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Investigating the Molecular Mechanisms Underlying Activity-Dependent Stabilization of Nascent Dendritic Spines

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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OFFICE OF GRADUATE STUDIES

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DAVIS

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Investigating the Molecular Mechanisms Underlying Activity-Dependent Stabilization of Nascent Dendritic Spines

ABSTRACT

Learning and memory are crucial cognitive processes that promote an organism's survival, allowing it to rapidly modify its behavior in response to changes in the environment and repeat patterns of behavior that enhance survivability and reproduction. At a cellular level, the phenomena of learning and memory are facilitated by the restructuring of plastic neural circuits that encode behaviors. The dynamic nature of dendritic spines, small, actin-rich, membranous protrusions on the dendrites of neurons, underlies this plasticity and the neural capacity for modification. Dendritic spine are the sites where neurons receive most of their excitatory inputs and several studies have linked the dynamic changes dendritic spines can undergo to learning and lasting memory. Amongst these changes are the spontaneous outgrowth and subsequent persistence of new dendritic spines. New spine outgrowth increases during learning paradigms, and the fraction of these new spines that are retained correlates with improved memory of a learned experience. Little is known about the mechanisms that determine whether a new spine stabilizes and persists, but recent work has demonstrated that strong synaptic stimulation, particularly that which induces long-lasting potentiation (LTP) of spine volume and synaptic strength at mature spines, enhances the survivorship of nascent dendritic spines. This phenomenon can be induced absent of presynaptic input using two-photon (2p) glutamate uncaging of 4-methoxy-7-nitroindolinyl (MNI)-caged glutamate at a high-frequency (HFU) that allows for the spatially restricted release of glutamate at a single dendritic spine. LTP-inducing glutamatergic stimulation initiates several molecular signaling cascades that occur within the

spine; which of these mechanisms exist in new spines and play a role in enhancing new spine stability remains a mystery. The interaction between the GluN2B subunit of the NMDA-type glutamate receptor (NMDAR) and CaMKII that is activated downstream of NMDAR-mediated Ca^{2+} influx is one molecular mechanism induced during LTP that has been implicated in new spine survivorship. This interaction was found to be necessary for activity-dependent new spine stabilization and genetically disrupting GluN2B-CaMKII binding blocks the stabilizing effects of glutamatergic stimulation. The work described in this dissertation follows up on these results by further defining the role that CaMKII plays in activity-dependent new spine stabilization. **Chapter 1** of this dissertation is a review of the current understanding of new spines, including their role in the dynamic restructuring of neural circuits to facilitate learning and memory, the mechanisms that govern their formation, and the current understanding of the mechanisms that promote their stabilization. **Chapter 1** also reviews what is known about the signaling cascades that are involved in nascent dendritic spine stability and the role that CaMKII and its myriad functions and interactions may play in enhancing new spine stabilization. **Chapter 2** of this dissertation describes experiments which further define the roles that CaMKII structural and enzymatic activity play in activity-dependent new spine stabilization. 2p imaging and HFU of MNI-caged glutamate was used to stimulate spontaneously formed nascent dendritic spines shortly after outgrowth on CA1 neurons in cultured organotypic hippocampal slices where CaMKIIα enzymatic and structural activities were altered. These experiments provided direct evidence that CaMKIIα structural and scaffolding interactions, but not its kinase activity, are required for the previously reported enhancement of new spine stability following 2p glutamate uncaging. **Chapter 3** of this dissertation contains additional analysis performed on the data described in **Chapter 2**. These analyses corroborate previous results that HFU at a new spine, which enhances survivorship, often results in new spine enlargement, but new evidence described in this chapter suggests that this enlargement is not necessary to stabilize new spines. Also shown in **Chapter 3** is evidence that CaMKIIα kinase activity inhibits spine enlargement at new spines,

despite it being necessary for spine enlargement at mature spines. **Chapter 4** describes preliminary experiments that further explore the non-enzymatic role that CaMKIIα performs at new spines by targeting two binding partners that do not require CaMKIIα to function: the 26S proteasome and synGAP-1α. **The first section of Chapter 4** describes experiments that aim to determine the role of the 26S proteasome in activity-dependent new spine stabilization. Pharmacological inhibition of the 26S proteasome was utilized to determine if the proteasome had a role in enhancing new spine survivorship rates following HFU. The preliminary results of these experiments show that inhibition of the 26S proteasome increased basal rates of new spine survivorship such that any changes or deficits in activity-induced stabilization could not be determined. **The second section of Chapter 4** focuses on the role of synGAP-1α, a negative regulator of synaptic stability, plasticity, and maturation, in activity-dependent new spine stabilization. Green fluorescent protein (GFP)-tagged synGAP-1α was overexpressed in CA1 neurons in cultured organotypic hippocampal slices and HFU of MNI-caged glutamate was used to stimulate individual new spines that had formed on these cells. The preliminary findings of these experiments show that synGAP-1α, which is enriched at lower levels in new spines compared to their mature counterparts, undergoes activity-dependent evacuation from new spines following HFU. This evacuation does not require CaMKIIα kinase activity, as preliminary results show that synGAP-1α dispersion from new spines still occurs during pharmacological inhibition of CaMKIIα. Together, my results demonstrate that non-enzymatic scaffolding activities of CaMKIIα, such as the interactions between CaMKII and the 26S proteasome or synGAP-1α, regulate new spine survivorship and volume changes, providing further insight into the molecular mechanisms that govern new spine stability and maturation.

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CHAPTER 1: LITERATURE REVIEW

Introduction

While evolution may act on the time scale of hundreds of millennia, organisms on this planet demonstrate the remarkable ability to rapidly adapt to changes in their environment and modify their behaviors to ensure their survival. This incredible ability to adapt is facilitated by the restructuring of existing neural circuits to encode salient information about the environment and strengthen connections to promote the execution of adaptive behaviors. How are these neural circuits modified to accommodate such adaptations? Synapses- the points of communication between neurons- are the key to understanding how the reorganization of neural circuits shape adaptive behaviors. In many mammals, a significant proportion of these synapses, particularly excitatory synapses, occur at dendritic spines, small membranous protrusions emanating from the shafts of neuronal dendrites. Dendritic spines were first observed and described by Santiago Ramón y Cajal in 1888 using a silver nitrate staining technique developed by his intellectual rival, Camilo Golgi (*for review, see* Garcia-Lopez, et al., 2007). Since their discovery a large body of evidence has implicated dendritic spines as the structural correlates of learning and memory, facilitating the dynamic restructuring of neural circuits required for adaptation. Bliss and Lømo found that strong electrical stimulation of the CA3 region of the rabbit hippocampus increased the synaptic responses recorded from neurons in the CA1 region of the hippocampus, which receives input from CA3 (1973). The patterns of stimulation used by Bliss and Lømo to induce long-lasting strengthening of the synaptic connections between CA3 and CA1 were later found to induce several changes at dendritic spines, including the addition of new synaptic glutamate receptors, the phosphorylation of existing glutamate receptors, and the enlargement of activated dendritic spines (*for review, see* Herring and Nicoll, 2016). These changes, along with the activity-induced outgrowth of new dendritic spines, are thought to underlie long-term learning and memory. New spines are particularly intriguing substrates of learning and memory,

as they are hypothesized to facilitate the formation of novel synaptic connections that are necessary to encode information of novel experiences.

Rates of new spine outgrowth are high during development and increase during learning experiences. Several studies have found that the persistence of these new spines correlates with learning and lasting memory (Roberts, et al., 2010; Yang, et al., 2009; Xu, et al., 2009). Still, these new spines remain somewhat mysterious. Little is known about their molecular composition and functional capabilities, which have been shown to differ from those of older spines (Zito, et al., 2009; Kwon and Sabatini, 2011; Lambert, et al., 2017) and the mechanisms determining which new spines persist or are eliminate are largely undetermined. In this literature review, I aim to highlight what is currently known about new spine stabilization, including recent work to identify patterns of activity that bias new spines towards persistence and the molecular mechanisms that have been found to be necessary for this activity-dependent stabilization. I also hope to highlight gaps in our current understanding of new spines and the mechanisms that stabilize them and contextualize my own dissertation work, described in Chapters 2,3, and 4, in the larger body of nascent dendritic spine stabilization research.

What is a new dendritic spine?

New spine outgrowth is typically observed using time-lapse imaging, a technique that requires taking images of dendrites *in vivo* or in *in vitro* neural tissue at regular intervals to identify instances of new spine growth or retraction over time. The experimental observation window and imaging frequency largely dictates when spines are considered “new”. *In vitro* studies in the hippocampus tend to utilize more frequent observations over an overall shorter experimental window (~24 hours) and therefore most *in vitro* studies consider new spines to be any protrusions that appear within a 30 min-2.5-hour window (Engert and Bonhoeffer, 1999; Hill and Zito, 2013; Lambert, et al., 2017; Zito, et al., 2009; De Roo, et al., 2008a) In contrast, many *in vivo* studies in the cortex monitor spine dynamic over periods of several days, usually imaging every 1-4 days (Trachtenberg, et al., 2002; Holtmaat, et al., 2005; Knott, et al, 2006). The

limited temporal resolution of many *in vivo* studies in the cortex therefore limits definition of a new dendritic spine to one appearing within the 1–4-day interval between images. Each definition of a new spine presents unique caveats and insight into our understanding of new dendritic spines. The narrower window for new spine outgrowth in i*n vitro* studies has led to discoveries about the molecular composition and morphology of spines shortly after outgrowth, while the longer experimental timeline afforded by *in vivo* studies provides a clearer understanding of the survivorship and maturation of new spines over time. Both *in vivo* and *in vitro* studies on new spines should be taken into account when reviewing our current understanding of new spines and their individual discoveries and caveats should be considered. In developing our understanding of new spines, many studies have used "mature" or "pre-existing" neighboring spines on the same dendrites as controls to compare their dynamics, morphology, survivorship, and molecular composition to that of new spines. It is highly probable that a portion of these supposedly pre-existing spines may be in fact be "new" by the experimentally relevant definition, having appeared within 30 minutes to 4 days prior to the first observation or image acquisition. It is difficult to determine which fraction of all observed "mature/pre-existing" spines may still fit the definition of "new" as described by each experimenter, and it is necessary to consider this fact when making comparisons of "old/mature/persistent" spines versus new spines, such as the comparisons I make in this dissertation. This also highlights the need to use additional evidence to strengthen our determinations of which spines are new. Such additional evidence that may be necessary to consider when determining the age of a dendritic spine, including morphology and molecular composition, will be discussed later in this chapter.

How are new spines formed?

The formation of new spines reflects their critical roles in development, learning and memory. The spontaneous outgrowth of new spines has been observed both *in vitro* and *in vivo* in the hippocampus (De Roo, et al., 2008b; Zito, et al., 2009; Gu, et al., 2014) and many cortical

regions including the primary somatosensory cortex, primary visual cortex, and motor cortex (Trachtenberg, et al., 2002; Holtmaat, et al., 2005; Yang, et al., 2009; Xu, et al., 2009). The rates of spontaneous new spine outgrowth appear to vary by developmental period, with higher rates of new spine outgrowth occurring earlier in development in a variety of different regions (Dunaevsky, et al., 2019; Holtmaat, et al., 2005). The developmental regulation of spontaneous new spine formation is hypothesized to control critical and sensitive periods necessary for sensory formation (Majewska and Sur, 2003) and species-relevant learning (Roberts, et al., 2010) and to facilitate the formation of preliminary neural circuits that can be refined with future maturation, experience, and learning (Dunaevsky, et al., 2019; Khanal and Hotulainen, 2021). Currently our understanding of the mechanisms that underliedevelopmentally regulated increases in new spine outgrowth is limited, as it is difficult to distinguish between the activity and experience-dependent new spine induction that also occur during early development. The rest of this section will further discuss activity and experience dependent new spine outgrowth and what is known about the mechanisms involved.

While initially difficult to distinguish between (to me, at least), experience-dependence and activity-dependence are discrete, but often intertwined, phenomena in terms of understanding synaptic plasticity. Experience-dependent changes in synaptic strength and number occur due to changes in an organism's environment or during skill acquisition and is necessary for the ability to adapt and modify behavior in environmentally relevant ways. Enriched environments and social housing increase cortical thickness by way of increasing both dendritic arborization and dendritic spine density in rats (*for review see* Bailey and Kandel, 1993). In mice, "chessboard deprivation", trimming of every other facial whisker in a pattern that resembles a chess or checkerboard, enhances spine densities in the barrel cortex over the course of several days, which correlates with the modification of receptive fields of neurons that initially responded to the trimmed whiskers (Trachtenberg, et al., 2002). A similar increase in spine outgrowth linked to compensatory retinotopic restructuring occurs following retinal lesions

and monocular deprivation (Keck, et al., 2008; Hofer, et al., 2009). Behavioral learning, such as species-relevant song learning in zebra finches or completely novel motor tasks like balancing on an accelerating rotarod or grasping pasta, also results in rapid increases in nascent dendritic spine outgrowth in task-relevant cortical regions (Roberts, et al., 2010; Yang, et al., 2009; Xu, et al., 2009). These experience-dependent changes in dendritic spine induction likely serve to facilitate the restructuring of neural circuits necessary for the acquisition of new skills and the refinement of sensory systems that an organism might need to survive in an ever-changing environment. Experience results in changes in synaptic activity in certain circuits (Tang and Zou, 2002; Artola, et al., 2006; Frenkel and Bear, 2004), and experience-dependent changes in activity and dendritic spine morphology, outgrowth, and pruning can be regulated by age and development (Hosseiny, et al., 2015), making it difficult to truly attribute changes in new spine outgrowth to the individual contributions of experience, activity, and development.

Synaptic activity is often used to approximate the conditions that might normally occur during learning in *in vitro* preparations, however, changes in synaptic activity may occur independent of learning experiences, regulated by myriad factors such as development, hormonal fluctuations, diet, and circadian rhythms (*some examples include* Maki and Resnick, 2001; De Silva, et al., 2011; Schwartzkroin, 1999; Shibata, et al., 1982). The formation of ocular dominance columns is one well-studied example of the activity-dependent changes in neural circuitry that can occur during developmentally regulated time periods. While experience does play an important role in the functional organization of the mammalian primary visual cortex (Le Vay, et al., 1980; Frenkel and Bear, 2004), it has also been shown that spontaneous activity preceding any form of visual experience is also crucial for self-organization of the visual system and retinotopy in the primary visual cortex (Ackman, et al., 2012; Winnubst, et al., 2015; Scholl, et al., 2017).

Artificial manipulations of synaptic activity, either locally at precise places on dendrites or globally through the application of drugs, toxins, or electrical stimulation, also promote robust

new spine outgrowth. An early study demonstrating this structural phenomenon used local superfusion, the application of a suitable solution to tissue exposed to air or an otherwise experimentally unsuitable solution (Gaddum, 1997), to show that strong electrical stimulation of Schaffer collaterals triggered rapid new spine outgrowth in CA1 (Engert and Bonhoeffer, 1999). Hippocampal slices were bathed in a moderate Cd^{2+}, low Ca^{2+} solution to block Ca^{2+}-dependent neurotransmitter release. Engert and Bonhoeffer then applied a small, spatially restricted stream of normal, extracellular solution to regions of CA1 innervated by a subset of the Schaffer collaterals and paired that superfusion with electrical stimulation of the Schaffer collaterals. New spine outgrowth was enhanced on superfused regions of CA1, where the Ca^{2+} entry into the presynaptic terminals was permitted and glutamatergic transmission occurred, but not on neighboring dendrites (Engert and Bonhoeffer, 1999). More refined techniques have corroborated this finding, showing that local neurotransmitter release can also induce the growth of a single new spine from the dendrite (Kwon and Sabatini, 2011; Hamilton, et al., 2012). Increasing synaptic activity *in vitro* with bicuculine incubation or theta-burst stimulation similarly increases the rates of new spine outgrowth on hippocampal neurons (Hamilton, et al., 2012; De Roo, et al., 2008a). In these studies, activity-induced new spine formation was more likely to occur in tissue from younger animals (P8-12 for acute slices, DIV 7-9 for cultured hippocampal slices). In my own work, which will be described in detail in Chapters 2-4, I found that to be true; the rates of observable new spine outgrowth were greatly reduced after only 10 days *in vitro*. This suggests that the mechanisms regulating spontaneous new spine outgrowth as well as experience-dependent and activity-dependent new spine formation may be further regulated by developmental time period. Our current understanding of new spine outgrowth has not yet determined if the underlying mechanisms differ between spontaneous, activity, and experience induced new spine outgrowth. It has been reported that NMDAR activity, Ca^{2+} signaling, cAMP/PKA-dependent signaling cascades, but not CaMKII, are necessary for activity-dependent new spine formation (Kwon and Sabatini, 2011). Spontaneous new spine outgrowth

has been shown to require NMDAR-CaMKII signaling, the 26S proteasome, and the RhoGEF ephexin 5, but not PKA (Hamilton, et al., 2012; Hamilton, et al., 2017), suggesting that there may in fact be differences in the mechanisms that promote new spine outgrowth, depending on the method of induction.

Characteristics of new spines

Given the variable definitions of “new” and “mature” dendritic spines and the caveats presented by the different definitions, it is necessary to try and distinguish new spines from their mature counterparts using methods other than the time at which they appear during a very limited observation window. Fortunately, work has been done to characterize new spines and identify key similarities and differences between new spines and their pre-existing neighbors. This work has been crucial for understanding the stabilization and maturation of new spines; while the age of a pre-existing neighboring spine cannot be fully ascertained, these differences can be used to strengthen the conclusions drawn from comparisons between new and “mature” dendritic spines.

Dynamics

New spine dynamics are perhaps the most obvious distinction between new and pre-existing spines. New spines are less stable than their mature counterparts, with some studies reporting that up to 50% of newly formed spines are eliminated within 1-7 days (Trachtenberg, et al., 2002; Knott, et al, 2006; Hill and Zito, 2013), despite striking differences in the cortical regions and ages of the animals used in these studies. Conversely, many groups have reported low levels of elimination for “mature” spines, those pre-existing prior to or at the start of the imaging series (Trachtenberg, et al., 2002; Holtmaat, et al., 2005). The impermanence of new spines may be a direct result of their proposed role in the restructuring of neural circuits. Rates of new spine outgrowth may increase during learning and experience to increase the probability of forming lasting synaptic connections that would serve to rewire existing neural circuits to accommodate memory of the learned experience (Trachtenberg, et al., 2002; Yang, et al., 2009

Xu, et al., 2009; Hofer, et al., 2009; Roberts, et al., 2010). These newly remodeled circuits need only a small fraction of the newly formed spines to persist to accommodate lasting memory (Yang, et al., 2009; Xu, et al., 2009) and new spines that fail to find appropriate presynaptic partners are likely eliminated without contributing any drastic changes to the revised or established neural networks.

