

**Mechanisms of synaptic homeostasis and their influence on Hebbian plasticity
at CA1 hippocampal synapses**

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Abstract

Information is transferred between neurons in the brain via electrochemical transmission at specialized cell-cell junctions called synapses. These structures are far from being static, but rather are influenced by plasticity mechanisms that alter features of synaptic transmission as means to build routes of information flow in the brain. Hebbian forms of synaptic plasticity – long-term potentiation and long-term depression – have been well studied and are considered to be the cellular basis of learning and memory, although their positive feedback nature is prone to instability. Neurons are also endowed with homeostatic mechanisms of synaptic plasticity that act to stabilize neural network functions by globally tuning synaptic drive. Precisely how neurons orchestrate this adaptive homeostatic response and how it influences Hebbian forms of synaptic plasticity, however, remains only partially understood. Using a combination of whole-cell electrophysiology, two-photon imaging and glutamate uncaging in organotypic hippocampal slices, I have expanded upon the known repertoire of homeostatic mechanisms that increase excitatory synaptic drive when CA1 hippocampal neurons experience a prolonged period of diminished activity. I found that the subunit composition of AMPA and NMDA receptors, the two major glutamate receptor subtypes at excitatory synapses, are altered which, in addition to increasing synaptic strength, are predicted to change the signaling and integrative properties of synaptic transmission. Moreover, I found that the amount of glutamate released from presynaptic terminals during evoked-transmission is enhanced and that this mechanism might, in part, underlie the uniform cell-wide homeostatic increase in synaptic strengths. Lastly, I found that homeostatic strengthening of synaptic transmission reduced the potential for CA1 synapses to exhibit long-term potentiation, and that this was caused by altered presynaptic release dynamics that impeded plasticity induction. Together, this work highlights several mechanistic strategies

employed by neurons to increase excitatory synaptic drive during periods of activity deprivation which, in addition to balancing cellular excitability, alters the metaplastic state of synapses.

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General Introduction

A defining feature of nearly all biological systems is the presence of homeostatic mechanisms that are continuously operant to provide balance and stability. In general, a biological homeostatic mechanism senses perturbations with respect to some physiological variable and triggers a response that re-establishes conditions back to a targeted set point – one that is optimal for the organism's function and/or survival. It is likely that biological homeostasis had an important role in early animal evolution, since even the simplest of organisms require strict intrinsic conditions for their viability. For instance, the single-celled *paramecium* evolved contractile vacuoles that eliminate water entering the cell through osmosis, which enabled them to maintain a constant internal fluid balance. Examples such as this are found everywhere in animal physiology, from the actions of single cells to the concerted action of tissues and organ systems, with a common theme being that they operate to maintain some physiological parameter in balance. What I hope to convey in this thesis is that the smallest functional units of the mammalian nervous system express a variety of homeostatic mechanisms that ensure an overall stability in the brain's functional dynamics.

The human brain contains nearly a hundred of billion (Azevedo et al., 2009) elaborately structured, functionally independent and electrically active cells called neurons, which are densely interconnected and have the innate ability to excite one another. Despite having these structural and functional properties one might predict would tend towards issues of electrical instability, our brains are typically in full control of their function, dictating and coordinating the numerous conscious and subconscious actions that keep us alive, healthy, and productive. What exactly stability in brain function means can be difficult to envision unless we consider obvious examples of when the brain clearly loses control of its stable state. For instance, an epileptic

seizure is characterized by waves of uncontrolled brain activity that can result in various symptoms including temporary involuntary convulsions and memory loss. These episodes remind us that active brain networks have innate stabilizing forces that are vulnerable to losing control, but are also effective at re-establishing stability once control is lost. The mechanisms through which active brain networks stabilize their functional dynamics such that they are able to coordinate tasks with precision, and in most cases, prevent the uncontrolled propagation of electrical activity throughout the brain, however, are only partially understood.

Since it is the collective activity of individual neurons that dictates large-scale network activity in the brain, it makes intuitive sense to search at the cellular level for homeostatic mechanisms that might stabilize the brain's functional dynamics. For whole brain networks to lose control, the activity many millions of individual brain cells must be hijacked and spun out of their functional equilibriums simultaneously. Since single neurons create functional assemblies with up to thousands of other neurons in the brain, the functional dysregulation of only a few cells can initiate a chain reaction that can propagate and disrupt the dynamics of many other connected brain regions. It is now increasingly understood that individual neurons are endowed with homeostatic mechanisms that direct their excitability to a set point that is optimal for them to participate in network functions and prevent their functional dysregulation, which is achieved by fine tuning various structural or functional features of their physiology. Collectively referred to as homeostatic plasticity mechanisms, these cellular phenomena are believed to provide an innate stability at the functional root of the complex and dynamic system that is our brain.

The purpose of this introduction is to guide you through the results and implications of decades of research that has focused on characterizing a unique strategy utilized by single neurons to maintain a homeostatic balance their function. To do so effectively, I will first

provide a brief overview of single neuron physiology, which will include a detailed description of the most important structures and processes that enable neurons to act as integrators and transmitters of neural information. I will then introduce the concept of neural plasticity, through which the structure and function of the brain's fine circuitry gets modified by experiences in order to form memories and associations. This concept of neural plasticity will then be expanded in the final section where I will introduce known forms of cellular homeostasis in the brain and discuss the specifics on how they influence neuronal excitability. Finally, I will end this introduction by highlighting several key conceptual gaps in our mechanistic understanding of how neurons express these cellular homeostatic mechanisms, which will set the stage for the three main chapters of this thesis.

Neurons are independent structural and functional units

The idea that each neuron is an independent structural and functional entity was met with heavy criticism when it first emerged prior to the turn of 19th century. Although the cell theory had been around for several decades, many anatomists still opposed that this theory applied to the nervous system, arguing instead that neuronal processes were continuous in their cytoplasm and thus physically connected with one another to enable electrical propagation (commented on in Cowan, 2001). The persistence of this *reticular theory* through till the early portion of the 20th century was accounted for by limitations in experimental tools that would enable visualization of the final terminations of neuronal processes. Despite these limitations, by the beginning of the 20th century the predominant view on this matter shifted and many began to accept the notion that neurons were morphologically discrete (Cowan, 2001). The most influential advocate for this *neuronal theory* was Santiago Ramón y Cajal, a Spanish neurohistologist at times referred to as the grandfather of modern neuroscience (Lopez-Munoz et al., 2006). Using a technique known

as the Golgi stain (Golgi, 1873), which enabled a sparse and apparently random labelling of neurons in brain slices, Cajal visualized and documented through his drawings the unique structures of single neurons across many different brain regions (Cajal, 1888c, b, a). His observations lead him to the idea that neuronal processes, including both axons and dendrites, ended freely onto what he proposed were the surfaces of other neurons (Cajal, 1889). Although explicit validation for this idea would not come until several years after his death, when the first images of these fine terminations were captured using electron microscopy (De Robertis and Bennett, 1955; Palay and Palade, 1955), Cajal had a tremendous influence on the neuroscience community of his era and swayed many into believing that this neuronal doctrine – that neurons were morphologically discrete entities – was likely factual.

Soon after, however, proponents of the neuronal theory debated over the mechanism through which electrical impulses were carried from one neuron to the next. In what was famously coined the *soup* vs *spark* debate, the two factions argued whether transmission of electrical impulses between cells of the nervous system required release of chemical messengers (soup) or rather there was direct electrical propagation from one cell to the next (spark) (Valenstein, 2005). The discovery of a synaptic delay in the propagation of electrical potentials between cells (Kuffler, 1942) – one that was longer than expected if transmission at the synapse was purely electrical – was the first major piece of physiological evidence against the spark theory and convinced many spark enthusiasts to accept that an alternative to purely electrical propagation was probable. With the discovery of acetylcholine being released at the neuromuscular junction (Dale et al., 1936; Feldberg, 1950), and the demonstration that when directly applied it could elicit muscle contraction (Brown et al., 1936), most of the remaining spark enthusiasts conceded and the debate over the mechanism of synaptic transmission was all

but settled. We now know that purely electrical transmission does occur at specialized synapses through ionic pores called gap junctions (for review, see Pereda, 2014), however, in general, neuron-neuron communication is achieved by releasing chemical messengers called neurotransmitters across the synapse.

The brain is thus built upon many uniquely positioned, structured and connected neurons that communicate with each other via electrochemical transmission. While there are a multitude of neuronal types, from a very simple perspective they all share a basic structure and enact a similar role. An individual neuron can receive information from thousands of other cells, and it is their task to be continuous integrators of this information and to relay relevant features of this information to target cells downstream. All neurons are able to carry out these fundamental functions thanks to their excitable plasma membrane, which typically has a negative resting membrane potential around -70 mV but can be modulated in space and time. Inputs arriving at specialized cell-cell contacts called synapses, which are found scattered across the neuron's complex and branched dendritic arbor, can drive depolarization (excitatory inputs) or hyperpolarization (inhibitory inputs) of the membrane potential, respectively. These synaptically-generated electrical potentials propagate throughout the dendritic arbor and get integrated at the cell soma, which is the central neuronal compartment containing the nucleus and other vital organelles. If neurons receive sufficient excitatory drive to depolarize their membrane above a threshold potential at the axon hillock, which is positioned directly adjacent to the cell soma, an all-or-none depolarizing event referred to as an action potential or 'spike' is discharged down their axon. When action potentials invade the final terminations of the axon, where synaptic contacts are made with other cells, they can trigger the release of chemical transmitters which notifies cells downstream that some features of their inputs were relevant.

Action potentials, much like the bit in digital communication, serves as the basic unit of information transfer in the brain, and it is believed that the precise patterning of these output signals is the basis for how all neurons communicate. It is now clear that, in addition to the all-or-none propagation down the axon, action potentials also propagate back into the dendritic tree where the majority of the input current originated (Spruston et al., 1995; Waters et al., 2005). While the functional relevance of these back-propagating action potentials (bAPs) is incompletely understood, there is mounting evidence that they might serve as a reinforcement cue that can stabilize or enhance the specific inputs that drove the neuron to fire an action potential (Lisman and Spruston, 2005). Thus, bAPs likely serve a very important role in synaptic refinement during neural plasticity, a process that will be introduced in detail shortly.

Structure and Function of the Excitatory Synapse

Much attention has been directed towards understanding the underlying mechanisms of chemical synaptic transmission, for its role is fundamental in nearly all brain functions and its dysregulation may be at the root of various neurological conditions (Volk et al., 2015; Lepeta et al., 2016). We now have a detailed understanding of the principle steps that underlie this process (extensively reviewed in Lisman et al., 2007), which can be summarized as follows. Neurotransmitter molecules are densely packaged into tiny vesicles that populate the presynaptic side of the synapse, and they can be released when an action potential invades the presynaptic terminal. Directly opposed to these presynaptic ‘release sites’ are neurotransmitter specific postsynaptic proteins which bind the neurotransmitter and convert these chemical messages back into either an excitatory or an inhibitory electrical potential depending on the nature of the neurotransmitter being release and the identity of postsynaptic receptors being activated. Excitatory and inhibitory synapses have unique pre-and postsynaptic structural specializations

that consist of hundreds of distinct synaptic proteins (Sheng and Kim, 2011; Harris and Weinberg, 2012; Heller et al., 2012; Wilhelm et al., 2014). It is important to mention that, although there exists many types of excitatory and inhibitory synapses in the brain (Walmsley et al., 1998; Fritschy et al., 2012; O'Rourke et al., 2012; Kubota et al., 2016), most of the basic machinery and components for each subclass are the same or at least broadly similar. I will describe only the key pre- and postsynaptic elements of excitatory synapses, since this thesis is centered upon their role in establishing a functional homeostasis at the level of individual neurons.

Most excitatory synapses are found on specialized postsynaptic structures called dendritic spines, which are tiny membrane protrusions that are studded along dendrites. Despite being discovered over a century ago by Cajal (Cajal, 1888c; Yuste, 2015), dendritic spines have been a challenge to study since their size ($< 1 \mu\text{m}^3$) nears the limits of what is observable using traditional forms of light microscopy. Nonetheless, based on a number of ultrastructural studies using electron microscopy, we know that dendritic spines typically harbor one excitatory postsynaptic domain within its spine head, which is often separated from the parent dendrite by a thin spine neck (Schikorski and Stevens, 1997; Shepherd and Harris, 1998). Visualization and active monitoring of spines neck physiology in living tissue has been achieved only recently using super-resolution imaging techniques. Spine neck diameter and length were found to be modulated by synaptic activity (Urban et al., 2011; Tonnesen et al., 2014), and these changes are proposed to differently affect spine biochemical and electrical compartmentalization features (Adrian et al., 2014). Thus, the morphological features of dendritic spines can compartmentalize and isolate the postsynaptic specialization of excitatory synapses, enabling them to act and be regulated somewhat independently from neighbouring synapses. The overall structure of

dendritic spines is maintained by cytoskeletal elements, especially actin filaments, which invade each spine and dictate features of its morphology (Hotulainen and Hoogenraad, 2010; Shirao and Gonzalez-Billault, 2013). Through the process of actin treadmilling, the actin network also contributes to the dynamic reconfiguration of dendritic spine structure in response to synaptic activity which, as we will see in the next section, plays an important role in the regulation of synaptic function and plasticity (Chazeau and Giannone, 2016; Lei et al., 2016). For a more detailed discussion on the relationship between dendritic spine form and function, and the compartmentalization features of dendritic spines, refer to Manuscript IV (Appendix A1), which contains a review manuscript that I co-authored on this topic (Lee et al., 2012).

The amino acid glutamate serves as the excitatory neurotransmitter at nearly all excitatory synapses in the central nervous system. Synaptically-released glutamate binds to and activates postsynaptic ionotropic glutamate receptors, which selectively permeate positively charged ions (typically Na^+ , K^+ , and Ca^{2+}) thereby delivering an excitatory depolarizing event. Although glutamate can also act on a metabotropic class of glutamate receptors (mGluRs), which are localized both to the presynaptic and postsynaptic specializations of excitatory synapses, their neuromodulatory effects will not be discussed here for the sake of brevity (for a recent informative review, see Rondard and Pin, 2015). In order to be in a position where they are maximally activated by glutamate release, ionotropic glutamate receptors get anchored in the postsynaptic membrane in direct opposition to the presynaptic active zone (*i.e.*, release sites) in a protein dense cluster referred to as the postsynaptic density (PSD; for a detailed molecular description of the PSD, see Boeckers, 2006). This specialized structure contains a diverse set of scaffolding proteins that bind to both the actin cytoskeleton and the intracellular domains of glutamate receptors, and thereby effectively trap surface (*i.e.*, plasma membrane bound)

glutamate receptors into a confined domain (Bard et al., 2010; Opazo et al., 2012; Chen et al., 2015). The postsynaptic density also tethers proteins that form trans-synaptic interactions, keeping presynaptic and postsynaptic specializations of the synapse in close proximity (Dean and Dresbach, 2006; McMahon and Diaz, 2011). In addition, this protein rich PSD contains a variety of other intracellular and transmembrane proteins that form signalling complexes with glutamate receptors (Zhang and Wang, 2003; Feng and Zhang, 2009; Lee et al., 2014a), which together serve a wide variety of plastic functions that will be described in more detail shortly. There are three known classes of ionotropic glutamate receptors, AMPA, NMDA and kainate, all being assembled as tetramers of pore forming receptor subunits that are uniquely encoded by separate genes (Traynelis et al., 2010). I will expand on the function and distribution of only the AMPA and NMDA subtypes since these are the predominate subtypes that are found at the specific excitatory synapses where my investigations were focused, which are those belonging to CA1 hippocampal pyramidal neurons (Lee et al., 2016).

AMPA receptors (AMPA Rs) are pore-forming protein complexes that are composed of different combinations of GluA1, GluA2, GluA3 and GluA4 subunits (encoded by the GRIA1-4 genes), and it is their precise subunit composition that dictates their distribution and exact biophysical properties (Traynelis et al., 2010). The expression profile of individual AMPAR subunits differs amongst neuronal types and across brain regions (Schwenk et al., 2014), and even within the same class of neuron, subunit expression changes can occur during development (Martin et al., 1998). In general, most AMPARs at excitatory synapses of hippocampal pyramidal neurons are thought to be diheteromeric combinations containing GluA1 and GluA2 subunits (~ 90%), whereas the rest are predicted to have the configuration of either homomeric GluA1 (*i.e.*, four GluA1 subunits) or diheteromeric GluA2/GluA3 (Beique and Huganir, 2009;

Lu et al., 2009). There are several key functional distinctions between GluA2-containing and GluA2-lacking AMPARs that, for the purposes of this thesis, are worth mentioning. First and foremost, the presence of GluA2 subunits impairs calcium permeability, rendering AMPARs permeable to only Na^+ and K^+ ions (Hollmann et al., 1991; Hume et al., 1991). In addition, unlike GluA2-lacking AMPARs which show outward rectification in their current-voltage (I-V) relationship (Nakanishi et al., 1990) due to a pore block at positive potentials by intracellular polyamines (Bowie and Mayer, 1995), the I-V relationship of GluA2-containing AMPARs is linear (Nakanishi et al., 1990). GluA2-lacking AMPARs, in addition to their calcium permeability and linear I-V relationship, also have increased conductance (Swanson et al., 1997). Both outward rectification and higher conductance are electrophysiological features often used to identify the presence of GluA2-lacking AMPARs at synapses (Shi et al., 1999; Shi et al., 2001; Goel et al., 2011; He et al., 2012; Morita et al., 2014). Moreover, GluA2-lacking AMPARs can be targeted by specific pharmacological antagonists such as 1-naphthyl acetyl spermine (NASPM) (Tsubokawa et al., 1995; Koike et al., 1997) and philanthrotoxin-466 (Washburn and Dingledine, 1996). As a brief aside, AMPARs associate with auxiliary subunits such as TARPs (transmembrane AMPA receptor regulatory proteins), cornichons, and several others (Jackson and Nicoll, 2011; Haering et al., 2014), which can also alter their biophysical properties and dictate their subcellular distribution. The interaction with auxiliary subunits in natural preparations offers a likely explanation for why measurements of AMPAR biophysical properties (eg. recovery from desensitization, channel conductance, degree of rectification) often differ in neurons as compared to in expression systems (Heine et al., 2008; Traynelis et al., 2010; Soto et al., 2014). Since synaptic AMPARs provide most of the excitatory synaptic drive that brings neurons to threshold for action potential discharge, these receptors can be thought of as

the general laborers in the overall task of encoding signals at the synapse. Differences in the number, type, and density of AMPARs in the postsynaptic domain will influence the postsynaptic response to glutamate release. Thus, the dynamic regulation of synaptic AMPARs plays an important role in the postsynaptic expression of synaptic plasticity, whereby the efficacy of synaptic transmission can be modulated in a synapse-specific manner (Huganir and Nicoll, 2013).

NMDA receptors (NMDARs), although related in structure to their AMPAR counterparts, have an entirely different role at the synapse by virtue of several unique functional characteristics. First, NMDARs are composed of two obligatory GluN1 subunits (encoded by the *GRIN1* gene) along with some combination of two other GluN2A-D or GluN3A-B subunits (encoded by *GRIN2A-D* and *GRIN3A-B* genes, respectively). Much like AMPARs, the composition of GluN2 and GluN3 subunits of NMDARs will dictate both their functional properties and their subcellular distribution (Traynelis et al., 2010; Paoletti et al., 2013). At excitatory synapses of hippocampal pyramidal cells, NMDARs are formed almost exclusively through combinations of GluN1, GluN2A and GluN2B subunits (Tovar et al., 2013), and the relative proportions of these NMDAR subunits changes during development. In most excitatory neurons, GluN1 and GluN2B expression is high at birth, whereas GluN2A expression is nearly absent (Monyer et al., 1994; Wenzel et al., 1997). During the first few postnatal weeks GluN2A expression elevates and this coincides with a progressive subunit switch of NMDARs at synapses. Specifically, homomeric GluN2B-type NMDARs (GluN1/GluN1/GluN2B/GluN2B) are predominant at naïve synapses (Paoletti et al., 2013), which have slower activation and deactivation kinetics and a lower open probability when bound to glutamate than most other NMDAR subtypes (Gray et al., 2011; Paoletti et al., 2013). As synapses mature, they become

increasingly dominated by GluN2A-containing NMDARs, likely of the triheteromeric GluN1/GluN1/GluN2A/GluN2B subtype (Rauner and Kohr, 2011; Tovar et al., 2013), which sharpens the kinetics of NMDAR-mediated postsynaptic responses (Flint et al., 1997; Bellone and Nicoll, 2007; Gray et al., 2011). For a brief review on the role of triheteromeric NMDARs at hippocampal synapses, refer to Manuscript VI (Appendix A3), which contains a short review on this topic (Soares and Lee, 2013). The overall reduction of GluN2B-containing NMDARs at synapses may offer some explanation as to why mature synapses are less plastic than naïve ones, since the intracellular tail of GluN2B subunits interacts directly with CAM Kinase II (CAMKII) (Bayer et al., 2001), and this interaction has a critical role in synaptic plasticity (Barria and Malinow, 2005; Lisman et al., 2012; Sanhueza and Lisman, 2013). Unlike other ionotropic glutamate receptors, there are several events in addition to glutamate binding that are required for the activation of NMDARs (Paoletti et al., 2013). First, their activation requires the coincident binding of two agonists, glutamate and glycine (and/ or D-serine) (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988), which bind to GluN2 and GluN1/GluN3 receptor subunits, respectively (Furukawa and Gouaux, 2003; Furukawa et al., 2005). Moreover, their activation is dependent on local membrane depolarization to relieve a voltage-dependent block of their ionic pore by magnesium ions (Mayer et al., 1984; Nowak et al., 1984). Thus, NMDAR activation requires both presynaptic activation (i.e., glutamate release) and postsynaptic depolarization in order to contribute to synaptic responses, which is typically offered by local AMPAR-mediated depolarization or in some cases by bAPs. It is because of this dual requirement that NMDARs are referred to as coincidence detectors at the synapse (Seeburg et al., 1995), responding exclusively to instances of coincident pre- and postsynaptic activity. Lastly, as a result of their intense calcium permeability and the long duration of their conductive glutamate-

bound state, NMDAR activation at synapses can trigger a multitude of intracellular calcium-dependent processes that are critical for neuronal refinement and development through mechanisms of synaptic plasticity (Paoletti et al., 2013).

On the presynaptic side of the synapse, axon terminals contain hundreds of glutamate containing vesicles, although at any given time only a small subset of these vesicles are set for release (for a review on vesicle pools, see Alabi and Tsien, 2012). These ‘ready releasable vesicles’ are already docked in close association with the presynaptic membrane (Schikorski and Stevens, 1997; Shepherd and Harris, 1998) where they wait for a final command (an invading action potential) to trigger their fusion with the membrane. The remainder of the glutamate packaged vesicles form a reserve pool that can be rapidly recruited into the ready releasable pool, should the number of release ready vesicles be exhausted (Sudhof, 2004; Fowler and Staras, 2015), although this recruitment takes time and very rapid stimulation patterns can deplete the ready releasable pool of synaptic vesicles (Stevens and Tsujimoto, 1995). In most instances, vesicle fusion relies on presynaptic calcium influx from voltage gated calcium channels that are gated by invading action potentials. There are numerous steps in this release process of which I will not go into much detail (for an informative review on this topic see Neher and Sakaba, 2008), the end result being that glutamate gets released into the synaptic cleft and can bind to postsynaptic glutamate receptors. There are two very important features of this overall process that I must highlight. First, at the majority of excitatory synapses in the central nervous system, release of neurotransmitter is probabilistic (Hessler et al., 1993; Murthy et al., 1997), meaning that in some instances, an invading action potential will fail to trigger vesicular release (Ribrault et al., 2011). In fact, the average release probability at hippocampal synapses is typically estimated to be quite low ($< 50\%$) (Allen and Stevens, 1994; Dobrunz and Stevens, 1997;

Emptage et al., 1999; Mainen et al., 1999; Hanse and Gustafsson, 2001; Oertner et al., 2002), but this unreliability can be overcome or at least modulated in several different ways, such as through presynaptic modification by neuromodulators or by patterned activity that induces a short-term plasticity of presynaptic release function. Second, when synaptic transmission does occur successfully, there is often only a single vesicle that gets released (Korn et al., 1981; Redman, 1990; Gulyas et al., 1993; Hanse and Gustafsson, 2001; Silver et al., 2003; Biro et al., 2005), despite there being, on average, 10 release ready vesicles docked at the presynaptic membrane (Schikorski and Stevens, 1997). While this ‘one-release site, one-vesicle’ hypothesis likely holds true in most instances at central synapses, there is now undebatable evidence that multiple vesicles can be released simultaneously at a single release site (Tong and Jahr, 1994; Oertner et al., 2002), for review see (Rudolph et al., 2015). As we will see in the next section, this unreliability in synaptic glutamate release offers synapses the potential to tweak this overall process to enhance the faithfulness of transmitter release, which is yet another way that synaptic contacts can alter their effectiveness during synaptic plasticity.

Endocytosis and exocytosis are continuously active processes that are important contributors to both presynaptic and postsynaptic function. As was already mentioned, release of neurotransmitter relies on the fusion of synaptic vesicles with the presynaptic membrane via exocytosis. However, because of the limited number of synaptic vesicles and the fact that vesicle fusion events add to the overall contents of the plasma membrane, there is also a constant retrieval of membrane components to form new synaptic vesicles through the process of endocytosis (reviewed in Kononenko and Haucke, 2015). Whereas synaptic vesicles fuse at the presynaptic active zone, which directly opposes postsynaptic machinery, the endocytic zones where vesicles are formed are typically outside of the ‘synaptic’ region (Gad et al., 1998;

Schikorski, 2014; Jahne et al., 2015). Upon clathrin-mediated endocytosis, vesicles at excitatory synapses get refilled with glutamate by action of vesicular glutamate transporters (vGluT1 and vGluT2) (Hisano, 2003; Takamori, 2006), and within minutes, these vesicles incorporate back into the pool of vesicles that can be recruited for release (Alabi and Tsien, 2012). There is emerging evidence, however, that a much faster recycling process can occur, which involves incomplete fusion of vesicles with the plasma membrane. Typically referred to as ‘kiss-and-run’, this form of exocytosis allows glutamate release through a small fusion pore, rather than the full collapse of the vesicle with the presynaptic membrane (Stevens and Williams, 2000; Richards et al., 2005; He et al., 2006). Once the contents have been released, the vesicular compartment remains intact and can be quickly refilled with neurotransmitter for subsequent release (Zhang et al., 2009). On the postsynaptic side, endocytosis and exocytosis act to regulate the surface expression of nearly all membrane proteins, including glutamate receptors. Synaptic activity patterns are thought to locally influence the balance between exocytosis-based insertion and endocytosis based removal of synaptic proteins from the plasma membrane (Kopec et al., 2006; Yudowski et al., 2007), leading some to believe that this property could underlie synapse-autonomous functions such as regulation of glutamate receptor type/number. While there are diffusional restraints imposed by long thin spine necks that can support the idea that synapses have access to an exclusive pool of surface receptors (Ashby et al., 2006; Lu et al., 2007a), it is generally believed that the extrasynaptic pool of receptors is shared amongst spines and serves as continuous source of synaptic proteins (Triller and Choquet, 2005). Thus, at both the transmitting and receiving side of excitatory synapses, vesicular trafficking events to and from the plasma membrane have important roles in regulating synaptic transmission.

In summary, the general structure of excitatory synapses in the central nervous system includes a transmitting presynaptic specialization and a receiving postsynaptic specialization that are built specifically for electrochemical transmission of neural information via glutamate. These domains are far from being static, but rather exhibit a continuous dynamic that can directly modify features of synaptic transmission. With this framework of excitatory synapse structure and function now planted, I will continue in the next section with a brief overview of the mechanisms through which synaptic activity patterns can modify presynaptic and postsynaptic elements of excitatory synapses to instill lasting changes in the efficacy of synaptic transmission. Prior to the discovery of synaptic transmission as being a malleable process, there was a general lack of understanding of how information was stored in the brain. Because the discovery of synaptic plasticity was monumental in shaping our ideas of how the brain develops and forms memories, I will provide brief historical perspective on earliest studies and descriptions of synaptic plasticity, which has become one of the most intriguing and popular areas of study in all of neuroscience.

Hebbian Synaptic Plasticity

Canadian psychologist Donald Hebb was keenly interested in the mechanisms through which neuronal activity in the brain could underlie the psychological processes of learning. In his famous book *The Organization of Behavior*, published in 1949, he proposed a theory of cellular learning in the nervous system that continues to dominate today. He proposed that when the repetitive activation of an axonal pathway leads to the repeated and persistent firing of the postsynaptic cell, there are metabolic changes occurring in both cells that enhance their functional connection. Experimental validation for Hebb's theory came nearly 25 years later, when colleagues Tim Bliss and Terje Lømo found that the persistent activation of a neural

pathway in the hippocampus of the anaesthetised rabbit caused a long term potentiation (LTP) of that same neural pathway, but importantly, not of a control test pathway (Bliss and Lomo, 1973). This idea of neural connections being strengthened through functional association has since been the basis for how we conceptualise how our brains encode information. In the decades that followed the initial discovery of synaptic plasticity, many other intriguing aspects of this phenomenon were revealed. First, synaptic plasticity was found to be bidirectional, in that mild stimulation of a neural pathway can lead to persistent functional depression (long term depression or LTD (Lynch et al., 1977; Dunwiddie and Lynch, 1978; Dudek and Bear, 1992), in some cases to the extreme of synapse elimination (Bastrikova et al., 2008; Wiegert and Oertner, 2013). Moreover, distinct forms of synaptic plasticity were observed across cell types and in nearly all brain regions. This universality of synapses being functionally plastic points to it having a truly fundamental role in nervous system functions. Despite over 50 years of an intense global effort to comprehend the multitude of synaptic plasticity mechanisms, there are many pathway specific intricacies that have yet to be unravelled. There are, however, several common pathways in the brain where synaptic plasticity mechanisms have been extensively characterized, many of which are found in the hippocampus, an area that has a long clinical history of being associated with certain types of memory formation.

The hippocampus is a highly plastic brain region that is bilaterally situated underneath the cerebral cortex and has an important role in the formation of spatial and episodic memories (Tulving and Markowitsch, 1998; Burgess et al., 2002). Its popularity in mammalian studies of synaptic plasticity stems from the fact that the hippocampus has a simple and defined circuit architecture that lends itself very nicely for repeated long-term electrophysiological recording of synaptic transmission, a technique that has been invaluable in pushing forward our understanding

of synaptic function and plasticity, both *in vivo* (Bliss and Lomo, 1973) and *in vitro* (Alger and Teyler, 1976). Unlike pyramidal cells from the cerebral cortex, which receive excitatory synaptic input from a multitude of other cortical and non-cortical regions, the apical dendrites of pyramidal cells in the CA1 region of the hippocampus are densely innervated by a direct excitatory pathway from the CA3 region of the hippocampus, along a well-defined track called the Schaffer's collaterals (Andersen et al., 1969; Neves et al., 2008a). By placing a stimulating electrode in the apical region of CA1, it is with near certainty that the stimulated excitatory inputs onto CA1 cells arise from axons from CA3 pyramidal cells, and thus, controlled studies of synaptic transmission and plasticity in the same neural pathway can be conducted repeatedly. Importantly, deficits in synaptic plasticity mechanisms in the CA3-CA1 pathway have often been associated with spatial learning deficits in animal behavior paradigms, for example (Auer et al., 1989; Tsien et al., 1996), suggesting that the functional plasticity between these two hippocampal regions is critical for hippocampal dependent forms of learning. Although it has been subject to a long history of debate, it is likely that there are both postsynaptic and presynaptic changes that occur during activity-dependent synaptic plasticity at CA1 synapses (Bliss and Collingridge, 2013), which will now be discussed separately. Importantly, both presynaptic and postsynaptic mechanisms that contribute to changes in synaptic efficacy, often referred to as 'expression mechanisms', are, at least at CA1 synapses, undoubtedly triggered by postsynaptic 'induction mechanisms' that typically require NMDAR activation (Harris et al., 1984; Dudek and Bear, 1992; Mulkey and Malenka, 1992).

Postsynaptically, it is both the activation and the regulated trafficking of glutamate receptors that underlies long-term changes in synaptic efficacy. LTP and LTD of synaptic transmission require strong and mild NMDAR activation for their induction, respectively, and

are expressed, at least in part, by dynamically altering the phosphorylation state, number, and/or composition of glutamate receptors in the postsynaptic membrane (Malinow and Malenka, 2002; Shepherd and Huganir, 2007). For instance, a synaptic stimulus sufficient to drive strong NMDAR-mediated calcium influx will: 1) drive the surface delivery of AMPARs which get incorporated specifically into those synapses that were strongly activated (Shi et al., 1999; Hayashi et al., 2000; Makino and Malinow, 2009), 2) transiently phosphorylate the intracellular tail of synaptic AMPARs to increase their conductance (Barria et al., 1997; Benke et al., 1998; Derkach et al., 1999; Lee et al., 2000). Together, this postsynaptic trafficking response of glutamate receptors enhances the sensitivity and magnitude of the postsynaptic response to the same amount of glutamate release, thereby leading to a potentiation of synaptic transmission. In addition, during early development, a subunit switch of NMDARs towards GluN2A-containing receptors occurs that accelerates the decay kinetics of NMDAR-mediated responses and shortens the integration window for plasticity, a feature that is believed to be relevant for mature network processing (Daw et al., 2006; Bellone and Nicoll, 2007). When synapses are only weakly activated and calcium elevations through NMDARs are minimal, the exact opposite can occur, whereby a synapse specific dephosphorylation and removal of AMPARs occurs at activated synapses (Mulkey and Malenka, 1992; Lee et al., 1998; Luscher et al., 1999; Beattie et al., 2000; Lee et al., 2000; Man et al., 2000). These opposing postsynaptic plasticity processes enable the bidirectional activity-dependent refinement of synaptic strengths. Specifically, they ensure that only when synapses are effective at generating relevant postsynaptic responses (i.e., strong and sufficient to get the postsynaptic neuron to fire) will they be strengthened, whereas synapses whose signals rarely get encoded as relevant (i.e, fail to generate a spike) will be diminished and possibly eliminated. There is also a dynamic alteration of dendritic spine structure that occurs in

conjunction with and may potentially dictate changes in postsynaptic glutamate receptors. First, while it is established that the overall size of the dendritic spine compartment correlates with synaptic efficacy, where large spines typically have more AMPARs (Matsuzaki et al., 2001), there are some exceptions to this rule (see Manuscript IV in Appendix A1 for an additional discussion on the structure function relationship of dendritic spines (Lee et al., 2012). This relationship between spine size and AMPAR content likely reflects the fact that the same induction stimulus that produces LTP of AMPAR transmission will also increase spine volume (Matsuzaki et al., 2004), and the opposite is also true, where LTD inducing stimuli will cause an overall reduction in spine volume (Zhou et al., 2004; Oh et al., 2013) and in some cases synapse elimination (Nagerl et al., 2004; Wiegert and Oertner, 2013). A popular model of synaptic plasticity posits that the postsynaptic density disassembles quickly following patterned synaptic activation to enable the dynamic reorganization of glutamate receptors, which can exchange rapidly with extrasynaptic pools of receptors, and then quickly reassembles to re-stabilize glutamate receptors with their intracellular signalling partners (Cingolani and Goda, 2008; Opazo and Choquet, 2011). When the PSD disassembles and reassembles, its components can be modified to favour the stabilization of more/less glutamate receptors, and potentially to alter the composition of glutamate receptors in the postsynaptic domain (Steiner et al., 2008; Xu et al., 2008), contributing to an overall change in postsynaptic responsiveness. Because of its close ties with functional potentiation (Bosch and Hayashi, 2012), actin-dependent changes in spine volume have often been used in imaging studies to infer synaptic potentiation (Harvey and Svoboda, 2007; Govindarajan et al., 2011; Lee et al., 2016) or depression (Oh et al., 2013; Oh et al., 2015). As a brief aside, I contributed to a study in which we sought to identify the role a particular protein kinase in the structural plasticity of dendritic spines during LTP. We found that

the palmitoylation of LIM domain containing kinase 1 (LIMK-1) was critical for its subcellular targeting to synapses and that the proper localization of this protein was critical for synapse specific actin-dependent structural plasticity of dendritic spines during LTP. For the full story please refer to (George et al., 2015), and for my specific contributions, refer to Appendix B1.

On the presynaptic side, synaptic efficacy can be modified through mechanisms that influence the reliability of glutamate release, which can occur both transiently or persistently. As was stated previously, release of neurotransmitter is unreliable, and the probability of glutamate release can be quite low at hippocampal synapses (Hessler et al., 1993; Rosenmund et al., 1993; Allen and Stevens, 1994; Dobrunz and Stevens, 1997; Murthy et al., 1997). However, when synapses are stimulated in quick succession, the probability of transmitter release of each proceeding stimulus will typically be enhanced (Creager et al., 1980; Wigstrom and Gustafsson, 1981; Manabe et al., 1993). Although the exact mechanism of presynaptic facilitation remains controversial, (reviewed in Burnashev and Rozov, 2005; Regehr, 2002) it is likely that this effect is mediated by some lasting effect produced from calcium entry preceding stimuli. Although this effect only lasts a few hundred milliseconds, facilitation can be extremely influential to increase the reliability of transmitter release when synapses receive a burst of action potentials, which is common at CA3-CA1 synapses (Lisman, 1997). Of course, synapses that exhibit a high release probability will not show strong facilitation, but rather, can exhibit short term depression, whereby the probability of transmitter release is reduced on subsequent stimuli (Chen et al., 2004; Regehr, 2012). When studying the activity of population of synapses using electrophysiological recording techniques, the average release probability across all synapses under investigation is often inferred based on whether the response behavior to multiple stimuli is facilitating or depressing (Stuart and Redman, 1991; Debanne et al., 1996). In addition to these

presynaptic forms of plasticity operating at relatively short (< 1 s) time scales, there are also more persistent changes that can influence vesicle release probability. For instance, strong stimulation sufficient to generate postsynaptic NMDAR activation can trigger, in some instances, a long-term increase of transmitter release probability at CA1 synapses (Bekkers and Stevens, 1990; Stevens and Wang, 1994; Emptage et al., 1999; Enoki et al., 2009). Although the specific mechanisms through which postsynaptic NMDAR activation can increase the probability of transmitter release remains unclear, it is likely that a retrograde messenger is involved. It is important to note that changes in release probability observed during LTP are complicated by the presence of silent synapses, which contain NMDARs but not AMPARs (Kerchner and Nicoll, 2008). This idea is strengthened by the fact that these naïve synapses are thought to be preferential sites of AMPAR insertion during LTP (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Beique et al., 2006; Kerchner and Nicoll, 2008), and could serve to explain observed changes in release probability. There is also some evidence that during long-term depression there is a reduction in vesicular release probability (Sjostrom et al., 2003; Castillo, 2012). Thus, the probability of vesicle fusion lies within a dynamic range that can be modified by synapse history, which is yet another strategy in which neurons are able to bidirectionally adjust synaptic strength.

It is important to mention that there is strong evidence for the existence of both presynaptic and postsynaptic forms of synaptic plasticity at CA3-CA1 synapses. For instance, LTP of synaptic transmission at CA1 synapses is sometimes observed as a change in transmitter release probability without a change in EPSC potency (i.e., the EPSC amplitude of successful release events) (Emptage et al., 1999; Enoki et al., 2009), arguing in favor of an entirely presynaptic change. In other instances, however, the reverse scenario has been observed,

whereby the potency of synaptic transmission is enhanced without changes in transmitter release probability (Kauer et al., 1988; Palmer et al., 2004). It is likely that the contingencies for plasticity at each side of the synapse may be different such that, in some cases, their expression can be observed separately. The details of this pre-post issue have been intensely studied and debated and are beyond the scope of this thesis. Ultimately, what is clear is that synapses have several means to alter their functional efficacy based on the patterns of stimuli they receive, and that these changes occur specifically at the activated synapses and not at unstimulated neighbouring synapses.

Now that the basic features of neuronal structure, function and plasticity have been introduced, I'd like to shift the focus back to the concept of neuronal stability and re-state the original problem, which is how do single neurons maintain an optimal functional balance despite being challenged by a number of destabilizing forces that are inherent in neural networks? As was already mentioned, a neuron's output (*i.e.* spikes) is dictated by its own excitatory / inhibitory drive, which is simply the collective influence of all synaptic inputs in time. Hebb's theory of synaptic plasticity would predict that the synapses that contributed to the generation of a spike would be selectively strengthened and thus would have greater influence on dictating the output of the neuron. Similarly, weak synapses that rarely play a meaningful role in driving spikes would eventually become functionally depressed. It is difficult to envision how this theory of synaptic plasticity by itself can explain how a single neuron can preserve the existence of many thousands of synapses, ranging in their functional efficacies from weak to strong. In an effort to understand how neurons would behave if their synapses were modified exclusively according to Hebb's rules, many groups have turned to *in silico* approaches and have modeled the behaviors in small integrated networks of neurons. The sole implementation of Hebbian

algorithms in even the most basic simulated network models have revealed inherent issues of functional instability, where cells fire uncontrollably because of the powerful influence of a few synapses that have been functionally strengthened, while the remaining synapses remain dormant and contribute inconsequentially (Miller and Mackay, 1994; Turrigiano, 2008; Lazar et al., 2009). Herein lies the ‘Hebbian instability problem’, which is that Hebbian mechanisms alone, which operate on positive feedback reinforcement, cannot explain the balanced functional dynamics of neural networks (Turrigiano, 2008). Thus, it has been proposed that other forms of synaptic plasticity must exist to provide functional balance to neuronal activity and counteract Hebbian instabilities. In the next section, I will describe in detail one candidate mechanism that is likely to enact such a role.

Homeostatic Synaptic plasticity

It has become increasingly clear that the activity output of individual neurons is subject to homeostatic regulation. This implies that neurons can actively sense their own activity levels and can bidirectionally adjust some cellular parameters such that their activity output remains within a computationally optimal range. Several mechanisms contribute to the homeostatic regulation of cellular excitability but, for the purpose of introducing my own work, I will focus almost exclusively on describing one that exists globally (i.e., across the entire neuron) to affect the functional strength of excitatory synapses (Turrigiano, 2008), which is referred to specifically as homeostatic synaptic plasticity (henceforth termed HSP). The synapse is not the only locus of homeostatic plasticity expression. There are a number of other expression mechanisms, many of which involve the differential expression non-synaptic ion channels, which can refine intrinsic excitability of neurons and alter spiking thresholds and dynamics (Desai et al., 1999; Turrigiano and Nelson, 2004). Overall, homeostatic plasticity mechanisms are of particular interest in the

context of neural stability since they provides a theoretically plausible solution to the instability problem of purely Hebbian networks.

Bidirectional HSP at excitatory synapses, which is often referred to as homeostatic scaling, was first described in a study where electrophysiological measurements of synaptic strengths were gathered from cultured neurons that had been exposed to long-term pharmacological manipulations that altered network activity (O'Brien et al., 1998; Turrigiano et al., 1998). Specifically, the authors were interested in the very basic question of what happens to the strength of synaptic connections when network activity is either chronically elevated or diminished. What the authors found was that chronic silencing of network activity lead to a compensatory increase in the overall strength of excitatory synaptic transmission, and conversely, when network activity was chronically enhanced by pharmacological removal of synaptic inhibition, the strength of excitatory synapses became weakened (O'Brien et al., 1998; Turrigiano et al., 1998). This fascinating finding indicates that neurons have a means to continuously sense their own levels of activity and can balance their functional output, at least in part, by a global 'scaling' of their own excitatory synaptic drive. Intriguingly, synapses were found to increase or decrease their efficacy by a single common factor. This multiplicative aspect of the homeostatic response has important implications, since it suggests that synaptic efficacies are tuned in a concerted manner that does not alter the relative weights of synapses, those that were likely inscribed through precise Hebbian modifications. Thus, by globally adjusting synaptic drive, neurons can balance their cellular excitability to maintain an optimal output frequency, which might offer some explanation as to how individual neurons, and by extension neural networks, generally exist in a functional equilibrium.

Since the discovery of homeostatic synaptic plasticity almost two decades ago, there has been an ongoing quest to identify the synaptic mechanisms that underlie this adaptive response. While there have been some indications that presynaptic adaptations have a role in the expression of HSP, most investigations have focused on postsynaptic mechanisms, since it was determined almost immediately that changes in the synaptic regulation of glutamate receptors (AMPA and NMDA) was an integral part of the homeostatic response (O'Brien et al., 1998; Turrigiano et al., 1998; Watt et al., 2000). When network activity is chronically silenced, the postsynaptic response to glutamate is enhanced. Conversely, when network activity is chronically enhanced, the postsynaptic response to glutamate is reduced (Turrigiano et al., 1998). The most parsimonious explanation for these findings is that the homeostatic plasticity directly affected the number of postsynaptic glutamate receptors, and a number of biochemical, electrophysiological, and imaging based experiments have provided support for this model (Lissin et al., 1998; O'Brien et al., 1998; Watt et al., 2000; Wierenga et al., 2005; Shepherd et al., 2006). It is, however, difficult to envision a mechanistic model that could account for the uniform scaling of quantal amplitudes (commonly referred to as synaptic strengths; measured electrophysiologically as miniature excitatory postsynaptic currents) that is based exclusively on the postsynaptic regulation of glutamate receptors, and thus the source of multiplicativity remains unclear. Nevertheless, it is generally agreed that this postsynaptic mechanism plays an important role and has thus been the focus of much attention. While several groups have made attempts to identify and characterize the precise mechanisms through which synapses remodel glutamate receptor content at synapses during homeostatic plasticity, there is a general lack of consistency in the overall outcomes of these studies (reviewed in Perez-Otano and Ehlers, 2005; Turrigiano, 2008). Since the majority of these experiments were performed in dissociated

cortical cultures, which are composed of a variety of different cell types, these inconsistencies may speak to there being distinct types of homeostatic regulations that exist amongst cell types. Alternatively, it is possible that these inconsistencies stem from the fact that synaptic properties are difficult to study dissociated neuronal cultures, since there is a dense and random connectivity pattern and it is nearly impossible to ensure that a common synapse type is studied across different experiments. Thus, many including myself have turned to studying mechanisms of homeostatic synaptic plasticity in cultured slices (Kim and Tsien, 2008; Vlachos et al., 2012; Arendt et al., 2013), which better preserves the *in situ* circuit architecture (De Simoni et al., 2003) and lends itself much more readily to controlled studies of synaptic transmission using electrophysiological techniques. Chronically manipulating network activity in cultured slices, as opposed to in dissociated neuronal cultures, enables a more comprehensive critique of the precise synaptic mechanisms underlying the homeostatic adaptive response in distinctly identifiable neuronal cell types and at specified synapses. Deciphering the precise mechanisms of HSP and how they might differ amongst cell types is critical if we are to unravel the mystery of whether and how homeostatic mechanisms play a role in stabilizing the brain's functional dynamics.

It is important to mention that, whereas this thesis is focused on describing mechanisms of *global* homeostatic synaptic plasticity, which is a cellular response to chronic perturbations of network activity, there exist several other potentially distinct forms of HSP. For instance, cell-autonomous forms of HSP are revealed when the firing activity of an individual neuron in an otherwise unperturbed network is chronically manipulated (Burrone et al., 2002; Ibata et al., 2008; Goold and Nicoll, 2010). Because network activity remains intact, this homeostatic response is independent of changes in synaptic drive but rather results directly from changes in the cell's firing activity. Since some but not all of the core features of global HSP are revealed

during cell-autonomous HSP, it is possible that they share some underlying mechanism. Nonetheless, based on several key distinctions, there is a clear mechanistic delineation that separates the overall outcome of these two HSP processes. To now add an additional layer of complexity, it is now clear that individual synapses can also act as homeostatic units (Hou et al., 2008; Lee et al., 2010; Beique et al., 2011; Hou et al., 2011), leading to a third and final form of HSP that is synapse-autonomous. Chronic enhancement or reduction of activity at a single synaptic input can lead to bidirectional changes in synaptic efficacy, indicating that even single synapses have the ability to autonomously sense their activity level and actively compensate for it in a homeostatic-like fashion. It is interesting to speculate that global forms of HSP might be the result of some interaction between cell-autonomous (i.e. response to reduced firing rate) and synapse-autonomous (i.e., response to reduced synaptic drive) homeostatic regulations, although this has yet to be verified experimentally. This discussion on the interaction between homeostatic synaptic regulations at different scales will be laid to rest for now, but because of its importance for the general theme of this thesis will be re-appear in the final discussion. For additional details on the diversity of homeostatic synaptic plasticity mechanisms, see Manuscript V (Appendix A2), which contains a review article I co-authored which is specific to this topic (Lee et al., 2014b).

A question that is often asked is whether or not there is clear evidence that HSP occurs in the living brain. While most demonstrations of HSP *in vivo* have come from experiments in the visual system, (Goel and Lee, 2007; Goel et al., 2011) reviewed in (Whitt et al., 2013), there is evidence that HSP mechanisms may exist across sensory cortices. In a seminal study by the group of Turrigiano and colleagues, the authors found that synaptic connections in the visual cortex are weakened within the first few days after eyelid opening, which they propose is a

homeostatic weakening that is triggered by the enhanced sensory input through the eyes (Desai et al., 2002). This was validated by showing that dark rearing animals during this critical period of eyelid opening prevents synapse weakening (Desai et al., 2002). When retinal activity in a single eye was chronically disrupted later in development by intraocular injections of TTX, electrophysiological measurements of synaptic strength from neurons in the contralateral monocular visual cortex were enhanced in a multiplicative fashion (Desai et al., 2002). Similar forms of HSP have been described in the auditory cortex following cochlear ablation (Kotak et al., 2005), and in the somatosensory cortex following resection of the infraorbital nerve, which is expected to abolish all tactile driven activity (Yu et al., 2012). Thus, it is likely that HSP plays a very important role in sensory cortices to balance synaptic drive during periods where sensory stimulation is altered.

Open questions concerning HSP mechanisms: A preface to Chapters I-III

The main body of this thesis is composed of three chapters, each of which addresses an unresolved and important topic in the field of homeostatic synaptic plasticity. In all of these investigations, HSP mechanisms were studied in CA1 hippocampal neurons from cultured hippocampal slices following 3-4 days of tetrodotoxin (TTX, a Na⁺ channel antagonist) exposure. In this model, network activity is nearly abolished since neurons are unable to fire action potentials, and as a result, neurons receive far less synaptic inputs. It is important to note, however, that in the absence of action potential firing, synaptic transmission still occurs stochastically at a low frequency, since synaptic vesicles can spontaneously fuse with the presynaptic membrane. Thus, whereas some small amount of synaptic drive remains intact in this silencing paradigm, most is abolished. In response to this manipulation, my work indicates that

neurons orchestrate a cell-wide strengthening of excitatory synaptic transmission that occurs progressively over a few days. While it is unlikely that neural networks will ever undergo such a prolonged period of silencing in the intact brain, this forceful manipulation nonetheless demonstrates that there are underlying homeostatic mechanisms in place to combat such changes in neuronal excitability, which points to them being continuously operant at a lower gain to provide a constant balance to synaptic drive. It is in describing the underlying cellular mechanisms and the functional implications of this adaptive response that I have devoted most of my attention in recent years. In the closing paragraphs of this introduction, I will provide a short preview of each of these three projects, highlighting the critical gaps in our current understanding of each topic, the main theme of questioning, and a general overview of the experiments performed.

As was summarized briefly before, there has been a monumental effort to describe the precise rules and regulations governing the targeting and trafficking of glutamate receptors during activity-dependent synaptic plasticity (i.e., LTP and LTD). Using hippocampal synapses as a model system, a general framework has been built to describe in detail how these mechanisms operate (Malenka and Bear, 2004; Citri and Malenka, 2008). It is now quite clear that synaptic efficacy can be altered, at least in part, through postsynaptic modification of glutamate receptor numbers, however, this postsynaptic response is much more complicated. For instance, the specific subtypes of glutamate receptors that get stabilized at synapses following plasticity induction are often changed (Rebola et al., 2010; Huganir and Nicoll, 2013). The dynamic reconfiguration of glutamate receptor subtypes during synaptic plasticity can alter features of synaptic transmission that extend well beyond changing the overall strength of synapses, based on the fact that receptor subtypes differ in their functional properties and can

interact with different groups of postsynaptic signalling proteins (Traynelis et al., 2010). One question that was of particular interest for me when I started my PhD studies was whether or not HSP could similarly alter the composition of glutamate receptors at synapses, because of the implications of what these changes would have on synaptic behaviors. With the guidance of my supervisor, I designed a research project that would directly address the question of whether there was a differential targeting of glutamate receptors, including both AMPARs and NMDARs, during the homeostatic response to activity deprivation. Our results, which can be found in the first chapter of this thesis, lead us to the important conclusion that CA1 synapses dynamically altered glutamate receptor composition during homeostatic strengthening in ways that are similar to those described during Hebbian strengthening. These findings have important implications with regard to how the signaling and integrative properties of synaptic transmission get refined as a consequence of homeostatic adaptations, which will be discussed in detail in Chapter 1.

One immediate prediction that stemmed from the results of the aforementioned study was that Hebbian plasticity rules would be altered at synapses following the homeostatic response to inactivity. The dynamic redistribution of synaptic glutamate receptor subtypes during homeostatic strengthening was in many ways similar to what occurs much more rapidly during Hebbian LTP (Plant et al., 2006; Bellone and Nicoll, 2007; Lu et al., 2007b; Morita et al., 2014). Since homeostatic forces are thought to continuously balance synaptic strengths, I reasoned that it was important to resolve the functional interaction between these two plasticity processes, which are likely operating in synchrony in the living brain. Specifically, I wondered what influence global homeostatic strengthening would have on the ability for synapses to undergo further synaptic potentiation during LTP. The use of organotypic slices (as opposed to dissociated neurons where HSP mechanisms are typically described) was particularly well suited

to explore this fundamental issue. What I found, which is described in detail in Chapter 2 of this thesis, is that prior homeostatic strengthening of synaptic connections impaired their ability of CA1 synapses to exhibit subsequent LTP via a common Hebbian induction protocol. Importantly, synapses that had undergone homeostatic refinement still showed the potential for synaptic strengthening by two-photon glutamate uncaging, indicating that this was not due to an occlusion of postsynaptic expression mechanisms. Rather, presynaptic dynamics of glutamate release were altered in ways that influence LTP induction. Thus, this work demonstrates that the metaplastic state of synapses is altered by the homeostatic process, which may be a critical feature that enables it to balance perpetually active brain networks.

In the final chapter of this thesis, the focus shifts towards homeostatic regulations that occur on the opposite side of the synapse. Very little attention has been directed towards understanding how properties of glutamate release at central synapses are affected by the homeostatic response to inactivity. Homeostatic scaling is often regarded as a purely postsynaptic phenomenon, although it has never been definitively shown that this is actually the case, leaving open the possibility that presynaptic factors could play a significant role. I approached this question using a novel genetically-encoded glutamate sensor, which can be transfected into neurons and can be used to visualise and quantify features of glutamate release events at single synapses (Marvin et al., 2013). This novel optical strategy enable me to revisit and expand upon some key concepts surrounding quantal glutamate release, such as the amount of variability in glutamate release at single synapses, the occurrence frequency of multivesicular release, and several others. Moreover, I found that synapses that had undergone prolonged silencing released more glutamate per successful release event than in control synapses, outlining a novel presynaptic contribution to HSP. What was fascinating about this finding that the amount

of glutamate released by individual synaptic vesicles increased by a single common factor, and thus, this increase was multiplicative. This finding sheds new light on the mechanisms that might underlie the multiplicative scaling of synaptic weights during homeostatic plasticity.

Found herein is the results of most of the experiments I have performed during my PhD tenure. For each of the three studies, which comprise the main body of this thesis, I will provide an initial disclosure statement indicating my contributions and importantly, the contributions made by my colleagues in the lab.

In order to preserve the common theme of homeostatic synaptic plasticity, I have resisted the inclusion of a fourth first-authored manuscript in the body of this thesis, which instead appears as Manuscript VII in Appendix A4. Prior to my transfer into the PhD program, I was interested in the role of glycine binding to NMDARs and how this affected their subcellular trafficking, specifically to and from the plasma membrane. The appendices of this thesis contain all other works that I have contributed to over the years, some of which are review manuscripts written in collaboration with other lab colleagues, while others are original research manuscripts that I have contributed to directly. In all cases, these manuscripts found in the appendices are preceded by disclosure statements indicating my contributions.

Manuscript I

Title: Differential subcellular targeting of glutamate receptor subtypes during homeostatic synaptic plasticity

Abbreviated Title: AMPA and NMDAR changes in homeostatic plasticity

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Statement of Contributions:

Kevin Lee and I are co-first authors on this manuscript. Kevin initiated the experiments for this paper in 2009-2010 when I was an undergraduate student in the lab. Some of the core observations described in Figures 1-3 and 6 were found during these early stages of the project. However, toward the end of 2010, he encountered problems maintaining healthy slice cultures which put the project on hiatus. Soon thereafter, I revitalized the project by troubleshoot the slice culture methods. I adopted this project as my primary research focus and led the project to its completion, acquiring and analyzing the majority of the data contained in the paper. Kevin assisted in the experimental design, and aided in collecting electrophysiological and morphological data shown in Figures 1-6, and contributed novel computational tools (in MATLAB) for the iterative multiplicative scaling analysis in Figure 1. Wissam Nassrallah, an undergraduate student at the time, performed and analyzed the Western Blot experiments shown in Figures 3 and 5. Both Kevin and I wrote the paper, contributing equal efforts in the interpretation of the data, formulation of the concepts for developing the narrative and discussion topics. Dr. Jean-Claude Béïque oversaw the entire process and provided major guidance for the writing/editing of the drafts leading to the publication of the paper.

Abstract

Homeostatic processes are believed to contribute to the stability of neuronal networks that are perpetually influenced by Hebbian forms of synaptic plasticity. Whereas the rules governing the targeting and trafficking of AMPA and NMDA subtypes of glutamate receptors during rapid Hebbian LTP have been extensively studied, those that are operant during homeostatic forms of synaptic strengthening are less well understood. Here, we used biochemical, biophysical and pharmacological approaches to investigate glutamate receptor regulation during homeostatic synaptic plasticity. We show in rat organotypic hippocampal slices that prolonged network silencing induced a robust surface upregulation of GluA2-lacking AMPARs, not only at synapses, but also at extrasynaptic dendritic and somatic regions of CA1 pyramidal neurons. We also detected a shift in NMDAR subunit composition that, in contrast to the cell-wide surface delivery of GluA2-lacking AMPARs, occurred exclusively at synapses. The subunit composition and subcellular distribution of AMPA and NMDARs are therefore distinctly regulated during homeostatic synaptic plasticity. Thus, because subunit composition dictates key channel properties such as agonist affinity, gating kinetics and calcium permeability, the homeostatic synaptic process transcends the simple modulation of synaptic strength by also regulating the signaling and integrative properties of central synapses.

Introduction

Hebbian forms of synaptic plasticity *i.e.*, long-term potentiation and long-term depression (LTP and LTD), exhibit features that are consistent with a synaptic encoding of information and, as such, have come to dominate our understanding of how memories are stored in the brain (Kessels and Malinow, 2009). However, neural network models that implement solely Hebbian plasticity are inherently unstable due to the positive feedback nature of LTP and LTD (Turrigiano, 2008; Lee et al., 2014). The discovery of homeostatic plasticity has been received with great interest in part because it provides a biologically plausible means to stabilize perpetually active and plastic neural networks (Turrigiano, 2008; Lazar et al., 2009; Lee et al., 2014). Homeostatic synaptic plasticity (HSP) enables neurons to adapt to sustained alterations in overall cellular activity by bidirectionally regulating the strength of excitatory and inhibitory synaptic transmission. For example, neurons respond to prolonged inactivity by a cell-wide enhancement of excitatory synaptic strength, and adapt to sustained hyperactivity by a global synaptic depression of excitatory synapses (Turrigiano et al., 1998).

Despite enacting fundamentally different roles in neuronal function, Hebbian and homeostatic synaptic plasticity share common synaptic loci of expression. Indeed, both forms of plasticity can manifest through the regulation of postsynaptic glutamate receptor number and/or function. Some, but not all, studies have reported that synaptic strengthening during HSP is mediated by the insertion of GluA2-lacking AMPARs (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006; Aoto et al., 2008; Groth et al., 2011), a calcium-permeable subtype of AMPAR expressed at very low levels under baseline conditions in pyramidal neurons (Beique and Huganir, 2009; Lu et al., 2009). Interestingly, this AMPAR subtype has also been implicated in LTP expression (Plant et al., 2006; Guire et al., 2008), although this remains controversial

(Adesnik and Nicoll, 2007; Gray et al., 2007). Furthermore, the subunit composition of synaptic NMDARs is also dynamically regulated. Recent work indicates that synaptic NMDAR subunit composition is highly regulated during LTP, with subunit switching occurring as rapidly as synaptic potentiation *per se* (Bellone and Nicoll, 2007). It remains unclear, however, whether such mechanisms also operate during homeostatic synaptic strengthening.

Here, we show that prolonged network inactivity in organotypic hippocampal slices leads to a cell-wide surface delivery of calcium-permeable GluA2-lacking AMPARs that populate both synaptic and extrasynaptic sites. Synaptic NMDARs, but not their extrasynaptic counterparts, undergo a switch from predominantly GluN2B-containing to GluN2A-containing NMDARs in response to prolonged inactivity. These results therefore expand the known repertoire of the cellular processes involved in the homeostatic regulation of excitability of CA1 pyramidal neurons. The synaptic homeostatic response is thus not merely limited to the regulation of synaptic strength as a means to control excitability, but encompasses broader alterations that fundamentally influence key features of excitatory synaptic transmission.

Materials & Methods:

Organotypic Slice Culture

Organotypic slice cultures were prepared using a modified method of the original interface technique (Stoppini et al., 1991). Male and female Sprague-Dawley rats (Charles River Laboratories; 6-8 days old) were anaesthetized by isofluorane (Baxter Corporation, Canada) inhalation and decapitated according to procedures approved by the University of Ottawa Animal Care Committee. Individual hippocampi were removed in ice-cold cutting solution containing the following (in mM): 119 choline chloride, 2.5 KCl, 4.3 MgSO₄, 1.0 CaCl₂, 1.0 NaH₂PO₄–H₂O, 1.3 Na-ascorbate, 11 glucose, 1 kynurenic acid, 26.2 NaHCO₃, saturated with 95% O₂ and 5% CO₂ (pH = 7.3; 295-310 mOsm/L) and hippocampal slices (400µm) were obtained using a MX-TS Tissue Slicer (Siskyou). Individual hippocampal slices were transferred to membrane inserts (Millipore, #PICM03050) and maintained in six-well plates at 34°C in 95% O₂ and 5% CO₂ containing a Neurobasal-A based culture media. Slice culture media was exchanged at 1 day in vitro (DIV) and then every 2-3 days thereafter. After 6-8 DIV, tetrodotoxin (1µM; TTX, Tocris Bioscience) was added to the treatment group.

Whole-Cell Electrophysiology

Whole-cell recordings were performed on CA1 pyramidal neurons from control slices or slices incubated for 3-4 days with TTX. For recording, one slice was removed from a culture insert, placed in a recording chamber and cells in stratum pyramidale of the CA1 subfield were visualized under differential-interference contrast (DIC) using a BX61WI upright microscope (with a 40x/0.8NA or 60x/1.0NA objective; Olympus, Melville, NY) or a Zeiss Axio Examiner D1 upright microscope (40x/0.75NA objective). All experiments were performed at room

temperature in Ringer containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1.0 NaH₂PO₄, 11 glucose, and 26.2 NaHCO₃ (or low Mg²⁺ Ringer containing 0.1 MgSO₄, 3.0 CaCl₂) saturated with 95% O₂ and 5% CO₂ (pH = 7.3; 295-310 mOsm/L). Additional drugs were added to the Ringer as follows (in mM), as indicated in the text: 0.001 TTX, 0.1 picrotoxin, 0.01 NBQX, 0.05-0.1 DL-APV, 0.003 ifenprodil; 0.02 1-Naphthyl acetyl sperminetrihydrochloride (NASPM), all purchased from Tocris Bioscience. Whole-cell recordings were carried out using an Axon Multiclamp 700B amplifier, filtered at 2 kHz, sampled at 10 kHz and digitized with an Axon Digidata 1440A digitizer. Borosilicate glass recording electrodes (World Precision Instruments, Florida or Sutter, California) were pulled using a Narashige PC-10 vertical puller (Narishige, Japan) and had resistances ranging from 3-5 MΩ. For voltage-clamp recordings, electrodes were filled with an internal solution containing (in mM): 115 cesium methane-sulfonate, 0.4 EGTA, 5 tetraethylammonium-chloride, 2.8 NaCl, 20 HEPES, 3 ATP-Mg, 0.5 GTP, 10 Na-phosphocreatine, all purchased from Life Technologies, and 5 QX-314 purchased from Abcam (pH = 7.2-7.3; 280-290 mOsm/L). For all voltage clamp recordings, access resistance was continuously monitored during the experiment by delivering a 5 mV hyperpolarizing step at the onset of every electrophysiological sweep. All recordings were analyzed using Clampfit 10.2 (Molecular Devices) and Origin 8 analysis software (OriginLab). All voltages were left uncompensated. All error bars represent SEM.

For AMPAR I-V curves, 0.1 mM spermine (Tocris Biosciences) was included in the internal solution. To calculate a rectification index for AMPAR I-V relationships, the slope was calculated for both the inward (-70mV to 0mV) and outward (0mV to +40 mV) portions of the curve, and the ratio of the outward slope over the inward slope was computed (Beique et al., 2011; Granger et al., 2013). The amplitudes of AMPA and NMDA currents for AMPA/NMDA

ratios were estimated using the EPSC recorded at +40 mV, based on their respective time-courses, as previously described (Beique et al., 2006). For decay analysis of NMDAR-mediated EPSCs (both evoked EPSCs at +40mV and uncaging EPSCs at -60mV), a bi-exponential fit was used to calculate a weighted tau value (τ_w) (Vicini et al., 1998). To ensure that all evoked currents were monosynaptic, extreme care has been taken to minimize polysynaptic activity. A glass patch electrode was used to electrically stimulate glutamate release from axons and was positioned close to the proximal apical dendritic arbour of the recorded neurons. Stimulation intensity was kept low, eliciting eEPSCs of small amplitude (<100 pA, typically around 50 pA). In some cases, 10-30 nM TTX was included in the Ringer solution to dampen polysynaptic activity.

Peak Scaled Non-Stationary Noise Analysis of AMPAR-mEPSCs

For estimates of mean AMPAR channel conductance (γ) and the number of channels exposed to glutamate (N), peak scaled noise analysis of AMPAR-mEPSCs was performed using Mini Analysis software (Synaptosoft). All recordings with less than 50 events were discarded from the analysis. An average mEPSC waveform from each recording was scaled to the peak of each mEPSC in the recording and the variance of current fluctuations around the mean for each point in time was calculated. The average current variance relationship was then binned into 30 time points (independent of the amplitude of the average mEPSC) and the data was fit with the parabolic equation:

$$(1) \quad \sigma^2 = iI - I^2/N + b$$

where σ^2 = variance, I = Mean Current, i = single channel current, N = Number of open channels at peak current, and b = background variance. From this equation, γ was calculated by dividing i

by the driving force (-80mV). Recordings were discarded if the parabolic fits of the current variance plots had $R^2 < 0.5$. Since R^2 values of the parabolic fits were generally greater than 0.75, our estimate of N (i.e., number of channels) was not affected by a skewed variance versus mean relationship (Traynelis et al., 1993; Hartveit and Veruki, 2007)

Two-Photon Imaging and Uncaging

Simultaneous two-photon imaging and glutamate uncaging was performed using two Ti:Sapphire pulsed lasers (MaiTai-DeepSee, Spectra Physics, USA) coupled to an Olympus MPE-1000 galvanometer scanning system. Prior to uncaging experiments, lasers were aligned to one another using fluorescent beads. One Ti:Sapphire laser was tuned to 810 nm to visualize morphology (Alexa Fluor 594) and the second laser was tuned to 720 nm for MNI-Glutamate uncaging. Synchronization of electrophysiological and optical equipment was accomplished using a Master-8 pulse generator (A.M.P.I, Israel).

