vary depending on the organism and the circuits in which they function and the specific induction stimulus protocol used.

A defining feature of LTP at many synapses is its reliance on N-methyl-D-aspartate receptors (NMDARs), a ligand-gated ion channel that requires simultaneous binding of glutamate and postsynaptic membrane depolarization to achieve maximal conductance and is thus thought to serve as a molecular coincidence detector. NMDA receptors provide a molecular basis for encoding correlative activity between neurons through the detection of two temporally close and spatially distributed signaling events, a key feature in Hebbian plasticity. Moreover, pharmacological disruption of NMDAR-signaling with APV (Morris et al., 1986) or MK-801 (Shapiro & Caramanos, 1990) inhibits LTP induction and impairs hippocampal-dependent spatial learning. Importantly, there is also sufficient evidence of synaptic LTP induction that does not require NMDAR activation. For example, 100 μM APV treatment during conditioning stimulus had no effect on the long-lasting potentiation of EPSPs recorded from layer II/III

neurons of kitten visual cortex (Komatsu et al., 1991). Additional evidence of NMDAR-independent LTP can be found within the hippocampus, where mossy fiber projections onto CA3 pyramidal neurons exhibit a presynaptic form of LTP that is independent of NMDAR activation (Dahl et al., 1990). Interestingly, it has been shown that within the same synapses in CA3-CA1 hippocampal neurons, varying stimulation protocols result in distinct forms of LTP; high-frequency stimulation (HFS) (Harris et al., 1984; Coolingridge et al., 1983; Morris et al., 1986) or theta-burst stimulation (TBS) (Nguyen & Kandel, 1997) induces NMDAR-dependent LTP whereas 200Hz tetanic stimulation (Grover & Teyler, 1990; Grover, 1998) or activation of metabotropic G-protein coupled receptors induces NMDAR-independent LTP.

We now know that the complexity of long-term plasticity extends past the immediate output of strengthening or weakening of synapses. As with short-term plasticity, LTP and LTD have also been dissected temporally with conclusive evidence indicating that these phenomena are multiphasic. It is widely accepted that LTP can be divided into at least three different phases: early LTP (E-LTP), intermediate LTP (I-LTP) and late LTP (L-LTP). E-LTP and L-LTP were first distinguished based on the observations that several high-frequency stimulus trains produced larger and more durable potentiation than a single stimulus train, an effect that was abolished in the presence of protein synthesis inhibitors (Krug et al., 1984; Frey et al. 1988). This late stage of LTP was later found to also depend on D1 dopamine receptors (Huang & Kandel, 1995; Frey et al., 1991), cAMP (Nguyen et al., 1994), PKA (Matthies & Reymann, 1993; Huang & Kandel, 1994; Abel et al., 1997), ERK (Winder et al., 1999; Schafe et al., 2008), and CREB phosphorylation (Impey et al., 1996). I-LTP was later described to require PKA but not protein synthesis (Winder et al., 1998). Collectively these data suggest that unique patterns of stimulation evoke multiple phases of long-lasting synaptic plasticity that can be distinguished by

both their persistence and underlying molecular mechanisms. In this respect, parallels can be drawn between LTP and STE in that both forms of plasticity exhibit unique temporal variants that are mechanistically distinct. This feature also seems to be highly conserved, as a variety of model synapses in a range of organisms exhibit distinct forms of enduring plasticity that persist for varying periods depending on the pattern of stimulation delivered for induction; long-term facilitation (LTF) in *Aplysia californica* following repeated spaced shocks or 5-HT pulses last >24hrs (Walters et al., 1983; Buonomano et al., 1990), LTF of miniature excitatory junctional potentials (mEJPs) in *Drosophila melanogaster* larvae NMJ with >5Hz stimulation persists for tens of seconds (Jan & Jan, 1978), and LTF of EJPs in the proximal muscle fibers of *Procambarus clarkii* elicited following 10 minute tetanic train at 20Hz last >1hr (Beaumont et al., 2001).

The role of calcium in long-term plasticity

Alike STE, the induction of LTP mechanisms is heavily reliant on Ca^{2+}-mediated signaling. Beyond its presence during neurotransmission, Ca^{2+} role as a second messenger is crucial in triggering complex signaling cascades required for both LTP and LTD. The directionality of plasticity is determined by three characteristics of Ca^{2+}: the amplitude, duration, and location, reviewed in (Evans et al., 2015). The calcium amplitude hypothesis, also known as the two-threshold hypothesis, was the first proposed (Lisman, 1989; Artola et al., 1990; Artola & Singer, 1993). It posited that a moderate but sufficient Ca^{2+} concentration is necessary for LTD and that higher levels are necessary for LTP. Evidence of this has been shown with imaging studies of Ca^{2+} concentration in layer 2/3 pyramidal cells of rat visual cortical slices during application of either HFS or LFS. It has been shown that calcium peak signals were the highest

during the HFS protocol and lower during the LFS protocol which resulted in LTP and LTD, respectively, synaptic changes that were completely abolished upon the addition of Ca^{2+} chelators (Hansel et al., 1996). The two-threshold hypothesis was more directly tested by manipulating extracellular calcium concentration in the hippocampus during frequency-dependent plasticity. Lowering extracellular calcium induced LTD in CA1 pyramidal cells despite using a stimulation protocol that previously elicited LTP (Mulkey & Malenka, 1992). Spike-timing dependent plasticity (STDP) provides further evidence for the Ca^{2+} amplitude model. LTP is elicited when pre-synaptic stimulation precedes post-synaptic action potential whereas LTD is induced when post-synaptic AP precedes pre-synaptic stimulation (Bi & Poo, 1998). The former was demonstrated to trigger a higher elevation in calcium concentration within single spines on basal dendrites of neocortical layer 5 pyramidal neurons (Koester & Sakmann, 1998).

This initial hypothesis was built upon with observations that Ca^{2+} mediated signaling is not as simple as just high or low concentration. The duration of calcium transients also plays a critical role in determining whether neural circuits undergo LTP or LTD. Evidence of this phenomenon emerged from Ca^{2+} uncaging experiments that allowed experimenters precise temporal control of Ca^{2+} elevation. Uncaging of a calcium compound, nitrophenyl-ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid, in CA1 pyramidal neurons, Yang et al., demonstrated that LTP can only be triggered by a brief and relatively high magnitude (>10μM for a few seconds) of Ca^{2+} elevation whereas LTD is induced by a prolonged and modest (~750nM for 1 minute) rise (Yang et al. 1999).

Lastly, there is evidence that the location of calcium entry also determines the direction of plasticity. Indication that the source of calcium is vital stems from experimental evidence that

demonstrates channel specificity during LTP and LTD. For example, within the striatum, Ca^{2+} influx through NMDARs is necessary for LTP whereas through L-type voltage gated calcium-channels (VGCCs) is necessary for LTD (Fino et al., 2010; Shindou et al., 2011). In contrast, within the hippocampus, NMDAR-mediated Ca^{2+} signaling is required for both LTP and LTD while L-type VGCCs is only responsible for the latter (Bi & Poo,1998). In addition to brain region differences, the cellular location of calcium influx is also crucial. Liu and colleagues demonstrated that TBS induces LTD in CA1 neurons of rat hippocampal slices by selectively blocking synaptic NMDARs with MK-801 (Liu et al., 2013). This piece of data suggests that the resulting plasticity is directly linked to which cohort of NMDARs are activated; activation of post-synaptic NMDARs induces LTP and activation of extrasynaptic NMDARs induces LTD despite equal electrical stimulation. Collectively, the accumulation of data over the span of several decades reveals the essential role of Ca^{2+} signaling in both short-term and long-term forms of plasticity although it has been suggested that glutamate binding to NMDARs alone and not Ca^{2+} influx is sufficient to induce LTD (Navabi et al., 2013). Moreover, these conclusions only scratch the surface. It is still unclear how calcium signaling through different calcium channels, in different brain regions, and even different compartments of the same neuron can have such profoundly different outcomes in synaptic efficacy.

Homeostatic control of neuronal excitability

Thus far, I have discussed forms of synaptic plasticity that are thought to play a role in information processing or storage in neural circuits. To provide consistent and stable representations, neural circuits must offset their malleability, crucial for information storage, with mechanisms that actively promote stability of network activity to maintain circuit function

in a useful dynamic range. This innate "homeostatic" control is not unique to the human brain as it has been a central concept of physiological systems, first described by Claude Bernade as 'la fixité du milieu intérieur', or the constancy of the internal environment (Claude, 1878). This concept was later coined, homeostasis (Cannon, 1926; Cannon, 1929), and has become a fundamental theme in modern biology that describes the tendency of readjustments such that an equilibrium or a steady state is achieved. Early studies of homeostatic mechanisms were primarily focused on the stability of blood constituents (Henderson & Haggard, 1918; Austin et al., 1922; Perlman, 1977) with further evidence of homeostasis developed later in studies of muscle excitability observed during denervation super sensitivity (Axelsson & Thesleff, 1959). Although originally proposed to account for excitability changes in muscle, it is now clear that homeostatic mechanisms are pervasive in the central nervous system and are key to maintain proper functioning in neural circuits.

Homeostatic synaptic scaling

The first indication that central synapses exhibit homeostatic regulation was provided in 1998, in a now classic paper from Turrigiano and colleagues (Turrigiano et al., 1998). In this study, networks of cortical excitatory neurons exhibited bidirectional synaptic adaptations in response to long-term changes in activity. They showed that chronic (48hrs) activity silencing, via inhibition of voltage-gated Na^+ channels with tetrodotoxin (TTX), induced a homeostatic increase in miniature excitatory post synaptic current (mEPSC) amplitudes, a measure of basal synaptic function (Katz & Miledi, 1963; Colomo and Erulkar, 1968; Brown et al., 1979). Conversely, chronic (48hr) hyperactivation of neuronal networks, via blockade of gamma-Aminobutyric acid A receptors (GABAA), resulted in a decrease in mEPSC amplitude

(Turrigiano et al., 1998). This homeostatic adaptation in response to deviations in firing rate was further found to be multiplicative in nature; the distribution of mEPSC amplitudes appeared to shift by a constant "scaling" factor, which has led the field to dub this form of homeostatic plasticity, "synaptic scaling." These early findings initiated a novel field of neuroscience devoted to understanding this and other forms of homeostatic synaptic plasticity (HSP), based on the hypothesis that homeostatic adjustments of synaptic properties play a key role in stabilizing neural firing rates in the face of dynamic changes in neural circuits.

Synaptic scaling is the most heavily studied homeostatic mechanism at central synapses, but it is now evidently clear that scaling only represents one of many processes that neurons utilize to stabilize activity levels. Neurons also homeostatically regulate its intrinsic excitability (Marder & Goaillard, 2006; Zhang & Linden, 2003), ion conductance (Golowasch et al., 1999; Frank, 2014) and inhibitory synaptic transmission (Kilman et al., 2002; Swanwick et al., 2006). In addition, while synaptic scaling is most likely driven by changes in neural firing rate, other forms of HSP have been describe that are tuned to local synaptic drive (Sutton et al., 2006; Branco et al., 2008; Jakawich et al., 2010a; Lindskog et al., 2010). Synaptic scaling can be distinguished from these "local" HSP processes as well as from Hebbian forms of plasticity by three main characteristics. First, synaptic scaling is thought to occur in a global, cell-autonomous, and multiplicative manner that serves to adjust cell excitability while retaining synapse-specific information previously stored through other forms of plasticity such as LTP or LTD (Turrigiano & Nelson, 2000; Turrigiano & Nelson, 2004; Nelson & Turrigiano, 2008; Turrigiano, 2008; Rabinowitch & Segev, 2008; Turrigiano, 2012). While many studies, some of which are listed here, have demonstrated relatively uniform shifts in synaptic weights during scaling, a recent study (Hanes et al., 2020) has suggested a novel "divergent scaling" of weaker

synapses relative to stronger synapses on the same neuron, a finding that complicates the canonical view of homeostatic scaling as purely multiplicative in nature.

A second characteristic feature of synaptic scaling is its slow time course. While activity manipulations generally alter network function immediately, the compensatory synaptic adaptations that ensue require at least 18 hours to first emerge (Turrigiano et al., 1998; Watt et al., 2000; Sutton et al., 2006; Ibata et al., 2008). In contrast to synaptic scaling, there is a wealth of evidence of other existing HSP mechanisms induced by changes in synaptic drive (instead of firing rate) and act on more rapid timescales. A brief (~3hrs) blockade of NMDARs coincident with AP blockade scales up synaptic strength orders of magnitudes faster than AP blockade alone (Sutton et al., 2006; Jakawich et al., 2010a; Henry et al., 2012). Additionally, pharmacological inhibition of glutamate receptors with a noncompetitive blocker, GYKI 53655, of mossy fiber synapses in the mouse cerebellum rapidly (minutes) modulated presynaptic exocytosis, a form of presynaptic homeostasis (Delvendahl et al., 2019). Rapid forms of HSP have also been studied in the Drosophila neuromuscular junction (NMJ) where the blockade of postsynaptic AMPARs induced an acute homeostatic increase in presynaptic quantal content within minutes to offset the decrease in postsynaptic drive (Frank et al., 2006). Although synaptic scaling and local HSP are clearly unique with distinct underlying mechanisms, the existence of both forms of homeostatic plasticity suggest that neural circuits can maintain stability during activity perturbations across a wide temporal range. Likely, forms of HSP mechanisms exist on a spectrum varying from rapid to long timescales and its induction is dependent on a myriad of parameters including brain region, neuronal type, and activity perturbation.

A third characteristic of synaptic scaling that has been intensely studied, is the postsynaptic mechanisms required for the regulation of synaptic efficacy. There is, however, extensive research detailing homeostatic changes in presynaptic function in response to activity perturbations during synaptic scaling (Murthy et al., 2001; Burrone et al., 2002; Thiagarajan et al., 2005; Jakawich 2010). A multitude of studies in varying model systems and varying pharmacological manipulations have demonstrated adaptations of postsynaptic glutamate receptors after activity perturbation. Hyperactivation of hippocampal networks with picrotoxin resulted in the NMDAR-dependent removal of GluA1 at synapses (Lissin et al., 1998). This finding was corroborated in cultured spinal neurons where decreases in glutamatergic (APV + CNQX) or GABAergic (picrotoxin + strychnine) signaling resulted in the homeostatic accumulation and loss of GluA1, respectively (O'Brien et al., 1998). Additionally, silencing of visual cortical neurons with TTX (Weirenga et al., 2005) or with monocular TTX injections (Gainey et al., 2009) both resulted in the robust increase in GluA2 expression. Together, these findings suggest that postsynaptic glutamate receptor protein expression levels are homeostatically regulated during neuronal activity perturbation.

An immense amount of experimental attention has been focused on homeostatic postsynaptic mechanisms, but an equal amount of effort has been placed in understanding presynaptic mechanisms and have branched off into an entirely new field of study, presynaptic homeostatic plasticity (PHP). Distinct from synaptic scaling, accumulating evidence within studies using the model system, *Drosophila melanogaster*, indicate a homeostatic response upon pharmacological inhibition of glutamatergic signaling, akin to alterations of synaptic drive studies in central synapses (Sutton et al., 2006; Aoto et al., 2008; Hou et al., 2008; Jakawich et al., 2010; Lindskog et al., 2010). An acute perturbation with a GluR antagonist, philanthotoxin-

433 (Frank et al., 2006), or sustained genetic glutamate receptor impairment (GluRIIA ablation; Petersen et al., 1997) induces a homeostatic increase in neurotransmitter release. This precise compensation has led to the hypothesis that PHP involves a retrograde, trans-synaptic signaling system (Delvendahl & Müller, 2019). These studies also suggest that functional compensations at synapses are not necessarily limited to regulation of postsynaptic protein expression and that both pre- and postsynaptic regions are subject to activity-dependent alterations.

Synaptic scaling in the pathological brain

Evidence amassed over the past decade have indicated the significance of homeostatic plasticity mechanisms in both healthy and pathological brain function. Dysfunction of HSP has been implicated in a growing number of neurological disorders, a topic that has been reviewed extensively elsewhere (Wondolowski & Dickman, 2013). For example, loss of the FMR protein (FMRP) causes Fragile X Syndrome, an inherited neurodevelopmental disorder characterized by autism, intellectual disability, and hyperactivity. FMRP is also critical for the expression of retinoic acid-mediated homeostatic increases in AMPAR expression (Soden & Chen, 2010; Sarti et al., 2013) and homeostatic changes in intrinsic excitability driven by chronic changes in network activity (Bülow et al., 2019). Likewise, Rett syndrome is caused by loss of MeCP2, and MeCP2 has been shown to be necessary for homeostatic synaptic upscaling (Blackman et al., 2012; Qiu et al., 2012; Zhong et al., 2012; Della Sala & Pizzorusso, 2014). Tuberous sclerosis, a neurodevelopmental disorder caused by hyperactivation of the mTORC1 pathway, is also associated with dysregulated homeostatic plasticity (Henry et al., 2012; Bateup et al., 2013). Finally, we recently examined the role of retinoic acid induced 1 (RAI1) in synaptic scaling, loss of which causes Smith Magenis Syndrome, and found that it played a critical role in suppressing

synaptic scaling during normal levels of network activation (Garay et al., 2020). Taken together, these studies suggest that dysregulated synaptic scaling is a pathological hallmark of numerous neurodevelopmental disorders.

In addition to neurological disorders, synaptic scaling has also gained traction as a potential target mechanism for neuropsychiatric treatments. Recent studies of ketamine, a NMDAR antagonist, and lithium, a common treatment for depression and bipolar disorder, respectively, have reported to trigger synaptic scaling-like alterations in synaptic efficacy. Acute (1-3hrs) application of ketamine increases mEPSC amplitudes globally whereas chronic (10-11 days) treatment with lithium results in a marked downscaling of synaptic strength (Kavalali & Monteggia, 2020). Although ketamine and lithium treatments have very different mechanisms of action, both appear to initiate scaling of synaptic strength, albeit, in opposite directions.

Synaptic scaling has also been found to play a role in drug addiction. The majority of studies until recently have been focused on rapidly-inducing Hebbian forms of plasticity and its involvement in addiction-motivated behavioral and synaptic adaptations (Kauer & Malenka, 2007). Regulation of AMPAR expression during LTP and LTD has also been implicated in drug-seeking behaviors during cocaine re-exposure in the nucleus accumbens (Kourrich et al., 2007; Brebner et al., 2005; Anderson et al., 2008). More recently, upscaling of GluA1 and GluA2 containing AMPARs in the nucleus accumbens has been proposed as a mechanism for the slowly developing and prolonged plasticity changes during cocaine withdrawal (Boudreau & Wolf, 2005).

Connections between homeostatic synaptic plasticity dysfunction and pathological brain function are only starting to be revealed. Importantly, the molecular mechanisms that link disorder phenotypes with defective HSP are far from fully understood, illustrating the need for

continued research into this area. There is a general consensus, however, that defining those key points of molecular regulation will open up novel avenues for designing effective therapeutics against these disorders.

Homeostatic synaptic plasticity is observed in many brain regions and neurotransmitter systems

It is becoming increasingly clear that neurons, depending on type and brain region, exhibit and utilize many different plasticity tools to adapt to a wide variety of activity-dependent perturbations. Homeostatic plasticity phenotypes have been described in the hippocampus (Sutton et al., 2006), the cortex (Turrigiano et al., 1998), the spinal cord (Chub & O'Donovan, 1998; Galante et al., 2001; Wenner, 2014; Gonzalez-Islas et al., 2018), the nucleus accumbens (Ishikawa et al., 2009; Sun and Wolf, 2009), the cerebellum (Iijima et al., 2009), the locus coeruleus (Cao et al., 2010), and the striatum (Azdad et al., 2009). It has also been heavily studied in a variety of neurotransmitter systems including glutamate (Turrigiano et al., 1998; Sutton et al., 2006; Goel et al., 2011), GABA (Bartley et al., 2008; Saliba et al., 2009; Keck et al., 2011), glycine (Ganser & Dallman, 2009), endocannabinoid (Kim & Alger, 2010), and dopamine (Azdad et al., 2009; Sun and Wolf, 2009). The flexibility HSP affords neurons allows for malleable neuronal circuits to learn and undergo ongoing plasticity while simultaneously buffer destabilizing forces. However, many challenges lie ahead in understanding the interplay between these different homeostatic mechanisms in maintaining proper brain function.