Morphology

At early developmental time points when rates of new spine outgrowth are highest, filopodial like outgrowths make up a significant proportion of all newly formed protrusions (De Roo, et al., 2008b; Fiala, et al., 1998; Aceti, et al., 2015). Filopodia are often not considered to be true spines, with many lacking a defined spine head and post-synaptic densities (Fiala, et al., 1998). The turnover rate for filopodia is high even in adulthood (Grutzendler, et al., 2002) and they are the least stable morphology of all protrusions, nascent or pre-existing (De Roo, et al., 2008b; Grutzendler, et al., 2002; Aceti, et al., 2015). It is speculated that filopodial protrusions are precursors to new spines (Parnass, et al., 2000), and there is evidence that a small proportion of filopodia do eventually become stable, mushroom-type dendritic spines (De Roo, et al., 2008b; Aceti, et al., 2015; Parnass, et al., 2000). Recent work utilizing advanced super-resolution imaging techniques has shown that filopodial outgrowths in the adult cortex are equipped with NMDARs and are often paired with functionally mature presynaptic partners, but lack AMPARs (Vardalaki, et al., 2022). Vardalaki and colleagues (2022) also found that inducing Hebbian plasticity by pairing electrical stimulation of presynaptic axons with postsynaptic depolarization at the soma can “unsilence” filopodial synapses on the postsynaptic cell by recruiting AMPARs. Surprisingly, this functional potentiation is not coupled to the structural or morphological changes that typically occur following similar Hebbian pairing protocols (Hill and Zito, 2013). Filopodia paired with presynaptic boutons may receive glutamatergic stimulation that allows for the stepwise recruitment of AMPAR and subsequent morphological changes that can further aid in maturation and stabilization into spines.

Other evidence suggests that the high turnover rate of filopodia serves as an indicator that many do not survive long enough to adopt more stable morphologies (Parnass, et al., 2000; Zuo, et al., 2005; De Roo, et al., 2008b; Aceti, et al., 2015). Additionally, filopodia have been shown to precede shaft synapse formation more than spine synapse formation (Fiala, et al., 1998), and the majority of stable, mushroom-type spines begin as mushroom type-spines, with very few filopodia undergoing morphological changes to become mushroom spines (Zuo, et al., 2005; De Roo, et al., 2008b; Aceti, et al., 2015). Rarely are distinctions made between thin spines and filopodial protrusions, increasing the likelihood that filopodia thought to adopt a more stable spine morphology might actually be thin spines. Full understanding of the role filopodia play in the restructuring of neural circuits remains elusive.

De Roo and colleagues (2008b) observed that mushroom-type spines make up the largest proportion of all new protrusions and are by far the most stable over a five-day period. Indeed, there is plenty of additional evidence demonstrating that stability favors the mushroom morphology for both new and preexisting spines (Kasai, et al., 2003). While a population of new spines may enjoy this privileged morphology, there still exist structural differences between them and their pre-existing neighbors. New spines are often smaller than their mature counterparts (De Roo, et al., 2008b; Hill and Zito, 2013) and their smaller volumes may contribute to their impermanence. Hill and Zito (2013) found that larger new spines were more stable, and manipulations that enhanced new spine survivorship only resulted in drastic differences in spine stability amongst smaller new spines, with survivorship rates of "large" and "medium" sized spines unaffected by their stabilizing manipulation. Similarly, an earlier study by De Roo and colleagues (2008b) reported that the majority of stable new spines had larger spine head volumes and underwent drastic increases in spine volume over the course of 24 hours. Even at later time points or in disease states, spine size and volume changes might still regulate stability. MacDonald, et al., (2017) reported that smaller spines were more likely to be targeted for elimination than their larger counterparts in models of schizophrenia. Spine length may also

serve as a predictor of spine stability. As previously mentioned, long thin, filopodial-type protrusions are most common in earlier developmental time points and are also the least stable type of protrusion (Zuo, et al., 2005; De Roo, et al., 2008b; Aceti, et al., 2015); Clement, et al., 2012). Thinner spines are also more susceptible to dynamic protrusive morphological changes, which are also correlated with decreased stability (Tashiro and Yuste, 2004; Vazquez, et al., 2004; Oray, et al., 2006).

Molecular composition

The differences in new spine stability and morphology raise the question of whether new spines, many of which are hypothesized to be excluded from functional neural circuits (Knott, et al, 2006, *but see* Zito, et al., 2009), also differ molecularly from their pre-existing counterparts. New spines, although reported to form functional asymmetrical synapses shortly after outgrowth (Zito, et al., 2009) are known to express PSD-family MAGUKs at lower levels than mature spines on the same cells (De Roo, et al., 2008b; Lambert, et al., 2017). PSD-family MAGUKs, particularly PSD95, play well-established roles in regulating dendritic spine size, stability, and functional strength, and maturation (Elias, et al., 2006; Ehrlich and Malinow, 2004; Taft and Turrigiano, 2014; Cane, et al., 2014), therefore, the low expression levels of PSD- family MAGUKs may contribute to the impermanence of most new spines. Intriguingly, the arrival times of PSD-family MAGUKs to new spines differs by family member. PSD95 is one of the last to arrive at new spines, taking up to 24 hours after new spine outgrowth to accumulate to mature levels (De Roo, et al., 2008b; Lambert, et al., 2017), consistent with the role that PSD95 is thought to have in synapse maturation (Vazquez, et al., 2004; Ehrlich and Malinow, 2004) and stability (Taft and Turrigiano, 2014; Cane, et al., 2014) and the relative instability of new spines. Lambert and colleagues (2017) reported that, shortly after outgrowth, SAP102 is one of the fastest arriving PSD-family MAGUKS at new spines, however, enrichment levels of SAP97 predicted stability. While the exact mechanism by which SAP97 enrichment is linked to stability in new spines has not yet been experimentally determined, “mature” levels of SAP97 may mark

sites of recent synaptic activity (Mauceri, et al., 2004). The relative enrichment of SAP97 at new spines may give us further insight into the mechanisms that stabilize new spines by marking new spines that are part of active, functional synapses, as synaptic activity is known to enhance new spine stability (Hill and Zito, 2013).

SAP102 is one of the earliest PSD-family MAGUKs to arrive at new spines, accumulating to mature levels within two hours (Lambert, et al., 2017), however the specific role this molecule plays in regulating new dendritic spine stability and maturation is currently unknown. During early-stage hippocampal development, SAP102 is expressed at higher levels than both PSD95 and PSD93 (Murata and Constantine-Paton, 2013; Sans, et al., 1996). New spines may first exist in a state that more closely resembles dendritic spines in the developing CNS, with high levels of SAP102 and low levels of PSD95, until other mechanisms and processes occur to stabilize the spines and recruit PSD95. Further studies are needed to determine the role that SAP102 plays at new spines.

Glutamatergic synaptic transmission

New spines are known to be rapidly functionally mature, with AMPAR and NMDAR mediated currents that are indistinguishable from those recorded from mature spines within a few hours after outgrowth (Zito, et al., 2009; Kwon and Sabatini, 2011). This is consistent with evidence that new spines form functional synapses within a few hours following their initial formation (Zito, et al., 2009). Still, new spines may not contribute to neural circuits to the same extent as their pre-existing counterparts. NMDAR mediated calcium influx at new spines is lower than that at mature or pre-existing spines (Zito, et al., 2009; Kwon and Sabatini, 2011). New spines also demonstrate greater diffusional coupling with the dendrite, meaning these reduced calcium transients may also diffuse from the spine head more rapidly and decrease the amount of calcium available for signaling in pathways that regulate synaptic strength, cytoskeletal changes, and stability (Hell, 2014; Bayer and Schulman, 2019). The potential for reduced calcium signaling at new spines may therefore be one factor that biases new spines towards

transience. Whether the maturation and stabilization of new spines is regulated by changes in calcium handling and compartmentalization is not yet known.

When is a new spine “stable”?

Rates of new spine formation increase during learning (Yang, et al., 2009; Xu, et al., 2009; Roberts, et al., 2010; Hayashi-Takagi, et al., 2015) and it is thought that this increase provides a structural basis for lasting memory retention. For new spines to serve as physical substrates of long-term memory storage, they would need to persist long enough to establish reliable circuits that can be reactivated during recall of experiences. Indeed, the stability of newly formed spines is key to retention of experience-based adaptations and behaviors. Across species and cortical regions, performance of a learned skill is directly correlated not only with the number of new spines that form during learning, but the fraction of those new spines that persist after the learning experience has ended (Yang, et al., 2009; Xu, et al., 2009; Roberts, et al., 2010). There is also evidence that the population of experience-induced stable new spines participates in functional neural circuits that are activated during recall or re-exposure. Monocular deprivation (MD) increases new spine outgrowth and stabilization of those new spines, which is believed to strengthen responses to the non-deprived eye (Hofer, et al., 2009). A subset of these new spines is retained after vision is restored, and, remarkably, a second MD of the same eye results in structural enlargement of the new spines gained during the first MD without the addition of new spines (Hofer, et al., 2009). This phenomenon, the re-activation of experience-acquired new spines following subsequent exposures and recall, has also been observed in the motor cortex of mice trained on novel motor skills (Hayashi-Takagi, et al., 2015).

Our current understanding of the role that new spines play in learning and memory posits that only a small fraction of new spines needs to survive to support lasting memory (Yang, et al., 2009; Xu, et al., 2009), yet the question of how that subset of spines is selected for stabilization remains. Recent work has identified synaptic stimulation as one mechanism for enhancing new spine survivorship. De Roo, et al, (2008a) found that new hippocampal spine

elimination was higher when NMDARs were blocked with APV; conversely, rhythmic theta burst stimulation enhanced the survivorship of new spines over a three-day period. It was also shown that blocking AMPA and NMDA receptor-mediated glutamatergic transmission decreased the fraction of new spines that accumulated fluorescently tagged PSD95 over the course of 24 hours (De Roo, et al., 2008b). This is consistent with the role PSD95 plays in regulating spine maturation and stability (Vazquez, et al., 2004; Ehrlich, et al., 2007; Taft and Turrigiano, 2014; Cane, et al., 2014) and provides additional evidence that synaptic activity likely induces molecular changes that stabilize new spines.

The most compelling evidence demonstrating that synaptic stimulation enhances new spine survivorship required the ability to simulate synaptic activity at a single new spine. Hill and Zito (2013) utilized two-photon (2p) glutamate uncaging to stimulate a single new dendritic spine within an hour after its formation. They found that, when stimulated by MNI-glutamate uncaging in a pattern that induces strong functional and structural potentiation at mature spines new spines survivorship was enhanced compared to unstimulated new spines on the same cells. Although LTP is known to initiate myriad signaling cascades and molecular processes, Hill and Zito (2013) identified the interaction between the GluN2B subunit of the NMDAR and CaMKII as one molecular mechanism downstream of strong glutamatergic stimulation that is required to enhance new spine survivorship. The work described in chapters 2-4 follows up on this finding and investigates the role of CaMKII in activity-dependent new spine stabilization.

Long-term potentiation (LTP)

The patterns of stimulation that enhance new spine stability are specifically those that induce lasting potentiation of glutamate-evoked currents and dendritic spine volume. These changes, the functional and structural potentiation at stimulated dendritic spines, are collectively known as long-term potentiation, or LTP. LTP was first described by Bliss and Lømo in 1973, when they recorded enhanced electrical responses from neurons in the dentate gyrus (DG) for several hours after applying bursts of high-frequency, tetanic electrical stimulation to axons in

the perforant pathway that synapsed onto the DG (Bliss and Lømo, 1973). These results were the first evidence supporting earlier ideas regarding the plastic potential of the mammalian brain, which can be summarized by Hebb's postulate: if a cells firing is temporally correlated with the firing of another cell, the connection between the two cells is strengthened (Hebb, 1949). Bliss and Lomo (1973) found that stimulating neurotransmitter release from perforant pathway afferents resulted in a population spike from the postsynaptic DG neurons, demonstrating that the firing of these axons correlated with the firing of DG neurons and therefore led to their potentiation by the mechanism proposed by Hebb.

Later work has shown that dendritic spines, which serve as the primary sites of excitatory neurotransmission in the mammalian brain, are the structural substrates of the phenomenon of LTP. Matsuzaki,,et al. (2004) used 2p imaging to monitor changes at individual dendritic spines on hippocampal CA1 neurons after the spines were stimulated with high frequency uncaging of MNI-caged glutamate to mimic the release of excitatory glutamate that occurs during experimental protocols like the tetanic stimulation of perforant pathway axons described in the previous paragraph. 2p glutamate uncaging at a single new spine resulted in spine-head enlargement that lasted for over an hour, known as structural LTP (sLTP) at the stimulated spine only, while spines neighboring the stimulated spine remained structurally unchanged (Matsuzaki, et al., 2004). This sLTP was accompanied by functional potentiation as well, as Matsuzaki and colleagues (2004) reported that stimulated spine sLTP also correlated with enhanced AMPAR-mediated currents evoked by brief pulses of glutamate uncaging after LTP was induced. Enhancement of AMPAR-mediated currents following LTP induction is regulated by the phosphorylation of AMPARs present at stimulated synapses (Barria, et al., 1997a; Kristensen, et al., 2011) and the recruitment of new AMPARs to sites of recent synaptic activity (Hayashi, et al., 2000; Makino and Malinow, 2009). This tightly-coupled functional and structural potentiation at new spines was shown to require CaMKII, calmodulin, the NMDAR, and actin polymerization, supporting previous reports implicating these molecules in structural

or functional LTP (Malinow, et al., 1989; Collinridge, et al., 1983; Fukazawa, et al., 2003). LTP is divided in to three stages, induction or initiation of LTP, early-phase LTP that lasts from 1-6 hours, and late-phase LTP that can last for days. and different molecular mechanisms are involved in each phase. For example, binding of CaMKII to the NMDAR is appears to be required for both LTP induction and early-phase maintenance (Barria and Malinow, 2005; Halt, et al., 2012), but CaMKII autophosphorylation, which will be discussed in-depth in the next section, is required only for induction, not maintenance (Buard, et al., 2010; Chang, et al., 2017). Late-phase LTP has been shown to require protein synthesis and the 26S proteasome (Frey, et al., 1988; Dong, et al., 2008).

LTP at dendritic spines appears to be a promising candidate for a mechanism that can support the dynamic and long-lasting restructuring of neural circuits to support learning and memory. This has resulted in decades of cutting-edge research dedicated to understanding and characterizing dendritic spine LTP and defining its role in learning and memory. I will refrain from discussing all the discoveries that have resulted from these studies in this chapter, but there are several reviews that cover the current body of literature (Segal, et al., 2017, Ma and Zuo, 2022), Many more discoveries are required to fully understand the extent of the molecular signaling required for LTP induction and maintenance at dendritic spines and how this signaling contributes to learning and memory. The remainder of this chapter will discuss CaMKII, one of the most important molecules regulating LTP and the most pertinent for contextualizing the findings described in chapters 2-4., Specifically, I focus on CaMKII's enzymatic and non-enzymatic roles in dendritic spines, and potential molecular interactors that may facilitate the role CaMKII plays in activity-dependent new spine stabilization.

CaMKII

Ca^{2+}/calmodulin (CaM) dependent protein kinase II, better known as CaMKII, is one of the most intriguing and important molecules involved learning and memory. The body of work

describing the structure, interactions, and activities of CaMKII spans nearly four decades and, resultantly, the ever-changing scientific understanding of this molecule and its myriad functions have been the subject of numerous review articles to synthesize our updated understanding. CaMKII has known roles in regulating glutamate receptor composition and function, cytoskeletal rearrangement, and stabilization, signaling molecule activation, and synaptic potentiation and depression. Several excellent reviews have summarized our current understanding of this molecule and its functions (Hell, 2014; Bayer and Schulman, 2019), so for the sake of this literature review, I will limit my discussion to functions of CaMKII that are pertinent to the discoveries I describe in my later chapters.

General overview

CaMKII is part of a larger family of Ca^{2+}/calmodulin (CaM) dependent protein kinases encoded by four separate genes (Hell, 2014). In the mammalian cortex and hippocampus, CaMKII is known to function as a dodecameric holoenzyme comprised of a 3:1 ratio of α and β subunits (Hell, 2014), although recent work using *in vitro* single molecule EM has determined that a small fraction of purified murine CaMKII forms 14-and-16mer holoenzymes (Buonarati, et al., 2021). It is unclear if these larger oligomers occur *in vivo* in humans or other mammalian model organisms, but this finding may have functional implications that could further our understanding of CaMKII's role in the brain. As CaMKIIα and β are the CaM kinase family members that are the most expressed in the mammalian brain (*for review, see* Hell, 2014; Bayer and Schulman, 2019), I will focus on these two members- primarily CaMKIIα- in this section.

Structurally, the CaMKIIα and β proteins are quite similar, with most of the variability occurring in the flexible linker domain (Cook, et al., 2018). The central hub/ association domain allows for subunits to interact with each other and form functional dodecamers. The regulatory segment contains the psuedosubstrate or regulatory domain that autoinhibits individual CaMKII subunits by binding to the kinase domain. Ca^{2+}/CaM binding to the regulatory domain induces a

conformational change that relieves the autoinhibition and frees up the kinase domain to allow for interaction with downstream substrates. The psuedosubstrate domain can phosphorylate neighboring CaMKII subunits following Ca^{2+}/CaM binding as well (**Fig. 1A-B)**.

CaMKII in learning and memory

Kinase activity, LTP, and learning and memory

Much work has been done to understand the role CaMKII plays in learning and memory. As described earlier in this chapter, LTP is the phenomenon by which learning or experience, simulated by strong synaptic stimulation *in vitro,* induces long-lasting synaptic strengthening in an input specific manner. The duration of these synaptic and structural changes and the requirement that these changes be maintained to support lifelong memory led to questions about the molecular mechanisms required to localize newly synthesized proteins to potentiated spines over time despite constant protein turnover (Lisman, 1985). Lisman (1985) predicted the existence of a “memory molecule” in the form of a kinase capable of alternating between active and inactive states by way of external stimulation and autophosphorylation, later identified CaMKII as a likely candidate for such a molecule (Lisman and Goldring, 1988). Other evidence has helped to establish the likelihood that CaMKII was the memory molecule that Lisman had described. For example, inhibition of CaMKII impairs LTP induction (Malinow, et al., 1989).