Glutamate uncaging experiments were performed with 2.5 mM MNI-glutamate trifluoroacetate (Femtonics, Hungary) and tetrodotoxin supplemented to the Ringer, while 0.03 mM Alexa Fluor 594 hydrazide (Na-salt; Invitrogen) was included in the internal recording solution. Whole-cell electrophysiological recordings were carried out as described above. Neurons were allowed to fill with the dye for a minimum of 10 minutes before onset of uncaging experiments. All two-photon uncaging EPSCs were generated from proximal secondary and tertiary apical dendrites to minimize issues of space clamp. Intensity of each laser was independently controlled using two independent acousto-optic modulators (AOMs). The intensity of the uncaging laser was tuned to generate a 10-20 pA AMPAR-mediated 2P-EPSC at a holding potential of -70 mV for AMPAR-IV curves. For NMDAR uncaging experiments, laser intensity was set to generate a NMDAR-mediated 2P-EPSCs smaller than 25pA when recorded

in low magnesium Ringer (0.1mM Mg^{2+} , 3 mM Ca^{2+}) at -60mV in the presence of 0.01 mM NBQX (Tocris) and 0.01 mM glycine. Because no particular care was taken to assure constant uncaging laser power between experiments in different neurons or slices (by normalizing for uneven light scattering at different tissue depth), we have not directly compared absolute amplitudes of 2P-EPSCs between experiments. Rather, we have compared metrics that are largely independent of laser power (*i.e.*, rectification properties and decay kinetics: see results section). Uncaging laser power was however kept constant when uncaging was performed on neighbouring spines and shaft regions of the same dendritic segment.

Image Analysis

For spine density and spine volume measurements, two photon image stacks of proximal apical dendrites were obtained after a minimum of 30 minutes of dye filling following whole-cell access. Image stacks were gathered in optical sections of 0.5-0.7 μm , with an X-Y resolution between 0.05 and 0.1 μm /pixel. Spine density was calculated after manually sectioning apical dendritic reconstructions into 10 μm segments. Spine volume measurements were calculated using an intensity-based method, as previously described (Matsuzaki et al., 2001; Beique et al., 2006). In cases where spine diameter (*i.e.*, FWHM) are shown (Figure 7-8), the intensity based method of determining spine volume was not applicable to do to limitations in the images gathered (*i.e.*, images of spines from uncaging experiments were not gathered for volume analysis, which necessitates a high resolution image containing many large and resolvable spines for calibrating the intensity-based methods). All measurements for spine volume and spine density were generated from unprocessed images.

Analysis of dendritic complexity was achieved using Neuron Studio analysis software (Computational Neurobiology and Imaging Center, Mount Sinai School of Medicine, New

York). Dendritic arbors of CA1 pyramidal neurons were modeled in Neuron Studio software and dendritic complexity measurements were extracted. The dendritic complexity metric reported is an underestimate of the total dendritic complexity, as only apical regions were included in the analysis. Complexity of Alexa-594 filled CA1 pyramidal neurons from control and TTX treated slices (postnatal day 7-8 + 9-10 days *in vitro*) were compared to age matched neurons from acute slices (post natal day 16-18).

Surface biotinylation Assay.

Organotypic slices were removed from the incubator and rapidly placed in ice cold TBS+ (in mM; 20 Tris, 0.5 KCl, 13.7 NaCl, 20 MgCl₂, 20 CaCl₂, pH 7.4) containing 0.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce/Thermo Scientific, Rockford, IL, USA) for 20 min. Unbound biotin was removed by washing slices with ice-cold TBS+. Slices were lysed in lysis buffer (in mM; 150 NaCl, 20 HEPES, 2 EDTA and, in %; 0.1 SDS, 1 Triton X-100) using a Dounce homogenizer, and sonicated (2 × 10 s, 25% on a Vibra-Cell, VCX130, Sonics, Newtown, CT, USA). The cleared supernatant was incubated with equilibrated NeutrAvidin beads (Pierce/Thermo Scientific, Rockford, IL, USA) at 4°C for 1.5 h. Beads were washed six times in a TBS solution with 0.05% SDS, and bound protein was eluted with elution buffer (in mM; 50 Tris-HCl, 1 DTT, in %; 2 SDS) and boiled at 100°C for 10 min.

Equal concentrations of internal and surface proteins were loaded on SDS-PAGE and Western blot was performed with the following antibodies: anti-GluN1 (1:3000, mouse monoclonal) and anti-GlyR (1:1000, mouse monoclonal) from Synaptic Systems (Goettingen, Germany); anti-GluN2A (1:1500, rabbit polyclonal), anti-GluN2B (1:1500, rabbit polyclonal), anti-GluA1 (1:1000 rabbit monoclonal) from Millipore (Billerica, MA, USA); anti-GluA2 (1:2000, Rabbit polyclonal) from Pierce Antibodies (Rockford, IL, USA) and anti-β-actin

(1:6000, mouse monoclonal) from Genscript (Piscataway, NJ, USA). The chemiluminescent intensities were recorded using an Odyssey Fc Imaging System (Li-COR, Lincoln, NE, USA). Quantitative analyses were performed by determining the intensity of each band with Image Studio software (Li-COR, Lincoln, NE, USA).

Results

Enhanced AMPAR conductance during multiplicative homeostatic synaptic strengthening in CA1 pyramidal neurons

We first sought to recapitulate key features of homeostatic synaptic plasticity (HSP) in an organotypic hippocampal slice preparation. Consistent with previous studies, CA1 pyramidal neurons from hippocampal slices incubated for 3-4 days with tetrodotoxin (TTX, 1 μ M) exhibited a robust increase in the amplitude (CTL: 16.51 ± 0.98 pA, $n=35$ cells; TTX: 22.50 ± 0.74 pA, $n=33$ cells; $p<0.01$) (Tyler and Pozzo-Miller, 2003; Kim and Tsien, 2008; Arendt et al., 2013) and frequency of AMPAR mediated miniature excitatory postsynaptic currents (mEPSCs) (CTL: 0.20 ± 0.03 Hz; TTX: 0.36 ± 0.06 Hz; $p<0.01$) as determined by whole-cell electrophysiological recordings (Figure 1A-B). A key feature of homeostatic synaptic strengthening is that the amplitude distribution of AMPAR-mEPSCs is scaled by a single common factor, likely reflecting cell-wide synaptic changes whereby each synapse scales proportionally to its original weight (Turrigiano et al., 1998; Lee et al., 2014). To establish whether such multiplicative scaling occurs in our experimental paradigm, we derived a scaling factor using two independent analytical methods (Turrigiano et al., 1998; Kim et al., 2012). First, we plotted the rank-order relationship of 3380 randomly selected AMPAR-mediated mEPSC amplitudes from both control and TTX-treated neurons (Figure 1C). As true multiplicativity necessitates the exclusion of additive components (Kim et al., 2012), the scaling factor was obtained from the slope of the linear relationship through the origin of the rank ordered plot. Consistent with multiplicative HSP, the distribution of mEPSC amplitudes from TTX-treated neurons overlapped well with the control distribution when divided (or “scaled”) by this scaling factor (scaling factor = 1.169; $p=0.34$, Kolmogorov-Smirnov [K-S] test; Figure 1D-E). Using a separate approach, we

iteratively tested 1000 scaling factors ranging from 0.5 to 1.5 (Figure 1C; see Materials & Methods) (Kim et al., 2012). We found perfect agreement in the scaling factors obtained from these different tests, indicating that prolonged TTX-treatment induced multiplicative scaling of synaptic AMPAR function in our experimental paradigm.

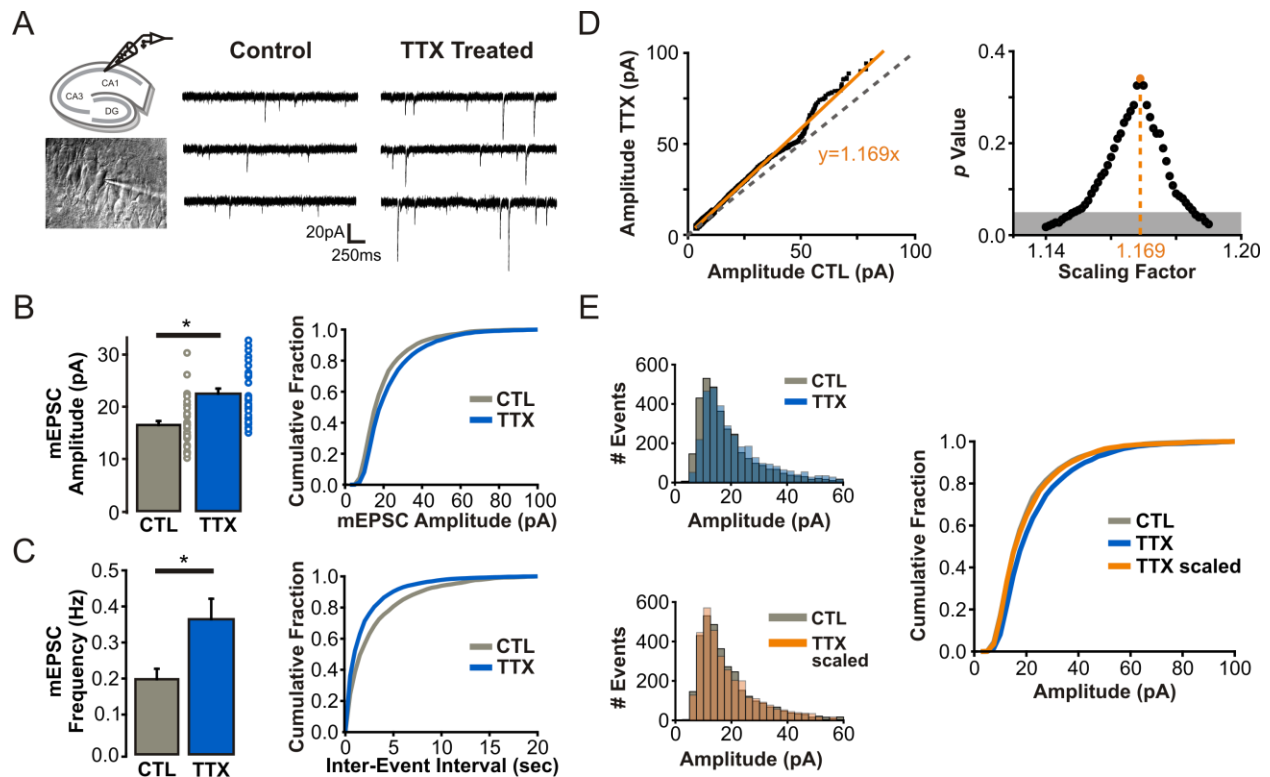


Figure 1: Multiplicative scaling of CA1 pyramidal neurons in response to prolonged TTX treatment. (A) Current traces (Vm: -70mV) of AMPAR-mediated mEPSCs from CA1 pyramidal neurons in control and TTX-treated hippocampal slices. (B) *Top panel*, Average mEPSC amplitudes (for each cell) and a cumulative distribution of all mEPSC amplitudes (cells pooled) from control and TTX-treated neurons. *Lower panel*, Average frequency of mEPSCs (for each cell) and a cumulative distribution of all mEPSC inter-event-intervals (cells pooled) from control and TTX-treated neurons. (C) *Top panel*, Rank ordered plot of pooled mEPSC amplitudes ($n=3380$ each, random subset of TTX event amplitudes) was fit through the origin with a linear function (orange line) where the slope (1.169) represents the scaling factor. *Lower panel*, the distribution of mEPSC amplitudes of TTX-treated neurons was scaled iteratively by increasing scaling factors from 0.5 to 1.5 (in bins of 0.01; only p values flanking the highest p value are shown; K-S test was used to calculate p -values). (D) *Top panel*, Amplitude distributions of AMPAR-mEPSCs recorded in control neurons (grey) and TTX-treated neurons (blue) ($p<0.01$, K-S test). *Lower panel*, Amplitude distributions of AMPAR-mEPSCs recorded in control neurons (grey) and a 'scaled' TTX-treated distribution (orange) using the scaling factor derived in panel C ($p=0.34$, K-S test). (E) Cumulative distribution of mEPSC amplitudes (as in Figure 1B, top panel) with an additional 'TTX-scaled' distribution (i.e., TTX-treated mEPSC amplitudes divided by the scaling factor).

We then performed a peak-scaled non-stationary noise analysis of AMPAR-mediated mEPSCs to determine whether prolonged network silencing alters unitary properties of AMPAR-mediated synaptic transmission (Figure 2A) (Hartveit and Veruki, 2007). Whereas the average number of synaptic AMPARs was unchanged (CTL: 12.44 ± 1.31 , $n=17$ cells; TTX: 12.72 ± 0.72 , $n=18$ cells; $p=0.33$; Figure 2B), we found that the mean AMPAR channel conductance was enhanced in TTX-treated neurons (CTL: 12.01 ± 1.12 pS; TTX: 17.65 ± 1.05 pS; $p<0.01$; Figure 2C). These changes in postsynaptic AMPAR function were not accompanied by alterations in the volume (CTL: $0.24 \pm 0.01 \mu\text{m}^3$, $n=227$ spines, 8 cells; TTX: $0.26 \pm 0.01 \mu\text{m}^3$, $n=271$ spines, 8 cells; $p=0.07$, K-S test; Figure 2E) or density of dendritic spines (CTL: 6.41 ± 0.27 spines/ $10 \mu\text{m}$; TTX: 6.18 ± 0.24 spines/ $10 \mu\text{m}$; $p=0.98$, K-S test; Figure 2F), as determined by two-photon imaging of neurons filled with Alexa-594. In principle, this increased AMPAR conductance could be mediated by post-translational modifications to AMPARs, or by changes in the pore-forming subunit composition of AMPARs. Although there is support for the latter possibility in dissociated neuronal cultures (Thiagarajan et al., 2005; Sutton et al., 2006; Groth et al., 2011), it is unclear whether the subunit composition of AMPARs is homeostatically regulated in CA1 pyramidal neurons from organotypic slices. We therefore sought to determine whether the subunit composition of synaptic AMPARs at CA1 synapses is altered following prolonged TTX-treatment.

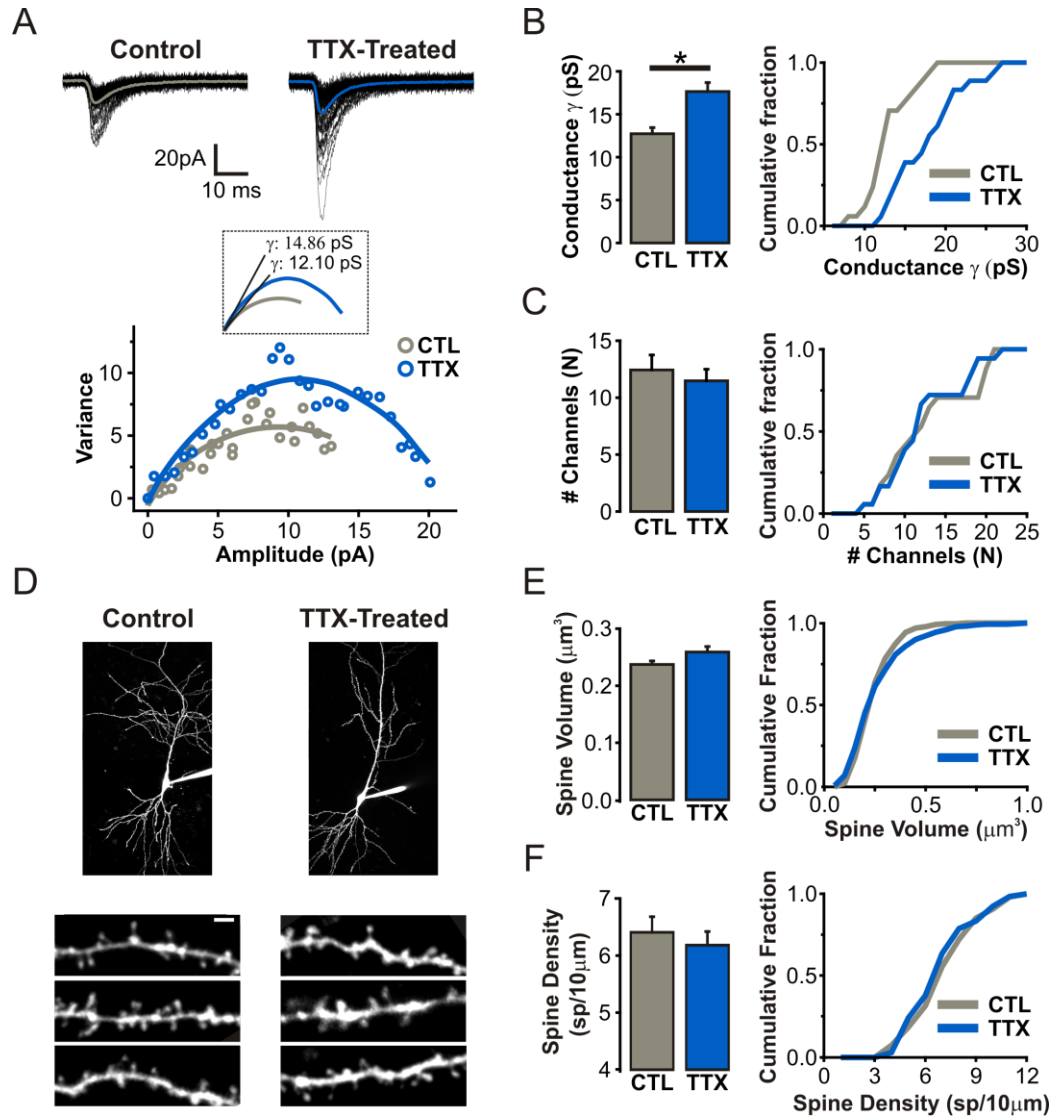


Figure 2: Recruitment of higher conductance synaptic AMPARs following prolonged TTX-treatment (A) *Top panel*, Traces of AMPAR-mediated mEPSC from a single voltage clamp recording ($V_m = -70\text{mV}$) of a control and TTX-treated CA1 pyramidal neuron. *Lower panel*, Current-variance plots from peak-scaled non-stationary noise analysis of AMPAR-mediated mEPSCs (see methods). *Inset*, Initial slope of the parabolic fit was used to calculate the mean AMPAR channel conductance (γ). (B) Number of channels ‘N’ open at the peak of cell-averaged mEPSC. Cumulative distribution of N is plotted for each condition. (C) Mean AMPAR channel conductance (γ) for mEPSCs recorded from CTL and TTX-treated neurons. Cumulative distribution of γ is plotted for each condition. (D) Two-photon images of CA1 pyramidal neurons filled with Alexa 594 (30 μM). All images were taken > 20 minutes after gaining whole-cell access to allow for adequate intracellular dye loading. Scale bar = 2 μm . (E) Volumes of dendritic spines on apical dendrites of control and TTX-treated neurons calculated using an intensity based method (see methods). Cumulative distribution of spine volumes for each condition is plotted. (F) Average Density of dendritic spines on apical dendrites of filled CA1 pyramidal neurons. Cumulative distribution of spine densities sampled in 10 μm dendritic segments.

Homeostatic upregulation of synaptic GluA2-lacking AMPARs

Western blot analyses of organotypic hippocampal slice lysates were performed to first determine whether changes in AMPAR subunit expression occurred in response to prolonged inactivity. TTX-induced inactivity caused an increase in the expression of GluA1, but not GluA2, AMPAR subunits (TTX/CTL ratio; GluA1: of 2.62 ± 0.30 , $n=3$; GluA2: 0.96 ± 0.07 , $n=4$; Figure 3A). Moreover, surface biotinylation experiments from these slices revealed a robust homeostatic enhancement of surface GluA1 with no change in the amount of surface GluA2 or glycine receptor subunits (TTX/CTL ratio; GluA1: 2.22 ± 0.26 , $n=9$; GluA2: 1.03 ± 0.07 , $n=10$; GlyR: 1.01 ± 0.10 , $n=7$; Figure 3B). These biochemical findings, combined with the increased AMPAR conductance detected by non-stationary noise analysis (Figure 2C), raise the possibility that prolonged inactivity triggers a specific upregulation of GluA2-lacking AMPARs. Since this hypothesis is consistent with some (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006; Aoto et al., 2008; Groth et al., 2011) but not all previously reported evidence (O'Brien et al., 1998; Gainey et al., 2009; Anggono et al., 2011) using broadly analogous manipulations in dissociated neuronal cultures, we further investigated this possibility in our hippocampal slice paradigm using biophysical and pharmacological methods.

AMPA receptors containing GluA2 subunits exhibit linear current-voltage (I-V) relationships, while those lacking GluA2 display inward rectification due to a pore block by intracellular polyamines at depolarized potentials (Bowie and Mayer, 1995). We thus used this biophysical signature to test the hypothesis that HSP involves an upregulation of GluA2-lacking AMPARs. We calculated rectification indices for I-V curves constructed from pharmacologically-isolated AMPAR-mediated evoked excitatory postsynaptic currents (eEPSCs) at Schaffer-collateral (SC) synapses in the presence of intracellular spermine (100 μ M). As expected, control AMPAR-

eEPSCs displayed linear I-V relationships (Rectification Index; 1.01 ± 0.09 , $n=13$ cells; Figure 3C-D), consistent with the presence of predominantly GluA2-containing AMPARs at these synapses (Beique and Huganir, 2009; Lu et al., 2009). In contrast, we detected a strong voltage-dependent block of AMPAR-eEPSCs in TTX-treated neurons (Rectification Index; TTX: 0.64 ± 0.11 , $n=8$ cells; Figure 3C-D), supporting the presence of synaptic GluA2-lacking AMPARs in response to inactivity.

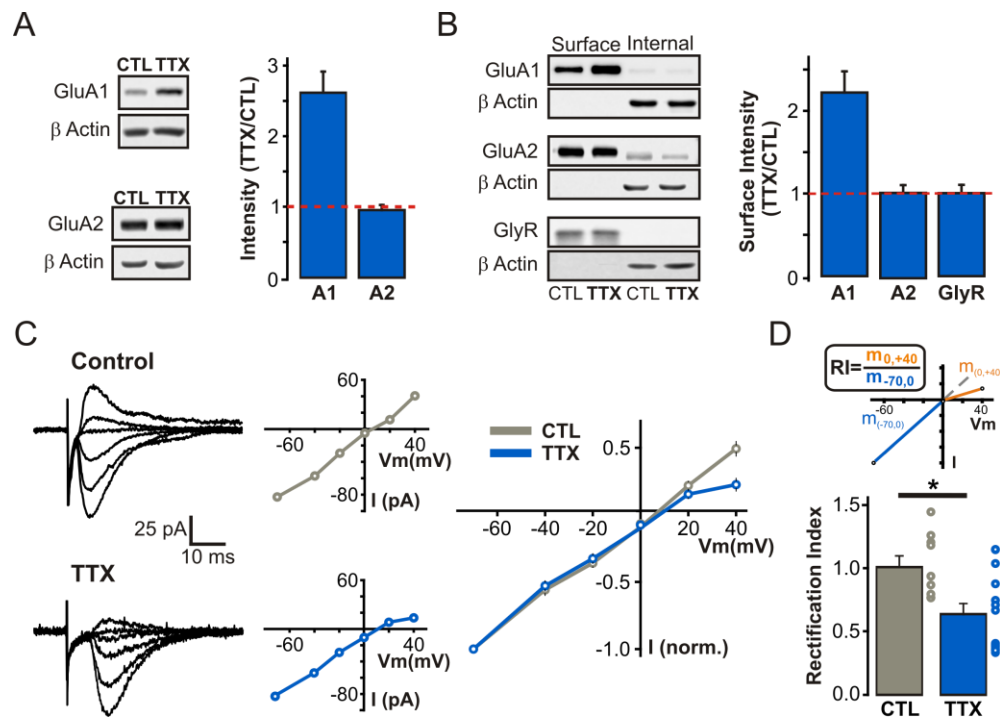


Figure 3: Increased surface expression of GluA1 and the emergence of inwardly rectifying AMPARs at SC-synapses following prolonged TTX-treatment. (A) Representative western blots and quantification for the change in AMPA receptor subunit expression, plotted as a TTX/CTL ratio of band intensity, in hippocampal lysates from control and TTX-treated slices. All bands were normalized to β -Actin before calculating the TTX/CTL ratio. (B) Representative western blots of biotinylated (surface) and non-biotinylated (internal) fractions from control and TTX-treated hippocampal slices. Quantification of the relative surface expression of AMPA and Glycine receptor subunits from control and TTX treated slices, plotted as a TTX/CTL ratio of band intensity. (C) Current-voltage relationship of evoked AMPAR-mediated EPSCs. *Left panel*, Current traces at different holding potentials (-70 to +40 mV; with 100 μ M intracellular spermine) and corresponding I-V curves from a control and a TTX treated neuron. *Right panel*, Average I-V relationship of AMPAR-mediated eEPSCs from control and TTX-treated neurons. (D) Rectification indices for control and TTX-treated neurons in panel B, computed as the ratio of the slope (m) of the outward portion of the I-V curve over that of the inward portion ($p < 0.01$, unpaired Student's t-test).

We then measured the sensitivity of AMPAR-eEPSCs to the selective antagonist of GluA2-lacking AMPARs, 1-naphthyl acetyl spermine trihydrochloride (NASPM, 20 μ M). Bath administration of NASPM robustly reduced the amplitude of AMPAR-eEPSCs recorded from TTX-treated neurons whereas AMPAR-eEPSCs from control slices were unaffected (NASPM inhibition; CTL: $-6.15 \pm 7.95\%$, $n=7$ cells, $p=0.47$; TTX: $69\% \pm 9.48\%$, $n=8$ cells, $p<0.01$; Figure 4A). To sample a larger population of synapses, we also measured the NASPM sensitivity of pharmacologically isolated spontaneous and miniature AMPAR-EPSCs (sEPSCs and mEPSCs). In agreement with the results outlined above, NASPM did not affect the amplitude of control sEPSCs (from 14.85 ± 1.50 pA to 13.98 ± 1.59 pA in NASPM; $p=0.12$; $n=8$ cells) or mEPSCs (from 13.41 ± 0.59 pA to 13.53 ± 0.86 pA in NASPM; $p=0.82$; $n=8$ cells), but significantly reduced the amplitude of these events recorded from TTX-treated neurons (sEPSCs: from 26.53 ± 2.26 pA to 19.85 ± 2.13 pA in NASPM, $p<0.01$; mEPSCs: from 18.71 ± 0.79 pA to 15.62 ± 0.64 pA in NASPM, $p<0.01$; Figure 4B-E). Together, these data are consistent with the expression of synaptic GluA2-lacking AMPARs during HSP.

Previous studies have demonstrated that synaptic strengthening during LTP is mediated by an increase in synaptic AMPAR number and is accompanied by robust dendritic spine enlargement (Matsuzaki et al., 2004; Harvey and Svoboda, 2007). Remarkably, we found that the synaptic strengthening during HSP was not accompanied by changes in the number of AMPARs at synapses (Figure 2B), nor in the volume of dendritic spines (Figure 2E). Rather, our biochemical, biophysical and pharmacological data strongly suggest that the postsynaptic manifestation of HSP is expressed in part through the direct replacement of synaptic GluA2-containing AMPARs with higher conductance GluA2-lacking AMPARs.

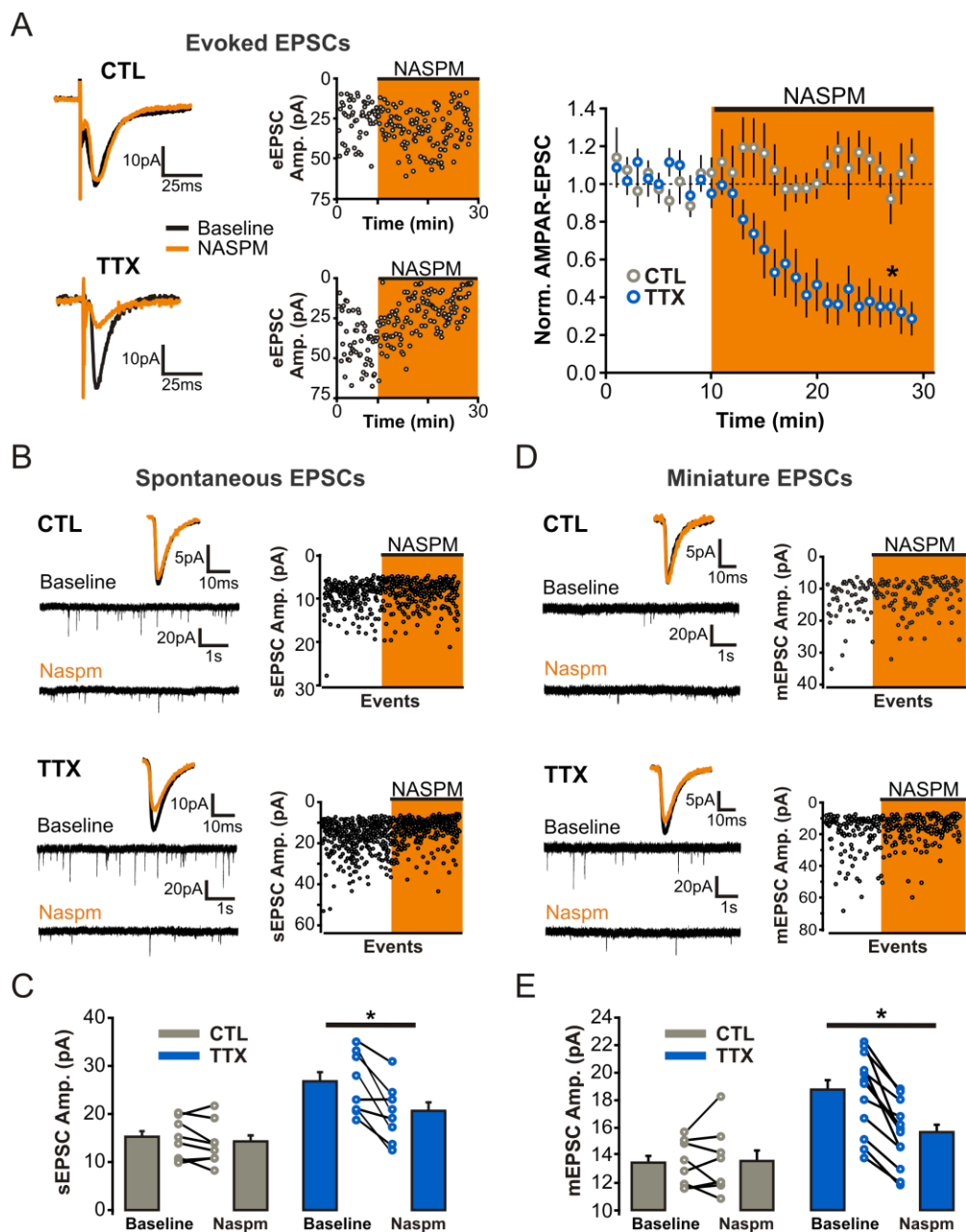


Figure 4: Synaptic incorporation of GluA2-lacking AMPARs in CA1 pyramidal neurons following prolonged TTX-treatment (A) *Left panel*, Representative traces and event amplitude scatter plots of evoked AMPAR-mediated EPSCs before and after NASPM administration (20 μ M). *Right panel*, Normalized average amplitude of evoked AMPAR-mediated EPSCs. (B) Current traces and event amplitude scatter plots of spontaneous AMPAR-mediated EPSCs (*i.e.*, no TTX during recording) before (baseline) and 15-20 minutes after onset of NASPM (20 μ M) administration. (C) Average sEPSC amplitudes before (baseline) and 15-20 minutes after NASPM administration. (D) Current traces and event amplitude scatter plots of mEPSCs (*i.e.*, in TTX) before and 15-20 minutes after onset NASPM administration. (E) Average mEPSC amplitudes before (baseline) and 15-20 minutes after NASPM administration.

Homeostatic switch in synaptic NMDAR subunit composition

The dynamic nature of NMDAR trafficking and targeting behaviour at rest, during postnatal development and during Hebbian plasticity has gained considerable appreciation over the past two decades (Lau and Zukin, 2007). In part because HSP has overwhelmingly been studied in dissociated neuronal cultures, a preparation that does not lend itself with ease to the study of NMDAR function, the homeostatic regulation of NMDARs has been far less extensively studied than that of AMPARs (Perez-Otano and Ehlers, 2005). To determine whether alterations in NMDAR function accompanied the homeostatic enhancement in AMPAR function outlined above, we evoked EPSCs while holding neurons at -70 mV and +40 mV to compute the ratio of AMPAR and NMDAR contributions to SC synapse function (see Methods). We found that the ratio of AMPA to NMDA receptor components of eEPSCs was not altered by prolonged inactivity (CTL: 0.87 ± 0.13 , $n=13$ cells; TTX: 0.87 ± 0.12 , $n=12$ cells; $p=0.98$; Figure 5A), consistent with previous findings in both neuronal cultures and organotypic slices younger than those used here (Watt et al., 2000; Arendt et al., 2013). Since AMPAR function was significantly enhanced during HSP in our experimental conditions (Figure 1B), this finding suggests a concomitant upregulation of both synaptic NMDA and AMPAR function during HSP. In line with this notion, Western blot analysis of hippocampal slice lysates (Figure 5B) and surface biotinylation experiments (Figure 5C) revealed an increase in the expression and surface delivery of all three major NMDAR subunits found in the hippocampus in TTX-treated slices (TTX/CTL ratio for hippocampal lysates; GluN1: 1.43 ± 0.09 , $n=4$; GluN2A: 1.52 ± 0.16 , $n=6$; GluN2B: 1.85 ± 0.13 , $n=3$; Figure 5B; TTX/CTL ratio for biotinylated samples; GluN1: 1.31 ± 0.10 , $n=10$; GluN2A: 1.86 ± 0.35 , $n=8$; GluN2B: 1.69 ± 0.24 , $n=15$; Figure 5C).

To further establish whether NMDAR function is homeostatically regulated in CA1 pyramidal neurons, we gathered two complementary electrophysiological readouts of NMDAR function. First, we reasoned that an upregulation of surface NMDARs could be revealed by examining the degree of tonic activation of these receptors by ambient levels of extracellular glutamate (Sah et al., 1989). To this end, we monitored changes in whole-cell current of CA1 pyramidal neurons induced by bath administration of the NMDAR antagonist DL-APV (50 μ M; see Materials & Methods). NMDAR blockade in control neurons induced a small but highly reproducible change in holding current (15.48 ± 10.86 pA, $n=5$ cells), thus revealing the presence of an ambient glutamate tone in organotypic hippocampal slices (Figure 5D). Interestingly, the magnitude of this tonic current was more than three times greater in TTX treated slices (56.15 ± 9.51 pA, $n=7$ cells) compared to that seen in controls. In principle, this difference could reflect an upregulation of surface NMDARs, an alteration in the regulation of ambient extracellular glutamate concentration, or a combination of both. To directly measure NMDAR function, we next monitored the whole-cell response to bath administration of NMDA (5 μ M for 3 minutes) and found that NMDA induced significantly larger whole-cell currents in TTX-treated neurons compared to control (CTL: 128.69 ± 15.44 pA, $n=16$ cells; TTX: 198.98 ± 21.42 pA, $n=16$ cells; $p<0.05$; Figure 5E). This enhancement was likely not due to an overall greater membrane surface area in TTX-treated neurons, since the dendritic arborisation between control and TTX-treated neurons was not different (CTL: 732.36 ± 86.43 μ m, $n=10$ cells; TTX: 787.48 ± 48.56 μ m, $n=12$ cells; $p=0.56$; Figure 5F). These functional and morphological measurements aligned well with our biochemical data (Figure 5B-C) and together demonstrate that prolonged inactivity induced a robust upregulation of surface NMDAR expression in CA1 pyramidal neurons.

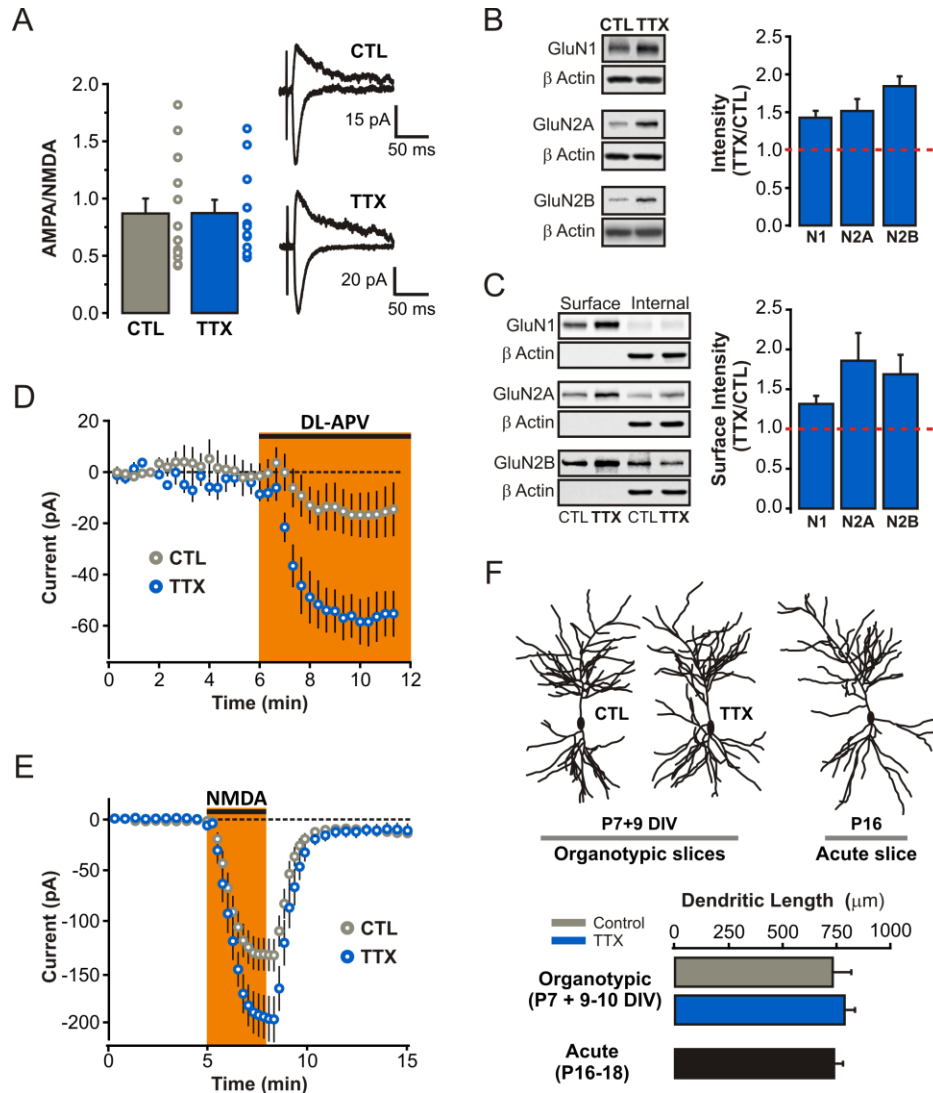


Figure 5: Homeostatic Upregulation of surface NMDARs in CA1 pyramidal neurons in response to prolonged TTX-treatment. (A) AMPA to NMDA ratio of evoked EPSCs (see methods). (B) Representative western blots and quantification of changes in NMDAR subunit expression, plotted as a TTX/CTL ratio of band intensity, in hippocampal lysates of control and TTX-treated slices. All bands were normalized to β -Actin before calculating the TTX/CTL ratio. (C) Representative western blots of biotinylated (surface) and non-biotinylated (internal) fractions from control and TTX-treated slices. Quantification of the relative surface expression of NMDA receptor subunits between control and TTX treated slices plotted as a TTX/CTL ratio of band intensity. (D) Amplitude of the current induced by bath administration of 50 μ M DL-APV while holding the neurons at +40mV ($p < 0.05$, unpaired Student's t -test). These experiments were performed with NBQX, picrotoxin and TTX in the Ringer solution. (E) Amplitude of the inward current induced by bath administration of NMDA (5 μ M; $V_m = -60$ mV in 0.1 mM Mg^{2+}). All NMDA bath administration experiments were performed with NBQX, picrotoxin, and TTX in the Ringer solution. (F) 2P-images of filled CA1 pyramidal neurons were reconstructed using Neuron Studio software (see methods). Dendritic length (μ m) is plotted for CA1 pyramidal neurons from TTX-treated or control organotypic slices (P 7 + 9-10 DIV) and from age-matched neurons from acute slices (P16-17 animals; $n=10$, 12, and 9 neurons for control, TTX-treated and acute slices, respectively).

During Hebbian LTP, synaptic NMDARs undergo a rapid switch in subunit composition, from predominantly NR2B-containing towards NR2A-containing NMDARs (Bellone and Nicoll, 2007). We therefore next wondered on the degree of mechanistic commonality between Hebbian and homeostatic synaptic strengthening and asked whether the subunit composition of synaptic NMDARs is likewise regulated during homeostatic synaptic plasticity. To this end, we probed the subunit composition of synaptic NMDARs by first analyzing the kinetics of pharmacologically isolated NMDAR-eEPSCs from SC synapses and found that TTX-treated neurons exhibited NMDAR-eEPSCs with faster decay kinetics compared to control (weighted tau; CTL: 366.03 ± 19.72 ms, $n=15$ cells; TTX: 315.44 ± 15.16 ms, $n=18$ cells; $p<0.05$; Figure 6A). Based on the well characterized subunit dependence of NMDAR kinetics (Vicini et al., 1998), these results suggest that prolonged inactivity led to synaptic enrichment of GluN2A-containing NMDARs. We further tested this possibility pharmacologically by measuring the sensitivity of NMDAR-eEPSCs to the GluN2B-containing NMDAR antagonist ifenprodil (3 μ M) and found that NMDAR-eEPSCs recorded from TTX-treated neurons were significantly less sensitive to ifenprodil than interleaved controls (ifenprodil inhibition; CTL: 56.67 ± 4.80 %, $n=12$ cells; TTX: 37.95 ± 3.65 %, $n=16$ cells; $p<0.01$; Figure 6B). Thus, despite a robust upregulation of all three major NMDAR subunits at the plasma membrane, we found that GluN2A-containing NMDARs are preferentially stabilized at synapses during HSP.

Subcellular distribution of AMPA and NMDAR subtypes following network silencing

The trafficking, targeting and stabilization of glutamate receptors at synapses occurs through a number of highly regulated intracellular and extracellular interactions (Shepherd and Huganir, 2007). An emerging model of synaptic AMPAR recruitment involves the trapping of freely diffusing extrasynaptic surface receptors as they enter the synaptic compartment (Opazo

and Choquet, 2011), and an analogous diffusional trapping mechanism has also been described for NMDARs (Groc et al., 2006; Bard et al., 2010). Moreover, the functional enhancement of AMPAR transmission during LTP is highly dependent on this reserve pool of non-synaptic receptors (Makino and Malinow, 2009; Granger et al., 2013). Although our electrophysiological data outlined above clearly demonstrate that the subunit composition of synaptic AMPARs (Figures 3 & 4) and NMDARs (Figure 6) are altered during HSP, it is unclear whether these changes reflect synapse-specific regulation or rather diffuse, cell-wide, changes in surface glutamate receptor expression. To specifically address this issue, we took advantage of subunit-specific biophysical signatures of AMPAR and NMDAR subtypes in combination with the ability afforded by 2P-uncaging of MNI-Glutamate (MNI-Glu) to activate glutamate receptors at defined subcellular compartments (Figure 7A-C).

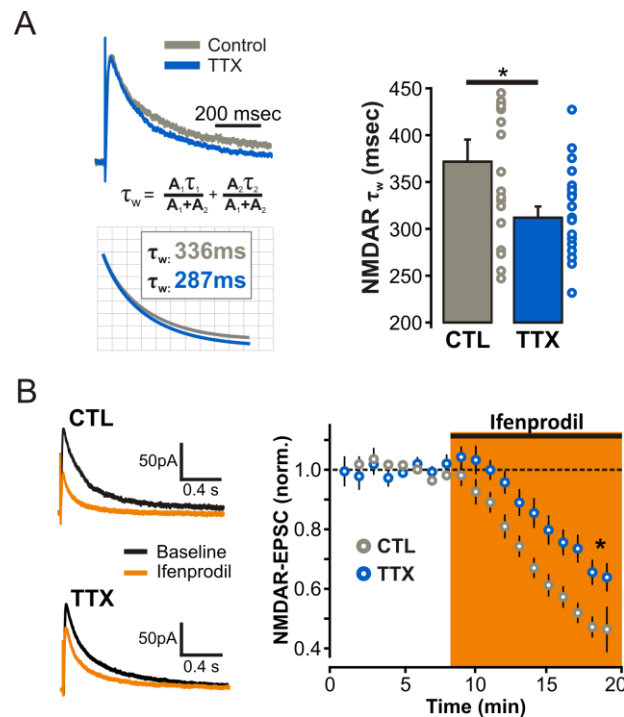


Figure 6: Homeostatic shift in synaptic NMDAR subunit composition in response to prolonged TTX-treatment. (A) Weighted tau values from bi-exponential fits of evoked NMDAR-EPSCs recorded at +40mV from control and TTX-treated neurons. (B) Amplitude of evoked NMDAR-EPSCs (Vm=+40mV) before (baseline) and after administration of the selective GluN2B-containing NMDARs antagonist Ifenprodil (3 μM)

To determine the spatial extent of GluA2-lacking AMPAR surface expression, we analyzed the I-V relationship of 2P glutamate uncaging-evoked AMPAR-mediated EPSCs (AMPAR-2P-EPSCs) at dendritic spines and nearby ($<5\mu\text{m}$) extrasynaptic shaft regions of secondary and tertiary proximal apical dendrites. In agreement with a previous study in neuronal cultures (Beique et al., 2011), uncaging of MNI-glutamate onto spines and onto extrasynaptic shaft and somatic regions of control neurons yielded AMPAR-2P-EPSCs exhibiting linear I-V relationships, although rectifying currents were occasionally encountered (Rectification Index; spine: 1.10 ± 0.06 , $n=16$; dendrite: 1.01 ± 0.08 , $n=9$; soma: 0.98 ± 0.04 , $n=6$; Figure 7D-F). Thus, GluA2-containing AMPARs appear to dominate both synaptic and extrasynaptic regions of control CA1 neurons. Consistent with the upregulation of synaptic GluA2-lacking AMPARs (*i.e.*, synaptically evoked-EPSCs; Figures 3-4) in response to prolonged inactivity, we observed strong inwardly-rectifying AMPAR-2P-EPSCs when uncaging pulses were directed onto the tips of dendritic spines in TTX-treated neurons (Rectification Index; 0.76 ± 0.09 , $n=12$ spines; Figure 7D-F). The changes in the rectifying properties of 2P-EPSCs from spines between control and TTX-treated neurons could not be accounted for by an experimental bias towards morphologically dissimilar spines in the treatment groups ($p=0.47$; Figure 7H). Interestingly, inwardly-rectifying AMPAR-2P-EPSCs were also detected when glutamate was uncaged onto dendritic shafts and somatic regions of TTX-treated neurons (Rectification Index; dendrite: 0.61 ± 0.11 , $n=10$; soma: 0.30 ± 0.04 , $n=4$; Figure 7D-F). Together, our data suggest that prolonged inactivity drives a robust cell-wide expression of GluA2-lacking AMPARs at both synaptic and extrasynaptic regions of CA1 pyramidal neurons.

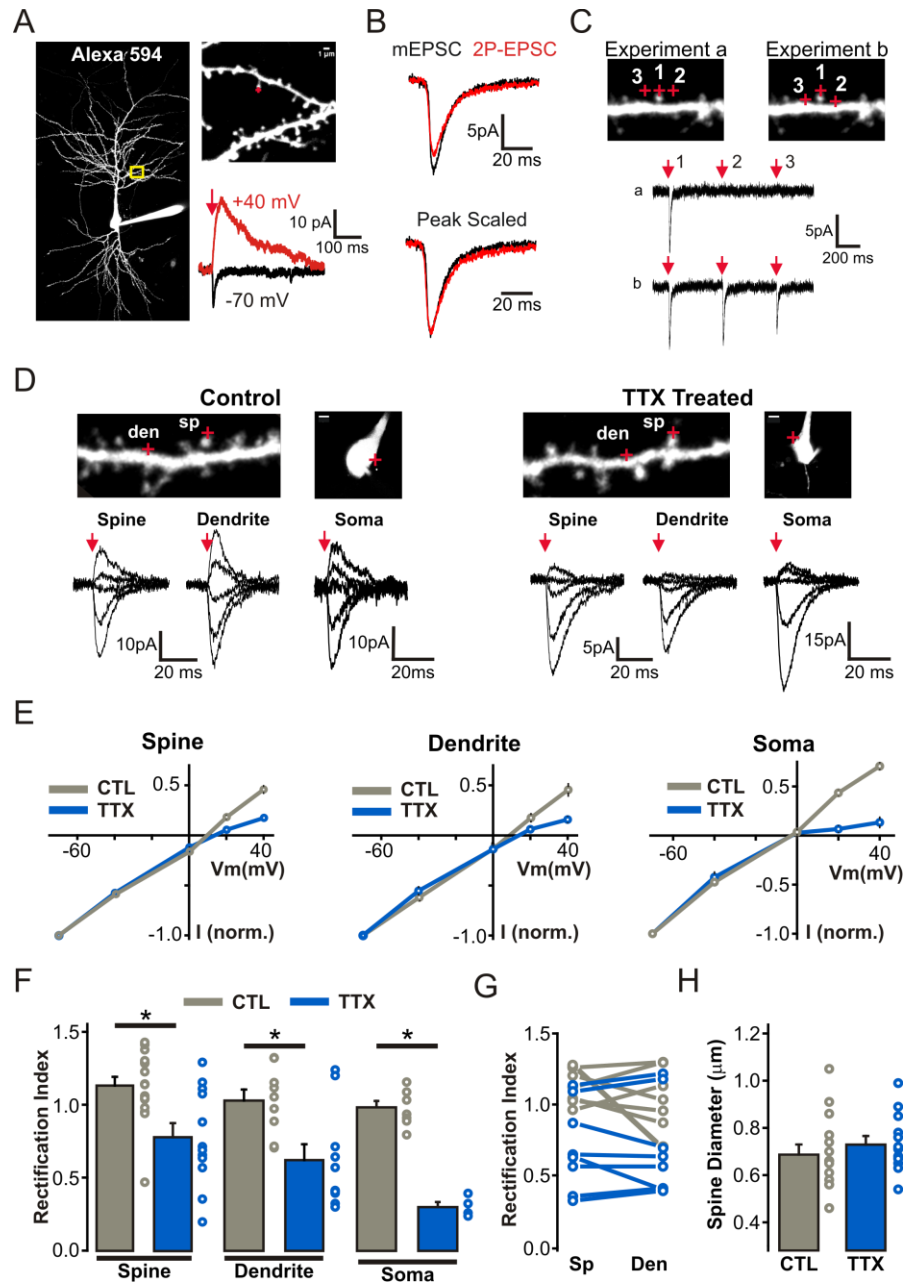


Figure 7: Cell-wide homeostatic upregulation of GluA2-lacking AMPARs in response to prolonged TTX treatment. (A) *Left panel*, Two photon (2P) image of a control CA1 pyramidal neuron filled with Alexa-594 to visualize dendritic morphology. Scale bar = 15 μ m. *Right panel*, Enlarged view of an apical dendritic segment with red crosshairs illustrating the site of two-photon glutamate uncaging (1ms at 720nm); scale bar: 2 μ m. At -70mV, glutamate uncaging elicits a postsynaptic AMPAR-mediated response whereas at +40mV, uncaging of glutamate also activates longer decaying postsynaptic NMDARs. (B) *Top panel*, in the same recording, AMPAR-mediated 2P-EPSCs can be generated to match the amplitude of AMPAR-mEPSCs. *Bottom panel*, Peak scaling the average traces of 2P-EPSCs and mEPSCs reveals a similar rise and decay time course. (C) A set of control experiments whereby three uncaging pulses (separated by 500ms) were elicited at each of the three points illustrated with red

crosshairs. In experiment b, the uncaging positions of sites 2 and 3 were brought closer to the dendrite to elicit a response mediated by extrasynaptic receptors. Scale bars = 1 μm . (D) Current voltage (I-V) relationship of AMPAR-mediated 2P-EPSCs generated at distinct subcellular locations. *Top panel*, Two-photon images of secondary apical dendritic segment show sites of glutamate uncaging (red crosshairs); Scale bars: 1 μm and 5 μm for images of dendrite and soma, respectively. *Lower panel*, Traces of AMPAR-mediated 2P-EPSCs at different holding potentials (-70 to +40 mV; with 100 μM intracellular spermine) with red arrow depicts the timing of the 1ms uncaging pulse. (E) Average I-V plots of 2P-EPSCs from each subcellular location in both conditions (F) Rectification indices for all spine, dendritic, and somatic IV curves presented in Figure 7E; $p < 0.01$; unpaired Student's t-test. (G) Rectification indices of 2P-EPSCs generated from 'pairs' of spine and neighbouring (<5 μm) extrasynaptic shaft regions. (H) Diameter (FWHM) of all dendritic spines probed for AMPAR-2P I-V relationships.

We last investigated the subcellular distribution of GluN2A- and GluN2B-containing NMDARs during HSP by analyzing the decay kinetics of NMDAR-2P-EPSCs elicited at either dendritic spines or nearby extrasynaptic shaft regions. Consistent with the inactivity induced enrichment of synaptic GluN2A-containing NMDARs (*i.e.*, synaptically evoked EPSCs; Figure 6), we found that NMDAR-2P-EPSCs from dendritic spines of TTX-treated neurons exhibited faster decay kinetics than from morphologically similar control spines (CTL: 191.71 ± 4.73 ms, $n=18$; TTX: 145.07 ± 16.44 ms, $n=26$; $p < 0.01$; Figure 8A-D). Strikingly, however, the decay kinetics of NMDAR-2P-EPSCs elicited from shaft regions of TTX-treated neurons were comparable to those elicited from shaft regions of control neurons (CTL: 209.72 ± 10.80 ms, $n=13$; TTX: 195.54 ± 11.83 ms, $n=19$; $p=0.32$) and significantly longer than those from nearby spines (Figure 8A-C). Whereas uncaging onto spines can in principle activate a mixture of synaptic and extrasynaptic of receptors, uncaging onto shaft regions overwhelmingly activates extrasynaptic receptor populations. Thus, in stark contrast to the cell-wide homeostatic upregulation of surface GluA2-lacking AMPARs at both synaptic and extrasynaptic membrane regions, the TTX-induced enrichment of GluN2A-containing NMDARs occurs selectively at synapses. Collectively, by discriminating between synaptic and extrasynaptic glutamate receptor

populations, our results expose intriguing differences in the regulatory mechanisms that dictate the synaptic targeting of AMPA and NMDARs of defined subunit composition during homeostatic plasticity (Figure 9A-B).

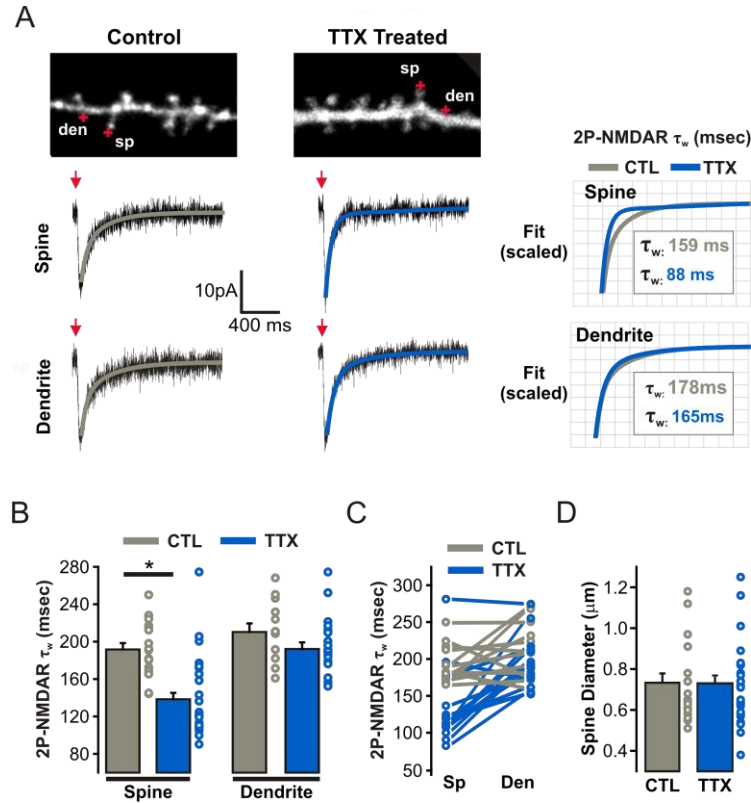


Figure 8: GluN2A-NMDARs are specifically targeted to synapses in response to prolonged TTX-treatment (A) *Left panel*, NMDAR-mediated 2P-EPSCs ($V_m = -60$ mV; 0.1 mM Mg^{2+}) elicited by uncaging glutamate onto the tips of dendritic spines and nearby isolated dendritic shaft segments. Bi-exponential fits of the current decay are overlaid on the current traces for spine and dendrite uncaging events; Scale bars = 1μ m. *Right panel*, Scaled bi-exponential fits of NMDAR-2P-EPSC decay from spines and dendrites are overlaid for clarity and the corresponding weighted tau values are indicated. (B) Weighted tau values of NMDAR-2P-EPSCs. (C) Weighted tau values of NMDAR-2P-EPSCs from ‘pairs’ of spine and nearby ($<5 \mu$ m) dendritic regions. (D) Diameters (FWHM) of all dendritic spines probed for NMDAR-2P-EPSCs ($p=0.97$, unpaired student’s t-test).

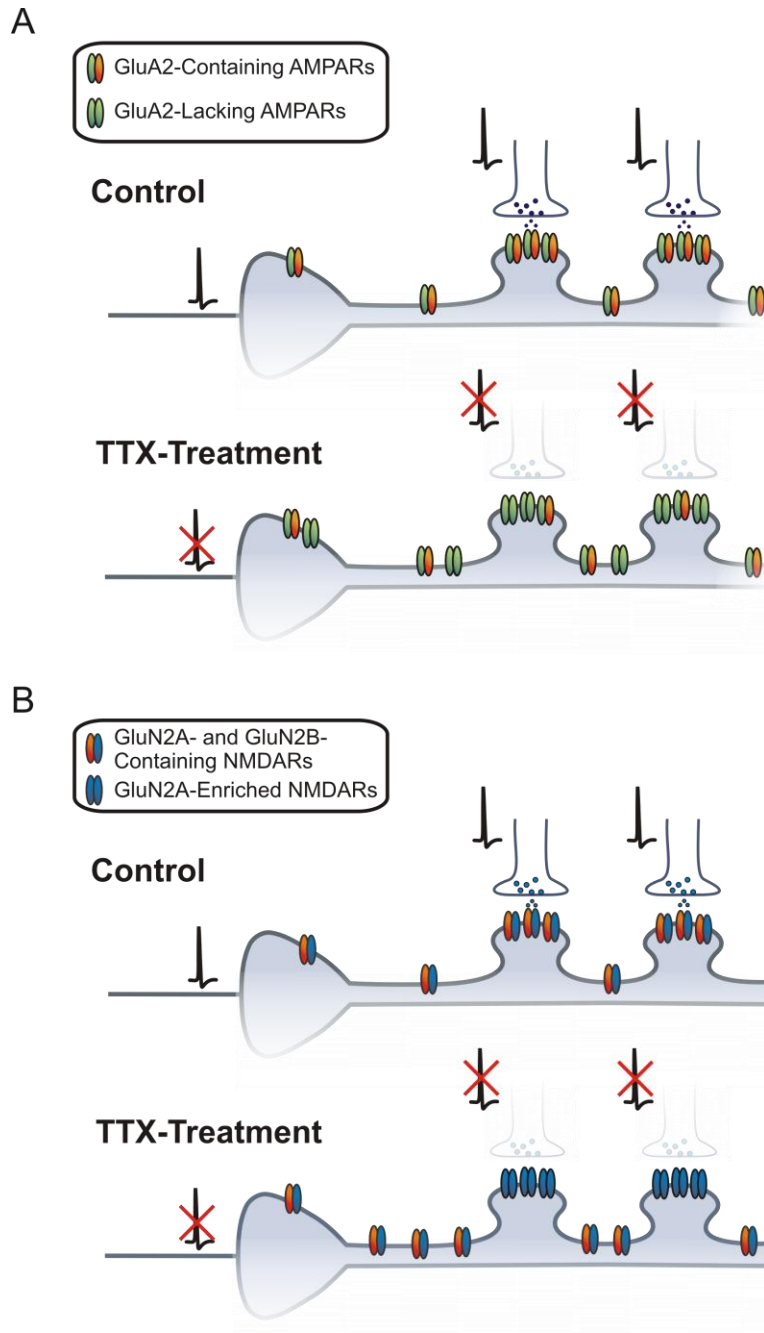


Figure 9: Differential subcellular targeting of glutamate receptor subtypes during HSP

(A) *Top panel*, AMPARs containing the GluA2 subunit predominate at both synaptic and extrasynaptic regions of CA1 pyramidal neurons. *Bottom panel*, When network activity is silenced by prolonged TTX-treatment, there is a homeostatic upregulation of GluA2-lacking AMPARs in both synaptic and extrasynaptic compartments of the neuronal membrane. (B) *Top panel*, CA1 pyramidal neurons display a mixed population of NMDARs containing both GluN2A and GluN2B subunits. *Bottom panel*, When network activity is silenced by prolonged TTX-treatment, there is an indiscriminate increase surface NMDARs subunits, however, GluN2A-containing NMDARs are preferentially localized/stabilized at synapses.

Discussion

Here, using a combination of biochemical, biophysical and pharmacological approaches, we found that prolonged TTX treatment altered the subunit composition of both synaptic AMPA and NMDARs in CA1 pyramidal neurons from organotypic hippocampal slices. Notably, we show that prolonged inactivity induced a robust upregulation of the AMPAR subunit GluA1 that resulted in a widespread, cell-wide, surface expression of GluA2-lacking AMPARs. Remarkably, despite inducing a robust and generalized upregulation of the three major hippocampal NMDAR subunits, network silencing triggered a switch in the subunit composition of solely the synaptic population of NMDARs, leaving unaltered the composition of their extrasynaptic counterparts. Altogether, these findings highlight the notion that the homeostatic mechanisms used by neurons to adjust their excitability levels regulate synapse function in ways beyond solely modifying synaptic strength *per se*.

A number of previous studies have reported conflicting evidence regarding the subunit composition of AMPARs involved in homeostatic synaptic potentiation. A recent review (Lee, 2012a) attempted to reconcile these discrepancies by documenting differences in the pharmacological paradigms used to induce HSP. Specifically, it was highlighted that the selective regulation of GluA1 occurred after prolonged blockade of both network activity (*i.e.*, TTX) and NMDARs (Ju et al., 2004; Sutton et al., 2006; Aoto et al., 2008), whereas both GluA1 and GluA2 expression was affected when neurons were treated with TTX alone (O'Brien et al., 1998; Gainey et al., 2009; Anggono et al., 2011). In contrast to this unifying picture, we provide here a number of complementary and converging lines of evidence indicating that a TTX treatment alone led to a robust and selective upregulation of GluA1 expression and formation of GluA2-lacking AMPARs in CA1 pyramidal neurons in an organotypic slice preparation. The

direct replacement of GluA2-containing AMPARs with higher conductance GluA2-lacking AMPARs during homeostatic plasticity offers an effective means to enhance synaptic strength without the need to increase receptor number or to increase spine volume. This scenario is consistent with homeostatic plasticity occurring at single synapses (Beique et al., 2011) and is in line with manifestations of homeostatic synaptic plasticity *in vivo* (He et al., 2012).

It is pertinent to compare and contrast the mechanistic underpinnings of Hebbian and homeostatic synaptic strengthening, including those involving subunit composition of glutamate receptors. While a transient insertion of GluA2-lacking AMPARs has been observed following LTP induction (Plant et al., 2006; Guire et al., 2008), the role of this particular subtype of AMPARs in LTP is controversial (Adesnik and Nicoll, 2007; Gray et al., 2007). Likewise, the implication of GluA2-lacking AMPARs in homeostatic synaptic strengthening is also debated, as outlined above. These divergences may reflect the presence of distinct synaptic plasticity mechanisms that are heavily dependent on subtleties in experimental conditions and paradigms. Nevertheless, the homeostatic switch in NMDAR subunit composition we report here is highly analogous to that previously shown to occur during Hebbian LTP (Bellone and Nicoll, 2007). Indeed, LTP was shown to be accompanied by a GluN2B-containing towards GluN2A-containing NMDAR subunit switch that exhibited a time course highly similar to that of the synaptic delivery of AMPARs. Although our results provide limited insights into the precise time course of the inactivity induced subunit switches for both AMPA and NMDARs, these homeostatic adaptive mechanisms might be occurring simultaneously. It is thus tempting to speculate that both Hebbian and homeostatic synaptic strengthening utilize common mechanisms involving the concerted upregulation of AMPARs and GluN2A-containing NMDARs. Future

studies will be required to substantiate this possibility and further establish the extent of the molecular commonalities between Hebbian and homeostatic synaptic plasticity.

The homeostatic adjustments reported here for both AMPA and NMDAR subunit composition likely influence Hebbian plasticity rules. Indeed, *in vivo* visual deprivation paradigms that lead to homeostatic upregulation of synapse function in visual cortex (Goel and Lee, 2007), impart a metaplastic influence that modifies the stimulus threshold for inducing LTP and LTD (Philpot et al., 2001), and spike-timing dependent synaptic plasticity (Guo et al., 2012). These changes appear to be caused by an increased proportion of GluN2B-containing NMDARs at synapses, in an apparent contrast to what we report here. Whereas the various *in vivo* visual deprivation paradigms reduce thalamic synaptic input into visual cortex, it is unclear to what extent they reduce overall network excitability in the visual cortex. Thus, it is possible that the GluN2A enrichment we report here following prolonged TTX treatment represents a homeostatic response to a prolonged postsynaptic neuronal silencing, whereas the GluN2B-enrichment observed following visual deprivation reflects a homeostatic response to a reduction in presynaptic activity. In support of this idea, selective presynaptic silencing of individual synapses has recently been shown to cause postsynaptic GluN2B enrichment (Lee et al., 2010). Conversely, the presence of calcium-permeable GluA2-lacking AMPARs following prolonged inactivity may also convey metaplastic influences to synapses, either by lowering the threshold for Hebbian synaptic potentiation, or even by imparting anti-Hebbian features (Lamsa et al., 2007). Future studies are required to better understand the influence of glutamate receptor composition on synaptic plasticity rules.

AMPA and NMDARs of different subunit composition are differentially localized to synaptic and extrasynaptic membrane compartments. For instance, AMPARs containing

GluA2/GluA3 subunits are found almost exclusively at synapses whereas GluA1/GluA2-AMPARs occupy both synaptic and extrasynaptic membrane regions (Beique and Huganir, 2009; Lu et al., 2009). Moreover, GluN2A-containing NMDARs are believed to be preferentially stabilized at synapses over GluN2B-containing NMDARs (Groc et al., 2006). These subcellular distribution profiles are thought to arise through preferential interactions of specific AMPAR and NMDAR subunits, and/or auxiliary subunits, with PSD scaffolding proteins at synapses (Lau and Zukin, 2007; Shepherd and Huganir, 2007; Jackson and Nicoll, 2011). The differential subcellular distribution of AMPA and NMDAR expression during HSP we have described can be traced, at least in part, to the changes in subunit protein expression. Specifically, the selective upregulation of GluA1 protein expression (over GluA2) was accompanied by a widespread enhancement of surface GluA2-lacking AMPARs, evident at both dendritic spines and extrasynaptic membrane regions. Such a cell-wide upregulation of AMPARs offers an effective means to account for the remarkable multiplicativity of homeostatic synaptic strengthening triggered by a somatic homeostatic sensing mechanism (Lee et al., 2014). Interestingly, the TTX-induced increase in NMDAR protein expression (both total and surface expression) was not subunit selective, as we detected an upregulation of GluN1, GluN2A and GluN2B. Despite this generalized increase in NMDAR surface expression, synapses were specifically enriched with GluN2A-containing NMDARs during HSP, likely reflecting the preferential synaptic stabilization of this subunit compared to GluN2B-containing NMDARs. Thus, whereas the synaptic incorporation of GluA2-lacking AMPARs likely results from the bulk loading of these receptors onto the plasma membrane, the selective synaptic stabilization of GluN2A-containing NMDARs during HSP emphasizes the competitive interactions of GluN2A subunits for synaptic anchoring/scaffolding proteins.

The functional importance of extrasynaptic receptors is increasingly being recognized. For instance, extrasynaptic AMPARs and NMDARs can be recruited to and/or exchanged with synaptic receptor populations in a dynamic and highly regulated manner. Recent studies have shown that extrasynaptic AMPARs can shape synaptic transmission (Heine et al., 2008) and are required for LTP (Makino and Malinow, 2009; Granger et al., 2013). Moreover, extrasynaptic NMDARs can powerfully influence synaptic integration (Chalifoux and Carter, 2011; Lee, 2012b) and differentially regulate neuronal survival and death signaling pathways (Hardingham and Bading, 2003). The homeostatic regulation of the number and subunit composition of extrasynaptic glutamate receptors described here will, in principle, influence all of the functions ascribed to this population of receptors, thus broadening the functional implications of the homeostatic process.