The role of calcium in homeostatic synaptic plasticity

Ca^{2+} has a particularly important role in excitable cells like neurons as an intracellular second messenger. At rest, the intracellular calcium concentration of most neurons fluctuates around 50-100nM and can transiently increase multiple magnitudes of orders higher during electrical activity (Berridge et al., 2000). Unsurprisingly, there exists a host of different buffering mechanisms which help stabilize the concentration of free calcium ions within cells. Intracellular Ca^{2+} buffers generally refer to members of the EF-hand protein family, proteins with a structural domain consisting of an α-helix–loop–α-helix motif of approximately 30 amino acids (Schwaller, 2020). Hundreds (>600) of family members have been discovered with the prototypical Ca^{2+} sensor being calmodulin (CaM; Chin et al., 2000). Importantly, the majority of calcium buffers have dissociation constants for Ca^{2+} at least an order of magnitude higher than the average Ca^{2+} concentration at rest, indicating that these buffer mechanisms are generally in the unbound state (Schwaller, 2020). For this reason, Ca^{2+} is the ideal sensor candidate for electrical activity and has been theorized to be how neurons "sense" changes in intrinsic activity levels.

In recent years, voltage-gated Ca^{2+} channels (VGCC) have gained considerable recognition for their potential role in homeostatic control of synapse function. VGCCs mediate voltage-dependent calcium entry and regulate activity-dependent processes such as neurotransmission, gene transcription, and intracellular signaling cascades (Murphy et al., 1991; Catterall, 2000; Zamponi et al., 2005; Lipscombe et al. 2013). This family of cation channels can be subdivided into several subfamilies based on their pharmacological and physiological properties: L-type (Ca_V1), T-type (Ca_V3), P/Q-type, R-type, and N-type (Ca_V2) (Catterall, 2000). P/Q/N channels of the Ca_V2 family have been shown to gate homeostatic changes in presynaptic function at hippocampal synapses driven by loss of postsynaptic excitatory synaptic drive

(Jakawich et al., 2010; Henry et al., 2018). Similarly, loss-of-function mutations in the Drosophila cacophony (cac) gene, the pore-forming α1 subunit of Ca_V2 channels, prevent the homeostatic increase in quantal content at the NMJ induced by pharmacological or genetic impairment of postsynaptic glutamate receptor function (Frank et al., 2006; Müller & Davis, 2012). More recently, Ca^{2+} influx through P/Q-type, but not N-type VGCCs has been shown to mediate bidirectional homeostatic regulation of neurotransmitter release and size of synaptic vesicle pools in hippocampal neuron cultures (Jeans et al., 2017). In addition to a role for P/Q/N channels in presynaptic forms of synaptic compensation, LTCCs are thought to be critical for encoding chronic changes in neuronal activity that drive synaptic scaling. Several studies have demonstrated that the pharmacological blockade of LTCC signaling with dihydropyridines is sufficient to drive homeostatic upscaling of synaptic strength (Thiagarajan et al., 2005; Ibata et al., 2008; Henry et al., 2012) and block upscaling (Sokolova & Mody, 2008). Dihydropyridines, however, have several off-target effects and do not distinguish between the two major types of LTCCs expressed in the brain. I address this issue using a genetic approach in Chapter 2.

Calcium signaling during synaptic scaling

Downstream of calcium ion entry, exists many Ca^{2+} signaling cascades, some of which we are only starting to link to HSP within neurons. Calcium/calmodulin-dependent protein kinase type IV (CaMKIV) has been implicated as a key sensor kinase in excitatory synaptic scaling that senses perturbations in firing through changes in intracellular Ca^{2+} concentration. The compensations are manifested in changes in excitatory quantal amplitude (Ibata et al., 2008; Goold & Nicoll, 2010). Moreover, expression of dominant-negative and constitutively-active forms of nuclear CaMKIV bidirectionally induced excitatory synaptic scaling and intrinsic

plasticity with no impact on inhibitory quantal amplitude (Joseph & Turrigiano, 2017). Interestingly, the addition of these exogenous CaMKIV constructs also shifted spontaneous firing rates suggesting CaMKIV activation acts as a negative feedback mechanism that controls neuronal activity (Joseph & Turrigiano, 2017). In parallel, knockdown of CaMKII function with shRNA or the non-selective CaM kinase inhibitor, KN-93, but not the inactive analog, KN-92, prevented synaptic inactivity-induced increase in GluA1 accumulation (Groth et al., 2010). Moreover, transfection of exogenous CaMKII but not a kinase-dead mutant, increased GluA1 expression on dendrites (Groth et al., 2010). Taken together, these results point to two critical calcium-activated kinases that work in coordination to adjust properties of synaptic neurotransmission in a homeostatic manner. Conversely, there is also evidence of a role for the calcium-dependent phosphatase, calcineurin, in the regulation of synaptic scaling (Kim & Ziff, 2014).

Calcium-dependent signaling has a pervasive role in synaptic plasticity. Calcium abundance in presynaptic terminals drives several distinct forms of short-term plasticity, and its abundance, duration, and location of influx at synapses can determine the form of long-term plasticity induced (but see Navabi et al., 2013). Lastly, Ca^{2+} influx through VGCCs and possibly other sources activate downstream signaling cascades critical for homeostatic forms of plasticity – maintaining proper neuronal function amidst activity perturbation. Studies contributed over decades and from many experimental groups help paint a picture for how neurons function in the face of ongoing fluctuations in activity, however, there remains many understudied areas. What role do other sources of Ca^{2+} such as internal stores within the endoplasmic reticulum (Karagas et al., 2019) or through NMDARs play in HSP? What other Ca^{2+}-dependent signaling cascades are activated during activity perturbation and which are responsible for inducing the

physiological compensations? How do these downstream mechanisms precisely regulate transcription, translation, and protein expression to alter physiology properly? Even more importantly, is calcium the only critical messenger or do neurons utilize other mechanisms to decode activity to help stabilize function within neural networks?

Gene transcription and translation during homeostatic synaptic plasticity

It is now well established that *de novo* transcription and translation are essential regulators of enduring forms of synaptic plasticity. The long-standing thought is that persistent changes in neuronal function underlying learning and memory are driven by changes in gene expression and modifications in protein synthesis. Prior to the advanced genome sequencing techniques that are readily available today, the necessity of transcription and translation in enduring forms of LTP and LTD have been largely studied using inhibitors such as actinomycin-D, DRB, anisomycin, cycloheximide, or emetine. Similarly, in HSP, it has been shown that the addition of the transcription inhibitor, actinomycin-D, in cortical neurons blocked TTX-induced increases in mEPSC amplitude (Ibata et al., 2008). Moreover, treatment with translation inhibitors, anisomycin and cycloheximide, or the transcriptional inhibitor, DRB, completely abolished homeostatic depression of AMPAR synaptic currents induced by optogenetic hyperactivation (Goold & Nicoll, 2010). This body of work, along with the emerging role of numerous chromatin regulators discussed below, suggests that regulation of activity-dependent transcription plays a key role in homeostatic synaptic scaling.

Technological advances in RNA-sequencing have allowed more recent experiments to precisely probe for alterations in gene expression during activity perturbation and homeostatic

plasticity. These new techniques have also enabled testing of specific epigenetic regulators and elucidating their roles in gene expression changes underlying homeostatic scaling. For example, Garay and colleagues (Garay et al., 2020), recently established a link between retinoic acid-induced 1 (RAI1), a chromatin regulator, and the suppression of synaptic upscaling in neuronal networks. This work showed that RAI1 knockdown with shRNA strengthens basal neurotransmission, while occluding TTX-induced upscaling of mEPSC amplitudes. This regulation of synaptic upscaling can be explained by the fact that many TTX-responsive genes are RAI1 targets and were basally dysregulated in response to RAI knockdown. Intriguingly, RAI1 KD did not impair BIC-induced synaptic downscaling and had a much weaker impact on BIC responsive genes (Garay et al., 2020). A handful of other chromatin regulators, TET3 DNA demethylase (Yu et al., 2015), EHMT1/2 histone H3K9 methyltransferases (Benevento et al., 2016), and L3MBTL1 methyl-histone binding factor (Mao et al., 2018) have similarly been shown to play a role in synaptic scaling, although all of these latter examples appear to promote, rather than suppress scaling. Although these subset of transcription regulators provide an initial framework for studying the role of epigenetic modifications in HSP, it is important to note that these molecules represent an infinitesimal fraction of the many chromatin regulators that have been discovered, many of which have genetic links to neurodevelopmental disorders (Gabriele et al., 2018).

Reconciliation of shared mechanisms between Hebbian and homeostatic plasticity; metaplasticity

Although Hebbian forms of plasticity such as LTP and LTD are thought to promote neural circuit formation in development and learning and memory in the mature brain, it is

important to note that the rules by which these forms of plasticity are implemented are themselves modifiable. For example, the recent history of pre- and postsynaptic activities plays a critical role in the nature of LTP/LTD that is induced subsequently, a phenomenon termed "metaplasticity" or the plasticity of synaptic plasticity (Abraham & Bear, 1996). A clear example of metaplasticity was demonstrated by Huang and colleagues (Huang et al., 1992) where they showed a history of NMDAR activation can inhibit subsequent LTP induction suggesting that LTP induction parameters are continually adjusted and is highly dependent on the activity history of a neural circuit. The involvement of NMDAR activation history and inhibition of LTP induction has been validated by other experimental groups (Coan et al., 1989; Youssef et al., 2006) and has also been shown to facilitate subsequent LTD (Christie & Abraham, 1992). With the rapidly increasing wealth of different synaptic plasticity mechanisms that have emerged over the last century, one of the biggest challenges is understanding how they work in concert to maintain a properly functioning central nervous system.

The interplay between Hebbian and homeostatic plasticity is one connection that has received significant focus. HSP, either through homeostatic regulation of synaptic weights or firing rate, has been theorized as the mechanism for constraining extreme and aberrant levels of neuronal activity while guiding activity-dependent circuit organization and maintaining synapse specific "learned experiences." An important feature, however, is that both forms of plasticity act on vastly different time scales. Hebbian mechanisms are synapse specific, are implemented over minutes to hours (LTP/LTD), and alone, are known to promote instability in neural circuits (Rochester et al., 1956; Miller, 1996). By contrast, synaptic scaling requires prolonged activity perturbation (~24hrs) and in some cases, days *in vivo* (Kaneko et al., 2008; Keck et al., 2013; Greenhill et al., 2015). This temporal paradox (reviewed in Zenke et al., 2017) points to the

possibility that homeostatic plasticity mechanisms may be too slow to compensate and stabilize the fast positive feedback instability of Hebbian plasticity. Moreover, computational models that have attempted to reconcile this time disparity found that speeding the temporal kinetics of homeostatic scaling was required to effectively stabilize network function (Zenke et al., 2013; Toyoizumi et al., 2014).

Is synaptic scaling subject to metaplasticity?

Given the disparity in temporal kinetics between Hebbian LTP/LTD and synaptic scaling, a mechanism thought to curtail the destabilizing forces of LTP/LTD, we were interested in whether the time-course of synaptic scaling was modifiable. Inspired by the psychological concept of priming, originally introduced by Karl Lashley (Lashley, 1951; Bargh, 2014) as a history-dependent mechanism to increase the probability of a behavioral response, we hypothesized that circuits may implement scaling mechanisms more rapidly if they have a prior history of synaptic scaling. In that way, circuits with a history of both Hebbian and homeostatic forms of plasticity would be better equipped to coordinate these processes to allow both information storage and stability. Since HSP mechanisms are thought to continuously active from early development to the mature brain, we proposed the idea that neural circuits with a previous scaling history would be able to initiate these same compensatory changes at a much quicker rate relative to naïve circuits. As described in Chapter 3, we not only disproved this hypothesis, but also uncovered a form of homeostatic metaplasticity, where circuits with a prior history of scaling become refractory to homeostatic adaptations initiated by subsequent changes in network activity. Moreover, our results suggest that lasting alterations in transcriptional regulation play a key role in homeostatic metaplasticity and underscore the importance of

understanding how the activity-dependent history of a circuit ultimately shapes the rules by which homeostatic adaptations are implemented.

CHAPTER II

Unique Roles of L-Type Calcium Channel Subtypes in Homeostatic Synaptic Scaling

Abstract

L-type voltage-gated Ca^{2+}-channels (LTCCs) provide a pivotal link between membrane depolarization, calcium influx, and activity-dependent changes in gene expression. Such activity-transcription coupling is critical for long-lasting forms of synaptic plasticity, including well-studied forms of homeostatic plasticity such as synaptic scaling. A role of LTCCs in synaptic scaling has been demonstrated by using dihydropyridine antagonists such as nifedipine, nimodipine, or verapamil, but these agents do not distinguish between the two LTCC subtypes $Ca_V1.2$ and $Ca_V1.3$ expressed in brain. To investigate the unique roles of these LTCC subtypes, we examined synaptic upscaling in cultured hippocampal neurons derived from $Ca_V1.2$ conditional and $Ca_V1.3$ constitutive knockout (KO) mice. We found distinct effects on homeostatic upscaling in each mutant, demonstrating that different LTCC subtypes play non-redundant and unique roles. Basal synaptic properties were unaffected by $Ca_V1.3$ deletion, but $Ca_V1.3$ deficient neurons exhibited no synaptic upscaling in response to chronic activity suppression with tetrodotoxin (TTX). By contrast, $Ca_V1.2$ deletion in excitatory neurons induced a significant increase in mEPSC amplitude and enhanced surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) at excitatory synapses, two signatures of homeostatic upscaling. Activity suppression in $Ca_V1.2$ deficient neurons produced no further increase in mEPSCs or synaptic AMPARs, suggesting that upscaling is occluded in

Cav1.2 KO neurons. Together, our results demonstrate unique roles for brain LTCC subtypes in homeostatic synaptic scaling and open up new opportunities to understand the activity-dependent sensor that couples chronic alterations in activity with changes in gene expression underlying synaptic adaptations.

Introduction

Homeostatic synaptic plasticity (HSP) has received considerable attention, largely because of its pivotal role in buffering destabilizing levels of activity in neural circuits. Synaptic scaling is one of the most intensely studied forms of HSP and is characterized by adaptations that involve most or all synapses on a given neuron and enhance or depress synaptic function proportionately in response to chronic changes in neuronal firing rate (Turrigiano et a., 1998; O'Brien et al., 1998; Thiagarajan et al., 2005; Ibata et al., 2008; Gainey et al., 2009; Goold & Nicoll, 2010; Garcia-Bereguiain et al. 2013; Keck et al., 2013). In excitatory cortical or hippocampal neurons, chronic silencing of activity *in vitro* with tetrodotoxin (TTX), a voltage-gated sodium channel blocker, leads to increased synaptic strength, or upscaling. Conversely, chronic elevation in activity with bicuculline, a competitive antagonist of gamma-Aminobutyric acid A (GABAA) receptors results in a homeostatic decrease in synaptic strength, or downscaling. Although widely studied in cultured neurons, synaptic scaling has been repeatedly observed *in vivo* in response to sensory deprivation (Desai et al., 2002; Goel et al., 2007; Keck et al., 2013; Barnes et al., 2017). Work primarily in cultured neurons has uncovered a wide range of molecular mechanisms underlying this form of homeostatic plasticity (Fernandes & Carvalho,2016), including changes in the abundance of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors at synapses (Turrigiano et al., 1998;

Sutton et al., 2006) and a clear dependence on Ca^{2+}-dependent gene transcription (Schaukowitch et al., 2017). Remarkably, however, we still lack a clear understanding of the mechanism that couples activity-dependent Ca^{2+}-signaling with functional homeostatic changes in synaptic strength.

Calcium influx through voltage-gated calcium channels (VGCCs) plays a key role in regulating important activity-dependent processes such as neurotransmission, gene transcription, and synaptic plasticity (Greer et al., 2008; Nanou et al., 2018). VGCCs can be subdivided into several subfamilies based on their pharmacological and physiological properties: L-type (Ca_V1), T-type (Ca_V3), P/Q-type, R-type, and N-type (Ca_V2) (Catterall, 2000). Numerous studies have demonstrated the importance of VGCCs in the regulation of HSP processes. P/Q/N channels of the Ca_V2 family have been shown to gate homeostatic changes in presynaptic function at hippocampal synapses driven by loss of postsynaptic excitatory synaptic drive (Jakawich et al., 2010; Henry et al., 2018). Similarly, loss-of-function mutations in the *Drosophila* cacophony (cac) gene, the pore-forming α1 subunit of Ca_V2 channels, prevent the homeostatic increase in quantal content at the neuromuscular junction (NMJ) induced by pharmacological or genetic impairment of postsynaptic glutamate receptor function (Frank et al., 2006; Müller & Davis, 2012). More recently, Ca^{2+} influx through P/Q-type, but not N-type VGCCs has been shown to mediate bidirectional homeostatic regulation of neurotransmitter release and size of synaptic vesicle pools in hippocampal neuron cultures (Jeans et al., 2017). Collectively, these results suggest the importance of calcium-signaling through Ca_V2 channels in presynaptic HSP mechanisms.

In the mammalian central nervous system (CNS), P/Q and N-type VGCCs are the primary presynaptic sources of Ca^{2+} influx (Wheeler et al., 1994; Christie et al., 1997), while L-

type VGCCs exhibit pronounced expression in cell bodies and dendrites (Hell et al., 1993; Lipscombe et al., 2004) and are important for coupling changes in neural activity with regulation of gene expression (Murphy et al., 1991; Bading et al., 1993; Finkbeiner & Greenberg, 1998), among other roles (Tachibana et al., 1993; Christie et al., 1997; Norris et al., 1998; Sand et al., 2001). LTCCs are classified into $Ca_V1.1$-4 based on the identity of the pore-forming α1 subunit, but only $Ca_V1.2$ and $Ca_V1.3$ are expressed significantly in brain (Hell et al., 1993; Lipscombe et al., 2004; Catterall, 2010). Ca^{2+} influx through LTCCs activates a number of downstream signaling cascades that impinge on gene regulation (Norris et al., 1998; Deisseroth et al., 1998; Dolmetsch et al., 2001; Weick et al., 2003; Wheeler et al., 2008; Wheeler et al., 2012). In addition, the C-terminal domains of both $Ca_V1.2$ and $Ca_V1.3$ have been shown to translocate into the nucleus and act as transcription factors (Gomez-Opsina et al., 2006; Lu et al., 2015). Several studies have demonstrated that the block of LTCC signaling with dihydropyridines such as nifedipine is sufficient to drive homeostatic upscaling of synaptic strength (Thiagarajan et al., 2005; Ibata et al., 2008; Henry et al., 2012). Additionally, nifedipine treatment has also been shown to prevent the homeostatic downregulation of both NMDAR and AMPAR-mediated responses after chronic excitation in Channelrhodopsin 2 (ChR2)-expressing CA1 pyramidal neurons (Goold & Nicoll, 2010). These findings suggest that reduced LTCC signaling might contribute to homeostatic upscaling. However, inhibition of LTCC-dependent signaling with nifedipine has also been shown to block TTX-dependent upscaling in hippocampal neurons (Sokolova & Mody, 2008), suggesting a more complex role that includes both negative and positive regulation of HSP.

$Ca_V1.2$ and 1.3 LTCCs exhibit differences in voltage-dependent activation, dihydropyridine sensitivity, and cellular localization (Hell et al., 1993; Koschak et al., 2001; Safa

et al., 2001; Scholze et al., 2001; Xu & Lipscombe, 2001; Lipscombe et al., 2004), raising the possibility that different LTCC subtypes play unique roles in homeostatic scaling. To test this idea, we examined how different LTCC subtypes support homeostatic synaptic scaling using hippocampal neurons isolated from $Ca_V1.2$ cKO and $Ca_V1.3$ KO mice, foregoing potential off-target effects with the use of pharmacological LTCC antagonists. If $Ca_V1.2$ and $Ca_V1.3$ play overlapping roles in upscaling, then deletion of either subtype should have little impact on the ability of activity suppression to induce compensatory increases in excitatory synaptic strength. Contrary to this prediction, we found that $Ca_V1.2$ and $Ca_V1.3$ play non-redundant and unique roles in homeostatic upscaling. $Ca_V1.3$ deletion in neurons did not alter basal synaptic properties but prevented upscaling of miniature excitatory postsynaptic currents (mEPSCs) induced by chronic TTX treatment. By contrast, neuronal $Ca_V1.2$ deletion induced a significant increase in mEPSCs and enhanced synaptic AMPAR expression, two signatures of homeostatic upscaling, in the absence of TTX. Activity suppression did not further alter mEPSCs or synaptic AMPAR content, suggesting that $Ca_V1.2$ deletion occludes further homeostatic upscaling. Taken together, our findings suggest that $Ca_V1.2$ and $Ca_V1.3$ are each important for homeostatic synaptic scaling but play unique roles in establishing adaptive alterations in synapse function.