Much of CaMKII’s known role in learning and memory pertains to its enzymatic activities, which are primarily executed by CaMKIIα. Inhibition of CaMKIIα with kinase inhibitors such as KN62, AIP, and CN21 impairs LTP. Intriguingly, there is some debate as to whether the primary function of CaMKII kinase activity is to promote LTP induction or maintenance. Chen, et al., (2001) found that the potent kinase inhibitor AIP blocked hippocampal LTP when applied prior to LTP-inducing tetanic stimulation but did not reverse LTP when applied after stimulation. Later studies corroborated this phenomenon (Matsuzaki, et al., 2004; Chang, et al., 2017; Murakoshi, et al., 2017). Conversely, other groups have shown that incubation in a different class of kinase inhibitor, CN19 or CN21, can block LTP maintenance and even reverse it (Sanhueza, et al.,

2011; Barcomb, et al., 2016). Consistent with this, Rosetti and colleagues (2017) found that injection with virus to transiently express a dominant-negative kinase-dead form of CaMKII containing the K42M mutation interfered with injected rats' ability to remember and avoid an area in an open field where a foot shock was delivered, even when the animal had previously learned to avoid that region. They postulated that the variety of CaMKII inhibitor targets and concentrations differentially affected different stages of LTP, either disrupting the earlier stages requiring substrate phosphorylation or the later stages where CaMKII binding to the NMDAR is necessary (Rossetti, et al., 2017). Indeed, it was found that low concentrations of the membrane-permeable tatCN21 was sufficient to block substate phosphorylation by CaMKII, but only at higher concentrations did it disrupt the interaction between CaMKII and the NMDAR (Sanhueza, et al., 2011) and reverse synaptic potentiation.

CaMKII's enzymatic activities regulate a number of molecular signaling cascades downstream of glutamatergic stimulation that contribute to LTP and long-lasting changes in synaptic strength. Ca^{2+}/CaM activated CaMKII phosphorylates the GluA1 subunit of AMPA-type glutamate receptors at the S831 residue (McGlade-Mcculloh, et al., 1993; Barria, et al., 1997a; Mammen, et al., 1997; Barria, et al., 1997b), which increases AMPAR channel conductance, thereby strengthening the postsynaptic response to subsequent glutamatergic stimulation (Barria, et al., 1997a; Kristensen, et al., 2011). CaMKII enzymatic activity also regulates AMPAR surface expression. Overexpressing a truncated form of CaMKII lacking a regulatory domain and thus rendering it constitutively enzymatically active (tCaMKII) increases AMPAR mediated synaptic transmission in hippocampal slices without the need for synaptic stimulation (Hayashi, et al., 2000; Pi, et al., 2010a), indicating that CaMKII kinase activity is sufficient to drive GluA1 containing AMPARs to synapses (Appleby, et al., 2011). CaMKII phosphorylation of the auxiliary protein stargazin traps GluA1 containing AMPARs at stimulated spines via interactions with PSD95 and stargazin (Opazo, et al., 2010) and may be one mechanism by which CaMKII kinase activity increases the surface expression of AMPARs.

The ability of adjacent subunits in the CaMKII holoenzyme to phosphorylate their neighbors, known as autophosphorylation, also plays an important role in LTP and synaptic memory. Calmodulin binding to neighboring subunits is required both to render the subunits enzymatically active and to induce the confirmational change that exposes the regulatory domain containing the T286 residue for autophosphorylation (Hanson, et al., 1994). Inter-subunit autophosphorylation at T286 enhances calmodulin trapping (Meyer, et al., 1992), which normally rapidly dissociates from CaMKII, and maintains CaMKII activity at 15-80% of its calmodulin-bound levels (Coultrap, et al., 2010). This autophosphorylation allows CaMKII to remain enzymatically active even in the absence of additional Ca^{2+} binding, known as autonomous activity. Autonomous CaMKII may be biased towards different substrates than fully active, calmodulin-bound CaMKII (Coultrap, et al., 2010), which is consistent with reports that overexpression of either autonomous and constitutively active CaMKII differentially affect synaptic strength and spine morphology (Pi, et al., 2010a; Pi, et al., 2010b). Thus, autophosphorylation greatly enhances the computational power of CaMKII, allowing for greater dynamic range of kinase activity in response to repeated spikes of Ca^{2+} and even after stimulation (*for review, see* Bayer and Schulman, 2019).

CaMKII kinase activity also initiates several molecular signaling cascades that are likely intrinsically connected to its role in both structural and functional potentiation at dendritic spines. CaMKII phosphorylation regulates the activity of several key signaling molecules following synaptic stimulation, including RhoA, Cdc42, Rac1, Rap1, and Ras (Murakoshi, et al., 2011; Saneyoshi, et al., 2019; Koeberle, et al., 2017; Walkup, et al., 2015, Harvey, et al., 2008). These signaling molecules regulate the dynamic organization of the actin cytoskeleton to accommodate the structural and functional changes required to support long-term potentiation and serve as another mechanism through which the enzymatic activity of CaMKII is required for LTP. In Chapters 2 and 3, I discuss work I have done to determine the role of CaMKIIα kinase

activity and its many downstream interactions in activity-dependent new spine stabilization and volume changes.

CaMKII phosphomutants and LTP

As suggested in the previous section, the enzymatic activity of CaMKII greatly contributes to its role in LTP, learning, and memory. Specifically, the ability for graded inter-subunit autophosphorylation that allows the CaMKII holoenzyme to maintain various states of activity even after the initial calcium influx may serve as a means of encoding stimulus intensity and facilitating later stages of LTP. Several studies investigating the role of CaMKII autophosphorylation at the T286 residue in the regulatory domain and the T305/306 residues in the calmodulin binding region of the CaMKIIα subunit (T287 and T306/307 on CaMKIIβ) have helped to solidify our understanding of the role CaMKII in LTP using phosphomutants.

Pi, et al., (2010b) found that overexpressing a full-length version of CaMKII containing the T286D mutation to mimic autophosphorylation decreased basal synaptic strength. Conversely, overexpression of the truncated, constitutively enzymatically active tCaMKII enhanced basal synaptic EPSCs. The role of autophosphorylation in LTP is well established; what then explains this paradoxical synaptic depression when pseudo-autophosphorylated CaMKII is overexpressed? Lou and Schulman (1989) found that, *in vitro*, autophosphorylated CaMKII sequentially phosphorylates at least three other serine and threonine residues on neighboring subunits, two of which were later identified as the T305/306 residues in the calmodulin binding footprint (Colbran and Soderling, 1990). Phosphorylation of these residues greatly reduces the affinity of CaMKII for calcium-bound calmodulin, thus preventing further calcium-induced potentiation (Colbran and Soderling, 1990; Elgersma, et al., 2002). Intriguingly, Pi and colleagues (2010b) found that pairing the T286D mutant with T305/306A mutations to prevent autophosphorylation of the calmodulin binding region resulted in a similar functional enhancement as seen when tCaMKII is overexpressed. It has been hypothesized that

autophosphorylation of the T305/306 residues contributes to cellular metaplasticity (Pi, et al., 2010b).

The discrete effects of T286D overexpression and tCaMKII overexpression points to another hypothesis discussed in the previous section: autonomous and Ca^{2+}/CaM bound CaMKII may be biased towards distinct molecular substrates that respectively contribute to different stages of LTP (Coultrap, et al., 2010). This is a likely possibility, as Jalan-Sakrikar and colleagues (2012) found that α-actinin binding to CaMKII can simulate Ca^{2+}/CaM binding and induce CaMKII activity only towards specific substrates. Consistent with this, Pi and colleagues (2010a) also reported that the same T286D mutant that depressed synapses enhanced basal dendritic spine size and density. tCaMKII had no effect on either spine size or density. When T286D/T305A/T306A was overexpressed, Pi, et al. (2010a) reported enhanced spine size, density, and synaptic strength. This suggests that autonomous CaMKII might be biased towards activating different substrates that may induce structural plasticity, but Ca^{2+}/CaM binding may be required to activate substrates involved in functional potentiation. Later studies have corroborated the need for subsequent calmodulin binding to enhance synaptic strength (Barcomb, et al., 2014). The effects of these phosphomutants have been shown to occlude further potentiation by typical LTP-inducing stimulation (Elgersma, et al., 2002; Pi, et al., 2010b), indicating that the pathways required for structural and functional potentiation are saturated by overexpression of tCaMKII or T286D containing CaMKII and that these various enzymatically active states of CaMKII are crucial for LTP. I discuss my use of the T286D phosphomutant and the interplay between autonomous CaMKIIα and kinase activity in activity-dependent changes to new spine volume and stability in Chapters 2 and 3.

It could be hypothesized that, due to the critical role autophosphorylated CaMKIIα plays in structural and functional plasticity, mutations to the T286 residue that block autophosphorylation may have an opposite effect on synaptic size and strength, similar to the reversal of LTP that can occur with some kinase inhibitors. Incontro and colleagues (2018)

found that overexpression of T286A, a CaMKII mutant where the T286 residue cannot be phosphorylated, rescues hippocampal NMDAR signaling but not AMPAR signaling in CaMKIIα/β double knock-out tissue. Overexpression of T286A also decreases basal hippocampal EPSCs without affecting spine volume or spine density (Pi, et la., 2010a). T286A also fails to rescue both functional and structural LTP (Incontro, et al., 2018). Interestingly, it was found that T286 phosphorylation does not regulate basal CaMKII localization to synapses (Barcomb, et al., 2014) nor GluN2B binding to CaMKIIα (O'Leary, et al., 2011). Thus, the use of mutant CaMKIIα containing a phosphodeficient T286A residue has revealed that CaMKIIα autophosphorylation at the T286 residue plays a significant role in LTP as well as basal AMPAR transmission at synapses, which is consistent with the role CaMKII is known to play regulating the synaptic accumulation and trapping of AMPARs.

Non-enzymatic activities of CaMKII and LTP

CaMKII performs several non-enzymatic functions that are equally crucial for structural and functional synaptic potentiation. Many of these functions are mediated by the β subunit. CaMKIIβ directly binds the actin cytoskeleton and contributes to actin bundling and cross-linking (Hell, 2014) and it is thought to contribute to localizing CaMKIIα/β heteromers to dendritic spines (Shen, et al., 1999). Though less well-studied than CaMKIIα, CaMKIIβ is indeed necessary for LTP and in addition to the role that its ability to bind actin plays (Kim, et al., 2015), it may also act as an inverse synaptic tag that marks inactive synapses near active ones for depotentiation or elimination (Okuno, et al., 2018; El-Boustani, et al., 2018). CaMKIIα kinase activity is required for inter-subunit phosphorylation of CaMKIIβ to trigger dissociation of the CaMKII holoenzyme from the actin-cytoskeleton, which may permit signaling molecule dependent rearrangement of actin to accommodate structural and functional potentiation (Hell, 2014).

CaMKIIα cannot directly bind the actin cytoskeleton but does so via interactions with the actin binding proteins α-actinin and densin-180. α-actinin help targets CaMKII to spines (Jalan-Sakrikar, et al., 2012), but binds the regulatory domain and is thus rapidly outcompeted for

binding by Ca^{2+}/CaM. This dissociation of the CaMKIIα-α-actinin-actin complex allows for both rearrangement of actin fibers and redistribution of CaMKII in response to synaptic activity (Hell, 2014). The role of CaMKIIα binding to α-actinin is crucial for AMPAR accumulation and basal synaptic maintenance (Hodges, et al., 2014). Densin-180 complexes with CaMKII and α-actinin and may also contribute to targeting CaMKII to spines via actin binding. Unlike α-actinin, Ca^{2+}/CaM binding and even phosphorylation by CaMKII does little to disrupt the interaction between CaMKII and densin-180, however autophosphorylation of CaMKIIα greatly enhances the affinity of densin-180 for CaMKIIα (Walikonis, et al., 2001). Carlisle and colleagues (2011) found that densin knock-out did not significantly affect CaMKII accumulation in hippocampal spines, however a double knock-out of GluN1 and densin decreased CaMKII tone at hippocampal synapses while a GluN1 knock-out did not. This finding suggests that densin may play a redundant or compensatory role in targeting CaMKII to synapses, but that the NMDAR plays the primary role in synaptic targeting of CaMKII (which will be discussed in the following section).

CaMKIIα may play other structural or non-enzymatic roles that are less well-defined. The work described in Chapter 2 suggests that CaMKIIα kinase activity is not required to enhance new spine stability following strong glutamatergic stimulation and the data I present suggests that other molecules besides α-actinin and densin-180 may be at play (which will be discussed in more detail in Chapter 4). The interaction between CaMKIIα and the NMDAR were briefly touched on earlier in this section, and I will go into more detail in the subsequent section. Additionally, the interactions with the 26S proteasome and the lesser-known multi-PDZ domain protein MUPP1, amongst other interactions with PSD-family MAGUKs, are promising candidates for understanding both LTP, basal synaptic maintenance, and activity-dependent new spine stabilization. The remainder of this chapter will focus on these other non-enzymatic interactions and their roles in LTP and putative roles in activity-dependent new spine stabilization.

The CaMKII-GluN2B interaction and LTP- implications for new spine stabilization

The interaction between CaMKIIα and the GluN2B subunit of the NMDAR has been well studied, largely in the context of LTP, however recent work has highlighted a role for this interaction in synaptogenesis, basal synaptic transmission, and regulating dendritic spine morphology (Gambrill and Barria, 2011; Incontro, et al., 2014). The NMDAR is an obligate heterotetrametric receptor, with receptors comprised of GluN1 and either GluN2A or GluN2B dominating NMDAR expression in the hippocampus (*for review, see* Mori and Mishina, 1995). The interaction between CaMKIIα and GluN2B likely regulates the synaptic localization of CaMKII (Leonard, et al., 1999) and places CaMKII at a prime location to rapidly respond to NMDAR mediated Ca^{2+} influx and regulate AMPAR recruitment and phosphorylation. Synaptic stimulation enhances CaMKIIα-GluN2B binding and contributes to an increase in the synaptic accumulation of CaMKII (She, et al., 2012; Halt, et al., 2012). This interaction is known to protect CaMKII from dephosphorylation at the T286 residue (Cheriyan, et al., 2012) and contribute to autonomous CaMKII activity independent of autophosphorylation of the T286 residue (Bayer, et al., 2001; Bayer, et al., 2006). As a result, activity-dependent and basal GluN2B- CaMKIIα binding is critical for AMPAR mediated synaptic transmission and LTP (Incontro, et al., 2018; Barcomb, et al., 2016). The GluN2B- CaMKIIα interaction is also necessary for activity-dependent spine enlargement (El-Gaamouch, et al., 2012). The exact molecular mechanisms through which the GluN2B-CaMKIIα interaction regulates basal synaptic transmission and LTP require further elucidation, but likely rely on concerted interactions with signaling molecules and scaffolding molecules such as PSD-family MAGUKs and α-actinin.

Due to the pivotal role that the GluN2B interaction plays in LTP, it was hypothesized that this interaction may be one mechanism by which LTP-inducing glutamatergic stimulation enhances new spine survivorship. Indeed, Hill and Zito (2013) found that activity-dependent new spine stabilization was impaired in tissue from GluN2B L1298A/ R1300Q KI mice in which LTP was shown to be reduced by ~50% (Halt, et al., 2012). The requirement for GluN2B-

CaMKIIα binding to enhance new spine stability suggests a role for CaMKIIα downstream of this interaction, although it does not exclude the requirement for CaMKII-independent NMDAR activity, such as non-ionotropic NMDAR signaling (Stein, et al., 2021; Park, et al., 2022). My work sought to further understand the role that CaMKIIα plays downstream of glutamatergic stimulation and GluN2B binding to enhance the survivorship of nascent dendritic spines. In Chapters 2 and 3 I investigate whether the enzymatic activity facilitated by NMDAR signaling and binding is required for new spine survivorship and enlargement and find that CaMKII kinase activity is not required to enhance spine stability or new spine volume. In Chapter 4, I investigate the role of proteins known to interact with CaMKII in a manner independent of its kinase activities as potential regulators of activity-dependent new spine stabilization. The remainder of this chapter will discuss the putative scaffolding partners and the role they may play in enhancing new spine survivorship.

Non-enzymatic scaffolding to the proteasome and synGAP

The 26S proteasome

The 26S proteasome is required for the degradation of ubiquitinated proteins which has been shown to be necessary for spinogenesis, AMPAR clustering at synapses, and later stages of LTP (Hamilton, et al., 2012; Dong, et al., 2008; Cai, et al., 2010; Ferreira, et al., 2015). Earlier work from Hamilton and colleagues identified proteasomal degradation of ephexin 5 as one of the molecular mechanisms required for activity dependent spinogenesis (Hamilton, et al., 2017) raising the possibility that the proteasome is recruited to sites of new spine formation where it may remain to play a role in activity dependent new spine stabilization as well.

The 26S proteasome is a protein complex consisting of a 20S proteolytic core and two 19S subunits that recognize ubiquitinated proteins and begin the process of protein unfolding for further deconstruction within the 20S core (Voges, et al., 1999) While protein degradation is known to be crucial for maintaining normal cellular function, it was unclear if proteolytic activity was also a critical part of normal synaptic transmission and synaptic plasticity. Several studies

have identified a role for the proteasome in the maintenance of LTP, particularly in establishing late-LTP. Dong, et al. (2008) found that inhibition of the 26S proteasome in hippocampal slices enhanced early phase LTP occurring within one hour of LTP induction, however maintenance of LTP at later time points (>2hr) was disrupted. Intriguingly, this impairment of late-phase LTP coincided with the well-established time frame at which transcription is required to maintain LTP (Frey, et al., 1988; Bosch, et al., 2014) and Dong and colleagues (2008) found that proteasome activity was required for sustained degradation of a CREB repressor that is likely hinders activity-dependent transcription of new proteins required for LTP maintenance. Other groups have indeed reported that inhibition of the 26S proteasome during LTP induction impairs late-LTP (Cai, et al., 2010). Surprisingly, it was found that CaMKII-dependent phosphorylation of the proteasome, which enhances its proteolytic activity (Djakovic, et al., 2009; Jarome, et al., 2013), is not necessary for LTP induction or maintenance (Scudder, et al., 2021), suggesting that the activity-dependent effects of the proteasome and its contributions to LTP may occur independently of enzymatic CaMKII-dependent signaling cascades. Indeed, Bingol and colleagues (2010) reported that while CaMKII is required to recruit the proteasome to activated synapses following LTP induction, its kinase functions are not necessary for this recruitment. Following a knock-down of endogenous CaMKII, molecular replacement with enzymatically inert CaMKII-K42R was sufficient to rescue activity-dependent recruitment of the 26S proteasome to stimulated spines (Bingol, et al., 2010). Constitutively active CaMKII containing the T286D mutation enhanced basal CaMKII-proteasome interactions without the need for synaptic stimulation even when paired with the K42R mutation (Bingol, et al., 2010) suggesting that the CaMKII facilitates this interaction in a non-enzymatic manner.