Both *in vitro* and *in vivo* manifestations of homeostatic synaptic plasticity have been documented using several experimental paradigms. Homeostatic synapse regulation operates continuously ‘online’ to enable tuning of cellular excitability in the face of perpetual alterations in neuronal firing activity. We have demonstrated that, in addition to triggering robust synaptic strengthening, the homeostatic process also involves changes in the subunit composition and subcellular distribution of both AMPA and NMDARs. Thus, the homeostatic adjustment of synapse function is not limited to the regulation of synaptic strength, but likely impacts synaptic properties such as temporal integration of synaptic input and calcium-dependent biochemical signaling. Future studies will be required to fully grasp the functional implications of these homeostatic regulations.

Manuscript II

Title: Metaplasticity at CA1 synapses by homeostatic control of presynaptic release dynamics

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At the time of thesis acceptance, this manuscript was in submission and therefore this work was unpublished.

Statement of Contributions:

I designed and performed almost all experiment in this manuscript. I analyzed all the data and wrote the paper. Kevin Lee provided helpful discussions performed the uncaging experiments found in Figure 4C-D, and edited final versions of the manuscript. Dr. Jean-Claude Béïque supervised the project and helped write the paper.

Abstract

Hebbian and homeostatic forms of synaptic plasticity operate on different time scales to regulate synaptic strength. The degree of mechanistic overlap between these plasticity processes, and their mutual influence, are still incompletely understood. Here, we found that the homeostatic synaptic strengthening that develops in response to prolonged network inactivity was accompanied by a compromised ability of CA1 synapses to exhibit LTP. This effect could not be accounted for by an obvious deficit in the postsynaptic capacity for LTP expression, since neither the fraction of silent synapses nor the ability to induce LTP by two-photon glutamate uncaging were reduced by the homeostatic process. Rather, optical quantal analysis revealed that homeostatically strengthened synapses displayed a markedly reduced capacity to maintain glutamate release fidelity during repetitive stimulation, ultimately impeding the induction, and thus expression, of LTP. By regulating short-term dynamics of glutamate release, the homeostatic process influences key features of dynamical network function and exhibits features of metaplasticity.

Introduction:

Several forms of plasticity operating over diverse spatial and temporal scales have been described at glutamatergic synapses. For instance, ‘Hebbian’ forms of synaptic plasticity, namely long-term potentiation and long-term depression (LTP and LTD), modulate synaptic transmission efficacy in response to activity patterns that occur on the order of seconds to minutes (Luscher and Malenka, 2012). Hebbian plasticity mechanisms have long served as a cellular substrate for learning and memory, although their implementation in models of active neural circuits often leads to unbalanced network functions (Miller and MacKay, 1994; Miller, 1996; Abbott and Nelson, 2000; Turrigiano, 2008). Neurons are endowed with a diverse set of cellular homeostatic mechanisms that are believed to counterbalance Hebbian instabilities (Turrigiano and Nelson, 2004; Lee et al., 2014). Mechanisms of synaptic homeostasis, which enable a bidirectional cell-wide refinement of synaptic strengths (Turrigiano et al., 1998), are particularly interesting because they share several mechanistic commonalities with Hebbian mechanisms (Pozo and Goda, 2010; Turrigiano, 2012; Huganir and Nicoll, 2013; Soares et al., 2013). While it is clear that Hebbian and homeostatic processes belong to two unique classes of synaptic plasticity, since they are induced by fundamentally distinct cues and operate over vastly different time scales, the extent to which their cellular expression mechanisms converge and interact with one another to regulate synaptic transmission remains incompletely understood.

In this study, we focused on hippocampal CA1 synapses and induced homeostatic and Hebbian forms of synaptic strengthening sequentially to examine whether and how these two plasticity processes interact. We applied a widely used homeostatic protocol consisting of several days of network silencing (Turrigiano et al., 1998; Watt et al., 2000; Kim and Tsien, 2008; Arendt et al., 2013; Soares et al., 2013) to hippocampal organotypic slices and found that it

prevented the ability of synapses to undergo significant LTP. Two-photon glutamate uncaging experiments unexpectedly showed that dendritic spine enlargement and spine AMPA receptor accumulation – two proxies of postsynaptic strengthening during LTP – appeared unaffected by the homeostatic process, thereby arguing against the idea that homeostatic synaptic strengthening occludes the subsequent expression of LTP. Rather, optical quantal analysis revealed that the homeostatic response to inactivity altered LTP induction by robustly reducing the faithfulness of glutamate release during repetitive stimulation. Thus, the homeostatic response to inactivity regulates plasticity rules at CA1 synapses by influencing the short-term dynamics of glutamate release, thereby exhibiting features of metaplasticity (Abraham and Bear, 1996). Moreover, since short-term synaptic plasticity critically governs input-output computations at synapses (Abbott and Regehr, 2004), our results suggest that the continuous homeostatic adaptations occurring in the brain regulate dynamical information flow in neural circuits.

Results

In order to examine the relationship between Hebbian and homeostatic forms of synaptic plasticity, we turned to hippocampal organotypic slices (Stoppini et al., 1991; Soares et al., 2014). This preparation is amenable to traditional synaptic physiology experiments, a means for examining Hebbian forms of plasticity, while enabling the induction of homeostatic plasticity by prolonged pharmacological treatments. Herein, we report the results of a series of whole-cell electrophysiological and two-photon imaging/uncaging experiments on control organotypic slices, or slices treated with the sodium channel blocker tetrodotoxin (TTX) for 3-4 days (**Figure 1A**). A wealth of previous studies, including several carried out specifically in organotypic slices (Kim and Tsien, 2008; Arendt et al., 2013; Soares et al., 2013), have demonstrated that the prolonged silencing of network activity induced by TTX treatment offers a robust paradigm to induce homeostatic forms of plasticity (O'Brien et al., 1998; Turrigiano et al., 1998; Desai et al., 1999).

Homeostatic synaptic strengthening is accompanied by a reduction in the magnitude of Long-Term Potentiation

We first validated our homeostatic plasticity induction protocol by whole-cell electrophysiological recordings of spontaneous miniature excitatory postsynaptic currents (mEPSCs) at -70 mV in 10 DIV hippocampal slices. Consistent with the well-described, cell-wide, homeostatic increase in synaptic strengths following prolonged periods of activity suppression (Turrigiano et al., 1998; Lee et al., 2014), we found that the amplitude of mEPSCs were significantly larger in neurons from TTX-treated slices (**Figure 1B**). We then compared the ability of a widely used protocol to induce LTP (Malinow and Tsien, 1990; Kullmann and Nicoll, 1992; Manabe et al., 1992; Chen et al., 1999) at synapses in control and TTX-treated

slices. After thoroughly washing the slices with prolonged perfusion with Ringer's solution, CA1 cells were targeted for whole-cell patch clamp recordings, voltage clamped at -70 mV, and a baseline of evoked AMPAR-mediated EPSCs was obtained by stimulating Schaffer collateral axons in stratum radiatum at low frequency (0.1 Hz). Particular care was devoted to obtain monosynaptic EPSCs of fixed latency. After 5 minutes of recording baseline EPSCs, we applied a pairing stimulation protocol, which consisted of a train of 180 electrical stimuli delivered at 3 Hz while clamping the membrane potential at 0 mV. In recordings from control neurons, this induction protocol was followed by an increase in the amplitude of evoked AMPAR-EPSCs, which persisted for greater than 40 minutes (**Figure 1C-D**), indicative of robust LTP in our recording conditions. In sharp contrast, AMPAR-EPSCs were not potentiated by the LTP induction protocol in recordings from TTX-treated neurons (**Figure 1C-D**). To determine whether this effect was specific to our induction protocol, we opted to repeat the experiment using an alternative LTP paradigm in a current clamp recording configuration. We established a baseline of evoked excitatory postsynaptic potentials (EPSPs) before pairing these stimuli with somatic current injection sufficient to fire 3-4 action potentials (60 pairings at 1 Hz) at a short delay. Consistent with our voltage-clamp experiments, evoked EPSPs from control neurons showed significant potentiation, while those from TTX-treated neurons failed to potentiate significantly (**Figure 1 - Supplement 1A-B**). Thus, the expression of homeostatic synaptic strengthening was accompanied in these conditions by a reduced ability of CA1 synapses to exhibit LTP. We next sought to identify a mechanistic underpinning that could explain this effect.

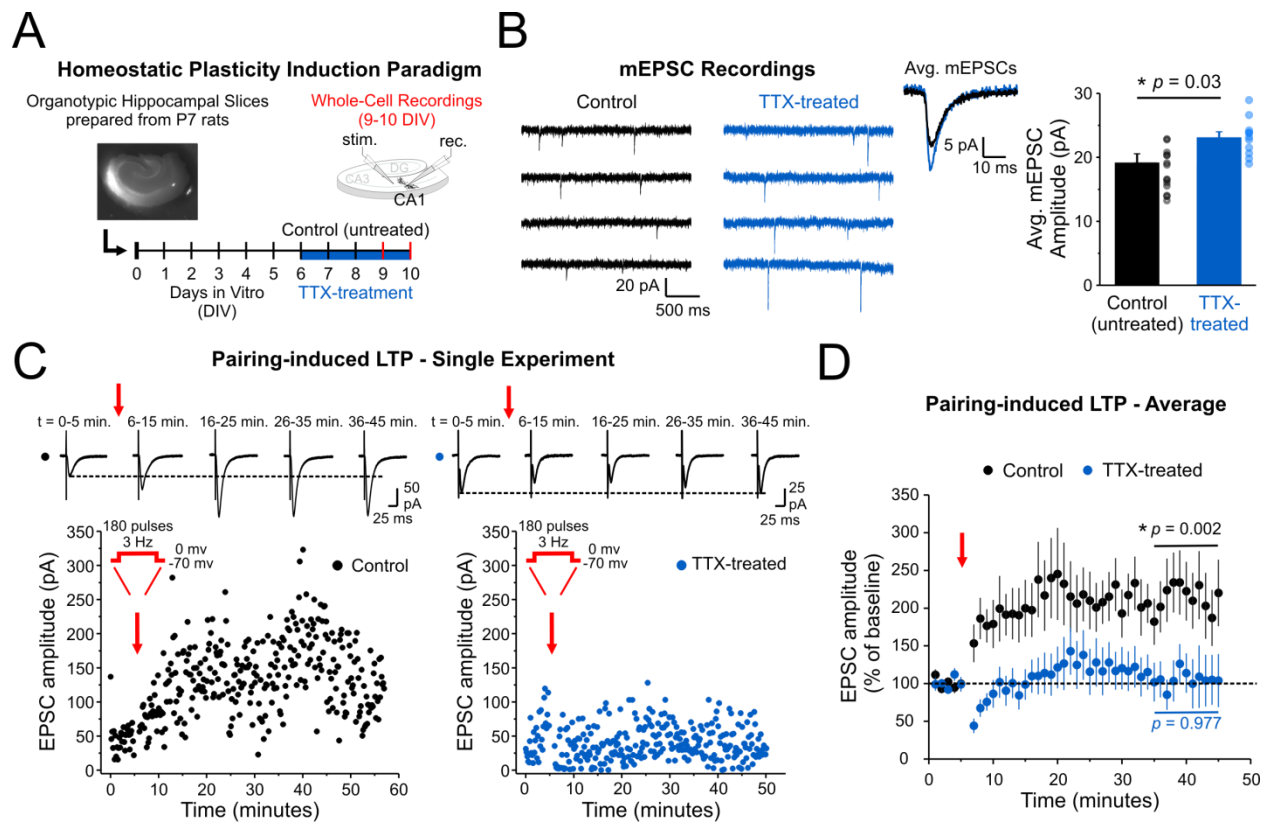


Figure 1: Homeostatic strengthening of CA1 synapses is accompanied by a reduction in the magnitude of LTP. (A) A schematic overview of our homeostatic synaptic plasticity induction protocol. Organotypic hippocampal slices (6 days in vitro) were subjected to 3-4 days of network silencing to induce homeostatic plasticity. Network activity was suppressed by addition of a Na⁺ channel antagonist, tetrodotoxin (TTX, 1 μ M) to the culture media, while control slices were left untreated. (B) Prolonged network inactivity leads to the functional strengthening of excitatory synapses. AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) measured in the continuous presence of TTX (1 μ M) and picrotoxin (100 μ M) were recorded at -70 mV. Average traces (> 50 events) were used to quantify the mean mEPSC amplitude for each cell ($p = 0.03$, student's t-test; $n = 15$ cells and 12 cells for CTL and TTX, respectively). (C) Example experiments where a pairing protocol (180 synaptic stimuli delivered at 3 Hz, while clamping the cell at 0 mV) was used to induce LTP of evoked AMPAR-mediated synaptic currents (measured at -70 mV). (D) Summary data for experiments where LTP was induced by a pairing protocol (Contol: $p = 0.002$ when compared to baseline, z-test, $n = 16$ neurons; TTX: $p = 0.977$ when compared to baseline, z-test, $n = 15$ neurons). All error bars represent the standard error of the mean (SEM).

Prolonged network inactivity delays synapse unsilencing in the emerging hippocampal network

Silent synapses are operationally defined as synapses harbouring functional NMDARs, but not AMPARs. Most prominent in emerging networks (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Busetto et al., 2008; Ashby and Isaac, 2011; Lee et al., 2016), this subtype of synapse has attracted considerable attention in part because it acts as a preferential site of AMPAR insertion during LTP (Isaac et al., 1996; Nicoll and Malenka, 1999). Indeed, the proportion of silent synapses is believed to be a meaningful determinant of the plasticity potential of synaptic networks (Beique et al., 2006; Kerchner and Nicoll, 2008). Because our experiments were carried out during a developmental epoch in organotypic slices wherein silent synapses may be prevalent, we next set out to: 1) provide a quantitative estimate of the fraction of silent synapses on CA1 pyramidal cells at different stages of slice development, and 2) determine whether this maturational trajectory is altered by a network silencing paradigm. We were also compelled to specifically carry out these experiments in light of a recent study relying on an analogous (but not identical; see below) experimental design that showed enhanced LTP following network silencing (contrary to what we observed here) that was surmised to be caused by the *de novo* emergence of silent synapses (Arendt et al., 2013).

We estimated the fraction of silent synapses by means of recording AMPAR- and NMDAR-mediated EPSCs evoked by two-photon uncaging of MNI-glutamate onto single spines, as we have done in the past (Beique et al., 2006; Lee et al., 2016). CA1 pyramidal neurons were filled with Alexa 594 to enable visualization of dendritic morphology (**Figure 2A**). Uncaging-evoked EPSCs (uEPSCs) were elicited by delivering brief laser pulses (1 ms at 720 nm) onto visually identified dendritic spine tips (**Figure 2A**). We computed an AMPA to NMDA

ratio for each spine using the peak AMPAR-mediated current amplitude measured at -70 mV, and the NMDAR-mediated current amplitude measured at +40 mV (35 ms after the AMPAR current peak at -70 mV; **Figure 2B**). Several precautionary measures were taken to ensure that our uncaging stimuli and electrophysiological recording conditions were appropriate for the detection of silent synapses. First, the laser power used for uncaging was calibrated for spines based on the ability to resolve outward currents of *ca.* 5-30 pA at +40 mV, matching the range commonly observed at single synapses during minimal stimulation experiments (Isaac et al., 1995; Liao et al., 1995; Hanse and Gustafsson, 2001). To determine the spatial spread of glutamate released by this range of uncaging stimulus intensities, we carried out a set of parallel uncaging experiments on dendritic spines of CA1 neurons that were biolistically-transfected with the fluorescent glutamate reporter iGluSnFR (Marvin et al., 2013). These experiments revealed that: 1) the spatial spread of glutamate was almost exclusively restricted to the spine head (**Figure 2C**), and 2) that the amplitudes of these uncaging-evoked spine iGluSnFR transients were within the range of those observed following electrical stimulation (see **Figure 5**). Finally, we experimented only with spines that were located within 150 μ m from the cell body to minimize errors in AMPA to NMDA ratio estimates due to poor voltage clamping of more distal spines. In this line, parallel experiments showed that the reversal potential of AMPAR-mediated uEPSCs, which provides a reasonable approximation of voltage-clamping performance, were highly consistent for spines located within 150 μ m of the cell soma (**Figure 2 - Supplement 1A-B**). Using this experimental approach, clear examples of silent synapses were observed, wherein uEPSCs were readily detected at +40 mV but not at -70 mV (**Figure 2D**). These silent synapses were flanked by regions of dendrite that contained functional AMPARs (**Figure 2D**).

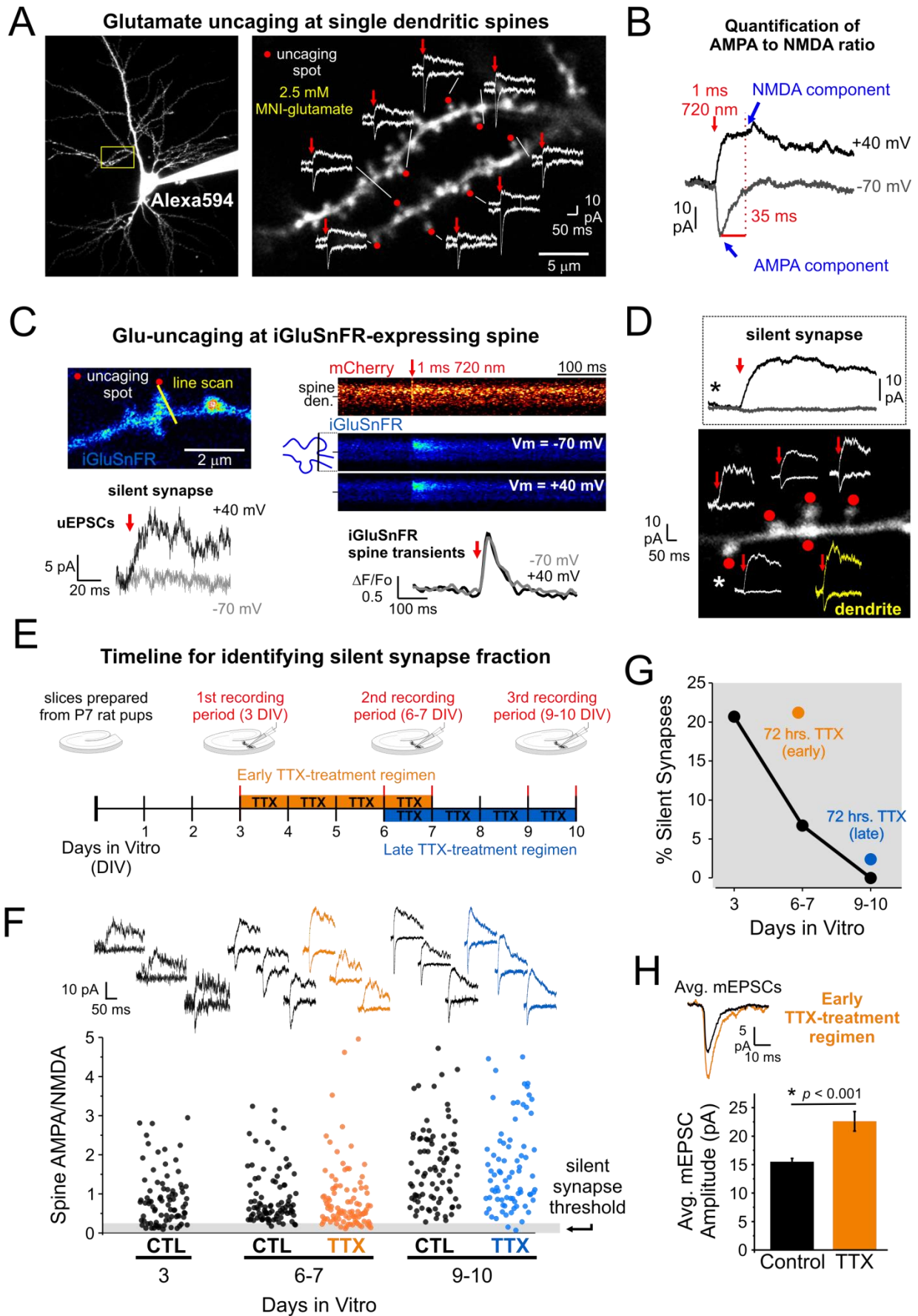


Figure 2: Abolishing network activity prevents the natural unsilencing of synapses in the developing hippocampal network. (A) Functional mapping of AMPA and NMDA-mediated EPSCs by two-photon uncaging of MNI-glutamate. CA1 pyramidal cells were filled with Alexa594 to enable the live visualization of dendritic morphology by two-photon imaging at 810 nm. Glutamate uncaging at dendritic spine tips (indicated by the red dots) yielded uncaging-evoked EPSCs (uEPSCs) recorded in voltage clamp at -70 mV (inward traces) and at +40 mV (outward traces). (B) A demonstration for how AMPA to NMDA ratio was calculated (see results and methods sections). (C) Silent synapse detection in neurons expressing the intensity-based glutamate sensing fluorescent reporter, iGluSnFR. Glutamate release following uncaging was largely restricted to the dendritic spine head. (D) An example uncaging experiment where two silent synapses (zero inward conductances at -70 mV) are neighboured by two active synapses. Yellow traces represent a similar experiment performed at a nearby ‘extrasynaptic’ dendritic location. (E) A schematic of the timeline for the uncaging experiments performed. Two separate TTX-treatment regimens (3-4 days) were used to determine how the silent synapse fraction was affected by prolonged network silencing. (F) Quantification of all uncaging AMPA to NMDA ratios across slice development and in conditions of network silencing. A silent synapse threshold was set (AMPA/NMDA < 0.25) which is indicated by the shaded region. (G) Percent of silent synapses measured at each developmental stage and in each treatment condition. (H) The average amplitude of mEPSCs recorded from neurons at DIV6-7 (CTL vs. TTX: $p < 0.001$; unpaired students t-test). Error bars reflect the SEM. Error Bars reflect the SEM.

To examine the temporal trajectory of synaptic maturation, we examined the fraction of silent synapses at 3 developmental epochs of our organotypic slice preparation (*i.e.*, 3 DIV; 6-7 DIV and 9-10 DIV; see **Figure 2E** for a schematic timeline of these experiments). The occurrence of silent synapses significantly dropped with development: whereas over 20% of the spines were found to be silent in the youngest slices tested (*i.e.*, 3 DIV), this proportion was less than 5% at 6-7 DIV and no silent synapses were detected at 9-10 DIV (**Figure 2F-G**, **Figure 2 - Supplement 2A-C**). Altogether, these results show that the proportion of silent synapses gradually wanes during network development in organotypic slices, with a time course that is remarkably close to that reported during postnatal development *in situ* (Durand et al., 1996; Busetto et al., 2008; Lee et al., 2016).

Having established the developmental trajectory of the proportion of silent synapses in our organotypic slice network, we next determined whether it was altered by a homeostatic silencing paradigm. To begin addressing this question, we first determined the effects of network

silencing in young slices, starting the TTX treatments at 3 DIV and assessing silent synapse fraction at 6-7 DIV (*early* TTX-treatment regimen; **Figure 2E**). In these conditions, and in close agreement with previous findings using a similar treatment regimen (Arendt et al., 2013), we found that the fraction of silent synapses was higher in slices treated with TTX (*ca.*, 20%) than that seen in control slices (**Figure 2F-G** and **Figure 2 - Supplement 2B**). Noteworthy, however, the fraction of silent synapses in the TTX condition at 6-7 DIV ($\sim 20\%$) was similar to that observed in the 3 DIV control slices (*i.e.*, at the time when the TTX treatment was initiated; **Figure 2G**). As such, these results raise the possibility that, rather than inducing a *de novo* emergence of silent synapses, as concluded by Arendt et al., a prolonged treatment with TTX would simply halt, or delay, the activity-dependent unsilencing of synapses that normally develops with increasing age.

To further explore this idea, we reasoned that beginning the TTX treatment at a later developmental stage when there are far fewer silent synapses would help to disambiguate these competing interpretations. Indeed, a *bone fide* homeostatically-induced increase in silent synapse fraction should be apparent if network silencing commenced at a time when there are nominally no (or very few) silent synapses present. We thus repeated the experiments, but started the TTX treatment at 6 DIV (*late* TTX-treatment regimen, **Figure 2E**), at a time when only a small fraction of synapses remained silent ($< 5\%$; **Figure 2G**). Recordings from TTX-treated neurons at 9-10 DIV revealed the presence of only a small fraction of silent synapses which was comparable to age matched controls (**Figure 2F-G** and **Figure 2 - Supplement 2C**). Altogether, the most parsimonious explanation accounting for these results is that the prolonged TTX treatment halts the developmental decrease in silent synapse fraction observed in control conditions, without causing a *de novo* formation of silent synapses. Yet, it is interesting to emphasize that both of the TTX treatment regimens (*i.e.*, early and late) studied here are

homeostatic plasticity paradigms inasmuch as they robustly increased the amplitude of AMPAR-mediated mEPSCs (**Figure 1B** and **Figure 2H**). As such, the global homeostatic strengthening exhibits an intriguing synapse specificity, in that the changes to synapse function (*i.e.*, increase in AMPAR-mediated transmission) appear to be restricted to functionally active (AMPAR-containing) synapses. Beyond this corollary interpretation, these results show that the compromised ability of inducing LTP at synapses that have been homeostatically strengthened (**Figure 1D** and **Figure 1 – Supplement 1B**) cannot be readily accounted for by an alteration in the proportion of silent synapses.

Homeostatic synaptic plasticity does not disrupt postsynaptic expression of LTP

In principle, the LTP deficit observed at synapses that have been homeostatically strengthened may reflect an occlusion-like phenomenon, whereby the homeostatic synaptic strengthening would prevent further AMPAR insertion at synapses during LTP. To test this idea, we examined the influence of prolonged network silencing on the expression of two cardinal features of postsynaptic plasticity – spine enlargement and synaptic insertion of AMPARs – in response to an LTP stimulus induced by glutamate uncaging (Matsuzaki et al., 2004; George et al., 2015). We thus biolistically transfected CA1 pyramidal neurons with mCherry to monitor structural plasticity of dendritic spines and with SEP-GluA1, a pH-sensitive GFP derivative tagged to an AMPAR subunit that allows to image AMPARs located in the plasma membrane but not those in acidic intracellular compartments (Ashby et al., 2004). A repetitive glutamate uncaging protocol (30 pulses, 0.5 Hz) onto single spines of control neurons induced both a rapid and sustained structural enlargement and accumulation of SEP-GluA1 at stimulated spines (**Figure 3A-B**). These changes were synapse-specific, as both the size and AMPAR content at neighbouring spines were unaltered (**Figure 3B**). Intriguingly, the magnitude of both structural

(*i.e.*, spine enlargement) and functional (inferred from SEP-GluA1 spine insertion) LTP were similar between spines of control and interleaved TTX-treated neurons (**Figure 3C-D**). Since these two hallmark features of Hebbian postsynaptic strengthening were unaffected by prior expression of homeostatic plasticity (**Figures 3E-G**), the deficits in LTP observed at homeostatically-strengthened synapses when studied by a classic pairing protocol (**Figure 1D**) was likely not due to an occlusion of postsynaptic strengthening mechanisms. These results suggest that homeostatic and Hebbian mechanisms of synaptic strengthening likely do not compete to an appreciable degree for a limited set of shared resources.

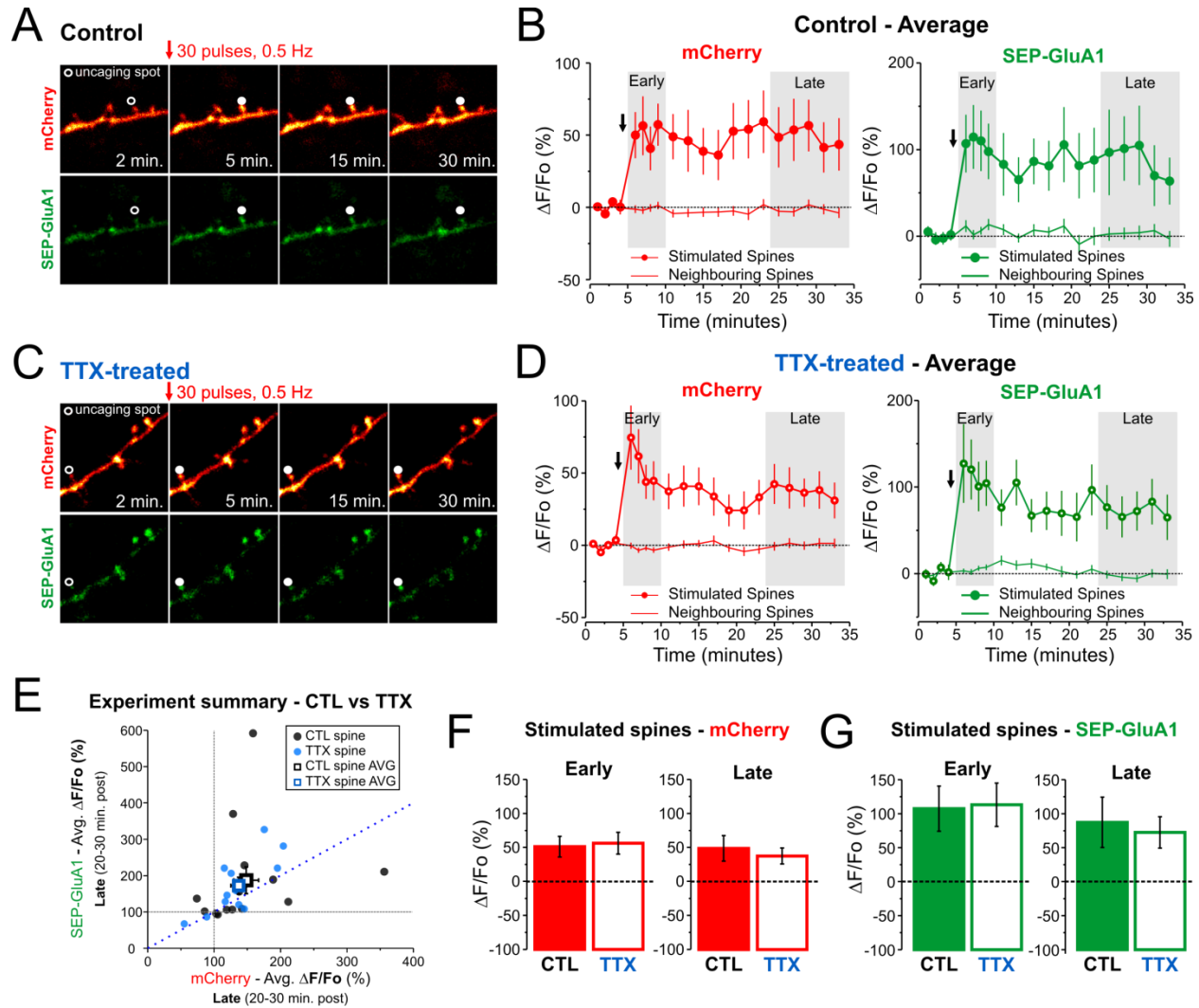


Figure 3: Structural and functional features of postsynaptic Hebbian LTP are unaltered by a prolonged silencing paradigm. (A) An example experiment whereby two-photon uncaging of MNI-glutamate was used to induce LTP at a single spine of a control (untreated) neuron. After a baseline imaging period, 30 uncaging pulses were delivered to a single spine to induce LTP. (B) Average summary data ($\Delta F/Fo$) for all 14 single spine uncaging-LTP experiments from control (untreated) neurons. Unstimulated neighbouring spines were also quantified as within-experiment controls. (C) Same experiment as in panel A, except that the slice had been treated with TTX for 3 days prior to imaging. (D) Average summary data ($\Delta F/Fo$) for all 13 uncaging-LTP experiments recorded from TTX-treated slices, including unstimulated neighbouring spines. (E) Overlaid summary of spine experiments from panels B and D, showing the mean change in the intensity of the mCherry signal plotted against the mean change in SEP-GluA1 signal at all stimulated spines, 20-30 minutes following LTP induction. (F) Summary of mCherry intensity changes at stimulated spines following glutamate uncaging (Early, $p = 0.82$; Late, $p = 0.62$; unpaired student's t-tests). (G) Summary of SEP-GluA1 intensity changes at stimulated spines following glutamate uncaging (Early, $p = 0.90$; Late, $p = 0.75$; unpaired student's t-tests). Error bars reflect the SEM.

Homeostatic plasticity affects LTP induction through altered presynaptic release dynamics

Thus far, our results show that the homeostatic response to network silencing neither decreased the proportion of silent synapses nor altered the potential for postsynaptic LTP expression, two outcomes that in principle may have explained the compromised LTP observed in TTX treated-slices. We next wondered whether the homeostatic influence on Hebbian plasticity might occur through alterations that affect the induction phase of LTP, rather than its expression.

As a first step to explore this possibility, we more closely examined the LTP experiments outlined in **Figure 1D** and noticed that the evoked EPSCs consistently exhibited a transient depression immediately following the pairing protocol in TTX-treated slices (in stark contrast to the immediate potentiation observed in control slices). In principle, this transient synaptic depression in the TTX condition could result from the LTP induction protocol (*i.e.*, prolonged depolarization and/or an effect downstream of NMDAR activation) or from a short-term plasticity-like phenomenon emanating solely from the temporal pattern of synaptic stimulation. To disambiguate between these possibilities, we first conducted experiments wherein we monitored AMPAR-mediated currents at -70 mV (instead of at 0 mV; **Figure 1D**) while keeping the same stimulation pattern (*i.e.*, 180 pulses at 3 Hz). Whereas the 3 Hz stimulation protocol induced a moderate depression in the amplitude of AMPAR-mediated EPSCs in control conditions (**Figure 4A-B**), the magnitude of this depression was much more pronounced in recordings from TTX-treated slices (**Figure 4A-B**). These results therefore suggest that the transient suppression of EPSCs observed in TTX-treated slices following the LTP induction protocol could be accounted for by the 3 Hz stimulation regimen alone, and was independent of the prolonged depolarization and/or postsynaptic NMDAR activation.

In principle, the 3 Hz synaptic stimulation protocol may induce a suppression of EPSC amplitude by either a presynaptic mechanism, or by a direct postsynaptic mechanism involving the internalization or desensitization of AMPARs. To directly address the latter possibility, we mimicked the 3 Hz stimulation protocol by direct postsynaptic stimulation of AMPARs by means of two-photon glutamate uncaging. A 3 Hz uncaging stimulation for 60 seconds at single spines (*i.e.*, 180 pulses; matching the upper bounds of the electrical stimulus frequency assuming a maximal release probability of 1.0) induced a moderate reduction in the amplitude of AMPAR-uEPSCs (~20%-25%; **Figure 4C-D**). The magnitude of this reduction was however indistinguishable between control and TTX-treated slices (**Figure 4D**). This finding thus argues against a role for AMPAR internalization and/or desensitization in mediating the transient depression observed during 3Hz stimulation in TTX-treated neurons, and therefore points towards a presynaptic mechanism. Such a scenario predicts that the rate of synaptic transmission failures developing during a stimulus train should be higher in the TTX-treated slices than in controls. Consistent with this idea, the rate of occurrence of EPSC failures only marginally increased throughout a 3 Hz stimulus train (*i.e.*, electrically-evoked synaptic release) in control conditions, but was robustly increased in recording from TTX-treated neurons (**Figure 4E**; see **Figure 4 - Supplement 1A** for additional examples). To determine whether the increased prevalence of transmission failures was dependent on the frequency of synaptic stimulation, we repeated the experiment at a reduced stimulus frequency (1 Hz), keeping the number of stimuli constant at 180 pulses. In these conditions, the magnitude of synaptic depression was greater in TTX-treated slices compared to controls (**Figure 4F₁**), which again was accompanied by an enhanced rate of transmission failures (**Figure 4F₂**; see **Figure 4 - Supplement 1B** for additional examples). Collectively, these results suggest that the homeostatic response to prolonged

inactivity profoundly alters the short-term dynamics of glutamate release during repetitive synaptic activation.

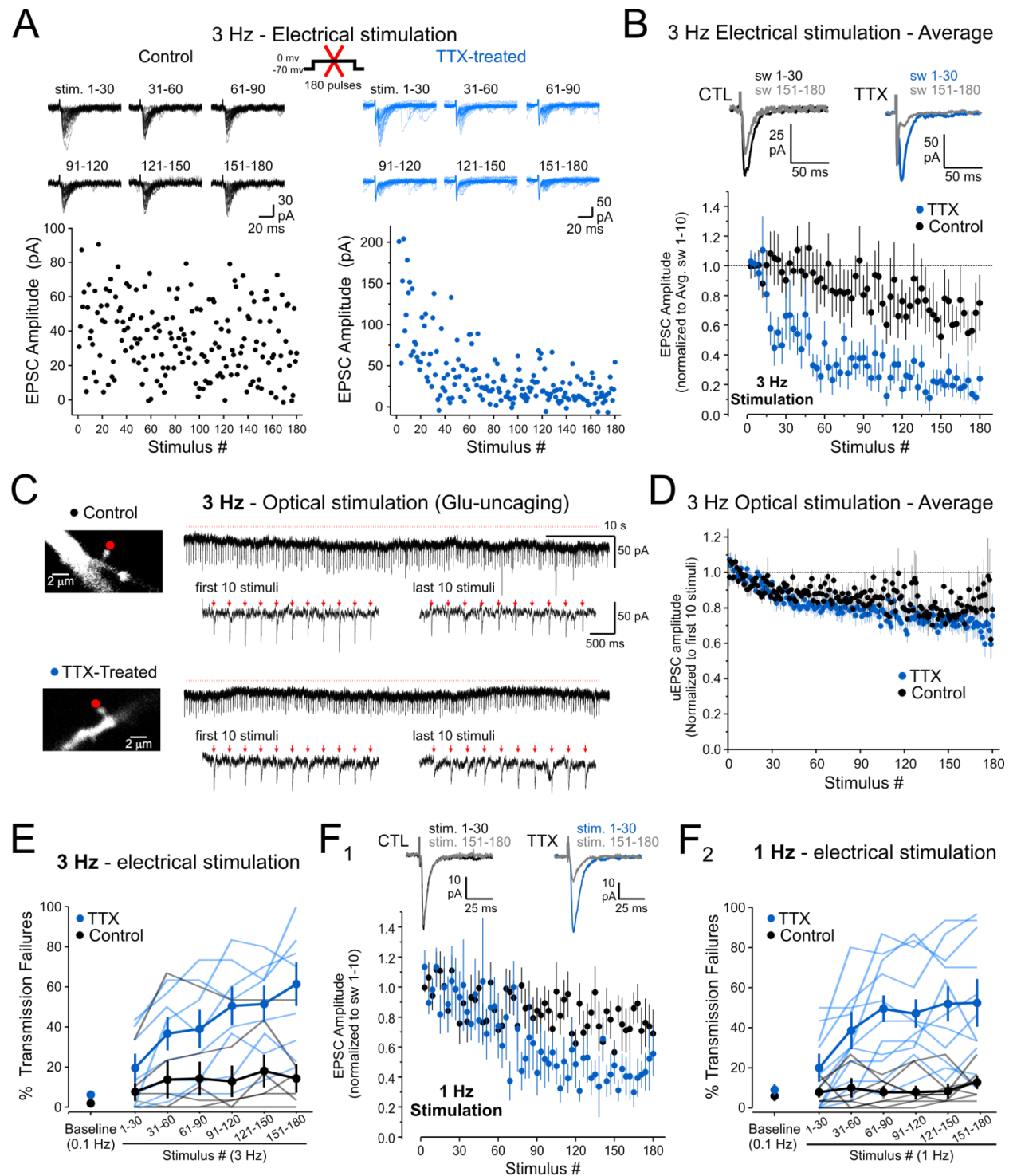


Figure 4: Prolonged network silencing renders synapses more susceptible to a frequency-dependent short-term depression. (A) Example experiments in which AMPAR-mediated EPSCs were evoked at 3 Hz while holding the cell at -70 mV. This stimulus frequency was chosen to match that of the LTP induction protocol from Figure 1D, but importantly, cells were not depolarized. (B) Summary data for all experiments where evoked AMPAR-mediated EPSCs were recorded at 3 Hz ($n = 10$ cells in each condition). (C) Example experiments in which glutamate uncaging was used to repetitively stimulate dendritic spines at 3 Hz. AMPAR-mediated uEPSCs were recorded at the soma while holding the cell -70 mV. Red circles indicate the location of glutamate uncaging, and the red arrows indicate the timing of the 1 ms laser pulses. (D) Summary data for all experiments where AMPAR-uEPSCs were evoked at 3 Hz at a single spine ($n = 11$ and 15 spines for control and TTX-treated, respectively). (E) Failure analysis of electrically-evoked EPSCs recorded at 3 Hz. Percentage of EPSC failures were quantified during a 5 minute baseline (30 pulses, 0.1 Hz), and then in bins of 30 stimuli during the 3 Hz stimulation protocol. Average data is plotted (circles) with the data from individual experiments overlaid (lines). (F₁) As in panel B, except that the stimulus frequency was reduced to 1 Hz ($n = 7$ and 10 cells in control and TTX-treated, respectively). (F₂) Failure analysis for the 1 Hz stimulation experiments, as described in panel E. All error bars represent the SEM.

Finally, we turned to an all-optical approach to directly visualize glutamate release onto individual dendritic spines to quantify the changes in presynaptic function that appear to arise during homeostatic strengthening. To this end, we biolistically transfected CA1 pyramidal neurons with the intensity-based glutamate sensor, iGluSnFR, along with the morphological marker mCherry (**Figure 5A**). We positioned a patch stimulating electrode close to an apical dendritic segment of a transfected CA1 neuron and delivered stimuli at low frequency (0.1 Hz) while surveying spines in the apical dendrites for time-locked glutamate release events (**Figure 5A-B**). The iGluSnFR-mediated fluorescence signal at single spines provided a reliable method to monitor the behaviour of endogenous glutamate release as it exhibited, consistent with the probabilistic nature of transmitter release, clearly distinguishable transmission successes and failures (**Figure 5B**). The fluorescence signals were usually restricted to the spine head, with little or no signal arising from the parent dendrite (**Figure 5B-C**). A total of 50 consecutive stimuli at low frequency (~ 0.1 Hz) were delivered to obtain a direct estimate of the probability of glutamate release at each spine (**Figure 5C-D**). We found no difference in the basal probability

of glutamate release at responsive spines between control and TTX-treated slices (**Figure 5E**). We then examined the effects of a higher-frequency stimulus train by administering 90 consecutive electrical stimuli at 3 Hz (**Figure 5F**), mimicking the stimulus frequency of our LTP induction protocol (**Figure 1D**). To analyze glutamate release probability during the 3 Hz train, we binned the events into 3 epochs, each containing 30 synaptic stimuli (**Figure 5G-H**). In control neurons, stimulation at 3 Hz induced a small ($\sim 20\%$) decrease in glutamate release probability by the end of the train (**Figure 5I**). However, the magnitude of this reduction was significantly more prominent at synapses from TTX-treated slices, dropping to almost 50% of the baseline level by the end of the train (**Figure 5I**). The amplitude of successful iGluSNFR transients (*i.e.*, potency) remained constant throughout the duration of train in both control and TTX-treated slices (**Figure 5G,J**). This argues against the possibility that the drop in release probability observed in TTX conditions reflected an artefactual increase in failure rates emanating from a suboptimal and partial sampling of smaller amplitude events (*i.e.*, below detection threshold). Thus, the homeostatic response to prolonged inactivity alters the ability of synapses to maintain transmission fidelity during trains of repetitive activity, thereby regulating the stimulus thresholds required to induce Hebbian plasticity at CA1 synapses.

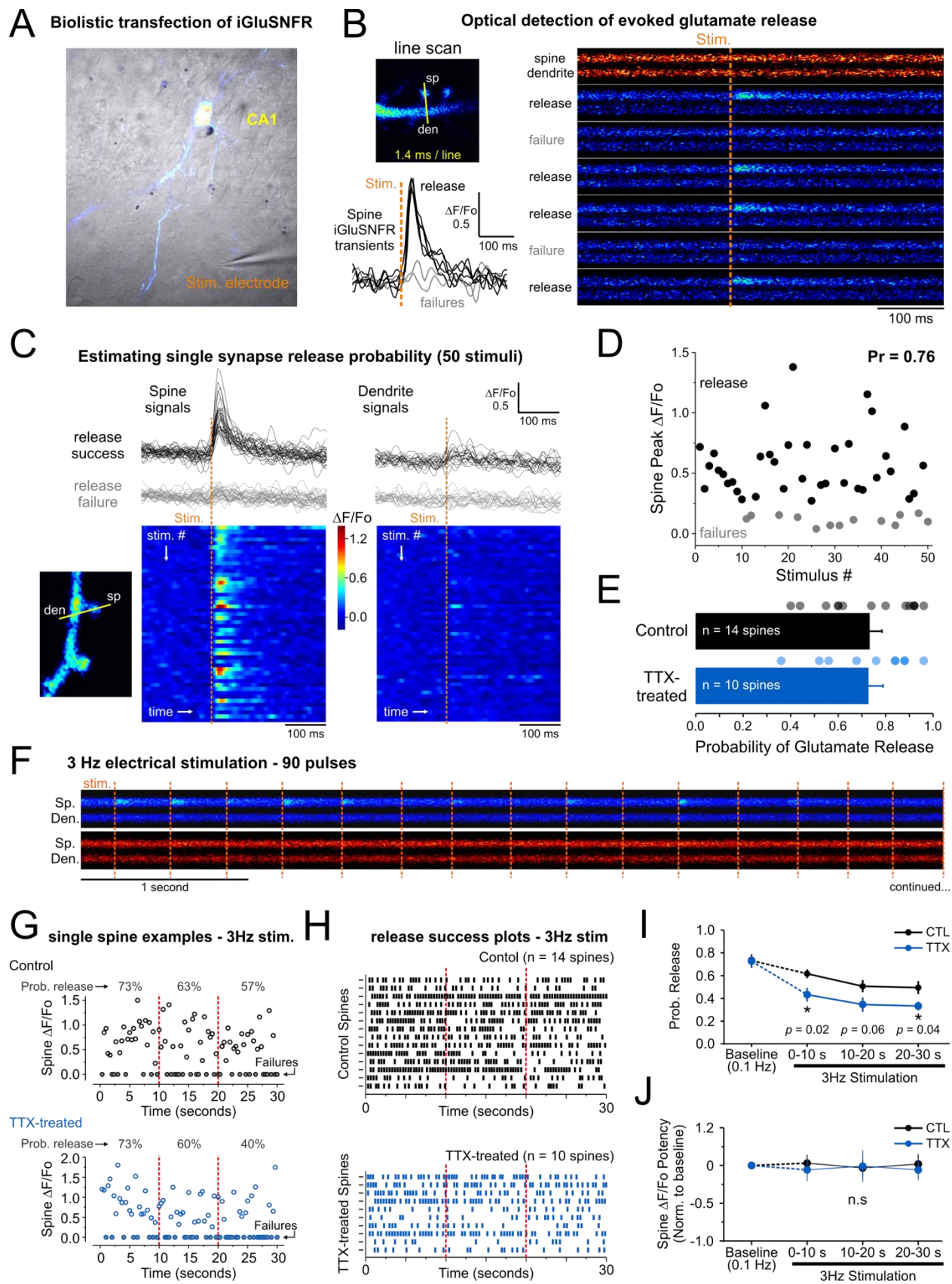


Figure 5: Prolonged network silencing renders synapses less capable of responding with fidelity to repetitive synaptic stimulation. (A) Two photon image (at 950 nm) of a CA1 pyramidal cell transfected with iGluSNFR, overlaid on a DIC image of the hippocampal slice. A patch electrode was placed in close proximity to the dendritic arbor of the transfected cell to evoke glutamate release. (B) Optical detection of evoked-glutamate release at single spines. A brief electrical stimulus caused a probabilistic release of glutamate onto a small subset of dendritic spines, which was detected as an increase in fluorescence intensity of the iGluSNFR signal. (C) 50 consecutive stimuli were delivered at ~ 0.1 Hz to estimate a single spine release probability (# successes / # pulses). Traces of spine and dendritic signals are shown above the summary data, which is presented as an intensity-based $\Delta F/F_0$ plot. Each row in the $\Delta F/F_0$ plot represents the baseline-normalized intensity of the iGluSNFR signal from a single line-scan experiment. Timing of the electrical stimulus is indicated by the orange line. (D) Quantification of the normalized peak amplitude in the spine compartment for each sweep, calculated between 0 – 100 ms after the electrical stimulus, for the experiment depicted in panel C. An amplitude threshold was set to distinguish between successes and failures (see methods). (E) Release probability at single spines was estimated based on the fraction of successes out of a total of 50 stimuli ($p = 0.95$, unpaired student's t-test). (F) A subset (5 seconds) of a prolonged line-scan (30 seconds total duration) at a dendritic spine during which 90 electrical stimuli were delivered at a frequency of 3 Hz (timing indicated by the orange lines). (G) Peak amplitude quantification for spine iGluSNFR signals from an example 3 Hz stimulation experiments in each condition (Control *top*, TTX-treated *bottom*). The amplitude of failures events were assigned to 0 for presentation purposes. Vertical red-dashed lines separate the three distinct time bins (10 s each) used in the quantification of release probability during the stimulus train. (H) Release success raster plot for all spines experiments where glutamate signals were recorded during high-frequency electrical stimulation. Each raster mark represents the peak time of a successful glutamate release event. (I) Average single spine release probability at baseline (same as panel E) and during each epoch of the 3Hz stimulus train. Unpaired student's t-tests were used for statistical analysis. (J) Quantification of the average spine potency (amplitude of successful release events only) during the 3Hz stimulus train, normalized to the mean potency during the baseline period. All cross comparisons between treatment conditions were not significant ($p > 0.05$, unpaired student's t-test). Error bars represent the SEM.

Discussion:

The degrees of mechanistic overlap between homeostatic and Hebbian synaptic plasticity at hippocampal synapses is incompletely understood. Here, we found that the homeostatic strengthening of CA1 synapses that develops in response to prolonged network silencing was accompanied by a reduced ability of synapses to exhibit LTP. This outcome was, at least in part, due to a homeostatic regulation of presynaptic glutamate release dynamics that ultimately impeded LTP induction. Hallmark features of postsynaptic LTP expression induced by single-

spine glutamate uncaging, which effectively bypass the requirement for presynaptic machinery, remained intact following the homeostatic process. Thus, despite an overlap in the postsynaptic mechanisms of Hebbian and homeostatic strengthening (*i.e.*, increased AMPAR function), these two processes do not occlude one another. By altering the short-term dynamics of glutamate release, however, the homeostatic response to inactivity constrains LTP and thus exhibits metaplasticity features.

The proportion of silent synapses is an important determinant of plasticity potential in synaptic networks (Isaac, 2003; Beique et al., 2006; Kerchner and Nicoll, 2008). In principle, a reduction in the fraction of silent synapses could have contributed, at least in part, to the compromised ability of synapses to undergo LTP that we observed in TTX-treated slices. However, this outcome runs contrary to a previous report (Arendt et al., 2013) that showed that a network silencing paradigm analogous to the one used herein increased the proportion of silent synapses. We reassessed this issue here by mapping the weights of individual synapses using two-photon glutamate uncaging and extended this analysis by determining the trajectory of silent synapse fraction during organotypic slice development. Whereas our results are formally consistent with those of Arendt et al., the interpretation from our developmental analysis shows that, rather than triggering an increase in the abundance of silent synapses, a prolonged TTX-treatment halted (or delayed) the progressive reduction of silent synapses that occurs naturally with increasing age. Thus, our results argue against the notion that the homeostatic response to inactivity leads to the *de novo* synthesis of silent synapses (Arendt et al., 2013). Irrespective of this interpretation point, our data show that an altered proportion of silent synapse fraction is unlikely to account for the compromised ability of synapses to undergo LTP that we observed in our more mature slices. Taken together with other related studies, these data further support the

idea that the homeostatic process, along with its interaction with Hebbian plasticity, is regulated during network development.

The multiplicative nature of global (*i.e.*, triggered by a network-wide manipulation) forms of homeostatic synaptic plasticity suggests that all synapses are equally affected by the homeostatic process (Turrigiano et al., 1998; Turrigiano, 2008; Lee et al., 2013; Soares et al., 2013). There is evidence, however, that global manipulations can lead to synapse-specific forms of homeostasis. For example, a prolonged silencing of network activity in hippocampal slices leads to a homeostatic strengthening of dentate-CA3 synapses but not of recurrent CA3-CA3 synapses (Kim and Tsien, 2008; Mitra et al., 2012). Our results extend this concept of synapse-specificity in the homeostatic response to a global manipulation by showing that silent synapses, unlike their functionally active counterparts, resist homeostatic strengthening during network silencing. Conceptually, these global forms of synapse-specific homeostasis differ from those that are triggered when prolonged activity manipulations are restricted to only a small subset of synapses (Hou et al., 2008; Lee et al., 2010; Beique et al., 2011; Hou et al., 2011). It will be important for future studies to identify the molecular cues that distinguish synapses that are destined for homeostatic regulation from those that are spared.

In summary, we found that the failure to induce LTP at activity deprived synapses was due to a homeostatic modulation of short-term plasticity, whereby synapses became unreliable at transmitting when presented with trains of successive stimuli – a condition that is often required to meet induction thresholds for LTP (Chen et al., 1999). By enacting an effective low-pass filter of synaptic transmission, this novel form of homeostatic adaptation regulates the rules for plasticity induction and therefore bears features reminiscent of metaplasticity (Abraham and Bear, 1996). Adjustments in short-term release dynamics might occur in cellular states of

prolonged low activity, resulting in the optimal detection of low-frequency, single spike, transmission (Rotman et al., 2011) rather than bursts. Since short-term plasticity rules govern input-output transformations at synapses, the homeostatic regulation of glutamate release dynamics will influence a wide and complex array of synaptic and network computations. Thus, the role of homeostatic plasticity extends well beyond the bidirectional control of cellular excitability by influencing dynamical information transfer in neural circuits.

Methods:

Organotypic Slice Cultures and Biolistic Transfection:

A detailed description of how slices were cultured and transfected was published previously (Soares et al., 2014). To summarize, organotypic hippocampal slices were prepared from both male and female postnatal day 7 Sprague Daley rats (Charles River Laboratories, Saint-Regis, Canada) according to protocols approved by the University of Ottawa's Animal Care Committee. Animals were anesthetized with isoflurane, decapitated and hippocampi were removed and placed into ice cold cutting solution containing (in mM): 119 choline chloride, 2.5 KCl, 4.3 MgSO₄-7H₂O, 1.0 CaCl₂, 1.0 NaH₂PO₄-H₂O, 1.3 Na-ascorbate, 11 glucose, 0.5-1 kynurenic acid, 26.2 NaHCO₃, saturated with 95% O₂ and 5% CO₂ (295-310 mOsm/L). Transverse hippocampal slices (400 µm) were obtained using a MX-TS tissue slicer (Siskiyou, Grants Pass, OR) and cultured on 0.4 µm millicell culture inserts (EMD Millipore, Etobicoke, Canada) maintained in 6 well plates. Culture media was exchanged on the first day in vitro, and then every 2-3 days thereafter. Unless otherwise indicated, network silencing was induced at 6 DIV by adding 1 µM tetrodotoxin (Abcam, Cambridge, UK) to the culture media while half of the culture wells were left untreated as controls. Experiments were typically performed 3-4 days later, unless otherwise indicated.

Biolistic transfection of hippocampal slices was performed using a hand held gene gun (Biorad, Hercules, CA). Gene gun cartridges were prepared by precipitating ~ 50 µg of cDNA plasmid (~ 40 µg of either iGluSNFR or SEP-GluA1 with ~10 µg of mCherry) onto 8-10 mg of gold microparticles (1.0 µm diameter; Biorad). The precipitation step was performed in a 0.1 M KH_2PO_4 buffer solution containing 0.05 mM protamine sulfate. The DNA-gold precipitate was then washed and suspended in 100% ethanol prior to loading into the tubing station. Once the cartridges were dried and cut, they were loaded into the revolver of the gene gun. The DNA-coated gold particles were delivered to the slice using ~180 psi of helium air pressure. A modified gene gun barrel was used to protect slices from helium blast (Soares et al., 2014). Slices were transfected at 6 DIV and were returned to the incubator for 4-5 days prior to imaging.

Whole-Cell Electrophysiology:

Slices were removed from culture and placed in a custom recording chamber under a BX61WU upright microscope (60X, 1.0 NA objective; Olympus, Melville, NY) and were continuously perfused with extracellular Ringer's solution containing (in mM): 119 NaCl, 2.5 KCl, 4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 CaCl_2 , 1.0 NaH_2PO_4 , 11 glucose, and 26.2 NaHCO_3 saturated with 95% O_2 and 5% CO_2 (295-310 mOsm/L). In all experiments where glutamatergic excitatory currents were measured, picrotoxin (100-150 µM; Abcam) was added to the extracellular solution to eliminate inhibitory GABAergic transmission. Whole-cell recordings were carried out using an Axon Multiclamp 700B amplifier, filtered at 2 kHz, sampled at 10 kHz and digitized with an Axon Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA). CA1 pyramidal neurons were targeted under differential interference contrast imaging, patched and voltage-clamped at the indicated membrane potentials using borosilicate glass recording electrodes (World Precision

Instruments, Sarasota, FL) with resistances ranging from 3-5 M Ω . For voltage clamp experiments, the intracellular recording solution contained (in mM): 115 cesium methane-sulfonate, 0.4 EGTA, 5 tetraethylammonium-chloride, 6.67 NaCl, 20 HEPES, 4 ATP-Mg, 0.5 GTP, 10 Na-phosphocreatine, (all purchased from Life Technologies, Carlsbad, CA) and 5 QX-314 purchased from Abcam (pH = 7.2-7.3; 280-290 mOsm/L). Evoked excitatory postsynaptic currents (EPSCs) were driven by electrical stimuli (0.1 ms) delivered to the slice via a glass pipette (open tip resistance 3-5 M Ω) placed nearby in stratum radiatum of the CA1 subfield. Spontaneous miniature excitatory post synaptic currents (mEPSCs) were recorded in the presence of tetrodotoxin (1 μ M). For all recordings, access resistance was continuously monitored during the experiment by delivering a 2.5-5 mV hyperpolarizing step at the onset of every sweep, and recordings were discarded if access resistance exceeded 20 M Ω or changed by more than 20%. For current clamp experiments where EPSPs were recorded, the intracellular solution contained (in mM): 135 K⁺-gluconate, 7 KCl, 10 HEPES, 4 ATP-Mg, 0.4 GTP, and 10 Na-phosphocreatine. For the spike-timing dependent LTP experiments, the magnitude of current injection (in pA) was calibrated to amount needed for the cell to fire 3-4 action potentials immediately after gaining whole-cell access (150 ms duration current injection). Electrical stimuli to generate EPSPs were elicited 10 ms prior to current injection during the LTP induction phase, where a total of 60 pairings at 1 Hz were used to induce LTP.

Two-Photon Imaging and Glutamate Uncaging:

For glutamate uncaging experiments where AMPA to NMDA ratios were mapped along dendritic segments, 2.5 mM MNI-glutamate-trifluoroacetate (Femtonics, Budapest, Hungary), 1 μ M tetrodotoxin and 10 μ M glycine were added the Ringer's solution, which contained a reduced concentration of MgSO₄·7H₂O and CaCl₂ (1.3 and 2.5 mM, respectively). The intracellular

recording solution was also modified to contain 10 mM BAPTA to reduce the potential for plasticity induction following activation of NMDARs at positive holding potentials. The modified intracellular solution contained (in mM): 77 cesium methane-sulfonate, 10 tetracesium-BAPTA, 5 TEA-Cl, 3 CaCl_2 , 20 HEPES, 4 ATP-Mg, 0.5 GTP, 5 QX-314, 10 Na-phosphocreatine, along with Alexa 594 (20 μM ; Molecular Probes, Eugene, OR) to visualize dendritic morphology. Two-photon imaging and glutamate uncaging were performed using two Ti:Sapphire pulsed lasers (MaiTai-DeepSee; Spectra Physics, Santa Clara, CA). The first laser was tuned to 810 nm to image Alexa594 and visualize dendritic morphology and the second was tuned to 720 nm to uncage MNI-glutamate. Uncaging events (1 ms) were directed towards a single spot at the tips of dendritic spines, and the resulting uncaging excitatory postsynaptic currents (uEPSCs) were recorded at the soma. Spines of secondary and tertiary apical dendrites that were proximal to the cell soma ($< 150 \mu\text{m}$) were selected to limit issues of space clamp. Neurons were first clamped at +40 mV and laser power was tuned to generate a 5-30 pA mixed AMPA- and NMDAR-mediated uEPSC. At the same uncaging stimulus intensity, uncaging pulses were delivered at 0 mV and -70 mV. The NMDA component of the EPSC was measured at +40 mV, 35 ms after the peak current of the -70 mV trace (the AMPA component), which was sufficient time for the AMPAR-component to fully decay. Synapses were deemed to be silent when the AMPA to NMDA ratio was less than 0.25 (Lee et al., 2016).

For LTP experiment induced by glutamate uncaging at single spines, 30 consecutive pulses of 4 ms duration were delivered at 0.5 Hz to a CA1 pyramidal cell spine expressing mCherry and SEP-GluA1. Short dendritic segments were imaged in 4D by taking small Z-stacks (8-12 slices at 0.75 μm steps) centered on the stimulated dendritic spine over time (1-2 minute intervals). The imaging laser was tuned between 930 and 970 nm to excite both SEP-GluA1 and

mCherry simultaneously. Emission photons were spectrally separated using a dichroic mirror (570 nm) and additionally filtered using separate bandpass filters (green 495-540; red 575-630). To quantify changes in spine structure and GluA1 intensity, summed intensity projections of entire Z-stacks were first corrected for X-Y drift using a StackReg function in ImageJ (NIH, Bethesda, Maryland). Regions of interest were then drawn around spine heads, pixel intensities were extracted, background subtracted, normalized to the average intensity of four baseline images taken prior to LTP induction (F_0), and expressed as $\Delta F/F_0$. All quantifications were done on raw unprocessed images. For figure presentation, images were thresholded and filtered using a 0.2 μm median filter (ImageJ).

Optical Quantal Analysis:

For experiments where glutamate transients were recorded using iGluSNFR, single line scans through dendritic spines (~ 1.4 ms / line, 1000 lines) and adjacent dendritic shafts were gathered using a 950 nm laser excitation. Slices were perfused with the same Ringer's solution used in the whole-cell electrophysiology experiments (see above) with the exception that picrotoxin was omitted. Short duration (0.1 ms) low intensity (5-25 μA) stimuli were delivered from a stimulating electrode placed in stratum radiatum and apical dendrites of CA1 pyramidal cells were surveyed for spines responsive to the stimulus. To facilitate the process of finding responsive spines, line scans were performed simultaneously through multiple nearby dendritic spines, and paired-pulse stimuli (100 ms inter stimulus interval) were delivered initially to increase the probability of glutamate release. Spines that were unresponsive to these electrical stimuli during this probe phase (typically 5-10 paired pulse stimuli) were ignored, while responsive spines (*i.e.*, showing time-locked responses) were selected for optical quantal analysis experiments. A MATLAB script was developed and used to automatically detect spine and

dendrite compartments based on the profile of mCherry signal intensity across the scanning line. Line scans were reduced post hoc by a factor of 4 by averaging to minimize noise, and pixel intensities across each of the identified compartments were summed at each time point. Signals were then background subtracted, and normalized to the baseline (F_0) and expressed as $\Delta F/F_0$. In all experiments, transmission successes were identified as having peak amplitudes (within 0 – 100 ms after the stimulus) that exceeded 4X the standard deviation of the baseline noise. To calculate release probability, 50 consecutive stimuli were delivered at 0.1 Hz, and the number of success was divided by the total number of trials. For the 3 Hz stimulation experiments, line scan duration was 30 s and 90 consecutive electrical stimuli were delivered. Event amplitudes were normalized to a 50 ms baseline period prior to each stimulus and, again, successful transmission was defined by fluorescent responses that exceeded 4x the standard deviation of baseline noise.

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Competing Interests:

The authors declare no competing financial interests.

Supplementary Figures:

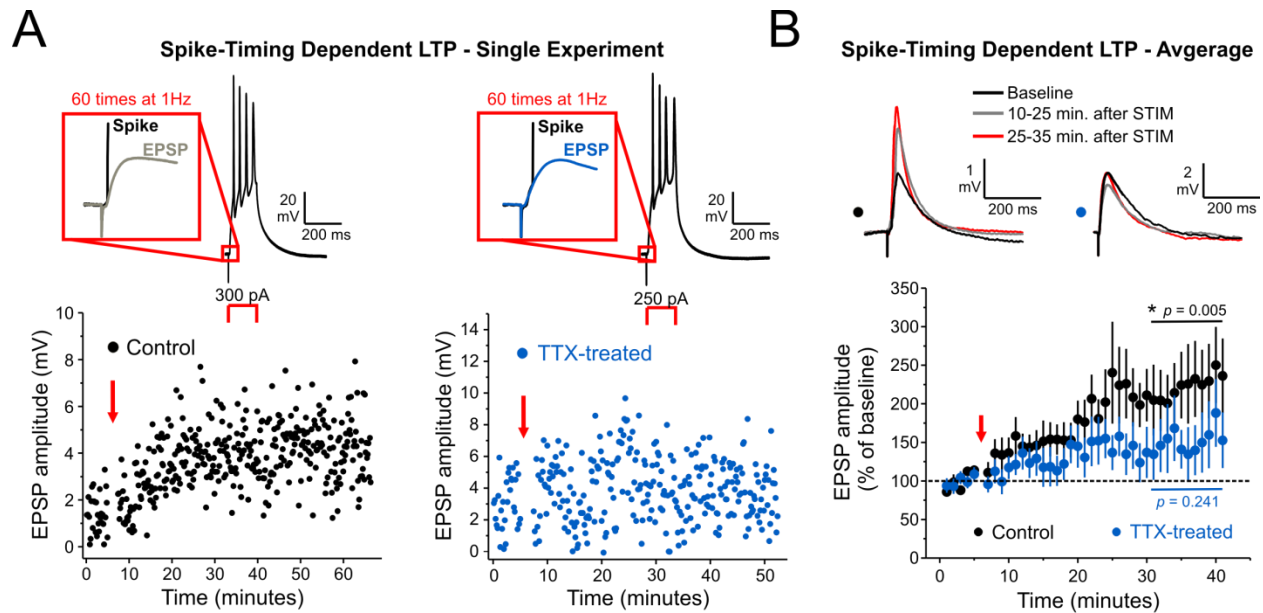


Figure 1 Supplement 1: Homeostatic synaptic strengthening restricts LTP induced by spike-timing dependent plasticity. (A) Example experiments where excitatory postsynaptic potentials (EPSPs) were paired with somatic current injection (150 ms duration starting 10 ms after the evoked stimulus; sufficient to evoke 3-4 action potentials; see methods), at a frequency of 1 Hz for 1 minute to induce LTP. (B) Summary data for all spike-timing dependent LTP experiments (Control: $p = 0.005$ when compared to baseline, z-test, $n = 10$ cells; TTX-treated: $p = 0.241$ when compared to baseline, z-test, $n = 11$ cells).

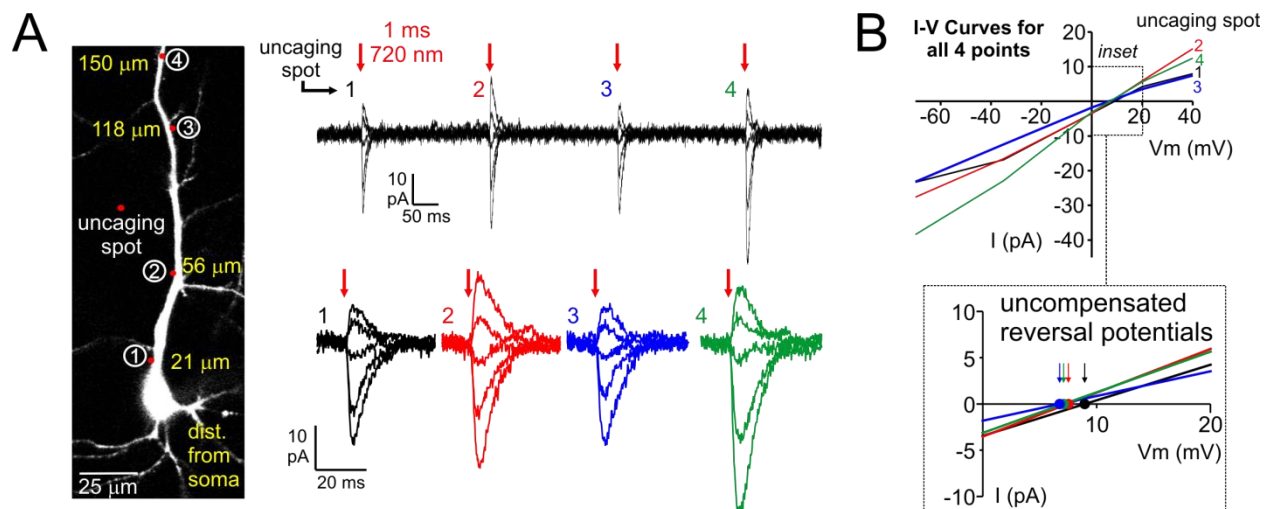


Figure 2 Supplement 1: Adequate clamping of membrane voltage within proximity to the cell soma. (A) A glutamate uncaging experiment whereby AMPAR-mediated uEPSCs were gathered at different holding potentials (-70, -35, 0, 20 and 40 mV, respectively) at regions along the main apical dendrite ranging from ~20 – 150 μ M away from the cell soma simultaneously. (B) Current-voltage (I-V) relationships are plotted for all four uncaging locations. Uncompensated reversal potentials, a practical means to evaluate the accuracy of voltage clamp, are indicated in the inset.