Materials and Methods

Animals

All animal use followed NIH guidelines and was in compliance with the University of Michigan Committee on Use and Care of Animals. For the $Ca_V1.2$ study, conditional knockout mice with a pan-neuronal deletion of $Ca_V1.2$ were used (Temme et al., 2016; Temme et al., 2017). For these experiments, mice heterozygous for the floxed $Ca_V1.2$ exon two allele were

maintained on a 129SvEv genetic background. Experimental animals were generated by crossing heterozygous floxed $Ca_V1.2$ mice ($Ca_V1.2^{f/+}$ mice) with C57BL/6 transgenic mice that expressed Cre-recombinase under the synapsin I promoter (Cui et al., 2008). Offspring from the F1 cross that were heterozygous floxed and Cre positive (i.e., $Ca_V1.2^{f/+}$, Synapsin-$Cre^{Cre/+}$) were then intercrossed (non-sibling) with mice heterozygous floxed and Cre negative (i.e., $Ca_V1.2^{f/+}$, Synapsin-$Cre^{+/+}$) to achieve homozygous conditional knockout mice ($Ca_V1.2^{f/f}$, Synapsin-$Cre^{Cre/+}$) and wild-type mice ($Ca_V1.2^{+/+}$, Synapsin-$Cre^{+/+}$) on an F2 129SvEv:C57Bl/6 hybrid background. For ease of reading, conditional knockout mice are referred to as $Ca_V1.2$ cKO and wild-type mice as WT throughout the text. For the $Ca_V1.3$ study, knockout mice with a global deletion of the $Ca_V1.3$ gene were used (Platzer et al., 2000; Clark et al., 2003; McKinney et al., 2006). Mice were maintained on a C57BL/6 background by successively crossing heterozygous offspring with C57BL/6 WT mice. Experimental animals were generated by crossing heterozygous $Ca_V1.3$ mice with WT 129SvEv mice. Heterozygous offspring from the F1 cross were then intercrossed (non-sibling) to achieve homozygous knockout mice ($Ca_V1.3^{-/-}$) and wild-type mice ($Ca_V1.3^{+/+}$) on an F2 129Sve:C57Bl/6 hybrid background (hereafter referred to as $Ca_V1.3$ KO and WT). All comparisons were made between knockout mice and WT littermates and the experimenter was kept blind to genotype throughout the experiment.

Cell Culture and Electrophysiology

Dissociated postnatal (P0-2) rat hippocampal neuron cultures were prepared as previously described (Sutton et al., 2006). Hippocampal neuron cultures were derived from both male and female knockout mice and WT littermates. mEPSCs were recorded from a holding potential of –70 mV with an Axopatch 200B amplifier from neurons bathed in HEPES-buffered saline (HBS)

containing: 119 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 30 mM Glucose, 10 mM HEPES (pH 7.4) plus 1 µM TTX and 10 µM bicuculine; mEPSCs were analyzed with Synaptosoft MiniAnalysis software. Whole-cell pipette internal solutions contained: 100 mM cesium gluconate, 0.2 mM EGTA, 5 mM MgCl2, 2 mM ATP, 0.3 mM GTP, 40 mM HEPES (pH 7.2). Statistical differences between experimental conditions were determined by ANOVA and post-hoc Fisher's LSD test. Shifts in cumulative probability curves were analyzed by Kolmogorov–Smirnov test.

Immunocytochemistry and Microscopy

Surface GluA1 (sGluA1) was labeled and imaged as described previously (Sutton et al., 2006). Neurons were live labeled for GluA1 with rabbit polyclonal anti-GluA1 (0.01 mg/mL; Millipore #ABN241) for 15 min at 37°C and fixed with 2% paraformaldehyde/2% sucrose in PBS with 2.0 mM $MgCl_2$ and 2.0 mM $CaCl_2$ (PBS-MC). Samples were blocked with 2% BSA in PBS-MC prior to labeling with Alexa555-conjugated goat anti-rabbit secondary antibody (Molecular Probes #A21429, 1:1000). Cells were then permeabilized with 0.1% Triton-X in PBS-MC followed by immunocytochemical labeling of PSD95 with mouse monoclonal anti-PSD95 (1 µg/mL; Millipore #) for 60 min at RT and Alexa488-conjugated goat anti-mouse secondary antibody (Molecular Probes #A11029, 1:1000). 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. Analysis was performed with custom written analysis routines for ImageJ and Matlab. sGluA1 content at synapses was determined by measuring the integrated sGluA1 signal at puncta

co-localized with PSD95. Statistical differences were assessed by ANOVA, then by Fisher's LSD post-hoc tests.

Results

Global Deletion of $Ca_V1.3$ Abolishes TTX-dependent Homeostatic Upscaling

We examined the role of distinct LTCC subtypes in homeostatic upscaling by measuring compensatory changes in synaptic function in WT and $Ca_V1.2/1.3$-deficient neurons induced by chronic activity suppression with TTX (1 μM; 24-48hrs). To study the role of $Ca_V1.3$ LTCCs, we performed whole-cell voltage clamp recordings from neurons isolated from 8 $Ca_V1.3$ KO mice (51 neurons) or their WT littermates (7 WT mice; 30 neurons). At DIV14, we found that basal synaptic properties were highly similar between WT and $Ca_V1.3$-deficient neurons — mEPSC amplitude, frequency, and decay kinetics were all statistically indistinguishable between WT and $Ca_V1.3$ KO neurons (**Figure 2.1A-D**). As expected, chronic silencing of WT cultures with TTX-treatment significantly increased mEPSC amplitudes ($F(3\ 77) = 3.895$, $p<.05$; post-hoc Fisher's LSD, $p<.005$) (**Figure 2.1B**), without altering mEPSC frequency or mEPSC decay time (**Figure 2.1C, D**). Interestingly, in neurons lacking $Ca_V1.3$, activity silencing with TTX did not significantly increase mEPSC amplitude, nor did we observe significant differences in mEPSC frequency or decay kinetics between TTX-treated and control $Ca_V1.3$ KO neurons (**Figure 2.1C, D**). The TTX-induced upscaling of mEPSC amplitudes in WT neurons can be seen in a significant (Kolmogorov–Smirnov test, $p<.05$) rightward shift in the cumulative probability curve of mEPSCs treated with TTX (**Figure 2.1E**). $Ca_V1.3$ KO neurons failed to demonstrate significant upscaling of mEPSC amplitudes (a significant rightward shift in the mEPSC distribution) after TTX treatment; in fact, a small leftward shift was observed indicating reduced

mEPSC amplitudes, though this was not statistically significant (**Figure 2.1E**). These results suggest that neurons lacking $Ca_V1.3$ channels develop with normal synaptic properties and that $Ca_V1.3$ channels are required for homeostatic upscaling induced by activity suppression in hippocampal neuron cultures.

Conditional Knockout of $Ca_V1.2$ Increases Basal Synaptic Strength

We next studied the role of $Ca_V1.2$ in regulating basal synaptic strength and TTX-dependent synaptic upscaling. Whole-cell voltage clamp recordings were made from neurons isolated from 12 $Ca_V1.2$ cKO mice (73 neurons) and littermate WT mice (12 mice, 66 neurons). Unlike $Ca_V1.3$-deficient neurons, we observed a significant baseline increase in mEPSC amplitude in neurons lacking $Ca_V1.2$ ($t(33) = 2.426$, $p<.05$) (**Figure 2.2A, B**), but no change in mEPSC frequency (**Figure 2.2C**) or mEPSC decay time (**Figure 2.2D**). This increase in basal synaptic function was associated with a robust increase in synaptic AMPAR content, as revealed by surface expression of the AMPAR subunit GluA1 at PSD95-labeled excitatory synapses (**Figure 2.2E, F**). As an increase in mEPSC amplitude and AMPAR content each accompany chronic activity suppression with TTX, these basal changes in $Ca_V1.2$ cKO neurons resemble synaptic upscaling. Indeed, WT neurons treated chronically with TTX exhibit a rightward shift in the mEPSC cumulative probability distribution that is superimposable with the mEPSC distribution from $Ca_V1.2$ cKO neurons recorded at baseline (**Figure 2.3E**).

Conditional Knockout of $Ca_V1.2$ Occludes Homeostatic Upscaling

Although the increased synaptic strength in $Ca_V1.2$ cKO neurons resembles a "pre-scaled" state, these synaptic features could arise through mechanisms independent of

homeostatic signaling. If, on the other hand, the synaptic changes are related to normal upscaling driven by activity suppression, then chronic silencing of $Ca_V1.2$ cKO neurons should not further increase mEPSC amplitude or synaptic AMPAR content. To test these possibilities, we treated both WT and $Ca_V1.2$ cKO neurons with TTX for 24-48hrs and recorded mEPSCs and sGluA1 content at PSD95-labeled excitatory synapses.

We observed robust TTX-dependent homeostatic upscaling in WT controls reflected by a significant increase in mEPSC amplitude ($F(3,132) = 2.920$, $p<.05$; Fisher's LSD, $p<.05$) (**Figure 2.3B**), without changes in mEPSC frequency or decay time (**Figure 2.3A-D**). By contrast, TTX was ineffective in further increasing the enhanced mEPSC amplitude in $Ca_V1.2$ cKO neurons. Moreover, while TTX induced a clear rightward shift in the WT mEPSC amplitude cumulative probability distribution (Kolmogorov-Smirnov test, $p<.05$), it failed to significantly shift the distribution of mEPSCs from $Ca_V1.2$ cKO mice (**Figure 2.3E)**.

Numerous studies have demonstrated a direct relationship between synaptic scaling and the regulation of surface AMPARs at postsynaptic sites (O'Brien et al., 1998; Thiagarajan et al., 2005; Sutton et al., 2006; Gainey et al., 2009; Garcia-Bereguiain et al., 2013; Chowdhury & Hell, 2018). We thus compared surface GluA1 intensity at synapses (co-localized with PSD95) between WT (3 mice, 80 neurons) and $Ca_V1.2$ cKO (3 mice, 79 neurons) neurons treated with TTX or vehicle (H_2O). TTX-treatment significantly increased surface GluA1 expression at synapses in WT neurons but not in $Ca_V1.2$ cKO neurons ($F(3,155) = 3.152$, $p<.05$; Fisher's LSD, $p<.05$) (**Figure 2.3F, G**). Consistent with previous studies (Turrigiano et al., 1998; Nanou et al., 2018) and our mEPSC frequency data, TTX treatment did not alter synapse density (PSD95 puncta density) in either WT or $Ca_V1.2$ cKO neurons (**Figure 2.3H**). Collectively, our studies reveal that $Ca_V1.3$ and $Ca_V1.2$ play non-overlapping roles in homeostatic synaptic scaling —

$Ca_V1.3$ channel function appears necessary for homeostatic upscaling even when neurons express $Ca_V1.2$, while loss off $Ca_V1.2$ induces an upscaling-like phenotype that occludes synaptic adaptations induced by activity suppression.

Discussion

L-type voltage-gated calcium channels are thought to play a role in decoding neural activity changes that ultimately drive compensatory synaptic adaptations underlying homeostatic scaling. Direct support for the role of LTCCs has come primarily from pharmacological inhibition using dihydropyridines, but these drugs have potential off-target effects and cannot readily distinguish between roles of different LTCC subtypes. In this study, we used a genetic approach to individually delete $Ca_V1.3$ and $Ca_V1.2$ to test if these LTCC subtypes play redundant or unique roles in homeostatic synaptic scaling. Redundant roles could be inferred if deletion of either $Ca_V1.2/1.3$ alone led to minimal or no alterations in synaptic scaling, implying that loss of a specific LTCC subtype can be mitigated by expression of the other. Instead, we found that deletion of either $Ca_V1.2$ or $Ca_V1.3$ had pronounced, yet distinct, effects on homeostatic upscaling thus demonstrating that these LTCC subtypes play unique roles in HSP.

Previous studies point to an important role of LTCC function in negatively regulating homeostatic upscaling, as application of LTCC antagonists drive compensatory increases in synaptic function that resemble upscaling driven by activity suppression (Thiagarajan et al., 2005; Ibata et al., 2008; Henry et al., 2012). Consistent with this role, we find that neuronal $Ca_V1.2$ deletion increases basal synaptic strength and synaptic AMPAR content, and these synaptic changes occlude homeostatic synaptic strengthening in response to activity suppression. These findings support the idea that a reduction in $Ca_V1.2$ LTCC activity is a key part of the

signaling network that drives homeostatic upscaling, though they are not definitive. Future studies are needed to validate the idea that loss of ongoing $Ca_V1.2$ LTCC activity is a normal trigger for homeostatic upscaling during reduced neural firing rates. For example, chemo-genetic approaches could be used to specifically disrupt $Ca_V1.2$ LTCCs to evaluate the extent to which transcriptional changes and synaptic adaptations overlap with that driven by activity suppression.

On the other hand, prior studies have also suggested that intact LTCC signaling is needed for HSP, as co-application of LTCC antagonists with TTX prevents synaptic upscaling (Sokolova & Mody, 2008). Our results raise the intriguing possibility that this positive regulatory role for LTCCs is mediated by $Ca_V1.3$ LTCCs, since $Ca_V1.3$ deletion also disrupted upscaling without altering basal synaptic properties. This result suggests that rather than aggregate LTCC activity being a simple cellular readout of neural activity, perhaps combinatorial signaling through $Ca_V1.2$ and $Ca_V1.3$ LTCCs might be important for homeostatic signaling during chronic changes in network activity. It is now of interest to determine if unique roles for LTCC subtypes are similarly evident during homeostatic downscaling induced by network hyperactivation, as previous studies have demonstrated that Ca^{2+} influx through LTCCs is necessary for structural and functional adaptations driven by persistent increases in firing rates (Goold & Nicoll, 2010; Siddoway et al., 2013). It is also important to determine how LTCC subtypes each contribute to downstream effectors that regulate transcription and/or translation to induce the functional changes during synaptic scaling. CaMKIV has been implicated as a downstream signaling protein that encodes the Ca^{2+} signal during activity perturbations (Goold & Nicoll, 2010; Joseph & Turrigiano, 2017), and $Ca_V1.2$ and $Ca_V1.3$ have been found to play distinct roles in pCREB signaling (Zhang et al., 2006). Our work suggests that unique roles of

$Ca_V1.2$ and $Ca_V1.3$ during synaptic scaling may provide a handle to better understand homeostatic signaling pathways that couple alterations in neural activity to gene-expression changes that ultimately drive synaptic adaptations to stabilize firing.

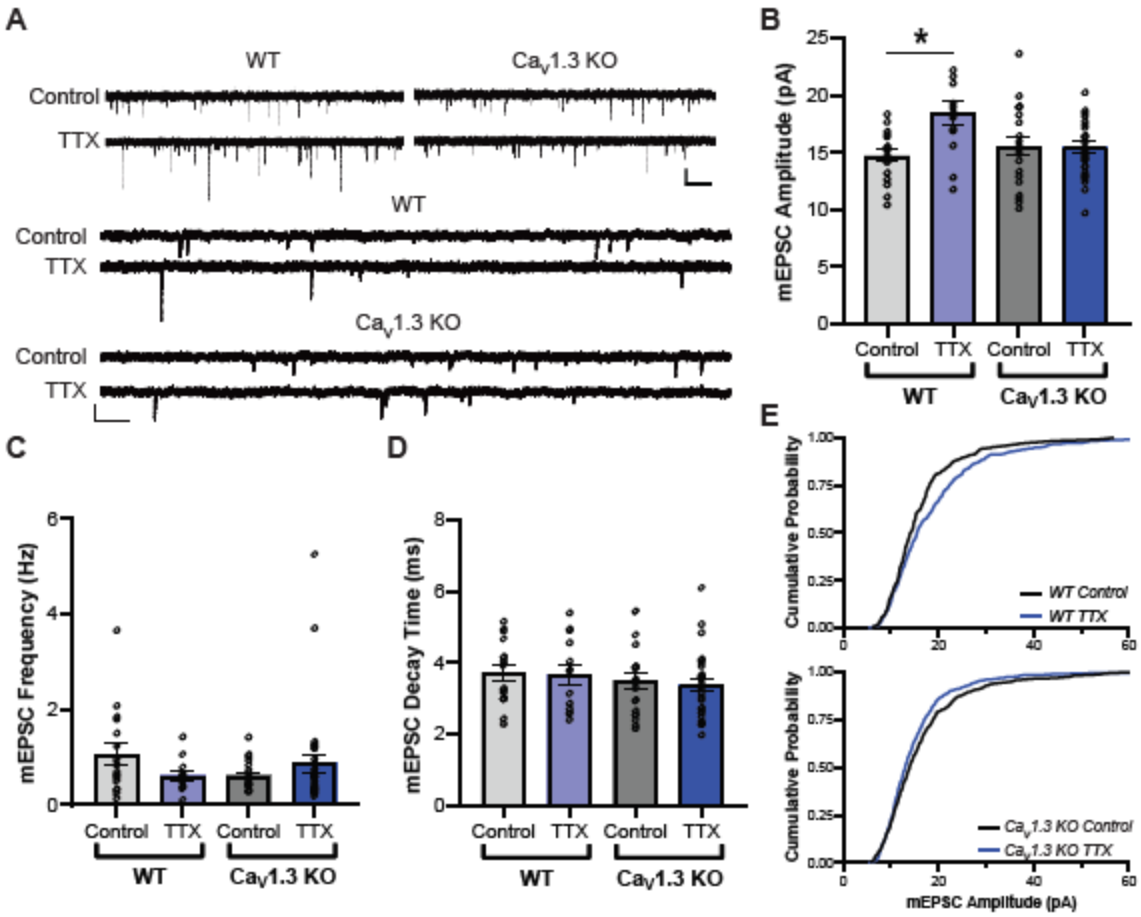


Figure 2.1 Global Deletion of Ca$_V$1.3 Abolishes TTX-dependent Homeostatic Upscaling (A) Representative whole-cell voltage clamp recordings of hippocampal cultured neurons derived from Ca$_V$1.3 KO mice and WT littermate controls; scalebar: 20pA, 5s (top), 20 pA, 500ms (bottom). **(B)** Mean (±SEM) mEPSC amplitudes recorded from WT and Ca$_V$1.3 KO neurons treated with TTX or vehicle; *p<0.05 by one-way ANOVA and Fisher's LSD. **(C)** Mean (±SEM) mEPSC frequencies from WT and Ca$_V$1.3 KO neurons treated as indicated. **(D)** Mean (±SEM) mEPSC decay times from WT and Ca$_V$1.3 KO neurons treated as indicated. **(E)** Cumulative probability distributions of mEPSC amplitudes in WT (top) and Ca$_V$1.3 KO (bottom) neurons treated with vehicle or TTX; *p < 0.05 by Kolmogorov-Smirnov's test.