What functions does the 26S proteasome perform at stimulated spines once recruited by CaMKIIα? It is likely that the proteasome plays a role in the degradation of proteins that negatively regulate plasticity, some of which may not be required during the earlier stages of LTP but are necessary for late-phase LTP, such as the CREB repressor ATF4 (Dong, et al.,

2008). Alternatively, the proteasome may serve as a brake on synaptic plasticity. The proteasome has been found to regulate the activity-dependent degradation of various synaptic proteins during plasticity (Ehlers, 2003), and some of these proteins, such as shank3, are implicated in autism spectrum disorder (ASD), synaptic maintenance, and LTP (Bozdagi, et al., 2010; Yang, et al., 2012). Intriguingly, synaptic stimulation has been shown to increase the activity of the deubiquitinase UPS8 towards shank3, enhancing the synaptic expression of shank3 and likely contributing to changes in spine density, volume, and synaptic strength (Campbell and Sheng, 2018). The proteasome is also involved in activity-dependent degradation of SAP102 (Ehlers, 2003), which is highly expressed in the developing CNS to regulate synapse formation (Murata and Constantine-Paton, 2013) and one of the first PSD-family MAGUKs to arrive at new spines (Lambert, et al., 2017). The proteasome may be involved in constitutive degradation of LTP molecules such shank3 and SAP102 to ensure that potentiation only occurs following strong synaptic stimulation, when other molecular mechanisms may be recruited to shield these plasticity molecules from degradation. Further work is needed to fully understand the mechanisms by which the proteasome regulates the expression levels of synaptic proteins involved in new spine stabilization and how that regulation may be facilitated by its non-enzymatic interactions with CaMKIIα. In my own work, I set out to determine if the proteasome plays a role in activity-dependent new spine stabilization, which I discuss in Chapter 4.

SynGAP -1α

SynGAP-1α is a Ras and RapGAP that is crucial for neural development, synaptic maturation, and synaptic plasticity, making it a promising candidate for regulating activity-dependent new spine stabilization via non-enzymatic interactions with CaMKIIα. In humans, mutations of synGAP-1α or haploinsufficiency cause ASD, intellectual disability, and epilepsy (*for review, see* Jeyabalan and Clement, 2016) and in mice synGAP-1α knock-out is embryonic lethal (Vazquez, et al., 2004). Vazquez, et al. (2004) found precocious accumulation of PSD95

in spines, synapse formation, and functional maturation in dissociated synGAP-1α KO neurons, indicating that synGAP-1α negatively regulates synaptic plasticity and maturation. Other groups using mouse models of synGAP-1α haploinsufficiency have found that spine dynamics and morphological changes during early development in heterozygous animals more closely mirror later stages of development in WT animals (Clement, et al., 2012; Aceti, et al., 2015). Thus, it appears that synGAP-1α negatively regulates synaptic maturation in the developing CNS. It is not yet known how regulation of synGAP-1α expression levels and localization changes during development to permit the structural, functional, and molecular maturation of synapses. SynGAP-1α also negatively regulates synaptic plasticity in the mature CNS. SynGAP -1α accumulates at dendritic spines and synaptic stimulation triggers a rapid and persistent evacuation of synGAP-1α from stimulated spines (Araki, et al., 2015). This dispersion is facilitated by CaMKIIα phosphorylation of synGAP-1α, which causes it to dissociate from PSD95. Blocking synGAP-1α evacuation from spines by preventing phosphorylation by CaMKIIα blocks functional and structural LTP and turns stable spines into unstable spines (Araki, et al., 2015).

CaMKIIα phosphorylation of synGAP-1α to disrupt its interaction with PSD95 may not be the only mechanism regulating activity-dependent synGAP-1α dispersion as there is evidence suggesting that interactions between synGAP-1α and the multi-PDZ domain protein MUPP1 can contribute to synGAP-1α evacuation in a manner independent of CaMKII kinase activity. Krapivinsky and colleagues (2004) found that CaMKII and synGAP-1α form a complex with MUPP1` facilitated by synGAP-1α binding to the PDZ13 domain and CaMKII binding to PDZ2 domain of MUPP1. Binding to the Ca^{2+}/CaM footprint of CaMKIIα, either by Ca^{2+} or the competitive inhibitor KN93, was sufficient to dissociate the MUPP1-synGAP-1α complex from CaMKII (Krapivinsky, et al., 2004). Thus, even when CaMKIIα kinase activity is inhibited, competitive binding to the calmodulin binding footprint may result in synGAP dispersion. Importantly, disruption of the CaMKII-MUPP1- synGAP-1α complex increased basal synaptic

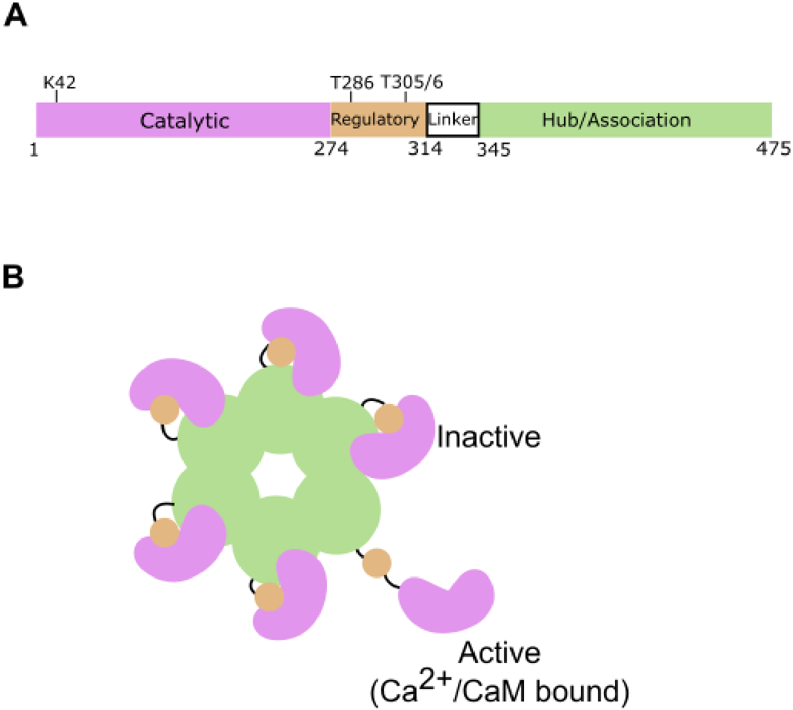
strength (Krapivinsky, et al., 2004), although in a manner that does not occlude further LTP (Rama, et al., 2008) but does enhance hippocampal LTP. This suggests that the CaMKII-MUPP1- synGAP-1α complex may regulate synaptic strength through a pathway that is redundant or parallel to typical NMDAR-mediated LTP. The CaMKII-MUPP1- synGAP-1α complex may also play a larger role in regulating activity-dependent changes in younger CNS or in new spines, where the levels of PSD95 are lower and disruption of the CaMKII-MUPP1-synGAP-1α complex may be the primary mechanism controlling synGAP-1α dispersion. In Chapter 4, investigate whether syngap-1α expression and dispersion predict nascent dendritic spine stability and whether syngap-1α dispersion at new spines requires CaMKIIα kinase activity.

FIGURES AND FIGURE LEGENDS

Figure 1: Overview of CaMKII structure

(A) Linearized representation of a single CaMKIIα subunit domains and key residues mutated for the experiments described in later Chapters of this dissertation. ***(B)*** Cartoon representation of the CaMKII holoenzyme showing inactive states of CaMKII, where the regulatory domain is bound to the catalytic/ kinase domain and the active, Ca^{2+}/CaM-bound state.

Figure 1



CHAPTER 2: ACTIVITY-DEPENDENT STABILIZATION OF NASCENT DENDRITIC SPINES REQUIRES NON-ENZYMATIC CaMKIIα FUNCTION

PREFACE

The following chapter was submitted as a manuscript under the title "Activity-dependent stabilization of nascent dendritic spines requires non-enzymatic CaMKIIα function" to The Journal of Neuroscience on July 18th, 2022, and the additional experiments requested by reviewers are currently in progress, after which the paper will be resubmitted. In addition to the content that was originally submitted to The Journal of Neuroscience, this chapter includes supplemental analyses that address some of the reviewer questions/ comments. In this chapter, I first show that genetic and pharmacological inhibition of CaMKIIα kinase activity does not disrupt the well-established phenomenon of enhancing dendritic spine stability following high-frequency glutamate uncaging. I then show that knocking down CaMKIIα does impair activity-dependent new spine stabilization, showing that structural interactions facilitated by CaMKIIα, but not enzymatic activities, are necessary to enhance new spine survivorship. Finally, I present evidence that genetic manipulations that render CaMKIIα constitutively autonomously active by mimicking activity-dependent autophosphorylation enhance basal spine survivorship without the need for further glutamatergic stimulation. This basal enhancement occurs even when further kinase activity is genetically inhibited, demonstrating that non-enzymatic CaMKIIα is both necessary and sufficient to enhance new spine survivorship. The authors on the submitted manuscript are Nicole Claiborne and Dr. Karen Zito, and the resubmitted version will include Dr. Margarita Anisimova as well as any other members of the Zito Lab who contribute to the manuscript. I acknowledge the intellectual contributions of my dissertation advisor, Dr. Karen Zito and my dissertation committee: Drs. Elva Diaz, John Gray, Kim McAllister, Johannes Hell, and Michael Ferns. I would also like to acknowledge the input of my peers in the lab, Sam Petshow, Juan Flores, and Dr. Margarita Anisimova. I thank Dr. Paul DeKoninck for his generous donation of all the GFP-tagged CaMKIIα constructs as well as the CaMKIIα shRNA.

ABSTRACT

The outgrowth and stabilization of nascent dendritic spines are crucial processes underlying learning and memory. Most new spines retract shortly after growth; only a small subset is stabilized and integrated into the new circuit connections that support learning. New spine stabilization has been shown to rely upon activity-dependent molecular mechanisms that also contribute to long-term potentiation (LTP) of synaptic strength. Indeed, disruption of the activity-dependent targeting of the kinase CaMKIIα to the GluN2B subunit of the NMDA-type glutamate receptor disrupts both LTP and activity-dependent stabilization of new spines. Yet it is not known which of CaMKIIα's many enzymatic and structural functions are important for new spine stabilization. Here, we used two-photon imaging and photolysis of caged glutamate to monitor the activity-dependent stabilization of new dendritic spines on hippocampal CA1 neurons from mice of both sexes in conditions where CaMKIIα functional and structural interactions were altered. Surprisingly, we found that inhibiting CaMKIIα kinase activity either genetically or pharmacologically did not impair activity-dependent new spine stabilization. In contrast, shRNA knock-down of CaMKIIα abolished activity-dependent new spine stabilization, which was rescued by co-expressing shRNA-resistant CaMKIIα. Notably, overexpression of phospho-mimetic CaMKIIα-T286D, which exhibits activity-independent targeting to GluN2B, enhanced basal new spine survivorship in the absence of additional glutamatergic stimulation, even when kinase activity was disrupted. Together, our results suggest that nascent dendritic spine stabilization requires structural and scaffolding interactions mediated by CaMKIIα that are independent of its enzymatic activities.

INTRODUCTION

The dynamic modification of neuronal circuitry underlies learning and memory and is crucial for adaptation and survival. Dendritic spines are the sites of most excitatory synaptic connections in the mammalian cerebral cortex, and the morphological and functional changes that occur at dendritic spines contribute to the neural circuit modifications that support behavior (Kasai, et al., 2021). Notably, the stabilization of newly formed spines in the cortex is tightly linked to lasting memory of learned experiences (Hayashi-Takagi, et al., 2015; (Roberts, et al., 2010; Xu, et al., 2009; Yang, et al., 2009). Interestingly, most new spines are transient (Berry and Nedivi, 2017; Holtmaat, et al., 2005)) suggesting that stabilization is precisely regulated to favor only a subset of new spines sufficient to support memory. Thus, defining the mechanisms that determine which new spines are stabilized will strengthen our understanding of learning and memory.

Previous studies have shown that synaptic activity enhances the stability of new dendritic spines in the hippocampus, and that the enhancement of new dendritic spine stability appears to be specific to patterns of synaptic activity that result in the coordinated long-term enhancement of synaptic strength and spine volume (Matsuzaki, et al., 2004) known as long-term potentiation (LTP) (De Roo, et al., 2008b; Hill and Zito, 2013). NMDA-type glutamate receptor (NMDAR) activation is required for LTP-induced nascent spine stabilization, and disruption of the interaction between the Ca^{2+}/calmodulin-activated kinase CaMKIIα and the GluN2B subunit of the NMDAR prevents activity-dependent new spine stabilization (Hill and Zito, 2013) . Notably, CaMKIIα-GluN2B binding facilitates a number of CaMKIIα enzymatic and structural functions that promote LTP induction and maintenance, including binding to densin-180 and α-actinin, activation of signaling molecules, and phosphorylation of AMPA-type glutamate receptors (Bayer and Schulman, 2019; Sanhueza and Lisman, 2013). Whether these enzymatic and structural activities of CaMKIIα and the downstream cascades they initiate are required for activity-dependent new spine stabilization is not yet known.

Here, we used time-lapse imaging and two-photon glutamate uncaging along with genetic and pharmacological manipulations to elucidate the role of CaMKIIα in activity-dependent new spine stabilization. We first demonstrated that CaMKIIα is present and enriched at mature levels in new spines shortly after outgrowth on CA1 neurons in hippocampal slice cultures, supporting that CaMKIIα could play an important role in nascent spine stabilization. Surprisingly, we found that high-frequency glutamate uncaging (HFU) enhanced new spine survivorship even when CaMKIIα kinase activity was genetically and pharmacologically inhibited. In contrast, shRNA-mediated knock-down of CaMKIIα blocked activity-dependent new spine stabilization, indicating that CaMKIIα is indeed required for new spine stabilization. Finally, we show that autonomous CaMKIIα-T286D enhanced new spine stability without further glutamatergic stimulation or kinase activity. Together, our results support a model whereby strong glutamatergic transmission at a subset of new spines facilitates new spine stabilization through structural and scaffolding functions of CaMKIIα.

METHODS

Preparation and transfection of organotypic slice cultures

Organotypic hippocampal slice cultures were prepared from postnatal day (P) 6-8 C57BL/6J wild-type mice of both sexes, as described (Opitz-Araya and Barria, 2010; Stoppini, et al., 1991). Neurons were transfected 2-3 days prior to imaging using particle-mediated gene transfer, as described (Woods and Zito, 2008), except 6-8 µg of DsRed-Express (Clontech) and 6 µg of mEGFP-tagged constructs or 5-10 µg of mEGFP were coated onto 6-7 mg of 1.6 µm gold beads. mEGFP-tagged constructs included: GFP-CaMKIIα, GFP-CaMKIIα-T286D, GFP-CaMKIIα-K42R/T286D (Pi, et al., 2010a), or GFP-CaMKIIα-K42R (Tullis, et al., 2020). CaMKIIα knockdown used 25 µg CaMKIIα-shRNA and rescue also contained 6 µg shRNA-resistant mEGFP-CaMKIIα* (Lemieux, et al., 2012).

Two-photon imaging

Image stacks (512 X 512 pixels, 1 μm z-steps) of 4-6 secondary and tertiary, apical and basal dendritic segments from CA1 pyramidal neurons (6-10 DIV) were acquired on a custom two-photon microscope with a pulsed Ti::Sapphire laser (930 nm, 0.5-3 mW at the sample; Spectra Physics, Newport). Data acquisition was controlled by ScanImage (Pologruto, et al., 2003) written in MATLAB (MathWorks). All images shown are maximum projections of 3D image stacks after applying a median filter (3 X 3). The first time point was acquired in slice culture medium at room temperature (RT) and the slice was maintained in the incubator (35°C) for 1 h between first and subsequent acquisitions. After 1 h, the slice was placed in a bath of recirculating, oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 127 NaCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 2.5 KCl, 25 D-glucose, ~310 mOsm, pH 7.2) with 2 mM Ca^{2+}, 0-0.1 mM Mg^{2+}, and 1 μM tetrodotoxin at 31°C. 2.5-3.5 mM of 4-methoxy-7-nitroindolinyl-caged L-glutamate (MNI-glutamate) was added for uncaging experiments. Staurosporine (1 μM) or an equivalent volume vehicle were added to the bath after new spine identification and 30 min prior to uncaging.

Identification of new spines and estimation of spine size

We defined new spines as any protrusion emanating from the dendrite that was present in the second and/or third images in the time-lapse series (60-90 min later) but not detectable in either the red or green channels in the first image. Persistent neighbor spines were defined as spines that were present in all images in the time-lapse series. Spines of ambiguous persistence or presence due to fluctuations in dendrite swelling, spine motility, or spine drift in the z-axis were excluded. Spine size was estimated from bleed-through-corrected and background-subtracted red (DsRed-Express) fluorescence intensity. Spine brightness measurements give an accurate estimate of relative spine size when compared with electron microscopy (Holtmaat, et al., 2005).

High frequency uncaging (HFU) stimulus

The HFU stimulus consisted of 60 pulses (720 nm, 8-10 mW at the sample) of 2 ms duration delivered at 2 Hz in the presence of 2.5-3.5 mM MNI-glutamate by parking the beam at a point ~0.5 μm from the spine head away from the dendrite.

Quantification of relative enrichment of GFP-tagged proteins

Relative enrichment of GFP-tagged proteins in dendritic spines was calculated using bleed-through-corrected and background-subtracted green (GFP) and red (DsRed-Express) fluorescence intensities from spines and dendrites, as described (Woods, et al., 2011). Briefly, the ratio of green fluorescence intensity to red fluorescence intensity (G/R) was calculated for each new spine, size-matched neighboring persistent spines (6-10), and three representative regions on the dendritic shaft (excluding regions dendrite swelling and GFP-puncta, which were indicative of the presence of a z spine). To quantify spine fluorescence intensities, boxes were drawn around whole spines and spine necks using custom software written in MATLAB. Background subtraction was done by drawing a box next to a target spine that was equal on the axis perpendicular to the dendrite as the box drawn around the spine head and neck. The average intensity of that box was multiplied by the number of pixels in the target spine box and subtracted from the integrated intensity from the target spine box. Relative enrichment of spines was calculated by normalizing the G/R ratio of the target spine to the mean G/R ratio of three locations on the adjacent dendrite.