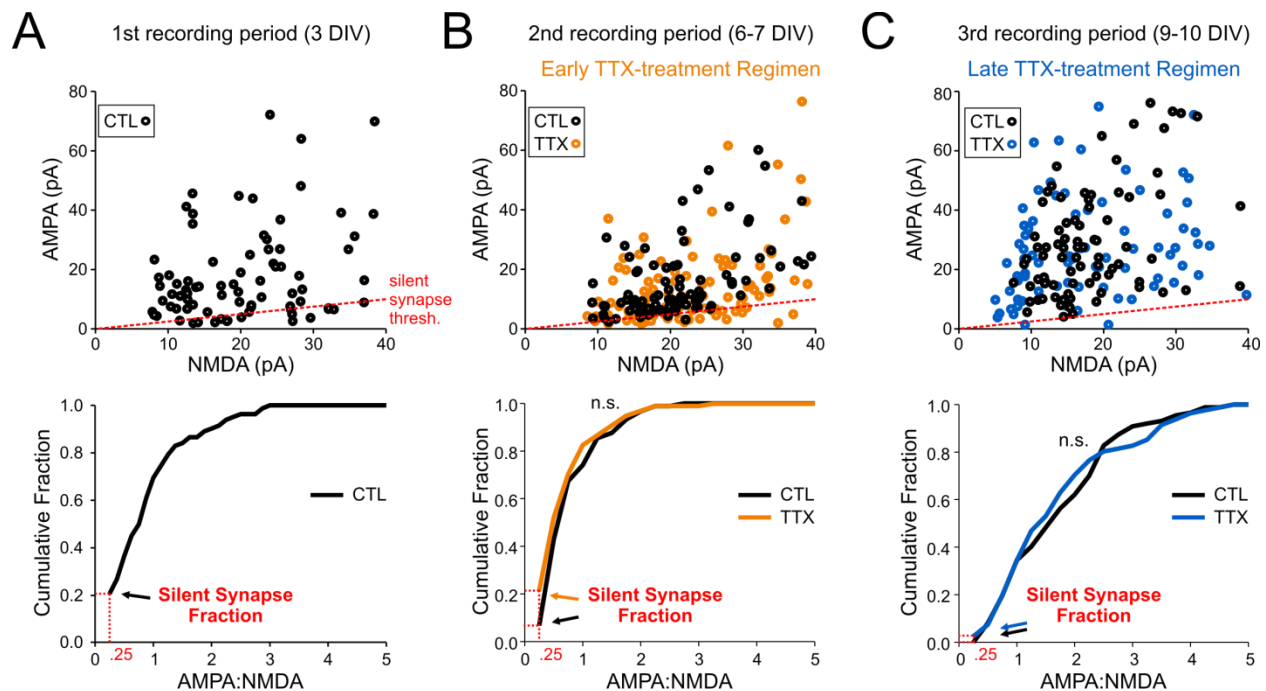


Figure 2 Supplement 2: Functional mapping synaptic weights at distinct stages of hippocampal slice development and following network silencing. (A) Summary of all uncaging-evoked AMPA to NMDA ratios recorded at single spines from control, untreated, neurons at 3 DIV. Raw values for the amplitude of the NMDA component is plotted against the amplitude of the AMPA component (top panel; $n = 79$ spines). Dotted line is overlaid to show the silent synapse threshold (AMPA to NMDA ratio < 0.25). Cumulative distribution of all AMPA to NMDA ratios (bottom panel), highlighting the fraction of synapses that were functionally silent (dotted red line). (B) Same plot description as panel A, except that recording were made at 6-7 DIV in slices that were either treated with TTX (orange; $n = 95$ spines) or left untreated (control, black; $n = 90$ spines). The cumulative distributions of AMPA to NMDA ratios were not significantly different between conditions ($p > 0.05$, two-sample Kolmogorov-Smirnov test). (C) Same plot descriptions as panels A and B, except that recordings were made at 9-10 DIV in slices that were either treated with TTX (blue; $n = 80$ spines) or left untreated (control, black; $n = 82$ spines). The cumulative distributions of AMPA to NMDA ratios were not significantly different between conditions ($p > 0.05$, two-sample Kolmogorov-Smirnov test).

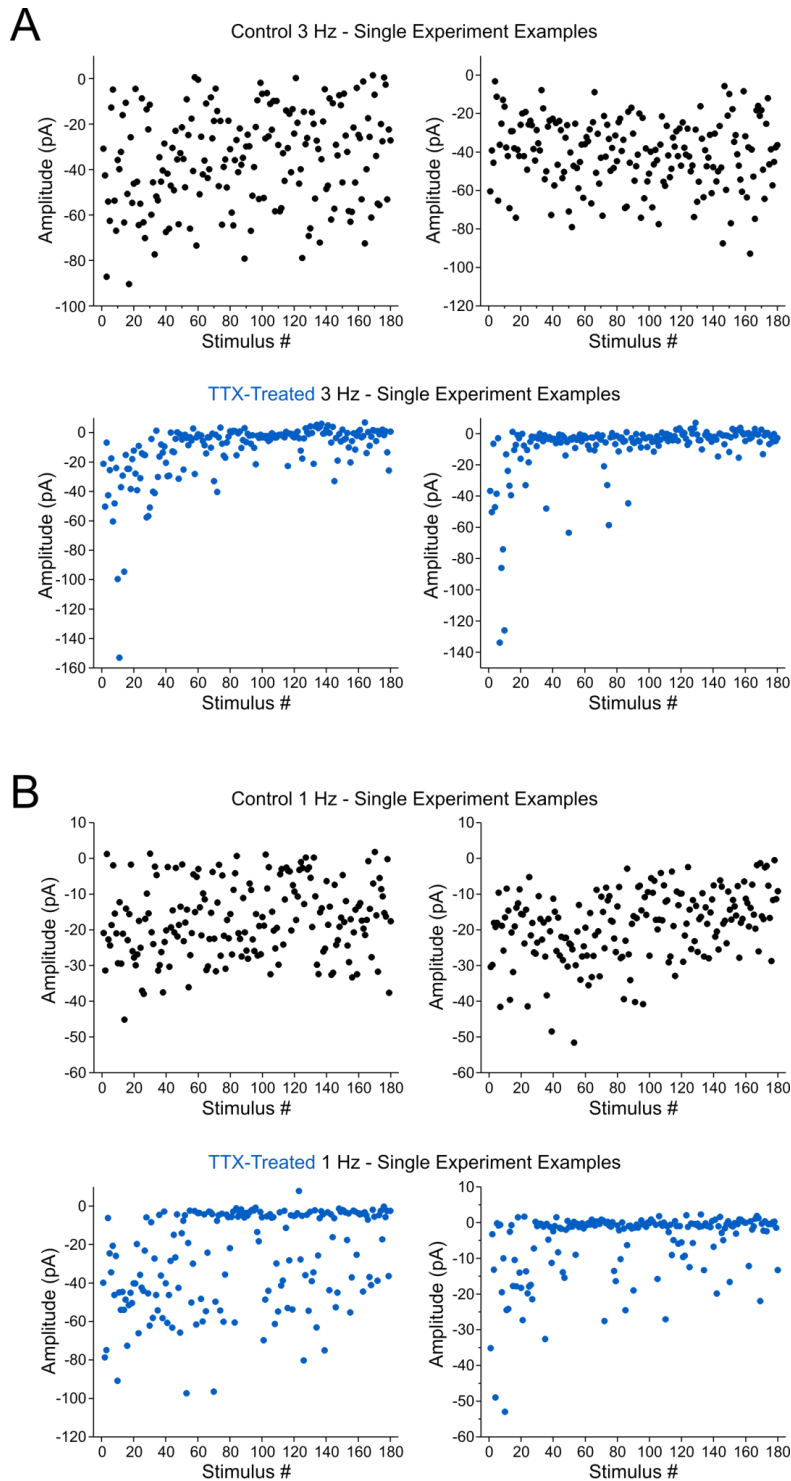


Figure 4 Supplement 1: Frequency-dependent shutdown of presynaptic release at synapses that have been homeostatically strengthened. (A) Additional examples of experiments where evoked AMPAR-EPSCs were recorded during a prolonged 3 Hz stimulation in control (top, black circles) or in TTX-treated (below, blue circles) slices. (B) Additional examples of experiments where evoked AMPAR-EPSCs were recorded during a prolonged 1 Hz stimulation in control (top, black circles) or in TTX-treated (below, blue circles) slices.

Manuscript III

Title: Quantal properties and plasticity of glutamate release at hippocampal CA1 synapses

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At the time of thesis acceptance, this manuscript was in preparation and therefore unpublished.

Statement of Contributions:

I designed, performed and analyzed all of the experiments. Dr. Jean-Claude Béïque supervised the project and helped write the paper.

Abstract:

Transmission at single synapses in the brain is stochastic, unreliable and variable. The full complement of factors that contribute to such synaptic noise, including their plasticity potential, still remains only partly understood. Here, we revisited, and expanded upon, key concepts of glutamate release at single dendritic spines of CA1 pyramidal neurons. Using whole-cell electrophysiology, two-photon imaging and glutamate uncaging, we show that the genetically-encoded optical glutamate sensor iGluSNFR is a linear reporter of glutamate concentration over physiological ranges, with kinetics comparable to that of ionotropic glutamate receptors. Using optical quantal analysis, we provide an estimate of both the variability in cleft glutamate concentration at single synapses and of the ability of postsynaptic glutamate receptors to decode such variability. Remarkably, we found that the rate of occurrence of multivesicular release was differentially regulated by distinct stimulus patterns and that the amount of glutamate release was regulated by a homeostatic form of plasticity. Thus, the amplitudes of individual quantal events may be part of the informational content transmitted at central synapses.

Introduction:

At most excitatory synapses in the brain, glutamate release occurs probabilistically upon arrival of a presynaptic action potential and is thus an unreliable means of information transfer (Raastad et al., 1992; Allen and Stevens, 1994). Such unreliability can however be computationally advantageous by providing synapses with the flexibility to bidirectionally adjust transmission fidelity to instill both rapid and lasting changes in synaptic efficacy (Manwani and Koch, 2001). When transmission occurs successfully, there is typically a single vesicle or ‘quantum’ of glutamate released into the synaptic cleft, which in turn activates postsynaptic glutamate receptors (Stevens and Wang, 1995; Lisman et al., 2007). A number of recent studies have demonstrated that postsynaptic responses to quantal glutamate release at single synapses can vary considerably in amplitude, indicating that transmission between neurons is not only probabilistic but also highly variable in nature (Liu and Tsien, 1995; Forti et al., 1997; McAllister and Stevens, 2000; Conti and Lisman, 2003). Identifying the full complement of stochastic molecular processes that underlie quantal variability, and determining how they are affected during synaptic plasticity, is critical if we are to fully appreciate the role of synaptic unreliability in neural information transfer.

Our understanding of the factors that contribute to the stochastic and variable process of synaptic transmission has improved steadily over the last few decades (for review see Ribault et

al., 2011). It is now generally agreed that, at most glutamatergic synapses, quantal release does not saturate postsynaptic receptors (Ebisawa et al., 1999; Liu et al., 1999; McAllister and Stevens, 2000; Liu, 2003; Nimchinsky et al., 2004) and that variability in quantal responses arises primarily due to presynaptic mechanisms that instill differences in the profile of glutamate released into the synaptic cleft (Ribrault et al., 2011). While several presynaptic mechanisms have been proposed to account for quantal amplitude fluctuations – uneven packaging of glutamate into synaptic vesicles, differences in release location within the active zone, mode of exocytosis (Choi et al., 2003; Franks et al., 2003; Wu et al., 2007; Richards, 2009) – technical limitations have rendered it challenging to independently investigate and quantify their relative contributions in intact preparations. A recently developed genetically-encoded optical glutamate sensor has been used to resolve glutamate transients at microscopic scales (Marvin et al., 2013), and thus may be suitable to interrogate fundamental features of quantal response variability at single glutamatergic synapses.

In this study, we combined whole-cell electrophysiology with two-photon glutamate uncaging and two-photon imaging of iGluSNFR-mediated fluorescence signals to revisit and expand on key features of glutamate release at single hippocampal synapses. We first characterized and calibrated the intrinsic properties of iGluSNFR at dendritic spines in response to exogenous glutamate release by glutamate uncaging and showed that its sensitivity, onset and decay kinetics are comparable to, and in some aspects exceeded, that of synaptic glutamate

receptors. Using optical quantal analysis, we observed a remarkably high variability in cleft glutamate at single synapses. Part of this variability in cleft concentration was attributable to multivesicular release, which occurred rarely in response to a single action potential but more frequently in conditions of presynaptic facilitation. Last, we characterized a novel presynaptic mechanism of homeostatic plasticity in response to prolonged network silencing in which CA1 synapses are strengthened through a multiplicative increase in the amount of glutamate released from presynaptic terminals. Thus, the amount of glutamate released during quantal transmission is a physiologically regulated variable that is dependent on synapse history, which might have important functional consequences for maintaining homeostasis.

Results:

We sought to investigate basic features of glutamate release at single hippocampal synapses and determine how they are affected by a homeostatic plasticity paradigm. For this purpose, we turned to an optical approach based on a recently developed intensity-based glutamate sensing fluorescent reporter (iGluSNFR), which is targeted to the plasma membrane and enables visualization of glutamate release onto individual dendritic spines (Marvin et al., 2013). Neurons in organotypic hippocampal slices were sparsely transfected with iGluSNFR and a cytosolic morphological reporter mCherry using biolistics (Soares et al., 2014). Two-photon imaging of transfected cells at 950 nm revealed a diffuse expression of iGluSNFR which readily invaded all dendritic compartments including the smallest dendritic spines (**Figure 1 Supplement 1A**). For all experiments, we focused on dendritic spines in the apical arbour of CA1 pyramidal neurons, since they are likely to receive glutamatergic input from CA3 Schaffer collaterals. Quantal properties at this CA3-CA1 synapse have been extensively characterized using both electrophysiological and optical methods (Sayer et al., 1989; Larkman et al., 1997; Hanse and Gustafsson, 2001; Oertner et al., 2002; Conti and Lisman, 2003; Raghavachari and Lisman, 2004), although many features of quantal glutamate release, including factors that contribute to its variability and whether they can be influenced by synaptic plasticity mechanisms, remain only partially understood.

iGluSNFR is a linear reporter of glutamate concentration at dendritic spines

Previous studies have demonstrated that iGluSNFR is a versatile and powerful optical reporter of glutamate release in both microscopic and macroscopic brain compartments (Borghuis et al., 2013; Marvin et al., 2013; Park et al., 2014; Parsons et al., 2016; Xie et al., 2016), although it has seldom been used to study properties of glutamate release at single synapses (but see Hikima et al., 2016). Thus, in order to determine its usefulness and shortcomings in reporting features of vesicular glutamate release at single synapses, we first sought to calibrate some biophysical properties of iGluSNFR using two-photon uncaging of MNI-glutamate (Matsuzaki et al., 2001). Whole-cell electrophysiological recordings were obtained from transfected CA1 pyramidal neurons expressing iGluSNFR (**Figure 1A**). Cells were voltage clamped at -70 mV and short duration laser pulses (1 ms at 720 nm) were directed to the tips of dendritic spines to induce glutamate release from a point source. Glutamate transients at stimulated spines were detected by line scan imaging of iGluSNFR (950 nm), and the corresponding uncaging-evoked excitatory postsynaptic currents mediated by AMPARs (AMPA-uEPSCs) were recorded at the soma (**Figure 1B,C**).

In order to calibrate the sensitivity and dynamic range of iGluSNFR activation, varying laser intensities were used to uncage glutamate onto the tips of dendritic spines. We observed a linear increase in the fluorescence amplitude of iGluSNFR transients in dendritic spines with

step-wise increases in uncaging stimulus intensity (**Figure 1C-E**). These uncaging-evoked iGluSNFR transients displayed no obvious signs of saturation, indicating that iGluSNFR in dendritic spines linearly reports changes in extracellular glutamate concentration, at least within the bounds of uncaging stimulus intensities tested (**Figure 1E**). In all instances, spine iGluSNFR transients were detected at lower uncaging stimulus intensities than was required to induce an AMPAR-uEPSC measureable at the soma (**Figure 1F**). Moreover, when similar uncaging power curves of AMPAR-uEPSCs were generated at dendritic spines of non-iGluSNFR-expressing CA1 cells, this range of weak uncaging stimulus intensities (5 - 7.5%) that were reliable at activating iGluSNFR (**Figure 1E**) rarely elicited a postsynaptic current (**Figure 1 Supplement 1B**). These experiments indicate that iGluSNFR is able to detect glutamate release at synapses with higher sensitivity than electrophysiological methods. At the upper end of stimulus intensities tested, AMPAR-uEPSCs were highly analogous in both their amplitude and kinetics to spontaneous mEPSCs recorded from a single cell (**Figure 1 Supplement 1C**), suggesting that optical glutamate release was within a physiological range. In response to the strongest uncaging stimuli, iGluSNFR signals were also detected in the immediately adjacent dendritic compartment (**Figure 1D,E, Figure 1 Supplement 1D**), although they were always of smaller amplitude (average $\sim 33\%$) than their spine counterparts (**Figure 1 Supplement 1E**), and their time to achieve maximal activation was slightly delayed (20-80% rise time, **Figure 1 Supplement 1F**). Altogether, the optical glutamate sensor, iGluSNFR, proved to have high sensitivity and could

linearly report transient changes in glutamate concentration in dendritic spines, some of which were below threshold of electrophysiological detection.

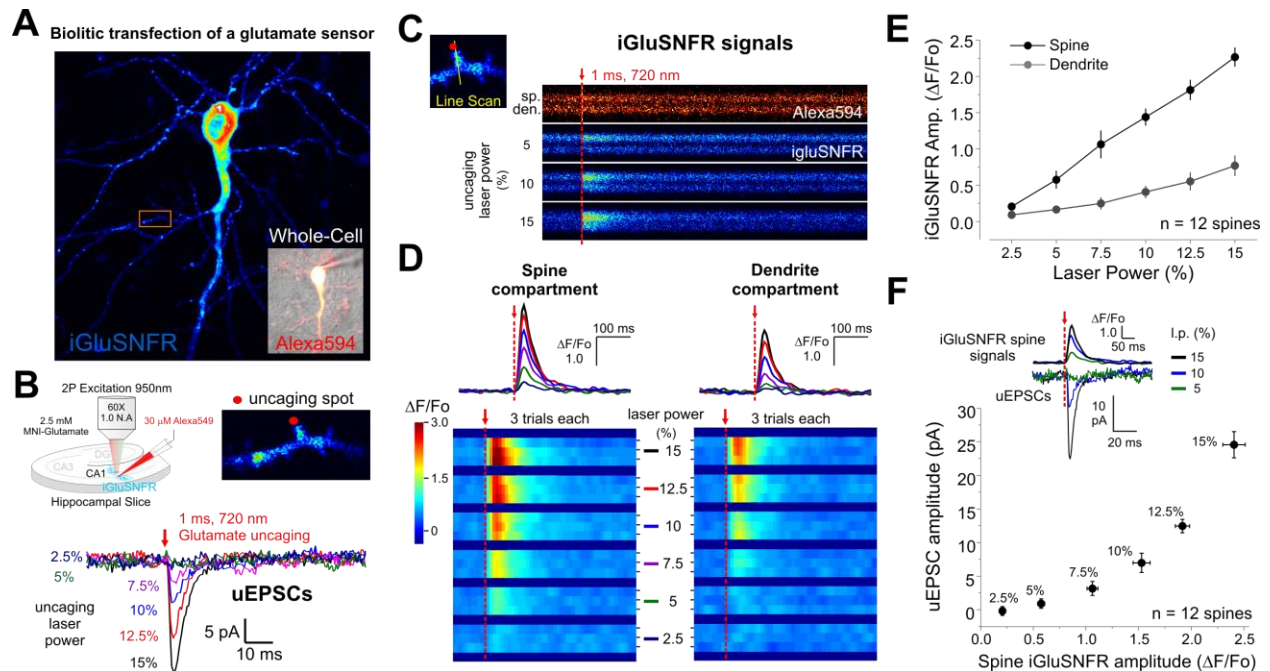


Figure 1: iGluSNFR linearly reports glutamate concentration at dendritic spines with high sensitivity. (A) CA1 neurons expressing the optical glutamate sensor iGluSNFR were targeted for whole-cell electrophysiological recordings. (B) Optical stimuli of increasing intensity were delivered to the tip of dendritic spines to uncage MNI-glutamate while the resulting AMPAR-mediated EPSCs were recorded at the soma (voltage clamp, -70 mV). (C) Two-photon imaging of iGluSNFR signals (950 nm excitation) was used to measure the amount and spatial spread of glutamate release at single spines following glutamate uncaging. (D) iGluSNFR signals from spine and dendrite compartments of the line scan were separated and analyzed independently of one another. (E) A step-wise linear increase laser intensity (% laser power) to uncage glutamate was reported by iGluSNFR at dendritic spines as a linear increase fluorescence intensity change ($\Delta F/F_0$). Dendritic iGluSNFR signals in response the same range of optical stimuli were smaller and in some cases undetectable. (F) A comparison of the amplitude of iGluSNFR spine transients versus the uEPSC amplitude recorded at the soma following two-photon uncaging of glutamate at single spines.

Rise and decay kinetics of iGluSNFR activation at dendritic spines

We next sought to characterize the temporal performance of this optical-based glutamate reporter in dendritic spines and compare these kinetic metrics to those of ionotropic glutamate receptors. Again, we used two-photon glutamate uncaging to minimize variability in our measurements by having precise spatiotemporal and quantitative control over glutamate presentation onto dendritic spines across experiments. To determine the activation kinetics of iGluSNFR in response to glutamate presentation, we calculated the rise time (20-80%) for uncaging-evoked iGluSNFR transients following a 1 ms uncaging stimulus. On average, the rise time of iGluSNFR spine transients was ~ 3.75 ms and was independent of uncaging stimulus intensity (**Figure 1 Supplement 2A**). Using the exact same stimulus conditions to elicit postsynaptic responses from ionotropic glutamate receptors, we found that the rise time of iGluSNFR activation was slower than that of AMPAR-EPSCs (resampled *post hoc*, to match the imaging acquisition rate; see methods) but faster than NMDAR-uEPSCs (**Figure 1 Supplement 2B**). The average decay of uncaging-evoked iGluSNFR transients, which were best fit by a single exponential function ($R^2 > 90\%$), was ~ 45 ms and also independent of uncaging stimulus intensity (**Figure 1 Supplement 2C**). In comparison to the decay kinetics of ionotropic glutamate receptors under similar stimulus conditions (Soares et al., 2013), iGluSNFR transients exhibited slower decay kinetics than AMPAR-uEPSCs, but were over twice as fast as NMDAR-uEPSCs. Altogether, by using two-photon glutamate uncaging to probe the kinetic properties of

iGluSNFR activation and decay, this optical sensor reports glutamate release with kinetics that are broadly comparable to that of endogenous ionotropic glutamate receptors.

Optical quantal analysis at single hippocampal synapses using iGluSNFR

Having established the sensitivity and the spatiotemporal performance of iGluSNFR, we next analyzed features of synaptically-evoked glutamate release using this optical sensor. To achieve this, we positioned a glass stimulating patch electrode in stratum radiatum adjacent to the dendritic arbor of a transfected CA1 pyramidal neuron (**Figure 2A**). Short duration (0.1 ms) and low intensity (10-50 μ A) pairs of electrical stimuli (100 ms inter stimulus interval) were delivered initially to maximize the potential for finding a responsive spine. Groups of spines were surveyed simultaneously by rapidly scanning a line across multiple adjacent spines. The majority of spines surveyed were unresponsive to consecutive ($\sim$ 10) paired-pulse stimuli and were not further analyzed. When a dendritic spine was found to be responsive to this initial survey, the stimulus intensity was gradually reduced to a minimum without losing time-locked iGluSNFR responses. Optical measurements of quantal glutamate release events were then gathered by line-scan imaging of iGluSNFR in both the spine and dendritic compartment in response to 50 consecutive single-pulse electrical stimuli delivered at low frequency (0.1 Hz).

Electrically-evoked iGluSNFR transients at dendritic spines displayed hallmark features of synaptic glutamate release, the most apparent of which being that release occurred

probabilistically (**Figure 2B-D**). The amplitude distribution of successful iGluSNFR transients were sufficiently separated from the recording noise as to allow unambiguous discrimination of successful release events from release failures (**Figure 2E**). In these cases, the release probability (Pr) at single synapses could be computed, and we found that it varied considerably between synapses (Range: 0.1 to 0.96; **Figure 2F-G**). There was no apparent relationship between the probability of glutamate release and the average success amplitude of iGluSNFR transients (henceforth referred to as *potency*, **Figure 2F**). Whereas the iGluSNFR fluorescent signals were typically restricted to the dendritic spine head (**Figure 2D,G, Figure 2 Supplement 1A**), at times smaller events could be detected in the dendritic shaft (**Figure 2 Supplement 1B**). Dendritic iGluSNFR signals occurred less frequently and were associated with the largest spine transients (**Figure 2 Supplement 2C**). Since both the occurrence and amplitude of the spine and dendritic transients co-varied (**Figure 2 Supplement 2D**), dendritic signals were likely the result of glutamate spillover (Kullmann et al., 1996; Asztely et al., 1997; Barbour and Hausser, 1997; Chalifoux and Carter, 2011) rather than from glutamate release at a distinct neighbouring synapse. Collectively, these results demonstrate that electrically-evoked iGluSNFR signals in dendritic spines result from direct release of glutamate onto the spine of interest and can thus be used to define features of glutamate release at putative single synapses.

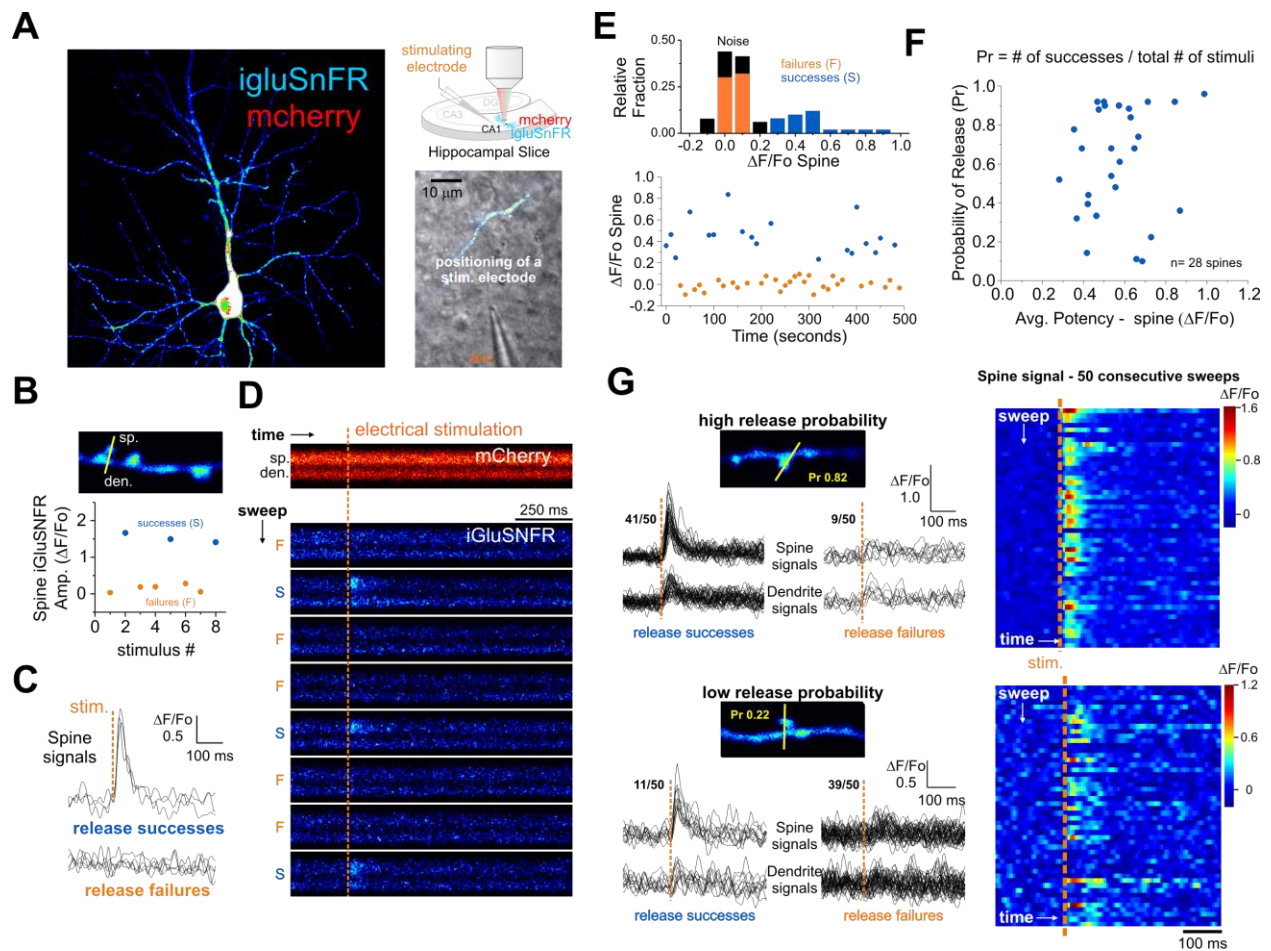


Figure 2: iGluSNFR reliably reports electrically-evoked glutamate release at single synapses. (A) Brief electrical stimuli were delivered from a glass patch electrode placed into the slice close to the dendritic arbor of a neuron transfected with iGluSNFR and mCherry. (B) 2P-imaging of iGluSNFR at single spines revealed probabilistic glutamate transients following electrical stimulation. (C) Release successes were readily distinguishable from release failures. (D) An example (also shown in panels B and C) of successive line scans at a spine that was response to electrical stimulation. A transient increase in the fluorescence intensity of iGluSNFR signals followed electrical stimulation, which was typically restricted to the spine head. (E) iGluSNFR amplitudes following evoked glutamate release successes were sufficiently high to be discriminated from optical noise, allowing unambiguous discrimination between release success and failures. In a typical experiment, 50 consecutive stimulations were delivered at 0.1 Hz. (F) Average potency of iGluSNFR transients (mean success amplitude) displayed no obvious relationship with the release probability. (G) Release probability estimates at single spines were highly variable. Optical traces of normalized iGluSNFR intensities in both the spine and dendrite compartment are shown on the left for two spines. On the right, a 2D intensity based rendering of the entire experiment is shown, where the x-axis is time, the y axis represent all 50 consecutive trials and the color depicts the amplitude of normalized (to baseline) iGluSNFR signals in the dendritic spine head.

Experimental approaches used commonly to describe features of quantal release at single synapses have relied on the activation of glutamate receptors and coincident measurements based either on their electrophysiological response (Liu and Tsien, 1995; Forti et al., 1997; McAllister and Stevens, 2000), or for the case of NMDARs, their calcium permeability (Mainen et al., 1999; Umekiya et al., 1999; Oertner et al., 2002). Since activation of ionotropic glutamate receptors can lead to synaptic plasticity, these techniques have the potential to alter synaptic properties during repetitive sampling. We reasoned that the use of iGluSNFR could negate such potential since it could be used to study glutamate release in the presence of glutamate receptor antagonists. To test this idea, we washed in the AMPAR antagonist NBQX (20 μ M) in a subset of experiments, and evaluated its effect on single spine iGluSNFR transients. We found that neither the probability of glutamate release nor the amplitude of iGluSNFR transients were affected by NBQX administration (**Figure 2 Supplement 2A-C**). Thus, one particular advantage of using iGluSNFR for studying quantal properties of glutamate release is that it can be used in conjunction with glutamate receptor antagonists, thereby preventing the potential induction of synaptic plasticity. Taken together, iGluSNFR is well suited for studying properties of glutamate release at single synapses, and has unique advantages over classical techniques, including calcium imaging, for performing optical quantal analysis.

Quantal variability at single synapses detected by iGluSNFR

Several factors contribute to the inherent variability of glutamatergic neurotransmission at central synapses, including: i) the all-or-none nature of vesicular release; ii) the stochastic nature of postsynaptic receptor opening and iii) the variability in cleft glutamate concentration following vesicular fusion (for a detailed review of all three sources, see Ribault et al., 2011). The importance of the latter is emphasized by the now generally accepted view that postsynaptic receptors are not saturated by single quantal release events (Ebisawa et al., 1999; Liu et al., 1999; Mainen et al., 1999; McAllister and Stevens, 2000; Nimchinsky et al., 2004). It has proven challenging, however, to study the presynaptic variability of glutamate release in isolation, in order to disambiguate its relative contribution to the variable nature of synaptic transmission. We reasoned that using iGluSNFR may offer a useful approach to directly study variability in glutamate release at single, visually-identified, CA1 synapses.

As a first step to estimate the variability of glutamate concentration released at single synapses, we sought to estimate the amount variability inherent to our method of optical detection (i.e., fluctuations of the glutamate sensor, instrument noise) by providing a relatively constant presentation of glutamate by two-photon glutamate uncaging onto dendritic spines (**Figure 3A**). By repeated stimulations at fixed and constant uncaging intensity, sufficient to elicit postsynaptic AMPAR-mediated responses within a physiological range (**Figure 1**

Supplement 1C), we found that the amplitude of uncaging-evoked iGluSNFR transients at a single spine had an average coefficient of variation (CV) of 0.12 (**Figure 3B,C**). We next examined the variability of electrically-evoked iGluSNFR transients induced by endogenous glutamate release at single synapses (**Figure 3E**). We excluded release failures, and focused only on instances of successful glutamate release (*i.e.*, potency). Intriguingly, the variability of electrically-evoked iGluSNFR transients at single synapses was over three times greater (CV = 0.38; **Figure 3F,G**) than uncaging-evoked iGluSNFR transients. Notably, whereas the pooled distribution of all uncaging-evoked spine amplitudes was narrow (standard deviation, $\sigma = 0.12$) and centered on the mean (**Figure 3D**), that of electrically-evoked spine amplitudes had a greater variance ($\sigma = 0.35$) and was skewed towards larger event amplitudes (**Figure 3G**).

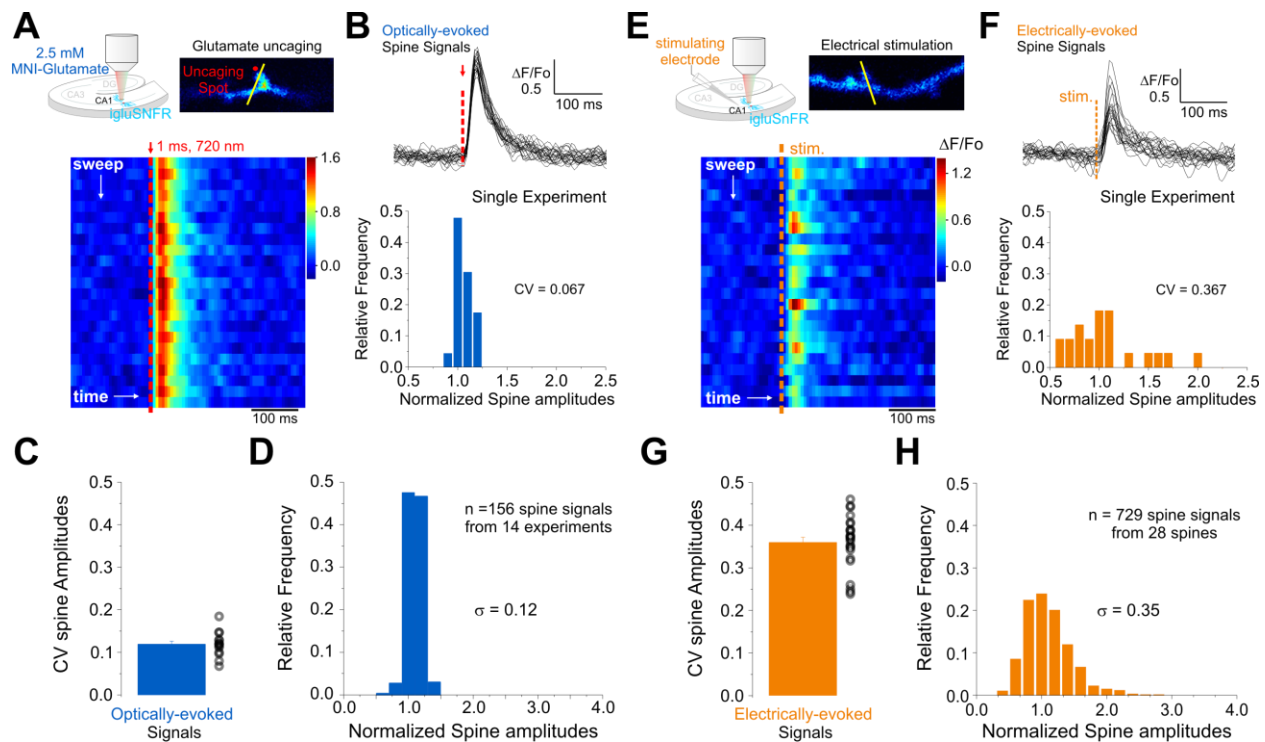


Figure 3: Quantitative estimate of variability in cleft glutamate concentration following evoked release. (A) Two-photon glutamate uncaging was used to deliver a relatively fixed concentration of glutamate to dendritic spines repetitively at low-frequency (0.1 Hz). (B) Optically-evoked spine signals varied minimally from trial-to-trial. (C) A coefficient of variation of spine iGluSNFR amplitude was calculated for each experiment where consecutive (10-25) stimulations were delivered at a fixed uncaging intensity. (D) A pooled distribution of normalized optically-evoked iGluSNFR amplitudes from 14 spine experiments, as shown in panels A and B. The standard deviation of this pooled distribution was 0.12. (E) An example experiment showing only spine iGluSNFR successes following electrical stimulation. (F) Electrically-evoked spine iGluSNFR signals displayed a broad distribution of event amplitudes. (G) A coefficient of variation of spine successes was calculated for each spine experiment. (H) A pooled distribution of normalized electrically-evoked iGluSNFR amplitudes from 28 experiments. The standard deviation of this pooled distribution was 0.38.

We then reasoned that a proper estimate of variability in cleft glutamate concentration would be the difference between electrically-evoked and uncaging-evoked transients, since glutamate presentation was relatively fixed during two-photon uncaging and was therefore an estimate of variability afforded through optical detection. Thus, the variability attributable to our method of optical detection, which was admittedly an overestimate due to an expected

contribution of other potential sources (*eg.* variability in the process of MNI-glutamate photolysis despite fixed stimulus parameters) that were difficult to define, was subtracted to generate a lower estimate of the variability in cleft glutamate concentration following evoked release. Using this simple arithmetic subtraction, we found that the variability of glutamate concentration in the synaptic cleft following evoked release, as defined by the coefficient of variation, was 0.26. Thus, through the repeated optical measurement of glutamate release at single hippocampal synapses, we provide a direct quantification of the variability in cleft glutamate concentration following evoked release. Because single release events at synapses are sub-saturating for glutamate receptor activation (Ebisawa et al., 1999; Liu et al., 1999; Mainen et al., 1999; McAllister and Stevens, 2000; Nimchinsky et al., 2004), variability in the amount of glutamate release, which we have defined experimentally, will translate to differences in quantal responses at single synapses.

The frequency of multivesicular release at CA1 synapses

The so-called ‘one release site, one fusion hypothesis’ predicts that synaptic transmission involves fusion of a single vesicle. While it is generally accepted that this is the dominant mode of release at canonical excitatory synapses, there is mounting evidence that under certain conditions, multivesicular release (MVR) can occur (Tong and Jahr, 1994; Oertner et al., 2002; Conti and Lisman, 2003; Christie and Jahr, 2006; Rudolph et al., 2015). The quasi-simultaneous

release of multiple synaptic vesicles would translate into a linear summation of cleft glutamate concentration (Raghavachari and Lisman, 2004) and, assuming lack of saturation, a proportional increase in the iGluSNFR response amplitude (depicted in **Figure 4A**). Since the amplitude distribution of electrically-evoked iGluSNFR transients was skewed towards large event amplitudes, which were visually evident in some cases (**Figure 4B**), we reasoned that MVR likely occurred at low frequency during our experiments. Since the frequency at which MVR occurs at hippocampal synapses is poorly defined, we next sought to estimate the frequency of MVR during baseline transmission, and determine whether this probability is altered by patterns of synaptic stimulation.

As a first step, we generated a normalized distribution of iGluSNFR amplitudes for each spine experiment by dividing by the median amplitude, instead of the mean (for additional details and discussion, see methods). The occurrence of more than one peak in these normalized amplitude distributions was at times readily apparent (**Figure 4B,C**). When data from all spine experiments were pooled, the iGluSNFR amplitude distribution showed a small but distinct second hump that was approximately twice that of the first peak (**Figure 4D**). This result suggested that MVR occurred at low frequency following a single electrical stimulus. In order to quantitatively infer the frequency of occurrence of MVR across all of our experiments, we generated a series of simulated iGluSNFR amplitude distributions, each with a different set probability of MVR, and tested for statistical significance against our actual amplitude

distribution. Amplitudes were randomly drawn from a Gaussian distribution (to simulate the distribution of univesicular release [UVR] amplitudes) with a mean and standard deviation that was based on experimental data (**Figure 4 Supplement 1A**). To simulate MVR, which was set to occur probabilistically, two amplitudes were randomly drawn from this expected UVR distribution and their values were summed. For each probability of MVR tested (0% – 50%), 50 simulated distributions were generated and tested against the actual distribution, enabling us to calculate an average p value (**Figure 4 Supplement 1B,C**). Using this iterative statistical method, we determined that the best estimate (highest p value; most similar distribution) for the frequency of MVR in our experimental dataset was 10% of all release successes (**Figure 4E**). In addition, this analysis revealed that simulated distributions based purely UVR could not predict our experimental distribution of spine iGluSNFR amplitudes (**Figure 4E, Figure 4 Supplement 1C**). Thus, by estimating the frequency at which MVR occurs under baseline conditions of evoked release, our results add to an expanding literature showing that MVR occurs at hippocampal synapses.

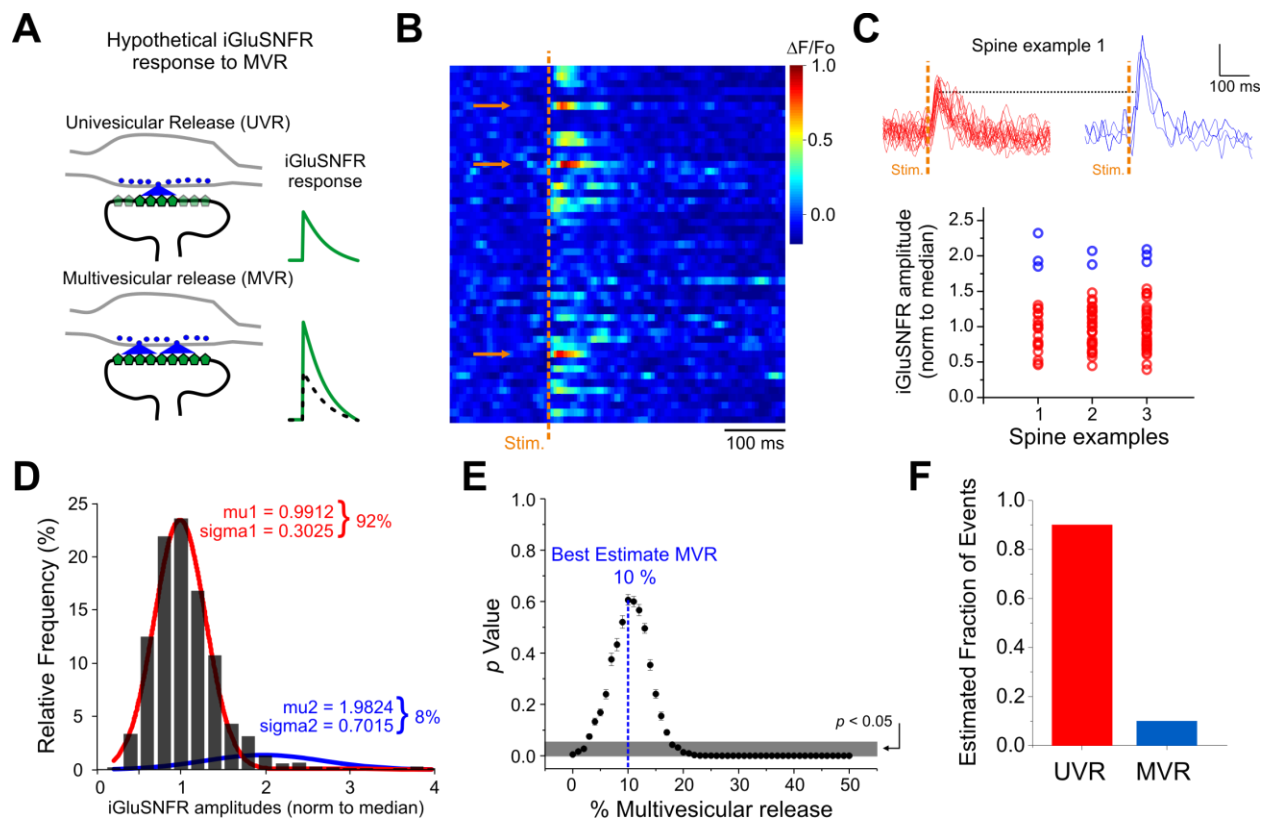


Figure 4: Multivesicular release occurs at low-frequency during evoked release. (A) A schematic description of the hypothetical iGluSNFR response to multivesicular release at a glutamatergic synapse. Since iGluSNFR linearly reports glutamate concentration over a wide range, the simultaneous release of two vesicles should translate to a doubling of cleft glutamate concentration and of iGluSNFR amplitude. (B) An example experiment (also depicted in **Figure 2 supplement 1A**) where several infrequent spine iGluSNFR transients were much larger than the typical response amplitude. (C) Normalized distributions of iGluSNFR spine transients from three spines. In each case, a group of events were observed that were twice the median amplitude of all events, and were well separated from the rest of the amplitude distribution. (D) A pooled amplitude distribution of electrically evoked spine iGluSNFR transients exhibited multimodality, suggestive of MVR occurrences at low frequency. (E) Results from an iterative statistical test where simulated distributions iGluSNFR amplitudes with set MVR probabilities were compared to the actual distribution of event amplitudes (panel D) to determine the best estimate for the frequency of multivesicular release following evoked stimulation. (F) Estimated fraction of glutamate transients that resulted from univesicular and multivesicular release, based on the results of the iterative statistical test shown in panel E.

We next questioned whether stimulus conditions could influence the frequency of MVR occurrences. Indeed, MVR was shown to increase with release probability, as occurs during paired pulse delivery (Tong and Jahr, 1994; Oertner et al., 2002; Christie and Jahr, 2006). We sought extend this concept by examining whether the occurrence of MVR would likewise increase during trains of successive electrical stimuli. A 3 Hz stimulus train (30 second duration) was delivered to the slice while line scans of iGluSNFR signals were used to monitor glutamate release at single dendritic spines (**Figure 5A**). Under these stimulus conditions, the amplitude distribution of events from single spines exhibited, in some cases, a very defined secondary peak (**Figure 5 B**). Intriguingly, the summed distribution of normalized iGluSNFR amplitudes across all spines displayed a more definitive secondary peak during 3 Hz stimulation (**Figure 5C**) as compared to low frequency stimulation (0.1 Hz; **Figure 4 D**), which again was centered at approximately twice the first peak. Using the same iterative statistical test described above, the best estimate for the frequency of MVR during these 3 Hz stimulation experiments was 22% (**Figure 5D**), which was over two times more frequent than the value predicted during lower-frequency stimulation. Thus our experiments revealed that trains of repetitive synaptic activation can increase the per-stimulus amount of glutamate released into the synaptic cleft due to an enhanced probability of multi-quantal glutamate release.

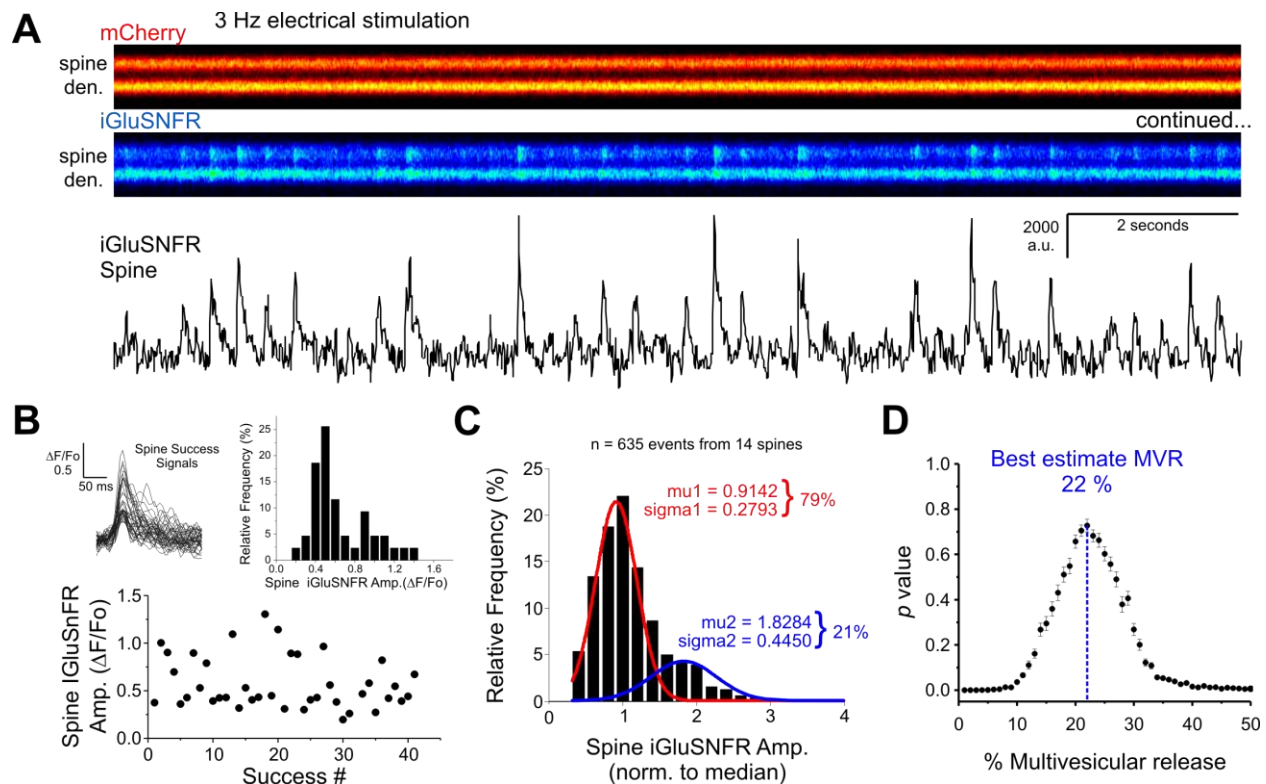


Figure 5: The occurrence of multivesicular release is enhanced during trains of electrical stimulation. (A) A line-scan through a single responsive spine and its parent dendrite during 3 Hz electrical stimulation. Below, a trace of spine intensities is shown. Only a subset of the entire 3 Hz experiment is shown so that individual events could be visually discriminated. (B) Quantification of spine iGluSNFR success amplitudes ($\Delta F/F_0$) during the entire 3 Hz stimulus train for the experiment shown in panel A. Shown above are traces of spine signals and the distribution of event amplitudes from the same experiment. (C) A pooled normalized distribution of event amplitudes across 14 spine experiments during 3 Hz stimulation. The overall distribution was fitted with two Gaussian functions using a maximum likelihood estimate where the second mean (μ_2) was equal to 2x the first mean. (D) Results from the iterative test for estimating the occurrence frequency of MVR is shown above.

Homeostatic scaling of quantal amplitudes through multiplicative changes in quantal glutamate release

Neurons actively maintain a set point of excitability through the expression of a variety of cellular homeostatic mechanisms. One such mechanism, termed homeostatic synaptic plasticity, involves the bidirectional tuning of excitatory synaptic drive as a means to compensate for changes in cellular activity (O'Brien et al., 1998; Turrigiano et al., 1998). This cellular phenomenon involves both presynaptic and postsynaptic modifications that together alter transmission efficacy (Turrigiano, 2008; Pozo and Goda, 2010; Davis, 2013; Lee et al., 2014). A fundamental feature homeostatic synaptic plasticity, at least in most cortical and hippocampal excitatory neurons, is that changes to synaptic efficacy occur in a multiplicative fashion, such that the relative strengths of synapses remain constant (Turrigiano et al., 1998; Goel and Lee, 2007; Kim et al., 2012; Soares et al., 2013). Our mechanistic understanding of how individual neurons manage this orchestrated homeostatic response such that all of synapses are synapses are equally affected, however, remains unclear. We next sought to exploit the ability of iGluSNFR to detect glutamate release at single synapses to explore homeostatic regulation of the presynaptic release processes. To this end, we used a well-validated homeostatic strengthening paradigm whereby network activity is pharmacologically reduced for 48-72 hours by including the sodium channel blocker tetrodotoxin (TTX; 1 μ m) in the culture medium.

To determine how homeostatic strengthening affects features of glutamate release, optical detection of evoked iGluSNFR transients was performed as described above. While a number of studies have documented a change in the probability of glutamate release during homeostatic strengthening (Thiagarajan et al., 2005; Zhao et al., 2011), we were unable to detect a difference in the baseline release probability of TTX-treated spines as compared to controls. Because our technique was inherently biased towards synapses with a high release probability (*i.e.*, based on a general surveying for responsive spines), we reasoned that a change in release probability was potentially masked. Next, we compared the potency of electrically-evoked iGluSNFR transients from dendritic spines in control slices TTX-treated slices. We found that the mean potency of spine iGluSNFR transients from TTX-treated slice cultures were significantly larger than those of interleaved untreated control cultures (**Figure 6A,B**). To account for the possibility that the silencing paradigm increased iGluSNFR sensitivity for glutamate release, we performed two-photon uncaging experiments to present fixed levels of exogenous glutamate onto dendritic spines in both conditions. We found no difference in the response amplitudes of iGluSNFR to fixed levels of optically-evoked glutamate release (**Figure 6 Supplement 1A**), arguing against altered sensitivity as a mechanism to explain the larger events observed in the TTX-treated condition. We then reasoned that the difference in iGluSNFR response potency could be due to a higher frequency of MVR in TTX-treated slices. Our estimates of the frequency of MVR events from TTX-treated neurons, however, was only slightly elevated as compared to controls (**Figure**

6 Supplement 1B). These experiments together suggested that the increased potency of iGluSNFR transients in TTX-treated slices reflected a *bone fide* increase in the amount of glutamate release from synaptic vesicles.

To test this possibility, the amplitudes of iGluSNFR transients from all spines were pooled to generate a single distribution of event amplitudes. As compared to controls, the distribution of event amplitudes from TTX-treated neurons was shifted towards larger amplitudes. This effect was strikingly reminiscent of what is commonly observed for quantal mEPSC amplitudes during homeostatic scaling (Turrigiano et al., 1998; Kim and Tsien, 2008; Soares et al., 2013). Remarkably, when we tested whether this shift in the amplitude distribution of iGluSNFR transients was multiplicative, by iteratively performing statistical comparisons between treatment groups using a range of possible scaling factors (**Figure 6E**, for additional details see methods), we found strong evidence that the amount of glutamate released from synapses following prolonged activity deprivation was increased by a single common factor (**Figure 6C-E**). We generated a scaled TTX distribution by dividing all raw iGluSNFR response amplitudes in the TTX condition by this single ‘best’ scaling factor (TTX scaled; **Figure 6E**), and found that it was nearly identical to the control distribution of iGluSNFR amplitudes. Taken together, these results indicate that glutamate release was globally increased by a factor of 1.27 at synapses that had been previously silenced. These results therefore reveal a novel mechanism through which the efficacy of synaptic transmission is globally refined during homeostatic

plasticity at hippocampal synapses. Since a uniform increase in the amount of glutamate release at synapses is expected to translate to elevated postsynaptic responses, this mechanism is likely to contribute to the uniform scaling of quantal amplitudes at CA1 synapses.

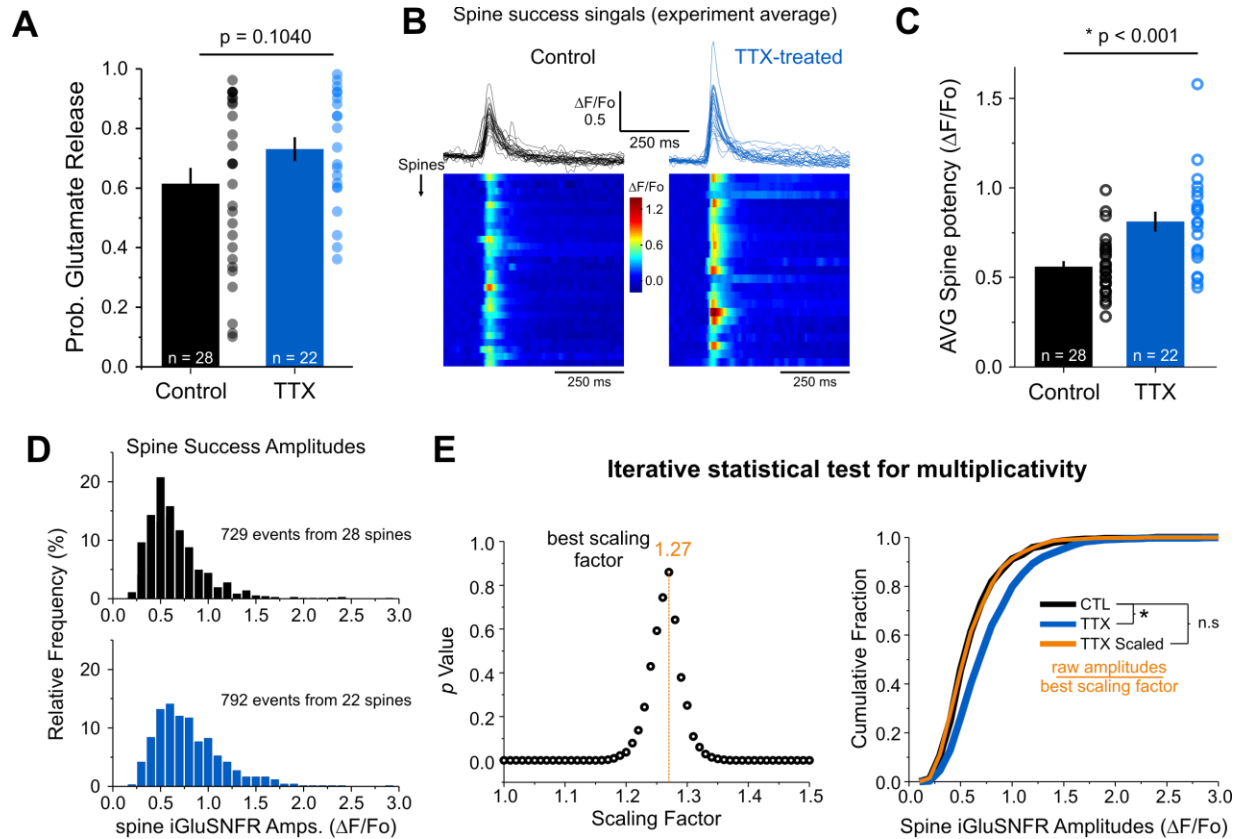


Figure 6: Multiplicative scaling of quantal glutamate release in response to prolonged activity deprivation. (A) Release probabilities at spine from control and TTX-treated neurons estimated by optical quantal analysis of evoked release using iGluSNFR. An unpaired t-test was used for statistical comparisons. (B) Average success traces were generated for spine experiment and overlaid to show the distribution of average spine iGluSNFR potency in both conditions. (C) Average amplitudes of iGluSNFR transients were increased at synapses from TTX-treated neurons. An unpaired t-test was used for statistical comparisons. (D) Raw spine iGluSNFR amplitudes pooled across all experiments in each treatment condition. (E) Results from an iterative statistical test to determine whether the increase in iGluSNFR event amplitudes in the TTX-treated distribution was multiplicative (see methods). A two-sample KS test was used for all statistical comparisons of ‘TTX-scaled’ distributions versus the control distribution.

Discussion:

In this study, we have used a recently developed genetically-encoded optical glutamate sensor to examine several features of glutamate release at single hippocampal synapses. We observed that the optical glutamate sensor, iGluSNFR, linearly reports extracellular glutamate concentration at dendritic spines within the dynamic range observed following vesicular release. Using optical quantal analysis, we estimated the trial-to-trial variability in glutamate release during low-frequency evoked stimulation. By estimating and calibrating the variability of both the iGluSNFR signal and AMPAR-mediated EPSCs in response to presentations of nominally fixed concentrations of glutamate release, we further infer that the variability in glutamate released at a single synapse is the dominant factor accounting for the variability of postsynaptic quantal responses, as previously suggested. The variability in the amount of glutamate release was, in part, attributable to multivesicular release, which occurred infrequently during low-frequency stimulation but more readily during periods of enhanced activity. Finally, we identified a novel mechanism of homeostatic strengthening whereby a multiplicative increase in the amount of glutamate released by presynaptic terminals contributes to the uniform scaling of synaptic strengths.

The optical glutamate sensor provides a direct estimate of glutamate release that is independent of ionotropic glutamate receptor activation. Using this approach, we observed a wide range of glutamate release probabilities at single spines (Range 0.1 to 0.96; **Figure 6A**).

Our estimate of P_r (*i.e.*, averaged over all spines sampled) is slightly higher than that previously reported using electrophysiological approaches. This may be accounted for by a general bias of optical quantal analysis techniques towards sampling synapses with higher release probabilities since these experiments hinge on an optical surveying of the dendritic arbor for responsive spines and, on average, will likely under sample synapses with very low P_r . Alternatively, it also remains a formal possibility that electrophysiological detection of release may underestimate true P_r because of the failure of release events of small amplitude to induce AMPAR-mediated currents, as previously suggested (Richards, 2009). In support of this scenario, we could readily induce by 2P-uncaging iGluSNFR-transients of small amplitudes that failed to induce any measureable AMPAR-mediated current recorded at the soma (**Figure 1F**). In principle, it is possible that a population of glutamate release events may not be detected by postsynaptic AMPARs. Given that 2P-uncaging of glutamate only approximates the temporal and spatial properties of vesicular release in the synaptic cleft, and given the limited ability of the iGluSNFR-transients to provide accurate estimates of glutamate concentration, future studies will be required to formally test this possibility.

The extent to which the variability in the amount of glutamate released contributes to quantal amplitude fluctuations at single synapse has been debated. Our optical detection of evoked glutamate release using iGluSNFR showed an average coefficient of variation of 0.25 in the trial-to-trial variability in the amount of glutamate released (**Figure 3**), which is slightly

lower than the quantal response amplitudes variability at the same synapses (Hanse and Gustafsson, 2001). By subtracting the variability induced by the noise of the iGluSNFR-mediated signal *per se* (by repeatedly presenting nominally equal concentrations of glutamate by 2P uncaging), our estimate of the variability in quantal amplitudes fluctuations was largely independent of the stochastic variability inherent in our method of optical detection. The factors that contribute to the variability in transmitter release at central synapses remain an outstanding question. For one, vesicle diameters can vary by approximately 11-13%: glutamate content, assuming equal volumetric vesicular filling, can thus vary by almost 40%. Second, the mode of vesicle fusion (*i.e.*, full collapse vs kiss-and-run) can impact the profile of glutamate release (Choi et al., 2003; Richards et al., 2005; Richards, 2009; Alabi and Tsien, 2013). Future studies will be required to further unravel the mechanistic underpinnings that contribute to quantal fluctuations in the amount of glutamate released.

The simultaneous release of multiple vesicles provides a mechanism to increase the dynamic range of synaptic transmission. While some synapses primarily depend on this mode of release for reliable transmission (vonGersdorff et al., 1996; Wadiche and Jahr, 2001), univesicular release mostly dominates at cortical excitatory synapses, including CA1 synapses (Stevens and Wang, 1995). Yet, a number of studies have shown that MVR can occur at these synapses, especially in scenarios where release probability is high, such as during paired-pulse facilitation (Oertner et al., 2002; Christie and Jahr, 2006). We extend this work by providing a

quantitative estimate of the frequency of MVR at CA1 synapses during single spike transmission that amounts to approximately 10% of all the vesicular release events we monitored. This fraction more than doubled during repetitive trains of presynaptic stimulation at 3 Hz (22% of vesicular release events) indicating a short-term plasticity of MVR occurrences. By generating similar quantitative estimates of MVR probability in neurons subjected to a prolonged silencing paradigm, we found a slight increase in the frequency of MVR (14%) during baseline transmission. Collectively, our findings thus contribute to an ever-growing literature that suggests that MVR may be a fundamental mechanism to increase the dynamic range and reliability of synaptic transmission, even at synapses that typically abide to the one-vesicle one release site hypothesis of synaptic transmission.

The multiplicative nature of homeostatic synaptic plasticity ensures that the overall tuning of synaptic drive occurs without altering the relative synaptic weight differences instilled through activity-dependent forms of synaptic plasticity (Turrigiano, 2008; Lee et al., 2014). While the regulation of AMPAR trafficking to synapses is undoubtedly a hallmark feature of homeostatic synaptic plasticity (O'Brien et al., 1998; Turrigiano et al., 1998; Thiagarajan et al., 2005; Shepherd et al., 2006; Turrigiano, 2012; Soares et al., 2013), it has been suggested that a distributed, proportional and tightly regulated increase in AMPAR content at synapses accounts for the multiplicative nature of synaptic scaling (Turrigiano, 2008). By turning our attention to the presynaptic terminal, we unexpectedly observed that the amplitude of evoked release of

glutamate was enhanced at CA1 hippocampal synapses following prolonged TTX-induced network silencing, a homeostatic paradigm that is known to multiplicatively scale quantal amplitudes in organotypic slices (Kim and Tsien, 2008; Soares et al., 2013). Remarkably, the increase in quantal glutamate release was multiplicative. Both mRNA and protein levels of vesicular glutamate transporter 1 (vGluT1) are directly modulated during homeostatic plasticity (De Gois et al., 2005), whereby network inactivity increases vGluT1 expression. Furthermore, manipulating vGluT1 expression in neurons alters vesicle filling glutamate (Wojcik et al., 2004; Wilson et al., 2005), a relationship that is shared by other vesicular transporter / neurotransmitter systems (Takamori, 2016). Thus, the amount of glutamate released at single synapses is homeostatically regulated. This process is likely an important contributor to the multiplicativity feature of homeostatic synaptic strengthening commonly observed.

Altogether, by implementing a quantal analysis relying on a genetically-encoded glutamate sensor, we have expanded upon various fundamental features of quantal glutamate release at single hippocampal synapses. At least one of these features, the vesicular packing of glutamate, is under homeostatic control and can be modified to increase transmission efficacy uniformly across all synapses in order to balance cellular excitability. The all-or-none stochastic nature of neurotransmitter release at central synapses has long been considered to be an inherent and computationally profitable feature used by neurons to transmit information. By showing that the amount of glutamate release is exquisitely regulated by an activity-dependent plasticity

mechanism, our results raise the intriguing possibility that the amplitude of individual quantal events may also be an integral determinant of the neural code.