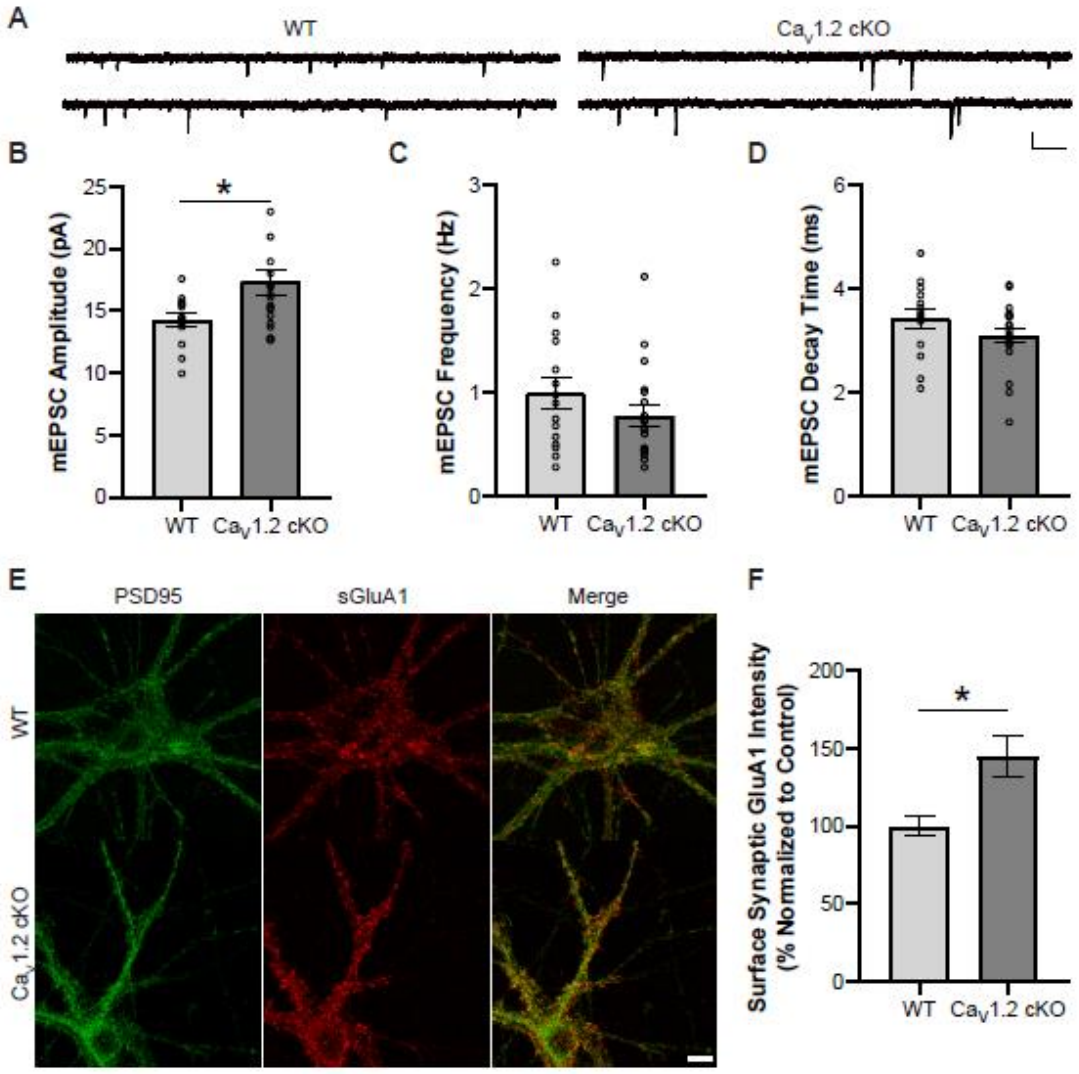


Figure 2.2 Conditional Knockout of $Ca_V1.2$ Increases Basal Synaptic Strength and Surface Expression of GluA1 at synapses (A) Representative whole-cell voltage clamp recordings of hippocampal cultured neurons derived from Cav1.2 cKO mice and WT littermate controls; scalebar: 5s, 20pA. **(B)** Mean (±SEM) mEPSC amplitudes recorded from WT and Cav1.2 cKO neurons; *p<.05 by unpaired t-test. **(C)** Mean (±SEM) mEPSC frequencies from WT and Cav1.2 cKO neurons treated as indicated. **(D)** Mean (±SEM) mEPSC decay times from WT and Cav1.2 cKO neurons treated as indicated. **(E)** Representative immunocytochemistry images of WT (top) and Cav1.2 cKO neurons (bottom) probed with antibodies against PSD95 (green) and surface GluA1 (red); scalebar = 10 μm. **(F)** Mean (±SEM) surface GluA1 expression at PSD95-puncta positive synapses; *p<.05 by unpaired t-test

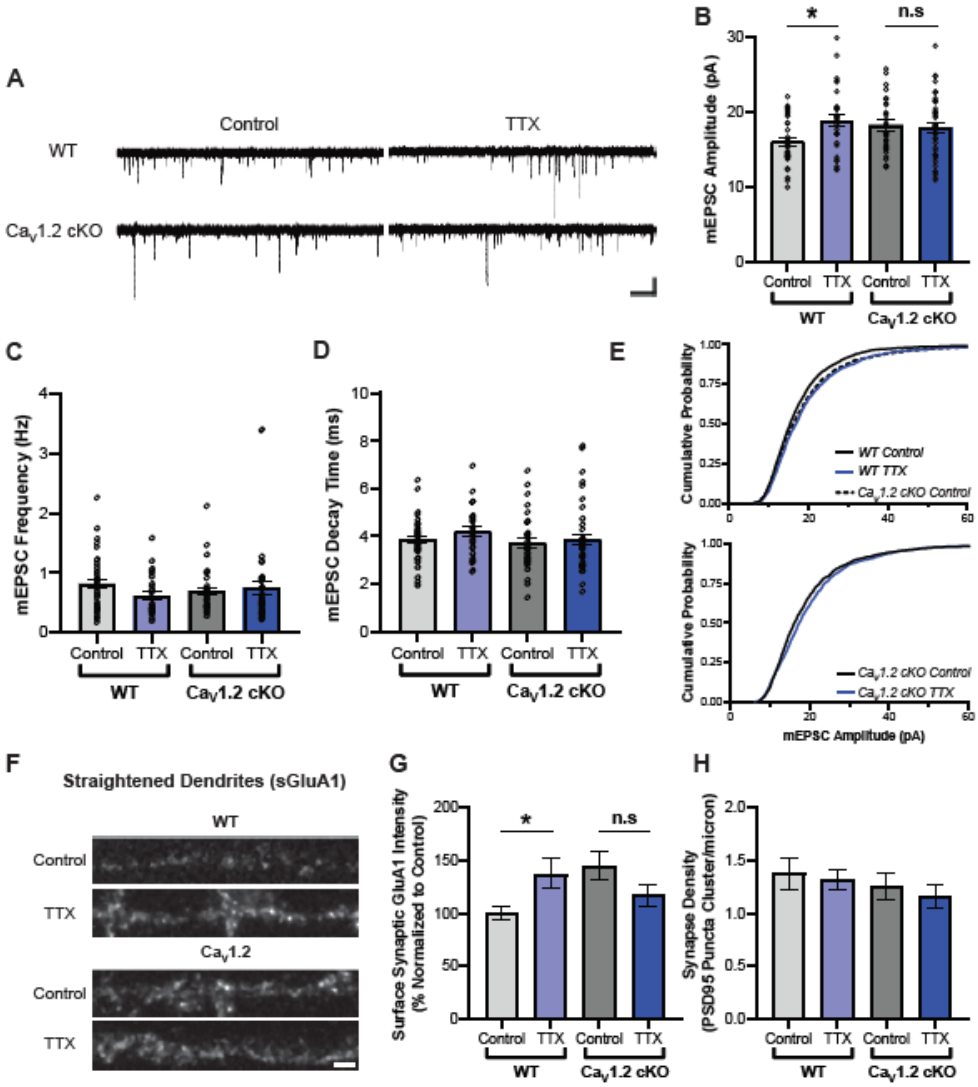


Figure 2.3 Conditional Knockout of $Ca_V1.2$ Occludes TTX-dependent Upscaling of mEPSC Amplitudes and Surface GluA1 Expression (A) Representative whole-cell voltage clamp recordings of hippocampal cultured neurons derived from Cav1.2 cKO (bottom) mice and WT (top) littermate controls; scalebar: 500ms, 20pA. **(B)** Mean (±SEM) mEPSC amplitudes recorded from WT and Cav1.2 cKO neurons treated with TTX or vehicle; *p<.05 by one-way ANOVA and Fisher's LSD. **(C)** Mean (±SEM) mEPSC frequencies from WT and Cav1.2 cKO neurons treated as indicated. **(D)** Mean (±SEM) mEPSC decay times from WT and Cav1.2 cKO neurons treated as indicated. **(E)** Cumulative probability distributions of mEPSC amplitudes in WT and Cav1.2 cKO neurons treated with TTX or vehicle; *p<.05 Kolmogorov-Smirnov's test. **(F)** Representative linearized dendrites of WT (top) and Cav1.2 cKO (bottom) neurons probed with antibodies against PSD95 (green) and surface GluA1 (red); scalebar = 5 μm) **(G)** Mean (±SEM) surface GluA1 expression at PSD95-puncta positive synapses; *p<.05 by one-way ANOVA and Fisher's LSD. **(H)** Mean (±SEM) synapse density (PSD95-positive puncta/length of dendrite) in linearized dendrites.

CHAPTER III

Activity-dependent History in Hippocampal Neurons Dictate Temporal Dynamics of Homeostatic Synaptic Scaling

Abstract

Neural circuits utilize a host of homeostatic plasticity mechanisms, including synaptic scaling, to maintain stability in circuits undergoing experience-dependent remodeling necessary for information processing. During synaptic scaling, compensatory adaptations in synaptic strength are induced after chronic manipulations in neuronal firing, but our understanding of this process is largely limited to its initial induction. How these homeostatic synaptic adaptations evolve when activity renormalizes and their impact on subsequent homeostatic compensation are both poorly understood. To examine these issues, we investigated whether a previous history of homeostatic scaling in networks of cultured hippocampal neurons altered their subsequent homeostatic responses to chronic activity manipulations. Unexpectedly, we found that a history of synaptic scaling strongly suppressed future scaling to the same, and even opposite, activity challenges. This history-dependent suppression was specific for future homeostatic compensation, as networks with a prior scaling history showed no deficits in the chemical induction of long-term potentiation (cLTP), a Hebbian form of synaptic plasticity. Hippocampal neurons with a prior scaling history exhibited normal engagement of activity-dependent signaling during subsequent activity challenges (as assessed by examination of the ERK/MAPK pathway) but demonstrated widespread alterations in activity-dependent transcriptional

regulation to further activity changes. We also investigated what features of synaptic scaling are most tightly associated with history-dependent suppression and found that the resetting of synaptic weights upon activity renormalization plays a key role: extending this resetting period or eliminating it altogether both abolished the suppressing effects of prior scaling history on future homeostatic scaling. Taken together, our data show that the history of homeostatic signaling in neural circuits plays a key role in shaping future compensatory adaptations to chronic changes in network activity.

Introduction

Homeostatic forms of synaptic plasticity are thought to promote long-term stabilization of neural circuit function by buffering aberrant increases or decreases in network activity. The most intensely studied homeostatic form of plasticity is synaptic scaling, characterized by compensatory increases (upscaling) or decreases (downscaling) in excitatory synaptic strength that counters network activity suppression or hyperactivation (Turrigiano et al., 1998; O'Brien, 1998; Lissin, 1998), respectively. Hebbian forms of plasticity, though critical for information storage in neural circuits, are thought to be a major source of instability for network activity through the positive feedback plasticity effects they promote (Keck et al., 2017; Zenke et al., 2017). Although synaptic scaling and other forms of homeostatic plasticity have been proposed as a mechanism to constrain instability driven by Hebbian forms of plasticity (Rabinowitch & Segev, 2006; Yger and Gilson, 2015), these have been found to act on vastly different timescales – Hebbian plasticity on orders of seconds to minutes while synaptic scaling is induced over hours or days (Zenke et al., 2017). This raises the question of whether the effectiveness of synaptic scaling in buffering changes in neural activity is necessarily limited to prolonged periods of time,

or whether the activity-dependent history of synapses might regulate the timescale over which homeostatic adaptations are implemented. Presumably, over the lifetime of a neural circuit, homeostatic mechanisms would be engaged repeatedly to offset destabilization, yet how the history of prior synaptic scaling affects subsequent homeostatic signaling remains completely unknown.

It is well established that the activity-dependent history in neural circuits can shape the rules and mechanisms by which plasticity is expressed. This concept of "metaplasticity," (Abraham & Bear, 1996) that synaptic plasticity is itself plastic, has been extensively studied in the context of Hebbian forms of plasticity (Huang et al., 1992; Cohen et al., 1999; Mellentin et al., 2007; Rosenberg et al., 2016), but is only beginning to be applied to homeostatic plasticity (Arendt et al., 2015). Given the effectiveness of synaptic scaling in buffering Hebbian-driven instability is theoretically enhanced by more closely aligning the temporal dynamics of the two (Yger & Gilson, 2015; Zenke et al., 2017), we reasoned that perhaps the dynamics of scaling might be accelerated in networks that have a prior history of synaptic scaling – in other words, that the features of homeostatic plasticity might be shaped by previous homeostatic signaling. To address this question, we examined how a previous round of synaptic scaling in networks of cultured hippocampal neurons altered homeostatic responses to a second set of activity challenges. Contrary to our prediction, we found that a history of synaptic scaling did not promote or accelerate subsequent homeostatic scaling, but rather potently suppressed it. This history-dependent suppression of synaptic scaling is specific, appears to be mediated largely by altered activity-dependent transcriptional regulation, and is closely tied to the resetting phase of scaling where synaptic strength returns to basal levels following a renormalization of network

activity. Together, our results demonstrate that homeostatic adaptations to altered network activity are potently regulated by the history of homeostatic signaling in neural circuits.

Materials and Methods

Animals

All animals followed NIH guidelines and was in compliance with the University of Michigan Committee on Use and Care of Animals.

Cell Culture

Dissociated postnatal (P0-2) hippocampal neuron cultures were prepared from both male and female Sprague Dawley rat pups as previously described (Henry et al., 2018) and maintained until DIV14 prior to experiments. Briefly, hippocampi were dissected in cold dissociation media (DM in mM; 82 Na2SO4, 30 K2SO4, 5.8 MgCl2-6H2O, .252 CaCl2-2H2O, 1 HEPES, 200 glucose, 0.001% w/v phenol red), and transferred to a 15 mL conical tube. Dissociation media (DM) was gently removed with enough to cover the tissue and replaced with 5mL of pre-warmed (37°C) cysteine-activated papain solution containing 3.2 mg l-cysteine (Sigma-Aldrich) and 500µL papain (Sigma-Aldrich) in 10mL DM, pH~7.2. The tissue was then incubated in the activated papain solution for 15min at 37°C to allow for tissue digestion, inverting the tube ~2-3 times halfway into the incubation. Papain inactivation was achieved with two washes in ice-cold DM containing 12.5% v/v fetal bovine serum, followed by two washes in DM alone. Dissociated cells were then washed twice in chilled normal growth medium [NGM; Neurobasal A (Invitrogen) supplemented with 2% v/v B27 (Invitrogen) and 1% v/v Glutamax (Invitrogen)], then titurated in 5mL NGM to obtain a single cell suspension. This single cell suspension was

incubated on ice for ~3-5min. The single cell suspension is transferred to a new 15mL conical tube and centrifuged at 67xg (0.5 x 1000 rcf) at 4°C. For plating, ~60K cells (in a volume of 150µL) were dispensed onto poly-D-lysine-coated glass-bottom Petri dishes (Mattek) and maintained at 5% CO2 and 37°C. Cells were then supplied with 2mL of NGM-GC (NGM supplemented with 15% v/v glial conditioned media and 10% v/v cortical conditioned media) approximately 4hrs after plating. The next day, cells were fed by replacing 50% of the total volume with fresh NGM-GC and fed every 4d. By DIV14, cells were then maintained with NGM alone and fed on the same schedule.

Electrophysiology

mEPSCs were recorded from a holding potential of -70mV with an Axopatch 200B amplifier from pyramidal neurons bathed in HEPES-buffered saline (HBS) containing (in mM): 119 NaCl, 5KCl, 2 $CaCl_2$, 2 $MgCl_2$, 30 Glucose, 10 HEPES (pH 7.4). Prior to recordings, neurons were treated with 1µM tetrodotoxin or 10µM bicuculline as indicated in experimental conditions. Before any pharmacological treatment, ~50% of the neuronal conditioned media were removed from dishes and saved at 5% CO2 and 37°C. Pharmacological agents were removed via 2x washes (leaving only enough media to cover the cells between washes, ~50µL) with fresh pre-warmed NGM and then incubated for 48hrs, unless indicated otherwise, in 50:50 saved media and fresh NGM.

Pharmacological induction of LTP in cultured hippocampal neurons was achieved via brief (5 min) exposure to a Mg^{2+}–free HBS solution supplemented with (in mM): 0.4 Glycine (Fisher, Waltham, MA), 0.02 Bicuculline (Tocris), and 0.003 Strychnine (Tocris, Bristol, UK) Neurons were immediately washed with warm HBS after glycine stimulation and recorded from.

Statistical differences between experimental conditions were determined by one-way ANOVA and post-hoc Dunnett's multiple comparisons test, unless indicated otherwise. Shifts in cumulative probability curves were analyzed by Kolmogorov-Smirnov test.

Immunocytochemistry and Microscopy

Surface GluA1 (sGluA1) was labeled and imaged as previously described (Sutton et al., 2006). Neurons were live labeled for GluA1 with rabbit polyclonal anti-GluA1 (0.01 mg/mL; Millipore #ABN241) for 15min at 37°C, washed with pre-warmed PBS-MC, and fixed with 2% paraformaldehyde/2% sucrose in PBS with 2.0 mM MgCl2 and 2.0 mM CaCl2 (PBS-MC). Samples were blocked with 2% BSA in PBS-MC prior to labeling with Alexa555-conjugated goat anti-rabbit secondary antibody (Molecular Probes #A21429, 1:1000). Cells were then permeabilized with 0.1% Triton-X in PBS-MC followed by immunocytochemical labeling of PSD95 with mouse monoclonal anti-PSD95 (1µg/mL; Millipore #MAB1596) for 60min at RT and Alexa4880conjugated goat anti-mouse secondary antibody (Molecular Probes #A11029, 1:1000). All imaging was performed on an inverted Olympus FV1000 laser scanning confocal microscope with identical acquisition parameters for each treatment condition. Normalized values to the Baseline condition were used to make comparisons between repeated experiments. Analysis was performed with custom written analysis routines for ImageJ and Matlab. sGluA1 content at synapses was determined by measuring the integrated sGluA1 signal at puncta co-localized with PSD95. Synapse density was measured by the number of PSD95 puncta normalized to the length of the straightened dendrite. Statistical differences were assessed by ANOVA, then by Fisher's LSD post-hoc tests.

Westernblotting

Samples were collected in lysis buffer containing (in mM) 100 NaCl, 10 NaPO4, 10 Na4P2O7, 10 lysine, 5 EDTA, 5 EGTA, 50 NaF, 1 NaVO3, 1% Triton-X, 0.1% SDS, and 1 tablet of Complete Mini protease inhibitor (Roche) per 7mL, pH 7.4. Protein concentrations of samples were determined using a spectrophotometer. Equal amounts of protein for each sample were loaded and separated on ~10-12% polyacrylamide gels, then transferred to PVDF membranes. Blots were blocked with Tris-buffered saline containing 0.1% Triton-X (TBST) and 5% nonfat milk for 60min at RT or overnight at 4°C, and incubated with polyclonal anti-rabbit Phospho-p44/42 MAPK (pERK1/2) (1:1000; Cell Signaling #9101) antibody for 60min at RT. After washing with TBST, blots were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:5000; Jackson Immunoresearch), followed by enhanced chemiluminescent detection (GE Healthcare). Phospho-p42/44 MAPK antibody was stripped and re-probed (https://www.novusbio.com/support/support-by-application/stripping-for-reprobing) with polyclonal anti-rabbit p44/42 MAPK (ERK1/2) (1:1000; Cell Signaling #9102). To confirm equal loading, protein concentrations of each sample were assessed using spectrometry. Additionally, all blots were probed with a mouse monoclonal antibody against α-tubulin (1:5000; Sigma-Aldrich) to confirm equal loading. Band intensity was quantified with densitometry with ImageJ and expressed relative to the matched control sample. Statistical differences between the treatment conditions were assessed by One-way ANOVA and comparisons were made to the average of the Baseline groups at 1, 2, and 4hrs.

Activity Alteration and BrU-seq Analysis

To achieve enough neurons, both cortical and hippocampal tissue were isolated as described. Neurons were plated onto poly-D-lysine-coated 6-well cell culture plates at a density of ~3 million cells/well. Cells were plated with ~1mL NGM per well and then supplemented with ~1mL of NGM-GC 2hrs after plating. Prior to experimental treatments, neurons were maintained at 5% CO2 and 37°C and fed NGM-GC every 4 days until DIV14. At the start of the experiment, each well contained ~3mL of media. Similarly, to our electrophysiology experiments, 1mL of initial conditioned media is removed and saved for the drug-washout period. After removal, neurons were treated with either TTX or Veh (1:1000; 1µM TTX) for 24hrs. The initial TTX/Veh treatment was thoroughly removed via 2x washes (leaving ~500µL of media to cover the cells each wash) and lastly maintained with a final 50:50 saved media and fresh NGM solution for 48hrs before secondary treatment. On DIV17, cells were treated with TTX or Veh. 3.5hrs post-treatment, bromouridine (BrU, Sigma, 18670, dissolved in PBS) was added to cultures at 2mM final concentration and incubated at 5% CO2 and 37°C for the final .5hrs. Cultures were harvested in Tri-reagent BD (Sigma, T3809) and frozen immediately. RNA was purified using phenol-chloroform extraction and isopropanol precipitation, treated with DNase-I (NEB, M0303) then fragmented by high-magnesium, high temperature incubation. Enrichment of BrU-containing RNA and library preparation were performed as previously described (Paulsen et al., 2013; Paulsen et al., 2014; Garay et al., 2020). After confirming the quality of sequencing data by FastQC, reads were mapped to rn6 reference genome using Bowtie2 (Langmead & Salzberg, 2012) and annotated with Tophat 2 (Kim et al., 2013). Adaptors were trimmed using BBDUK (https://jgi.doe.gov/data-and-tools/bb-tools/), when 2-30 bp on the left of the read matched the predicted adaptor (k = 30, mink = 2, minlength = 15, hdist

= 1). Bru-seq signals were quantified by FeatureCounts (Liao et al., 2014). Differentially expressed genes were identified using DESeq2 (Love et al., 2014). Gene ontology analysis of the top 100 (padj < 0.05) DE-genes were assessed using PANTHER (http://geneontology.org/; Ashburner et al., 2000; Gene Ontology Consortium, 2021).