Several criteria were used to ensure that analyzed data were of high quality. Cells that exhibited lower green fluorescence intensity than the background ROI were excluded. Cells with extremely high levels of GFP-tagged protein expression such that synaptic enrichment was lost were excluded. Cells were also excluded if, after background and bleed-through subtraction: (1) the value of the mean green pixel intensity (G) from neighbor spines was less than 3.23 a.u., (2) the value of the mean neighbor spine G/R was less than 0.01, or (3) the ratio of the square of the mean persistent spine G/R to the absolute value of the mean dendrite G/R was less than 0.05. These criteria allowed unbiased exclusion of cells that returned negative pixel intensity

values after background and bleed-through subtraction. Cells that exhibited significant photobleaching (a decline in average integrated fluorescence intensity in the dendrite greater than 20% compared to the first time point) in either the red or green channels were excluded.

Statistical Analysis

Survivorship curves were compared using the log-rank task. To compare survivorship at individual time points, we used Fisher's exact test. For comparisons of spine volumes at a given time point to baseline, 2-way ANOVAs with appropriate *post-hoc* tests for multiple comparisons were used. Between-group comparisons of spine baseline volume were performed using a two-tailed unpaired heteroscedastic Student's *t* test unless otherwise noted. Error bars represent standard error of the mean (SEM).

RESULTS

GFP-CaMKIIα enrichment in new spines is comparable to that in size-matched neighboring spines

To understand the role of CaMKIIα in activity-induced new spine stabilization, we first needed to determine whether CaMKIIα is expressed in new spines and in what time frame. This experiment was an important first step, as we and others have reported that several members of the PSD-MAGUK family of postsynaptic scaffolding molecules are expressed at very low levels in new spines and can take up to 24 h to accumulate to mature levels (De Roo, et al., 2008b; Lambert, et al., 2017), indicating that the molecular composition of new spines and their persistent neighbors is distinct, particularly in the earliest stages after new spine outgrowth. We used time-lapse, 2-photon imaging to observe spontaneous new spine outgrowth on the dendrites of hippocampal CA1 neurons in slice culture biolistically-transfected with mEGFP-tagged CaMKIIα (GFP-CaMKIIα) and a DsRed-Express cell fill (**Fig. 2A**). We found no difference in the enrichment of GFP-CaMKIIα in new spines as compared to size-matched neighboring control spines (new: 1.5 ± 0.2; neighbor: 1.7 ± 0.1; $p = 0.14$; **Fig. 2B, C**). We

conclude that CaMKIIα rapidly accumulates at new spines and therefore could play an important role in even the earliest molecular signaling events that support new spine stabilization.

Genetic and pharmacological inhibition of CaMKIIα kinase activity does not impair activity-dependent new spine stabilization

To investigate the role of CaMKIIα in activity-dependent new spine stabilization, we tested whether interfering with CaMKIIα kinase activity would disrupt the robust activity-dependent stabilization of new spines induced by high frequency uncaging (HFU) of MNI-caged glutamate (MNI-glutamate) at individual new spines (Hill and Zito, 2013). We first chose to use a genetic approach by overexpressing GFP-CaMKIIα containing the K42R point mutation that inhibits CaMKIIα kinase activity (Pi, et al., 2010a; Tullis, et al., 2020; Yamagata, et al., 2009). This CaMKIIα-K42R mutant has been shown to act in a dominant-negative manner (Pi, et al., 2010; Rossetti, et al., 2017). The average number of new spines that formed per cell was not significantly different between cells overexpressing GFP-CaMKIIα or GFP-CaMKIIα-K42R (**Supp Fig. 1A;** CaMKIIα: 3.4 ± 0.4; K42R: 3.4 ± 0.5; p=*0.86*). Importantly, enrichment of GFP-CaMKIIα-K42R in new spines was comparable to that in size-matched mature neighboring spines (new: 1.7 ± 0.1; neighbor: 1.8 ± 0.1; $p = 0.39$) and basal spine enrichment levels of GFP-CaMKIIα-K42R in new spines were comparable to those of GFP-CaMKIIα ($p = 0.37$; **Fig. 2D-F**).

We proceeded to test whether expression of GFP-CaMKIIα-K42R would disrupt stabilization of nascent dendritic spines. We used time-lapse imaging of dendrites on neurons expressing dsRed-Express and either GFP-CaMKIIα or GFP-CaMKIIα-K42R to identify multiple new spines that spontaneously grew on each cell. One new spine per cell was exposed to HFU stimulation (**Fig. 3A**). Survivorship of stimulated and unstimulated new spines on the same cell was monitored through time-lapse imaging. Consistent with our observations for cells transfected with GFP alone (Hill and Zito, 2013), our HFU protocol significantly enhanced stimulated new spine survivorship compared to unstimulated new spines on cells expressing GFP-CaMKIIα (**Fig. 3B-D**; stim: 94%, unstim: 62%; $p = 0.03$). Surprisingly, we found that HFU

also robustly enhanced new spine stabilization on cells expressing GFP-CaMKIIα-K42R (**Fig. 3B-D**; stim: 100%, unstim: 68%; $p = 0.02$), suggesting that CaMKIIα kinase activity is not necessary for activity-induced new spine stabilization. Indeed, the rate of stimulated and unstimulated new spine survivorship were not different between the GFP-CaMKIIα or GFP-CaMKIIα-K42R conditions (stim: GFP-CaMKIIα vs GFP-CaMKIIα-K42R: $p = 0.99$; unstim: GFP-CaMKIIα or GFP-CaMKIIα-K42R: $p = 0.79$). Importantly, we confirmed that GFP-CaMKIIα-K42R was acting as a dominant negative, as GFP-CaMKIIα-K42R-transfected neurons exhibited impaired HFU-induced long-term growth of mature spines (K42R: 138 ± 16%; $p = 0.11$), which is intact in neurons expressing GFP-CaMKIIα (WT: 188 ± 23%; $p = 0.01$; **Fig. 3E, F**).

While the K42R mutation acts in a dominant-negative manner, it retains residual kinase activity in response to glutamatergic stimulation (Rossetti, et al., 2017;Tullis, et al., 2020), and we were also concerned that transfected cells might contain fully endogenous CaMKII holoenzymes lacking the mutant subunit. Residual levels of CaMKII activity could be sufficient to promote the enzymatic interactions and signaling cascades necessary to stabilize new spines. As an independent means to test the role CaMKIIα enzymatic activity in activity-induced new spine stabilization, we pharmacologically inhibited CaMKIIα using staurosporine, a potent, broad-spectrum kinase inhibitor that competitively binds the ATP-binding pocket of CaMKIIα. Unlike many of the more widely used CaMKIIα kinase inhibitors with higher specificity, staurosporine does not interfere with the interaction between activated CaMKIIα and the GluN2B subunit (Barcomb, et al., 2013). Using staurosporine to inhibit CaMKIIα thus allowed us to distinguish between the requirement for GluN2B binding (Hill and Zito, 2013) and the potential requirement for kinase activity in activity-induced new spine stabilization.

Using time-lapse 2-photon imaging of dendrites on hippocampal CA1 neurons expressing GFP, we identified multiple new spines that spontaneously grew on each cell (**Fig. 4A**). New spine outgrowth rates did not differ between cells incubated in vehicle or staurosporine, as new spine outgrowth occurred prior to pharmacological application (**Supp Fig.**

1B; vehicle: 4.7 ± 0.8; staurosporine: 4.8 ± 0.4; p=*0.86*). We then added staurosporine (final concentration of 1 μM) or an equivalent volume of vehicle for the remainder of the experiment. One new spine per cell was exposed to HFU stimulation and survivorship was monitored for stimulated and unstimulated new spines on the same cell. We found that stimulated new spines were significantly more stable than unstimulated new spines on the same cells after incubation in either vehicle (stim: 100%; unstim: 65%; $p = 0.04$) or staurosporine (stim: 100%; unstim: 70%; $p = 0.04$) (**Fig. 4B, C**). Furthermore, the rate of stimulated and unstimulated new spine survivorship was not different between the vehicle and staurosporine conditions (stim veh vs sta: $p = 0.99$; unstim veh vs sta: $p = 0.80$). Importantly, we confirmed that HFU-induced long-term growth of mature spines was blocked by staurosporine (101 ± 9%; $p = 0.03$) but intact in vehicle (140 ± 11%; $p = 0.99$), indicating the effectiveness of staurosporine as a kinase inhibitor (**Fig. 4D, E**). Our results with staurosporine are consistent with our finding that genetic inhibition of CaMKIIα kinase activity did not impair activity-induced new spine stabilization. Together, these results strongly support that CaMKIIα kinase activity is not necessary for activity-dependent new spine stabilization.

Knock-down of CaMKIIα blocks activity-dependent new spine stabilization

We next set out to test whether kinase-independent functions of CaMKIIα are required for nascent spine stabilization. Beyond its enzymatic activities, CaMKIIα plays a number of structural and scaffolding roles, independent of those performed by CaMKIIβ, most of which are facilitated by interactions with other synaptic proteins such as α-actinin, densin-180, the GluN2B subunit of the NMDAR, the proteasome, and PSD MAGUKs (Bingol, et al., 2010; Krapivinsky, et al., 2004; Walikonis, et al., 2001). Some of these scaffolding and structural roles of CaMKIIα are distinct from its enzymatic roles and do not require CaMKIIα kinase activity (Barcomb, et al., 2013; Bingol, et al., 2010; Krapivinsky, et al., 2004; Pi, et al., 2010a). These interactions would require precise regulation of the amounts of available CaMKIIα and its physical interactions with potential binding partners. Thus, decreased levels of endogenous

CaMKIIα would likely interfere with these structural and scaffolding activities, some of which may be necessary for activity-dependent new spine stabilization.

We tested whether structural and/or scaffolding activities of CaMKIIα are needed to support activity-dependent nascent spine stabilization using an shRNA-mediated knock-down of endogenous CaMKIIα with an shRNA that was designed and validated in previous work (Lemieux, et al., 2012). We validated this shRNA in our preparation by demonstrating that HFU-induced long-term growth of mature spines was blocked by knock-down of CaMKIIα (98 ± 10%; $p = 0.99$) and rescued by co-expression of shRNA-resistant GFP-CaMKIIα* (200 ± 26%; $p = 0.04$; **Fig. 5A, B**). We next applied this shRNA to test HFU-induced new spine stabilization. We found that knock-down of CaMKIIα disrupted HFU-induced new spine stabilization (**Fig. 5C-E**; stim: 60%, unstim: 61%; $p = 0.98$). To rule out possible effects of non-specific shRNA activity, we rescued the knock-down by co-expressing an shRNA-resistant form of GFP-CaMKIIα (GFP-CaMKIIα*). shRNA knock-down of CaMKIIα caused a slight but non-significant reduction in the average number of new spines that grew per cell **(Supp. Fig. 1C;** shRNA: 4.2 ± 0.9; CaMKIIα*: 5.9 ± 0.6; $p=0.21$)**.** Rescuing CaMKIIα levels restored activity-dependent new spine stabilization, as new spines that received the HFU stimulus were again significantly more stable than unstimulated control new spines (**Fig. 5C-E**; stim: 100%, unstim: 67%; $p = 0.03$). These results confirm a role for CaMKIIα in activity-induced new spine stabilization. Together with our previous results, we conclude that non-enzymatic CaMKIIα function is required to enhance new spine stabilization downstream of strong glutamatergic stimulation.

Overexpression of pseudo-autophosphorylated CaMKIIα enhances basal spine survivorship independent of kinase activity

We next probed whether CaMKIIα's non-enzymatic structural and/or scaffolding activities are not only necessary, but sufficient to enhance activity-dependent new spine stabilization. We took advantage of phospho-mimetic CaMKIIα mutants that increase basal levels of CaMKIIα-GluN2B binding (Barcomb, et al., 2014), specifically the replacement of threonine 286 with an

aspartic acid, or CaMKIIα-T286D (Pi, et al., 2010a). The T286D mutation renders CaMKIIα constitutively autonomously active, allowing interactors and substrates access to the kinase and regulatory domains. Pairing this mutation with the K42R point mutation generates increased autonomous CaMKIIα interactions, while blocking CaMKIIα enzymatic activities.

To determine the effect of autonomous CaMKIIα on new spine survivorship with and without kinase activity, we expressed the GFP-CaMKIIα-T286D or GFP-CaMKIIα-K42R/T286D with a dsRed-Express cell fill in organotypic hippocampal slice cultures. For these experiments where we wanted to determine whether constitutively autonomous CaMKIIα was sufficient to enhance new spine survivorship, we did not expose new spines to our HFU protocol, instead we monitored basal new spine stability over a period of 70 min using time-lapse imaging. T286D overexpression with or without the K42R mutation to inhibit kinase activity enhanced new spine outgrowth rates compared to DsRed and wild-type (WT) GFP-CaMKIIα controls **(Supp. Fig. 1D;** DsRed: 5.0 ± 0.9; WT: 5.2 ± 0.6; T286D: 7.7 ± 0.8; T286D/K42R: 7.6 ± 1.2; $p=0.20$). We found that new spines were more stable on cells expressing either GFP-CaMKIIα-T286D or GFP-CaMKIIα-K42R/T286D compared to new spines on cells expressing only DsRed-Express (**Fig 6**; DsRed: 63%; T286D: 83%, $p = 0.02$; K42R/T286D: 85%, $p = 0.01$). Importantly, expression of GFP-CaMKIIα did not alter new spine survivorship as compared to dsRed-Express alone (DsRed: 67%; WT: 65%; $p = 0.84$), so increased survivorship was due to pseudo-autophosphorylated CaMKIIα, independent of kinase activity. Our findings support a model in which CaMKIIα-GluN2B binding facilitates non-enzymatic CaMKIIα functions that are both necessary and sufficient for enhancing new spine stabilization.

DISCUSSION

Molecular composition of nascent dendritic spines

There is substantial evidence indicating that the formation of new spines and their ability to persist and integrate into functional synaptic circuits is crucial to learning (Albarran, et al., 2021; Hayashi-Takagi, et al., 2015; Roberts, et al., 2010; Xu, et al., 2009; Yang, et al., 2009).

Despite this vital role, the molecular composition and signaling pathways at play in new spines remain largely unexplored. New spines do share some molecular and functional properties with mature spines; new spine AMPAR currents are comparable to those recorded from mature spines of similar size (Kwon and Sabatini, 2011; Zito, et al., 2009) and ultrastructural evidence shows that a subset of new spines are found directly apposed to presynaptic boutons (Knott, et al, 2006; Trachtenberg, et al., 2002; Zito, et al., 2009), suggesting that new spines are rapidly equipped to respond to glutamatergic stimulation and incorporated into neural circuits.

Still, new spines differ from mature spines in several key ways. Most notably, the very low expression levels in new spines of the PSD-family MAGUKs (De Roo, et al., 2008b; Lambert, et al., 2017), key scaffolding molecules that regulate synaptic strength, maturation, and stability (Boehm, et al., 2006; Cane, et al., 2014; Ehrlich and Malinow, 2004; Elias, et al., 2008; Taft and Turrigiano, 2014). NMDAR currents are also smaller in new spines (Kwon and Sabatini, 2011; Zito, et al., 2009), where they demonstrate greater diffusional coupling to the dendrite (Zito, et al., 2009). PSD-family MAGUKS, NMDAR-mediated signaling, and spine morphologies associated with a high degree of compartmentalization are all thought to regulate synapse stability (Cane, et al., 2014; De Roo, et al., 2008a; De Roo, et al., 2008b; Lambert, et al., 2017; Taft and Turrigiano, 2014), suggesting that the low basal survivorship rates of new spines may be due to their distinct molecular composition and signaling capabilities. Identifying the molecular signaling pathways at play in new spines is therefore crucial to understand the mechanisms involved in their stabilization.

Here, we show that, unlike GFP-tagged PSD-family MAGUKs, new spines express GFP-CaMKIIα at levels comparable to those in mature spine levels, independent of CaMKIIα kinase activity. CaMKIIα's presence in new spines supports that CaMKIIα signaling could play a critical role in new spine function. Indeed, evidence supports a requirement for the CaMKIIα-GluN2B interaction not only in activity-dependent new spine stabilization (Hill and Zito, 2013), but also in

spontaneous and activity-dependent new spine outgrowth (Hamilton, et al., 2012), suggesting that CaMKIIα's functions at new spines may precede any form of synaptic stimulation.

Role of CaMKIIα kinase activity in new spine stabilization

Despite our finding that CaMKIIα is present at mature levels in new spines, we were surprised to find that CaMKIIα kinase activity is not required for enhanced new spine stabilization induced either by strong glutamatergic stimulation at single spines or by overexpression of the CaMKIIα-K42R/T286 phospho-mutant. Our results in new spines are in contrast with what is known regarding the important role of CaMKIIα kinase activity in stabilization of the long-term growth of mature spines (Araki, et al., 2015; Koeberle, et al., 2017). However, major changes to the molecular composition of new spines occur during the maturation process, including the recruitment of PSD-family MAGUKs (De Roo, et al., 2008b; Lambert, et al., 2017), no doubt creating a vastly different biochemical signaling environment in the new spine as it develops. Indeed, it is possible that, while CaMKIIα kinase activity is not required to enhance new spine stabilization on the time scale of 70-130 min after new spine growth, as we observed in our experiments, it may be necessary at later times, for example following the delayed recruitment of other synaptic proteins, such as PSD-family MAGUKS. Notably, on the very earliest time scales (<40-100 min), even non-enzymatic CaMKIIα function does not appear to be required for activity-dependent new spine stabilization, as we show that stimulated new spines are initially stabilized even on cells where CaMKIIα is knocked down. Overall, our data demonstrate that CaMKIIα kinase function is not required for the early steps of new spine stabilization, within the first few hours following new spine outgrowth.

Role of GluN2B-CaMKIIα binding in new spine stabilization

Our finding that CaMKIIα kinase activity is not required for activity-dependent new spine stabilization leaves an undefined role for the required CaMKIIα-GluN2B interaction. This interaction has long been known to be important in the regulation of basal synaptic transmission and LTP maintenance (Barcomb, et al., 2014; Barria and Malinow, 2005; Halt, et al., 2012;

Incontro, et al., 2018), where it is thought to play a role in bringing Ca^{2+}/CaM-activated CaMKII closer to its targeted substrates to alter synaptic transmission and synaptic strengthening in a kinase-dependent manner. In new spines, our results instead support a non-enzymatic role for CaMKIIα in new spine stabilization. Indeed, we show that, although CaMKIIα kinase activity is not required, knockdown of CaMKIIα disrupts activity-dependent new spine stabilization. Altogether our results suggest that the interaction between GluN2B and CaMKIIα is required to support a primarily structural or scaffolding role for CaMKIIα in new spine stabilization.