Supplemental Figures:

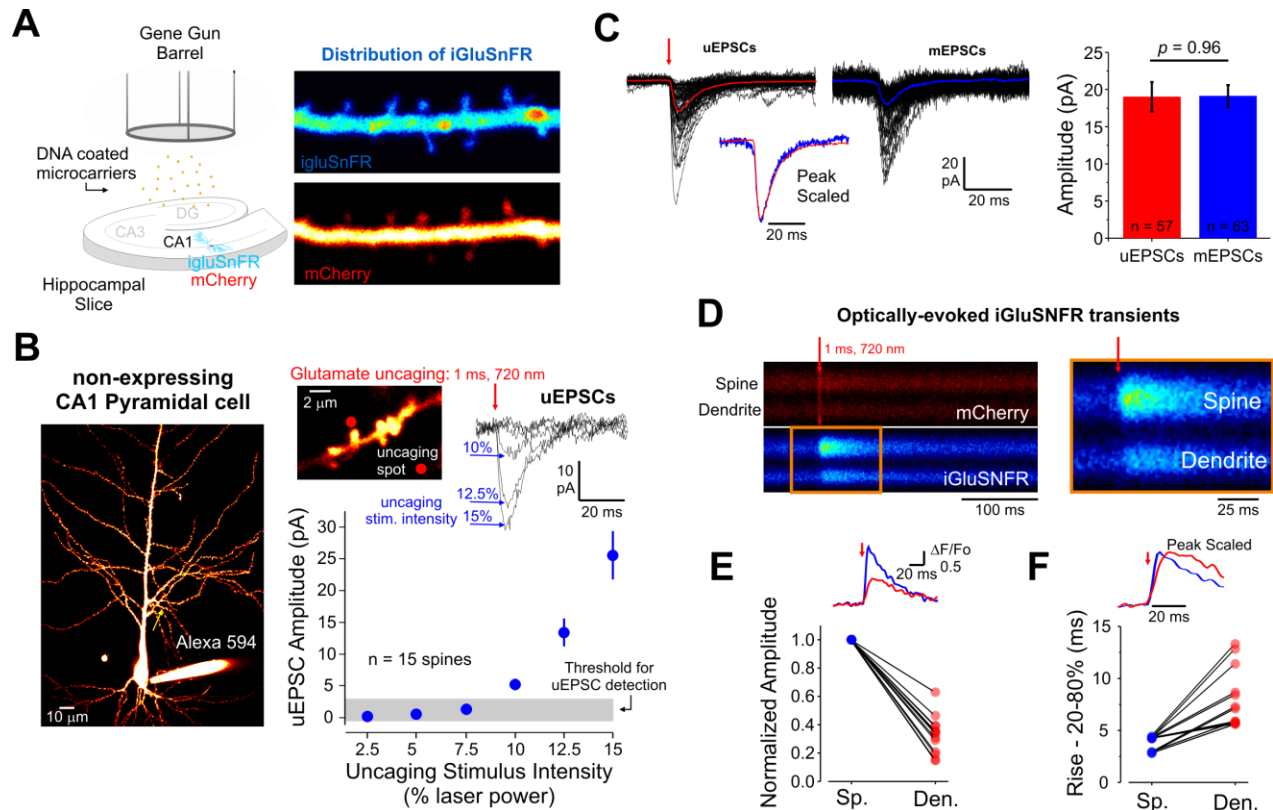


Figure 1 Supplement 1: (A) Subcellular distribution of iGluSNFR and morphological marker mCherry in a short segment of a CA1 pyramidal neuron. (B) Measurements of uncaging-evoked EPSCs recorded in voltage clamp (-70 mV) at dendritic spines of non-transfected CA1 pyramidal neurons. Alexa 594 (20 μ M) was added to the internal solution to enable visualization of dendritic morphology by two-photon imaging at 810 nm. (C) A comparison of all uEPSCs recorded from all transfected and non-transfected cells (at 12.5 and 15% l.p.) and mEPSCs from a single voltage clamp recording. Cells were voltage clamped at -70 mV. Unpaired student's t-test was used for statistical comparison. (D) Enlarged view iGluSNFR signals resulting from two-photon glutamate uncaging. (E) Paired comparison of optically-evoked spine and dendrite iGluSNFR amplitudes. (F) Paired comparison of optically-evoked spine and dendritic iGluSNFR rise times (20-80%).

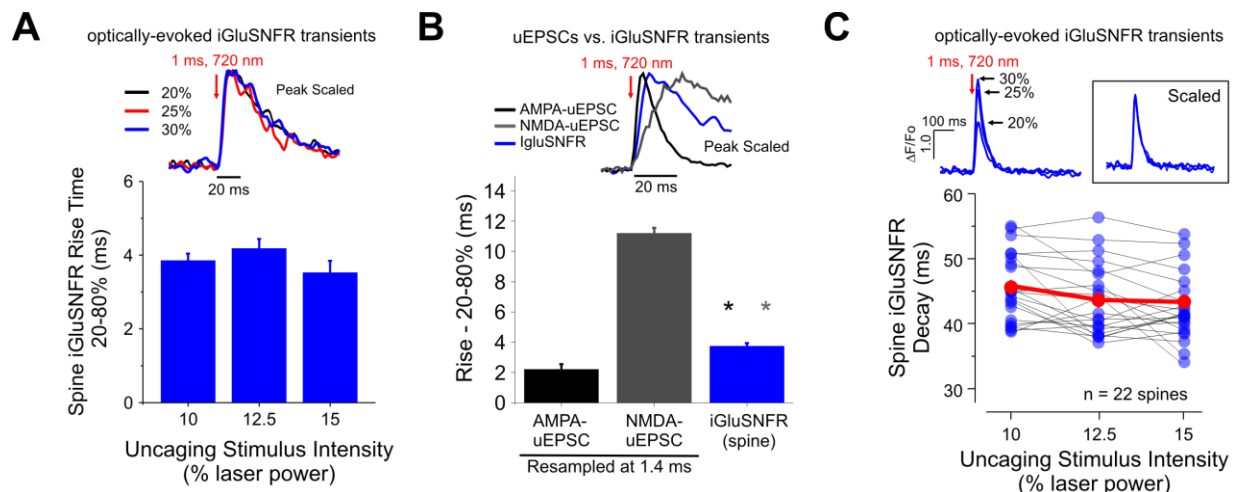


Figure 1 Supplemental 2: Biophysical properties of iGluSNFR activation. (A) Average rise times (20-80%) of optically-evoked iGluSNFR transients in dendritic spines resulting from different uncaging stimulus intensities. None of the paired comparisons were statistically significant (all p values > 0.05, paired student's t-test). (B) Average rise time of optically-evoked uEPSCs (AMPA and NMDA-mediated) and spine iGluSNFR transients elicited by uncaging at a fixed stimulus intensity. AMPA and NMDA-uEPSCs were resampled to match the acquisition rate of our optical experiments (1.4 ms sampling frequency). Rise kinetics of iGluSNFR activation were significantly slower than that of AMPARs ($p < 0.001$) but faster than that of NMDARs ($p < 0.001$). (C) Decay kinetics of spine iGluSNFR signals. Decays were best fit by a mono-exponential function.

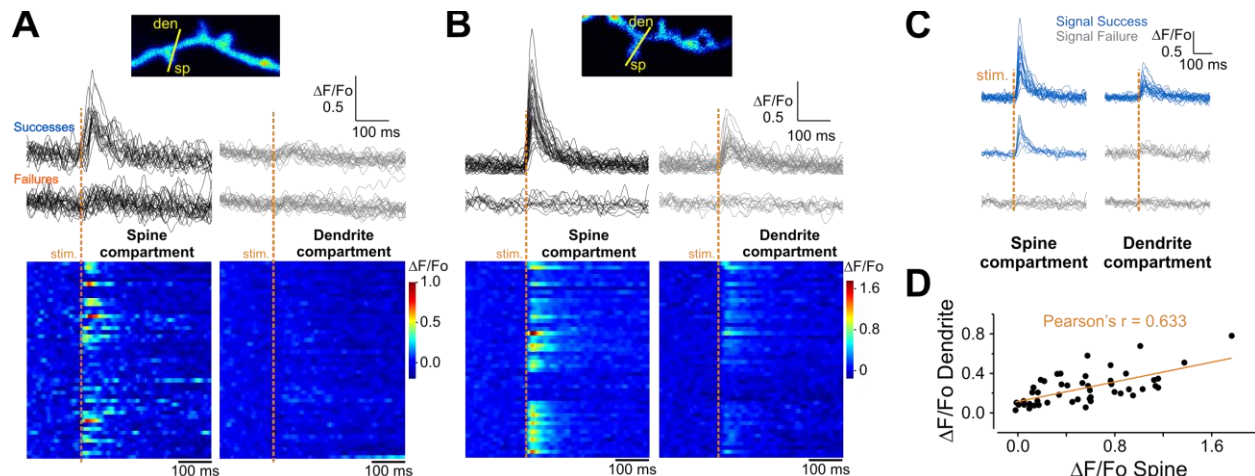


Figure 2 Supplemental 1: Dendritic iGluSNFR signals are the result of glutamate spillover. (A) An example experiment where iGluSNFR transients were restricted to the spine compartment. (B) An example experiment where iGluSNFR transients were observed in both spine and dendrite compartments. (C) iGluSNFR traces for the example shown in panel B. Dendritic signal successes were typically associated with the largest spine transients. (D) Same spine example shown in panel B. Spine iGluSNFR amplitude is linearly correlated with dendritic amplitudes.

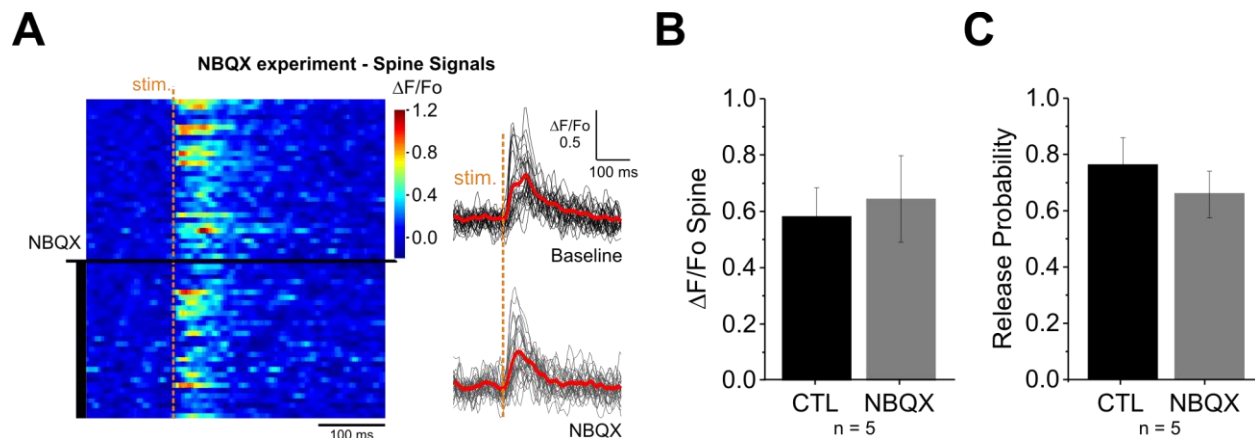


Figure 2 Supplemental 2: Features of glutamate release are unaltered following NBQX administration. (A) An example experiment where non-competitive AMPA receptor antagonist, NBQX (20 μ M) was administered while recording evoked iGluSNFR transients at a single spine. (B) NBQX administration did not alter the potency of iGluSNFR signals at dendritic spines ($p = 0.45$, paired student's t-test). (C) NBQX administration did not alter the estimated release probability ($p = 0.33$).

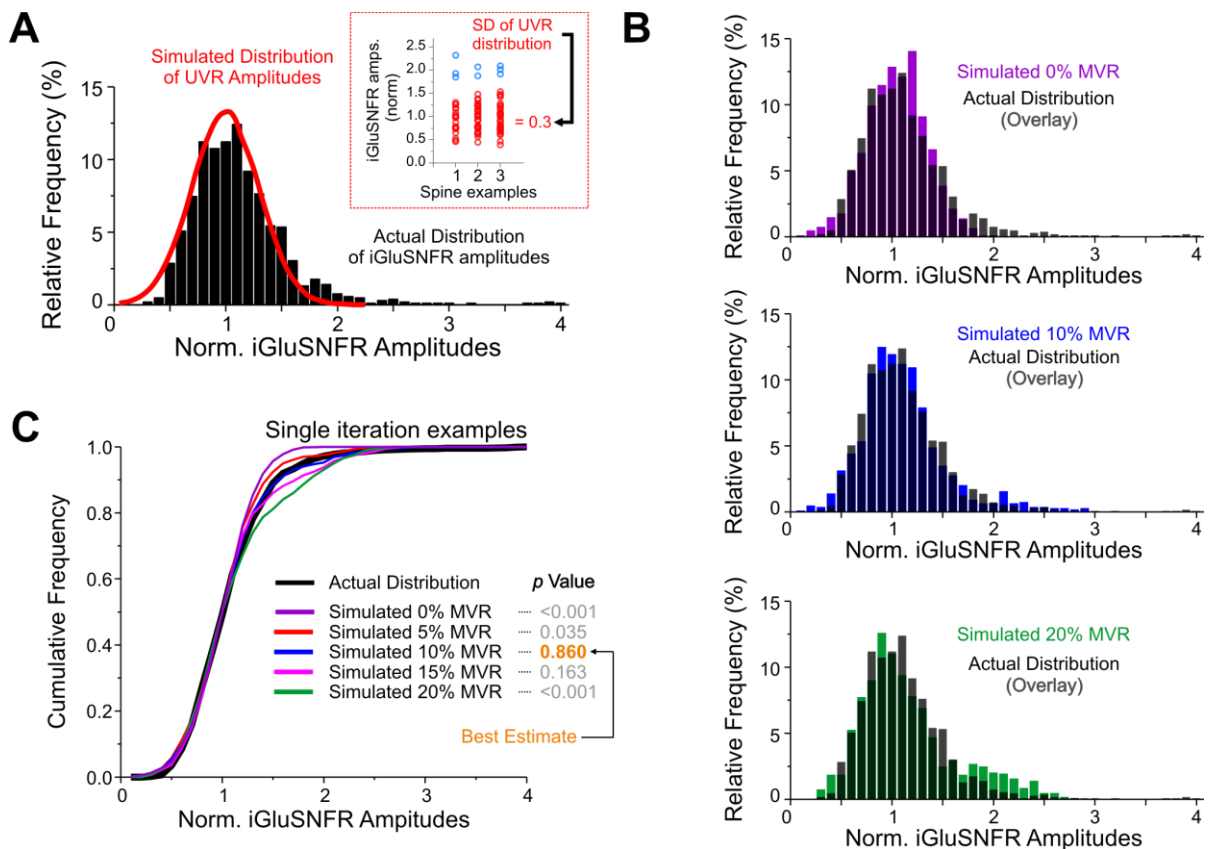


Figure 4 Supplemental 1: An iterative statistical test to estimate the frequency of multivesicular release. (A) A series of simulated distributions, each with a distinct probability of multivesicular release were generated by randomly drawing event amplitudes from the expected univesicular release distribution, which is shown overlaid (red) on the actual experimental distribution of normalized event amplitudes. This normal distribution had a standard deviation (0.3) that was calculated based on three experiments where the UVR and MVR distribution were sufficiently separated to allow unambiguous discrimination. MVR was simulated by summing two randomly drawn event amplitudes. (B) Example simulated distributions ($n = 729$ event amplitudes; matched to the experimental dataset) generated by setting the probability of MVR at 0% (purple) 10% (blue) and 20% (green), respectively. (C) Examples of simulated distributions of event distributions at varying set probabilities of MVR. A two-way KS test was used for statistical comparisons. A total of 50 simulated distributions were generated and tested for each probability of MVR tested, and an average p value was calculated to determine the best estimate.

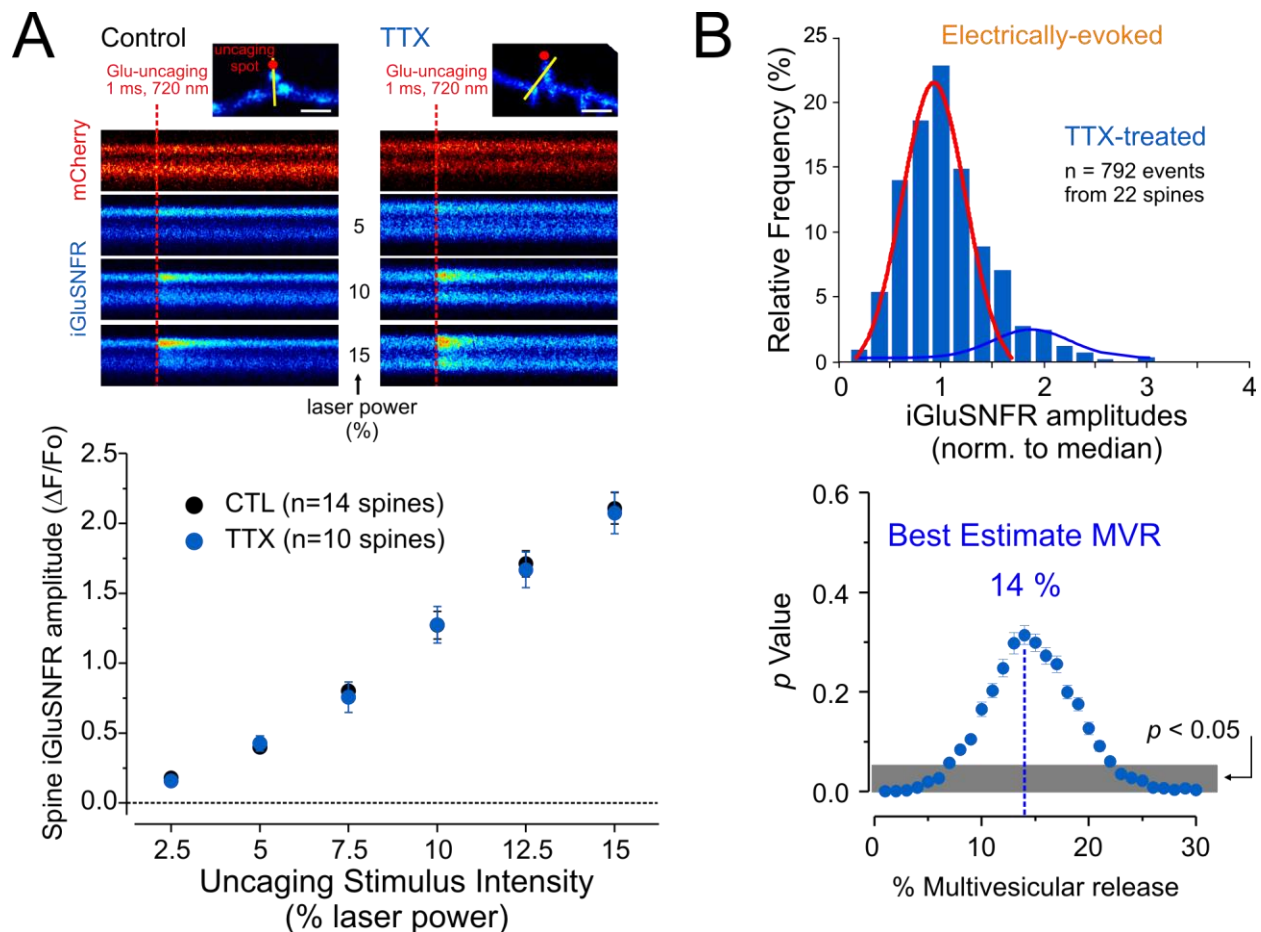


Figure 6 Supplemental 1: The increase in iGluSNFR event amplitudes during homeostatic plasticity was not attributable to either and increased sensitivity or increase in the frequency of MVR. (A) Set levels of glutamate were presented to dendritic spines in each treatment condition by increasing the stimulus intensity for glutamate uncaging. (B) Estimation of the frequency of MVR following network silencing based on the pooled distribution of normalized iGluSNFR event amplitudes across all spine experiments.

Materials and Methods:

Organotypic Slice Culture and Biolistic Transfection

Organotypic hippocampal slices were prepared as described previously (Soares et al., 2014), which is a modified method based on the original interface technique (Stoppini et al., 1991). Briefly, male and female Sprague Daley rats (Charles River Laboratories, USA; 6-8 d old) were sacrificed according to procedures approved by the University of Ottawa Animal Care Committee. Hippocampi were removed and placed in ice cold cutting solution. Transverse hippocampal slices (400 μm) were obtained using a MX-TS Tissue Slicer (Siskyou, USA), and transferred to membrane culture inserts (Cat. # PICM03050, Millipore) in a 6-well plate. Slice culture media was exchanged on the first day after dissection, and every 2-3 days thereafter. Network silencing was induced at 6-7 DIV by adding 1 μM tetrodotoxin to the culture media.

Biolistic transfection of hippocampal slices was performed at 6-7 days in vitro using a hand held gene gun (Bio-Rad Laboratories). Details for the preparation of gene gun cartridges and the biolistic transfection technique are previously described in detail (Soares et al., 2014). Briefly, 30-50 μg of cDNA plasmid (~80% iGluSNFR, ~20% mCherry) was added to 100 μl of 0.1 M KH_2PO_4 buffer solution containing 8-10 mg of gold microcarriers (1.0 μm diameter, Bio-Rad) and 0.05 mM protamine sulfate. The DNA-gold mixture was then washed and suspended in ethanol for uptake into hollow tubing designed for the gene gun (Bio-Rad). After cartridge

preparation, cartridges were loaded into the revolver of the gene gun attached to a pressurized helium gas tank. Slices were removed from incubator and shot using 150-180 psi through a modified gene gun barrel (Soares et al., 2014) and returned to the incubator for 3-5 days prior to experiments.

Two-photon imaging of glutamate transients in dendritic spines

Slices were transferred to an imaging chamber on a BX61WI upright microscope (60x/1.0 NA objective; Olympus, Melville, NY) and continuously perfused at room temperature in ringer solution containing (in mM): 119 NaCl, 2.5 KCl, 4 MgSO₄, 4 CaCl₂, 1.0 NaH₂PO₄, 11 glucose, 26.2 NaHCO₃ and 1 Na-ascorbate. Dendritic spines in the apical arbor of transfected CA1 pyramidal neurons were used exclusively in this study. The imaging laser (Mai Tai, Spectra-Physics) was tuned to 950 nm to excite both iGluSNFR and mCherry. Emissions were separated using a 570 nm dichroic mirror and additionally filtered using separate bandpass filters (green, 495-540; iGluSNFR; and red, 575-630nm; mCherry). For two-photon uncaging experiments, 2.5 mM MNI glutamate-trifluoroacetate (Femtonics, Hungary) and 1.0 μ M tetrodotoxin (Tocris) were added to the ringer solution, and a second laser (Mai Tai, Spectra-Physics) was tuned to 720 nm and delivered brief (1 ms) pulses to the tips of dendritic spines to uncage glutamate. Glutamate transients were detected by imaging line scans (~1.2 - 1.5 ms / line, 1000 lines) through the spine of interest and adjacent dendritic compartment. For evoked experiments, a glass stimulating electrode was positioned in stratum radiatum of CA1 adjacent to

the cell of interest. The orientation of the setup was such that the stimulating electrode was always positioned between the transfected cell and the CA3 hippocampal subfield. Short duration (0.1 ms) low intensity (10-50 μ A) stimuli were delivered from the electrode at a frequency of 0.1 Hz using an Isoflex stimulus isolator (A.M.P.I., Israel) and line scans were performed simultaneously on multiple nearby dendritic spines. Only responsive spines, determined by whether they displayed a glutamate transient in at least one of 5-10 paired pulse stimuli (100 ms inter stimulus interval), were used for analysis. Once responsive spines were identified, the stimulus intensity was gradually reduced to a minimum without losing time-locked glutamate responses. Synchronization of imaging and optical or electrical stimuli was achieved using an Axon Digidata 1440A (Molecular Devices, USA).

Whole-cell electrophysiology and analysis of synaptic currents

For simultaneous whole-cell recordings and two-photon imaging experiments, transfected CA1 pyramidal neurons were targeted with glass microelectrodes and cells were voltage-clamped at -70 mV. Borosilicate glass recording electrodes were pulled using a PC-10 vertical puller (Narishige, Japan) and had resistances ranging from 3-5 M Ω . Electrodes were filled with an internal solution (pH = 7.2-7.3; 270-280 mOsm/L) containing (in mM): 115 cesium methane-sulfonate, 0.4 EGTA, 5 tetraethylammonium-chloride, 2.8 NaCl, 20 HEPES, 3 ATP-Mg, 0.5 GTP, 10Na-phosphocreatine (all purchased from Life Technologies), and 5 QX-314 (Abcam).

Whole-cell recordings were acquired using an Axon Multiclamp 700B amplifier (Molecular Devices, USA), filtered at 2 kHz and digitized at 10 kHz using an Axon Digidata 1440A (Molecular Devices). Uncaging-evoked, AMPAR-mediated excitatory post synaptic currents (AMPA-uEPSCs) were recorded at the soma following brief optical stimuli (1 ms at 720 nm) delivered to the tips of the dendritic spines. For all whole cell recordings of uncaging-evoked EPSCs, 1 μ M tetrodotoxin was added to the Ringer solution. For uncaging power curves, 5-15 optical stimuli were delivered at each uncaging intensity and electrophysiological signals were averaged prior to amplitude analysis. Uncaging-evoked AMPAR-mediated EPSCs were analyzed in Clampfit (Molecular Devices).

Analysis of iGluSNFR signals

Images were immediately imported into MATLAB for processing. A custom was used to automatically detect spine and dendrite compartments based on the profile of mCherry signal intensity across the line. Line scans were reduced by a factor of 4 by averaging successive points to minimize optical detection noise. Pixel intensities across the identified compartments were summed at each binned time point. Signals were then background subtracted, and normalized to the baseline (F_0) and expressed as $\Delta F/F_0$. In all experiments, transmission successes were identified as having peak amplitudes (0 – 100 ms after the stimulus) that exceeded 4X the standard deviation of the baseline noise. To calculate release probability, 50 consecutive stimuli

were delivered at 0.1 Hz, and the number of success was divided by the total number of trials. For 3 Hz stimulation experiments, line scan duration lasted 30 s and 90 consecutive stimuli were delivered. Each event amplitude was normalized to a 50 ms baseline period prior to each stimulus presentation, and again, success amplitudes were those that were higher than 4x the standard deviation of baseline noise.

To quantitatively infer the frequency of multivesicular release (MVR) based on the amplitudes of single spine iGluSNFR transients, amplitudes for each spine experiment were normalized to their median amplitude. This was done for several reasons. First, there was variability in the average potency of iGluSNFR transients across spine experiments (see **Figure 6B**), which justified including a normalization step before pooling the data into a single distribution. Because the distribution of event amplitudes for most spine experiments was skewed towards larger events, we reasoned that the median value (rather than the mean) would be more representative of the average univesicular release (UVR) response amplitude. Once a distribution of normalized events was generated, it was then tested for significance (Two-sample Kolmogorov-Smirnov test) against a series of simulated distributions each conferring a distinct frequency of probabilistic MVR (0 – 50 %, 50 simulated distributions for each frequency). These distributions were generated in MATLAB using a *normrnd* function that randomly selects a value from a normal distribution with a customized mean and standard deviation. This was based on the assumption that a distribution of event amplitudes following purely UVR would be

normally distributed. The standard deviation of this normal distribution was set to 0.3, which was calculated based on clear spine examples where putative multivesicular release events were well separated from the distribution of UVR (see **Figure 4C**), and therefore, the standard deviation of UVR could be measured directly. To simulate MVR, two event amplitudes were drawn from this normal distribution and their values were summed. The sample size of these simulated distributions was chosen to match the experimental dataset. By generating an average p value for simulated distributions (50) at each frequency of MVR, we were able to determine which frequency of MVR best estimated (highest p value, most similar) the occurrence frequency in our experimental distribution of iGluSNFR amplitudes.

The iterative test for multiplicativity, which was adopted from our previous work where multiplicative scaling was tested on distributions of miniature quantal EPSCs (Soares et al., 2013), was performed on raw iGluSNFR amplitudes (**Figure 6D**). For this analysis, a range of scaling factors (1 – 1.5) were tested. iGluSNFR amplitude in the TTX-treated condition was divided by the scaling factor to produce a ‘TTX-scaled’ distribution, which was then compared to the control distribution of iGluSNFR amplitudes using a two sample Kolmogorov-Smirnov test.

General Discussion:

In the introduction of this thesis, I explained why the brain bears features that would seem to render it a particularly vulnerable substrate for issues of electrical instability. Yet somehow, despite being composed of hundreds of billions of functionally independent, interconnected and electrically excitable cells, our brain typically operates with functional precision, enabling us to consciously think and coordinate tasks, all the while sensing and intervening subconsciously when vital physiological variables sway too far away from their targeted set points. So what are the active mechanisms that ensure that our brain operates with functional stability, and avoids the undesirable consequences of losing control of its stable state? It was this general line of questioning that guided me towards the experiments that I performed while pursuing my PhD in Neuroscience, which you as the reader have just absorbed in reading the main body of this thesis. What I hope to have demonstrated is that individual neurons have adopted several strategies to balance their own functional dynamics, and that these cellular adaptations play an important role in stabilizing neuronal excitability so that neurons can optimally participate in complex brain functions. In the closing paragraphs of this thesis, I will provide an extended discussion on the main findings and expand on what I believe to be the most important and exciting questions that have emerged from this work that will be relevant for future studies.

Activity-dependent changes in the strength of synaptic connections between neurons is widely assumed to be the mechanism through which our brain encodes and stores information. According to this ‘synaptic plasticity and memory’ hypothesis (Martin et al., 2000; Neves et al., 2008b; Takeuchi et al., 2014), patterns of synaptic activity that are driven by our sensory experiences will cause persistent alterations to the brain’s fine circuitry such that a ‘memory

trace' is formed. Furthermore, memory recall requires the selective reactivation of neural circuits that were originally involved in the formation of the memory trace (Han et al., 2009; Liu et al., 2012). An overarching implication of this theory is that the functional strength of any given synapse in the brain is relevant in that it stores some form of information that was inscribed through experience-driven Hebbian modifications. An intriguing aspect of the homeostatic response to inactivity is that the refinement of synaptic transmission appears to occur without altering the relative differences in synaptic strengths on any given neuron, but rather, preserves these differences by increasing synaptic efficacies in a global and multiplicative fashion (Turrigiano et al., 1998; Kim and Tsien, 2008; Soares et al., 2013). Thus, through mechanisms of multiplicative homeostatic synaptic scaling, neurons are able to actively tune their excitability by altering synaptic drive in a way that ensures that memory traces that were engrained through Hebbian plasticity processes are preserved.

A central question that has been difficult to address experimentally and remains largely unresolved is what are the precise mechanisms that drive the multiplicative scaling of excitatory synaptic strengths? A longstanding theory, one that continues to dominate at present, is that synaptic scaling is achieved by an orchestrated response involving the postsynaptic trafficking of glutamate receptors to or from synapses, depending on whether the synapses are scaling up or down, respectively (Turrigiano, 2008; Wang et al., 2012). By most accounts, network silencing does lead to an increased surface expression and a dynamic reconfiguration of glutamate receptors at excitatory synapses, however, there is little indication that this mechanism is causally related to the enhanced efficacy of synaptic transmission. While it seems obvious that having more glutamate receptors in the postsynaptic domain will increase synaptic responsiveness to glutamate release, there is evidence to suggest that this may not necessarily be

the case. We know from a number of experiments that quantal transmission does not saturate postsynaptic receptors (Mainen et al., 1999; McAllister and Stevens, 2000; Ishikawa et al., 2002; Nimchinsky et al., 2004). Moreover, based on the results of some very intricate models of quantal transmission, synaptic AMPARs further than ~250 nm away from the release site will not be activated since glutamate concentration drops dramatically with distance from the release site (Franks et al., 2003; Raghavachari and Lisman, 2004). What this means is that increasing the total number of AMPARs in the postsynaptic domain, without increasing the density at which they are stabilized, may not necessarily contribute to a measureable difference in the postsynaptic response to glutamate release. Findings such as these argue against the current dogma that the postsynaptic regulation of AMPAR numbers *per se* could explain the homeostatic scaling of synaptic strengths. It will thus be important for future studies to determine whether the clustering of glutamate receptors in the postsynaptic domain is influenced during homeostatic plasticity, since changes in receptor density alone might be sufficient to underlie changes in synaptic efficacy (Raghavachari and Lisman, 2004; MacGillavry et al., 2013; Tang et al., 2016). At this point, I would like to state that my purpose is not to discredit the vast literature showing an altered postsynaptic regulation of glutamate receptors during the homeostatic response to inactivity, but rather, to simply highlight that, at least to my knowledge, it has never been demonstrated explicitly that this orchestrated trafficking response of glutamate receptors causes the multiplicative scaling of excitatory synaptic strengths.

Over the course of my experience in graduate school, my opinion on the matter of what mechanism drives the multiplicative scaling of excitatory synaptic strengths following network silencing has matured. Initially, my assumption was that the scaling of synaptic strengths following network silencing was caused by a homeostatic increase in the number of postsynaptic

AMPA receptors. This changed however, when gathering the data that is presented in Chapter 1. I found that the postsynaptic response to inactivity involved the surface trafficking and synaptic targeting of higher conducting AMPAR subtype than observed in control conditions (Lu et al., 2009; Soares et al., 2013). This change in AMPAR subunit composition was observed in all subcellular compartments, including at synapses, along extrasynaptic regions of the dendritic space, and at the soma. Based on these findings, I reasoned that the synaptic incorporation of higher conducting AMPARs could, in theory, explain synapse strengthening during HSP. Much like other groups before us, however, I failed demonstrate that the effect we characterized (the upregulation of higher-conducting GluA2-lacking AMPARs) was causally related to the multiplicative scaling-up of synaptic strengths. It was reported at nearly the same time that my work was published that the multiplicative scaling-up of excitatory synaptic strengths was independent of a change in AMPAR subunit composition (Altimimi and Stellwagen, 2013), since synaptic strengthening was observed in cortical cultures prepared from GluA1^{-/-}, GluA2^{-/-}, and GluA2^{-/-}GluA3^{-/-} mice, arguing against this mechanism as a model that can account for multiplicative scaling. It was not until I began analyzing data found in Chapter 3 of this thesis, which represents experiments that were carried out most recently, that I came to my current view which is that a presynaptic mechanism might be a principal contributor to the multiplicative scaling of synaptic strengths. Indeed, I found that prolonged network inactivity increased the amount of glutamate released per vesicle onto CA1 synapses, and that this increase showed features of multiplicativity. Since postsynaptic glutamate receptors at a single synapse are not saturated during quantal release (Forti et al., 1997; Liu et al., 1999; McAllister and Stevens, 2000; Conti and Lisman, 2003), a homeostatic increase in the amount of glutamate released by synaptic vesicles would translate to an enhanced postsynaptic response, irrespective of changes

postsynaptically. Again, while our results fail to demonstrate causality between these two metrics (*i.e.*, enhanced glutamate release and uniform scaling of quantal EPSCs), the fact that quantal glutamate release increases by a common factor across all synapses following network silencing is convincing that this effect contributes to the multiplicative scaling-up of synaptic strengths. This mechanism is consistent with biochemical reports in which both mRNA and protein expression of vesicular glutamate transporters (vGluTs) are subject to homeostatic regulation. Specifically, vGluT1 mRNA and protein in cortical cultures increases during network silencing and this is expected to translate directly to enhanced quantal packing of glutamate (De Gois et al., 2005; Wilson et al., 2005). Collectively, these data suggest an alternative model that can account for the multiplicative scaling of quantal EPSCs during HSP. Through homeostatic alterations in vGluT expression, synaptic drive is bidirectionally influenced by the amount of glutamate packaged and released from synaptic vesicles, which in turn alters synaptic efficacies in a direction that is homeostatic in nature. It will be important to elucidate how changes in presynaptic glutamate packaging interact with known homeostatic adaptations occurring postsynaptically, to ultimately dictate functional changes that are expressed uniformly across all synapses.

For what purpose do CA1 neurons globally upregulate GluA2-lacking AMPARs during the homeostatic response to inactivity, if it does not contribute to functional strengthening? One possible explanation is that neurons are trying to manage an intracellular calcium homeostasis. Calcium is a ubiquitous second messenger in neurons that serves a wide range of physiological functions. Of relevance here, local changes in intracellular calcium triggered by synaptic activity can cause various rapid forms of pre- and postsynaptic plasticity (Zucker, 1999; Catterall et al., 2013), and over much wider spatiotemporal scales, intracellular calcium levels are thought to

play a role in the transcriptional regulation of genes involved in neuronal growth, development and survival (West et al., 2001). Neurons actively maintain cytosolic calcium levels at a low concentration (Berridge et al., 2000) by extrusion mechanisms that involve pumping it into various intracellular stores such as the endoplasmic reticulum or mitochondria, or into the extracellular space, using various types of calcium pumps (eg. endoplasmic reticulum localised SERCA pumps) and exchangers (eg. the plasma membrane bound $\text{Na}^+/\text{Ca}^{2+}$ exchanger). External calcium enters the cell via two major pathways (Grienberger and Konnerth, 2012). The first of is through voltage-gated cation channels (eg. the CaV family of calcium channels), which can be gated by depolarizations driven by either synaptic activity or from back-propagating action potentials. The other source of external calcium influx is from synaptic receptors which, at CA1 synapses, comes almost exclusively from NMDARs since the majority of AMPARs in baseline conditions are calcium impermeable (i.e., predominantly GluA2-containing). Although there are other mechanisms through which calcium can enter the cell, what this implies is that at least two of the major sources of external calcium entry depend on membrane depolarization. Thus, during periods of activity deprivation that result in both reduced cell firing and reduced synaptic activity, both external sources of calcium entry are effectively reduced. GluA2-lacking AMPARs exhibit calcium permeability (Hollmann et al., 1991), and while the majority of network driven synaptic activity is abolished during TTX-silencing, some small amount of synaptic activity persists due to the spontaneous fusion of synaptic vesicles. The upregulation of GluA2-lacking AMPARs may therefore be part of the homeostatic response to inactivity to provide neurons with a much needed source of extracellular calcium in the absence of membrane depolarizations, suggesting that this cellular adaptation is directed towards calcium, rather than electrical, homeostasis. There is now an emerging literature linking impairments in calcium homeostasis to

neurodegeneration during normal ageing and in models of neurological disease (Gleichmann and Mattson, 2011; Nikolettou and Tavernarakis, 2012; Brini et al., 2014). Homeostatic synaptic plasticity and its impact on intracellular calcium regulation may thus be a relevant avenue to pursue experimentally to both identify the cellular etiology of these diseases and to develop therapeutic strategies that might combat disease progression.

It is generally believed that the multiplicative scaling of quantal EPSCs is indicative that all synapses increase their function by a common factor (Turrigiano et al., 1998; Turrigiano, 2008), however, this does not necessarily mean that all synapses are affected equally. In chapter 2, I showed that silent synapses, which are synapses that contain NMDARs but do not contain AMPARs, are resistant to functional changes during the homeostatic response to inactivity. Silent synapses are present on CA1 neurons during early development but disappear during the first few postnatal weeks (Isaac et al., 1995; Beique et al., 2006; Busetto et al., 2008; Lee et al., 2016). Through activity-dependent forms of plasticity, silent synapses are thought to acquire AMPARs and thus become functionally active (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Kerchner and Nicoll, 2008). Despite the fact that most investigations directed towards understanding the cellular basis of homeostatic plasticity were conducted in developing networks (i.e., dissociated or slice cultures prepared from early postnatal rodents) which are likely to contain some proportion of silent synapses, there was no prior indication on what was their fate during the homeostatic response to inactivity. If synaptic scaling is achieved through a concerted trafficking response of AMPARs, then our results suggest that silent synapses resist AMPAR accumulation during the homeostatic response to inactivity. While it is easy to imagine this being achievable, since silent synapses by definition avoid anchoring AMPARs despite there being a vast number of AMPARs diffusing freely in the nearby extrasynaptic membrane (Matsuzaki et

al., 2001; Groc et al., 2004; Soares et al., 2013), the mechanisms through which synapses avoid AMPAR accumulation are unclear. Silent synapses might lack some critical anchoring machinery that is required to tether AMPARs in the postsynaptic domain, which are only recruited following activity-dependent synaptic strengthening. In an alternative scenario, whereby synaptic scaling is achieved through a multiplicative increase in the amount of glutamate released (Chapter 3), silent synapses may resist functional strengthening simply because, unlike synapses that contain AMPARs, more glutamate released would not contribute to a change in functional efficacy. Irrespective of the precise mechanism, the fact that silent synapses resist functional strengthening during HSP indicates that the multiplicative homeostatic strengthening of CA1 synapses, at least in developing networks where silent synapses are present, can operate with some degree of synapse-specificity.

Finally, it is important to reflect on the fact that, while I have spent my entire graduate tenure studying a truly multiplicative form of HSP at CA1 synapses, non-multiplicative forms of homeostatic synaptic plasticity exist. For instance, using a nearly identical silencing strategy that I have used (i.e., organotypic hippocampal slices incubated in TTX for several days), Kim and Tsien observed a similar multiplicative scaling at CA1 synapses but observed a non-multiplicative form of HSP at CA3 synapses (Kim and Tsien, 2008). CA3 inputs arising from the dentate gyrus (a feed-forward pathway) are homeostatically strengthened following network silencing, while recurrent CA3 inputs formed between pairs of CA3 neurons are not. CA3 neurons might have adopted this unique pathway-specific homeostatic response to balance their own activity levels, since homeostatic strengthening of the CA3 self-excitation loop would likely promote epileptogenesis (Kim and Tsien, 2008).

Whereas the aforementioned study demonstrates that synapse-specific homeostatic alterations can occur in response to a global manipulation, there are examples that demonstrate pathway specific forms HSP exist. In an elegant *in vivo* study in the optic tectum of the *Xenopus* tadpole, Deeg and Aizenman showed that optic tectal neurons display a pathway-specific form of HSP (Deeg and Aizenman, 2011) that emerges following chronic activation or suppression of a specific set of inputs. The optic tectum in the tadpole is a multisensory area which receives, amongst others, visual and mechanosensory excitatory inputs. Specifically, by chronically enhancing sensory stimulation in either the visual or mechanosensory pathway for 48h, they observed a homeostatic weakening of synaptic connections in only the manipulated pathway. Thus, while truly multiplicative forms of HSP exist in certain cell types and in response to specific extrinsic cues, there are several examples where this multiplicative rule simply doesn't hold.

These non-multiplicative forms of HSP are intriguing since they offer clues that homeostatic refinement of synaptic efficacies likely occurs at multiple levels. While some groups have argued that global homeostatic scaling results specifically from changes in neuronal firing rate (Ibata et al., 2008; Goold and Nicoll, 2010), others have argued that integrated levels of synaptic activity plays the major role (Fong et al., 2015). When we consider that synapse-specific and non-multiplicative forms of HSP exist, such as those presented above, it becomes difficult to imagine how the cell's firing rate can serve as the universal trigger for all known forms of HSP. A number of more recent studies have shown that individual synapses can sense their own levels of activity and actively compensate for it in a homeostatic-like fashion (Hou et al., 2008; Lee et al., 2010; Beique et al., 2011; Hou et al., 2011). Chronically silencing or enhancing the activity at single inputs drives the homeostatic strengthening or weakening of

synaptic transmission, respectively, in ways that are similar to what is observed when activity manipulations target the entire network (reviewed in Manuscript V, Appendix A2). Since network activity blockade by prolonged TTX-treatment both reduces synaptic activity and cell firing rates, it is likely that both cell-autonomous and synapse-specific forms of HSP are engaged. It will be important for future studies to unravel how distinct forms of HSP interact, and whether there is a mechanistic overlap in their expression mechanisms. Furthermore, it will be interesting to revisit the notion of pathway specificity and investigate how it is that CA3-CA3 recurrent synapses resist functional strengthening during global network silencing. Based on fact that multiplicative changes cannot describe all forms of synaptic homeostasis and that in some cases, homeostatic refinements may occur in small subsets of synapses, we may need to revise the common interpretation that homeostatic plasticity mechanisms act without altering the relative differences in synaptic strengths.

A case can be made that the methods used in this thesis to induce homeostatic plasticity are non-physiologic. While it is true that it is unlikely that hippocampal neurons will ever experience such a prolonged state of functional inactivity, this forceful manipulation has nonetheless revealed a diverse set of homeostatic adaptations are likely to operate continuously in the living brain. Using a ‘real world’ analogy, one might easily verify that the thermostat and furnace in their household is operating by opening all the exterior windows at a time when the temperature outside is below freezing, because this event will cause considerable disturbance to air temperature that will certainly trigger the furnace to turn on. In the absence of such a large disturbance, air temperature within the household will still remain constant. This does not imply that the same mechanisms of temperature control are dormant, but rather that they operate continuously in a more subtle fashion to maintain a constant temperature. I believe the same

rules apply for mechanisms of homeostatic plasticity. By pushing the limits of what may be considered ‘physiologic’, I forced neurons to reveal their homeostatic regulation at an extreme expression level that is enabling for us to precisely identify its underlying cellular mechanisms. I would even argue that there are neurological conditions that might induce a prolonged state of functional inactivity not too distant from what I have induced using pharmacological antagonists. For instance, an ischemic stroke that permanently damages a particular brain region is predicted to lead to a prolonged state of reduced excitatory drive in densely innervated downstream regions. Neurons in these downstream affected regions would thus respond to their diminished excitatory drive by triggering a compensatory homeostatic response similar to what I have described in my thesis. In fact, a similar form of denervation-induced homeostatic synaptic plasticity is described in hippocampal granule cells that have lost excitatory inputs arising from the entorhinal cortex following axonal severing (Vlachos et al., 2012; Becker et al., 2013). Therefore, while there are some neurological conditions that might trigger a strong homeostatic response that parallels what I have measured experimentally, I would argue that these findings have broader implications since, much like the regulation of household temperature, mechanisms of homeostatic plasticity likely act continuously, albeit at lower gain, to provide a constant balance to neuronal excitability.

Concluding Remarks:

During the course of my PhD studies, I have contributed to an ever-growing literature that suggests that neurons actively sense and maintain their excitability within a computationally optimal regime through mechanisms of homeostatic plasticity. By extension, since it is the collective activity of individual neurons that drive the global patterns of activity in the brain, these mechanisms likely contribute to the fact that our brains typically operate in a functional

equilibrium. By examining under a magnifying lens (no pun intended!) and characterizing the specific strategies utilized by single neurons to adapt to conditions of prolonged inactivity, I have uncovered several novel forms of homeostatic plasticity. This work has lead me to the ultimate conclusion that there is no single mechanism by which neurons manage their excitability, but a diverse set of cellular mechanisms that all contribute functional homeostasis.

Several outstanding questions remain, some of which I have outlined above. I'd like to end this thesis by stating the fact that, while I have spent many years describing expression mechanisms of this global homeostatic response to inactivity, I have always been curious of what are the biological sensor mechanisms that exist in neurons that enable them to sense their own levels of activity, whether it be activity at a single synapse in the case of synapse-specific forms of HSP, or neuronal output in the case of cell-autonomous forms of HSP. Neurons, of course, are not conscious of their behaviors and thus must have evolved strategies that coordinate their homeostatic regulations. It will be important for future studies to identify the biological processes that underlie homeostatic sensing and induction, which are ultimately needed to develop a complete mechanistic framework for how homeostatic regulations operate to tune the excitability of single neurons in the brain.

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Statement for Appendix A:

This appendix contains 5 additional manuscripts to which I have contributed, all of which have been published between 2012 and 2016, with the exception of Manuscript VII which is currently unpublished. Manuscripts IV and V are review articles that I helped write during the early stages of my graduate school tenure. Because they provide an additional overview of many of the topics and concepts that were discussed in this thesis, I've decided to include them in this section. Manuscript VI is a much shorter review manuscript that I wrote that summarizes and provides additional discussion on a specific scientific report published in the *Journal of Neuroscience*. Again, because of its relevance to topics discussed in this thesis, I've chosen to include it in this section. Manuscript VII is an unpublished study that comprised the majority of the experiments I performed before transferring to the PhD program. Because the work is distant from the overall theme of this thesis, being homeostatic mechanisms of synaptic plasticity, I've chosen to include it as part of this appendix, despite the fact that I am the lead author of the study. Finally, Manuscript VIII is a methods chapter that summarizes an experimental technique that I established in the lab. This chapter provides a step by step procedure that includes many helpful tips that are typically left out of a methods section. Each of the manuscripts is preceded by a disclosure statement outlining all of the author's contributions. Permissions to include these manuscripts as part of my thesis can be found on pages xi-x (see Table of Contents)

A.1

Manuscript IV

Title:

Examining form and function of dendritic spines

Authors:

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Statement of Contributions:

I contributed to writing this review manuscript from start to finish, although Kevin Lee was the lead author on this project who provided most of the final text. I participated in the creating the original outline, helped to formulate and develop the main topics discussed, reviewed prior literature and laid out many draft paragraphs. In addition, I constructed both figures, and edited draft manuscripts. Dr. Jean-Claude Béïque provided guidance to our discussion throughout the preparation of this manuscript, and edited the paper at multiple stages of its development.

Abstract

The majority of fast excitatory synaptic transmission in the central nervous system takes place at protrusions along dendrites called spines. Dendritic spines are highly heterogeneous, both morphologically and functionally. Not surprisingly, there has been much speculation and debate on the relationship between spine structure and function. The advent of multi-photon laser-scanning microscopy has greatly improved our ability to investigate the dynamic interplay between spine form and function. Regulated structural changes occur at spines undergoing plasticity, offering a mechanism to account for the well-described correlation between spine size and synapse strength. In turn, spine structure can influence the degree of biochemical and perhaps electrical compartmentalization at individual synapses. Here, we review the relationship between dendritic spine morphology, features of spine compartmentalization and synaptic plasticity. We highlight emerging molecular mechanisms that link structural and functional changes in spines during plasticity, and also consider circumstances that underscore some divergence from a tight structure-function coupling. Because of the intricate influence of spine structure on biochemical and electrical signalling, activity-dependent changes in spine morphology alone may thus contribute to the metaplastic potential of synapses. This possibility asserts a role for structural dynamics in neuronal information storage and aligns well with current computational models.

Introduction

Ever since the first description of *espinas* on Purkinje cells by Cajal more than 100 years ago (Cajal, 1888; Yuste, 2010), these tiny, femtolitre-sized, structures have been found on dendrites of a wide variety of neuronal cell types and the role of these minute structures in neuronal function has been the subject of considerable attention, speculation and debate. These discrete dendritic protrusions form a rich structural scaffold for the majority of excitatory synapses in the brain, harbouring a complement of biochemical signalling machinery as well as a proteinaceous postsynaptic density (PSDs) containing, amongst others, ionotropic glutamate receptors of the AMPA and NMDA subtypes (Traynelis et al., 2010). These receptors mediate the bulk of fast excitatory neurotransmission in the brain. During postnatal development, dendritic spines acquire AMPARs and undergo structural enlargement, resulting in a positive correlation between spine size and AMPAR function. Interestingly, the high degree of heterogeneity in dendritic spine structure and function at maturity suggests that spine growth is regulated in a synapse-specific manner and not simply a consequence of *en masse* spine development.

In the past decade or so, a number of technological developments in fluorescence microscopy and molecular techniques have greatly accelerated our understanding of the relationship between structure and function at dendritic spines. For one, the induction of synaptic plasticity at single synapses was found to result in changes in spine structure, providing a plausible mechanism to explain the concurrent developmental changes in spine size and function (Matsuzaki et al., 2004). Furthermore, recent studies have elaborated a mechanistic and molecular framework to suggest that spines function as discrete compartments, offering a basis for computationally-relevant synaptic autonomy. Based on the robust concordance between

structural and functional plasticity, and on the similarities in the molecular underpinnings that drive these two processes, there is a growing trend in synaptic physiology to infer synaptic strength based on characteristics of spine morphology. However, the dissociation of spine structure and function under some experimental conditions suggests an important mechanistic divergence in the regulation of spine form and function. In this review, we will provide an outline of the dendritic spine as a discrete functional compartment, discuss new developments in structural and functional plasticity at single spines and highlight key aspects of our understanding of the relationship between spine structure and function.

Two-photon Microscopy and the Investigation of Individual Dendritic Spines

Despite unsheathing fundamental properties of various forms of synaptic plasticity (Malenka and Bear, 2004), investigations based solely upon electrically evoked synaptic events left a number of open questions. Although minimum-stimulation methods allow the functional study of single synapses in isolation (Allen and Stevens, 1994; Dobrunz and Stevens, 1997; Isaac et al., 1996; Stevens and Wang, 1995), the inherent technical challenges of these experiments hinder the ability to efficiently amass data and resolve spatial parameters such as the morphology and location of the activated synapses (relative to each other and to the soma). The advent of two-photon (2P) laser scanning microscopy circumvented a number of these experimental limitations and has contributed considerable depth to our understanding of spine function and plasticity.

The longer-wavelengths and lower excitation energy used in 2P imaging increases imaging depth in scattering tissue (such as brain) while also reducing photo-damage/toxicity compared to 1P imaging (Denk et al., 1994; Helmchen and Denk, 2005; Svoboda and Yasuda,

2006). Furthermore, the 2P excitation event is highly restricted in physical space with an excitation volume that roughly approximates the diffraction limits of the optical system (Svoboda and Yasuda, 2006). This small excitation volume thus confers the ability to photoactivate molecules with high spatial precision, thereby providing novel opportunities for the study of synaptic physiology. For instance, 2P “uncaging” of caged forms of neurotransmitters (for eg., MNI-Glutamate) provides the ability to selectively activate spatially discrete glutamate receptors in a number of experimental preparations *in vitro* (Araya et al., 2006; Ashby and Isaac, 2011; Beique et al., 2006; Beique et al., 2011; Bloodgood et al., 2009; Busetto et al., 2008; Harvey and Svoboda, 2007; Harvey et al., 2008; Losonczy and Magee, 2006; Matsuzaki et al., 2001; Matsuzaki et al., 2004; Matsuzaki and Kasai, 2011; Tanaka et al., 2008), and *in vivo* (Noguchi et al., 2011). Pioneering work by Kasai and colleagues used 2P imaging and glutamate uncaging to probe AMPAR content and induce LTP at individual dendritic spines on hippocampal CA1 pyramidal neurons, generating key insight into single synapse plasticity (Matsuzaki et al., 2001; Matsuzaki et al., 2004). For instance, the induction of LTP at single dendritic spines via 2P glutamate uncaging circumvented the presynaptic component of synaptic transmission and provided unequivocal support to the notion that, at least under certain conditions, synaptic plasticity can be mediated by solely postsynaptic mechanisms (Kerchner and Nicoll, 2008; Matsuzaki et al., 2004).

In addition to providing important information regarding plasticity at single synapses, advances in 2P imaging and related optical techniques have been instrumental in generating novel understanding of other neuronal mechanisms and properties such as the spatial distribution of synaptic weights, the autonomy of the spine as a functional compartment, the integrative

behaviour of dendritic branches and the recurrent connectivity of cortical circuits (Ashby and Isaac, 2011; Beique et al., 2011; Losonczy and Magee, 2006; Smith et al., 2003).

A Compartmental Model of Dendritic Spines

Dendritic spines are specialized structures exhibiting a high degree of molecular organization and exist in a wide range of morphologies. Although a number of nomenclatures have been proposed to describe the breadth of morphologies that individual spines can adopt, they can be broadly summarized as follows: “Mushroom-like”, identified by a round dendritic spine head connected to the parent dendrite by a spine neck; “Stubby” spines, which are short, stout protrusions or bumps with no definitive spine neck; and filipodial/long spines, which appear as thin, finger-like protrusions (Arellano et al., 2007; Harris et al., 1992). There has been considerable speculation on the specific role imparted by these varying morphologies on aspects of spine function. For one, substantial attention has been given to the role of the spine neck and accumulating experimental evidence suggests that it serves to compartmentalize the dendritic spine head. This compartmental model is particularly attractive in light of the synapse-specificity of the structural and functional changes that take place over development and during plasticity. The compartmentalization of dendritic spines can be broadly divided into two functional domains: i) the biochemical compartment, which describes the spatial confinement of biochemical signalling due to diffusional restriction and physical segregation of proteins and signalling molecules; and ii) the electrical compartment, where spine neck morphology can impact the kinetics and propagation of synaptic potentials in a spine-specific manner. Here, we will sequentially review these two functional domains.

The Biochemical Compartment

Postsynaptic induction and expression of several forms of synaptic plasticity requires calcium influx through NMDARs and the initiation of calcium-dependent biochemical signalling in the dendritic spine. The development of calcium-sensitive fluorescent indicators and imaging techniques has greatly facilitated the study of calcium dynamics during synaptic activity. Specifically, calcium imaging experiments demonstrate that NMDAR-mediated calcium influx elicited during synaptic transmission is tightly restricted to the spine head, with minimal calcium diffusion into the parent dendrite (Busetto et al., 2008; Connor et al., 1994; Muller and Connor, 1992; Noguchi et al., 2005; Oertner et al., 2002). Given the key role of calcium as a second messenger in the regulation of synaptic plasticity, highly compartmentalized calcium signalling at dendritic spines is likely critical for providing the synapse-specificity of synaptic plasticity. As a result, it has been proposed that the primary function of the dendritic spine structure is to compartmentalize signalling molecules such as calcium (Noguchi et al., 2005; Yuste et al., 2000). Many factors can influence the intracellular diffusion of calcium. For instance, the presence of a spine neck has been suggested to restrict calcium diffusion and also appears to limit the diffusion of other molecules such as GFP and fluorescein dextran (Bloodgood and Sabatini, 2005; Muller and Connor, 1992; Noguchi et al., 2005; Svoboda et al., 1996; Zito et al., 2009). In addition, calcium pumps such as PMCAs and SERCA, calcium-binding molecules such as calmodulin (CaM) or calbindin, and differential cytosolic viscosities at individual spines can all contribute to regulate free-calcium concentrations (and its dynamics) and influence intracellular diffusion (Kenyon et al., 2010; Majewska et al., 2000; Smith et al., 2007). Together, these diverse mechanisms indicate that dendritic spines utilize multiple strategies to

compartmentalize biochemical signals and promote autonomous synaptic function (See **Figure 1**).

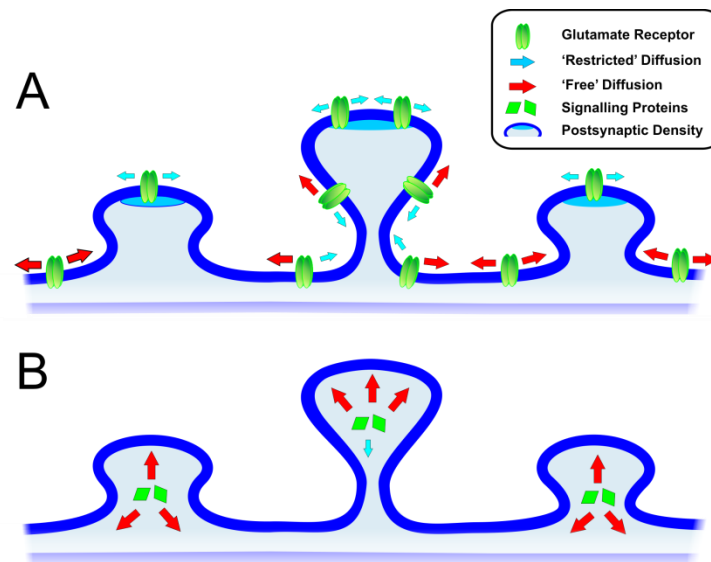


Figure 1: Biochemical compartmentalization in dendritic spines. The spine neck may offer enhanced compartmentalization of biochemical signalling at synapses. In **A**, the lateral mobility of surface glutamate receptors is attenuated across longer spine necks and at the postsynaptic density. In **B**, the spine neck imposes diffusional constraints on cytosolic signalling proteins. These mobility restraints imposed by the spine neck create spine-specific compartmentalization of cytosolic signalling and surface receptor dynamics.

Dendritic spines must also communicate with protein synthesis machinery located in the parent dendrite to sustain late phases of LTP (Abraham and Williams, 2008; Govindarajan et al., 2011; Redondo and Morris, 2011; Tanaka et al., 2008). Thus, the movement of signalling molecules to-and-from the dendritic spine must not be fully compartmentalized but conforms to some degree of regulation. An illustration of such regulation is provided by recent experiments showing that calcium/calmodulin-activated kinase II (CaMKII) and Ras, two important molecules for synaptic plasticity, exhibit differential displacements from activated spines into the parent dendrites during synaptic plasticity (Harvey et al., 2008; Lee et al., 2009). Recent work by Murakoshi, Wang and Yasuda (2011) extended these findings using a FRET based approach (Murakoshi et al., 2011). The authors assessed the spatial spread of two synaptically-activated

Rho-GTPases, RhoA and Cdc42. Whereas single-spine LTP induced by 2P glutamate uncaging lead to sustained activation (up to 30 min) of both RhoA and Cdc42, only activated RhoA readily traversed the spine neck into the parent dendrite, with activated Cdc42 restricted to the stimulated spine (Murakoshi et al., 2011). Since the measured diffusional properties of these proteins were similar, it was proposed that mechanisms localized to the spine head were likely required to spatially restrict Cdc42 activation, thereby enforcing the notion that spines are highly regulated biochemical compartments. Taken together, the spatial compartmentalization of key regulatory molecules (*eg.*, protein kinases) may also offer powerful constraints that impact the spread of intracellular signals from the spine to the parent dendrite.

Surface (plasma membrane-bound) AMPARs and NMDARs exist at both synaptic and extrasynaptic locations. These surface receptor populations are not rigidly fixed, but in perpetual diffusional flux laterally through the membrane (Borgdorff and Choquet, 2002; Groc et al., 2004; Heine et al., 2008). Similar to intracellular diffusion, the lateral mobility of proteins in the plasma membrane can also be influenced by morphological parameters of spines (Ashby et al., 2006; Choquet, 2010; Holcman and Triller, 2006). For instance, FRAP analysis demonstrated that spine necks restrict the lateral diffusion of surface AMPARs. Specifically, AMPARs at spines connected to the parent dendrite by a spine neck exhibit a two-fold slower rate of lateral mobility compared to those at spines without a distinguishable spine neck (**Figure 1**). Similar results were obtained using membrane-bound GFP, indicating that the impedance of lateral mobility was dictated by morphological parameters of the spine, and not by intrinsic properties of AMPAR trafficking *per se* (Ashby et al., 2006). Furthermore, AMPARs also undergo constitutive vesicular cycling via endo- and exocytosis. Evidence from both electron microscopy and fluorescence imaging indicates the presence of endocytic and exocytic zones within dendritic

spines (Blanpied et al., 2002; Gerges et al., 2006; Lu et al., 2007; Racz et al., 2004). Interestingly, the dynamic balance of endo- and exocytosis modulates synaptic strength and underlie certain forms of plasticity. Indeed, LTP induction results in an enhancement of AMPAR exocytosis (Lin et al., 2009; Luscher et al., 1999; Park et al., 2004; Petrini et al., 2009; Yudowski et al., 2007). Taken together, the strategic clustering of signalling proteins, the development of narrow spine necks and the organization of vesicular cycling machinery can all contribute to biochemical compartmentalization of spines. This compartmentalization provides individual spines with the autonomous capacity to dynamically regulate the surface expression of distinct pools of AMPARs to promote the synapse-specificity of synaptic plasticity.

The Electrical Compartment

In addition to providing the morphological substrate for bestowing features of biochemical compartmentalization, spines may also function as electrical compartments capable of modulating the amplitude, kinetics and integration of synaptic potentials. Early estimations based on Rallian cable theory and measurements of molecular diffusion indicated that only modest ohmic resistances would be provided by spine necks, and therefore largely dismissed the notion that spines behave as electrical compartments (Koch and Zador, 1993; Svoboda et al., 1996). However, recent experimental evidence suggests that electrical compartmentalization can take place in at least a subset of dendritic spines (Araya et al., 2006; Bloodgood et al., 2009; Tsay and Yuste, 2004). A combination of current-clamp recordings, 2P uncaging and second harmonic membrane potential measurements provided evidence that long spine necks attenuate synaptic potentials between spine head and the parent dendrite (Araya et al., 2006). In this line, it is interesting to note that calcium transients induced by activation of NMDARs can be readily

detected by 2P calcium imaging in physiological extracellular magnesium concentrations (~1.0mM) in slices (Chalifoux and Carter, 2011; Grunditz et al., 2008; Kovalchuk et al., 2002) and in vivo (Chen et al., 2011; Jia et al., 2010; Varga et al., 2011), despite the presence of the voltage-dependent magnesium block of NMDARs near resting membrane potentials. Furthermore, calcium transients mediated by voltage-sensitive calcium channels (VSCCs) can also be visualized upon synaptic activation, indicating unexpectedly large depolarizations at the spine head (Bloodgood et al., 2009). Together, these data suggest that spine necks may impart an appreciable degree of electrical resistance – and charge accumulation in spine heads – and thus electrically compartmentalize dendritic spines (Tsay and Yuste, 2004). An intriguing and functionally powerful idea is that the degree of both electrical and biochemical compartmentalization might be dictated by active modifications in spine morphology. This possibility is becoming increasingly prominent given the dynamic structural changes which accompany the expression of synaptic plasticity (see below).

Structural and Functional Plasticity at Spines

The development of 2P glutamate uncaging to stimulate and induce LTP at single dendritic spines has been instrumental in providing key insights on the structural and functional changes that take place during plasticity. In 2004, Kasai and colleagues induced LTP at individual dendritic spines by 2P glutamate uncaging and showed that the expression of LTP is associated with spine enlargement (Matsuzaki et al., 2004). Furthermore, smaller spines carried an inherently higher propensity for LTP expression compared to larger spines, which were functionally and structurally more stable. Interestingly, some of the molecular mechanisms underlying structural plasticity have been found to closely parallel those for synaptic plasticity.

For instance, LTP-induction stimuli involving strong synaptic input and large postsynaptic rises in calcium facilitates actin branching and polymerization, providing a protrusive force to mediate spine enlargement (Fukazawa et al., 2003; Kasai et al., 2010; Matsuzaki et al., 2004; Okamoto et al., 2004; Patterson and Yasuda, 2011). Conversely, LTD-inducing stimuli leads to actin depolymerization, spine shrinkage and retraction (Okamoto et al., 2004). Moreover, similar to the expression of long-lasting phases of LTP, the temporal stability of structural plasticity requires the synthesis of new proteins (Govindarajan et al., 2011; Tanaka et al., 2008). These fundamental similarities in the induction of both structural and functional plasticity indicate an intimate relationship between these two processes.

One critical molecular link is CaMKII, a highly abundant protein in spines with an established role in synaptic plasticity (Barria et al., 1997; Lee et al., 2009; Lisman et al., 2002; Malinow et al., 1989; Matsuzaki et al., 2004). At rest, CaMKII directly bundles and stabilizes actin filaments and is involved in the structural stability of spines (Okamoto et al., 2007). CaMKII is activated by LTP-inducing stimuli, remaining persistently active and spatially compartmentalized to the stimulated spine – properties that correlate well with the spatiotemporal characteristics of structural and functional plasticity (Lee et al., 2009). Moreover, active CaMKII dissociates from the actin cytoskeleton causing structural destabilization, thus permitting structural modifications of the spine to take place (Okamoto et al., 2007). Downstream, CaMKII activates a number of signalling molecules such as members of the Rho-GTPase family (RhoA, Cdc42, Rac1 and Rnd1) to mediate changes in spine structure (Cingolani and Goda, 2008; Murakoshi et al., 2011; Patterson and Yasuda, 2011). For instance, Cdc42 becomes activated during LTP induction and interacts with p21-activated kinase (PAK) proteins to stabilize structural modifications (Murakoshi et al., 2011). Mice expressing a dominant-

negative PAK (dnPAK) transgene in the forebrain show abnormal dendritic spine morphology, defects in both LTP and LTD, and impairments in the consolidation of spatial and fear memory (Hayashi et al., 2004). Whereas it is difficult to attribute the behavioural deficits exhibited by dnPAK mice to synaptic impairments alone, these experimental strategies help to elucidate the interplay between structural and functional plasticity.

Although structural and functional changes rely on common signalling molecules, is it possible for these changes to occur independently of one another? Some evidence suggests that structural and functional plasticity are mutually stabilizing processes. For instance, in CA1 pyramidal neurons, the temporal stability of LTP expression is dependent on actin polymerization (Krucker et al., 2000). Subsequent investigations have expanded on these findings, underscoring a critical role for cytoskeletal actin dynamics in the directed trafficking of AMPARs (Chen et al., 2007; Fukazawa et al., 2003; Hayashi et al., 2004; Okamoto et al., 2004; Ramachandran and Frey, 2009). Conversely, the insertion of AMPARs during LTP not only acts to increase synaptic strength, but has also been suggested to stabilize structural changes of the spine (Kopec et al., 2007). These data suggest that the molecular components that drive structural changes in dendritic spines during plasticity also act to stabilize AMPAR insertion and vice versa. This dynamic interplay thus provides a basis for the tight association between changes in spine volume and AMPAR content during LTP.