Results

A history of synaptic scaling in hippocampal cultures prevent subsequent activity-dependent changes in synaptic strength

Chronic silencing of neuronal activity (>24hrs) initiates robust compensatory upscaling of excitatory synaptic function, typically revealed as an increase in the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Turrigiano et al., 1998; Lissin et al., 1998; O'Brien et al., 1998; Thiagarajan et al., 2005; Sutton et al., 2006; Jakawich et al., 2010; Garay et al., 2020). Presumably, over the life-time of a neural circuit, synaptic up- and downscaling are engaged repeatedly to buffer potentially destabilizing levels of activity, raising the question of how a previous history of synaptic scaling affects subsequent homeostatic responses to alterations in network activity. Here we sought to test the resiliency of synaptic upscaling mechanisms focusing on whether networks of cultured hippocampal neurons exhibit changes in homeostatic synaptic adaptations depending on their prior scaling history. Since mEPSC recordings are not amenable to repeated measurements over days, we measured mEPSC changes a single time in four experimentally distinct groups subjected to the same 96hr experimental timeline where two separate 24hr exposures to TTX (1µM) or vehicle were applied with a 48hr washout period in between: 1) Baseline (n = 14): neurons that received only vehicle treatment; 2) Upscaled (n = 15): neurons treated with vehicle first, followed by TTX from 72-96hrs; 3) Reset

(n = 21): neurons treated with TTX from 0-24hrs, then with vehicle from 72-96hrs; and 4) Rescale (n = 17): neurons treated with TTX at both 0-24hrs and 72-96hrs, separated by a 48hr washout period (**Figure 3.1A**). This paradigm allowed us to compare the efficacy of upscaling in naïve neurons (Upscaled) relative to those with a prior history of upscaling (Rescale). As is well-established, we found that naïve neurons (Upscaled) exhibited robust compensatory increases in mEPSC amplitude after 24hr TTX relative to baseline ($F(3,63) = 11.65$, $p < 0.0001$; post-hoc Dunnett's multiple comparisons test, $p < 0.0001$) (**Figure 3.1B-C**). This compensatory increase in synaptic strength reset to basal levels upon removal of TTX for 72hrs (Baseline vs Reset: $p = 0.9969$) (**Figure 3.1C**). Surprisingly, however, neurons with a prior history of upscaling showed no evidence of upscaling when challenged with TTX a second time; mEPSC amplitudes were nearly identical to baseline levels (Baseline vs Rescale: $p = 0.9999$) (**Figure 3.1C**). The TTX-induced upscaling in naïve networks (Upscaled) can be seen in a significant (Kolmolgorov-Smirnov test, $p<.05$) rightward shift in the cumulative probability curve with mEPSC amplitudes, a shift that is not evident in the Rescale group (**Figure 3.1F**). Consistent with postsynaptic mechanisms, we did not observe any significant changes in mEPSC frequency or decay time in any of the groups (**Figure 3.1D-E**).

We next sought to test whether these history-dependent effects would also be evidence with synaptic downscaling induced by chronic network hyperactivation. We used a similar experimental paradigm (**Figure 3.2A**), where two separate 24hr exposures of GABA-A receptor antagonist, bicuculline (Bic, 10µM), were delivered – spaced by a 48hr wash period to cultured networks of hippocampal neurons and recorded mEPSCs in 4 groups: 1) Baseline (n = 13): neurons that received only vehicle treatment; 2) Downscaled (n = 8): naïve networks receiving vehicle from 0-24hrs and activated with Bic at 72-96hrs; 3) Reset (n = 11): neurons treated with

Bic from 0-24hrs, then vehicle from 72-96hrs; and 4) Rescale (n = 11): neurons exposed to Bic twice, at 0-24hrs and 72-96hrs. As expected, we observed robust synaptic downscaling in naïve networks treated with Bic from 72-96hrs (Downscaled) reflected by a significant reduction in mEPSC amplitudes relative to Baseline ($F(3,39) = 5.205$, $p = 0.0040$; post-hoc Dunnett's multiple comparisons test, $p = 0.0494$) (**Figure 3.2C**), as well as a significant leftward shift in the cumulative distribution of mEPSC amplitudes (**Figure 3.2F**). This compensatory depression in synaptic strength returned to basal levels following removal of Bic (Baseline vs Reset: $p = 0.2967$) (**Figure 3.2C**). As we observed with synaptic upscaling, we found a marked suppression of synaptic downscaling in cultures with a prior downscaling history (Baseline vs Rescale: $p = 0.7285$) (**Figure 3.2C, F**). No significant changes in mEPSC frequency or decay times were evident in any of the experimental groups ($F(3,39) = 6.338$, $p = 0.5677$; $F(3,39) = 6.780$, $p = 0.5708$, respectively) (**Figure 3.2D-E**). Together, these results indicate a prior history of synaptic scaling suppresses further homeostatic scaling to later changes in network activity.

Scaling-history dependent suppression of subsequent scaling events is directionally independent

We observe suppression of homeostatic changes in mEPSC amplitude in pre-scaled neuronal networks when the circuit is challenged repeatedly. We next asked how scaling history affects homeostatic responses to the opposite pattern of network alteration – for example, do previously downscaled networks exhibit altered homeostatic responses to upscaling induced by activity silencing or are these history effects only evident with scaling in the same direction? To address this question, we again exposed neurons to scaling epochs (24hrs) separated by a 48hr washout period and performed whole-cell mEPSC recordings in control neurons receiving vehicle alone (Baseline, n = 9), downscaled neurons exposed to Bic from 0-24hrs followed by

washout and vehicle treatment (Reset, n = 8), and previously downscaled neurons chronically silenced with TTX from 72-96hrs (Rescale, n = 10). We compared this Rescale group with neurons exposed to vehicle from 0-24hrs and TTX a single time from 72-96hrs (Naïve TTX) (**Figure 3.3A**). As shown in Figure 3B-E, downscaled mEPSCs in neurons return to basal levels following washout ($F(2,24) = 0.1362$, $p = 0.8733$; Baseline vs Reset: $p = 0.8402$), but later silencing with TTX for 24hrs is ineffective at upscaling mEPSCs (Baseline vs Rescale: $p = 0.9930$), while naïve neurons exhibit robust upscaling to the same TTX exposure (**Figure 3.3C**). We found similar results when switching the order of up- and downscaling regiments (**Figure 3.3F**): a previous history of upscaling also suppressed downscaling in response to later Bic challenge ($F(2,18) = 0.2526$, $p = 0.7795$) (**Figure 3.3H**). Collectively, these data suggest that, regardless of direction, a previous history of synaptic scaling suppresses subsequent homeostatic adaptations to chronic changes in activity.

Prior scaling is required for the suppression of subsequent scaling events and this suppression does not crossover to Hebbian forms of synaptic plasticity

We next asked whether a previous history of sustained network activity per se, rather than a synaptic scaling history, can suppress future homeostatic responses. To address this question, we substituted the initial 24hr TTX exposure which induces reliable upscaling with a 4hr TTX treatment, a sustained period of activity silencing that is insufficient to induce synaptic scaling (Turrigiano et al., 1998; Sutton et al., 2006) (**Figure 3.4A**). We included the same positive and negative control groups as in our previous experiments: vehicle-treated (Baseline, p = 7), vehicle-treated neurons exposed to TTX over the last 24hrs (Upscaled, p = 9), neurons previously treated with 4hrs of TTX followed by a 48hr washout period (Reset, p = 9), and

neurons exposed to TTX from 0-4hrs and 52-76hrs (4hr TTX-Upscaled, p = 9). As expected, we observed robust upscaling of mEPSC amplitudes in naïve neurons ($F(3,30) = 4.468$, $p = 0.0104$; Baseline vs Upscaled: $p = 0.0208$). Neurons with a prior 4hr TTX history also exhibited robust upscaling in response to the second TTX challenge (Baseline vs 4hr TTX-Upscaled: $p = 0.0125$) (**Figure 3.4C**), suggesting that history-dependent suppression of homeostatic scaling requires preconditioning with activity sufficient to induce synaptic scaling. Surprisingly, while a previous 4hr TTX exposure did not inhibit future induction of synaptic scaling, it did lead to a significant decrease in mEPSC frequency in both the Reset and Rescale groups. The interpretation of this specific result is not obvious but reflects a more complex effect of preconditioning on synaptic function.

Are neurons with a history of synaptic scaling refractory to all forms of synaptic plasticity or is this suppression specific for homeostatic plasticity? To address this question, we examined whether neurons with a prior history of synaptic upscaling had altered induction of Hebbian long-term potentiation (LTP). We used a well-established glycine-based chemical LTP (cLTP) induction protocol (400µM glycine for 10min in a low Mg^{2+}, HBS-based, stimulus solution, see materials and methods) and recorded mEPSCs in naïve (Baseline, n = 10) or previously upscaled neurons after resetting (Reset, n = 11), and compared synaptic efficacy to cultures treated with glycine (Naïve cLTP, n = 12; Pre-scaled cLTP, n = 13) (**Figure 3.4F**). We found that both naïve and previously upscaled neurons show robust increases in mEPSC amplitude ($F(3,41) = 4.005$, $p = 0.0137$; Baseline vs Naïve cLTP: $p = 0.0472$; Baseline vs Pre-scaled cLTP: $p = 0.0201$), but not frequency ($F(3,41) = 2.217$, $p = 0.1006$) or decay times ($F(3,41) = 1.793$, $p = 0.1637$) after cLTP induction (**Figure 3.4H-J**). Together, this data suggest

that the history-dependent suppression of synaptic scaling is not due to a general inhibition of all forms of synaptic plasticity but is rather specific for homeostatic synaptic plasticity.

Deficits in synaptic upscaling also occur in the surface expression of GluA1

What mechanism underlies history-dependent suppression of synaptic scaling? As an initial inroad to this question, we considered that suppression could be levied at 3 general mechanistic levels: 1) At trafficking of AMPARs to synapses, as bi-directional changes in synaptic AMPAR abundance are a hallmark of both synaptic up- and downscaling (O'Brien et al., 1998; Sutton et al., 2006; Jakawich et al., 2010; Groth et al., 2011); 2) At the activity-dependent signaling that engages downstream mechanisms necessary for synaptic scaling; and 3) At activity-dependent regulation of gene expression, as scaling is known to be critically dependent on new transcription (Ibata et al., 2008; Goold & Nicoll, 2010) and chromatin regulation (Yu et al., 2015; Benevento et al., 2016; Mao et al., 2018; Garay et al., 2020). AMPARs accumulate a synaptic sites during synaptic upscaling, so we asked whether suppression of scaling is associated with altered synaptic AMPAR expression or whether AMPARs traffic normally to synaptic sites but other mechanisms (e.g., post-translational modifications of receptors) account for the inhibition. We measured the surface levels of the AMPAR subunit GluA1 (sGluA1) at excitatory synapses by live-labeling with an anti-GluA1 antibody specific to an extracellular epitope, then fixing, permeabilizing and labeling for the synaptic marker, PSD95. Comparisons were made between the same four experimental groups Baseline, Upscaled, Reset, and Rescale (n = 90 neurons/group) (**Figure 3.5A**). Consistent with previous observations, we found a significant increase in sGluA1 at PSD95-labeled synapses in neurons silenced with 1μM TTX for 24hrs (Upscaled), and sGluA1 expression largely recovered

to basal levels following the 48hr washout period (Reset). Unlike neurons exposed to TTX a single time, those with a history of prior upscaling showed no increase in sGluA1 relative to controls (**Figure 3.5B-D**). These changes in sGluA1 were not associated with addition or removal of synaptic sites, as synaptic density (PSD95 puncta/μm) was similar across groups ($F(3,356) = 0.4646$, $p = 0.7072$) (**Figure 3.5E**). These results indicate that the history-dependent suppression of synaptic scaling is reflected in synaptic AMPAR abundance, suggesting that the suppression is mediated at some point upstream of AMPAR trafficking to synaptic sites.

Activity-dependent signaling is unaltered by a prior history of synaptic scaling

Might the history-dependent suppression of homeostatic scaling be due to altered encoding of chronic activity changes? This is a difficult question to fully address as the precise molecular mechanisms that encode chronic changes in activity remain poorly understood. However, considerable insight can be gained by asking whether activity-dependent signaling is regulated as a consequence of previous scaling experience. One of the most strongly activity-regulated pathways is the extracellular signal-regulated/mitogen-activated protein kinase (ERK/MAPK) signaling pathway, which is known to play a key role in linking neural activity with transcriptional regulation, among other critical neural processes (Treisman, 1996; Blüthgen et al., 2017). We addressed the potential regulation of altered activity signaling by measuring levels of phosphorylated ERK1/2 during the initial 1, 2, and 4hrs of activity manipulation in naïve and pre-scaled hippocampal cultures (**Figure 3.6A, E**). In naïve hippocampal neurons, activity silencing with TTX significantly reduced ERK1/2 phosphorylation without changing overall ERK1/2 levels or levels of tubulin (loading control), while network hyperactivation with Bic significantly enhanced ERK1/2 phosphorylation (**Figure 3.6C, D, G, H**). These patterns

were virtually identical in neurons with a previous history of upscaling (**Figure 3.6C, D**) or downscaling (**Figure 3.6G, H**) at all three timepoints examined. These data suggest that activity-dependent signaling is largely intact following a history of synaptic scaling implying that the history-dependent suppression is levied at a point downstream from initial activity decoding, perhaps at the level of transcriptional control.

Synaptic upscaling induces a persistent transcriptional program

As transcription plays a critical role in synaptic scaling, we next asked whether history-dependent suppression of homeostatic scaling might relate to altered activity-dependent regulation of gene expression. To examine transcriptional dynamics directly, we employed bromouridine RNA sequencing (BrU-seq) (Paulsen et al., 2013; Paulsen et al., 2014) to monitor newly generated transcripts over the first 4hrs of activity silencing. This genome-wide nascent transcript profiling technique provides an unbiased account of newly transcribed genes during TTX silencing of neurons. We prepared primary hippocampal and cortical neuron co-cultures from brain tissue isolated from P1-P2 rats and allowed them to mature for 14 days *in vitro* (DIV). We adopted the same paradigm we have used in functional studies, where neurons are treated with either vehicle or TTX from 0-24hrs, followed by a 48hr washout period, then either vehicle or TTX again for 4hrs. During the last 30mins of this second TTX treatment, we added BrU to the culture medium to label newly synthesized transcripts which are then isolated using magnetic beads coated with anti-BrU antibody and processed for next-generation sequencing (**Figure 3.7A**).

Our BrU-seq dataset revealed high levels of intronic reads, confirming that the detected transcripts were recently generated and had yet to be spliced into mature RNA (**Figure 3.7B**). Differential statistical analysis (DESeq2) of gene transcripts in response to TTX silencing of

naïve neurons revealed an abundance of down- (916, padj < 0.05) and up-regulated genes (202, padj < 0.05). Comparing transcriptional responses to 4hr TTX with or without a prior scaling history revealed that previous scaling dampened the overall transcriptional response to activity silencing, as perhaps most easily appreciated in the volcano plots shown in **Figure 3.7C**. The number of TTX response genes that surpass the significance threshold is greatly reduced in pre-scaled neurons (Downregulated: 299, padj < 0.05; Upregulated: 57, padj < 0.05) relative to naïve neurons. Moreover, the degree TTX-induced regulation of critical genes in synaptic plasticity such as brain-derived neurotrophic factor (*Bdnf*), neuronal pentraxin (*Nptx1*), and histone deacetylase 9 (*Hdac9*), are severely dampened in pre-scaled neurons (**Figure 3.7C**). This data suggests that a history of chronic activity silencing within neurons has long-lasting effects on future TTX-dependent gene transcription.

Although TTX-regulation of gene transcription is altered in pre-scaled neurons, we do not know at what stage these deficits start to arise – during the initial TTX challenge (Naïve TTX), the reset period where TTX is removed (Reset), or during the secondary TTX challenge (Pre-scaled TTX). As an initial probe into this issue, we looked at the FPKM (fragments per kilobase of transcript per million mapped reads) of widely known activity-dependent IEGs (immediate early genes) across our 4 experimental conditions (**Figure 3.7D**). Relative to Baseline, silencing of activity upon initial TTX treatment (Naïve TTX) results in a characteristic drop in FPKM in all 6 IEGs (*Junb*, *Homer1*, *Egr1*, *Bdnf*, *Arc*, and *Fos*). Unexpectedly, we found that these genes did not fully recover after removal of TTX in the Reset group, despite the full recovery of synaptic compensation (**Figure 3.1**). These genes were responsive to a second TTX treatment (Pre-scaled TTX), though this response is noticeably diminished. This incomplete recovery was an effect expressed by a number of genes though a number of others recovered

completely and responded nearly identically to a TTX challenge regardless of their activity-dependent history (**Figure 3.7G**). To analyze these trends further, we categorized gene responses by finding the overlap of pairwise differentially regulated genes (DESeq2; $p < 0.05$) with genes that were significantly different between Baseline, Naïve TTX, Reset, and Pre-scaled TTX (Chi-square test). From this analysis, 4 distinct groups emerged: resets and rescales, resets without rescale, no reset but rescales, and no reset or rescale (**Figure 3.7G; Figure 3.9B**). Gene ontology analysis (Ashburner et al., 2000; The Gene Ontology Consortium, 2021) of the top differentially regulated genes (padj < 0.05) between Baseline and Reset groups revealed regulation of activity-dependent molecules including genes involved in the activity of voltage-gated cation channels, growth factors, and protein serine phosphatases. As expected, DE-genes were localized to neuronal cellular components with some of the highest hits being neuron projection, glutamatergic synapse, pre- and postsynapse (GO:0043005; GO:0098978; GO:0098793; GO:0098794) (**Figure 3.9A**).

The reset phase plays a unique refractory role in the regulation of synaptic scaling

The heterogeneity in transcriptional response behavior identified in our BrU-seq dataset suggests that prior scaling impacts activity-dependent transcription at multiple levels. Moreover, the ongoing transcriptional impact following TTX washout suggest a potential role for the resetting phase in history-dependent suppression of scaling. To examine this possibility, we undertook two experiments where we specifically manipulated the features of the resetting phase to ask whether this impacted history-dependent suppression of synaptic scaling. First, we looked at extending the drug-free period from 48hrs to 96hrs (**Figure 3.8A**). Interestingly, the extension of the Reset phase completely rescued synaptic upscaling of mEPSC amplitudes in pre-scaled

hippocampal neurons ($F(3,37) = 15.69$, $p < 0.0001$); Baseline vs Rescale: $p = 0.0014$) (**Figure 3.8B**). These data suggest that neurons are refractory to future homeostatic scaling during the reset phase and that this inhibition is time-limited. These results are consistent with a role for the reset phase in suppressing homeostatic scaling, but they do not rule out the possibility that the initial scaling event imposes a time-limited suppression of future scaling that just happens to coincide with the reset phase in our experiments. If the reset phase is truly deterministic, then neurons should show no suppression of scaling if resetting is eliminated altogether. To test this possibility, we examined whether neurons could upscale directly from a downscaled state. We treated neurons with Bic for 24hrs to induce downscaling, then immediately exposed them to TTX to suppress firing (**Figure 3.8E**) and monitored mEPSCs 48hrs later. Under these conditions, we observed no history-dependent suppression of upscaling – TTX treated neurons exhibited a significant increase in mEPSC amplitudes ($F(2,20) = 17.09$, $p < 0.0001$; Baseline vs Pre-scaled TTX (48hrs): $p = 0.0138$) with no detectable changes in mEPSC frequency or decay time. Collectively, this data indicates there exists a unique reset phase and that it plays a critical refractory role in the regulation of synaptic scaling.

Discussion

Homeostatic forms of plasticity have been intensely studied due to their putative role in conferring long-term stability on network activity in neural circuits. A key question that has not been addressed is how such homeostatic regulation might be shaped by the history of activity within the circuit. We have examined this issue in networks of cultured hippocampal neurons and document several novel features of homeostatic synaptic scaling. First, our results demonstrate that functional homeostatic adaptations induced by synaptic scaling “reset” during

renormalization of network activity levels (**Figure 3.1-3.3**). While this finding is clearly predicted by current thinking in the field, it has surprisingly never before been empirically demonstrated. In an early study of synaptic protein changes during scaling, Ehlers found that changes in synaptic protein expression that accompany up- and downscaling do reverse to basal levels 48hrs after activity normalization (Ehlers, 2003), which is consistent with the changes in synapse function we report here. Second, we demonstrated that a prior history of synaptic scaling exerts profound effects on future homeostatic changes in response to the same activity perturbations. Hence, a history of upscaling prevented later upscaling, when induced 48hrs following renormalization of activity. Similarly, prior induction of Bic-induced downscaling suppressed future downscaling in response to the same Bic exposures. This history-dependent suppression of synaptic scaling was also evident when the activity changes from the initial event to the second event were opposite – that is, prior upscaling also suppressed future downscaling and vice versa. These history effects do not reflect a general dampening of synaptic plasticity, since a previous history of scaling did not alter the induction of cLTP at these synapses. Collectively, these results implicate a key role for synaptic scaling history of a network to shape future homeostatic adaptations to alterations in neuronal activity.