While we found that CaMKIIα-T286D, which enhanced the interaction between GluN2B and CaMKIIα, also increased basal spine stabilization, survivorship rates for CaMKIIα-T286D were lower than observed for new spines that received HFU stimulation. At mature spines glutamatergic stimulation initiates a number of concurrent signaling cascades and molecular changes, such as NMDAR and mGluR activation and downstream signaling mechanisms (Bosch, et al., 2014; H.-K. Lee, et al., 2003; Malinow, 2003; Murakoshi, et al., 2011; Stein, et al., 2021) that are not replicated by overexpression of the CaMKIIα-T286D phospho-mutant. It is likely that at least a subset of these mechanisms acts in conjunction with GluN2B-CaMKIIα binding to enhance activity-dependent new spine stabilization. In addition, the GluN2B-CaMKIIα interaction may serve to bring CaMKIIβ, which complexes with CaMKIIα at a 3:9 ratio in the hippocampus, within optimal proximity to its binding partners in order to regulate cytoskeletal stability (Kim, et al., 2015, Kim, et al., 2019; Okamoto, et al., 2007).

Non-enzymatic CaMKIIα function in new spine stabilization

Although CaMKIIβ is perhaps more well recognized than CaMKIIα for its non-enzymatic functions in regulating the spine actin cytoskeleton, CaMKIIα has also been shown to participate in several functionally important scaffolding and structural interactions that are distinct from those made by CaMKIIβ (Bayer and Schulman, 2019; Hell, 2014). Some of these interactions are less likely to be relevant for the earliest stages of new spine stabilization, such as roles with stargazin, TARP γ-8, or the Rac-1 activating RAKEC (Opazo, et al., 2010, 2012; Park, et al.,

2016; Saneyoshi, et al., 2019), as they require either binding to PSD-family MAGUKs or CaMKIIα kinase activity. However, other known CaMKIIα interactions are independent of these requirements and therefore would make attractive candidates for roles in new spine stabilization, such as the activity-dependent binding of CaMKIIα directly to the 26S proteasome, and indirect interactions of CaMKIIα with SynGAP-1α via the multi-PDZ domain protein MUPP-1 (Bingol, et al., 2010; Krapivinsky, et al., 2004).

Indeed, CaMKIIα's non-enzymatic interactions with the proteasome and the MUPP1-SynGAP-1α complex appear particularly promising in the context of understanding new spine stabilization. SynGAP-1α is a negative-regulator of synapse maturation, and its exclusion from synapses contributes to synaptic strengthening, precocious PSD-95 accumulation, and increased spine volume (Aceti, et al., 2015; Araki, et al., 2015; Clement, et al., 2012; Vazquez, et al., 2004). Interestingly, activity-dependent dissociation of the MUPP1-SynGAP-1α complex from CaMKIIα does not require CaMKIIα kinase activity (Krapivinsky, et al., 2004) and thus may provide a mechanism for SynGAP-1α dispersion (Araki, et al., 2015) from new spines, independent of PSD-family MAGUKs and kinase activity. Furthermore, activity-dependent new spine formation requires the proteasome (Hamilton, et al., 2012), which may remain accumulated at sites of new spine formation, where it could play a role in the activity-dependent degradation of negative regulators of synapse stability and maturation. Indeed, there is evidence that the proteasome mediates degradation of SynGAP (Zhang, et al., 2020) and Ephexin5 (Hamilton, et al., 2017), which both have roles in regulating dendritic spine stability. The elucidation of the role of these two proteins and of other non-enzymatic functions of CaMKIIα downstream of GluN2B-CaMKIIα to promote new spine survivorship is an intriguing and compelling avenue for future study.

FIGURE LEGENDS AND FIGURES

Figure 2. GFP-CaMKIIα enrichment in new spines is comparable to that in size-matched neighboring spines.

(A) Images of dendrites from hippocampal CA1 neurons in slice culture (DIV 7-9) expressing GFP-CaMKIIα (green) and DsRed-Express (red) before (open arrowhead) and after (filled arrowhead) spontaneous new spine outgrowth. ***(B)*** Enrichment (spine: dendrite ratio) of GFP-CaMKIIα in new spines (n = 33 spines/16 cells) was comparable to that in size-matched neighboring spines (n = 21 spines/16 cells). ***(C)*** Neighboring spines used for enrichment calculations in ***B*** were size-matched to new spines ($p = 0.62$). ***(D)*** Images of dendrites from CA1 neurons expressing GFP-CaMKIIα-K42R (green) and DsRed-Express (red) before (open arrowhead) and after (filled arrowhead) spontaneous new spine outgrowth. ***(E)*** Enrichment of GFP-CaMKIIα-K42R in new spines (n = 39 spines/ 14 cells) was comparable to that in size-matched mature neighboring spines (n = 39 spines/14 cells). Importantly, no difference in relative enrichment was found between new (filled bars) or size-matched neighboring spines (open bars) in the WT (black) and K42R (blue) conditions (new: $p = 0.37$; neighbors: $p=0.99$). Data for CaMKIIα-WT new spine enrichment is from ***B***. ***(F)*** Neighboring spines used for enrichment calculations in ***E*** were size-matched to new spines ($p = 0.99$). No difference in new spine size between WT (black) and K42R (blue; $p=0.99$). Data for WT new spine size is from ***C***. Paired two-tailed t-test in ***C***, two-way ANOVA with Dunnet's test to baseline in ***F*** and with Bonferroni multiple comparisons test in ***D*** and ***G***. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Figure 2

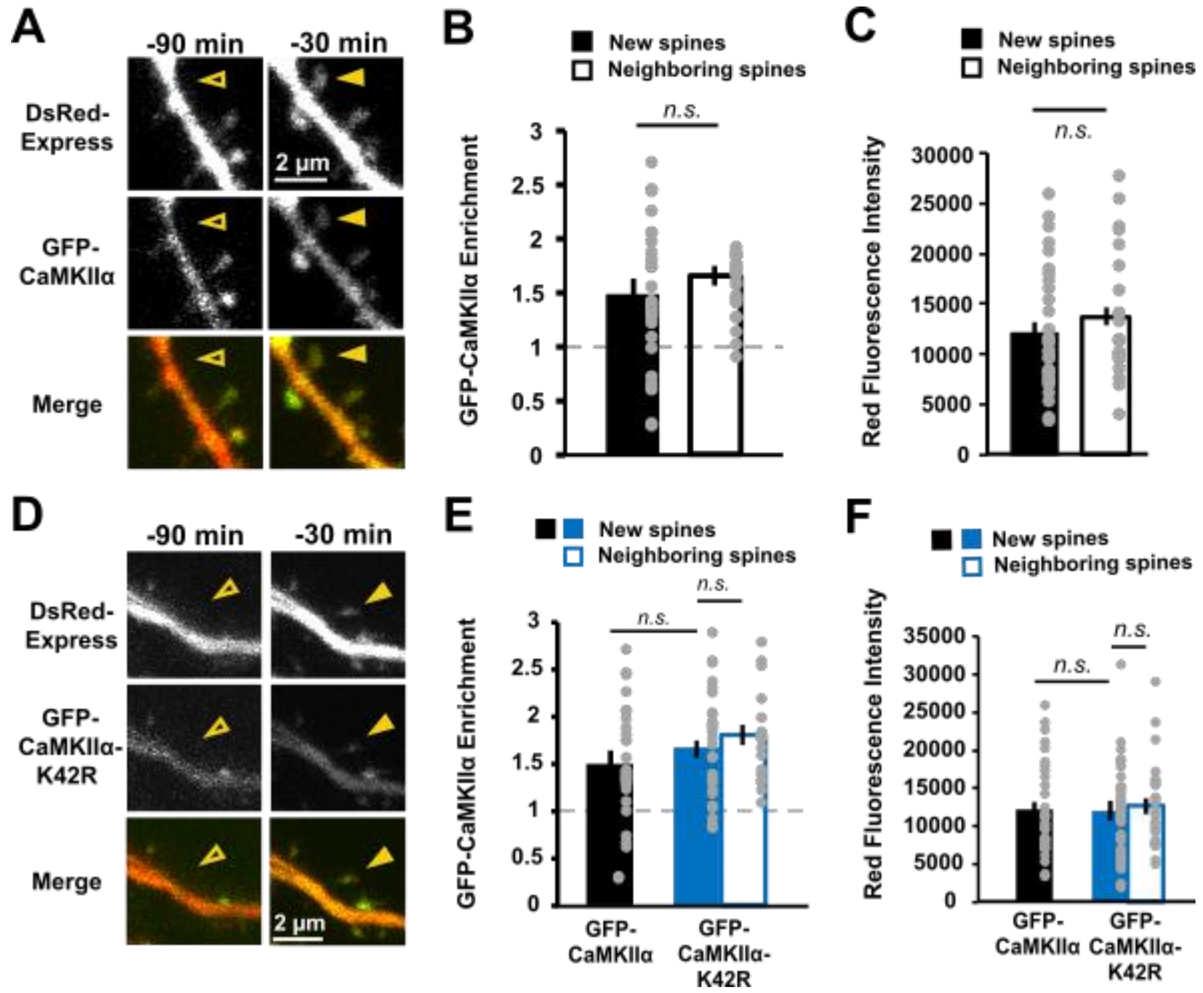


Figure 3. GFP-CaMKIIα-K42R does not impair activity-dependent new spine stabilization. ***(A)*** Images (red channel) of a hippocampal CA1 neuron expressing DsRed-Express and GFP-CaMKIIα. Three new spines appeared (solid arrowheads), one of which was stimulated with HFU. One unstimulated spine was eliminated (open arrowhead). ***(B)*** Images (red channel) of dendrites from CA1 neurons (DIV 7-9) expressing DsRed-Express and either GFP-CaMKIIα (top row) or GFP-CaMKIIα-K42R (bottom row) showing new dendritic spines stimulated at 1 min with HFU that were stable until 70 min. ***(C)*** HFU stimulation enhanced new spine survivorship (filled blue and black circles; WT: n=16 spines/16 cells; K42R: n=14 spines/14 cells) relative to unstimulated new spines (open blue and black circles; WT: n=31 spines/16 cells; K42R: n=32 spines/14 cells) on the same cells for both GFP-CaMKIIα-WT (black) and GFP-CaMKIIα-K42R (blue) conditions. ***(D)*** Survivorship of HFU-stimulated new spines (filled bars) at 70 min was increased compared to unstimulated new spines (open bars) on the same cells for both GFP-CaMKIIα-WT (black bars) and GFP-CaMKIIα-K42R (blue bars). ***(E)*** Images (red channel) of dendrites of CA1 neurons (DIV 7-9) before HFU at mature spines, immediately after HFU, and at 30 min after HFU. ***(F)*** GFP-CaMKIIα-K42R expression impaired HFU-induced long-term growth of mature spines (filled blue; n= 8 spines/8 cells) that is retained in cells expressing GFP-CaMKIIα (filled black; n= 8 spines/8 cells). Log-rank task was used in ***C*** and Barnard's exact test was used in ***D***, two-way ANOVA with Dunnet's test to baseline in ***E*** and with Bonferroni multiple comparisons test in ***F***. *p <0.05, **p < 0.01, ***p < 0.001.

Figure 3

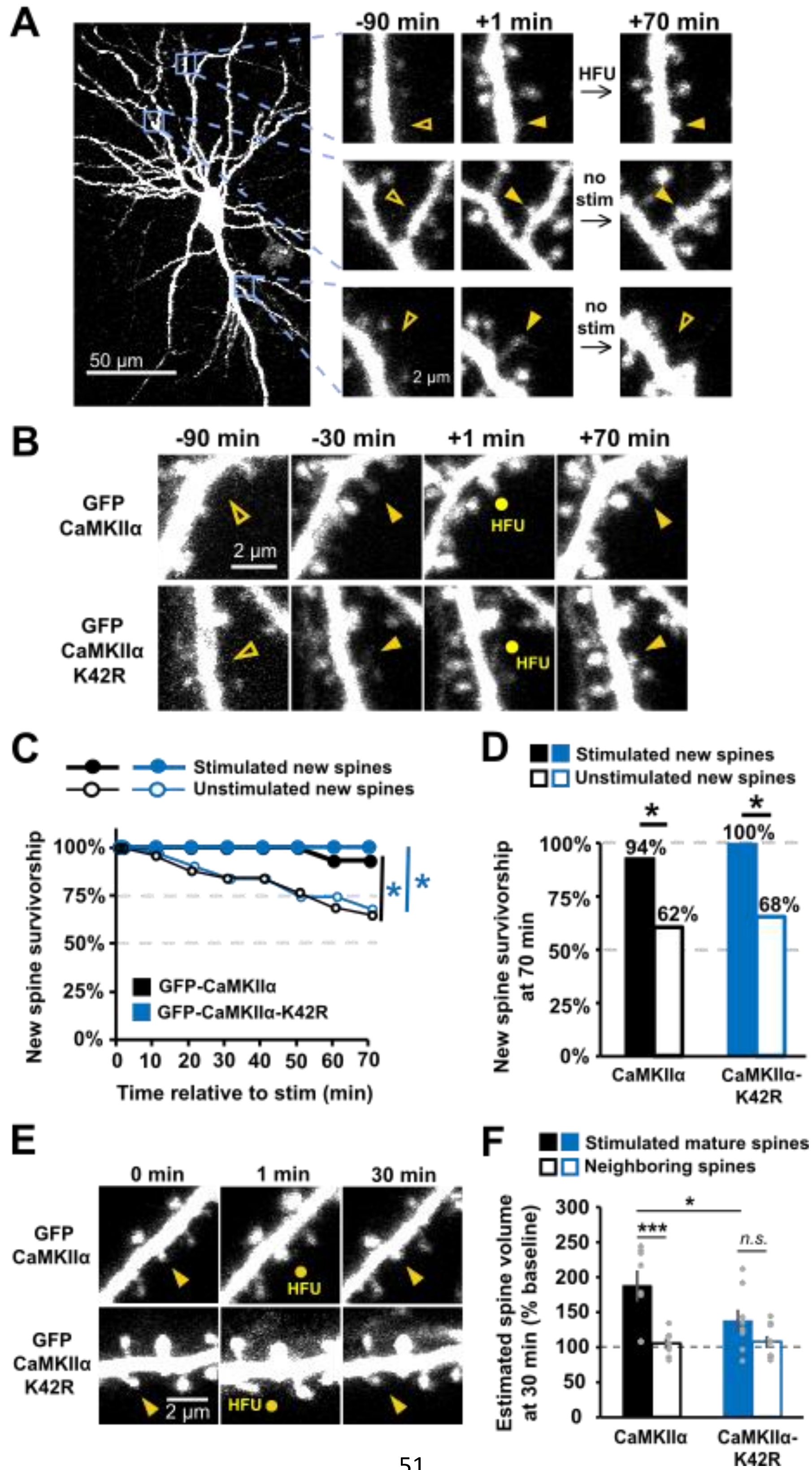


Figure 4. Inhibition of CaMKIIα kinase activity with staurosporine does not impair activity-dependent new spine stabilization.

(A) Images (green channel) of spontaneous new spine outgrowth (filled arrowhead at 0 min) on dendrites of GFP-transfected hippocampal CA1 neurons (DIV 7-9). One new spine per neuron was stimulated with HFU following 30 min pre-incubation in either vehicle (top row) or 1 μm staurosporine (Sta; bottom row). ***(B)*** Survivorship of stimulated new spines (filled circles; veh: 9 spines/9 cells; Sta: 11 spines/11 cells) was enhanced relative to unstimulated new spines on the same cells (open circles; veh: 33 spines/9 cells; Sta: 43 spines/11 cells) in both vehicle (black) and staurosporine (blue) conditions. ***(C)*** Survivorship of HFU-stimulated new spines (filled bars) at 70 min was increased compared to unstimulated new spines (open bars) on the same cells in both vehicle (black) and staurosporine (blue) conditions. ***(D)*** Images of dendrites (green channel) on GFP-transfected CA1 neurons before HFU, immediately after HFU (yellow circle), and at 30 min. ***(E)***. Incubation with 1 μM staurosporine (sta; filled blue; n=10 spines/10 cells) blocked HFU-induced long-term growth of mature spines (101 ± 9%; $p = 0.03$), which was intact in vehicle conditions (veh; filled black; n=12 spines/12 cells; 140 ± 11%; $p = 0.99$). Volume of unstimulated neighbors was unchanged (open bars; veh: 104 ± 3%, $p = 0.99$; K42R: 96 ± 7%, $p = 0.99$). Log-rank task was used in ***B***, Barnard's exact test was used in ***C***, and two-way ANOVA with Bonferroni multiple-comparisons test was used in ***E***. *p <0.05, **p < 0.01, ***p < 0.001.

Figure 4

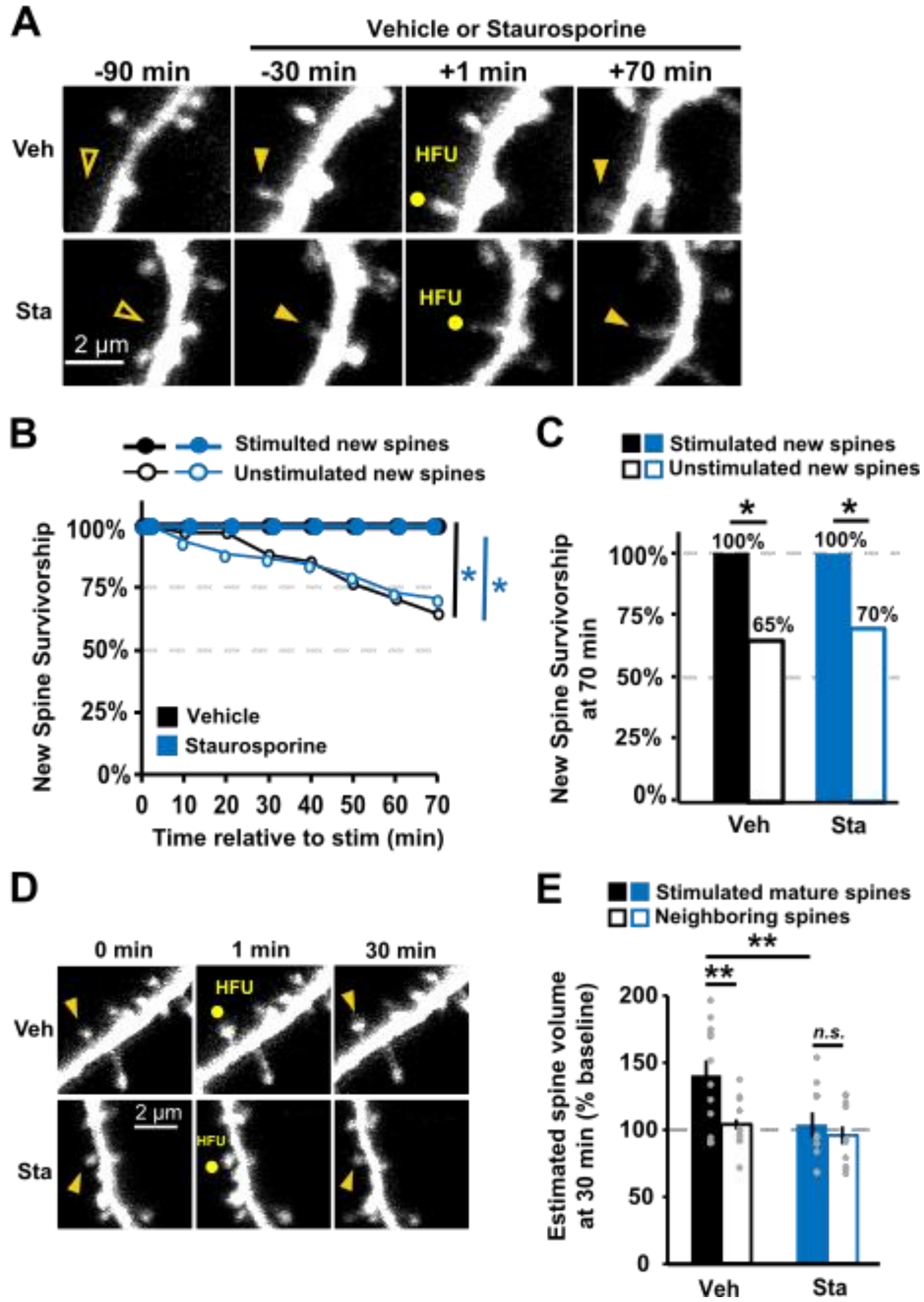


Figure 5. CaMKIIα knock-down blocks activity-dependent new spine stabilization.