Structure versus Function

Dendritic spines on pyramidal cell dendrites number in the thousands and exhibit a high degree of morphological heterogeneity. High resolution electron microscopy studies provided the first indication that spine form and function were related by demonstrating that the size of the

PSD and number of AMPARs positively correlates with the size of spines (Baude et al., 1995; Katz et al., 2009; Kharazia and Weinberg, 1999; Takumi et al., 1999). A number of recent studies provided functional support to these ultrastructural findings by showing a strong positive correlation between dendritic spine size and AMPAR function (i.e., size of AMPAR-mediated current), as determined by 2P glutamate uncaging (Ashby and Isaac, 2011; Beique et al., 2006; Matsuzaki et al., 2001; Noguchi et al., 2011; Zito et al., 2009). Considering the parallel changes observed in both structure (i.e., spine volume) and function (i.e., AMPAR content) during activity-dependent plasticity, it is perhaps not at all surprising that such a correlation exists. However, a more detailed and in depth look at the literature, as outlined below, reveals that spines, at least in certain conditions, have the ability to significantly depart from such a tight structure/function coupling.

One of the first indications pointing to a divergence of spine form and function was provided by Smith et al. (2003) while describing the distance-dependent scaling of synaptic AMPARs in hippocampal CA1 pyramidal neurons (Smith et al., 2003). Using a combination of whole-cell and dendritic recordings with 2P glutamate uncaging, they showed the synaptic weights of spines were progressively larger with increasing distances from the soma. However, this apparent increase in spine function was not accompanied with measurable changes in spine volume. Non-stationary fluctuation analysis on 2P glutamate uncaging currents further revealed that this increase in function with dendritic distance reflected a higher density of spine AMPARs, and not an enhanced single-channel conductance. Together, these data provide a convincing illustration that spines of similar size can express a strikingly wide range of AMPAR density.

A disconnect between dendritic spine structure and function is further evidenced in studies of ‘silent’ synapses. Silent synapses contain detectable NMDARs but are devoid of

AMPA receptors and are therefore largely 'silent' at rest (ought to the magnesium-dependent blockade of NMDA receptors at resting membrane potential). They are thought to represent immature glutamatergic synapses in part because their expression drastically diminishes during postnatal development (Isaac et al., 1997; Kerchner and Nicoll, 2008; Zhu and Malenka, 2002). Not surprisingly, early 2P-glutamate uncaging investigations described the presence of silent synapses on thin, filopodial-like spines in developing CA1 pyramidal neurons (Beique et al., 2006). Interestingly, however, subsequent investigations in the rodent somatosensory cortex reported that silent synapses can be detected at spines spanning a broad range of morphologies (Ashby and Isaac, 2011; Busetto et al., 2008). Although providing indirect support to this notion, work in PSD-95 KO mice also documented the presence of a structure/function uncoupling for spines. At an age where silent synapses were no longer detected in WT mice, PSD-95 KO mice displayed a high proportion of silent synapses onto large spines that otherwise appeared mature (Beique et al., 2006). Collectively, these data indicate that although there is a clear correlation between spine size and function, there is also room for a significant departure from this tight structure/function coupling.

Studies on the dynamical nature of spine structure during LTD also indicate a divergence in the signalling pathways that regulate spine size and function. For instance, Zhou et al (2004) reported that LTD and spine shrinkage at hippocampal synapses show differential requirements for protein phosphatase signalling via PP1 and calcineurin, despite sharing a similar requirement for NMDAR activation (Zhou et al., 2004). Furthermore, while the actin-binding protein cofilin was involved in spine shrinkage, it seemed to play no role in the expression of LTD. More recent investigations have also indicated that clathrin-mediated endocytosis is not required for spine shrinkage, despite being essential for LTD expression (Wang et al., 2007). Finally, LTD studies

in cerebellar Purkinje cells also reported dissociation between spine structure and function. Indeed, Sdrulla and Linden reported that LTD at cerebellar parallel fiber-Purkinje cell synapses was not associated with changes in either spine number or volume. In an interesting twist, the authors further identified a manipulation that lead to a dramatic and global retraction of spines on Purkinje neurons that, surprisingly, was not associated with significant changes in synaptic strength (Sdrulla and Linden, 2007). Thus, evidence obtained from LTD experiments in two distinct brain regions underscores a mechanistic divergence of spine structure and function.

Lastly, this divergence is further exemplified in a model of single-synapse homeostatic plasticity in dissociated cortical neuronal cultures. Homeostatic plasticity refers to the ability of a neuron to bi-directionally tune synaptic AMPAR content in response to changes in overall network activity (Turrigiano, 2011). Recent experiments have expanded these findings by showing that homeostatic regulation of synaptic strength can be achieved at the level of individual dendritic spines (Beique et al., 2011; Lee et al., 2010). In one experimental paradigm, chronic suppression of presynaptic glutamatergic input onto single spines lead to an enhancement of postsynaptic AMPAR function, as determined by 2P glutamate uncaging (Beique et al., 2011). Interestingly, despite a marked increase in AMPAR currents, there were no discernable changes in spine volume (see **Figure 2**). By comparing the current-voltage (I-V) relationship of AMPARs at these two populations of dendritic spines, activity-deprived synapses were found to express AMPARs with a distinct subunit composition (AMPARs lacking the GluA2 subunit). Because this AMPAR subtype has an inherently higher conductance, this switch in subunit composition provides a mechanistically plausible model to account for the increased synaptic strength onto spines of similar volume.

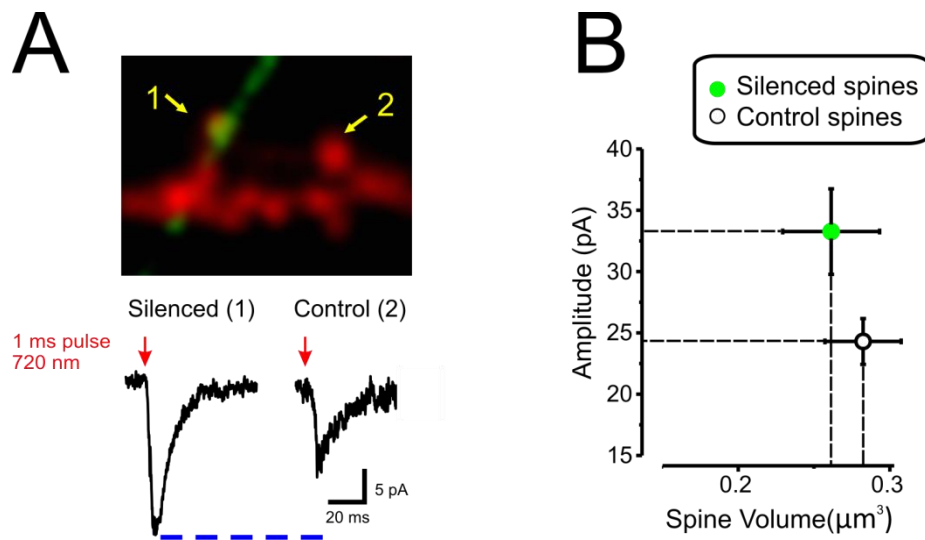


Figure 2: Dissociation of spine size and synaptic strength. In **A**, the release of glutamate was reduced for 48 hours specifically on the spine marked (1). This led to a homeostatic enhancement of synaptic strength as assessed by 2P-uncaging of MNI-Glutamate. The size of the synaptic currents induced by 2P-uncaging are shown in the bottom panel. In **B**, the significant enhancement of the amplitude of synaptic currents onto ‘silenced’ spines was not accompanied by any measureable changes in spine volume. Adapted from (Beique et al., 2011).

Conclusion

As a major component of excitatory synapses, spines are strategically poised to support important modulatory roles in synaptic transmission and neuronal function. Although still subject to debate, an emerging notion posits that spines provide a structural scaffold to act as biochemical and electrical compartments. Interestingly, discrete differences in dendritic spine morphology may directly influence the degree of functional compartmentalization (Figure 1).

In addition, the dynamic nature of spine structure (Blanpied and Ehlers; Blanpied et al., 2008) may generate parallel changes in the compartmentalization features of individual spines. One can speculate that these morphologically-dependent degrees of compartmentalization lead to distinct states of metaplasticity at individual synapses. This notion aligns well with emerging theoretical models of synaptic learning that demonstrate that synapses exhibiting a gradation of states, each bridged by distinct metaplastic transitions, bestow neural networks with enhanced information

storage capacity (Fusi and Abbott, 2007; Fusi et al., 2005). Altogether, these considerations highlight the rich computational potential afforded by the yet to be completely understood relationship between form and function of dendritic spines.

A.2.

Manuscript V

Title:

Tuning into diversity of homeostatic synaptic plasticity

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Kevin Lee was the main author of this manuscript, having a final impact on the entire text. I contributed to all aspects of this manuscript's preparation, including literature review, development of the outline, laying down draft paragraphs and sections, and editing versions of the manuscript during its preparation. I prepared the sole figure. Dr. Jean Claude Béïque guided our thoughts, helped to establish the overall outline of the manuscript and provided a full critique including suggestions and revisions at several stages.

Abstract

Neurons are endowed with the remarkable ability to integrate activity levels over time and tune their excitability such that action potential firing is maintained within a computationally optimal range. These feedback mechanisms, collectively referred to as “homeostatic plasticity”, enable neurons to respond and adapt to prolonged alterations in neuronal activity by regulating several determinants of cellular excitability. Perhaps the best-characterized of these homeostatic responses involves the regulation of excitatory glutamatergic transmission. This homeostatic synaptic plasticity (HSP) operates bidirectionally, thus providing a means for neurons to tune cellular excitability in response to either elevations or reductions in net activity. The last decade has seen rapid growth in interest and efforts to understand the mechanistic underpinnings of HSP in part because of the theoretical stabilization that HSP confers to neural network function. Since the initial reports describing HSP in central neurons, innovations in experimental approaches have permitted the mechanistic dissection of this cellular adaptive response and, as a result, key advances have been made in our understanding of the cellular and molecular basis of HSP. Here, we review recent evidence that outline the presence of distinct forms of HSP at excitatory glutamatergic synapses which operate at different sub-cellular levels. We further present theoretical considerations on the potential computational roles afforded by local, synapse-specific homeostatic regulation.

Introduction

Defined patterns of pre- and postsynaptic activity can induce input-specific changes in synaptic strength. The two most studied of these activity-dependent synaptic plasticity processes are long-term potentiation (LTP) and long-term depression (LTD). These processes exhibit many of the features described in a model postulated by Donald Hebb more than 50 years ago to account for the ability of a neuronal network to store information (Hebb, 1949). As a result, tremendous efforts have been devoted to define the cellular and molecular mechanisms of LTP and LTD, and to understand their role as substrates of learning and memory (Kerchner and Nicoll, 2008; Kessels and Malinow, 2009; Lisman and Raghavachari, 2006; Lisman, 2009; Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Whitlock et al., 2006). However, the simple implementation of Hebbian-type LTP and LTD processes in different neuronal network models soon revealed an inherent stability problem for network function (Lazar et al., 2009; Miller and MacKay, 1994; Turrigiano and Nelson, 2004).

Destructive instabilities of both synapse and network function are readily apparent in exclusively Hebbian neural network models (Lazar et al., 2009; Miller and MacKay, 1994; Shouval et al., 2002; Turrigiano and Nelson, 2004). Specifically, these models demonstrate that the positive-feedback nature of Hebbian plasticity favors unconstrained synaptic potentiation and depression, thus leading to synapses which hit their functional ‘ceiling’ (for instance, by reaching maximum AMPA receptor number and density), or synapses that are driven toward functional demise by depressive mechanisms. An important consequence of such positive-feedback behavior in neural circuits is runaway excitation and epileptogenic neural activity (Lazar et al., 2009; Turrigiano and Nelson, 2004). During early childhood, the brain experiences intense growth and development and these normal processes have been linked to enhanced susceptibility

to seizure in young children (Wong, 2005). However, the overall risk of pediatric seizure remains relatively low considering the breadth of developmental changes at play, prompting some to hypothesize that homeostatic mechanisms exist to stabilize neural networks during development (Davis, 2006; Turrigiano and Nelson, 2004) and during mature brain function (Lazar et al., 2009; Sullivan and de Sa, 2006, 2008; Toyoizumi et al., 2005; Turrigiano, 2012; Turrigiano, 2008; Watt and Desai, 2010; Yeung et al., 2004), but see (de Vries and van Slochteren, 2008; Gilson and Fukai, 2011; Houweling et al., 2005; Thivierge and Cisek, 2008).

Several distinct homeostatic plasticity mechanisms have been described, each in principle providing neurons the means to tune and maintain overall levels of spiking activity within biologically-determined set points. Neurons accomplish this by actively regulating several determinants of cellular excitability, including intrinsic excitability (Grubb and Burrone, 2010; Turrigiano, 2011) and synaptic strength (Turrigiano, 2012; Turrigiano et al., 1998; Turrigiano and Nelson, 2004). In particular, the discovery of homeostatic synaptic plasticity (HSP) has received considerable interest because it provides a theoretically plausible solution to the instability problem of Hebbian networks described above. With features that closely resemble the well described denervation supersensitivity at the neuromuscular junction (Cannon, 1949), homeostatic synaptic plasticity (HSP) is characterized by the bidirectional regulation of synaptic strength in response to prolonged alterations in network activity (O'Brien et al., 1998; Turrigiano, 2008; Turrigiano et al., 1998).

Borrowing from the widely-used distinction between the induction and expression of Hebbian forms of synaptic plasticity (LTP/LTD), one can conceptualize a loosely analogous distinction between the *induction* and *expression* of HSP. Determining key mechanistic features of both these processes, in addition of determining how they interact with classic Hebbian

synaptic plasticity, is necessary for developing a thorough understanding of the role played by HSP in neuronal computation. Here, we review recent studies that reveal fundamental mechanistic insights in the *induction* (eg., cell-autonomous vs. non-cell-autonomous) and *expression* (cell-wide vs. local) of HSP and consider conceptual refinements for the role of local forms of HSP in stabilizing neuronal information storage and processing at excitatory glutamatergic synapses.

The Locus of Homeostasis

Whereas Hebbian LTP and LTD occurs within seconds to minutes in response to relatively short bouts of synaptic stimulation, the presence of HSP is experimentally revealed when neuronal activity is altered over longer periods of time (*i.e.*, hours to days). For instance, in perhaps its simplest and most intuitively tractable form, HSP is revealed when neuronal network activity is globally suppressed for a prolonged period of time (*eg.*, by applying tetrodotoxin to the culture media for many hours; see Figure 1B). In response, neurons exhibit a compensatory increase in cellular excitability, in part through a cell-wide up-regulation of synaptic AMPAR function (Turrigiano et al., 1998). This slow-acting regulation of excitability is also bidirectional: when neuronal activity is enhanced for many days (*eg.*, by pharmacological network disinhibition), neurons adapt by a cell-wide down-regulation of synaptic AMPAR function.

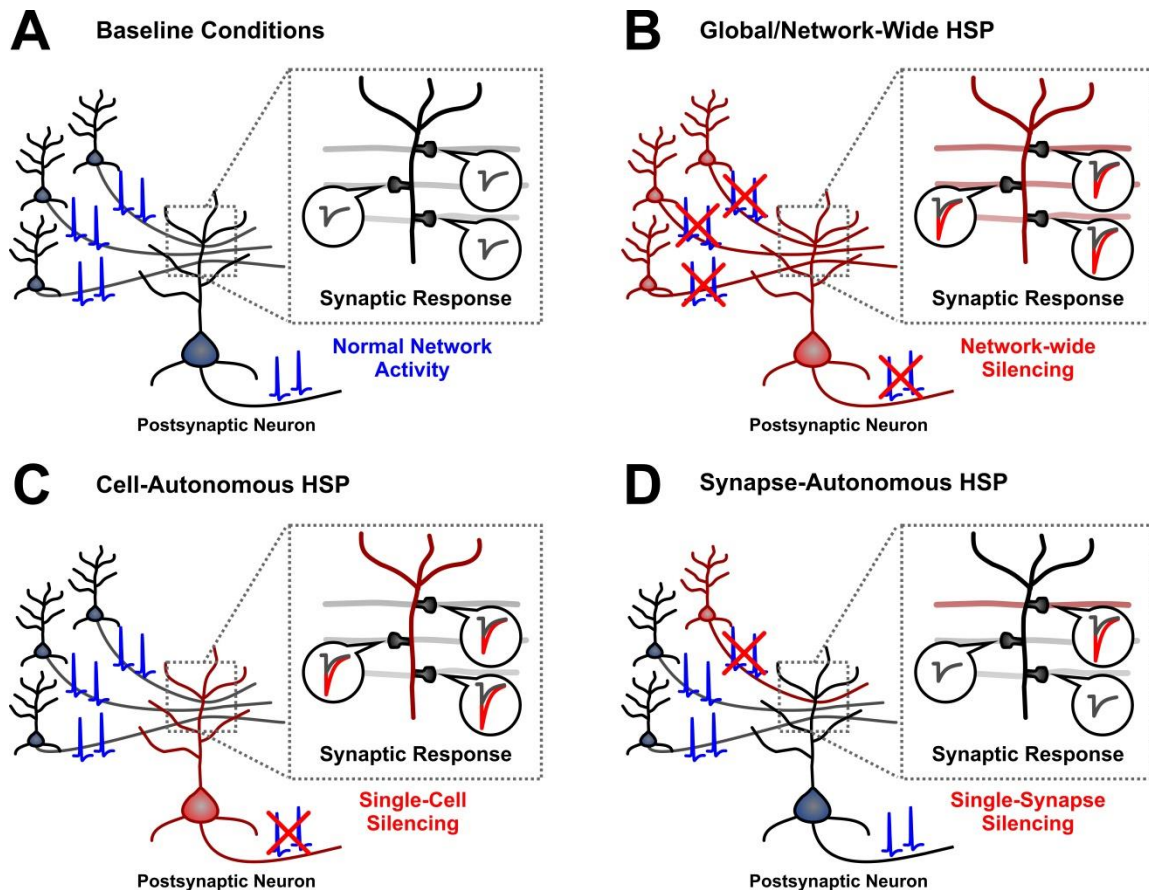


Figure 1: Diversity of Homeostatic Synaptic Plasticity (HSP). (A) A postsynaptic neuron receives excitatory synaptic input from presynaptic neurons in a neuronal network. Inset: The amplitude of the excitatory postsynaptic current (EPSC) schematizes the strength of each synapse. (B) HSP is revealed when network activity is altered for a prolonged period of time. In this example, action potential firing is abolished in all neurons by prolonged exposure to TTX. In response to the global reduction in activity, HSP mechanisms enhance synapse function in a cell-wide manner by increasing synaptic AMPA receptor content at all synapses (depicted by an increase in synaptic EPSC amplitude). Despite revealing HSP in its most intuitively-tractable form, the roles of pre- and post-synaptic activity cannot be distinguished with this global/network-wide experimental paradigm. (C) Cell-Autonomous HSP. Single-cell strategies that alter the activity of individual neurons (eg. via somatic TTX-perfusion) have revealed the cell-autonomous nature of HSP induction in glutamatergic pyramidal cells. Here, reducing the firing activity of a single neuron in an otherwise normal neural network results in cell-wide expression of HSP. (D) Synapse-Autonomous HSP. Suppression of firing activity in a single neuron reveals synapse-autonomous HSP at its postsynaptic target neuron. Here, the ‘silenced’ synapse is selectively strengthened over its nearby unperturbed neighbors. This demonstrates the ability of individual synapses to actively monitor and homeostatically respond to prolonged changes in activity.

These core features of homeostatic plasticity entail a number of conceptual postulates: 1) Neurons are endowed with mechanisms that monitor, and integrate over time, some parameters of neuronal activity; 2) These ‘sensing’ mechanisms are coupled to cellular ‘effectors’ that operate within a feedback loop to tune neuronal excitability in a direction that is homeostatic in nature (*eg.*, upregulation of excitatory glutamate receptors following prolonged suppression of neuronal activity); 3) whereas HSP’s activity sensing and integrating mechanisms are likely continuously operating ‘on line’, the feedback loop acts over a relatively long time course (*i.e.*, usually requiring several hours for expression). This conceptual framework has helped to guide the mechanistic dissection and understanding of HSP in the last several years (Lee, 2012a; Turrigiano, 2011; Turrigiano, 2008).

One feature of HSP that has received particular attention is its “multiplicative” nature. This refers to the observation that during some forms of HSP, the entire amplitude distribution of synaptic strength scales up (or down) by a single common factor, hence the term ‘scaling’, often used to denominate HSP (Kim et al., 2012; Turrigiano, 1999, 2008; Turrigiano et al., 1998). A common interpretation of the multiplicativity of HSP is that the relative strengths between synapses are maintained during the cell-wide homeostatic scaling process. As such, multiplicative HSP provides a means to tune neuronal excitability without disrupting the previously encoded catalogue of Hebbian synaptic engrams. A parsimonious cellular model to account for multiplicativity in HSP posits that a cell-wide mechanism drives the upregulation of AMPAR content across all synapses, with each synapse capturing (or stabilizing) AMPARs in a manner that is proportional to its original strength. However, since global/network-wide pharmacological manipulations (*eg.*, TTX treatment) alter the activity of each neuron within the network, a number of mechanistic details of HSP are left largely intractable. For instance, it is

impossible to determine the trigger for HSP induction since two broadly distinct changes take place. First, all neurons in the network are deprived of their ability to generate action potentials. Second, all synapses are deprived of presynaptic input. As such, where is the locus of homeostasis? Do neurons monitor and integrate activity over time by counting action potentials? Or rather, do individual synapses monitor and integrate activity over time by tallying presynaptic inputs? Recent studies have provided interesting insight into this important facet of HSP.

Cell-Autonomous HSP

In a first-step to distinguish between these possibilities, a key series of studies examined the ability of individual neurons to autonomously exhibit HSP (cell-autonomous HSP) by specifically modulating the firing activity of a single neuron embedded within an otherwise normal neural network (i.e., receiving normal ongoing synaptic input; see Figure 1C) (Burrone et al., 2002; Goold and Nicoll, 2010; Iwata et al., 2008). Borrowing from a previous study at the neuromuscular junction (Paradis et al., 2001), Burrone et al. (2002) developed a single-cell silencing strategy by overexpressing an inwardly rectifying potassium channel, Kir2.1, in a small subset of dissociated hippocampal neurons in culture. Single-cell silencing induced a homeostatic upregulation of glutamatergic transmission as evidenced by a robust enhancement of the frequency of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) in transfected neurons. It was concluded that the reduction of action potential firing by Kir2.1 overexpression induced a homeostatic increase in synaptic function, and thus demonstrated that individual neurons can autonomously express HSP. However, it was later argued that because the Kir2.1-mediated silencing strategy used by Burrone et al. (2002) caused cell-wide hyperpolarization, it was not possible to discriminate the involvement of local dendritic excitability from that of somatic action potentials in triggering HSP. Thus, to specifically

ascertain the role of somatic spiking activity in HSP induction, Ibata et al. (2008) used prolonged local perfusion of TTX over the soma of individual neurons. The authors found that suppression of action potential firing for 4 hours was sufficient to induce a significant increase in mEPSC amplitude and accumulation of synaptic EYFP-tagged GluA2-containing AMPARs, and that these changes were likely due to an alteration of somatic calcium-dependent signaling by the reduction in action potential firing. Together, these results support the notion that individual cells can autonomously respond to a reduction in action potential firing by expressing HSP.

HSP has been widely shown to operate bidirectionally and, as such, Goold & Nicoll later asked whether prolonged periods of enhanced activity in a single neuron could drive a homeostatic depression of glutamatergic transmission. To this end, the authors developed an elegant optogenetic approach whereby CA1 pyramidal neurons in organotypic hippocampal slice culture were biolistically transfected to express the light-activated cation channel Channelrhodopsin-2 (ChR2). This strategy allowed the authors to predictably and chronically enhance action potential firing of individual neurons by flashing blue light while the slices were incubated. After prolonged photostimulation (>24 hours), ChR2-expressing neurons exhibited a dramatic reduction in both synaptic AMPAR and NMDAR function – an effect which, similar to the findings of Ibata et al. (2008), was also suggested to be mediated by somatic calcium-dependent signaling.

Altogether, by modulating the firing activity of single-cells in otherwise unperturbed networks, these studies reveal a fundamental conceptual advancement for HSP: at least one parameter of neuronal activity that is homeostatically regulated is action potential firing rate *per se*, which neurons monitor over time by calcium-dependent signaling at the soma. Interestingly, these findings contrast with HSP at inhibitory GABAergic synapses, which rely not on cell-

autonomous signals, but upon network-wide activity levels (Hartman et al., 2006). Therefore, cell-autonomous functions likely act in concert with additional network-level mechanisms to regulate neural circuit function and stability (Maffei and Fontanini, 2009).

Synapse-Specificity of HSP

As outlined above, multiplicative HSP is generally interpreted to reflect that all synapses onto a neuron undergo homeostatic scaling by a single common factor. Although it has been well documented and observed by several groups in a number of experimental preparations, multiplicative HSP has not been universally observed (Echegoyen et al., 2007; Goel and Lee, 2007; Thiagarajan et al., 2007; Thiagarajan et al., 2005), suggesting some degree of synapse-specificity in HSP expression under certain conditions. This was perhaps best illustrated by a series of experiments studying HSP in TTX-treated organotypic hippocampal slice cultures (Deeg, 2009; Kim and Tsien, 2008). In response to prolonged suppression of activity by TTX treatment, CA1 neurons exhibit uniform, cell-wide multiplicative scaling (Kim and Tsien, 2008; Kim et al., 2012). However, the expression of HSP on CA3 neurons was not uniform, but rather synapse-specific. Despite being located on the same CA3 neuron, only throughput, feed-forward synapses exhibited HSP, while recurrent CA3-to-CA3 synapses became functionally depressed. Thus, HSP expression was dependent on the precise role of the synapse in information flow through the hippocampal network. Due to the feed-forward/feedback architecture of the hippocampal circuit, such synapse-specificity was found to impart network stability during HSP by preventing reverberating epileptogenic activity (Kim and Tsien, 2008). These results raise the intriguing possibility that local mechanisms exist to govern, or gate, the expression of HSP at individual synapses in response to cell-wide homeostatic signals. This idea is further illustrated by findings from *in vivo* HSP studies in rodents. Whereas visual sensory deprivation by dark

rearing of young animals resulted in the expression of multiplicative HSP in layer II/III visual cortical neurons, non-multiplicative HSP was observed following dark rearing of older animals (Goel and Lee, 2007). These results may therefore reflect an age-related divergence in the potential for HSP expression in a subset of synapses. Future investigations, likely requiring methodological and technological developments, will be necessary to thoroughly define the factors that impact HSP expression at individual synapses during prolonged changes in global network activity.

Local Synapse-Autonomous HSP

A corollary, but distinct, line of questioning relates to whether individual synapses, like neurons, can monitor and integrate their own activity over time and autonomously perform homeostatic adjustments. In other words, are individual synapses autonomous homeostatic units? One way to experimentally address this question is to determine the consequences of reducing (or blocking) neurotransmitter release onto a very small number of synapses - ideally as few as one - such that the overall excitability of the postsynaptic neuron is left unaltered. To this end, studies have used site-directed perfusion of drugs over discrete regions of dendritic segments to locally suppress activity (Ibata et al., 2008; Sutton et al., 2006). Ibata et al. (2008) micro-perfused TTX or CNQX+APV onto defined dendritic regions of cultured neurons for up to 5 hours, but did not observe any measurable changes in the accumulation of EYFP-tagged GluA2 subunits within the ‘silenced’ dendritic region. These results however do not preclude the possible accumulation of GluA2-lacking AMPARs, as occurs in cell-wide HSP (Groth et al., 2011; Lee, 2012a; Lindskog et al., 2011; Soden and Chen, 2010; Sutton et al., 2006), but see (Anggono et al., 2011; Gainey et al., 2009). In this respect, Sutton et al. (2006) previously found that the expression of HSP (induced by global TTX application) could be locally accelerated by

site-directed perfusion of APV over defined dendritic regions, leading to an increase in GluA1-containing AMPARs within the site of APV perfusion. These latter results therefore suggest that mechanisms can ‘sense’ local synaptic activity and engage local machinery to homeostatically regulate synapse function. However, since local perfusion strategies indiscriminately disrupt presynaptic activity, glutamate receptor function and/or dendritic excitability over broad dendritic segments, it is difficult to substantiate, or refute, the homeostatic autonomy of individual synapses with these methods alone.

To circumvent this problem, molecular-based experimental strategies were developed to modulate the activity of a small number of synaptic inputs onto an otherwise unaltered postsynaptic neuron such that the AMPAR content of ‘silenced’ synapses can be compared to their neighbors (see Figure 1D). One such strategy involved the overexpression of Kir2.1 in single neurons along with the synaptic marker Synapsin-YFP (Syn-YFP), to label and suppress the activity of a small number of presynaptic terminals onto neurons in culture. Immunocytochemical detection revealed that postsynaptic AMPAR content was enhanced specifically at synapses that were presynaptically suppressed by Kir2.1 overexpression (Beique et al., 2011; Hou et al., 2008). Moreover, electrophysiological recordings showed that the amplitude of AMPAR-mediated postsynaptic currents were larger on ‘silenced’ synapses, as determined by two-photon glutamate uncaging onto Kir2.1/Syn-YFP-apposed synapses compared to closely neighboring control spines (Beique et al., 2011). This synapse-autonomous form of HSP was mediated by the insertion of inwardly-rectifying calcium-permeable GluA2-lacking AMPARs and, furthermore, was shown to be dependent on the immediate early gene *Arc*. Altogether, these studies support the notion that individual synapses are endowed with the ability to autonomously sense, and integrate over time, their own level of activity and adapt to it

(likely bidirectionally (Hou et al., 2011)) in a homeostatic fashion. Interestingly, because GluA2-lacking AMPARs (Groth et al., 2011; Lee, 2012a; Lindskog et al., 2011; Soden and Chen, 2010; Sutton et al., 2006) (but see (Anggono et al., 2011; Gainey et al., 2009)) and Arc (Shepherd et al., 2006) have previously been shown to be involved in cell-wide HSP, these results reveal at least some degree of mechanistic convergence in the expression mechanisms of local and cell-wide forms of HSP.

Membrane hyperpolarization induced by Kir2.1 overexpression reduces, but does not abolish, action potential firing in the presynaptic neuron - especially in conditions similar to those during incubation of neuronal cultures (Beique et al., 2011). As such, the results outlined above indicate that individual synapses are homeostatically sensitive to relatively small changes in incoming activity and can autonomously undergo HSP. Interestingly, the complete blockade of glutamate release at single synapses by presynaptic overexpression of tetanus toxin light chain (TeTx) does not induce a compensatory homeostatic upregulation of postsynaptic AMPAR content. Indeed, several studies using this approach have either reported no change (Lee et al., 2010) or decreases (Ehlers et al., 2007; Harms et al., 2005; Okuno et al., 2012) in AMPAR function and/or content at TeTx-silenced synapses. Because TeTx blocks both spontaneous and action potential-dependent forms of vesicle release (Ehlers et al., 2007; Harms et al., 2005), these results collectively suggest that spontaneous glutamate release is required for synapse-autonomous HSP. This notion is fully consistent with the well documented role of spontaneous synaptic events in maintaining several aspects of synapse integrity and function, including glutamate receptor stability and clustering at synapses (Bouwman et al., 2004; Craig et al., 1994; Ehlers et al., 2007; Harms et al., 2005; Mateos et al., 2007; McKinney et al., 1999; Saitoe et al.,

2001; Verstreken and Bellen, 2001, 2002), thus revealing an interesting subtlety in activity-detection for HSP at single synapses.

Dating back to the late 1970's, several investigations have demonstrated that prolonged *in vivo* blockade of spinal cord motoneuron activity results in enhanced synaptic transmission (Gallego et al., 1979; Manabe et al., 1989; Webb and Cope, 1992), with some suggesting that these homeostatic adaptations are synapse-specific (Webb and Cope, 1992). More recently, by manipulating sensory stimuli to *Xenopus* tadpoles, Deeg & Aizenman (2011) provided evidence supporting the presence of such synapse-specific forms of HSP *in vivo* (Deeg and Aizenman, 2011). Neurons of the optic tectum in *Xenopus* tadpoles receive distinct synaptic inputs from both visual and mechanosensory pathways. By dark-rearing tadpoles, the authors observed a compensatory homeostatic increase in synaptic strength specifically at synapses of the visual pathway. In a complementary manner, prolonged mechanosensory hyperstimulation by persistent ambient vibration caused homeostatic synaptic depression specifically at mechanosensory synapses. These important findings thus demonstrate *in vivo* manifestations of synapse-autonomous HSP.

A Computational Paradox?

One immediate computational appeal of the multiplicative (uniform) nature of cell-wide HSP is that neurons can tune spiking activity to an optimal range without imposing an informational cost since the relative strength between synapses (presumably encoded by prior synaptic activity) would be left unaltered. As a result, it has been argued that local forms of HSP, such as those described above, are computationally costly and paradoxical as they would in essence erase information stored by Hebbian-forms of plasticity (Ibata et al., 2008; Turrigiano,

2012). We provide in this final section some alternative interpretations and speculate on the functional role of local HSP for stabilizing information storage and processing.

First, the informational cost intuitively associated with local HSP exists only within a model whereby information (or memory) is encoded by stable modifications solely of synaptic *strength*. Despite its historical popularity, this model is now believed to be unsatisfactory and too simplistic, as it necessitates unrealistic assumptions and fails to account for many complexities of synapse function (Fusi and Abbott, 2007; Fusi et al., 2005). While imposing realistic bounds on synaptic strength, one recent theoretical model rather suggested that Hebbian changes in synaptic strength are accompanied by biochemical alterations which permit transitions between cascades of metaplastic ‘states’ (Fusi et al., 2005). Thus, information can be encoded not solely through changes in synaptic strength, but in a more complex synaptic engram involving subtle biochemical alterations that can span various time scales (enzymatic activity, phosphorylation states, calcium dynamics etc.) (Shouval and Gavornik, 2011; Shouval et al., 2010). This progressive view of Hebbian plasticity is particularly attractive because it enriches the capacity of realistically bounded synapses to store information and ascribes richer computational roles to the known diverse biochemical environments of synapses. Due to the powerful actions of calcium in biochemical signaling, the insertion of calcium-permeable GluA2-lacking AMPARs during cell-wide and synapse-autonomous forms of HSP (Beique et al., 2011; Lee, 2012a; Lindskog et al., 2011; Thiagarajan et al., 2005) may directly enact one such metaplastic state-transition and, in effect, efficaciously encode recent synaptic history (here, prolonged inactivity). In addition, the insertion of this particular AMPAR subtype in HSP of hippocampal neuron cultures has recently been shown to trigger the release of a retrograde messenger to modulate presynaptic release probability (Lindskog et al., 2011), in principle providing further

computational potential to local HSP. Future studies will be required to define key features of synapse autonomous HSP such as its long-term stability, functional and temporal upper/lower bounds and effects on Hebbian plasticity rules.

Additional conceptual insight into the possible functions of local HSP can be established in the broader context of dendritic information processing. For instance, it is well established that spatiotemporal integration of synaptic inputs can evoke dendritic non-linearities such as NMDA spikes and dendritic spikes (Larkum and Nevian, 2008; London and Hausser, 2005). Of note, NMDA spikes in dendrites are well positioned to control the throughput of synaptic information by amplifying spatially-clustered and temporally-succinct inputs, while ignoring uncorrelated synaptic noise (Chalifoux and Carter, 2011; Larkum et al., 2009; Lee, 2012b; Polsky et al., 2009; Schiller et al., 2000). In a prescient commentary published a decade ago, it was suggested that the interplay between such dendritic non-linearities and Hebbian plasticity could lead to functional instability, as subsets of dendrites containing strong synapses would dominate neuronal firing at the expense of dendrites containing weaker synapses (Goldberg et al., 2002). This idea has gained considerable traction in light of recent evidence revealing local dendritic mechanisms that spatially influence Hebbian plasticity (Golding et al., 2002; Govindarajan et al., 2011; Govindarajan et al., 2006; Harvey and Svoboda, 2007; Holthoff, 2004; Kampa et al., 2007; Larkum and Nevian, 2008; Remy and Spruston, 2007). In principle, these mechanisms would favor the emergence of spatially clustered engrams encoded by groups of neighboring synapses, rather than spatially-distributed engrams encoded throughout the dendritic arbor (Kleindienst et al., 2011; Larkum and Nevian, 2008; Makino and Malinow, 2011; Winnubst and Lohmann, 2012). As such, the destructive instabilities inherent to Hebbian networks may also be applicable at the single-cell level by the destabilization of dendritic computation. It was thus suggested that

a powerful solution may be offered by HSP mechanisms operating locally to regulate synaptic weight and dendritic excitability (Goldberg et al., 2002). Local HSP mechanisms may therefore function to homeostatically normalize information throughput across all dendrites, providing a level playing field to ensure no single dendrite is too “loud” or too “quiet” during synaptic communications. The plausibility of such mechanism was supported *in silico* using a CA1 pyramidal neuron model, adding that homeostatic adjustments induced by prolonged local changes in activity could be shared between (likely a subset of) neighboring synapses without informational cost (Rabinowitch and Segev, 2008). Extending this concept even further, one can thus conceptualize multiplicative scaling of a group of synapses located on a dendritic segment. In essence, the compartmentalized nature of dendritic computation imparts a spatial attribute to neuronal information processing and storage that, in turn, shifts the theoretical problem of instability found in Hebbian circuits from a cell-wide level, to a local, dendritic level. Thus, in the context of a compartmentalized neuron model with active dendrites and a synaptic environment that is perpetually influenced by Hebbian plasticity mechanisms, this reformulation of the original instability problem suggests that local HSP could serve a fundamental role in stable information storage and processing by neurons.

Concluding Remarks

A predominant view of HSP posits that the chief parameter of activity that is subjected to homeostatic regulation is action potential firing *per se*. As such, prolonged alteration of neuronal firing would drive uniform cell-wide homeostatic adaptation across all synapses of a neuron. However, in light of recent studies demonstrating that HSP can also operate at a synapse-specific level, it is conceivable that multiple and distinct homeostatic mechanisms exist, each operating

with their specific feedback gain and dynamic range, as well as within their own contingencies for induction and expression. Borrowing from the concept that potentially every major electrical neural process will, in time, be shown to exhibit some form of plasticity (Kim and Linden, 2007), it is perhaps equally likely that many other neuronal processes are also governed by homeostatic regulatory mechanisms. The current understanding of these homeostatic mechanisms is still rudimentary and, as such, one overarching challenge is to not only uncover the cellular and molecular details of these processes, but to also account for the diversity of such mechanisms within the broader framework of neuronal function. This undertaking will ultimately require the synergistic implementation of *in vitro* and *in vivo* investigations in tandem with theoretical mathematical modeling *in silico*. The construction of such a framework will lead to a better appreciation of how information processing and storage occurs in the brain, in both health and disease.

A.3.

Manuscript VI

Title:

A prominent role for triheteromeric GluN1/GluN2A/GluN2B NMDARs at central synapses

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Statement of Contributions:

This manuscript was written to provide an extended discussion on a paper that was published in the *Journal of Neuroscience*. I came up with the main points discussed in this brief review manuscript, and wrote the paper. Kevin Lee provided ideas that shaped the paper during lengthy discussions on the topic and edited versions of the manuscript prior to its submission.

NMDA receptors (NMDARs) are a major subclass of ionotropic glutamate receptors at excitatory synapses in the brain. These receptors are critical for activity-dependent synaptic plasticity and therefore play an important role in network development and information storage in the brain. NMDARs are tetrameric assemblies composed of two obligatory GluN1 subunits together with varying combinations of GluN2(A-D) and GluN3(A-B) subunits. The expression of GluN2 and GluN3 subunits is tightly regulated during development and varies across brain regions, leading to the formation of NMDARs with distinct subunit composition and thus with distinct biophysical and pharmacological properties (Paoletti et al., 2013). The predominant expression of GluN2A and GluN2B subunits in cortical and hippocampal pyramidal neurons, for example, leads to the formation of both diheteromeric (GluN1/GluN2A, ‘A-type’ and GluN1/GluN2B, ‘B-type’) and triheteromeric (GluN1/GluN2A/GluN2B, ‘AB-type’) NMDARs (Tovar and Westbrook, 1999). Whereas the biophysical and pharmacological properties of diheteromeric A-type and B-type NMDARs are well characterized, very little is known about triheteromeric AB-type NMDARs. Nonetheless, recent evidence indicates that AB-type receptors are likely the predominant NMDAR subtype at synapses of the adult hippocampus (Rauner and Kohr, 2011), and may contribute to synaptic responses in other cortical and subcortical regions (Delaney et al., 2013). Because NMDARs are implicated in a variety of neuropsychiatric disorders such as schizophrenia, addiction and stroke, it is important to develop a comprehensive understanding of the function and distribution of different NMDAR subtypes, including triheteromeric AB-type NMDARs, in order to design novel and effective therapeutic strategies for these debilitating diseases (Paoletti et al., 2013). Biophysical properties such as deactivation kinetics and probability of channel opening have long been used to determine NMDAR subunit composition in neurons. Studies based on these properties have led to several

models of subunit-dependent NMDAR function both during normal synaptic transmission and in pathological states (Paoletti et al., 2013). However, interpretation of these data is complicated by the unknown properties of AB-type NMDARs, since limitations in experimental strategies have rendered these NMDARs notoriously difficult to study. Although AB-type NMDARs retain sensitivity to zinc and ifenprodil, which bind with high affinity to GluN2A and GluN2B subunits respectively, these antagonists fail to produce maximal inhibition of AB-type receptors even when administered simultaneously (Hatton and Paoletti, 2005). Moreover, the isolation of triheteromeric NMDAR currents in heterologous systems has relied on subunit mutagenesis, which alters their biophysical properties (Hatton and Paoletti, 2005). Therefore, a method to isolate native AB-type NMDAR currents in neuronal preparations is necessary to better understand their intrinsic kinetic properties and roles in synaptic transmission.

In a recent issue of *The Journal of Neuroscience*, Tovar et al. (2013) provide the first biophysical characterization of triheteromeric AB-type NMDARs in an intact neuronal culture preparation (Tovar et al., 2013). Autaptic hippocampal synapses were repetitively stimulated in the presence of two antagonists: 1) NVP-AAM007 (NVP), a competitive and reversible antagonist that displays approximately ten-fold greater selectivity for A-type over B-type NMDARs (Neyton and Paoletti, 2006); and 2) MK-801, a use-dependent and irreversible NMDAR open-channel blocker. Using this approach, B-type receptors, along with some proportion of AB-type receptors, were irreversibly blocked by MK-801. Those sensitive to NVP (*i.e.*, A-type and some fraction of AB-type), however, were reversibly blocked and recovered from inhibition following washout of both antagonists. A lower dose of NVP was then applied, sufficient to block >80% of A-type receptors (Tovar et al. 2013, Figure 3A), leaving a residual excitatory postsynaptic current (EPSC) dominated by AB-type NMDARs (Tovar et al., 2013;

Figure 6A). The AB-type receptors exhibited deactivation kinetics that more closely resembled that of fast A-type receptors than of the long-decaying B-type receptors (Tovar et al., 2013; Table 1). These results imply that the presence of a single GluN2A subunit in the triheteromeric configuration is sufficient to accelerate the kinetics of NMDAR deactivation by hundreds of milliseconds. Interestingly, AB-type NMDARs display kinetics leading to the open states that are similar to that of B-type receptors (Tovar et al., 2013; Table 1). Thus, AB-type NMDARs exhibit qualities of both diheteromeric A- and B-type NMDARs.

The results of Tovar et al. (2013) clarify our understanding of synaptic NMDAR function and help to resolve a long-standing inconsistency in the literature of NMDAR pharmacology. The GluN2B-selective antagonist ifenprodil and its derivatives have been widely used to characterize the “GluN2B-fraction” of synaptic NMDAR EPSCs. However, while some reports demonstrate that ifenprodil alters both the amplitude and deactivation kinetics of NMDAR currents (Bellone and Nicoll, 2007; de Marchena et al., 2008; Wang et al., 2008), others have shown that the change in amplitude by ifenprodil is not accompanied by a change in kinetics (Gray et al., 2011). Importantly, Tovar et al. convincingly demonstrate that ifenprodil reduces the amplitude but does not alter the kinetics of AB-type NMDAR EPSCs (Tovar et al., 2013; Figure 7A). Collectively, these results suggest that the acceleration of NMDAR deactivation kinetics by ifenprodil likely reflects the presence of a high proportion of diheteromeric B-type NMDARs. Consistent with this notion, ifenprodil significantly reduces the amplitude and accelerates the kinetics of NMDAR EPSCs from synapses in early developing hippocampal circuits, which are known to express a high proportion of B-type receptors (Bellone and Nicoll, 2007; de Marchena et al., 2008; Gray et al., 2011). Furthermore, NMDAR EPSCs from pyramidal neurons in the adult prefrontal cortex, which maintain a high proportion of B-type receptors into adulthood, also

display ifenprodil-accelerated kinetics (Wang et al., 2008). Finally, ifenprodil reduces the amplitude of NMDAR EPSCs from mature neurons, without altering their kinetics (Bellone and Nicoll, 2007; Gray et al., 2011), in line with the developmental reduction of B-type NMDARs at synapses. Together, these results support the notion that AB-type NMDARs underlie a large fraction of NMDAR EPSCs at mature hippocampal synapses (Rauner and Kohr, 2011; Tovar et al., 2013) and further suggest that the classic NMDAR subunit switch during development may not reflect a B-type to A-type replacement *per se*, but rather a change from B-type to predominately AB-type. Furthermore, these data highlight that changes in both amplitude and kinetics of NMDAR currents should be examined when using ifenprodil to more accurately determine the contribution of B-type and AB-type receptors. It is necessary to note here that the efficacy of ifenprodil is highly dependent on extracellular Mg^{2+} , with maximal inhibition achieved under Mg^{2+} -free conditions (Rauner and Kohr, 2011). This important detail should be considered in the design of future experiments using ifenprodil and its derivatives, as well as in the interpretation of previous results.

The hybrid nature of AB-type NMDARs, which exhibit features of both A-type and B-type NMDAR function, raises interesting possibilities regarding their role in synaptic transmission and plasticity. First, NMDAR kinetics can powerfully influence the temporal characteristics of synaptic integration (Paoletti et al., 2013). Long-decaying B-type NMDARs extend, whereas GluN2A-containing NMDARs shorten, the temporal window for synaptic integration and the initiation of regenerative dendritic and NMDA spikes (Larkum et al., 2009). The relatively rapid decay kinetics of AB-type receptors can thereby enhance the temporal precision of coincidence detection and stimulus representation during fast excitatory neurotransmission. Furthermore, GluN2A and GluN2B subunits, although very similar in

structure, exhibit key differences in amino-acid sequence of their intracellular C-terminal tails. These regions are sites of interaction with intracellular scaffolds and distinct downstream effector molecules, thereby conferring specific signaling properties to GluN2A- and GluN2B-containing NMDARs (Paoletti et al., 2013). For instance, the preferential interaction between GluN2B subunits and calcium/calmodulin-dependent kinase II (CaMKII) is critical for the expression of long-term potentiation (Barria and Malinow, 2005). Interestingly, during the so-called ‘critical period’ in early cortical development, neurons more readily express synaptic plasticity compared to mature neurons (Crair and Malenka, 1995). Since B-type NMDARs predominate during early development and preferentially associate with plasticity-related machinery, the reduction of synaptic GluN2B-containing NMDARs is generally thought to underlie this developmental change in synaptic plasticity (Paoletti et al., 2013). By virtue of their GluN2B subunit, the expression of AB-type NMDARs may help to explain why mature synapses generally retain some degree of ifenprodil sensitivity and synaptic plasticity. Future studies will likely shed light on these intriguing possibilities.

Because of its relatively poor subunit-selectivity compared to GluN2B-selective antagonists, the use of NVP to distinguish GluN2A- from GluN2B-containing NMDARs has been challenged (Neyton and Paoletti, 2006). Tovar et al. (2013) have demonstrated the utility of this GluN2A-selective antagonist in a series of well-designed pharmacological experiments to isolate AB-type NMDARs and have provided important insight into their intrinsic biophysical properties (Tovar et al., 2013). Based on these data, we have outlined a potential procedure for determining the relative contributions of NMDAR subtypes to central synapse function (*i.e.*, by examining the effects of ifenprodil on both amplitude and kinetics of NMDAR EPSCs). Although NMDARs are implicated in several neuropsychiatric disorders, with subunit-

composition hypothesized to play a critical role in their pathogenesis, several subunit-selective NMDAR-based clinical therapies have largely failed (Paoletti et al., 2013). It is tempting to speculate that these clinical shortcomings resulted in part from complexities associated with the presence of AB-type NMDARs, in light of the prominent role these receptors play in synaptic transmission (Rauner and Kohr, 2011; Tovar et al., 2013). The coming years will undoubtedly witness refinements in our understanding of both the function and subcellular distribution of distinct NMDAR subtypes, and perhaps the development of novel therapeutic strategies that accommodate for the enigmatic AB-type NMDARs.

A.4.

Manuscript VII

Title:

Subunit-selective modulation of NMDAR trafficking by glycine in central neurons

Authors:

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This paper has been in this near final form since October 2014. To be submitted in the fall of 2016.

Statement of Contributions:

This project was my main project for approximately one year when I started graduate studies, prior to my transfer to the PhD program and becoming interested in investigating mechanisms of homeostatic plasticity. I designed (with the help of all authors) and performed all of the electrophysiological and imaging experiments, performed all of the analyses and wrote the paper. I had some experimental support from Kevin Lee, and Sean Geddes who made some of the electrophysiological recordings in some of the earliest experiments (Figures 1B and 2B-D). Prakash aided in the preparation of the dissociated neuronal cultures and made sure that we had fresh DNA to work with for transfections. Richard Bergeron and Jean-Claude provided guidance and support throughout and Jean-Claude made edits and helped write the manuscript.

Abstract:

N-methyl-D-aspartate receptors (NMDARs) are an atypical subtype of glutamate receptor since they are gated by the coincident binding of a second agonist, either glycine or D-serine. Extracellular concentrations of these agonists rapidly rise in pathological states of excitotoxicity and, through sustained activation of NMDARs, may exacerbate neurotoxicity. Since both the subcellular localization and precise subunit composition of NMDARs are believed to impact excitotoxic signalling, we sought to characterize the subcellular trafficking of distinct NMDAR subtypes following sustained NMDAR activation. Using whole cell electrophysiology, we found that repetitive synaptic stimulation in conditions of elevated glycine induced NMDAR internalization from synaptic sites. In addition, using an optical approach that enabled us to monitor specific NMDAR-subunits, we found this glycine-induced internalization is preferential for GluN2A-containing NMDARs. These results highlight an intriguing disparity in the subcellular trafficking of distinct NMDAR subtypes at central synapses, which bears importance for the complex cellular response that proceeds periods of excitotoxicity.

Introduction:

N-methyl-D-aspartate receptors (NMDARs) are present at excitatory synapses where they participate in a myriad of signalling pathways that are critical for proper brain functioning (Hunt and Castillo, 2012; Lau and Zukin, 2007; Paoletti et al., 2013). These channels are tetrameric assemblies of two obligatory GluN1 subunits and varying combinations of two GluN2(A-D) and/or GluN3(A-B) subunits (Furukawa et al., 2005; Traynelis et al., 2010). Although each subunit has a distinct expression profile that differs across neuronal cell types and through development, most pyramidal neurons of hippocampal and cortical origins express primarily GluN1, GluN2A and GluN2B (Monyer et al., 1994; Paoletti et al., 2013). In response to the coincident binding of co-agonists glutamate (binding to GluN2 subunits) and glycine (or D-serine; binding to the GluN1 subunits), along with membrane depolarization to remove a voltage-dependent magnesium block, NMDARs permeate cations and contribute to slow excitatory neurotransmission. Due in large to their permeability to calcium, these channels gate various forms of structural and functional synaptic plasticity that are intimately tied to complex brain functions such as learning and memory (Lau and Zukin, 2007; Paoletti et al., 2013). In pathological conditions of excitotoxicity, however, excessive cell-wide NMDAR activation is linked to several of the acute and delayed aspects of neurotoxicity (Szydlowska and Tymianski, 2010; Villmann and Becker, 2007).

Although classically believed to be stable entities at synapses, NMDARs have proven to be much more dynamic in neuronal membranes than previously thought (Bard and Groc, 2011; Lau and Zukin, 2007). The surface population of NMDARs is in flux with intracellular pools through mechanisms of regulated and constitutive endo- and exocytosis (Groc and Choquet, 2006; Nong et al., 2004; Roche et al., 2001). Once in the plasma membrane, NMDARs move

laterally and are stabilized at synapses through intracellular interactions with scaffolding proteins embedded within the postsynaptic density (Bard et al., 2010; Groc et al., 2006). Interestingly, subtypes of NMDARs have distinct targeting and trafficking phenotypes that are guided by differences in the intracellular domain of receptor subunits (Bard and Groc, 2011; Ryan et al., 2008). Furthermore, the subunit composition of NMDARs confers unique biophysical signatures and pharmacological sensitivities, which have been especially relevant in ascribing subunit-dependent differences in NMDAR function (Sanz-Clemente et al., 2013). Amongst the most interesting and hotly debated topics in this regard has been the dichotomy of NMDAR signalling with respect to both subunit composition (primarily GluN2A-containing versus GluN2B-containing) and subcellular distribution (synaptic versus extrasynaptic) of receptors (Hardingham and Bading, 2010; Hardingham et al., 2002; Ivanov et al., 2006; Liu et al., 2007). Although this discriminate model of ‘pro-life’ (GluN2A; synaptic) versus ‘pro-death’ (GluN2B; extrasynaptic) NMDAR-mediated signalling has been challenged (Cepeda and Levine, 2012; Martel et al., 2012; Sattler et al., 2000; von Engelhardt et al., 2007; Zhou et al., 2013), there remains a loose understanding of how the excitotoxic environment influences the surface trafficking of distinct NMDAR subtypes.

Using whole-cell electrophysiology in acute hippocampal slices, we found that extracellular glycine elevations caused a rapid depression of evoked NMDAR-EPSCs at Shaffer’s collateral synapses which was dependent on postsynaptic NMDAR internalization. Using a live-cell imaging strategy, which offered molecular specificity to monitor the surface distribution of specific NMDAR subunits, we found subunit dependent differences in the synaptic regulation of NMDARs as a consequence of their sustained activation. Whereas GluN2A-containing NMDARs in dendritic spines were highly sensitive internalization, GluN2B-

containing receptors were largely resistant. Importantly, we validated the phluorin-based approach as a reliable reporter of surface NMDARs specifically in dendritic spines. Altogether, our results demonstrate an intriguing subunit-selective NMDAR trafficking phenotype that is triggered by an extracellular accumulation of glycine. Since subunit composition influences key biophysical and signalling properties of NMDARs, the glycine-induced trafficking phenotype we describe may be particularly important for understanding the complex nature of NMDAR-mediated signalling during excitotoxicity.

Materials and Methods:

Acute Slice Preparation

Acute hippocampal slices were prepared from Sprague Daley rats (Charles River) at post natal day (PND) 21-28, unless otherwise specified. Rats were anesthetized by isofluorane (Baxter Corporation, Canada) inhalation and sacrificed by decapitation, in accordance with methods approved by the University of Ottawa Animal Care Committee. The brain was rapidly removed and placed in an ice-cold cutting solution composed of (in mM): 119 choline chloride, 2.5 KCl, 1 CaCl₂, 4.3 MgSO₄·7H₂O, 1 NaH₂PO₄·H₂O, 1.3 sodium L-ascorbate, 26.2 NaHCO₃, and 11 glucose, saturated with a 95% O₂, 5% CO₂ gas mixture (pH 7.3; 290-300 mOsm/L). Coronal slices (300-350 µm thick) were sectioned in ice cold cutting solution using a table-top vibratome and were transferred to a recovery chamber pre-warmed at 37°C containing a ringer solution composed of (in mM): 119 NaCl, 2.5 CaCl₂, 1.3 MgSO₄·7H₂O, 1 NaH₂PO₄·H₂O, 26.2 NaHCO₃, and 11 glucose, saturated with a 95% O₂,/5% CO₂ gas mixture. Slices were recovered at room temperature for at least 1 hour before being transferred to the recording chamber of an electrophysiology setup for whole-cell recordings.

Whole-Cell Electrophysiology

CA1 hippocampal neurons were visualized under an upright microscope (either an Examiner D1 with a 40x/0.75NA objective - Carl Zeiss, Germany; or a BX61WI with a 40x/0.8NA or 60x/1.0NA objective – Olympus, New York) equipped with differential interference contrast (DIC) imaging. Whole-cell recordings were carried out using an Axon Multiclamp 700B amplifier, sampled at 10 kHz, filtered at 2 kHz and digitized with an Axon Digidata 1440A digitizer. Borosilicate glass patch electrodes (3-6 MΩ; World Precision Instruments, Florida or Sutter, California) were pulled on a Narishige PC-10 pipette puller (Narishige, Japan). All recordings were performed at room temperature in a ringer solution containing (in mM): 119 NaCl, 2.5 KCl, 0.1-1.3 MgSO₄·7H₂O, 2.5-3 CaCl₂·2H₂O, 1.0 NaH₂PO₄·H₂O, 11 glucose, 26.2 NaHCO₃, saturated with 95% O₂ and 5% CO₂ (pH = 7.3-7.4; 295-305 mOsm/L). When specified, additional drugs were added to the ringer solution at a final concentration of (in mM): 0.01-0.02 NBQX, 0.05 strychnine, 0.03 ifenprodil and 0.1 DL-APV. An intracellular recording solution containing (in mM): 115 cesium methane-sulfonate, 5 tetraethylammonium-Cl, 10 sodium phosphocreatine, 20 HEPES, 2.8 NaCl, 5 QX-314, 0.4 EGTA, 3 ATP(Mg²⁺ salt), and 0.5 GTP, pH 7.25 (adjusted using CsOH; osmolarity, 280–290 mOsmol/L) was used in all voltage clamp recordings. MK801 (1 mM) and Dynasore (100 μM) were added to the intracellular solution in a subset of recordings. Access resistance was continuously monitored by applying a 250 ms, 5 mV hyperpolarizing pulse at the beginning of each sweep. Recordings were discarded when the access resistance changed by >20%.

Synaptic excitatory postsynaptic currents (EPSCs) were evoked by placing a stimulating electrode proximal to the recorded cell (towards CA3) in stratum radiatum of CA1. For isolation of NMDAR-mediated EPSCs, neurons were voltage clamped at -60 mV in a low-magnesium ringer solution (0.1 mM Mg²⁺, 3.0 mM Ca²⁺), and NBQX was added to block AMPARs. For

isolation of AMPAR-EPSCs, experiments were performed at a holding potential of -70 mV in a standard ringer solution (1.3 mM Mg^{2+} , 2.5 mM Ca^{2+}) or at -60 mV in a low magnesium ringer solution (0.1 mM Mg^{2+} , 2.5 mM Ca^{2+}) by inclusion of MK801 in the recording electrode. In all experiments, picrotoxin (0.1 mM) was used to block GABAergic transmission. Amplitude of EPSCs were extracted using Clampfit 10.3 (Molecular Devices, USA) analyzed and plotted in Origin 8.5 (OriginLab, USA). Traces for NMDAR-EPSC decay analysis were transferred into Origin and a bi-exponential weighted tau values (ref) were calculated as fits (Vicini et al., 1998).

Two-Photon Imaging and Glutamate Uncaging

Glutamate uncaging experiments were performed in a ringer solution containing (in mM): 2.5 MNI-glutamate trifluoroacetate (Femtonics, Hungary), 119 NaCl, 2.5 $CaCl_2$, 1.3 $MgSO_4 \cdot 7H_2O$, 1 NaH_2PO_4 , 26.2 $NaHCO_3$, and 11 glucose, 0.01 glycine, 0.01 NBQX, and 0.001 tetrodotoxin (Tocris), which was continuously bubbled at room temperature with a 95% O_2 /5% CO_2 gas mixture. Whole cell recordings were made from CA1 pyramidal neurons using an intracellular solution containing (in mM): 37 cesium methane-sulfonate, 20 tetraethylammonium-BAPTA, 5 tetraethylammonium-Cl, 3 calcium-chloride, 10 sodium phosphocreatine, 20 HEPES, 5 QX-314, 4 ATP (Mg^{2+} salt), and 0.5 GTP, 0.03 Alexa 594 hydrazide (Na-salt; Invitrogen). Neurons were allowed to fill with the dye for a minimum of 10 minutes before onset of uncaging experiments.

Simultaneous two-photon imaging and glutamate uncaging was achieved using two Ti:Sapphire pulsed lasers with pre-chirp compensation (MaiTai DeepSee, Spectra Physics, USA) coupled to an Olympus MPE-1000 galvanometer scanning system. Image acquisition was performed through Olympus FV-1000 software (Olympus). One Ti:Sapphire laser was tuned to 810 nm to excite Alexa 594 and visualize dendritic morphology, and the second laser was tuned

to 720 nm for MNI-Glutamate uncaging (2 ms pulses directed at the tips of dendritic spines). Synchronization of electrophysiological and optical equipment was accomplished using a Master-8 pulse generator (A.M.P.I, Israel). Spines selected for 2P-uncaging experiments were on proximal secondary and tertiary apical dendrites in order minimize issues of space clamp, and were well segregated from the parent dendrite in the X-Y plane. The intensity of each laser was independently controlled by separate acousto-optic modulators (AOMs). The intensity of the uncaging laser was tuned to generate a 10-30 pA NMDAR-mediated EPSC at a holding potential of -30 mV.

Dissociated Neuronal Culture and Transfection

Hippocampi were isolated from embryonic day 18 (E18) rats, dissociated in a papain solution at 37°C, and plated on poly(L-lysine)-coated glass coverslips. Cultures were maintained in a neurobasal-based culture medium at 37 °C in 5% CO₂ incubator for 15–21 days prior to transfection. Neurons were transfected using lipofectamine 2000 as directed in the manufacturer's instructions (Invitrogen). 1-2 µg of cDNA plasmid (~ 20% mCherry reporter and ~ 80% SEP-tagged glutamate receptor subunit) was added to each well of a 12 well culture dish and the neurons were returned to the incubator for 1-2 hours. Neurons were then removed from the lipofectamine-containing solution and transferred back into normal culture medium for 48-96 hours prior to live cell imaging. SEP-GluN2A, SEP-GluN2B and SEP-GluA2 cDNA plasmids were all purchased from Addgene. The mCherry cDNA plasmid was kindly provided by Dr. Richard Huganir, and the SEP-GluA2ΔC49 was offered by Dr. Kenneth Madsen.

Live Cell Imaging and Image Analysis

Coverslips were removed from culture and placed in a recording chamber continuously perfused (~ 2mL / minute) in a ringer solution containing (in mM): 150 NaCl, 3 KCl, 0.25-0.75 MgCl, 2.5 CaCl₂, 10 HEPES, 1 sodium L-ascorbate, and 10 glucose. Dendritic segments of transfected neurons were imaged in time-lapse using laser-scanning microscopy (512X512 or 640x640 pixel reconstructions at 0.1-0.15 µm / pixel). Emission signals of SEP-GFP and mCherry were separated using a low-pass 560 nm dichroic mirror and additionally filtered using separate bandpass filters (500-550 for GFP and 565-595 for mCherry emissions, respectively). Each optical section was a kalman average of 2 consecutive frames. All data analysis was performed on raw unprocessed images. For figure purposes, representative images were manually thresholded and filtered using a 0.2 pixel median filter in ImageJ (NIH).

For spine enrichment indices (SEI), line intensity profiles through individual spines and their respective dendrite were extracted for each channel using the line plot function in ImageJ, and were imported into MATLAB. Only spines that were well separated from the dendrite were selected for SEI analysis. A custom script was written to extract the absolute peak intensities for both the spine and dendrite segments, and average peak intensities in each compartment were calculated as the mean of the absolute peak along with flanking pixels (3 pixel averages). S.E.I. for each spine was calculated as:

$$SEI = avgPeak\left(\frac{G_{sp}}{R_{sp}}\right) / avgPeak\left(\frac{G_{den}}{R_{den}}\right)$$

To estimate the percentage of ER signal contributing to spine associated SEP-NMDAR measurements (%spER), the dendritic peaks of all SEP-GluN2A and SEP-GLuN2B expressing spines were extracted from the line intensity profiles used in the SEI analysis. Next, we made the assumption that half the dendritic SEP signal was emanating from the surface and half was

emanating non-acidic ER compartments, based on previous evidence that SEP-signals in dendrites are contaminated by ER by approximately 50% (Rathje et al., 2013). Next, we calculated an expected spine ER intensity ($\text{spER}_{\text{expected}}$) based on the average SEI measurement of a putative ER signal (SEP-GluA2 Δ C49; average SEI = 0.33). Last, we divided the $\text{spER}_{\text{expected}}$ value by the average peak intensity of the SEP signal of each spine, and this value was multiplied by 100 to generate %sp ER estimates.

For time lapse imaging experiments (**Figure 6**), sequential frames were aligned in the X-Y plane using a stackreg function in ImageJ, and regions of interest (ROIs) were manually drawn around dendritic spines that were well separated from the parent dendrite. The summed pixel intensity from each ROI (for both red and green channels) was calculated by multiplying the area of the region by the average pixel intensity, and background contributions (average background pixel intensity multiplied by the area of the ROI) were subtracted. Baseline averages (F_0) were calculated by averaging the spine intensity of the first four frames, and the integrated spine intensity at each frame was then normalized to the average baseline ($\Delta F/F_0$). For each experiment, an average spine $\Delta F/F_0$ was calculated, and cell average behaviors were pooled, plotted (**Figure 6B-E**), and quantified as a % reduction in spine SEP signal (**Figure 6G**). Peak spine $\Delta F/F_0$ was calculated for each spine as the average intensity after glycine treatment. Internalization positive (Int.^+) spines were classified as spines whose intensity was reduced $>2 \times \text{SD}$ from the baseline average, which was typically between 84-86% of baseline. The percent spine internalization calculation was based on cell averages behaviors (i.e., % spine internalization was calculated for each experiment, and then averaged within each experimental condition). Pseudocolored $\Delta F/F_0$ plots were generated in ImageJ using the ‘delta F down’

plugin. An average of two consecutive frames after agonist treatment ($t = 10$ -12 minutes) was compared to the average baseline intensities ($t = 0$ -6 minutes).

Results

Rapid and transient depression of NMDAR-mediated synaptic currents by extracellular glycine

Glycine has been shown to internalize NMDARs from the plasma membrane, although there is little evidence to suggest that these mechanisms are operant at synapses (Han et al., 2013; Nong et al., 2003). To determine whether synaptic NMDARs are sensitive to elevations in extracellular glycine, we determined the effects of varying concentrations of extracellular glycine (1 – 1000 μ M) on NMDAR-mediated excitatory post-synaptic currents (NMDAR-EPSCs). CA1 pyramidal neurons from acute hippocampal slices (P21-28) were voltage clamped at a membrane potential (V_m) of -60 mV in a low magnesium (0.1 mM) ringer solution containing picrotoxin (100 μ m) and NBQX (10-20 μ m) to reduce magnesium block of NMDARs and eliminate the contribution of non-NMDA receptor channels. Schaffer's collateral axons were stimulated at low frequency (0.1 Hz) using a glass patch electrode positioned in the stratum radiatum of CA1.

Bath administration of glycine (5 min) in the low micromolar range (10 – 100 μ M) invariably potentiated the amplitude of evoked NMDAR-EPSCs ($128.8 \pm 4.5\%$ of baseline, $n = 6$ cells; **Figure 1A**), consistent with the notion that glycine binding sites of synaptic NMDARs are not saturated upon glutamate release (Bergeron et al., 1998; Johnson and Ascher, 1987; Wilcox et al., 1996). In contrast, bath administration of glycine at a dose of 1 mM lead to a robust reduction in the amplitude of NMDAR-EPSCs ($52.8 \pm 6.2\%$ of baseline, $n = 11$ cells), which slowly recovered upon glycine washout (**Figure 1B**). The magnitude of this glycine induced

depression of NMDAR-EPSCs was unaltered by strychnine (5 μ M), a competitive antagonist for inhibitory glycine receptors (without strychnine: $44.5 \pm 7.1\%$ of baseline; with strychnine: $38.9 \pm 4.7\%$ of baseline; $n = 5$ cells; **Figure 1C**). These results demonstrate a dose-dependent, bidirectional, influence of extracellular glycine on NMDAR-mediated transmission at hippocampal synapses - whereas small elevations in extracellular glycine potentiate NMDAR responses, larger elevations lead to a depression of NMDAR function (**Figure 1D**).