What is the mechanism by which initial scaling events suppress future scaling? We explored the underlying molecular mechanism at 3 general mechanistic levels. First, we demonstrate that neurons with a history of prior upscaling showed no increase in sGluA1 after 24hrs of activity silencing, a well-established homeostatic response (Sutton et al., 2006; Jakawich et al., 2010; Groth et al., 2011) observed in neurons exposed to TTX a single time (**Figure 3.5**). This deficit in TTX-dependent AMPAR accumulation cannot be explained by a loss of synapses as synaptic density, determined by PSD95 puncta density, was similar across all

experimental groups (**Figure 3.5E**) suggesting that the suppression is mediated upstream of AMPAR trafficking at synapses. It is noteworthy that post-translational modifications of AMPAR play a critical role in synaptic scaling (Goel et al., 2011) and that future experiments are needed to validate whether or not altered post-translational regulation of AMPARs contributes to the suppression seen in our functional studies.

Second, we examined the possibility that activity-dependent signaling might be altered as a consequence of the initial scaling event, and in that way, the mechanisms that decode chronic activity changes would be impaired. To address this question, we analyzed activity-dependent ERK1/2 phosphorylation (TTX silencing and Bic hyperactivation) in naïve neurons and neurons with a prior upscaling history since ERK/MAPK signaling is one of the most tightly linked to activity modulation. Our results clearly demonstrate that activity-dependent ERK1/2 signaling is largely intact following scaling, as we observed characteristic decreases and increases in phosphorylated ERK1/2 in hippocampal neurons after TTX and Bic treatments, respectively, without changes in overall ERK1/2 or tubulin protein levels regardless of activity-history (**Figure 3.6**). This result implicates that ERK-dependent encoding of neuronal activity remains unaltered, however, there exists an extensive list of postsynaptic scaffolding proteins, intracellular signaling pathways, transcriptional and translational regulators, cell-adhesion molecules, and soluble released factors that have been found to be critical during homeostatic synaptic plasticity (reviewed in Fernandez & Carvalho, 2016) and could contribute to the suppression of scaling.

As new transcription has been shown to play a key role in synaptic scaling (Ibata et al., 2008; Goold & Nicoll, 2010), we asked whether transcriptional dynamics are altered by a scaling history in neurons. Using a nascent mRNA sequencing approach (BrU-seq), we monitored newly

generated transcripts over 4hrs of activity silencing. Comparisons between transcriptional responses in neurons with or without a prior upscaling history revealed prior scaling severely dampened overall transcription of TTX-response genes (**Figure 3.7B**). In stark contrast to the resetting of functional changes we observed, many activity-dependent IEGs did not fully recover after renormalization of activity (**Figure 3.7D**). Although these genes were responsive to a second TTX treatment, the degree of expression was often noticeably diminished (**Figure 3.7D**). Collectively, our results suggest that history-dependent suppression of synaptic scaling associated with persistent changes in the activity-dependent transcriptional program of neurons. After further analysis of transcriptional trends, we found that gene responses fell into four distinct patterns: resets and rescales, resets without rescale, no reset but rescales, and no reset or rescale (**Figure 3.7F, G, 3.9B**). The emergence of these groups suggest that synaptic scaling does not alter the transcription of all genes equally; the recovery after initial upscaling and response to a secondary TTX treatment vary depending on the gene. Which gene products are responsible for the functional changes during scaling and which underlie the refractory suppression of subsequent scaling events? Our gene-ontology analysis of the top significantly TTX-regulated genes may provide qualitative insight (**Figure 3.9A**) and future experiments will be needed to elucidate the potential mechanisms involved. An important limitation of our BrU-seq approach is the lack of cell type specificity. The present study does not distinguish transcriptional effects between excitatory or inhibitory neurons or neurons vs astrocytes. Future studies should examine cell-type specific transcriptional profiles using approaches such as INTACT or single-cell transcriptomics to address this issue. Nevertheless, our study provides evidence of enduring changes in transcriptional regulation that significantly outlast the initial scaling event and likely contribute to altered homeostatic regulation in the future.

In summary, our results introduce another level of complexity when considering how homeostatic signaling regulates the properties of neural circuits. We show that synaptic scaling is not iteratively induced in a stereotyped manner, but rather, can be profoundly influenced by the activity-dependent history of the network. Surprisingly, this effect of activity history seems to require the resetting phase of synaptic scaling where scaled synaptic weights revert to basal levels when network activity renormalizes. Extending the resetting phase from 48 to 96hrs eliminated history-dependent suppression of scaling, revealing that the refractory period where future scaling events are suppressed is time-limited. More telling, eliminating the resetting phase altogether, by moving network activity directly from a hyperactive state to a suppressed state, also abolished history-dependent scaling. It is now of interest to determine what cellular and molecular mechanisms that accompany the resetting phase are key to both renormalizing synaptic weights and conferring suppression of future scaling. These findings illuminate distinct temporally-dependent molecular mechanisms that work in concert to determine homeostatic responses to chronic activity changes in neural circuits.

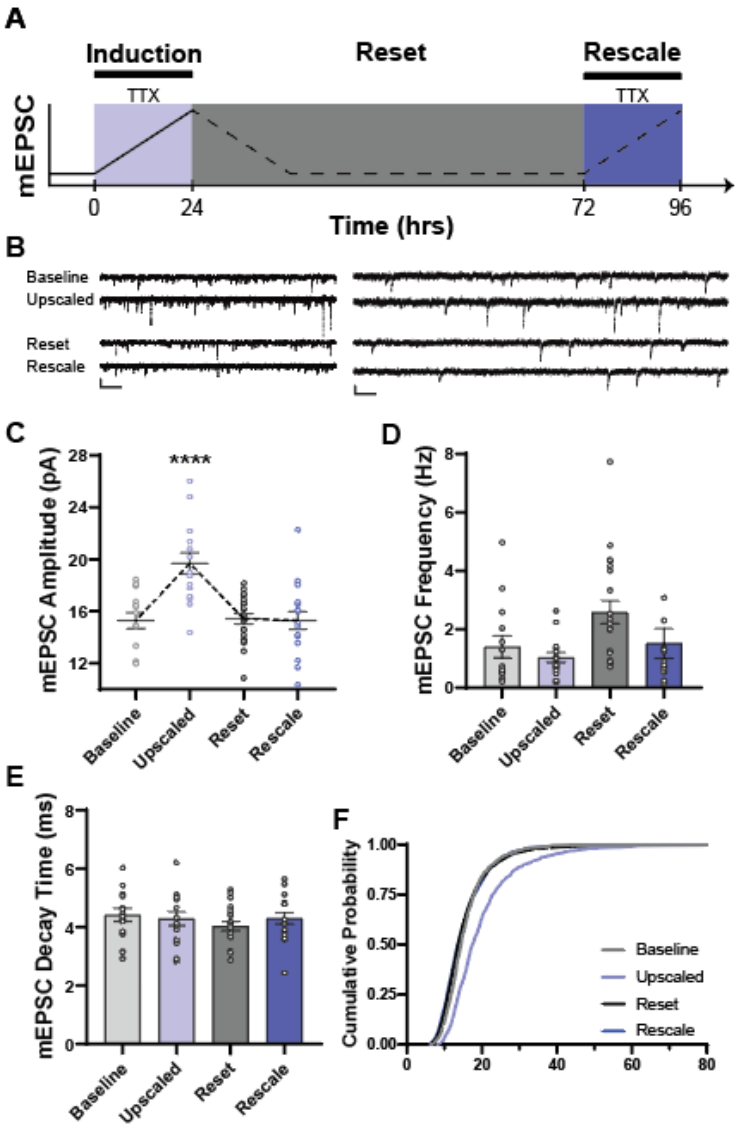


Figure 3.1 TTX-induced synaptic upscaling of mEPSC amplitudes is suppressed in hippocampal networks with a prior upscaling history (A) Experimental timeline representing the Rescale experimental group where hippocampal neurons are chronically silenced with TTX for 24hrs twice, given a 48hr drug-free period (dashed lines represent theoretical and untested changes in mEPSC amplitude). **(B)** Representative whole-cell voltage clamp recordings of hippocampal cultured neurons treated with only Veh (Baseline), Veh first and TTX second (Upscaled), TTX first and Veh second (Reset), TTX for both trials (Rescale); compressed (left; scalebar: 5pA, 1.25s) and expanded (right; scalebar: 10pA, 125ms). **(C)** mEPSC amplitude mean ± SEM, **(D)** mEPSC frequency, and **(E)** mEPSC decay time of the four experimental groups: Baseline, Upscaled, Reset, and Rescale. **(F)** Cumulative probability distributions of mEPSC amplitudes in Baseline, Upscaled, Reset, and Rescale experimental groups. All graphs represent mean ± SEM. For **(C-E)**, one-way ANOVA, followed by post hoc Dunnett's multiple-comparisons test relative to Baseline were performed; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

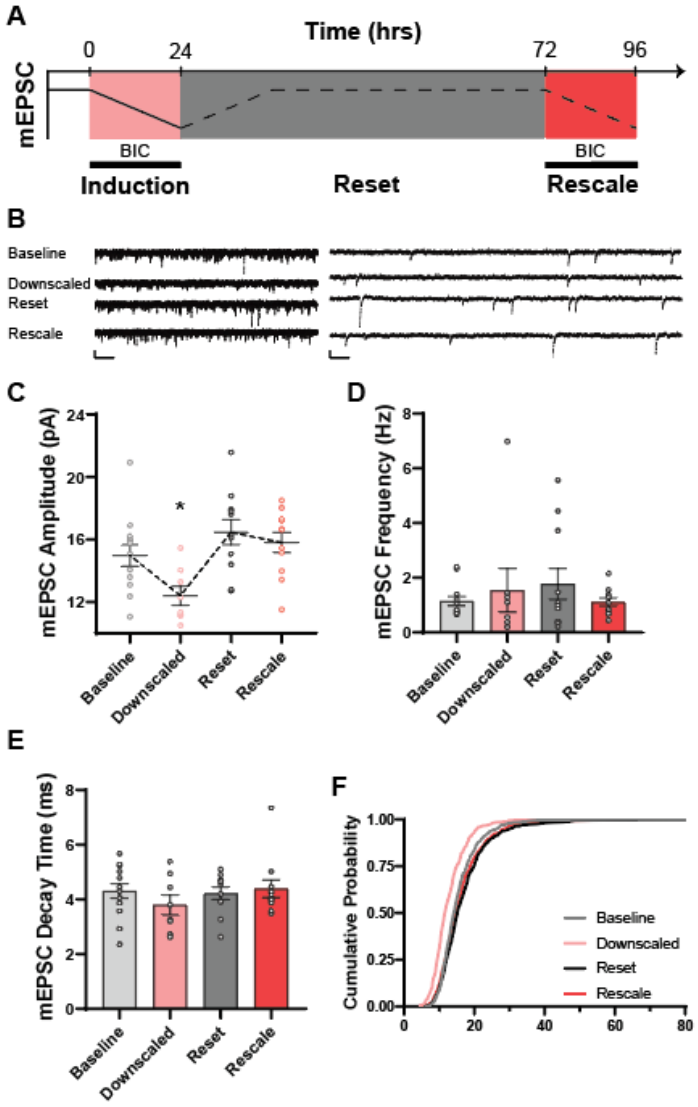


Figure 3.2 BIC-induced synaptic downscaling of mEPSC amplitudes is suppressed in hippocampal networks with a prior downscaling history (A) Experimental timeline representing the Rescale experimental group where activity levels in hippocampal neurons are chronically elevated with BIC for 24hrs twice, given a 48hr drug-free period (dashed lines represent theoretical and untested changes in mEPSC amplitude). **(B)** Representative whole-cell voltage clamp recordings of hippocampal cultured neurons treated with only Veh (Baseline), Veh first and BIC second (Downscaled), BIC first and Veh second (Reset), BIC for both trials (Rescale); compressed (left; scalebar: 5pA, 1.25s) and expanded (right; scalebar: 10pA, 125ms). **(C)** mEPSC amplitude mean ± SEM, **(D)** mEPSC frequency, and **(E)** mEPSC decay time of the four experimental groups: Baseline, Downscaled, Reset, and Rescale. **(F)** Cumulative probability distributions of mEPSC amplitudes in Baseline, Downscaled, Reset, and Rescale experimental groups. All graphs represent mean ± SEM. For **(C-E)**, one-way ANOVA, followed by post hoc Dunnett's multiple-comparisons test relative to Baseline were performed; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

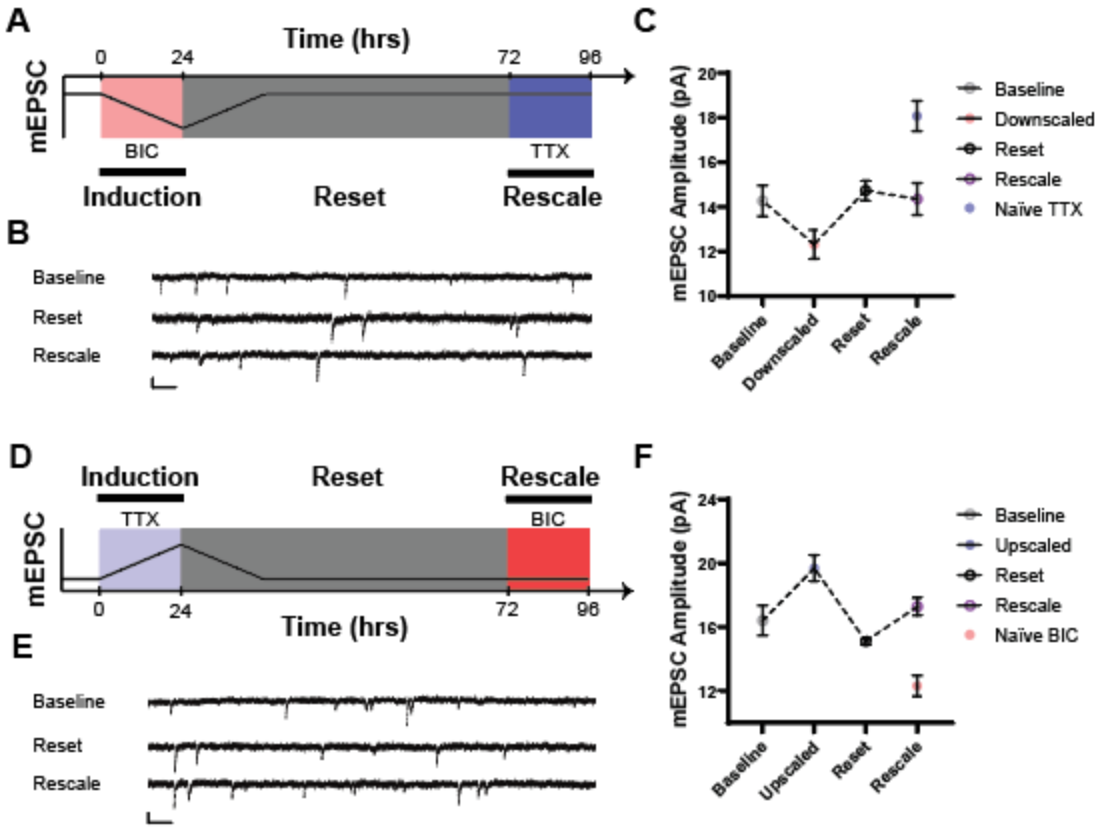


Figure 3.3 The suppression of synaptic scaling in previously scaled hippocampal networks is bidirectionally independent (A) Experimental timeline representing the Rescale experimental group where hippocampal neurons are chronically activated with BIC for 24hrs, reset for 48hrs, and then chronically silenced with TTX for 24hrs. **(B)** Representative mEPSC traces of Baseline, Reset, and Rescale (scalebar: 10pA, 125ms). **(C)** mEPSC amplitude **(D)** mEPSC frequency **(E)** mEPSC decay time **(F)** Experimental timeline representing the Rescale group where neurons are first silenced with TTX, reset with a drug-free period of 48hrs, and then chronically activated with BIC. **(G)** Representative mEPSC traces for Baseline, Reset, and Rescale (scalebar: 10pA, 125ms). **(H)** mEPSC amplitude **(I)** mEPSC frequency **(J)** mEPSC decay time. All graphs represent mean ± SEM. For **(C-E, H-J)**, one-way ANOVA, followed by post hoc Dunnett's multiple-comparisons test relative to Baseline were performed; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

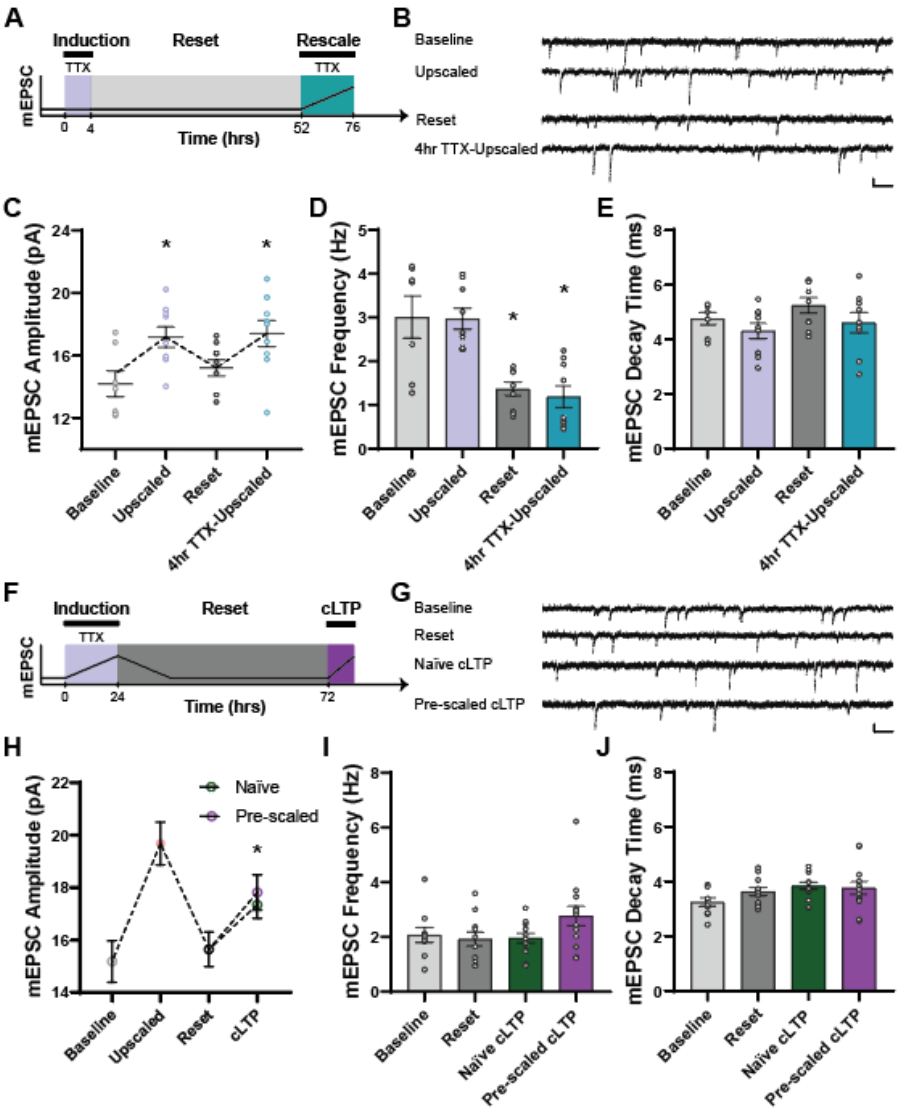


Figure 3.4 Prior scaling is required for the suppression of subsequent scaling events and this suppression does not crossover to Hebbian forms of synaptic plasticity (A) Experimental timeline showing the rescaling of mEPSCs in hippocampal neurons previously silenced with 4hrs of TTX. **(B)** Representative mEPSC traces for hippocampal cultured neurons treated with only Veh (Baseline), Veh first and TTX second (Upscaled), TTX for 4hrs first and Veh second (Reset), TTX for 4hrs first and TTX for 24hrs second (4hr TTX-Upscaled) (scalebar: 10pA, 125ms). **(C)** mEPSC amplitude **(D)** mEPSC frequency **(E)** mEPSC decay time **(F)** Experimental timeline of the induction of cLTP in hippocampal neurons previously upscaled with 24hrs of TTX. **(G)** Representative whole-cell voltage clamp recordings of neurons treated with only Veh (Baseline), TTX first and Veh second (Reset), Veh first and glycine-stimulus second (Naïve cLTP), TTX first and glycine-stimulus second (Pre-scaled cLTP). **(H)** mEPSC amplitude **(I)** mEPSC frequency **(J)** mEPSC decay time. All graphs represent mean ± SEM. For **(C-E, H-J)**, one-way ANOVA, followed by post hoc Dunnett's multiple-comparisons test relative to Baseline were performed; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