(A) Images of dendrites on organotypic hippocampal CA1 neurons (DIV 7-9) before HFU, immediately after HFU (yellow circle), and at the final 30 min time point. ***(B)*** shRNA-mediated knockdown of CaMKIIα (filled blue; n=8 spines/8 cells) impaired HFU-induced long-term spine growth that was restored with co-expression of shRNA-resistant GFP-CaMKIIα* (black; n=8 spines/8 cells). ***(C)*** Images of dendrites (red channel) on CA1 neurons (DIV 7-9) expressing a DsRed-Express cell fill and either CaMKIIα shRNA (top) or CaMKIIα shRNA + shRNA-resistant GFP-CaMKIIα* (bottom). Spontaneous new spine outgrowth (filled arrowheads) was observed in both conditions. HFU-induced new spine stabilization failed following knock-down of CaMKIIα (open arrowhead at 70 min) but was rescued with expression of shRNA-resistant GFP-CaMKIIα*. ***(D)*** Knockdown of CaMKIIα disrupted the stabilization of new spines (filled circles) as compared with unstimulated new spines (open circles) at times beyond 30-40 min (blue; stimulated: 10 spines/10 cells; unstimulated: 36 spines, 10 cells). Rescuing CaMKIIα restored activity-dependent new spine stabilization (black; stim: 11 spines/11 cells; unstim: 53 spines/11 cells). ***(E)*** Activity-dependent new spine stabilization at 70 min (filled bars) was not significantly different from that of unstimulated new spines (open bars) following knockdown of CaMKIIα (black) but was restored when CaMKIIα is rescued with shRNA-resistant CaMKIIα* (blue). Two-way ANOVA with Bonferroni multiple-comparisons test in ***B,*** log-rank task was used in ***D***, and Barnard's exact test was used in ***E.*** *p <0.05, **p < 0.01, ***p < 0.001.

Figure 5

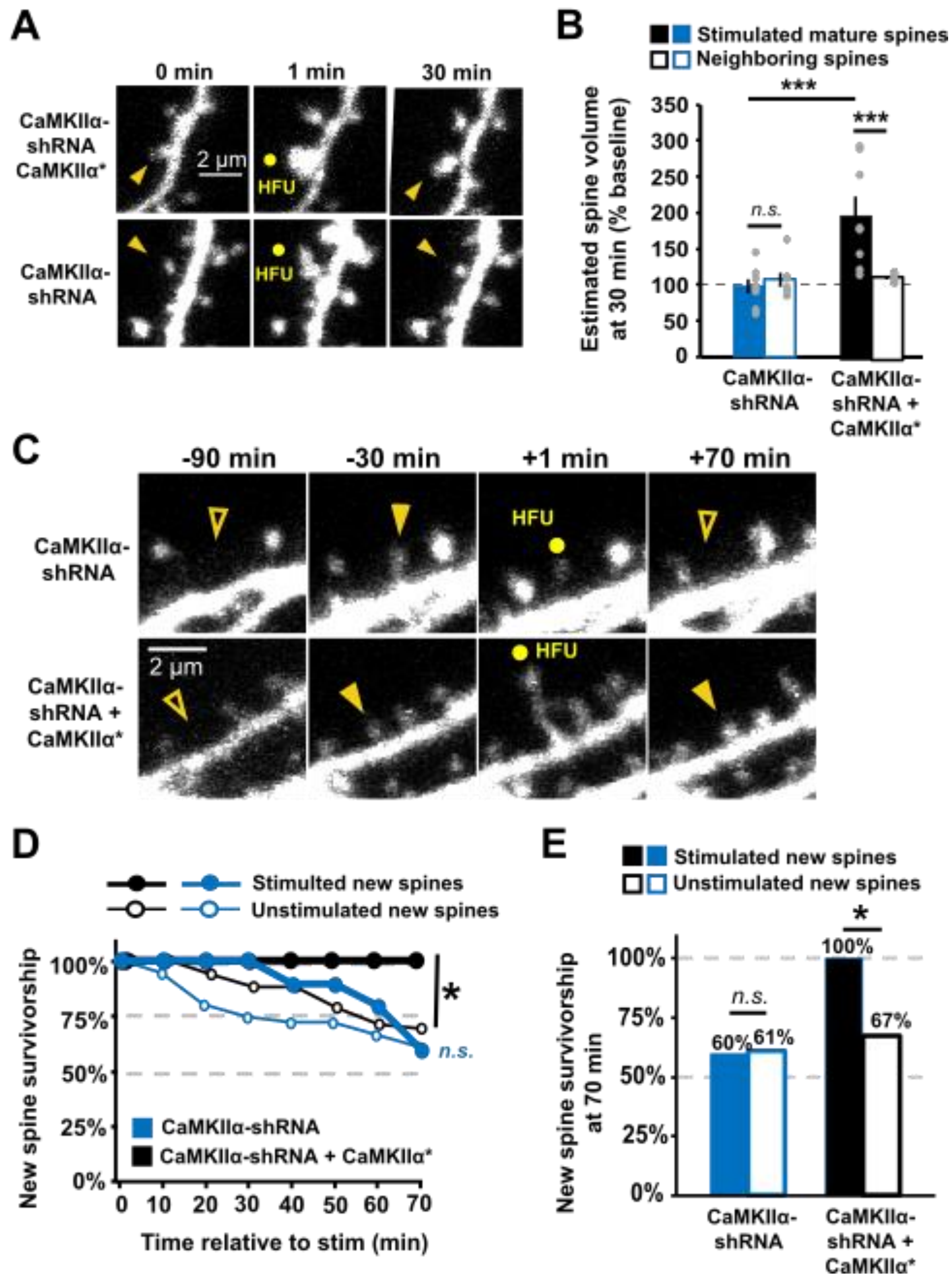
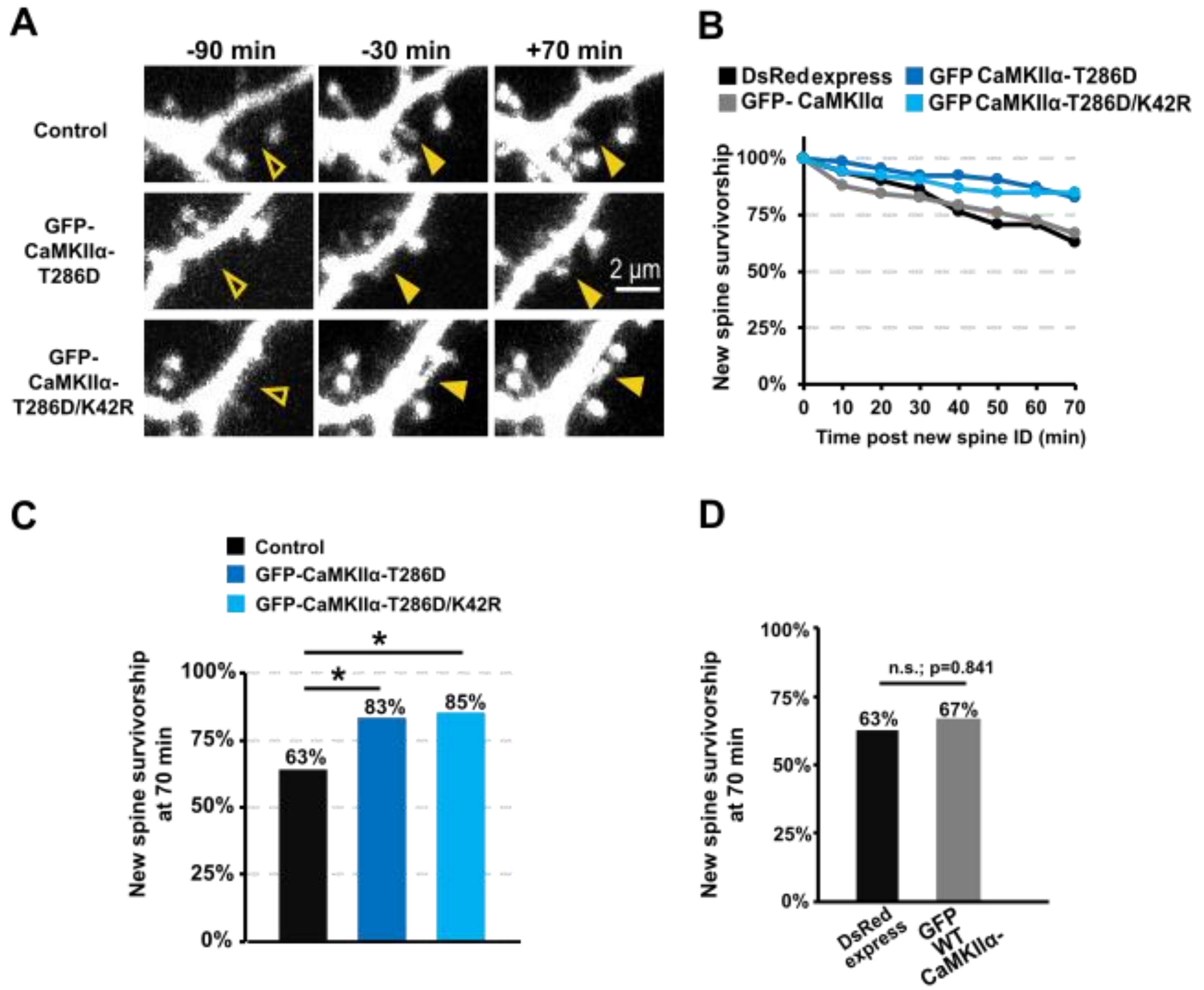


Figure 6. Overexpression of constitutively autonomous CaMKIIα enhances basal spine survivorship independent of kinase activity.

(A) Images (red channel) showing spontaneous new spine outgrowth (filled arrowhead at 0 min) and stabilization (filled arrowhead at 70 min) on dendrites of hippocampal neurons (DIV 7-9) expressing DsRed-Express alone (top row) or co-expressed with GFP-CaMKIIα-T286D (middle row) or GFP-CaMKIIα-K42R/T286D (bottom row). ***(B)*** New spine survivorship curves over 70 min following new spine identification. (C) Basal spine survivorship rates were higher on cells expressing GFP-CaMKIIα-T286D (dark blue; 63 spines/ 10 cells) and GFP-CaMKIIα-K42R/T286D (light blue; 53 spines/ 7 cells) compared to survivorship rates on cells expressing only DsRed-Express (black; 51 spines/ 10 cells). **(D)** Basal new spine survivorship rates did not differ between cells expressing only DsRed-Express (black bar) or (WT) GFP-C CaMKIIα (gray bar; 58 spines/ 10 cells). Barnard's exact test was used in *C and D*. *p <0.05, **p < 0.01, ***p < 0.001.

Figure 6

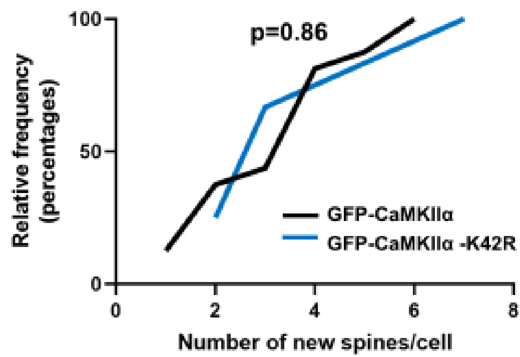


Supplemental Figure 1.

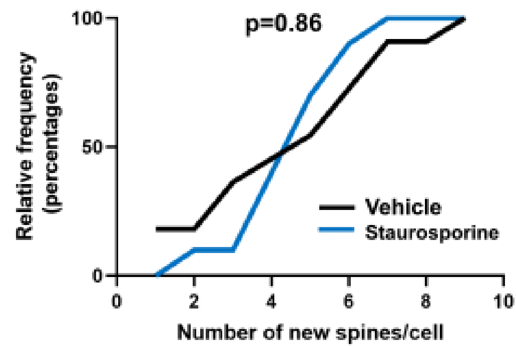
(A) Relative frequency distribution of number of newly formed spines observed on cells expressing GFP-CaMKIIα (black) and GFP-CaMKIIα-K42R (blue). There was no statistical difference in the new spine outgrowth rates between conditions. *(B)* There was no difference in observed new spine outgrowth rates on eGFP transfected neurons prior to incubation in either vehicle (black) or staurosporine (blue). *(C)* Relative frequency distribution of the number of newly formed spines on the same cells in Fig. 4. shRNA knock-down of CaMKIIα (blue) decreased the rate of new spine outgrowth compared to outgrowth rates on cells expressing shRNA resistant CaMKIIα* (black). *(D)* Overexpression of GFP-CaMKIIα containing the T286D point mutation with (light blue) or without (blue) the kinase-dead K42R point mutation increased rates of new spine formation compared to cells expressing DsRed Express alone (black) or with GFP-CaMKIIα (gray). Kolmogorov-Smirnov test used in A-C. Kruskal-Wallis test used in *D*.
*p <0.05, **p < 0.01, ***p < 0.001.

Supplemental Figure 1

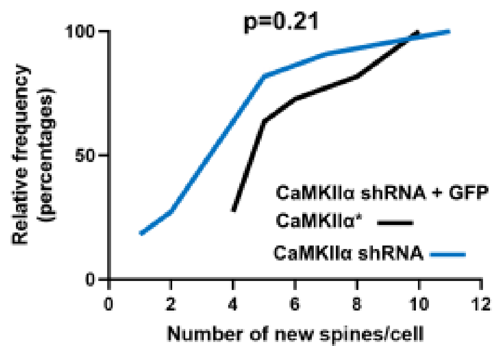
A



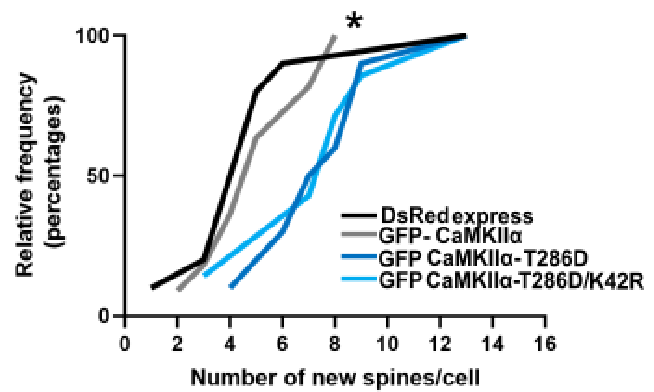
B



C



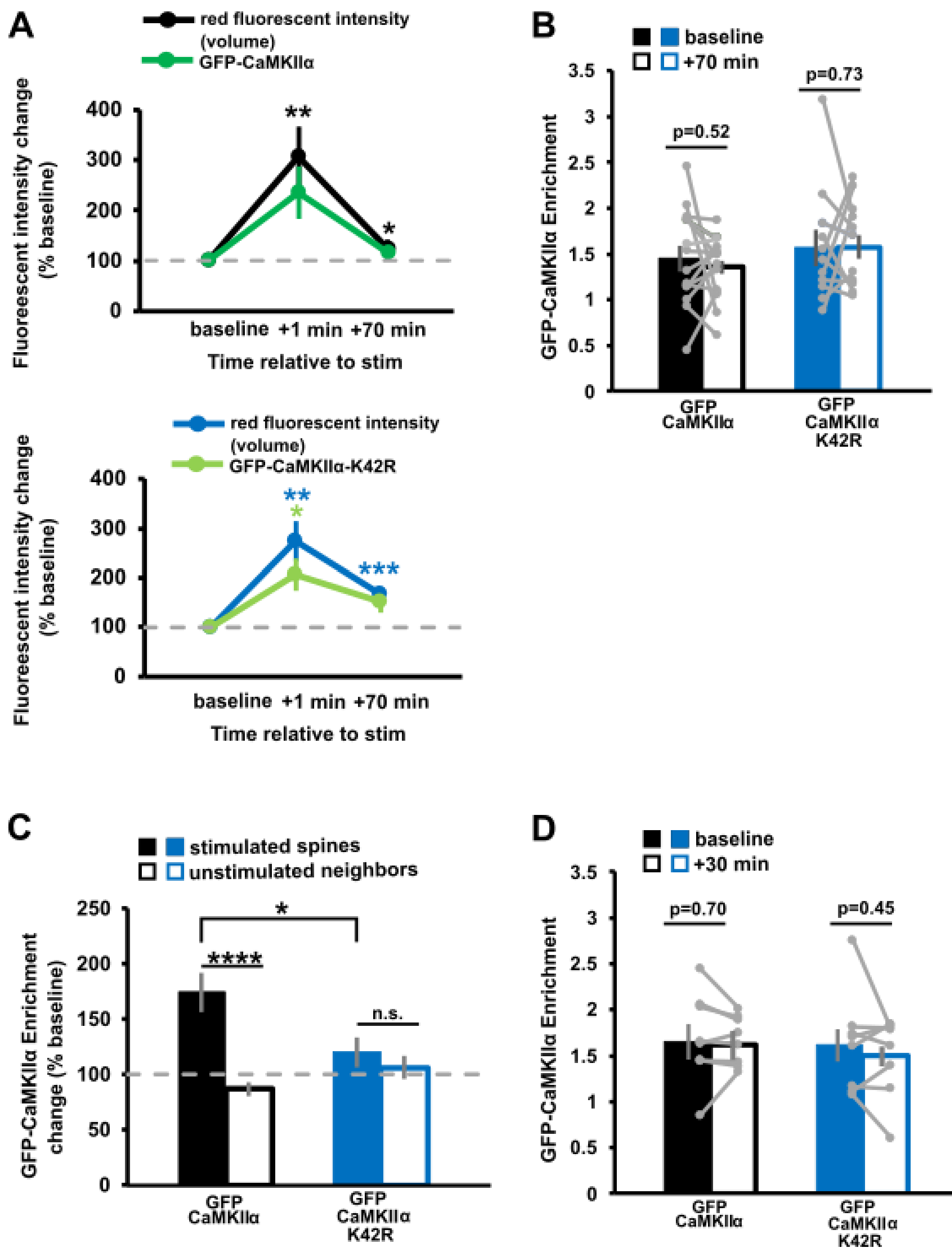
D



Supplemental Figure 2.