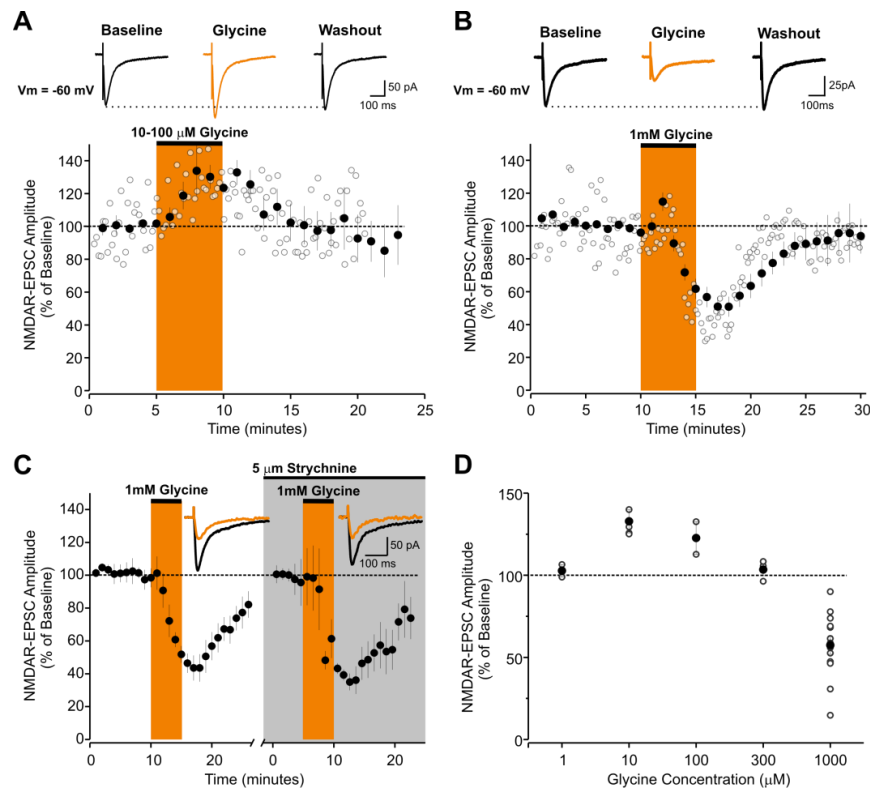


Figure 1: Dose-dependent bidirectional influence of extracellular glycine on the amplitude of NMDAR-mediated synaptic currents. **A**, Bath administration of glycine (5 minutes) in the low micromolar range (10-100 μ M) transiently potentiates evoked NMDAR-mediated EPSCs from Schaffer's collateral synapses. NMDAR-EPSCs were recorded at a holding potential (V_m) of -60 mV in a low magnesium ringers to reduce magnesium dependent block of NMDARs. Empty circles represent normalized amplitudes of a single experiment (100 μ M glycine) whereas filled circles represent the mean $\pm$ standard error (SEM). **B**, At an elevated dose of 1 mM, extracellular glycine caused depression of evoked NMDAR-EPSCs (empty circles: single experiment; filled circles: mean $\pm$ SEM). **C**, In the same recording, glycine induced NMDAR-depression was observed both in the absence and in the presence of extracellular strychnine (5 μ M), an antagonist of inhibitory glycine receptors. **D**, Peak changes in NMDAR-EPSC amplitude (normalized; % of baseline) in response to elevations in extracellular glycine concentration (1-1000 μ M; empty circles: single experiments; filled circles: mean $\pm$ SEM).

We next sought to determine whether the reduction in NMDAR-EPSC amplitude induced by high glycine could be accounted for by changes in presynaptic release probability. As a first test, we determined the effects of glycine on AMPAR-mediated EPSCs, reasoning that a change in presynaptic release probability should indiscriminately affect both subtypes of ionotropic glutamate receptor (Kauer et al., 1988). In extracellular conditions of normal Mg^{++} (1.3 mM, without NBQX), 1 mM glycine, did not reduce the amplitude of evoked AMPAR-EPSCs ($108.8 \pm 7.4\%$ of baseline; $n = 6$ cells; **Figure 2A**), arguing against the possibility that high glycine reduced release probability. However, it remained formally possible that high glycine modulates presynaptic release probability only in conditions that permit NMDAR gating (*i.e.*, in 0.1 mM Mg^{++}). In order to test for this, while maintaining the ability to discriminate AMPAR-EPSCs, we repeated these experiments in a low magnesium ringer and blocked postsynaptic NMDARs by inclusion of MK801 (1 mM), a use dependent NMDAR antagonist, in the recording electrode. In these conditions, the decay kinetics of evoked EPSCs progressively became faster, consistent with a use-dependent rundown of specifically a long decaying NMDAR-mediated contribution (**Figure 2B**). Once the amplitude and decay of the evoked EPSC stabilized to a steady-state (~ 10 minutes), the extracellular concentration of glycine was transiently raised to 1 mM, which affected neither the paired pulse ratio (PPR baseline: 1.45 ± 0.10 ; PPR glycine: 1.54 ± 0.14 ; $n = 4$ cells, $p = 0.338$, paired two-sample t-test; **Figure 2C**) or amplitude of evoked EPSCs ($105.6 \pm 8.6\%$ of baseline; $n = 4$ cells, **Figure 2D**). These results thus favored a postsynaptic modification as the basis for glycine-induced NMDAR depression. In a separate series of experiments, we directly tested our internalization hypothesis by including a dynamin inhibitor, dynasore (100 μM), in the recording electrode, and repeated the experiment with high extracellular glycine. Remarkably, in the presence of intracellular dynasore, the amplitude of evoked NMDAR-EPSCs

remained stable upon glycine administration ($98.4 \pm 9.7\%$ of baseline; $n = 7$ cells), whereas NMDAR-EPSCs from interleaved control neurons without dynasore in the pipette showed clear synaptic depression ($56.7 \pm 7.3\%$ of baseline; $n = 4$ cells, $p = 0.016$; **Figure 2E-F**). Bath application of the non-competitive NMDAR antagonist DL-APV ($100\ \mu\text{M}$) abolished evoked EPSCs (Figure 2F) and thus confirmed that they were mediated by NMDARs. These results strongly suggest that accumulation of extracellular glycine leads to the internalization of postsynaptic NMDARs at CA1 hippocampal synapses.

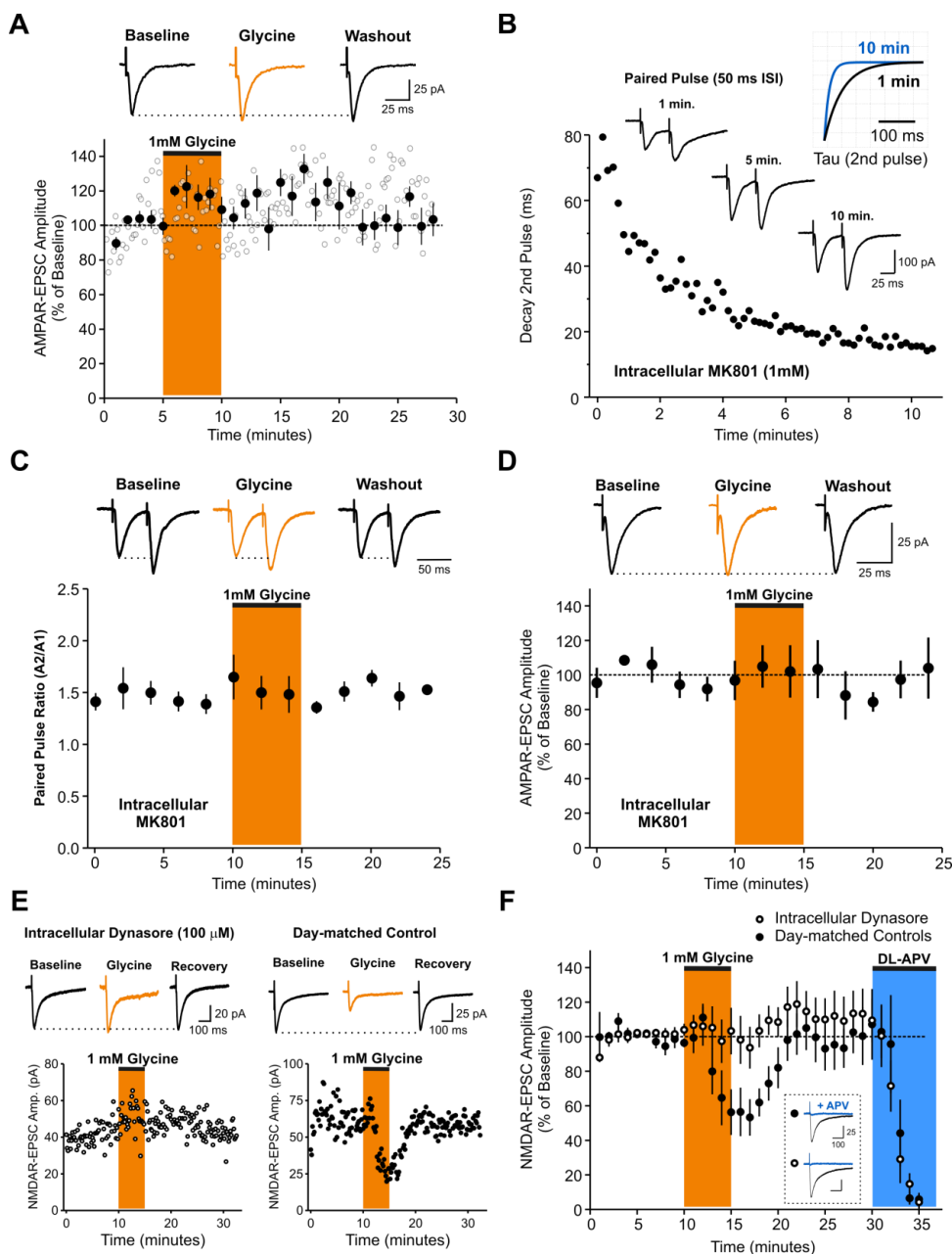


Figure 2: Glycine-induced depression of NMDAR-EPSCs results from postsynaptic receptor internalization. **A**, Bath administration of 1 mM glycine does not alter the amplitude of evoked AMPAR-mediated EPSCs (empty circles: single experiment; filled circles: mean $\pm$ SEM). AMPAR-mediated currents were recorded in conditions where NMDARs are largely blocked ($V_m = -70$ mV in standardringer solution containing 1.3 mM magnesium). **B**, Isolation of AMPAR-mediated EPSCs in a low magnesium (0.1 mM) ringer solution by inclusion of the use dependent NMDAR-antagonist MK801 in the recording electrode. Mono exponential decay of mixed AMPA/NMDA-EPSCs (tau, 2nd pulse) becomes accelerated in a stimulus-dependent manner, consistent with the use-dependent rundown of a NMDAR-mediated component. **C**, Paired pulse ratio (peak amplitude 2nd pulse / peak amplitude 1st pulse; 50 ms inter stimulus interval) of evoked AMPAR-EPSCs was unchanged in the presence of 1mM glycine. AMPAR-mediated EPSCs ($V_m = -60$ mV, low magnesium ringer) were isolated by intracellular MK801

addition, as in panel B. **D**, The amplitude of AMPAR-EPSCs (1st pulse) were stable during acute 1mM glycine administration (same recordings as panel C). **E**, Representative examples and **F**, average summary of NMDAR-EPSC amplitudes from experiments in which dynasore (100 μ M), an inhibitor of dynamin mediated endocytosis, was included in the recording electrode. Glycine induced NMDAR depression was compared to day matched interleaved control neurons (without dynasore). NMDAR specific antagonist (DL-APV; 100 μ M) was administered at the end of the experiment to validate the recording of NMDAR-mediated synaptic currents (for traces, see *inset* in panel F).

Glycine-induced internalization of synaptic NMDARs requires channel gating

The effect of extracellular glycine on synaptic NMDAR trafficking could have resulted from intracellular NMDAR-dependent signalling or rather by a metabotropic-like transmembrane signal (*i.e.*, occurring independent of ion flux). Previous studies suggest that glycine ‘primes’ surface NMDARs for internalization through recruitment of the endocytic adaptor protein AP-2, although subsequent channel activation was deemed necessary for their removal from the plasma membrane (Han et al., 2013; Nong et al., 2003). As a first step to determine whether channel activation was required to internalize synaptic NMDARs, we determined whether accumulation of glycine alone, without concurrent activation of NMDARs by endogenous glutamate release, was sufficient to induce synaptic NMDAR internalization. To this end, we halted synaptic stimulation for the first 5 minutes of a prolonged (10 minute) glycine administration, and then resumed it for the last 5 minutes (**Figure 3A**). In these conditions, the amplitude of NMDAR-EPSCs immediately after the pause in synaptic stimulation was comparable to baseline (89.0 ± 14.5 % of baseline, $n = 6$ cells) indicating that treatment with high glycine in the absence of synaptic stimulation did not cause NMDAR internalization. When synaptic stimulation was resumed in the continuous presence of high glycine, however, there was a pronounced reduction in the amplitude of NMDAR-EPSCs that was of comparable magnitude to that observed in previous experiments (60.2 ± 14.0 % of baseline; **Figure 3A**). These results

therefore suggest that synaptic NMDARs remain anchored at synapses in the presence of high glycine, and that subsequent glutamate binding is required for their internalization.

Although our initial estimates based collectively on the data above suggest that the time course of this glycine induced NMDAR internalization can occur within minutes, due to the probabilistic release of glutamate in response to electrical stimulation, this duration was likely an overestimate. To more specifically characterize the time course and number of synaptic stimuli required to internalize NMDARs in the presence of high extracellular glycine, we delivered precisely timed glutamate release onto individual, visually-identified, dendritic spines using two photon microscopy and glutamate uncaging. CA1 pyramidal neurons were filled with Alexa 594 to visualise neuronal morphology under two-photon microscopy (at 810 nm; **Figure 3B**), and glutamate receptors on individual dendritic spines were activated by two-photon uncaging of MNI-glutamate (Beique et al., 2006; Harvey and Svoboda, 2007; Matsuzaki et al., 2001; Soares et al., 2013a). In the presence of NBQX, short duration light-pulses (2 ms; 720 nm) delivered at the tips of dendritic spines induced small inward currents when neurons were voltage clamped at -30 mV, but not at -70 mV (**Figure 3C**), consistent with the voltage dependent block of NMDARs by extracellular magnesium. In the absence of glycine, the amplitude of 2P-uncaging-induced NMDAR-EPSCs (u-NMDAR-EPSC) remained stable for at least 20 consecutive pulses delivered at 0.33Hz (**Figure 3D**). However, following pre-incubation of a high concentration of extracellular glycine (at $V_m = -70$ mV, > 5 minutes), consecutive activation of NMDARs by MNI-Glu uncaging (at $V_m = -30$ mV) lead to a rapid reduction in the amplitude of u-NMDAR-EPSCs (**Figure 3D**). In several instances, we were able to monitor the amplitude of u-NMDAR-EPSCs on the same dendritic spine before and after high glycine administration to the bath

(**Figure 3E-F**). These experiments not only emphasize that the reduction of u-NMDAR-EPSCs occurred exclusively in the presence of high glycine (for example, **Figure 3E**) but also showed that glycine did not alter the amplitude of NMDAR-mediated currents in the absence of stimulation (at V_m -70mV; **Figure 3F**). As additional support that consecutive synaptic stimulation by two-photon uncaging of glutamate does not alter synaptic NMDARs in control conditions, we found the decay kinetics u-NMDAR-EPSCs to be unchanged during synaptic stimulation (weighted tau, '*ctl start*': 105.1 ± 6.4 ms, '*ctl end*': 114.3 ± 6.5 ms; $n = 28$ spines, $p = 0.17$). Interestingly, in addition to the reduction in amplitude, the kinetics of u-NMDAR-EPSCs were significantly prolonged following stimulation in the presence of high glycine ('*gly start*': 117.9 ± 8.3 ms, '*gly end*' 156.3 ± 15.6 ms; $n = 27$ spines, $p = 0.02$). Overall, these experiments show that very few consecutive NMDAR gating events are required to reduce NMDAR function in the presence of a high glycine. Moreover, by bypassing the need of glutamate release from presynaptic terminals, these uncaging data provides further support to the notion that the glycine-induced alterations in NMDAR function are mediated by postsynaptic mechanisms.

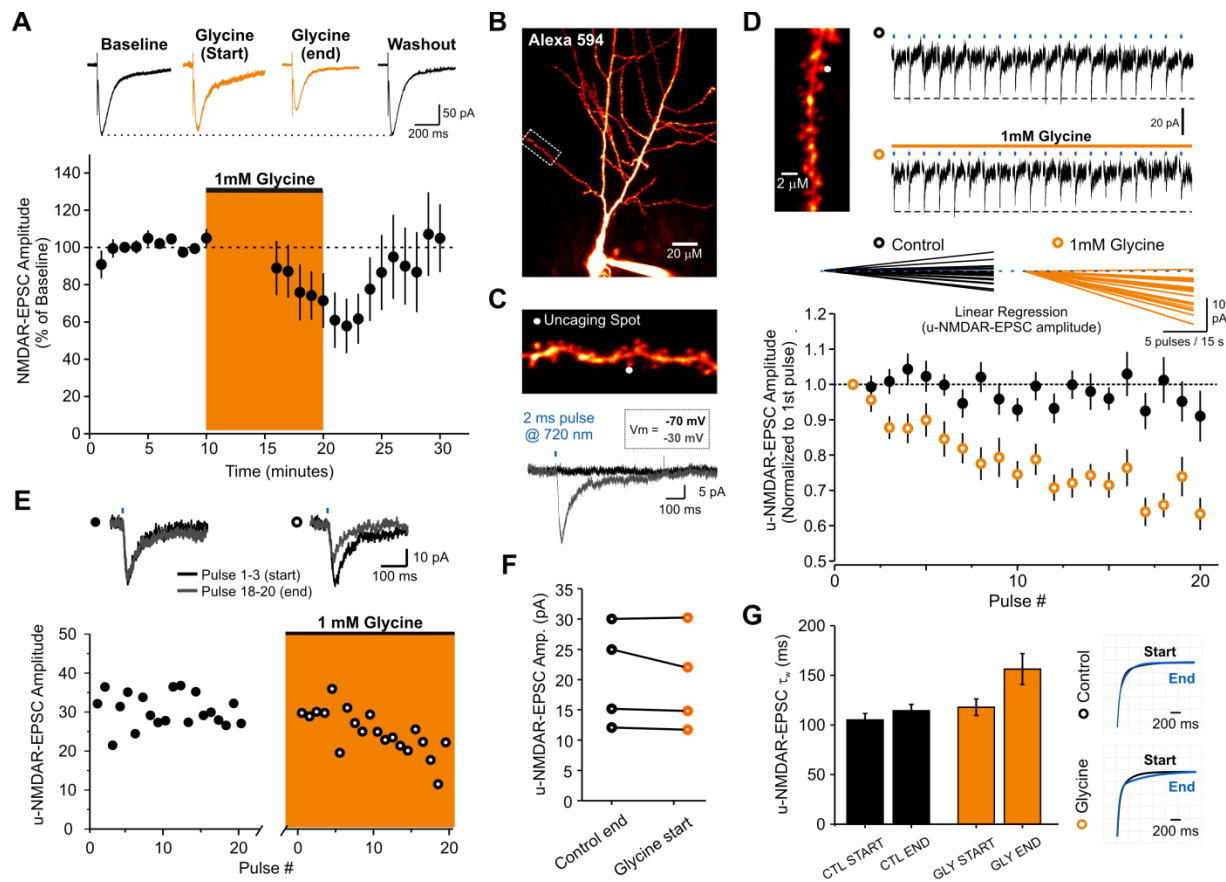


Figure 3: Glycine-induced internalization of synaptic NMDARs requires channel gating. **A**, Normalized NMDAR-EPSC amplitudes from recordings in which synaptic stimulation was transiently paused during the first 5 minutes of a prolonged (10 minutes) 1 mM glycine administration. Representative trace for 'glycine (start)' is a 1 minute average of NMDAR-EPSCs upon which synaptic stimulation was resumed ($t = 15\text{--}16 \text{ min.}$), and the trace for 'glycine end' is an average of NMDAR-EPSCs at the peak of the synaptic depression. **B**, A two-photon image of a live CA1 pyramidal neuron filled with Alexa594 via the recording electrode (taken at 810 nm). **C**, An enlarged view of a single dendritic segment of the neuron shown in panel B. A brief light stimulus (2 ms, 720 nm) directed at the tip of the indicated spine (filled circle) in the presence of 2.5 mM caged MNI-glutamate activates NMDARs at a holding potential of -30 mV but not at -70 mV, consistent with the voltage dependent block of NMDARs in extracellular magnesium. All uncaging experiments were done in the presence of the AMPA specific antagonist NBQX (20 μM). **D**, Repetitive synaptic stimulation (0.33 Hz, 20 pulses; $V_m = -30 \text{ mV}$) at a single spine by two-photon glutamate uncaging generates NMDAR-EPSCs of consistent amplitude (black circle), whereas the same stimulus paradigm in the presence of 1 mM extracellular glycine leads to a depression of uncaging-evoked NMDAR-EPSCs (orange circle). For uncaging experiments in the presence of extracellular glycine, neurons were voltage clamped at -70 mV during glycine addition ($\geq 5 \text{ minutes}$), and were subsequently clamped at -30 mV during glutamate uncaging. **E**, Average normalized u-NMDAR-EPSC amplitudes of single spine repetitive uncaging experiments, as described in panel D (black circles: without glycine; orange circles: in 1mM glycine). Linear regressions of u-NMDAR-EPSC amplitude from single spine experiments are plotted together and normalized to the 1st peak. **F**, A representative example where the consecutive uncaging paradigm (as in panel d) was done on the same spine before and after glycine addition. **G**, In experiments where the

consecutive uncaging experiment was done on the same spine both in the absence and presence of glycine ($n = 4$), there was no difference in amplitude of u-NMDAR-EPSCs prior to and after glycine addition. **H**, Weighted taus (τ_w) calculated from biexponential fits of u-NMDAR-EPSCs both at the start (first 3 sweeps) and end (last 3 sweeps) of the 20 pulse consecutive uncaging experiment described in panels D-E.

Subunit bias in glycine-induced NMDAR internalization

Given the known influence of NMDAR subunit composition on channel kinetics, protein-protein interactions, and subcellular distribution (Paoletti et al., 2013; Sanz-Clemente et al., 2013) – the latter of which being debated for its potential impact on excitotoxic signalling (Hardingham and Bading, 2010; Wroge et al., 2012; Zhou et al., 2013) – we questioned whether the glycine-induced internalization of synaptic NMDARs exhibits subtype specificity. As a first approach to explore this possibility, we recorded NMDAR-EPSCs from SC-synapses of young hippocampal slices (PND 8-9), since CA1 synapses at this early developmental epoch are dominated by GluN2B-containing NMDARs (Kirson and Yaari, 1996; Monyer et al., 1994; Paoletti et al., 2013). Indeed, the decay kinetics of NMDAR-EPSCs were slower in the early time period (P8-9; **Figure 4A**), consistent with a greater contribution by GluN2B-containing NMDARs. Bath administration of 1 mM glycine reduced the amplitude of NMDAR-EPSCs in recordings from young neurons (to $80.4 \pm 4.2\%$ of baseline; $n = 6$ cells; **Figure 4B**), although to a lesser extent than that observed in recordings from older neurons ($p = 0.016$; unpaired two-sample t-test). Given the developmental increase in GluN2A expression that occurs at hippocampal synapses (Kirson and Yaari, 1996; Monyer et al., 1994), we reasoned that the increase in the magnitude of the glycine-induced internalization during development (*i.e.*, from P8-9 to P21-28) could be accounted for by a preferential internalization of GluN2A-containing NMDARs.

As an additional test to probe for a potential subunit bias in the effects of glycine, we reasoned that we could enrich the contribution of GluN2A-containing NMDARs to the evoked NMDAR-EPSCs by pharmacologically removing an ifenprodil-sensitive GluN2B-containing population of NMDARs and, subsequently, determine the magnitude of glycine-induced internalization. In recordings from P21-28 slices, bath administration of ifenprodil, a GluN2B-selective antagonist (Williams, 1993), reduced the peak amplitude of NMDAR-EPSCs by $22.4 \pm 9.3\%$ (**Figure 4C**), outlining the presence of a relatively small fraction of GluN2B-containing NMDARs. Interestingly, the ifenprodil-insensitive component of the NMDAR-EPSCs exhibited an enhanced depression in response to glycine compared to age-matched control conditions (ifenprodil insensitive EPSCs, renormalized: $23.5 \pm 8.6\%$ of baseline; $n = 5$ cells, $p = 0.024$, unpaired two sample t-test; **Figure 4C**). Indeed, the summed suppression of NMDAR-EPSCs induced by both ifenprodil and glycine is far greater than that induced by glycine alone, suggesting that these two pharmacological manipulations target largely non-overlapping populations of NMDARs. These results provided additional support for a subunit selective bias in glycine-induced NMDAR internalization.

The subunit composition of synaptic NMDARs can be broadly inferred from the NMDAR-EPSC kinetics since NMDARs of different composition have different gating behaviours (Gray et al., 2011; Rauner and Kohr, 2011; Tovar et al., 2013; Vicini et al., 1998). To gain further insight to the possibility of a subunit selective bias in NMDAR internalization, we analyzed the decay kinetics of NMDAR-EPSCs prior to, during, and after 1 mM glycine administration in recordings from both old (PND 21-28) and young (PND 8-9) hippocampal slices. Whereas the kinetics of NMDAR-EPSCs from older neurons were not noticeably altered during glycine application ($p = 0.344$, paired two sample t-test; **Figure 4D**), the kinetics of those

from younger neurons were prolonged in the presence of glycine ($p = 0.016$, paired two sample t-test; **Figure 4D**). The slowing of NMDAR-EPSC kinetics by high glycine in young tissue in principle could be explained by a preferential internalization of GluN2A-containing NMDARs at a time when there is an overall larger fraction of long-decaying synaptic GluN2B-diheteromers (Kirson and Yaari, 1996; Monyer et al., 1994) compared to later in development (Rauner and Kohr, 2011; Tovar et al., 2013). Collectively, the results from this electrophysiological dissection (i.e., developmental regulation; ifenprodil sensitivity and kinetic analysis) of the glycine-induced suppression of NMDAR-EPSCs all suggest the presence of a subunit-selective bias in the ability of glycine to induce NMDAR internalization that points to GluN2A-containing receptors.

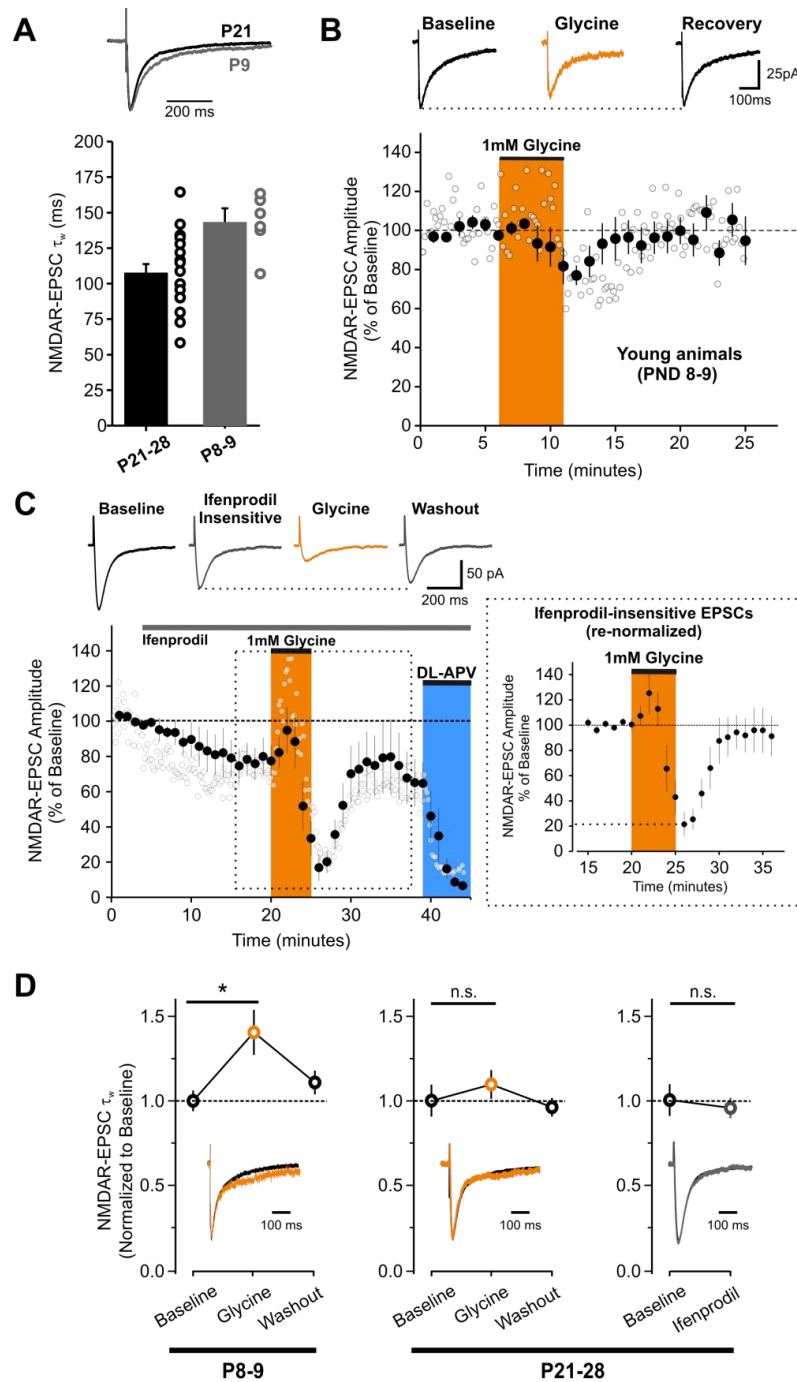


Figure 4: Glycine-induced internalization of synaptic NMDARs exhibits subunit selectivity. **A**, Acceleration of evoked NMDAR-EPSC decay kinetics at Schaffer's collateral synapses during development. Weighted taus calculated from biexponential fits of evoked NMDAR-EPSCs ($V_m = -60$ mV, low magnesium ring) are faster at postnatal day (P) 21-28 as compared to P8-9. **B**, Bath administration of 1 mM glycine reduces the amplitude of evoked NMDAR-EPSCs in slices from younger animals (P8-9; empty circles: single experiment; filled circles: mean $\pm$ SEM). **C**, Enrichment of the GluN2A-mediated contribution to the evoked NMDAR-EPSC by removal of a GluN2B-mediated, ifenprodil sensitive, component. Ifenprodil (3 μ M) was administered for 15 minutes prior to glycine

addition (empty circles: single experiment; filled circles: mean $\pm$ SEM). *Inset*, renormalized plot of ifenprodil insensitive NMDAR-EPSCs (*i.e.*, renormalized to the steady state at 10 and 15 minutes after ifenprodil addition). **D**, Decay kinetic analysis of evoked NMDAR-EPSCs. Weighted biexponential taus of NMDAR-EPSCs were calculated prior to, during, and after the glycine-induced depression in recordings from both younger (P8-9; Figure 4B) and older (P21-28; from Figures 1B, and 2F, controls) neurons. Weighted taus in glycine and washout bins were normalized to those of NMDAR-EPSCs during the baseline period for each experiment. Ifenprodil addition (from Figure 4B) did not alter the kinetics of NMDAR-EPSCs.

Expression of phluorin-tagged GluN2 subunits reports surface NMDARs in dendritic spines

To further elucidate whether the subunit composition of NMDARs influences their susceptibility to glycine-induced internalization, we moved to a culture preparation that would allow us to track the surface distribution of distinct NMDAR subunits by live-cell imaging. Super ecliptic phluorin (SEP), a pH-sensitive GFP fluorophore, has been used extensively as a reporter of surface ion channels (Ashby et al., 2004; Di Biase et al., 2011; Kopec et al., 2006; Lin and Huganir, 2007). Briefly, this technique is based on extracellular tagging of specific receptor subunits with an pH-sensitive GFP (SEP), the fluorescence of which becoming rapidly quenched upon receptor endocytosis due to the luminal acidification of sorting endosomes (**Figure 5A**) (Ashby et al., 2004). Dissociated neurons in culture were transfected at 15-18 DIV with N-terminally tagged SEP-GluN2A or SEP-GluN2B subunits, along with mCherry as a morphology reporter, and were subsequently transferred to a recording chamber for live-cell imaging experiments.

As our first attempt to assess the validity of this technique to report solely the surface population of tagged receptors, we briefly treated neurons with an acidic extracellular solution (pH = 6.0), resting on the assumption that only SEP-NMDARs at the surface of the plasma membrane are sensitive to a rapid (90 s) acid wash (Ashby et al., 2006; Wilkinson et al., 2014).

As expected, we observed a rapid and reversible quenching of SEP-signal upon brief extracellular acidification, which was not observed for mCherry (**Figure 5B**). Interestingly, the degree of acid-induced quenching was dependent on the subcellular localization of SEP emission. Whereas SEP-NMDAR signals in dendritic spines were almost completely quenched by the brief acidic wash (94.5 ± 1.0 % reduction from baseline fluorescence, **Figure 5B-C**), those emanating from dendritic and somatic compartments exhibited substantially less acid quenching (77.9 ± 3.0 % and 37.5 ± 1.4 % reduction, respectively; **Figure 5B-C**). This residual fluorescence likely emanates from tagged receptors in non-acidic intracellular compartments (Khiroug et al., 2009; Rathje et al., 2013). In support of this, longer duration acid-wash experiments (pH 6.0; 240 s) resulted in a more pronounced signal reduction in the dendritic shaft and somatic compartments ($81.3 \pm 1.7\%$ and $71.5 \pm 3.1\%$ reduction, respectively), but not in dendritic spines ($92.5 \pm 1.8\%$ reduction). Thus, the use of SEP-GFP as a reporter of surface-bound NMDARs was deemed potentially valid only for signals emanating from dendritic spines.

Despite a near complete quenching of SEP-NMDAR signal in dendritic spines during a brief extracellular acidification, we were cautious in our interpretation and reasoned that the fluorescence emanating from the spine compartment could still be, at least partially, contaminated by receptor subunits located intracellularly, notably in the endoplasmic reticulum (ER) network. Indeed, ER can either transiently or stably invade dendritic spines, although only a small fraction of spines are believed to harbour stable ER (Blumer et al., 2014; Cui-Wang et al., 2012; Rathje et al., 2013; Toresson and Grant, 2005). To discern whether spine-associated SEP-NMDAR signals were contaminated by receptors in intracellular ER, we estimated the extent of ER invasion in spines in our conditions. To this end, we computed a spine enrichment index (see Methods) for SEP-GluA2 Δ C49, an ER-trapped AMPA receptor subunit (**Figure 5D-**

F). Co-transfection of this construct with mCherry showed that the SEP-GluA2 Δ C49 signal was highly restricted to the dendritic compartment, with very little signal emanating from dendritic spines (spine enrichment index: 0.33 ± 0.05 , $n = 11$ cells, **Figure 5D-F**). In contrast, signals from WT SEP-GluA2 were readily detected in spines (spine enrichment index: 1.66 ± 0.29 , $n = 11$ cells; **Figure 5D-F**), indicating that the majority of spine SEP-GluA2 signal was emanating from surface receptors. Notably, the spine enrichment indices for both the SEP-GluN2A- and SEP-GluN2B fluorescent signal were significantly higher than that of SEP-GluA2-expressing spines, (cell averages: SEP-GluN2A: 6.50 ± 0.71 , $n = 8$ cells; SEP-GluN2B: 5.77 ± 0.87 , $n = 7$ cells; SEP-GluA2: 1.66 ± 0.29 , $n = 11$ cells; **Figure 5E-F**). Altogether, these results show that a very small proportion of spines have a discernable ER compartment containing SEP-signal in our conditions, and that SEP-NMDAR signals are enriched in dendritic spines.

Although the results outlined above indicate that SEP contamination from receptors in the ER is largely negligible in dendritic spines, we next sought to provide a quantitative estimate of the extent of SEP signal contamination by receptors in the ER in dendritic spines of SEP-GluN2A and SEP-GluN2B transfected neurons (see methods). Specifically, a metric based on the dendritic peaks of SEP-NMDAR signals and the average spine enrichment index of a putative ER-signal (SEP-GluA2 Δ C49) was used to calculate an expected ER intensity value ($\text{spER}_{\text{expected}}$) for each spine compartment of SEP-GluN2A and GluN2B expressing neurons. We then divided the $\text{spER}_{\text{expected}}$ intensity by the true intensity value of each spine compartment in order to estimate the percentage of signal contamination from receptors in spine ER (% sp ER; **Figure 5G**). On average, $\sim 5\%$ of spine-associated SEP-NMDAR signals were found to be attributable to signal emanating from the ER (SEP-GluN2A: $5.8 \pm 0.3\%$, $n = 438$ spines from 8 cells; SEP-GluN2B: $5.9 \pm 0.3\%$, $n = 294$ spines from 7 cells; **Figure 5G**), whereas the majority of SEP-

NMDAR signal were predicted to emanate from surface receptors. In combination with the results we obtained from the acid wash experiments, we conclude that monitoring florescent signals of SEP-tagged NMDAR subunits can reliably report changes in the number of surface-bound NMDARs in dendritic spines of live neurons.

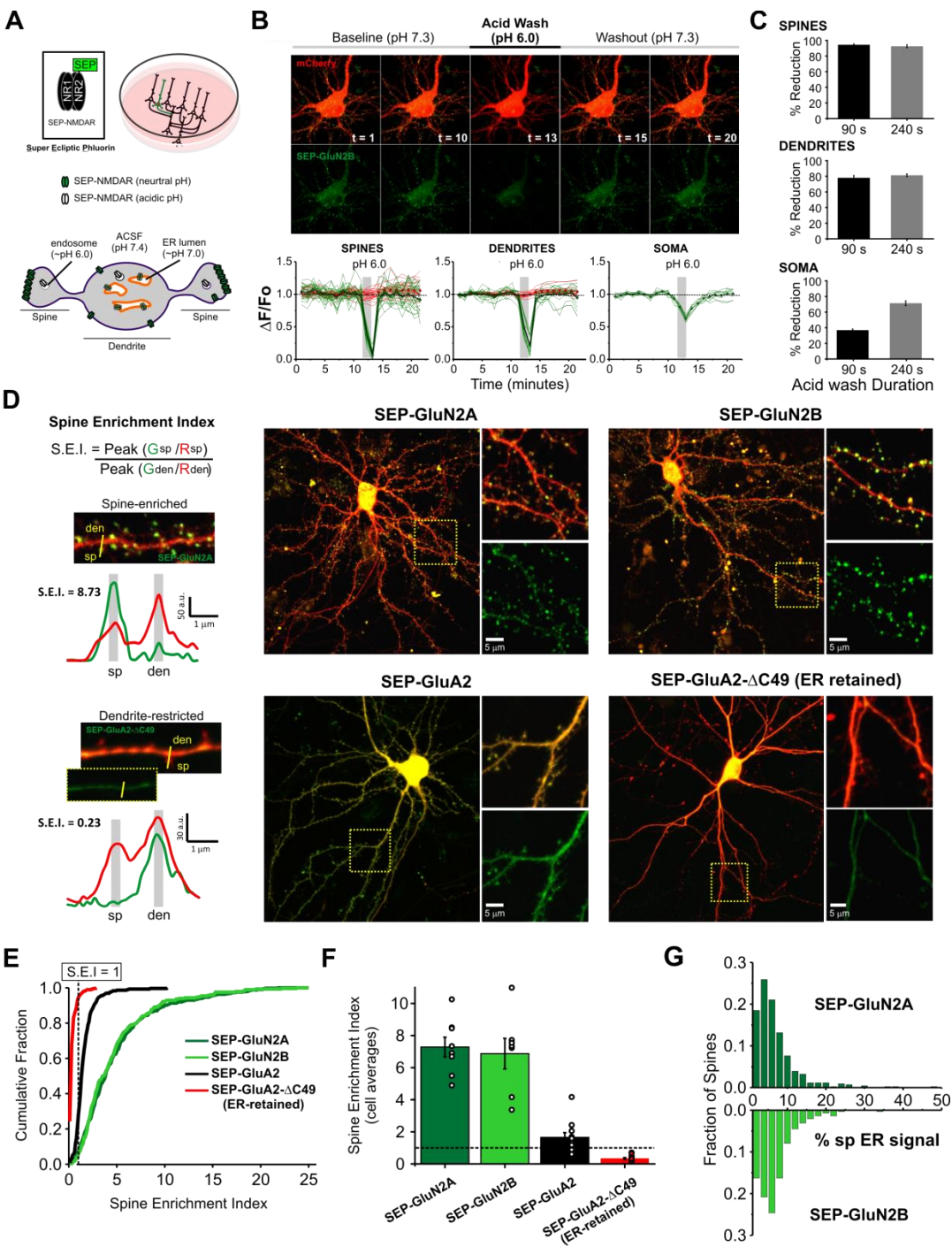


Figure 5: Monitoring surface NMDARs in dendritic spines using live cell imaging of SEP-tagged NMDAR subunits. **A**, Dissociated hippocampal neurons in culture (15-18 DIV) were transfected with phluorin-tagged glutamate receptor subunits to monitor the subunit specific surface trafficking of receptors. The fluorescence of super-ecliptic phluorin (SEP)-GFP is quenched when in an acidic environment, including the lumen of a number of intracellular trafficking compartments, making it a reporter of surface bound receptors. **B**, Time lapse images of an experiment in which a brief acid wash (extracellular pH = 6.0; 90 seconds) rapidly and reversibly quenches SEP-GluN2B signal. Normalized quantification of changes in fluorescence intensity ($\Delta F/F_0$) is plotted for individual (green) and average (black) regions of interest in each subcellular compartment. The normalized intensity of mCherry signals remained stable during the brief acid wash in both the spine and dendritic compartments, however, changes in mCherry intensity was unquantifiable in the somatic compartment due to a signal saturation. **C**, The average reduction in SEP-NMDAR intensity by both brief (90 seconds) and prolonged (240 s) extracellular acidification (pH = 6.0) in each subcellular compartment. **D**, A spine enrichment index (SEI) was used to compare the relative spine enrichment of SEP-tagged glutamate receptor subunits. SEIs were calculated based on the peak intensities of the spine and dendrite compartment for both the SEP and mCherry signal (for additional details, see methods). Example images of transfected neurons expressing one of either SEP-GluN2A, SEP-GluN2B, SEP-GluA2 or ER-retained SEP-GluA2 Δ C49 subunits, along with cytosolic mCherry as a dendritic reporter. **E**, Cumulative distribution all spines and **F**, cell averages of the spine enrichment values calculated for each plasmid condition. **G**, The percentage of ER signal contributing to the spine-associated SEP-NMDAR measurements was estimated for each spine of SEP-GluN2A and SEP-GluN2B expressing neurons (see methods).

Selective internalization of SEP-GluN2A NMDARs by glycine

Since SEP-NMDAR signals in spines were found to be predominately from surface receptors (**Figure 5**), we restricted our analysis to this subcellular compartment and reasoned that alterations in the intensity of the spine SEP signals reflect the gain or loss of surface NMDARs. The phluorin based live-cell imaging approach thus offered the ability to monitor with molecular specificity the trafficking behaviours of GluN2A and GluN2B NMDAR subunits in response to a glycine challenge.

Pilot imaging experiments were first conducted to determine the ‘baseline dynamics’ of spine-associated SEP-NMDAR signals in dendritic spines, initially focusing on SEP-GluN2A. In the absence of agonist treatment (**Figure 6A₁**), the average integrated spine SEP-GluN2A signals was stable over the duration of a typical imaging experiment (at least 30 minutes; $97.8 \pm$

2.2 % of baseline, 136 spines, $n = 6$ cells; **Figure 6B**), indicating stable imaging conditions. A closer inspection revealed that whereas the fluorescence intensity of the vast majority of spines were stable ($> 85\%$ of dendritic spines (117 of 136 spines; Figure 5B), that of a small fraction of spines showed significant deviation from baseline over this time frame, where approximately 8 % of spines showed an increase, (11 of 136 spines) and 6 % showed a decrease in SEP-GluN2A signal (8 of 136 spines; internalization positive spines, *int.*⁺; reduction of $> 2*SD$ of average baseline intensity, see methods; **Figure 6B**). Overall, these experiments described the behavior of SEP-GluN2A signals across a population of dendritic spines in control conditions.

Next, we monitored spine SEP-GluN2A signals during a brief glycine administration (1 mM, 4 minutes; **Figure 6A₂**). We observed minimal change in the spine-associated SEP-GluN2A intensities (93.0 ± 1.1 % of baseline, 91 spines, $n = 3$ cells; **Figure 6C**), although a slightly larger than expected fraction of spines met the criteria for internalization ($\sim 19\%$, 17 of 91 spines). In contrast, the co-application of glycine and NMDA (20 μ M) lead to a rapid and marked reduction in the intensity of spine SEP-GluN2A signals (77.4 ± 3.7 % of baseline, 238 spines, $n = 8$ cells; **Figure 6A₃, D**), and a far greater proportion of dendritic spines met the criteria for receptor internalization (~ 80 %, 191/238 spines). This widespread internalization behaviour was not observed when NMDA was administered alone (91.2 ± 0.6 % of baseline, 56 spines, $n = 3$ cells; 11/56 spines exhibiting internalization; **Figure 6D**). Strikingly, the same co-treatment of NMDA and glycine triggered only a modest reduction in spine fluorescent signal from SEP-GluN2B transfected neurons in interleaved experiments (94.6 ± 2.4 % of baseline, 174 spines, $n = 6$ cells; **Figure 6A₄, E**), with only a small fraction of spines showing internalization of SEP-GluN2B (24%, 42/174 spines), which was indifferent from administration of NMDA alone (90.5 ± 1.7 % of baseline, 122 spines, $n = 6$ cells; 33/122 spines exhibiting internalization;

Figure 6E). To further depict the spine behaviour of SEP-GluN2A signal in response to glycine, a pseudocolored image was generated to illustrate the pixels that exhibited the largest decrease in intensity following co-agonist treatment. Whereas the vast majority of SEP-GluN2A-expressing spines showed marked reduction in intensity following co-agonist stimulation, this was not the case for most SEP-GluN2B-expressing spines (**Figure 6F**). Altogether, these experiments demonstrate that GluN2A-containing NMDARs at dendritic spines are particularly sensitive to glycine-induced internalization.

As binding of both glutamate and glycine are required for NMDAR activation, administration of glycine alone was not expected to fully activate synaptic NMDARs. Thus, the dramatic enhancement of SEP-GluN2A internalization by co-agonist application (Glycine + NMDA) suggests that NMDAR activation facilitated the internalization of GluN2A-NMDARs. Consistent with this notion, pre-treatment of the competitive NMDAR antagonist DL-APV (100 μ M) abolished SEP-GluN2A internalization in response to co-agonist application (spine SEP-GluN2A intensity: 101.7 ± 2.4 % of baseline, 67 spines, $n = 2$ cells; 3/67 spines exhibiting internalization; **Figure 6G**). Moreover, the preferential antagonism of GluN2B-containing NMDARs by pre-treatment of ifenprodil (3 μ M) did not interfere with the glycine-induced internalization of GluN2A-NMDARs (avg. spine intensity: 81.5 ± 5.6 % of baseline, 52 spines, $n = 5$ cells; 40/52 spines exhibiting internalization) nor did pre-treatment with 5 μ M strychnine (66.4 ± 2.8 % of baseline, 35 spines, $n = 2$ cells; **Figure 6G**). Furthermore, the sodium channel blocker tetrodotoxin (1 μ M) did not reduce the extent of glycine-induced SEP-GluN2A internalization (spine SEP-GluN2A intensity: 79.3 ± 2.1 % of baseline, 107 spines, $n = 5$ cells; 70/107 spines exhibiting internalization; **Figure 6G**). Taken together, our live-cell imaging data suggests that NMDAR internalization induced glycine priming is selective for GluN2A-

containing NMDARs, requires channel activation, and can occur in conditions where GluN2B-containing receptors are blocked.

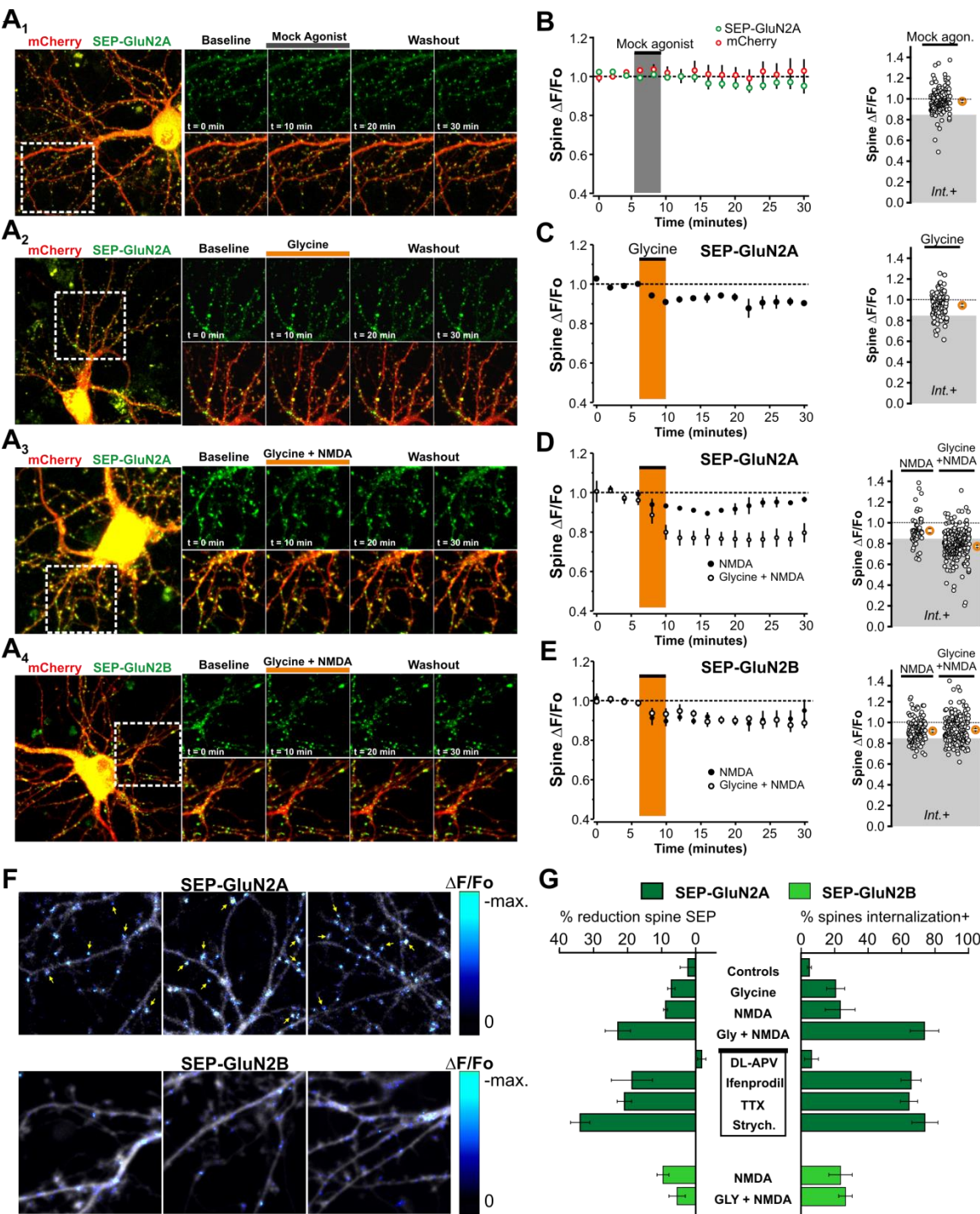


Figure 6: Subunit-selective internalization of GluN2A-containing NMDARs by high glycine. **A**, Representative time-lapse image sequences of experiments in which SEP-GluN2A expressing (A_1 - A_3) or SEP-GluN2B expressing (A_4) neurons were subjected to extracellular agonist treatment (4 minutes; A_2 - A_4), or a mock agonist treatment (A_1). Glycine was either administered alone (A_2) or in combination with co-agonist NMDA (A_3 - A_4). **B**, *left*, normalized average change in spine-associated SEP-GluN2A and mCherry signals following a mock agonist treatment. All spines traces from an individual experiment were normalized to baseline images, pooled and averaged. The plotted data depicts the average of all the cell average spine behaviors (avg. Spine $\Delta F/F_0$), $\pm$ SEM. *Right*, Normalized peak change in fluorescence (peak spine $\Delta F/F_0$) was calculated for each spine. A decrease in spine SEP signal that was greater than 2X the standard deviation of the baseline (reduction of $\sim 85\%$ of baseline average), spines were considered to have lost SEP-signal due to receptor internalization (internalization positive ; *Int +*). **C-E**, same as in panel B, except that agonist treatments (as indicated) replaced the mock agonist treatment. **F**, A comparison of interleaved experiments in which Glycine + NMDA was administered to SEP-GluN2A (top) or SEP-GluN2B (bottom) expressing neurons. mCherry signals from multiple dendritic segments are shown (grey), and are overlaid with a pseudocolored image depicting the pixels in the SEP-NMDAR channel that show the largest reduction in intensity following co-agonist administration (relative to baseline). SEP-GluN2A expressing neurons show strongest reduction in dendritic spines (arrows), whereas few spines of SEP-GluN2B expressing neurons show signal reduction. **G**, Summary of pooled 'cell average' behaviors of the % of spine SEP signal reduction (left) and the % of spines meeting the criteria for internalization (right), for all time lapse imaging experiments. Agonist conditions are shown in the middle column. All pharmacological agents displayed within the box (APV 100 μ M, ifenprodil 3 μ M, Tetrodotoxin 1 μ M, Strychnine 5 μ M) were pretreated for at least 10 minutes prior to experiment onset, and in each case, co-application of glycine (1 mM) and NMDA (20 μ M) were administered during the agonist treatment. All data represent cell averages $\pm$ SEM.

Discussion:

Using a combination of whole-cell electrophysiological recordings, two-photon uncaging of MNI-glutamate and live-cell imaging, we show that a high concentration of extracellular glycine leads to the rapid internalization of NMDARs at hippocampal synapses. This effect of glycine was independent of changes in presynaptic function, was mediated by a dynamin-dependent internalization process and required channel activation. Moreover, we show that the stimulated trafficking of synaptic NMDARs by high glycine was subunit selective. Because subunit composition of NMDARs dictates key biophysical and signalling properties of synapses, this subunit selective trafficking phenotype of NMDARs may be consequential in pathological conditions of elevated glycine such as epilepsy and stroke.

Significant efforts have been devoted to understanding the origin, release and binding of NMDAR co-agonists, in particular, those which bind to the glycine site of GluN1 subunits (*eg.* glycine, D-serine, amongst others). In agreement with several reports (Bergeron et al., 1998; Johnson and Ascher, 1987; Wilcox et al., 1996), we found that the glycine modulatory sites of NMDARs at CA1 synapses are not saturated during basal synaptic transmission, since low micromolar increases in extracellular glycine concentration transiently potentiated evoked NMDAR-mediated responses. Intriguingly, we also show that higher elevations of extracellular glycine induced a robust and rapid reduction in the amplitude of NMDAR-mediated currents, which is also in line with several previous reports (Han et al., 2013; Nong et al., 2003; Zhang et al., 2014). Although in principle this outcome could reflect a reduction in presynaptic release probability, we carried out a number of experiments that directly ruled out this possibility. Changes in release probability would be expected to alter the amplitude of AMPAR- and NMDAR-mediated post synaptic currents to similar degrees (Kauer et al., 1988), however, we found that high glycine altered neither the amplitude nor the paired pulse ratio of evoked AMPAR-EPSCs. Rather, when NMDAR-EPSCs were probed by 2P-uncaging of MNI-glutamate, thereby bypassing presynaptic release, the suppression of NMDAR-EPSCs by glycine was still observed, indicating that this effect was likely of postsynaptic origin. In agreement, the depression of evoked NMDAR-EPSCs by glycine was blocked by inhibiting postsynaptic, dynamin-mediated, receptor internalization, and furthermore, live-cell imaging experiments in cultured hippocampal neurons confirmed that surface NMDARs were indeed internalized by elevated extracellular glycine. Collectively, these results indicate that NMDAR function can be bidirectionally influenced by mechanisms and/or pathologies that alter extracellular glycine concentration. It is also possible that the combined concentration of all glycine site agonists

would contribute to this bidirectional influence, since it has been shown previously that D-serine, like glycine, can act to potentiate (Chen et al., 2003; Martina et al., 2003) or depress (Nong et al., 2003) NMDAR transmission depending on extracellular concentration.

The subunit composition of NMDARs confers distinct biophysical and signaling properties, and as such, the regulation of NMDAR subtypes has increasingly become recognized as a defining feature of synaptic physiology. For instance, it is now well established that synaptic NMDARs in many forebrain regions undergo a robust switch in their subunit composition during development, with GluN2B-containing receptors being predominant in early postnatal development followed by a gradual replacement by GluN2A-containing NMDARs with increasing age (Bellone and Nicoll, 2007; Flint et al., 1997; Kirson and Yaari, 1996). Moreover, both Hebbian and homeostatic forms of synaptic plasticity have been shown to dynamically regulate the subunit composition of synaptic NMDARs (Bellone and Nicoll, 2007; Lee et al., 2010; Soares et al., 2013a). By documenting a preferential internalization of GluN2A- over GluN2B-containing NMDARs in high glycine, our results outline an unexpected means by which the subunit composition of synaptic NMDARs can be regulated. It is pertinent here to note that the molecular rules governing the trafficking and targeting of specific NMDAR subtypes are still only partially understood. For instance, there is evidence that suggest GluN2A-containing NMDARs are more stable in postsynaptic compartments than GluN2B-containing NMDARs (Bard et al., 2010; Groc et al., 2006). While members of the MAGUKs superfamily of scaffolding proteins are thought to be involved in the synaptic stabilization of NMDARs, in some cases with subunit specificity (Beique et al., 2006; Cousins et al., 2008; Elias et al., 2008; Elias and Nicoll, 2007; Wei et al., 2015). Moreover, subunit-specific rules in the constitutive cycling of NMDARs have been identified, with critical internalization motifs being identified for

GluN2B subunits (Lavezzari et al., 2004; Roche et al., 2001), while those for the GluN2A are less well defined. These considerations stress the importance of developing a molecular framework of subunit specific NMDAR targeting and trafficking to compare and contrast the mechanisms of constitutive versus different forms stimulus-dependent NMDAR trafficking.

The use of super-ecliptic phluorin (SEP) as a selective reporter of surface protein in live neurons has been challenged of late (Rathje et al., 2013; Wilkinson et al., 2014). Traditionally, the extracellular tagging of surface proteins with SEP, a pH-sensitive fluorophore, was thought to discriminate tagged receptors at the surface from those in recycling endosomes, since the lumen of these intracellular vesicular compartments are acidic ($\text{pH} < 6.0$) (Ashby et al., 2004). This does not exclude other intracellular compartments, however, that are only mildly acidic, such as the endoplasmic reticulum (ER) and Golgi apparatus. Thus, interpreting SEP signals as emanating predominantly from surface protein is complicated by the degree of signal contamination from fluorophores in non-acidic intracellular compartments (Rathje et al., 2013). Here, we provide evidence that many of these shortcomings may not translate to SEP-tagged NMDARs in dendritic spines of live pyramidal neurons. First, the near complete ($\sim 95\%$) and rapidly reversible quenching of spine SEP-NMDAR fluorescence by a brief (90 s) extracellular acidification, which does not cause significant intracellular acidification (Ashby et al., 2006; Wilkinson et al., 2014), suggest a predominant surface signal of SEP-NMDARs in dendritic spines. In addition, a spine enrichment analysis indicated that SEP-NMDARs were exceedingly enriched dendritic spines as compared to SEP-GluA2 AMPAR subunits which were more uniformly expressed in spine and dendritic compartments. Since dendrites harbour the majority of non-somatic ER (Cui-Wang et al., 2012; Rathje et al., 2013; Toresson and Grant, 2005), as was evidenced in our own culture conditions by the emission profile of a SEP-tagged, ER-

retained, AMPAR subunit (SEP-GluA2-ΔC49), we concluded that there was likely minimal contribution (~ 5%) of an ER-retained signal in dendritic spines of SEP-NMDAR expressing neurons. Thus, we provide thorough evidence that the punctate SEP-NMDAR signals we observed in dendritic spines of cultured hippocampal pyramidal neurons are predominantly from surface receptors, and that this approach can be extremely valuable in monitoring the dynamics of NMDA receptor surface diffusion, endocytosis, and recycling at synapses of live neurons.

The sustained activation of NMDARs has long been implicated in both acute and delayed aspects excitotoxic signalling (Szydlowska and Tymianski, 2010; Villmann and Becker, 2007). Very little is known, however, about how the excitotoxic neuronal environment influences the surface trafficking, distribution and subunit composition of NMDARs. This is of particular interest since these properties of NMDARs have been proposed to influence the direction of downstream signalling between the dichotomous pro-death and pro-survival signalling cascades (Hardingham and Bading, 2010; Ivanov et al., 2006; Liu et al., 2007; von Engelhardt et al., 2007; Zhou et al., 2013). Several pathological conditions including ischemia and epilepsy display a dramatic buildup of excitatory amino acids in the extracellular space, including those which bind to the glycine modulatory site of NMDARs (Rossi et al., 2000; Szatkowski et al., 1990) (refs for epilepsy?). The results outlined here suggest that these conditions may fundamentally alter the subunit composition of NMDARs and, as a consequence, alter the ratio of pro-survival and pro-death signalling pathways. Future studies will be required to determine whether changes in NMDAR composition during excitotoxicity enact a beneficial or rather detrimental consequence to neurons affected by excitotoxic insults.

A.5.

Manuscript VIII

Title:

A cost-effective method for preparing, maintaining, and transfecting neurons in organotypic slices

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Statement of Contributions:

I was invited by the editor (Dr. Martina Marzia) to write this chapter which is a detailed protocol to perform an experimental procedure that I spent years perfecting in the lab. Culturing and transfecting organotypic slices was my initial project as an undergraduate student in the lab between 2009 and 2010. I developed many novel techniques and strategies that optimized some of the original protocols that Jean-Claude had given me when I started, which was a protocol that he used when he did similar experiments during his post-doctoral training. I wrote this entire paper based on how, exactly, I performed these experiments in 2014. Kevin Lee, Denise Cook, and Jean-Claude helped to edit the manuscript and provided helpful guidance and support. Kevin Lee took many of the images that are found in Figure 1.

Abstract

The cellular and molecular mechanisms that underlie brain function are often difficult to study in the living brain. The development of organotypic slices has provided a welcomed addition to our arsenal of experimental brain preparations by allowing both genetic and prolonged pharmacological manipulations in a system that, much like the acute slice preparation, retains several core features of the cellular and network architecture found *in situ*. Neurons maintained in organotypic slices can survive in culture for several weeks, can be molecularly manipulated by transfection procedures and their function can be interrogated by traditional cellular electrophysiological or imaging techniques. Here, we describe a cost effective protocol for the preparation and maintenance of organotypic slices and also describe a protocol for biolistic transfection that can be used to introduce plasmids in a small subset of neurons living in an otherwise molecularly unperturbed network. The implementation of these techniques offers a flexible paradigm that can be used to study a multitude of neuronal mechanisms.

Introduction

For over half a century, the acute brain slice preparation has offered neurophysiologists a means to study cellular processes that underlie nervous system function (Li and McIlwain, 1957; Yamamoto and McIlwain, 1966). This *in vitro* experimental preparation retains much of the three dimensional architecture and local connectivity of neural circuitries *in situ*, and has thus gained widespread popularity in modern neuroscience laboratories. Despite the development of new technologies that permit the interrogation of circuit function and cellular physiology *in vivo*, there still remain overarching barriers that restrict the implementation of certain methodologies in the intact brain.

Notwithstanding their widespread acceptance and practicality, the limited lifespan of acute slices restricts their use in experiments involving prolonged pharmacological and/or genetic manipulations (Gahwiler et al., 1997). The development of organotypic slices has thus opened an important avenue to circumvent these limitations (Gahwiler, 1981; Stoppini et al., 1991). This technique allows for thick sections of brain tissue to be maintained in culture for several weeks to months, enabling their use in experiments involving prolonged molecular and/or pharmacological intervention (Barria and Malinow, 2005; Beique and Andrade, 2003; Beique et al., 2007; Gahwiler et al., 1997; Hayashi et al., 2000; Noraberg, 2004; Schnell et al., 2002; Soares et al., 2013a). Importantly, unlike dissociated neurons, organotypic slices retain several of the key features of network and cellular physiology found *in situ* (Buchs et al., 1993; De Simoni et al., 2003; Gahwiler et al., 1997; Mellentin et al., 2006; Muller et al., 1993).

Combining organotypic slices with a reliable transfection method, such as biolistic-mediated gene transfer (Johnston, 1990), provides a powerful method for investigating the

effects of molecular interventions on cellular functions. The biolistic method involves the bombardment of brain slices with DNA-coated micron-sized gold particles, leading to a sparse transfection of neurons located in an otherwise undisturbed network (McAllister, 2000). This transfection method thus allows traditional molecular manipulations to be applied in a visually identified (*i.e.*, by inclusion of a fluorescent reporter gene) subset of neurons, whose function can be studied by electrophysiological or optical approaches. In addition, organotypic slices provide a highly controlled environment for evaluating the prolonged effect of pharmacological treatments on various neural functions (Gahwiler et al., 1997; Noraberg, 2004; Soares et al., 2013a).

Here, we describe a cost effective method for preparing organotypic slices, as well as provide several tips based on our experience to facilitate their use in electrophysiological experiments. Although described here for hippocampal tissue, this approach can be modified to culture tissue from other parts of the brain. We also describe a biolistic approach that has been optimized for transfection of neurons in hippocampal organotypic slices. Collectively, these techniques enable electrophysiological and/or optical recordings of genetically modified neurons and, as such, provide a cost-effective paradigm to capitalize on the ever expanding repertoire of available genetically encoded biosensors, reporters, and molecular tools to manipulate the expression of endogenous proteins in living cells.

Materials

Solutions

Culture Media:

Two culture media recipes have been used successfully in this protocol. We tend to prefer a Neurobasal based solution composed of (per 50 mL): 42.5 mL of 1X Neurobasal-A (without phenol red or glutamine; with 26mM sodium bicarbonate), 0.25 mL of 200 mM L-glutamine, 1 mL of B27 supplement (50X), 0.5 mL of penicillin/streptomycin (100X), 0.75 mL of 45% D-Glucose and 5 mL of heat inactivated horse serum. An alternative culture media (50 mL) is composed of: 25 mL of Minimal Essential Media (1X), 12 mL of Hanks Balanced Salt Solution (1X), 0.25 mL of 200 mM L-glutamine, 0.5 mL of penicillin/streptomycin (100X), 11.75 mL of heat inactivated horse serum and 0.5 mL of 45% D-glucose in solution. The pH of the culture media is adjusted to 7.15 using a 1M HCl solution, filtered with a sterile 0.22 μ m filter and kept at 4°C prior to use. Culture media should be prepared routinely and should not be stored for longer than two weeks.

Dissection solution:

For brain dissection and slice preparation, we use a choline-based dissection solution, although other dissection solution recipes can be appropriate. This solution is made fresh on the day of dissection and is composed of (in mM): 119 choline chloride, 2.5 KCl, 4.3 MgSO₄·7H₂O, 1 CaCl₂·2H₂O, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 Glucose and 1.3 Na-Ascorbate. To adjust pH, the solution is bubbled with 95/5% carbogen for 20-30 minutes (equilibrates to pH: 7.3-7.4) and then frozen at -80°C until a slush-like consistency is achieved.

Materials and Equipment for tissue culture:

Slices for organotypic cultures can be made using any conventional vibratome or tissue chopper. For hippocampal slice cultures, we use a MX-TS tissue slicer (Siskiyou, Oregon). Slice cultures are maintained on small rectangular islands of 0.4 μ m Biopore membrane (Millipore

#BGCM00010) placed on top of membrane culture inserts (0.4 μm pore) in a six well culture plate (see **Figure 1A**). An incubator that can be equilibrated to 34°C with 5% CO₂ is required for the culture technique, and to avoid contamination, part of this procedure should be performed in a sterile biosafety cabinet. For biolistic transfection of organotypic slices, we use the Helios Gene Gun System (Bio-Rad, California).

Methods

Preparation of organotypic slices

This is a modified version of an organotypic slice protocol originally described by Stoppini et al (Stoppini et al., 1991), which has been optimized to reduce cost and improve the transfer of slices to an electrophysiological or imaging chamber. One important difference is that individual brain slices are not directly cultured on the surface of the porous membrane inserts. Rather, slices are maintained on small rectangular pieces of biopore membrane that are placed on the surface of the membrane inserts prior to dissection (see **Figure 1A**). This additional piece of membrane at the slice-media interface provides practical advantages by facilitating the transfer of brain slices from culture conditions to experimental (electrophysiological or imaging) chambers, and an economical advantage by enabling recycling and repeated use of costly membrane inserts.