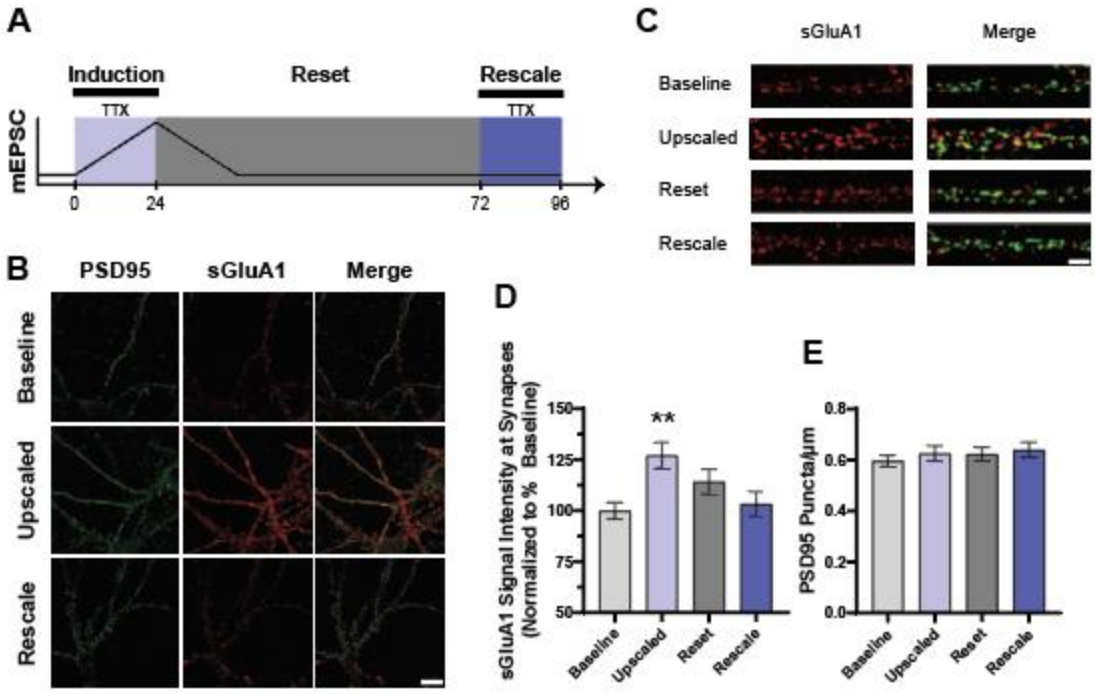


Figure 3.5 A scaling history prevents subsequent upscaling of surface AMPAR expression at synapses (A) Experimental paradigm representing the Rescale group. **(B)** Full frame representative images of hippocampal neurons fixed after only Veh treatment (Baseline), Veh first and TTX second (Upscaled), TTX silencing for both trials (Rescale), immunostained for antibodies against the surface epitope of GluA1 (red) and synaptic marker, PSD95 (green) (scalebar: 20µm). **(C)** Representative images of straightened dendrites immunostained with sGluA1 antibody (red) colocalized with PSD95 puncta (green); colocalization represented in yellow puncta within the Merge column (scalebar: 5µm). **(D)** Bar graphs of the mean ±SEM sGluA1 intensity at PSD95 puncta normalized to % baseline. **(E)** Mean ±SEM number of PSD95 puncta per µm of dendrite. **(D-E)** one-way ANOVA, followed by post hoc Dunnett's multiple-comparisons test relative to Baseline were performed; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

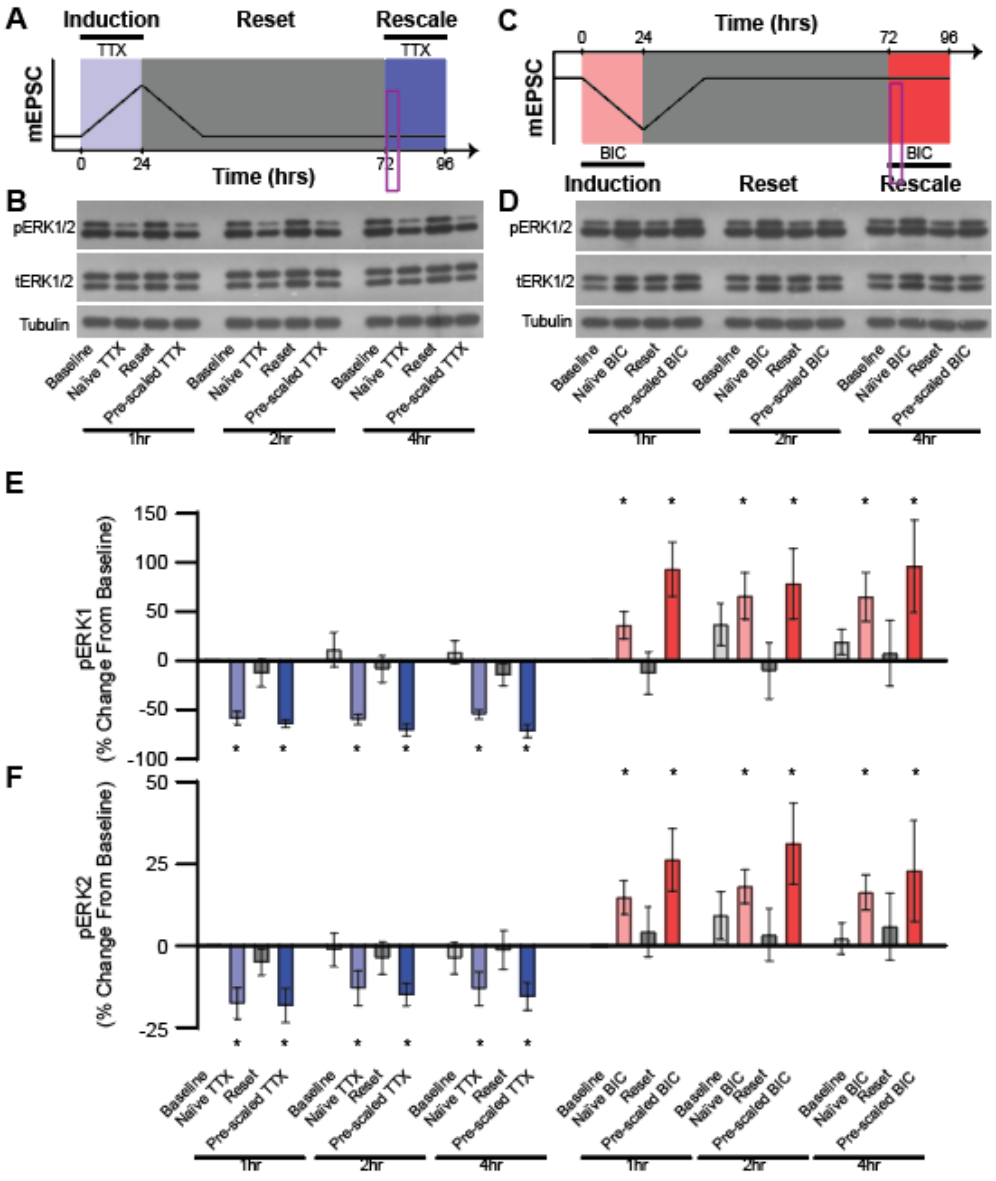


Figure 3.6 Activation of the ERK/MAPK signaling cascade via ERK1/2 phosphorylation remains intact in previously up- and downscaled hippocampal neurons (A, C) Experimental timeline representing the Rescale group; neurons were collected 1hr, 2hr, and 4hr post second Veh/TTX/BIC treatment. **(B, D)** Representative immunoblots of phosphorylated ERK1/2, total ERK1/2, and tubulin proteins collected from hippocampal neurons only treated with Veh (Baseline), Veh first and TTX/Bic second (Naïve TTX/Bic), TTX/Bic first and Veh second (Reset), and TTX/Bic for both trials (Pre-scaled TTX/Bic). **(E)** Bar graphs represent the mean ± SEM of pERK1 normalized to percent change from Baseline at 1hr. **(F)** Bar graphs represent the mean ± SEM of pERK2 normalized to percent change from Baseline at 1hr. One-way ANOVA and post-hoc LSD Fisher's test were used to compare pERK1/2 protein level differences between TTX or BIC treated groups in naïve and pre-scaled neurons relative to the average of Baseline groups.

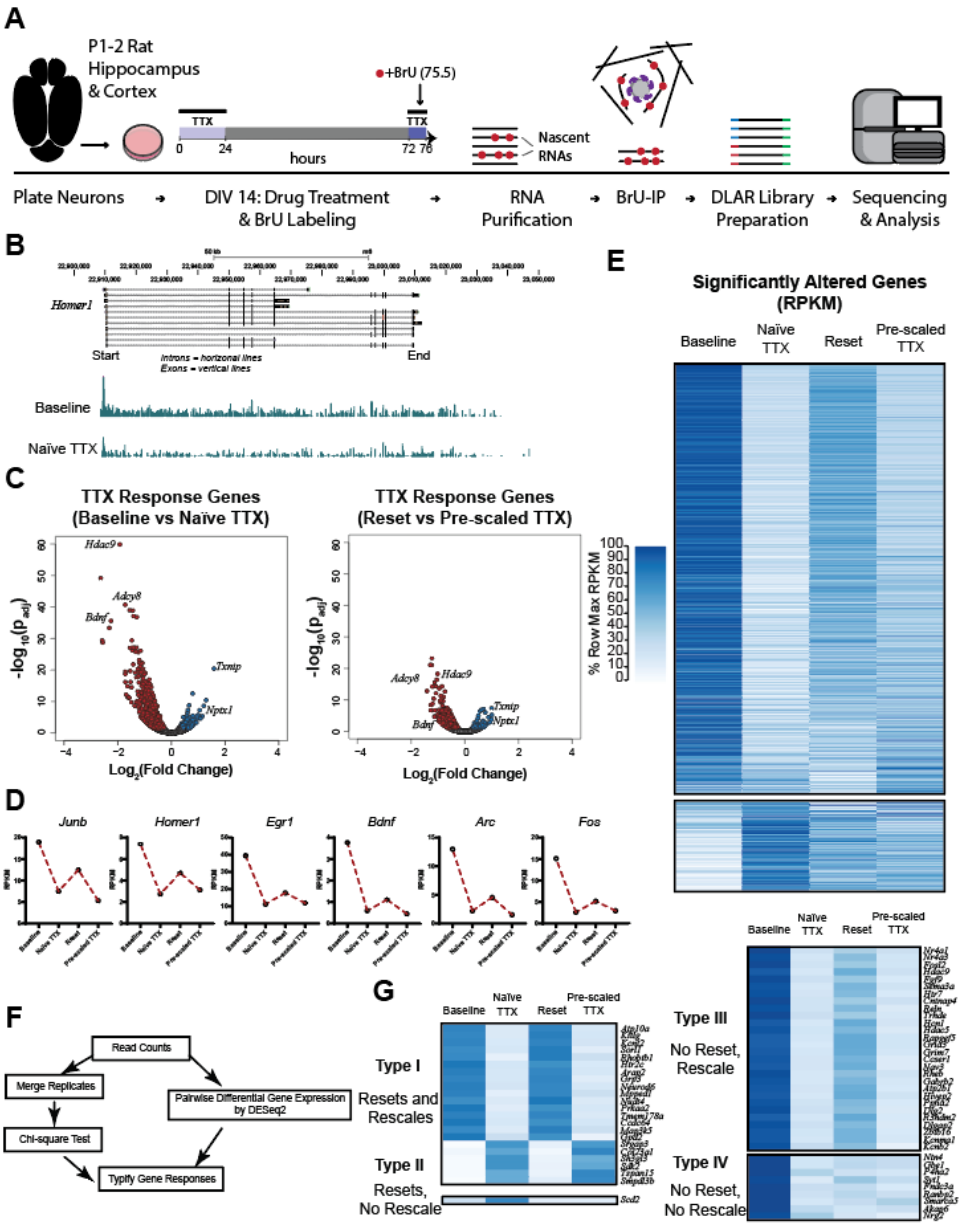


Figure 3.7 A history in synaptic upscaling in hippocampal neurons has a lasting role in transcription regulation (A) Experimental procedure using BrU-seq as a tool for analysis of nascent transcription. **(B)** Genome browser views of Homer1 isoform tracks with mapped reads of Baseline and Naïve TTX groups (bottom). Intronic reads are characteristic of nascent RNA. **(C)** Differential gene expression analysis (DESeq2) reveals widespread transcriptional changes in response to TTX in naïve hippocampal cultured neurons. The number and degree of differential regulated in response to TTX is dampened in hippocampal neurons with a prior TTX-induced upscaling history. **(D)** Graphs displaying fragments per kilobase of transcript per million mapped reads (FPKM) values of example activity dependent IEGs for the indicated conditions. **(E)** Heatmap of all significantly altered genes in response to TTX, padj < .05. **(F)** Schematic diagram of analysis to typify gene responses. **(G)** Heatmaps of typified gene transcriptional profiles.

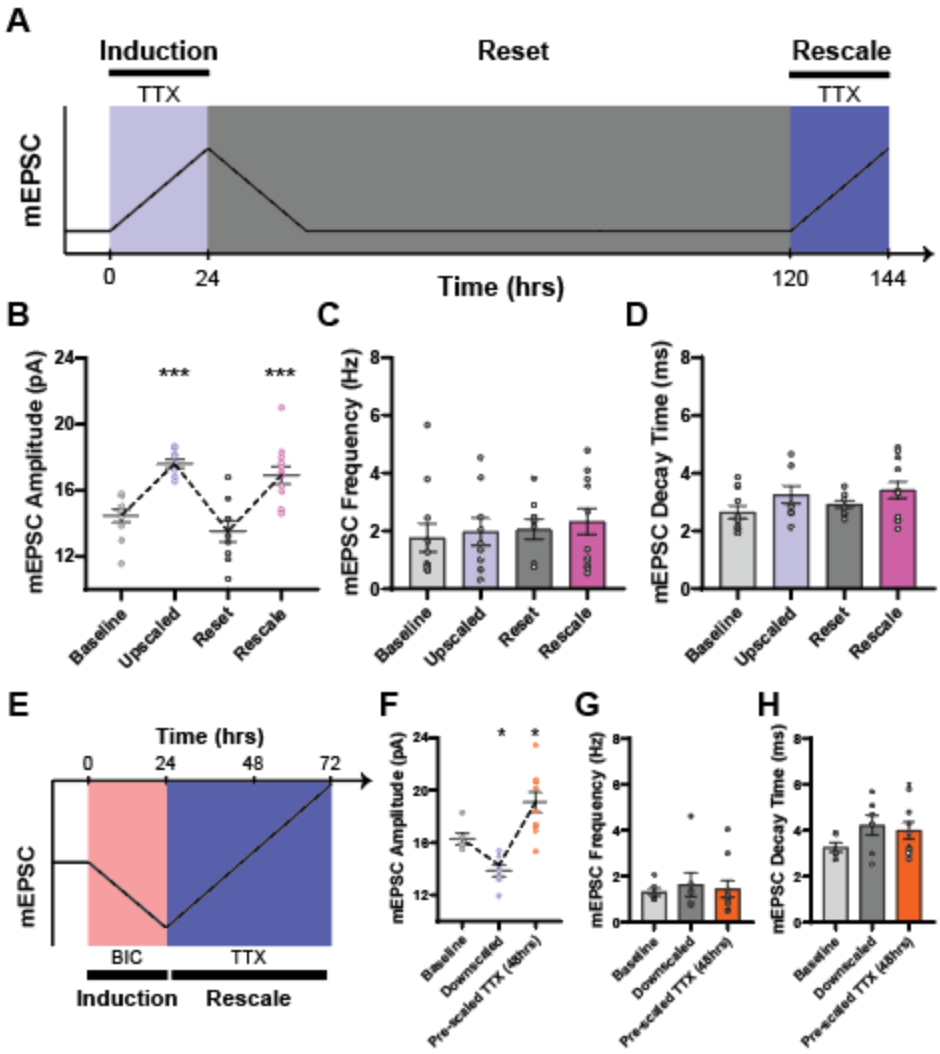
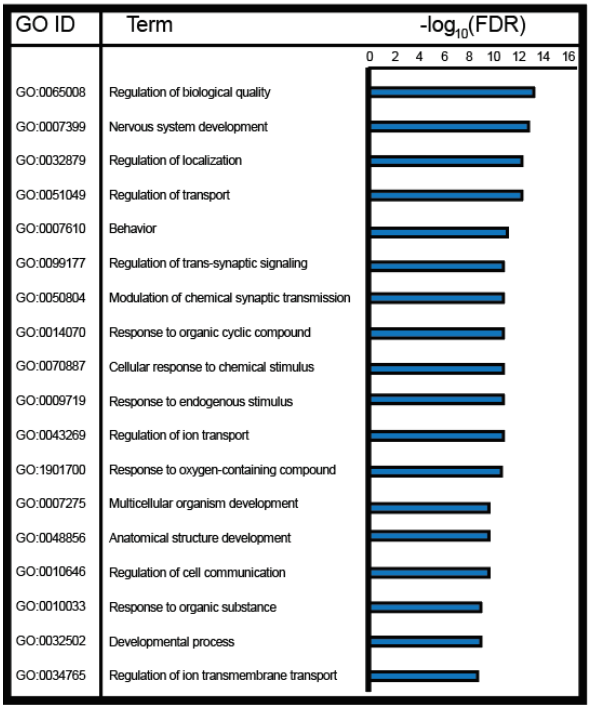


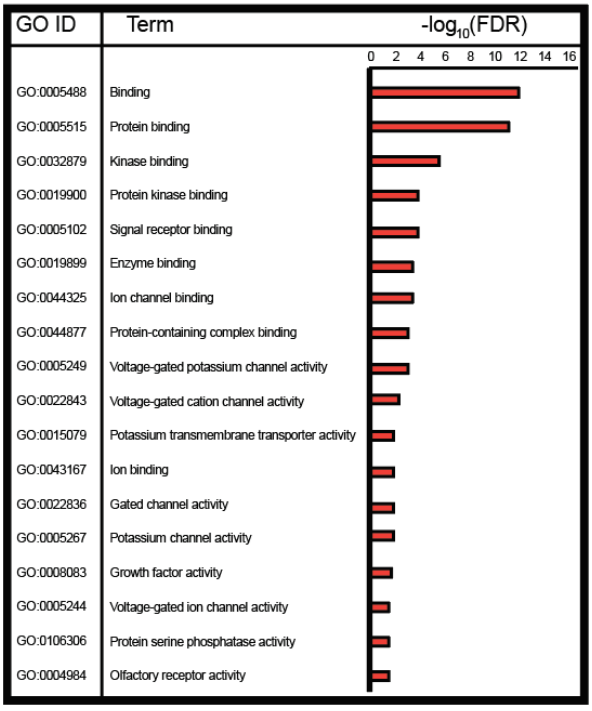
Figure 3.8 The reset phase plays a unique refractory role in the regulation of synaptic scaling. (A) Experimental timeline of the Rescale experimental group where the Reset drug-free phase was extended to 96hrs. **(B)** mEPSC amplitude **(C)** mEPSC frequency **(D)** mEPSC decay time represented as mean ± SEM of the indicated conditions. **(E)** Experimental timeline where hippocampal cultured neurons are downscaled with 24hr BIC treatment and immediately silenced with TTX for 48hrs. **(F)** mEPSC amplitude **(G)** mEPSC frequency **(H)** mEPSC decay time represented as mean ± SEM of the indicated conditions. **(B-D, F-H)** one-way ANOVA, followed by post hoc Dunnett's multiple-comparisons test relative to Baseline were performed; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

A)

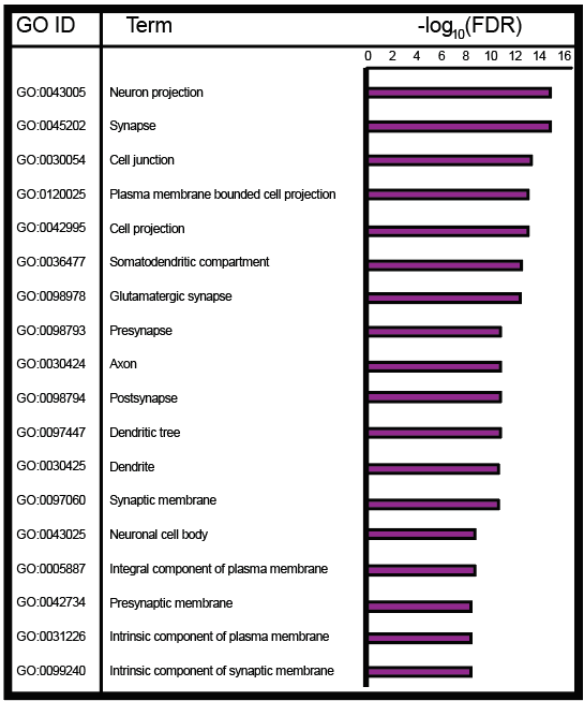
Biological Process



Molecular Function



Cellular Component



B)