- 1- Biopore membrane is acquired as a large sheet and is cut into small pieces using scissors prior to the dissection day. The dimensions of the cut membrane will depend on size of the slice intended for culturing (see **Note 1**).
- 2- On the morning of dissection, transfer the pieces of biopore membrane into a 10 cm petri dish containing 3 mL of 100% ethanol. Leave membranes to dry in a biosafety cabinet.

- 3- Prepare culture media (for recipe and directions, see **Section 2.1**) and add 1 mL of media into 5 wells of a 6 well plate. In the last remaining well, add 3 mL of culture media.
 - 4- Using round forceps, place a sterile membrane insert in each of the 5 wells containing 1mL of culture media. Avoid air bubbles from forming underneath the membrane insert by slowly lowering the insert into the media diagonally from one edge. If membrane inserts are being recycled (see **Note 2**), be sure to remove them from ethanol storage in advance so they are dry when used.
 - 5- Using rounded forceps, dip the dry rectangles of biopore membrane in culture media (use the well with 3 mL of media) and place them on top of the membrane inserts. Three to four pieces of biopore membrane will fit on a single membrane insert, and each small piece of membrane will accommodate a single hippocampal slice (see **Figure 1A**).
 - 6- Once the plates are prepared place them in a 5% CO₂ incubator set at 34 °C for a minimum of 1 hour to allow for proper temperature and pH equilibration of the culture media.
 - 7- Prepare dissection solution (for recipe, see **Section 2.1**) and bubble with 95/5% carbogen gas for a minimum of 30 minutes to equilibrate pH (should reach 7.2-7.3). Store at -80 °C until partially frozen. Slices should be prepared in an icy slush of dissection solution.
- Steps 7- 17 describe our dissection protocol for generating organotypic hippocampal slices using a MX-TS tissue slicer. Organotypic slices can be prepared from other brain regions using a vibratome and following standard acute slice preparation protocols (see **Note 3**). If slices are prepared using a vibratome, skip steps 8-18.

- 8- Wind tissue chopper frames with teflon coated tungsten wire according to the manufacturers protocol. We use a thread spacing of 400 μm . Rinse with 70% ethanol and let dry in a sterile biosafety cabinet. Once dry, attach the frame to the MX-TS tissue slicer (see **Figure 1B**).
- 9- A batch of 1% bacto-agar plates are made in advance and are required for the hippocampal dissection using an MX-TS tissue slicer. Make a solution of 1% bacto-agar in water (100 mL, autoclaved), microwave on high for two minutes, and let the agar solution cool at room temperature until warm. Next, aliquot 20-25 mL of the warm bacto-agar solution into 10 cm petri dishes and let the gel solidify at room temperature. The gel should be 1-2 cm thick. Seal edges with parafilm and store agar plates at 4°C until dissection (can be stored for several months).
- 10- Remove one bacto-agar plate from the refrigerator and cut the gel with a razor blade to fit the base of the tissue slicer. This provides a raised base for the hippocampi to rest which will allow the threads of the tissue slicer to fully traverse the tissue (see **Figure 1B**).
- 11- Remove the dissection solution from the freezer and let it thaw partially, and mix at room temperature until slushy. Meanwhile, clean dissection tools with 70% ethanol.
- 12- Rats or mice (post natal day 6-12) should be sacrificed according to local institutional animal care guidelines. We anesthetize animals by isoflurane inhalation prior to decapitation.
- 13- Remove the whole brain from the cranial cavity as quickly as possible and let it cool in a 100 mL beaker filled with ice-cold dissection solution for a few minutes. Multiple whole brains can be extracted simultaneously (see **Note 4**).

- 14- Use cyanoacrylate glue to adhere the brain(s) to the base of a dry 10 cm petri dish, and fill the dish with ice-cold dissection solution to completely immerse the brain(s).
- 15- Use a scalpel to make a shallow cut down the midline of the brain, severing the corpus callosum to separate the two cerebral hemispheres.
- 16- Expose the hippocampi by everting each hemisphere laterally with a spatula. Transect both the septal and temporal most regions along the longitudinal axis of the hippocampus with fine forceps. Once cut, use a spatula to flip the hippocampi out from the overlying cerebral cortex (see **Figure 1C**). Remove the hippocampi, which should have a ‘banana-like’ shape, using a scalpel.
- 17- Use a spatula to transfer the hippocampi to the bacto-agar gel at the base of the tissue slicer (see **Figure 1B**). It is critical that the longitudinal axis of the hippocampus be perpendicular to the threads of the tissue slicer (see **Note 5**). Release the tissue slicer and ensure that the threads have sufficiently penetrated into the gel (see **Note 6**). Transfer slices into a new 10 cm dish containing fresh ice-cold dissection solution.
- 18- Under a sterile dissection microscope, separate slices in cold dissection solution using two pairs of sterile dumont forceps, or other fine dissection tools according to preference (see **Note 7** and **Figure 1D**). All three major regions of the hippocampal circuit should be readily visible in each slice (see **Note 8** and **Figure 1E**). Transfer the best slices to a new petri dish with ice-cold dissection solution using a plastic Pasteur pipette (see **Figure 1D**).
- 19- Remove one six-well plate at a time from the incubator. Using a fine tip plastic pasteur pipette, transfer the slices first to the well containing extra culture media (to minimize transfer of dissection solution to culture inserts), and then onto the biopore membrane

(see **Note 9**). These steps require patience and finesse, as it is critical that the slices are carefully positioned on the small rectangular islands of membrane (see **Figure 1A**). Aspirate excess media surrounding each slice with a 100 μ l pipette (see **Note 10**) and return finished plates to the incubator.

20- Culture media should be changed the day after dissection (~24h) and then every 2-3 days thereafter. To change culture media, raise each membrane insert using rounded forceps and replace the entire volume in the culture well with fresh, equilibrated, culture media (see **Note 11**).

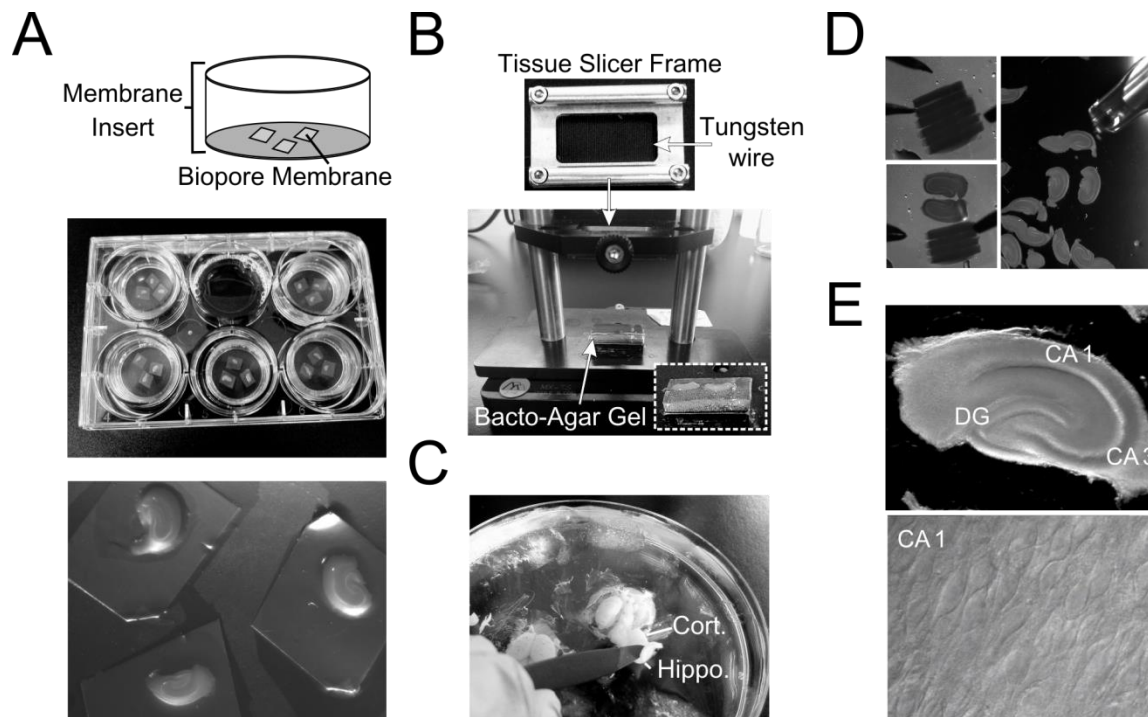


Figure 1: Protocol for generating organotypic hippocampal slices. **A** Small pieces of biopore membrane are placed on membrane inserts, each of which will accommodate a single hippocampal slice, which allows for easy transfer of slices to the experimental chamber. **B** The tissue slicer frame for the MX-TX tissue chopper is threaded with tungsten wire to cut hippocampal tissue into 400 μm slices. *Inset*, hippocampi are positioned on the bacto-agar base such that the longitudinal axis is perpendicular to the threads of the tissue slicer. **C** Once the septal and temporal most regions of the hippocampus are cut, the hippocampus (Hippo.) should easily fold out from the overlying cortex (Cort.). **D** Hippocampal slices under a dissection microscope. Slices can remain adhered to one another following dissection, resembling a loaf of sliced bread. Using a pair of forceps, the slices can be carefully separated from one another. A plastic Pasteur pipette with cut tips are used to transfer slices to membrane inserts. **E** A single hippocampal slice under the dissection microscope. All three major hippocampal regions (CA1, *Cornu Ammonis* 1; CA3, *Cornu Ammonis* 3; DG, Dentate Gyrus) should be intact and readily recognized in each slice.

Preparation of gene gun cartridges

Gene gun cartridges are prepared largely according to the manufacturer's protocol and previously published protocols (McAllister, 2000; Woods and Zito, 2008), although several steps have been modified to improve particle transfer and transfection efficiency of neurons in

organotypic slices. This section contains the steps required to precipitate DNA onto gold microcarriers and coat these particles uniformly along tubing, which is then cut into separate cartridges for the gene gun.

- 1- Cut tefzel tubing (outer diameter: 0.125"; inner diameter: 0.093") to fit the tubing apparatus, allowing a short overhang at the open end. Dry the tubing by commencing flow of 100% nitrogen gas through the tubing at > 0.5 L/min for 20 to 30 minutes.
- 2- Weigh 8-10 mg of 1 μ m gold microcarriers and transfer to a 1.5 mL test tube. Add 100 μ L of 0.05M spermidine solution (see **Note 12**).
- 3- Briefly sonicate and vortex the gold/spermidine solution to suspend the microcarriers, then immediately add DNA plasmids (see **Note 13**). The total amount of DNA required will vary between constructs and will also depend on the number plasmids added (see **Note 14**). For expression of a cytosolic marker (*eg.*, mCherry or EGFP) 10-15 μ g of DNA is often sufficient. Although we routinely keep total DNA to about 50 μ g, we have used on occasions up to 80 μ g of total DNA for expression of multiple plasmids (see **Figure 2B**).
- 4- Vortex the tube at a slow rate and add 100 μ L of 1M CaCl₂ drop wise to the gold/DNA mixture. This will precipitate the DNA onto the gold microcarriers.
- 5- Allow the gold to settle in the tube at room temperature for 10 minutes. Meanwhile, transfer 3.3 mL of fresh 100% ethanol to a 15 mL conical tube, and set aside until step 8. In the manufacturer's protocol, polyvinylpyrrolidone (PVP) is added to this ethanol solution; however, we avoid this step (see **Note 15**).
- 6- Once the gold has settled, place the 1.5 mL tube in a table top centrifuge and spin at 8,000-10,000 rcf for 10 seconds to form a pellet.

- 7- Remove and discard the supernatant, then add 1 mL of 100% fresh ethanol to the gold pellet. Resuspend the microcarriers in the ethanol solution (see **Note 16**) and repeat the centrifuge step to reform the pellet. Repeat this washing step three times.
- 8- After the final wash, remove the ethanol and add 500 μ l of 100% ethanol from the 3.3 mL set aside in **Section 3.2., Step 5**. Re-suspend the gold in the ethanol solution and transfer this mixture to a new 15mL conical tube. Repeat this step until all 3.3mL of 100% ethanol, including the gold microcarriers, are transferred to the new 15 mL conical tube.
- 9- Stop nitrogen flow at the tubing station and connect a 10 mL syringe to one end of the dry tefzel tubing.
- 10- Briefly vortex the gold/ethanol solution to maximize suspension. Immediately after, insert dry tubing to the bottom of the gold/ethanol suspension. Apply negative pressure to the 10 mL syringe to draw the microcarrier solution up into the tubing. The gold will settle in the 15 mL conical tube quickly, so speed will ensure an even transfer of gold along the length of tubing.
- 11- Insert tefzel tubing back into the tubing apparatus and allow the gold to settle for approximately 5 minutes.
- 12- Slowly draw the ethanol solution from the tubing by applying negative pressure to the 10 mL syringe until all the ethanol has been removed (see **Note 17**). Disconnect the syringe and commence rotation of the tubing apparatus without nitrogen flow for approximately 30 seconds, which should promote an even distribution of microcarriers around the inner circumference of the tubing (see **Note 18**).

- 13- Start flow of 100% nitrogen at a rate of 0.3-0.4 L/min to dry the tubing while maintaining rotation for an additional 5 minutes
- 14- Terminate the flow of nitrogen, stop the rotation, and remove tubing from the apparatus.
- 15- Cut tubing into individual cartridges using the BioRad tubing cutter. Bullets are stored in small plastic vials with desiccation pellets at 4 °C, and can be kept for several months.

Biolistic transfection of organotypic brain slices

The biolistic method described below is an adapted version of the manufacturer's protocol that has been modified to preserve slice vitality and improve transfection efficiency in organotypic brain slices. Mounting the hand held gene gun to a vertical support (see **Figure 2A**) has enabled us to systematically optimize the transfection conditions (*i.e.*, shooting distance and pressures, *see below*) and is strongly recommended for the implementation of this technique.

- 1- Slices can be transfected as early as the day of dissection, and as late as several weeks in culture. Typically, we wait at least one week to allow the slices to recover and adapt to culture conditions (*see Note 19*).
- 2- Remove the vial containing gene gun cartridges from the 4 °C refrigerator and let stand at room temperature for at least 10 minutes prior to opening.
- 3- Insert cartridges into the revolver cylinder using rounded forceps.
- 4- Attach the revolver cylinder and barrel to the mounted gene gun. It is important to use separate barrels if more than one DNA construct is being transferred (*see Note 20*).
- 5- Connect the gene gun to a compressed tank containing 100% helium. Raise the pressure at the regulator to 180-200 psi.

- 6- Fire a blank shot to remove debris from the chamber.
- 7- Remove plates from the incubator one at a time. Under a biosafety cabinet, carefully move slices into the center of each membrane insert using rounded forceps.
- 8- The gene gun barrel should be level with respect to the table and positioned above each well. The height is ideal when the tip of the barrel is in line with the height of the culture plate, ~1.5-2.5 cm from the table (*see Figure 2A*).
- 9- Fire the gene gun. Label each well of the culture plate appropriately after shooting and return slices to the incubator.
- 10- The amount of time required for maximal protein expression will vary between constructs (*see Note 21*).

Electrophysiological recordings from single neurons in organotypic slices

In this last section, we provide a set of experimental tips for obtaining whole-cell patch-clamp recordings from neurons maintained in organotypic slices. Recording techniques are largely the same as those from acute slices; however, there are some differences, which may need to be taken into account to facilitate recordings.

- 1- On an experiment day, remove an individual slice with its respective membrane from the insert (in a 6 well plate) and transfer it to a drop of equilibrated culture media in a small petri dish. The slice is then taken immediately to an electrophysiological and/or imaging chamber for experimentation.
- 2- A prerequisite for obtaining reliable electrophysiological recordings is to consistently have healthy tissue to work with. If tissue health degrades rapidly after the dissection protocol, several approaches can be taken to improve slice viability (*see Note 22*). If

tissue degeneration tends to be a problem only after transfection, the biolistic conditions may need to be optimized (see **Note 23**).

- 3- It is often appropriate to run experiments within a defined age range (*i.e.*, days in vitro) to reduce potential artifacts from studying slices at different developmental stages (see **Note 24**).
- 4- For whole-cell recordings, we prefer tissue that has been cultured for 7-14 days, as over time, the tissue flattens leading to improved visualization of cell morphology (see **Figure 1D**) (**Buchs et al., 1993; Miller et al., 1993**).
- 5- As the slice matures in culture, glial coverage will make it increasingly difficult to access neuronal cell bodies with glass microelectrodes. Approach the tissue with sufficient positive pressure to penetrate the glia while descending into the slice. Once the electrode is in proximity to the targeted neuron, reduce but maintain positive pressure to navigate toward the cell body of the neuron of interest prior to gigaohm seal formation.
- 6- For paired recordings (see **Figure 2D**), it is best to obtain a gigaohm seal in both cells before gaining whole cell access (see **Note 25**).
- 7- For studies of evoked excitatory transmission (*i.e.*, with pharmacological blockade of inhibitory GABA_A transmission), polysynaptic activity is often more prevalent and problematic in organotypic slices than in acute slices (see **Figure 2E**). In these conditions, electrical stimulation of axons is best achieved by positioning a monopolar glass patch electrode (rather than a bipolar electrode) filled with ACSF placed in proximity to the recorded neuron. This will not only minimize the number of axon fibres stimulated, but will also enable the experimenter to easily maneuver the position of the stimulating electrode to reduce the occurrence of evoked polysynaptic events. Several

methods have been developed to minimize polysynaptic activity during electrical synaptic stimulation (see **Note 26**).

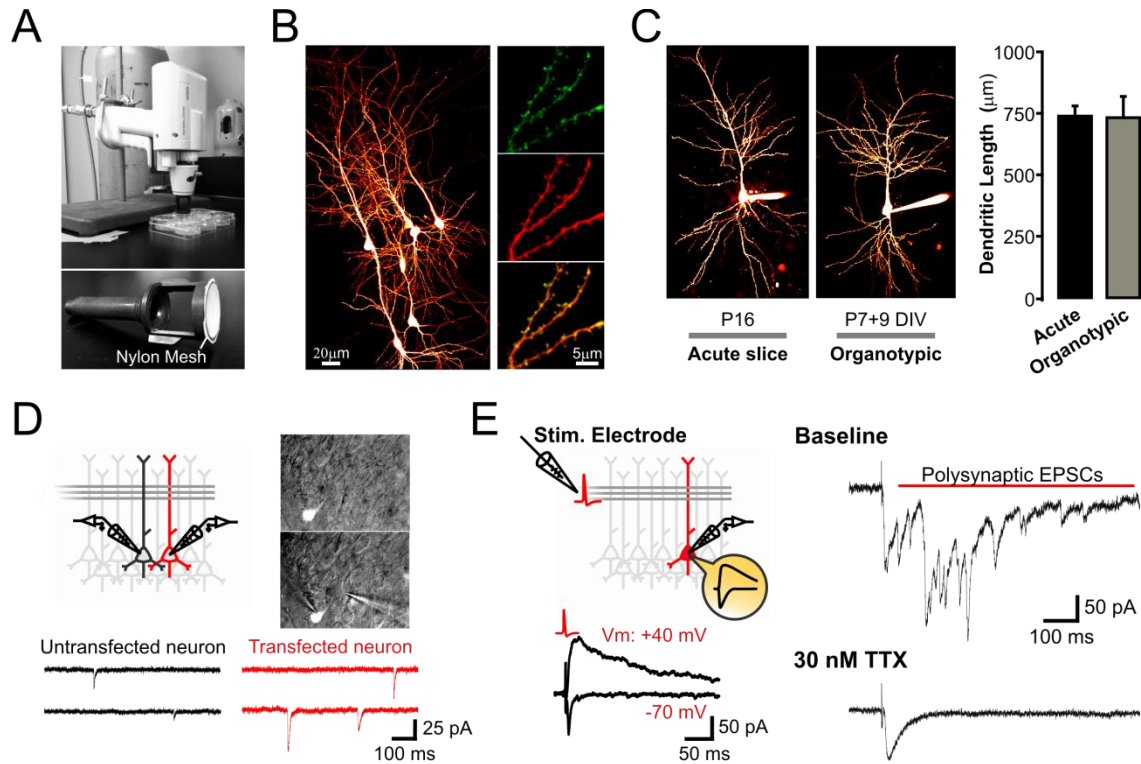


Figure 2: Biolistic transfection for single cell expression of DNA plasmids in hippocampal slice culture. **A** Mounting the gene gun to the lab bench has enabled the optimization of biolistic conditions. The addition of a nylon mesh (90 μm pore) to the end of the manufacturer's barrel shunts the blast of helium coming through the chamber and allows for shooting the gene gun at higher pressures. **B** A confocal image stack of hippocampal CA1 pyramidal neurons biolistically transfected with mCherry and a GFP-tagged protein of interest. **C** Two-photon images of age matched CA1 pyramidal neurons in an acute and organotypic slice filled with Alexa594 through the recording electrode. Measurement of dendrite development provides evidence that neurons in slice culture develop as they would *in situ*. **D** Paired recordings from a transfected and untransfected 'control' neuron is an effective strategy to examine the effect of single cell genetic manipulation. In this example, both the transfected and untransfected neuron are voltage clamped at -70 mV and miniature excitatory post synaptic currents (EPSCs) are recorded simultaneously in both cells. **E** Studies of evoked synaptic transmission in organotypic slices can be compromised by the occurrence of polysynaptic events. We suggest using a monopolar glass stimulating electrode to stimulate axons in organotypic slices. Several methods have been developed to dampen polysynaptic activity (Barria and Malinow, 2005; Schnell et al., 2002). A low concentration of tetrodotoxin (10-30 nM) in the extracellular solution is one approach we have taken to reduce the polysynaptic activity in organotypic slice recordings.

Notes:

- 1- To accommodate hippocampal slices, biopore membrane is cut into ~0.5 cm rectangles (see **Figure 1A**).
- 2- Used membrane inserts can be stored in a large beaker of distilled water until enough are gathered for cleaning. To clean, transfer the membrane inserts into a beaker containing a 10% acetic acid solution and let stand at room temperature for 10-15 minutes. Rinse the inserts by transferring them to a separate beaker containing 1-2 L of milliQ double distilled H₂O. Replace the water every 10 minutes for a total of three washes to dilute the residual acetic acid solution. After the third wash, store membrane inserts in a beaker filled with 100% ethanol until subsequent use. Use parafilm to seal the beaker to minimize ethanol evaporation.
- 3- Although the details of this procedure are for culturing slices of hippocampal tissue sectioned with a MX-TS tissue slicer, we have also had success culturing coronal / horizontal cortical brain sections (300-400 μ m) using a standard vibratome-based technique. Ensure that the vibratome chamber and blade are properly cleaned and sterilized prior to dissection.
- 4- To save time, sacrifice 2-3 animals simultaneously. This is not recommended, however, for individuals that are new to this protocol as best results are obtained when the duration between sacrificing the animal to completion of the culture procedure is minimized. Once the adeptness in the dissection technique is established, sacrificing multiple animals at once provides an efficient way to prepare many slices without the need to perform repetitions of the protocol.
- 5- The longitudinal hippocampal artery, which runs along the longitudinal axis of the hippocampus (**Coyle, 1976**), should be readily observable with the naked eye. Orient the

hippocampi such that the longitudinal axes are perpendicular to the threads of the tissue slicer and position the longitudinal hippocampal artery face-up, towards the tissue slicer frame (*see* **Figure 1C**).

- 6- In advance, adjust the final height of the tissue slicer frame such that the threads will pass into the bacto-agar gel block once released. This will ensure complete sectioning of the hippocampal tissue.
- 7- In some cases, slices will remain partially-adherent to one another following tissue-slicer sectioning. These blocks of tissue will resemble a loaf of bread. Use two pairs of Dumont forceps with a 45° tip to gently separate the slices from one another (*see* **Figure 1D**).
- 8- In each slice, the main hippocampal sub regions should be clearly identifiable (CA1, CA3, dentate gyrus) (*see* **Figure 1 E**). If this is not the case, there may have been a problem with the orientation in which the slices were cut (*see* **Note 5**).
- 9- Sterile, individually wrapped, plastic pasteur pipettes are used to transfer slices. Tips are cut to accommodate the size of the slice, but not too large, as larger pipette bores make it difficult to manipulate single slices when transferring onto membrane inserts. Furthermore, smaller pipette bores reduce the amount of dissection media that accompanies the slice on to the biopore membrane (*see* **Section 3.1., Step 19**).
- 10- To remove excess media from the slices once they are transferred to the biopore membrane, use a 100 µl pipette. Slices suspended in a drop of media will tend to follow the direction of solution flow while aspirating. To prevent the slice from entering the pipette, make physical contact between the pipette tip and the base of the biopore membrane, and maintain this contact while aspirating excess media. This will help draw the slice downward toward the membrane.

- 11-** The culture media needs to be warmed and equilibrated to the conditions of the incubator prior to media exchange. On a ‘feeding’ day, add fresh culture media (5 mL) to the empty well of the six well plate and return the plate to the incubator for at least an hour before transferring to the slices.
- 12-** Store spermidine aliquots (100 μ l, 0.05 M) at -20°C for no longer than 2-3 months. If aliquots are used past their shelf life, the transfection efficiency of the biolistic procedure can drop significantly.
- 13-** Once the DNA has been added to the gold/spermidine mixture, sonication should be avoided, as this can lead to sheering of DNA (O'Brien and Lummis, 2002).
- 14-** We prefer using DNA with a concentration above 1 μ g/ μ l, although this is not a requirement. Effective co-transfection of plasmids can be problematic, and needs to be optimized for each set of DNA constructs. As a typical starting point, a total of 50 μ g of DNA is used, of which 30-40 μ g are of the DNA for the protein of interest and 10-15 μ g are of that coding a cytosolic morphological reporter (*eg.*, mCherry). When possible, expressed constructs should be tagged with a distinguishable fluorescent reporter, to ensure that co-transfection has been achieved. Although co-transfection rates are typically high, they are inevitably not 100%.
- 15-** In the manufacturer’s protocol, the ethanol solution that is used to transfer the gold microcarriers to the tefzel tubing includes a low concentration of polyvinylpyrrolidone (PVP), which helps adhere the gold microcarriers to the tubing. PVP is, however, not used in our protocol. In our experience, when PVP is used, even at half the recommended concentration, a significant amount of gold microcarriers remain adhered to the tubing

cartridges after shooting. We find that excluding PVP enhances microcarrier delivery into tissue, which in turn provides higher transfection efficiency.

16- Gently flicking the tube should be sufficient to re-suspend the pellet. If this does not work, it is likely that the gold pellet is too compact and the speed/duration of the centrifuge step should be reduced.

17- Here, timing is important. Removal of ethanol should be done quickly, but slow enough to prevent re-suspending the gold microcarriers. The ethanol removal should take about 30 seconds, and if performed well, no gold should be visible in the ethanol that is drawn into the syringe. However, if this step is done too slowly, the tubing will dry and the gold will non-uniformly adhere to the inside wall of the tubing. Timely removal of ethanol from the tubing and immediate rotation of the tubing apparatus is the best way to achieve uniform coating of the microcarriers.

18- If uneven coating of microcarriers persists, try knocking the apparatus against the surface of the bench during the first few rotations, prior to resuming nitrogen flow. The impact will promote a more uniform distribution of gold particles around the inner surface of the tubing.

19- During the first week of culturing, the slices will flatten from a thickness of 400 μm to less than 200 μm (**Buchs et al., 1993**). During this time, healthy cells will persist while those damaged during the cutting process will gradually be sloughed away. Between one and two weeks after dissection, the slices tend to appear the healthiest and most appropriate for biolistic transfection. We obtain excellent transfection efficiency when slices are shot between 8 and 10 days in culture.

- 20-** We have optimized transfection conditions by modifying the original gene gun barrel to include a nylon mesh at the tip (see **Figure 2A**). The mesh has a 90 μm pore diameter and is glued to the end of the barrel using cyanoacrylate glue. We found that inclusion of the mesh prevents tissue damage when shooting at high pressures (180-200 psi), since it shunts the blast of helium coming through the chamber. At these high pressures, we found enhanced penetration of the microcarriers into the tissue resulting in higher transfection efficiency. A separate barrel / nylon mesh is used for each set of DNA/gold microcarriers to avoid cross contamination.
- 21-** Depending on the constructs being expressed, the amount of time required for maximal protein expression will vary. For example, when protein expression is suppressed using shRNA constructs, more time is typically required to accommodate for turnover of existing proteins.
- 22-** In the event that slices are obviously unhealthy early in the culture period (*i.e.*, first 2-3 days) it is likely that there is a problem with the dissection itself, or with culture media formulation. If this is consistently a problem, inclusion of a broad glutamate receptor antagonist (*eg.*, 0.5-1mM kynurenic acid) in the dissection solution may help.
- 23-** If problems with tissue health arise only after transfection, the biolistic approach can be modified to reduce slice damage. It is possible that the pressure blast coming from the gene gun while shooting is physically damaging the tissue. One way to circumvent this is to reduce the pressure or move the gene gun further away from the sample. It is also possible that the density of microcarriers in the tissue is too high which can be detrimental to slice health. To reduce microcarrier density, either reduce the amount of gold used in the protocol, or increase the shooting distance from the slice.

- 24-** Neurons in slice culture continue to develop largely as they would *in situ* (eg. see **Figure 2C**, and (Buchs et al., 1993; De Simoni et al., 2003; Gahwiler et al., 1997; Mellentin et al., 2006; Muller et al., 1993). Since organotypic slices are typically prepared from young neonatal animals during a period of rapid neuronal and circuit development, it may be difficult to compare results obtained from neurons at 1 and 3 weeks in culture. We suggest that a defined experimental time course should be established during experimental design to avoid potential complications associated with ongoing development of neurons in slice culture.
- 25-** Movement of the electrode in the slice will inevitably displace tissue in the local environment, especially when positive pressure is being applied. By obtaining a seal on the transfected neuron first allows the experimenter to decide which control neuron is most accessible during the second approach.
- 26-** Several methods have been described to reduce polysynaptic responses when evoking excitatory synaptic transmission in organotypic brain slices. Increasing the divalent ion concentration (eg., to 4 mM Ca^{2+} , 4 mM Mg^{2+}) and/or including the adenosine A1 receptor agonist 2-chloro adenosine in the extracellular recording solution has been used in several studies to minimize polysynaptic activity (Barria and Malinow, 2005; Schnell et al., 2002). In our hands, we find that inclusion of a low concentration of tetrodotoxin (10-30 nM), a Na^+ channel blocker, dampens network excitability while maintaining the ability to evoke synaptic transmission, thereby effectively minimizing polysynaptic responses (see **Figure 2E**). For hippocampal slice cultures, we also routinely cut the CA1 flanking regions with a scalpel prior to mounting the slice on the microscope (*i.e.*, at both the CA3:CA1 and CA1:subiculum interface).

Statement for Appendix B:

This appendix contains experimental work that I contributed to two published manuscripts. Because my contributions were significant, I've chosen to include them as part of this appendix. However, because these studies comprise a large amount of experimental data that I cannot claim responsibility for, I've decided to exclude the full text and only include the experiments that I carried out specifically. Instead, I will include only the project's abstract along with a detailed rationale for the experiments I performed, which can be found below the author's contribution statement that precede each section.

B.1.

Contributions to Manuscript IX

Title:

Palmitoylation of LIM Kinase-1 ensures spine-specific actin polymerization and morphological plasticity

Authors:

Joju George ^a, Cary Soares ^{b,c}, Audrey Montersino ^a, Jean-Claude Béïque ^{c,d,e,f}, Gareth M. Thomas ^{a,*}

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Statement of Contributions:

In the spring of 2013, I was asked to contribute a set of experiment to what was already a somewhat mature project. I performed only the two-photon glutamate uncaging and time lapse imaging experiments found in Figure 5 and Figure 5 supplement 1 and 2. I wrote, with the help of Jean-Claude, the corresponding results and discussion sections of the paper, and commented on and edited the full manuscript several times before it was accepted for publication. Most of the experiments were performed by Dr. Joju George, a postdoc in Dr. Thomas' Lab, and Dr. Thomas himself. After the original submission, Joju George left the lab and some final immunocytochemical experiments were performed by Audrey Montersino, a graduate student. I've chosen to include only the data that I contributed directly, rather than the full manuscript.

Rationale for experiments performed:

This project was focused at identifying the role of a known actin regulatory protein, LIM kinase 1 (LIMK1) in dendritic spine plasticity. Specifically, Dr. Thomas's group identified two cysteine residues on the N-terminal portion of LIMK1 that were sites of posttranslational modification via palmitoylation. He generated several mutant constructs that rendered these sites unable to be palmitoylated, and found that this disrupted the typical spine enriched localization of this protein in hippocampal neurons. He hypothesized that, by altering the subcellular localization of this protein, this mutation would disrupt dendritic spine actin dynamics.

Having an already established expertise in long-term imaging of dendritic spine plasticity, I was asked to carry out a set of experiments that would put this hypothesis to the test. I transfected hippocampal neurons in organotypic slices with cytosolic mCherry, a red fluorescent protein that enabled us to visualize the dendritic morphology of single neurons by

two-photon imaging. In the initial experiment, we co-transfected with a second DNA plasmid which encoded a shRNA for LIMK1, thereby reducing the endogenous expression of LIMK1 in a cell-specific manner. We found that removal of LIMK1 impaired spine structural plasticity in response to a glutamate uncaging protocol that, in control neurons, produced a lasting increase in the overall size of the stimulated spine. We went on to show, using various shRNA resistant constructs, that this effect could be rescued by reintroducing a WT-LIMK1 but not the palmitoylation mutant version of LIMK1. Together with other major results of this paper, we concluded that the palmitoylation of LIMK1 was critical for its proper localization at excitatory synapses and that its presence there was critical for the actin dynamics necessary to produce long-lasting structural spine plasticity. Below you will find the abstract along with the three full figures I contributed to this study, and the detailed description of the experimental methods.

Abstract

Precise regulation of the dendritic spine actin cytoskeleton is critical for neurodevelopment and neuronal plasticity, but how neurons spatially control actin dynamics is not well defined. Here, we identify direct palmitoylation of the actin regulator LIM kinase-1 (LIMK1) as a novel mechanism to control spine-specific actin dynamics. A conserved palmitoyl-motif is necessary and sufficient to target LIMK1 to spines and to anchor LIMK1 in spines. ShRNA knockdown/rescue experiments reveal that LIMK1 palmitoylation is essential for normal spine actin polymerization, for spine-specific structural plasticity and for long-term spine stability. Palmitoylation is critical for LIMK1 function because this modification not only controls LIMK1 targeting, but is also essential for LIMK1 activation by its membrane-localized upstream activator PAK. These novel roles for palmitoylation in the spatial control of actin dynamics and kinase signaling provide new insights into structural plasticity mechanisms and strengthen links between dendritic spine impairments and neuropathological conditions.

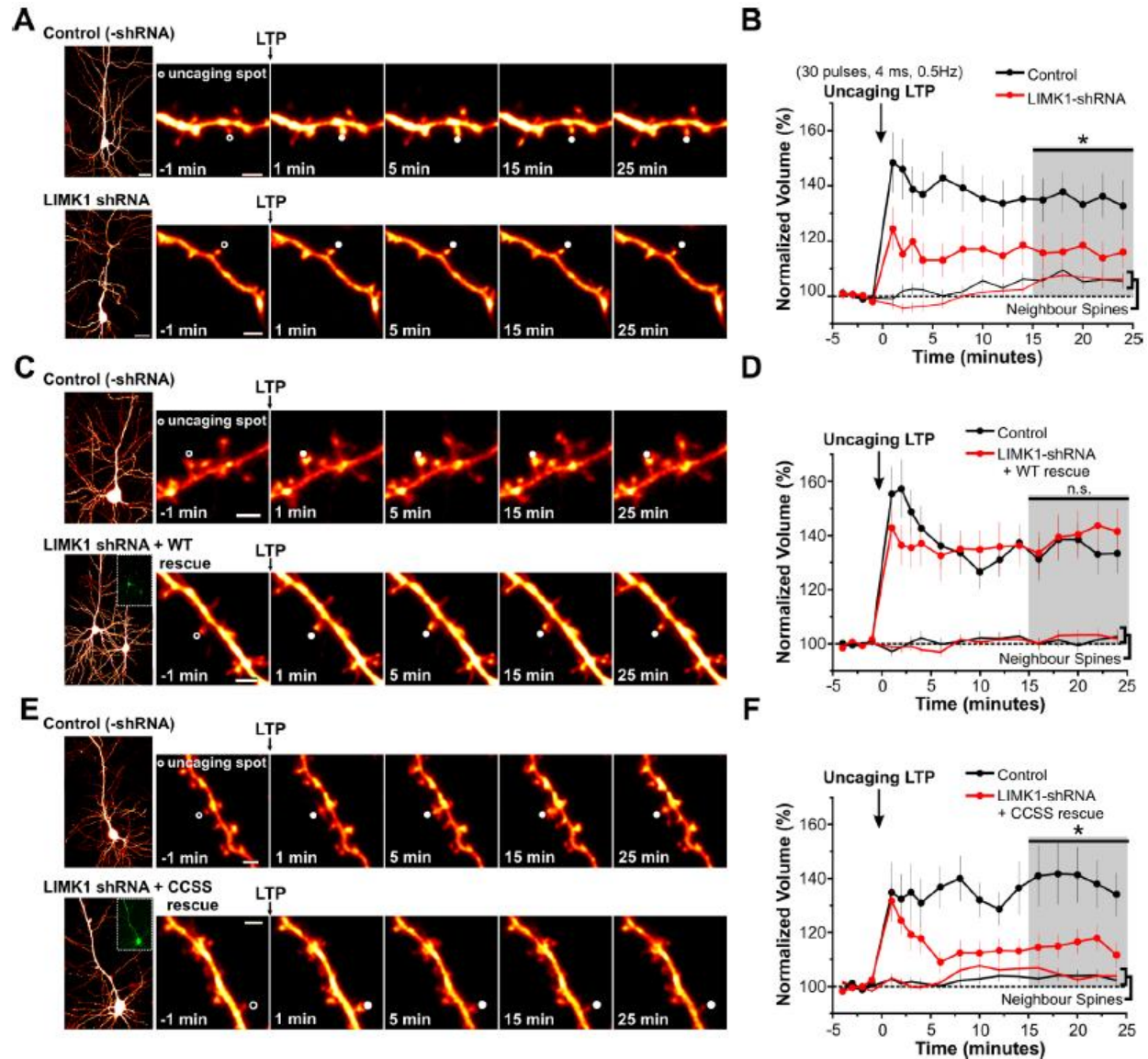


Figure 5: Palmitoyl-LIMK1 is required for spine-specific activity-dependent morphological plasticity. **A:** *Left panels:* Images of individual neurons in organotypic hippocampal slices expressing mCherry with or without LIMK1 shRNA. Magnified images of individual dendrites were acquired at the indicated times prior to and following uncaging of MNI-Glutamate on the head of the indicated spine (white circles). Scale bars: low magnification, 20 μ m, magnified, 2 μ m. **B:** Time course of normalized spine-head volume (mean $\pm$ SEM) of stimulated and neighboring spines (control: 24 stimulated spines, 72 neighbors, 6 neurons; LIMK1 shRNA: 28 stimulated spines, 84 neighbors, 6 neurons). Average normalized spine volume (15-25 mins post-uncaging) was plotted and used for statistical comparison (*: $p < 0.05$, Mann-Whitney U test, details in main text). **C, D:** Images and time courses plotted as in **A, B** for neurons expressing mCherry (Control; 21 stimulated spines, 63 neighbors, 6 neurons) or mCherry, LIMK1 shRNA and shr-wt-LIMK1-GFP (LIMK1-shRNA + WT rescue; 27 stimulated spines, 81 neighbors, 8 neurons). **E, F:** Images and time courses plotted as in **A, B** for neurons expressing mCherry (Control; 23 stimulated spines, 69 neighbors, 6 neurons) or mCherry, LIMK1 shRNA and shr-CCSS-LIMK1-GFP (LIMK1-shRNA+ CCSS rescue; 28 stimulated spines, 84 neighbors, 8 neurons).

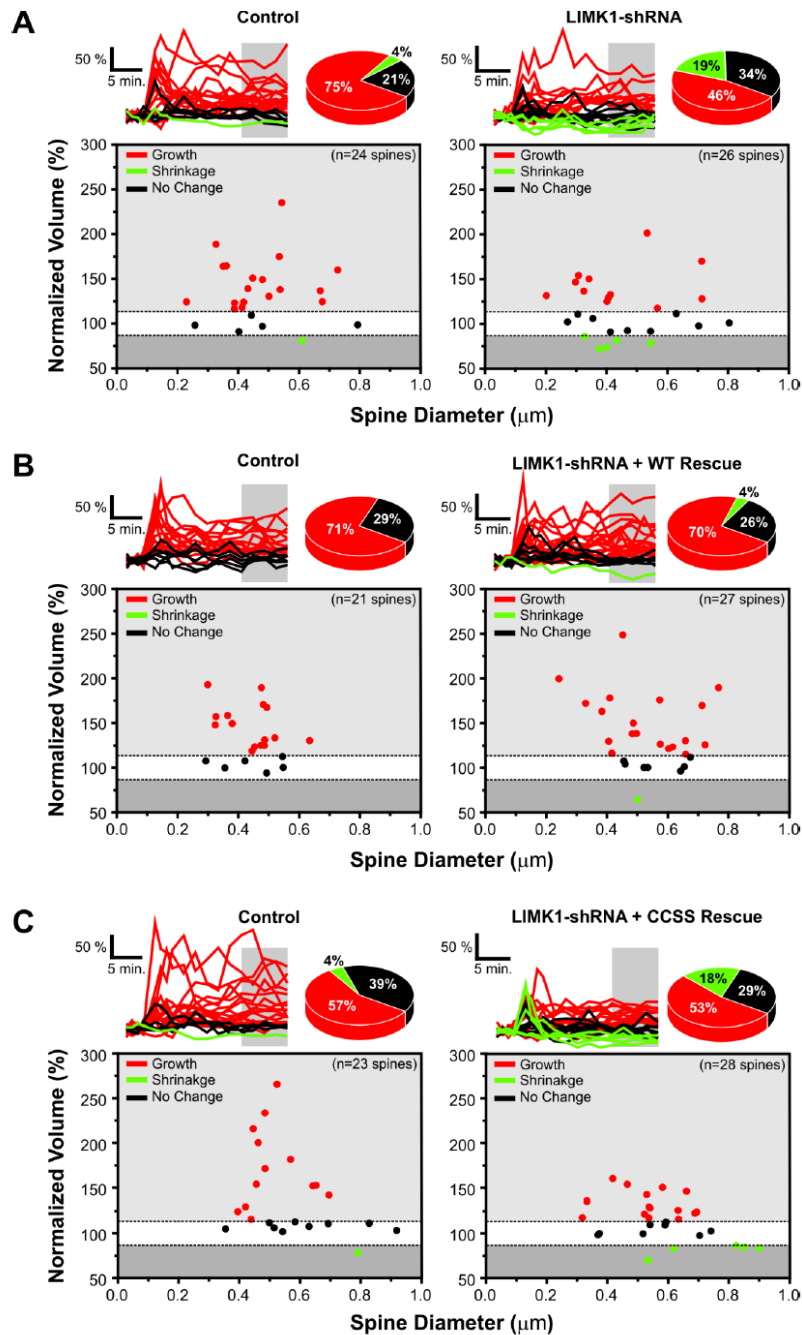


Figure 5 - figure supplement 1. Probability of spine growth or shrinkage in response to glutamate uncaging. **A:** *Upper left:* Individual traces for the average data in Fig. 5A for Control (vector-transfected) neurons. Normalized volumes for each spine, color-coded based on whether spine volume increased (red), decreased (green), or did not change (black) post-uncaging. Pie chart summarizes percentage of spines in each category. *Lower left:* Average normalized spine volume (15-25 min post-uncaging), plotted against initial spine diameters for each spine. *Right panels:* Same as left panels but for spines from LIMK1 ‘knockdown’ neurons. LIMK1 knockdown not only reduced the magnitude of activity-dependent spine enlargement (Fig 6A), but also decreased spine growth success (12/26 spines; 46%) compared with control neurons (18/24 spines; 75%). LIMK1 knockdown also more frequently caused spine shrinkage

(5/26 spines; 19%) compared with control neurons (1/24 spines; 4%). **B:** Individual traces for average data from Fig. 5C, plotted as in A for spines from interleaved control and shRNA-LIMK1 + WT ‘rescue’ neurons. Spine growth success was similar in wt-LIMK1 rescue neurons (19/27 spines; 70%) and controls (15/21 spines; 71%). **C:** Individual traces for the average data in Fig. 5E, plotted as in A for spines from interleaved control and shRNA-LIMK1 + CCSS ‘rescue’ neurons. CCSS-LIMK1 ‘rescue’ more frequently caused spine shrinkage (5/28 spines; 18%) compared with controls (1/23 spines; 4%).

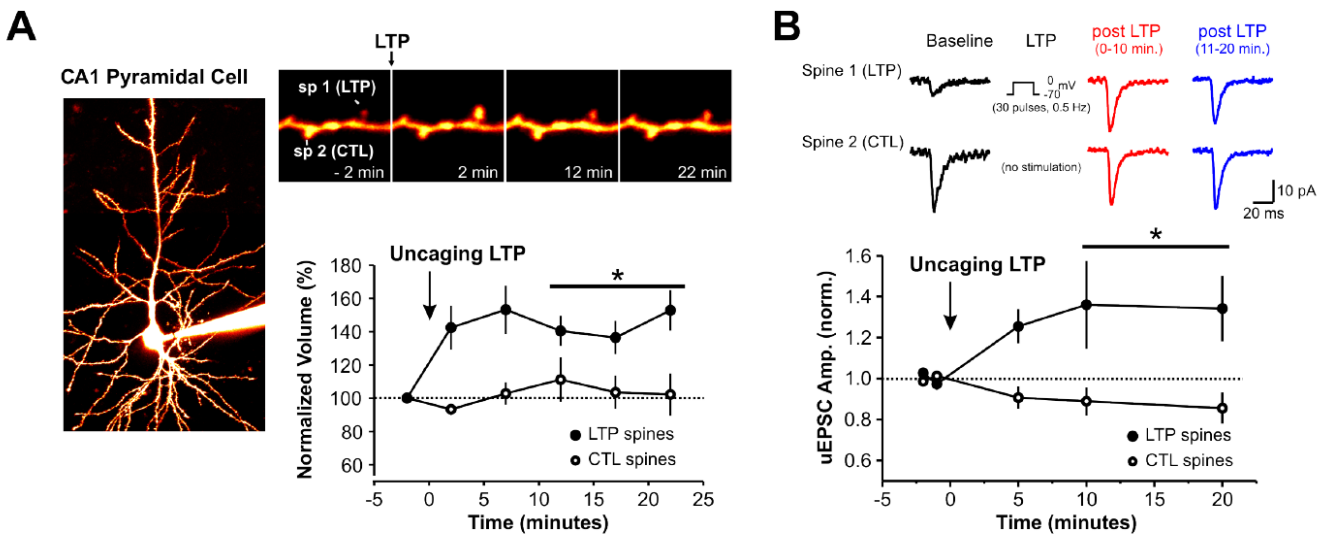


Figure 5 - figure supplement 2. 2-photon uncaging of MNI-glutamate induces spine-specific increases in both spine volume and synaptic strength. **A:** A CA1 pyramidal neuron in an organotypic hippocampal slice is filled with Alexa594 dye via the patch pipette. Repetitive focal uncaging of MNI-glutamate (4 ms pulses at 720 nm, 30 pulses, 0.5 Hz) when neurons are clamped at 0 mV results in structural enlargement of stimulated spine but not unstimulated control spines (spine volume at 10-20 minutes post LTP; stimulated spines: 141.3 ± 7.4 % of baseline, $n = 11$ spines, $P < 0.01$; unstimulated spines: 106.4 ± 9.6 % of baseline, $n = 11$ spines, $P = 0.519$). **B:** Amplitude of uncaging-evoked excitatory postsynaptic currents (uEPSCs) from stimulated spines (at -70 mV) was also significantly increased following single spine repetitive uncaging at 0 mV (normalized uEPSC amplitude at 10-20 minutes post LTP; stimulated spines: 1.31 ± 0.13 , $n = 11$ spines, $P < 0.05$; unstimulated spines: 0.89 ± 0.05 , $n = 11$ spines, $P = 0.09$)

Two Photon Glutamate uncaging

Transfected organotypic slices were transferred to an imaging chamber on a BX61WI upright microscope (60x/1.0 NA objective; Olympus) and continuously perfused at room temperature in ringer solution containing (in mM): 119 NaCl, 2.5 KCl, 0.1 MgSO₄, 3.0 CaCl₂, 1.0 NaH₂PO₄, 11 glucose, and 26.2 NaHCO₃, 0.01 glycine, 0.001 TTX and 2.5 MNI-Glutamate (Femtonics). For the morphological plasticity experiments, mCherry signal was imaged at 950nm using a Ti:Sapphire pulsed laser (MaiTai-DeepSee, Spectra Physics) and MNI-Glutamate was uncaged with a second laser tuned at 720nm. Short segments of secondary and/or tertiary apical dendrites were continuously imaged at 1-2 min intervals by gathering z-stacks (0.75 μ m steps) centered on the spine of interest. The glutamate uncaging protocol consisted of 4 ms pulses delivered at 0.5 Hz for 1 min (Harvey and Svoboda, 2007). Laser power of the uncaging beam was fixed across experiments at 30 mW, measured at the back aperture of the objective. Parallel electrophysiological experiments (n>50) showed that uncaging at this laser power consistently yields AMPAR-mediated inward currents between 5-30 pA. Experiments were performed on spines at approximately constant depth to minimize uneven light scattering between experiments.

To monitor changes in uncaging-evoked excitatory postsynaptic currents (uEPSCs) during single-spine LTP, CA1 neurons were voltage clamped at -70 mV with an internal solution containing (in mM): 115 cesium methane-sulfonate, 5 tetraethylammonium-Cl, 10 sodium phosphocreatine, 20 HEPES, 2.8 NaCl, 5 QX-314, 0.4 EGTA, 3 ATP(Mg²⁺ salt), and 0.5 GTP, and 0.02 Alexa 594 (pH 7.25, 280–290 mOsmol/L). Neurons were continuously perfused in a ringer solution containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 1.0 NaH₂PO₄, 11 glucose, and 26.2 NaHCO₃, 0.01 glycine, 0.001 TTX, and 2.5mM MNI-Glutamate. Ti-sapphire

lasers were tuned to 810 nm and 720 nm for imaging of Alexa 594 and uncaging of MNI-glutamate, respectively (Soares et al., 2013b). Shortly after gaining whole cell access (<5 minutes), short duration light pulses (4 ms) were delivered at low frequency (0.05 Hz) to the tips of two adjacent dendritic spines to uncage MNI-Glutamate and establish a baseline uEPSC amplitude for each spine. Neurons were then voltage clamped at 0 mV and 30 consecutive light pulses were delivered to a single spine at 0.5 Hz to induce LTP (Harvey and Svoboda, 2007). This pairing protocol was performed less than 6 minutes after gaining whole cell access to avoid potential issues associated with washout (Malinow and Tsien, 1990). uEPSCs (at -70 mV) were then monitored for an additional 20 minutes at 0.05 Hz. Two-photon image stacks (810 nm) of the dendritic segment was sampled approximately every 5 minutes. To monitor changes in spine volume during these experiments, we measured the intensity of Alexa594 signal in both the stimulated and unstimulated spine. Because the intracellular dye concentration was typically not at a steady state under these conditions, we normalized spine intensities to the intensity of a nearby dendritic segment at each time point.

Analysis of 2P uncaging-induced changes in Spine Volume:

For MNI-glutamate uncaging experiments, a stackreg function in ImageJ (NIH) was used to align maximum intensity projected images from each time series to correct for X-Y drift. Changes in spine volume were estimated using an intensity-based method by summing pixel intensities from spine regions of interest at each time point, as described previously (Harvey and Svoboda 2007). The average summed intensity of the first four baseline images (prior to uncaging) was used as F_0 and all intensity values are plotted as $\Delta F/F_0 \times 100$. All summed intensity measurements were background subtracted and gathered from raw unprocessed images. Spine ‘growth success’ and spine ‘shrinkage’ events reflect instances where the average

normalized intensity values remained above, or below, 2X the SD of the baseline ($\pm 16\%$ for growth success and spine shrinkage, respectively) for the duration of the experiment after glutamate uncaging. Spine diameter measures (Figure S5) were estimated based on a full width at half maximum value (in μm), calculated by applying a Gaussian fit to the intensity profile across the spine head of the first baseline image.

B.2.

Contributions to Manuscript X

Title:

Correlated synaptic inputs drive dendritic calcium amplification and cooperative plasticity during clustered synapse development

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Statement of Contributions:

This was the main PhD project of my colleague and good friend Kevin Lee. Nearly all experiments were performed by him (with the exception of those mentioned below). Initially, when the paper was first submitted, my contributions were limited to devoting time and ideas during lengthy late night discussions. I also helped Kevin with analysis and figure preparation, and helped edit and finalize versions of the paper. Once the reviewers comments came back, additional experiments were requested, many of which I helped to carry out experimentally. Specifically, I helped gathered electrophysiological and two- photon imaging data that contributed to Figures 1B-E and Figure 7. Jean Phillipe aided with statistical analyses and Jean Claude provided helpful support and guidance through the project's entire timeline. Jean-Claude also had a major role in editing the final version of the text.

Rationale for experiments performed:

Kevin found that during the development of CA1 hippocampal neurons, when dendritic spines and synapses first start to emerge, synaptic inputs could drive the local dendritic release of calcium stores, which flooded the dendrites and spine heads (of even unstimulated spines) with calcium. This coupling between NMDARs and ryanodine-sensitive calcium induced calcium release waned after the first few weeks, to the point where spine calcium signals were much more restricted in dendritic space and did not cause the regenerative activation of calcium stores. It was predicted that these propagating calcium waves played a role in the clustered development of synaptic inputs along regions of dendrite.

One of the reviewers comments asked whether these 'propagating waves' were triggered naturally in freely behaving hippocampal networks. I performed several experiments

with Kevin, some of which now appear in Figure 1 (see below), where we filled CA1 neurons with a calcium sensitive dye via a recording electrode and simply surveyed the dendritic arbor for these propagating calcium events. We found several instances of these propagating waves, whose features matched what Kevin had densely characterized following optical (glutamate uncaging) and electrical stimulation. Thus, my contributions helped to show that these calcium events were not artefactual but are triggered in developing hippocampal neurons.

Another of the reviewer's comments questioned the role of these propagating calcium waves in synapse plasticity and development. Together with Kevin, we designed and carried out a set of experiments that would test the hypothesis that calcium waves triggered from one spine would facilitate plasticity at a neighboring spine. To do this, we found both a reliable and subthreshold glutamate uncaging protocol to induce structural spine plasticity (spine enlargement), which is a structural correlate associated with long-term potentiation. When the subthreshold uncaging protocol at one spine was paired in quick temporal succession with activation of a neighboring spine, the subthreshold stimulus became reliable at inducing lasting spine enlargement. These results therefore suggested that the triggering of propagating calcium waves in developing dendrites helped facilitate the establishment of fine structural connectivity.

Below, you will find both the abstract and the figures that I contributed to directly. For more details, please refer to the published manuscript.

Abstract

The mechanisms that instruct the assembly of fine-scale features of synaptic connectivity in neural circuits are only beginning to be understood. Using whole-cell electrophysiology, two-photon calcium imaging and glutamate uncaging in hippocampal slices, we discovered a functional coupling between NMDA receptor activation and ryanodine-sensitive intracellular calcium release that dominates the spatiotemporal dynamics of activity-dependent calcium signals during synaptogenesis. This developmentally regulated calcium amplification mechanism was tuned to detect and bind spatially-clustered and temporally-correlated synaptic inputs, and enacted a local cooperative plasticity rule between coactive neighboring synapses. Consistent with the hypothesis that synapse maturation is spatially regulated, we observed clustering of synaptic weights in developing dendritic arbors. These results reveal developmental features of NMDA receptor-dependent calcium dynamics and local plasticity rules that are suited to spatially guide synaptic connectivity patterns in emerging neural networks.

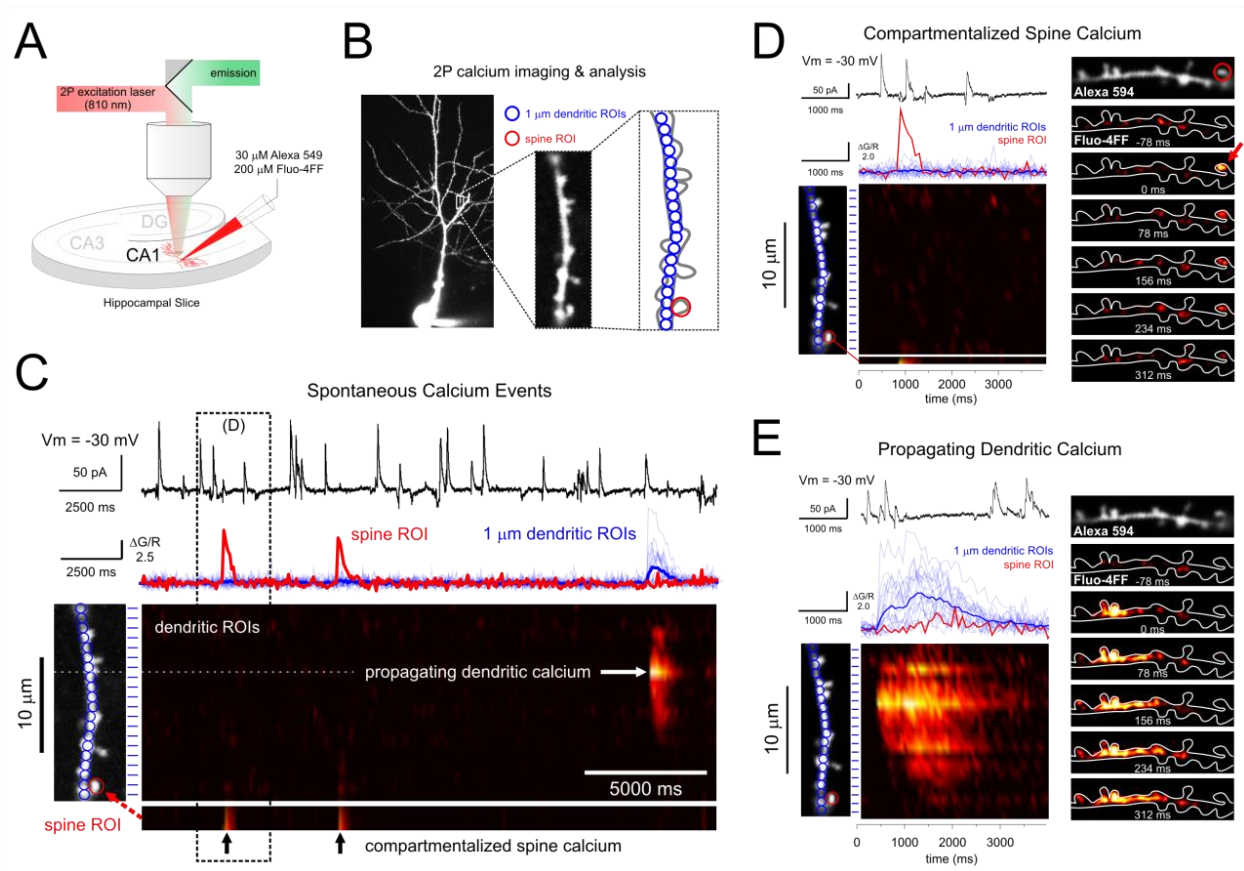


Figure 1. 2P imaging of spontaneous synaptic calcium signals at CA1 pyramidal neurons (A) Schematic illustration of whole-cell electrophysiology and 2P calcium imaging in hippocampal slice. (B) A CA1 pyramidal neuron in organotypic slice, filled with 30 μ M Alexa 594 and 200 μ M Fluo-4FF. Calcium signals were imaged by 2P frame scanning (12-15 Hz), and analyzed at 1 μ m circular ROIs positioned along dendrites and at ROIs placed over spines. (C) Spontaneous calcium events were diverse, even along the same dendrite. Example calcium signal traces from ROIs along a dendrite and at one spine are shown (blue and red, respectively), aligned with a pseudo-color map of dendritic calcium fluorescence (bottom). The spine showed two calcium events with no elevations in dendritic calcium. Later in the sweep, a large dendritic calcium signal was observed spreading from nearby dendritic spines. Glutamatergic and GABAergic transmission were identified in the simultaneously-recorded electrophysiological current trace (top) as inward and outward events, respectively. (D) Example of a spontaneous calcium signal that was compartmentalized at a dendritic spine (dashed box from C), shown with the simultaneously-recorded current (top, black trace). (Right) Time-series of individual imaging frames. (E) Example of a spontaneous dendritic calcium signal that propagated from a pair of neighboring spines. Propagating dendritic calcium signals comprised $75 \pm 8.9\%$ of all detected events ($n = 11$ dendrites from 5 neurons), and spread on average 8.7 ± 0.4 μ m.

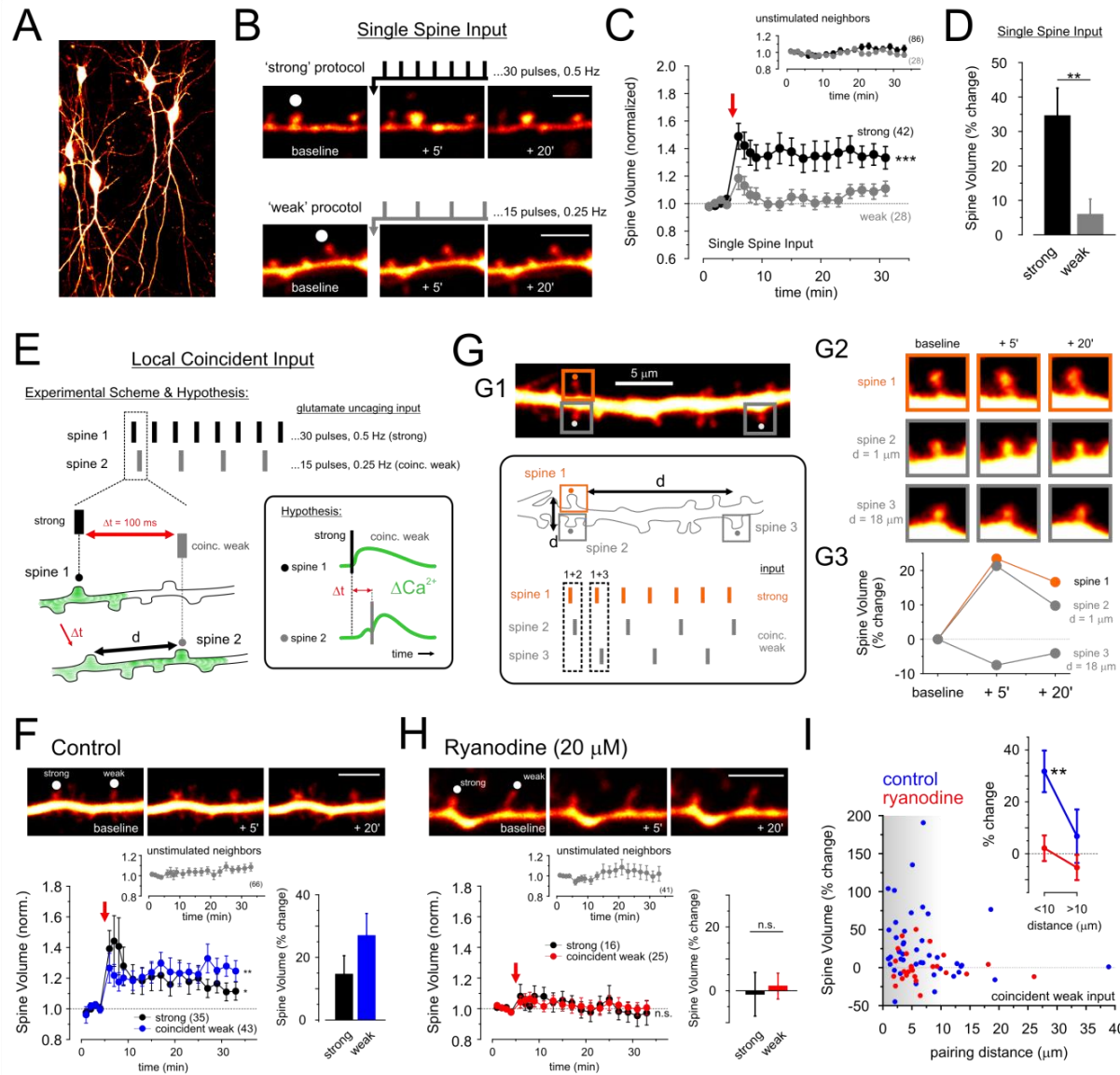


Figure 7. Ryanodine receptor-dependent intracellular calcium release supports local, distance-dependent cooperative synaptic plasticity at co-active dendritic spines. (A) 2P image stack of CA1 pyramidal neurons in organotypic slice expressing mCherry. To study dendritic spine plasticity at 'early' and 'late' developmental time points, slices were prepared at P7, biolistic transfection was performed at 2-3 or 6-7 DIV, and experiments were performed 3-5 days after transfection. **Figure 7** shows data from 'early' developing neurons. Analysis of spine plasticity at 'late' developing neurons can be found in **Figure S6**. (B) Two different glutamate uncaging protocols were used at single spines in 0.1 mM extracellular Mg^{2+} . A 'strong' protocol of 30 uncaging pulses (4 ms) delivered at 0.5 Hz (top) and a 'weak' protocol of 15 uncaging pulses (4 ms) at 0.25 Hz (bottom). Uncaging laser power was constant for all experiments. 'Baseline', '+5', and '+20' refers to the pre-stimulus period, and 5 and 20 minutes post-stimulus, respectively. Scale bars: 2 μm . (C) Time course of spine volume changes ($\Delta F/F$) in response to 'strong' (black) and 'weak' (grey) uncaging stimuli (red arrow). The strong uncaging protocol induced significant spine growth ($p = 0.0000779$) but the weak protocol did not ($p = 0.16845$). Unstimulated neighbor spines did not show changes in volume (inset). (D) Bar graph summary of spine volume change

15-25 minutes after the uncaging stimulus. ($p = 0.00223$). (E) Experimental scheme for probing the effect of input coincidence on plasticity at neighboring spines. (Top) Raster sequence showing the timing of uncaging at two neighboring spines: every second uncaging pulse at spine 1 (strong protocol) was followed by an uncaging pulse at spine 2 ($\Delta t = 100$ ms, 'coincident weak' protocol). (Bottom) Hypothesized local calcium summation behavior based on the features of NMDAR-dependent CICR (**Figures 1-5**). (F) (Top) Example experiment showing the effect of pairing 'strong' and 'weak' input at neighboring spines (scale bar: 5 μm). Significant spine growth was detected at both spines. (Bottom, left) Summary time course of spine volume in response to local coincident input (red arrow) at spines receiving 'strong' (black) and 'coincident weak' (blue) uncaging stimuli. Both uncaging protocols triggered significant spine growth (strong, $p = 0.01536$; weak, $p = 0.000329$). Unstimulated neighbor spines did not change (inset). (Right) Summary graph of spine volume changes (15-25 mins post-stimulus). (G) (G1) Example experiment involving three spines along the same dendritic segment. Spine 1 (orange box) received the 'strong' uncaging input while Spines 2 and 3 (grey boxes) received 'coincident weak' inputs. Uncaging pulses at Spines 2 and 3 alternated with every second pulse at Spine 1 ($\Delta t = 100$ ms). (G2) Enlarged views of Spines 1, 2 and 3 showing the effect of coincident activation on spine structure. (G3) Spine volume changes at the time points shown in (G2). (H) (Top) Example experiment showing local coincident input on spines with 20 μM ryanodine in the bath. Glutamate uncaging stimuli failed to induce spine growth in ryanodine (scale bar: 5 μm). (Bottom, left) Summary time course of spine volume changes in response to 'strong' and 'coincident weak' uncaging stimuli in ryanodine. Unstimulated neighbor spines did not change (inset). (Right) Summary graph of spine volume changes (15-25 mins post-stimulus). (I) Magnitude of spine volume changes (15-25 mins post-stimulus) in response to 'coincident weak input' plotted as a function of the inter-spine pairing distance. (Inset) Summary of spine volume changes at proximal (<10 μm) and distal (>10 μm) pairing distances. In control conditions (blue), coincident weak input triggered significant spine growth when the co-activated neighbor spine was <10 μm away ($p = 0.000367$), but not >10 μm ($p = 0.5346$). On average, spine growth was not observed in ryanodine (red; $p > 0.3$). Data are shown as mean $\pm$ SEM. Number of spines indicated in parentheses. Spine growth was determined by a one-way t-test compared to a mean effect of no change. Two-sample t-test was used for between-group comparisons. * $p < 0.05$, ** $p < 0.001$. See also **Figure S6**.