Type I	Baseline	Naïve TTX	Reset	Rescale		Type III	Baseline	Naïve TTX	Reset	Rescale
Arap2	0.7700	0.3714	0.7473	0.3733		*Atp2b1*	5.8847	3.0330	4.4232	2.9069
Atp10a	0.1881	0.0459	0.1864	0.0517		*Ccser1*	1.1187	0.5132	0.7968	0.4756
Ccdc64	2.1879	1.4901	2.0695	1.5016		*Cntnap4*	1.6866	0.5982	0.8700	0.6106
Col23a1	0.1026	0.1590	0.1018	0.1764		*Dlg2*	2.4437	1.3047	1.7023	1.2008
Gpd2	1.2884	0.9965	1.2305	0.9149		*Dlgap2*	2.8529	1.6554	2.0992	1.5252
Gpr63	1.3252	0.6692	1.3297	0.6241		*Fgf9*	1.7336	0.4924	0.8308	0.4005
Htr2c	0.3606	0.1738	0.3285	0.1677		*Fosl2*	4.1349	0.9579	1.7874	0.9233
Kcnt2	0.9607	0.4376	0.9583	0.4561		*Gabrb2*	1.6209	0.8071	1.1846	0.8070
Kitlg	0.6540	0.2225	0.6811	0.2462		*Gria3*	1.6144	0.7336	1.2237	0.7819
Map3k5	0.5107	0.3507	0.5242	0.3741		*Grm7*	2.0505	0.9369	1.5261	0.9411
Mpped1	2.3472	1.3320	2.2990	1.5312		*Hcn1*	1.4390	0.5917	1.0469	0.6109
Neurod6	9.2229	4.7765	8.8900	4.6394		*Hdac5*	4.7568	2.0258	3.1805	1.8474
Nudt	1.0969	1.1325	0.7626	1.3012		*Hdac9*	1.7272	0.4130	1.0813	0.4960
Prkaa2	1.6861	1.0987	1.5937	1.1391		*Hivep2*	4.2673	2.2259	3.3057	2.3136
Rhobtb1	0.4431	0.2062	0.4656	0.2551		*Htr7*	0.4729	0.1484	0.2778	0.1324
Sdk2	0.9902	1.5448	1.0426	1.4535		*Kcnb2*	1.9895	1.2098	1.4441	0.9977
Sh3gl3	1.4127	2.1997	1.4804	2.0374		*Kcnma1*	2.7545	1.6199	2.1585	1.6075
Smpdl3b	0.3825	0.8414	0.4851	0.9745		*Nav3*	2.8098	1.3166	2.0754	1.2263
Sorl1	1.2358	0.5739	1.3217	0.5186		*Nr4a1*	22.6568	1.4120	5.2923	1.6190
Srgap3	2.8854	3.9629	3.0421	3.9259		*Nr4a3*	4.6064	0.5100	2.0655	0.6189
Tmem178a	2.5596	1.6946	2.5321	1.6879		*Ppfia2*	3.0756	1.6192	2.2141	1.5192
Tspan15	0.3660	0.6681	0.3942	0.7641		*R3hdm2*	4.5161	2.4332	3.5656	2.3784
						Rapgef5	3.1517	1.3547	2.3354	1.4144
Type II						*Reln*	1.4654	0.5709	0.9259	0.4963
Scd2	45.3346	55.6647	43.0188	43.0680		*Rheb*	4.8807	2.3671	3.3062	2.4729
						Sema3a	0.5165	0.1506	0.3175	0.1700
Type IV	**Baseline**	**Naïve TTX**	**Reset**	**Rescale**		*Trhde*	0.6602	0.2618	0.3514	0.2256
Akap6	3.0678	2.4519	2.3581	2.4114		*Zbtb16*	2.1245	1.2351	1.6498	1.1835
Fndc3a	1.7690	1.1767	1.1940	1.0970						
Gbe1	0.2958	0.1522	0.1726	0.1650						
Nrg2	1.8194	1.4780	1.3584	1.3056						
Ntn4	0.4619	0.2157	0.2464	0.2372						
P4ha2	1.0303	0.5940	0.5024	0.4776						
Ranbp2	5.7824	3.9609	4.1933	4.0098						
Smarca5	4.9968	3.5451	3.7113	3.8673						
Syt1	5.7216	3.4359	4.2308	3.8069						

Figure 3.9 Gene Ontology and FPKM Table (A) Gene Ontology Analysis of top differentially regulated genes comparing Baseline and Reset groups. **(B)** Table of FPKM reads for typified significantly regulated genes identified by the overlap of DESeq2 and Chi-square analyses.

CHAPTER IV

Discussion

This dissertation examined two unique aspects of homeostatic synaptic scaling in networks of hippocampal neurons – the role of specific LTCC subtypes in the induction of synaptic upscaling and how synaptic up- and down-scaling are influenced by the history of homeostatic signaling in the network. The results suggest that homeostatic synaptic scaling is a mechanistically complex form of plasticity that depends on Ca^{2+}-signaling through LTCCs and can be dissected into multiple distinct phases, an area that remains highly understudied in the field. Our results suggest that LTCC subtypes play distinct and non-redundant roles during the induction of synaptic upscaling. Moreover, our work demonstrates a complex interplay between homeostatic signaling and the history of activity in the network, revealing a form of homeostatic metaplasticity, where prior synaptic scaling suppresses future homeostatic adaptations in response to similar activity challenges. Further investigation into this complexity will help elucidate how homeostatic mechanisms confer stability and proper function in the face of on-going activity fluctuations within neural circuits.

Roles of unique LTCC subtypes in homeostatic synaptic scaling?

Two major LTCC subtypes are expressed in the brain, Cav1.2 and Cav1.3, and it has remained unknown how each contributes to homeostatic forms of synaptic plasticity. Our results

indicate that $Ca_V1.2$ and $Ca_V1.3$ play unique roles in the regulation of both basal neurotransmission and homeostatic scaling. Removal of $Ca_V1.2$, but not $Ca_V1.3$, enhances basal synaptic strength (**Figure 2.1B, 2.3B**). This increase, resembling synaptic upscaling, is consistent with studies that show that pharmacological LTCC blockade of dihydropyridines is sufficient to drive homeostatic upscaling of synaptic strength (Thiagarajan et al., 2005; Henry et al., 2012). Further evidence that $Ca_V1.2$ removal enhances synaptic strength through a scaling-like mechanism comes from the fact that it drives multiplicative changes in the mEPSC amplitude distribution and occludes further enhancement by TTX-induced activity silencing. One the other hand, removal of LTCC-dependent signaling with nifedipine has been shown to interfere with synaptic upscaling (Sokolova & Mody, 2008), which resembles the synaptic phenotype accompanying $Ca_V1.3$ removal. While having no impact on basal transmission on its own, genetic deletion of $Ca_V1.3$ prevents synaptic upscaling induced by chronic TTX treatment (**Figure 2.1B**). Taken together, these findings suggest that LTCCs play a complex role in synaptic scaling that includes both positive and negative regulation of homeostatic signaling.

How does removal of LTCCs result in two antagonizing regulatory roles in synaptic strength? One likely possibility is that, rather than aggregate LTCC activity being the cellular readout of neural activity, perhaps the combinatorial signaling through both $Ca_V1.2$ and $Ca_V1.3$ LTCCs, play important roles for homeostatic signaling during chronic changes in network activity. Our results suggest that $Ca_V1.2$ LTCC disruption may act as a trigger for increased basal synaptic strength, a resulting phenotype that cannot be compensated for by signaling through other Ca^{2+} sources. Our data also suggest the possibility that $Ca_V1.3$-mediated signaling plays a positive regulatory role during HSP since $Ca_V1.3$ deletion disrupted upscaling without altering basal synaptic properties. However, since TTX-dependent upscaling is also occluded in

hippocampal neurons lacking $Ca_V1.2$, this homeostatic regulatory role may not be exclusively mediated by $Ca_V1.3$ LTCCs. It is important to consider that the "occlusion" of upscaling we observed in $Ca_V1.2$ lacking neurons may, in part, be due to a ceiling effect attributable to the increased basal mEPSC amplitude. This possibility seems unlikely given that mEPSC amplitudes exhibit a wide dynamic range (~5-80pA) and the average basal mEPSC amplitude observed in $Ca_V1.2$ cKOs (<20pA) is within the lower end of this range. Additionally, work in other genetic models have documented larger changes in basal mEPSC amplitudes that still exhibit robust homeostatic upscaling in response to chronic silencing with TTX (McCartney et al., 2014). Another important variable that may play a role in distinguishing $Ca_V1.2$ and $Ca_V1.3$ knockout effects on HSP is the spatial distribution of the two LTCC subtypes within hippocampal neurons. CNC1 and CND1 antibody immunoreactivity within the hippocampus demonstrated region-specific expression of both $Ca_V1.2$ (CA1: cell body only; CA2/3: cell body and dendrites; DG: dendrites only) and $Ca_V1.3$ (cell body and dendrites throughout the hippocampus) (Hell et al., 1993). The mixed hippocampal neuron cultures in our experiments do not exclude potential spatial distribution effects of $Ca_V1.2$ and $Ca_V1.3$ during homeostatic synaptic adaptations. Future experiments, possibly with chemo-genetic approaches that can be used to specifically disrupt one LTCC subtype at specific hippocampal regions, are needed to validate the roles of individual LTCC subtypes in HSP.

LTCCs are localized to somatodendritic domains of neurons and gate calcium influx in response to relatively large depolarizations in membrane potential. As such, they have long been though to play a critical role in decoding sustained changes in neuronal activity necessary for synaptic scaling. Of relevance, chronic hyperactivation can have differential effects of $Ca_V1.2$ and $Ca_V1.3$-driven signaling owing to their different activation thresholds; $Ca_V1.2$ requires larger

depolarizing shifts relative to Cav1.3 (Helton et al., 2005). Studies using double KO or gain-of-function overexpression of Cav1.2 and Cav1.3 will be important in elucidating how alterations in LTCC expression affect HSP in neurons. It is also important to note that in each of our genetic systems, Cav1.2 and Cav1.3 were removed from both excitatory and inhibitory neurons. Future studies should explore the potential unique roles of each LTCC subtype in excitatory and inhibitory neurons, to determine whether homeostatic scaling of mEPSCs reflects action in excitatory neurons. A related question worth exploring is whether Cav1.2 and Cav1.3 play similar roles in the homeostatic scaling of inhibitory synapses.

Our work now opens up a framework to explore how LTCC subtypes each contribute to downstream effectors that regulate transcription or other processes needed to induce functional changes during synaptic scaling. One attractive potential effector is CaMKIV, a nuclear downstream signaling protein that encodes Ca^{2+} signals during activity perturbations and is known to be required for synaptic scaling (Goold & Nicoll, 2010; Joseph & Turrigiano, 2017). Finding overlaps in transcriptional programs with RNA-seq between Cav1.2 and Cav1.3 KO may also shed light on downstream gene products that are LTCC-mediated and required for homeostatic adaptations to alterations in network activity.

Physiological implications of homeostatic resetting?

The study outlined in Chapter 2 describes the fundamental role of a specific LTCC subtypes in TTX-dependent of mEPSCs (**Figure 2.1B, 2.3B**) and surface synaptic AMPAR expression (**Figure 2.2F**). Like virtually every other study in the homeostatic synaptic plasticity field, these experiments focused on mechanisms required during the induction of synaptic scaling. Given the presumed pervasive role of homeostatic signaling in stabilizing activity over

the lifetime of a neural circuit, there has been a general assumption that these processes can be recruited iteratively in circuits to buffer destabilizing patterns of activity at different times. However, whether such homeostatic adaptations in previously scaled networks can be repeatedly induced in the future has never been empirically tested. In fact, we describe experiments in Chapter 3 that indicate a "resetting" of scaled synaptic weights when network activity returns to basal levels, a widely presumed feature of synaptic scaling that has surprisingly never been demonstrated. The most relevant work on this issue came from Ehlers (Ehlers, 2003), who documented opposing and reversible changes in numerous PSD proteins upon chronic TTX or BIC treatment and drug washout in cortical neurons. His results imply a "resetting" like process at the level of protein expression, but no previous study has demonstrated that such a process occurs at the level of functional changes in synaptic strength.

Our work provides the first direct evidence that suggests that scaled synaptic weights, can be reset upon renormalization of network activity. While these findings are not surprising, they do underscore the notion that synaptic scaling can be temporally dissected into functionally distinct phases similar to Hebbian forms of plasticity; LTP can be partitioned into at least two phases, E-LTP and L-LTP (Huang, 1998). Moreover, as discussed in more detail below, these observations also raise questions about how the resetting phase of synaptic scaling impacts functional properties of the network. It is also relevant to consider that the induction phase of homeostatic scaling alone may also encapsulate distinct phases defined by unique molecular footprints that are needed in different time domains to instantiate homeostatic synaptic adaptations. The concept that temporal phases can be distinguished based on distinct biochemical interactions exists for LTP. It is generally thought that Ca^{2+} signaling through NMDARs and subsequent activation of Ca^{2+}-dependent kinases such as CaMKs play crucial roles in the initial

induction of LTP but *de novo* gene transcription and protein translation is required for L-LTP (Frey et al., 1988). Further research is needed to evaluate whether similar molecular checkpoints exist during homeostatic synaptic scaling, but recent research from our lab is consistent with this possibility. Our unpublished studies reveal that the transcriptional coactivator and histone H3 lysine 4 (H3K4) methyltransferase, KMT2A, plays a key role in regulating a secondary round of gene expression changes needed for synaptic downscaling. Curiously, however, KMT2A is required for downscaling during only the first 4hrs of network hyperactivation, indicating a time-dependent transition in the mechanistic underpinnings of this form of homeostatic scaling (Tsukahara, Chen, Iwase, and Sutton, unpublished data).

Is there a functional role for history-dependent suppression of synaptic scaling?

As discussed earlier, homeostatic scaling is thought to functionally complement Hebbian forms of plasticity in neural circuits, but these two forms of plasticity typically act on vastly different time scales. We initially hypothesized that circuits may implement scaling mechanisms more rapidly if they have a prior history of synaptic scaling. This would allow for more efficient coordination between Hebbian and homeostatic forms of plasticity in neural circuits for both information storage and stability. However, our results actually demonstrated the opposite – a history of synaptic up- or down-scaling in hippocampal cultures, not only failed to accelerate future scaling events, but rather suppressed those homeostatic adaptations. This history-dependent suppression was evident for both upscaling and downscaling and most intriguingly, future scaling was suppressed regardless of whether the initial event was in the same or opposite direction. We confirmed this refractory mechanism requires an initial scaling induction event as an acute (4hr) silencing of neurons with TTX, insufficient for scaling, had no appreciable

inhibition of scaling after resetting (**Figure 3.4C**). Interestingly, we demonstrated robust cLTP of mEPSC amplitudes in previously upscaled neurons suggesting the suppression of scaling does not generalize to all forms of synaptic plasticity (**Figure 3.4H**). Our data thus suggest that hippocampal neurons that have recently undergone scaling, enter a novel state that renders them refractory to homeostatic scaling of synaptic strength. A key question that now arises is whether other local forms of HSP driven by changes in synaptic drive (Sutton et al., 2006; Frank et al., 2006; Jakawich et al., 2010), or a homeostatic adaptation in intrinsic excitability (Zhang & Linden, 2003; Marder & Goaillard, 2006), are also subject to history-dependent regulation. In particular, if local forms of HSP are similar inhibited by a previous history of synaptic scaling, this would reveal potential master homeostatic regulators and mechanistic cross-talk between the homeostatic signaling pathways tuned to global (firing rate) and local (synaptic drive) features of neuronal activity.

What features of homeostatic scaling confer the history-dependent suppression of future events? While this remains an open question, our data do point to a critical role for the resetting phase. Extending the resetting period from 48hrs to 96hrs eliminates history-dependent suppression indicating a finite lifetime of the homeostatic "refractory" period. Even more telling, removing the resetting phase altogether, by switching directly from network hyperactivation to suppression without an intervening reset period, also eliminates history-dependent suppression of synaptic scaling. Together, these findings implicate a key role for the resetting phase of synaptic scaling in suppressing future homeostatic scaling in the same network. The full functional implications of this relationship are still unknown. The suppression could reflect some incompatibility between "scaled" and "resetting" synaptic states, where the process of initiating a resetting program actively turns off homeostatic signaling pathways and/or transcriptional

regulatory events. Perhaps a deeper question is what functional role, if any, does this history-dependent suppression confer? This too remains an open question, but one interesting possibility is that history-dependent suppression acts as a check on potential "runaway" homeostatic adaptations, where compensatory adaptations might overcorrect and bring the system into a potential pathological state. In response to cortical denervation, for example, some studies have demonstrated epileptic-like activity surrounding the damaged region, possibly due to homeostatic overcompensation in response to the initial insult (e.g., Paz et al., 2010; Takahashi et al., 2016). By suppressing a potentially additive homeostatic scaling response, resetting-induced suppression of synaptic scaling may be a check on such potential pathological instances of overcompensation in neural circuits.

What can genomics and proteomics reveal about functional homeostatic adaptations during synaptic scaling?

It is now evident that activity patterns required for synaptic scaling have profound effects on gene transcription and translation. As noted earlier, the transcriptional profile of RAI1 knockdown neurons closely resemble the transcriptional program driven by decreased network activity (Garay et al., 2020). Additionally, loss of RAI1 increased basal synaptic strength and inhibited upscaling but had no effect on downscaling of mEPSCs (Garay et al., 2020). A proteomic study using dynamic SILAC labeling in combination with mass spectrometry demonstrated a reduction in both protein degradation and protein synthesis during TTX-upscaling and BIC-downscaling relative to control (Dörrbaum et al., 2020). Interestingly our BrU-seq experiments demonstrated that there were more TTX responsive downregulated genes than upregulated genes in both naïve and previously upscaled neurons (**Figure 3.7C**), a finding

consistent with other RNAseq studies (Benevento et al., 2016; Yu et al., 2015; Garay et al., 2020). These studies suggest an overall downregulation in activity-dependent transcription during synaptic upscaling, but it is important to distinguish this profile from a generalized suppression of transcription. In addition to downregulated genes, several genes show specific upregulation during chronic activity silencing suggesting a more complex regulation of gene expression. Interestingly, we find that a history of synaptic scaling suppresses response to both up- and down-regulated TTX response genes, in addition to other dysregulated features such as incomplete recovery. Our gene ontology analysis reveals that these genes encompass several distinct functional categories – including voltage-gated cation channels, protein serine phosphatases, and trans-synaptic signaling regulators. Future studies will be needed to test the functional roles for specific subsets of this regulated pool.

Final thoughts and future directions

Over the past two decades, our understanding of homeostatic synaptic plasticity has increased exponentially with a particular intensive focus on the underlying molecular mechanisms of its induction. The study in Chapter 2 makes an important contribution in defining unique roles for $Ca_V1.2$ and $Ca_V1.3$ LTCC subtypes in its induction mechanism. However, our study outlined in Chapter 3 reveals that there is a considerable knowledge gap in our understanding of the temporal dynamics of synaptic scaling, beyond its induction. Our studies have revealed a novel resetting phase that alters the rules of future homeostatic scaling events. Future studies aimed at these complex aspects of activity-dependent homeostatic mechanisms are needed for a more complete understanding of network dynamics and maintenance of stability within the healthy brain. Insights from this basic knowledge will also lead to a better

understanding of neurological and neuropsychiatric disorders and potentially help efforts to develop novel therapeutics.

A crucial challenge for the field going forward is to better understand how homeostatic mechanisms operate within intact neural circuits *in vivo*. Although there is general agreement, from *in vitro* studies and theoretical models, that synaptic scaling is required for maintaining functional networks, we lack a clear understanding of how aberrant circuit dynamics are buffered by homeostatic synaptic scaling and other forms of homeostatic plasticity. For example, we do not y et know how different classes of homeostatic plasticity work together to stabilize network activity or even if they can be simultaneously expressed in the same synaptic sites *in vivo*. Despite that, evidence of synaptic scaling in several *in vivo* model systems (e.g.: visual cortex, Keck et al., 2013; motor cortex, Knogler et al., 2010; embryonic spinal cord, Garcia-Bereguiain et al., 2013; barrel cortex, Jamann et al., 2021) has emerged and this plasticity does share several important features with synaptic scaling studied in *in vitro* systems. The challenge now is to understand how more subtle changes in neural activity, rather than the extreme cases that have been studied to date, engage homeostatic signaling pathways in the context of dynamic neural circuits. With the rapid advancement of experimental tools capable of detecting and monitory neuronal activity with high spatial and temporal resolution (Ca^{2+}-imaging, multi-electrode arrays, iGluSnfr, and genetic voltage indicators), there will be improved methods to forge the link between activity and HSP in behaving animal systems. Ultimately, continued efforts in identifying homeostatic adaptations during experience-dependent changes in neural activity will be key to a more complete and developed understanding of physiological neural circuit function.

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