(A) HFU induced new spine volume changes (red fluorescent intensity; black and blue traces) and GFP- CaMKIIα (green and light green traces) translocation on cells expressing either GFP- CaMKIIα (top panel) or GFP- CaMKIIα- K42R (bottom panel). Changes in GFP- CaMKIIα fluorescent intensity closely matched changes in red fluorescent intensity. (B) GFP- CaMKIIα enrichment at stimulated new spines was not different +70 following HFU. (C) HFU at mature spines induced a significant increase in GFP- CaMKIIα fluorescent intensity that was abolished by GFP- CaMKIIα-K42R expression. (D) GFP- CaMKIIα enrichment at stimulated n spines was not different +70 following HFU. Two-way ANOVA with Dunnett's test to baseline used in A, Unpaired two-tailed t-test used in B and D, and Two-way ANOVA with Bonferroni multiple-comparisons test in C.

Supplemental Figure 2



CHAPTER 3: INVESTIGATION OF THE RELATIONSHIP BETWEEN NEW SPINE VOLUME CHANGES AND STABILITY

PREFACE

The following chapter contains unpublished analyses of the data used in Chapter 2 to investigate the role of new spine volume changes in activity-dependent new spine stabilization. I determined that while CaMKIIα knock-down disrupts activity-dependent new spine volume changes, CaMKIIα kinase inhibition appears to enhance new spine volume changes. I also show that these volume changes are not necessary to enhance new spine stability, as manipulations that inadvertently block activity-dependent new spine volume changes still stabilize new spines (Chapter 2). Finally, I demonstrate that manipulations that enhance mature spine volume and increase new spine stability without the need for further glutamatergic stimulation (Chapter 2) also induce spontaneous new spine enlargement. I acknowledge the intellectual contributions of my dissertation advisor, Dr. Karen Zito and my dissertation committee: Drs. Elva Diaz, John Gray, Kim McAllister, Johannes Hell, and Michael Ferns. I would also like to acknowledge the input of my peers in the lab, specifically Sam Petshow and Juan Flores. I thank Dr. Paul DeKoninck for his generous donation of all the GFP-tagged CaMKIIα constructs as well as the CaMKIIα shRNA. I have chosen to include images of different cells from the data sets acquired during the experiments described in Chapter 2 because I wanted representative images that better reflected the volume changes described in this Chapter and I did not feel as though the images included in Chapter 2 accomplished this.

INTRODUCTION

Learning and memory require the restructuring of neural circuitry in response to our experiences and our environment. One way that this restructuring can occur is through the redistribution of synaptic weights. *In vivo* learning paradigms and *in vitro* manipulations that mimic the excitatory activity that occurs during learning and memory suggest that only a subset of dendritic spines is activated during learning (Araki, et al., 2015; Hayashi-Takagi, et al., 2015). This subset of spines undergoes a rapid and persistent enhancement of synaptic strength (Araki, et al., 2015) mediated by local AMPA-type glutamate receptors (AMPARs) phosphorylation and recruitment. This functional enhancement of dendritic spines occurs in tandem with spine enlargement (Matsuzaki, et al., 2004), collectively known as long-term potentiation (LTP), which is also implicated in learning and memory. *In vivo*, a subset of spines enlarges in response to learning paradigms and disrupting this enlargement by molecular destabilization of the actin cytoskeleton blocks motor learning and memory, suggesting that concurrent spine enlargement and functional enhancement are required for learning and memory. Interestingly, while the activity-dependent functional and structural enhancement of dendritic spines are tightly coupled (Matsuzaki, et al., 2004), these changes can be artificially and experimentally de-coupled, suggesting that spine enlargement and functional strengthening may not be interdependent (Kopec, et al., 2007; Pi, et al., 2010a). It remains to be seen whether the spine enlargement is truly necessary for learning and memory, or if only the simultaneous functional changes are required.

Another contributor to activity-dependent circuit rewiring is the outgrowth of new dendritic spines. *In* vivo imaging has revealed that learning paradigms, such as motor learning in mice and song learning in zebra finches, enhances the outgrowth of new dendritic spines (Roberts, et al., 2010; Xu, et al., 2009; Yang, et al., 2009). Simulating excitatory activity *in vitro* results in similar increases in new spine outgrowth (De Roo, et al., 2008a; Hamilton, et al., 2012; Kwon and Sabatini, 2011). The majority of these new spines are transient, lasting

anywhere from a few minutes to a few hours (De Roo, et al., 2008a; Hill and Zito, 2013) however, a subset of these new spines do stabilize via unknown molecular mechanisms. Critically, performance is strongly correlated with more of these new spines stabilizing, indicating a role for new spine outgrowth and stabilization in learning and memory (Roberts, et al., 2010; Xu, et al., 2009; Yang, et al., 2009). Recent studies have revealed a mechanism for enhancing new spine stability: further glutamatergic stimulation using paradigms that normally induce LTP can enhance the survival of new dendritic spines (Hill and Zito, 2013). While the molecular mechanisms that enhance new spine survivorship following activity require further elucidation, these studies suggest that spine volume changes may play a role in enhancing new spine stability. Most new spines that receive strong glutamatergic stimulation enlarge to roughly 160% of their initial volume (Hill and Zito, 2013). Among new spines that do not receive stimulation, the larger spines tend to outlast their smaller neighbors and among all stimulated new spines, a significant increase in spine survivorship is only seen amongst smaller spines that undergo activity-dependent volume changes (Hill and Zito, 2013).

These findings suggest that new spine size may predict stability, and that glutamatergic stimulation might enhance new spine survivorship by causing new spines to enlarge to a more stable volume. However, due to the tight coupling of spine enlargement and functional potentiation, as well as the link between basal spine size and synaptic strength (Matsuzaki, et al., 2004; Zito, et al., 2009) it is possible that the relationship between spine size and stability really reflects the relationship between stability and synaptic strength, which also correlates with spine size. The same genetic and pharmacological manipulations that affect new spine survivorship may also be used to investigate the requirement for new spine enlargement in activity-dependent new spine stabilization. In Chapter 2, I investigated the roles that CaMKIIα enzymatic and non-enzymatic scaffolding functions have in new spine stabilization. Similar manipulations, such as dominant-negative kinase-dead and phospho-mutant overexpression (Kabakov and Lisman, 2015; Pi, et al., 2010a), pharmacological CaMKIIα inhibition, and

CaMKIIα knock-down, are known to affect spine volume changes both basally and in response to glutamatergic stimulation (Matsuzaki, et al., 2004). It is not yet known if these manipulations also alter activity-dependent new spine volume changes, and whether any such changes play a role in activity-dependent new spine stabilization.

Here, I analyzed the spine volume changes of new spines exposed to high-frequency uncaging of MNI-caged glutamate (HFU) to test the hypothesis that new spine volume changes are necessary for activity-dependent new spine stabilization. I first show that inhibition of CaMKIIα does not impair activity-dependent new spine enlargement, despite blocking structural potentiation at mature spines on the same cells (Chapter 2). Intriguingly, my data provides evidence that, at new spines, CaMKIIα kinase-activity negatively regulates activity-dependent spine volume changes. New spine enlargement following HFU is significantly greater in conditions where CaMKIIα kinase-activity is blocked either genetically or pharmacologically. I also show that new spine volume changes are not requisite for enhanced stability, as manipulations that inadvertently block new spine volume changes do not interfere with activity-dependent new spine stabilization. I further demonstrate that new spine volume changes require non-enzymatic CaMKIIα functions with an shRNA knock-down, which I show blocks activity-dependent new spine enlargement. Finally, I show that overexpression of constitutively autonomous CaMKIIα-T286D mutant induces spontaneous new spine enlargement, and that this enlargement may be enhanced by introducing an additional K42R point mutation. While new spine enlargement is not required to enhance new spine stability within the first few hours after outgrowth, my results provide insight into the unique and distinct roles CaMKIIα structural and enzymatic functions play at new spines.

METHODS

All data presented in this chapter are the result of additional statistical analyses performed on the same data sets collected during the experiments described in Chapter 2.

Estimation of spine size

Spine size was estimated from bleed-through-corrected and background-subtracted red (DsRed-Express) fluorescence intensity. Spine brightness measurements give an accurate estimate of relative spine size when compared with electron microscopy (Holtmaat, et al., 2005).

Statistical Analysis

For comparisons of new spine volumes at a given time point to baseline, 2-way ANOVAs with appropriate *post-hoc* tests for multiple comparisons were used. Comparisons of new spine volumes at baseline and 70 min required a one-way ANOVA with appropriate *post-hoc* tests for multiple comparisons. Between-group comparisons of 70' spine volume were performed using a two-tailed unpaired heteroscedastic Student's *t* test unless otherwise noted. Error bars represent standard error of the mean (SEM). The Kolmogorov-Smirnov (K-S) test was used to compare cumulative distributions of new spine volume changes.

RESULTS

CaMKIIα knock-down blocks activity-dependent new spine enlargement

My work demonstrates that CaMKIIα is required for activity-dependent new spine stabilization in a primarily non-enzymatic, structural/ scaffolding role (Chapter 2). In addition to enhancing new spine stability, the HFU protocol used to enhance new spine survivorship also results in an increase in new spine volume (Hill and Zito, 2013), indicating a possible relationship between new spine volume changes and enhanced new spine stability. To test the hypothesis that activity-dependent new spine volume changes are required to enhance survivorship following HFU, I analyzed image data acquired from the time-lapse imaging experiments conducted in Chapter 2.

I first examined whether manipulations that impaired activity-dependent new spine stabilization also impaired new spine enlargement following HFU. shRNA knock-down of CaMKIIα prevents the HFU induced enhancement of new spine survivorship, which can be rescued by overexpression of shRNA-resistant WT- CaMKIIα (Chapter 2).

Hippocampal CA1 neurons in organotypic slice cultures were sparsely transfected with either CaMKIIα- shRNA or CaMKIIα- shRNA and GFP-shRNA resistant WT- CaMKIIα (GFP-WT- CaMKIIα*) as a control. Spontaneous new spine outgrowth was observed and 2-photon HFU was used to stimulate a single new spine per cell (**Fig. 7A**). I monitored new spine volume changes with time-lapse imaging 1 min and 70 min after stimulation.

On cells where CaMKIIα was knocked down, I observed a non-significant transient increase in spine volume 1 min after HFU (transient: 360.5 ± 144.9%; $p=0.22$) that returned to baseline by the final 70 min time point (93.2 ± 9.8%; $p=0.73$) (**Fig. 7B**). This was predicted since shRNA knock-down of CaMKIIα blocks activity-dependent new spine stabilization (Chapter 2). Expectedly, the 70 min spine volume change was rescued by GFP-WT- CaMKIIα* ($p=0.01$) (**Fig. 7C)**. HFU of new spines on cells where CaMKIIα was rescued by overexpression of GFP-WT- CaMKIIα* resulted in a non-significant transient increase in spine volume (367.9 ± 70.1%; $p=0.08$) as well as a significant increase in spine volume from baseline at the 70 min time point (182.9 ± 22.6%; $p=0.008$) (**Fig. 6B**). It is important to note that GFP-WT- CaMKIIα* overexpression also rescues activity-dependent new spine stability. These results suggest that new spine volume changes requires either structural, enzymatic, or both functions of CaMKIIα. They also demonstrate the correlation between new spine volume changes and new spine stability. I decided to further probe the relationship between new spine enlargement and stability, as well as the role of CaMKIIα enzymatic activity in new spine volume changes by analyzing HFU induced spine volume changes at spines when CaMKIIα kinase activity is blocked.

Genetic and pharmacological inhibition of CaMKIIα kinase activity-enhances activity dependent new spine enlargement

As activity-dependent new spine stabilization is not impaired by overexpression of dominant-negative GFP-tagged CaMKIIα-K42R (Chapter 2) therefore, I predicted that activity-dependent new spine enlargement would be unaffected as well. To analyze changes in spine

volume, I calculated changes in bleed-through-corrected and background- subtracted red fluorescent intensity of new spines on the dendrites of organotypic hippocampal CA1 neurons at the -30', +1', and +70' time points in the time-lapse series of data acquired for the experiments described in Chapter 2. As predicted, the stabilizing HFU (described in Chapter 2) at new spines on cells overexpressing my GFP-WT- CaMKIIα control construct induced robust transient new spine enlargement (307.3 ± 52.5%; *p=0.004*). New spine volume remained significantly elevated, although to a smaller degree than the transient growth, by the 70' time point (129.6 ± 12.1%; *p=0.04*) (**Fig. 8B**). Importantly, we found that stimulation at new spines overexpressing GFP-CaMKIIα-K42R still induced a large, transient increase in volume (273.4 ± 39.8; *p=0.003)* followed by a smaller long-term increase from the baseline spine volume at the 70 min time point + (168.1 ± 13.1%; *p=0.0004*) (**Fig. 8B**).

Although there was no difference in the magnitude of transient enlargement 1 min after HFU between new spines on cells expressing either GFP-WT-CaMKIIα or GFP- CaMKIIα-K42R (*p=0.91*), I found that the increase in activity-dependent new spine volume was greater for new spines in the GFP- CaMKIIα-K42R than in the GFP-WT-CaMKIIα (WT: 129.6 ± 12.1%; K42R: 168.1 ± 13.1%; *p=0.03*) (**Fig. 8C**). The magnitude of sLTP at mature spines is well-documented, stereotyped, and comparable across various control conditions (*for examples, see* Matsuzaki, et al., 2004; Bosch, et al., 2013; Stein, et al., 2021). Comparisons are often made between the magnitudes of transient and persistent spine growth across different studies using similar stimulation protocols. I therefore thought to compare the magnitude of spine enlargement of the stimulated new spines in my experiments to the volume changes observed by others in the lab. Hill and Zito (2013) observed a ~60% increase in new spine volume, which is comparable to the volume change I observed on cells expressing GFP- CaMKIIα-K42R, suggesting that overexpression of WT- CaMKIIα reduces new spine volume changes in a manner that is prevented by blocking CaMKIIα kinase activity. This is the inverse of the known role that CaMKIIα enzymatic activity plays at mature spines; inhibiting CaMKIIα kinase activity abolishes

sLTP (Chapter 2; Matsuzaki, et al., 2004; Tullis, et al., 2020; Yamagata, et al., 2009). The role that CaMKIIα enzymatic activity plays in regulating new spine volume changes may be yet another molecular marker that distinguishes new spines from their mature counterparts.

In Chapter 2, I used pharmacological inhibition of CaMKIIα to determine the role of CaMKIIα kinase activity in activity-dependent new spine stabilization without potential overexpression effects. This pharmacological approach can also be used to address the caveats presented by CaMKIIα overexpression, such as the reduction in new spine enlargement on GFP-WT-CaMKIIα when investigating the contribution of new spine volume changes to activity-dependent new spine stabilization. Using the same data set acquired in Chapter 2, I calculated the bleed-through-corrected and background- subtracted red fluorescent intensity of new spines from images captured during -30, +1, and +70 min timepoints in the time-lapse series. I found that vehicle treatment blocked both transient and persistent growth at stimulated new spines (1 min: 112.7 ± 1.9%, *p=0.69*; 70 min: 105.1 ± 0.7%, *p=0.99*) (**Fig. 8E**). Surprisingly, stimulated new spines on cells treated with staurosporine retained robust transient enlargement 1 min after HFU and their volume remained enhanced at the 70 min time point (1 min: 143.0 ± 28.4%, *p=0.47*; 70 min: 172.8 ± 35.9%, *p=0.01*) (**Fig. 8E**). The change in spine volume at the 70 min time point was significantly larger for stimulated new spines incubated in staurosporine than those incubated in vehicle (p*=0.02*) (**Fig. 8F**). I attribute the disruption of structural growth at new spines to their greater diffusional coupling to the dendrite and reduced NMDAR currents (Zito, et al., 2009), which may make them more susceptible to the effects of the vehicle (DMSO), which is known to reduce glutamatergic responses (Lu and Mattson, 2001). Inhibiting CaMKIIα kinase activity with staurosporine does not prevent activity-dependent enlargement at new spines, and the increase in new spine volume is comparable to the amounts observed by Hill and Zito (2013) as well the GFP- CaMKIIα-K42R condition.

While vehicle treatment blocked activity-dependent new spine enlargement, it did not affect activity-dependent new spine stabilization (Chapter 2). I can therefore conclude that

activity-dependent new spine stabilization does not require new spine volume changes. I was surprised to find both genetic and pharmacological inhibition of CaMKIIα retained activity-dependent new spine enlargement, even when control conditions abolished new spine volume changes. Based on these findings, I decided to test the hypothesis that CaMKIIα kinase activity may serve as a brake on activity-dependent volume changes further by evaluating my data from overexpression of CaMKIIα phospho-mutants.

Overexpression of constitutively autonomous CaMKIIα promotes spontaneous new spine enlargement

Phospho-mutants of CaMKIIα can induce spine volume changes independent of CaMKIIα kinase activity, suggesting that CaMKIIα regulates dendritic spine size in a nonenzymatic manner Pi, et al., 2010a. My results suggest, that at new spines, CaMKIIα may negatively regulate activity-dependent spine volume changes. The dominant-negative T286D CaMKIIα phospho-mutant mimics the activity-dependent autophosphorylation of CaMKIIα (Ouyang, et al., 1997), leading to enhanced basal spine volume (Pi, et al., 2010a; Chang, et al., 2017) I decided to see if overexpression of GFP-CaMKIIα-T286D could also increase basal new spine volume. Pairing this mutation with the kinase-dead K42R mutation allowed me to observe changes in basal new spine volume while also testing my hypothesis that CaMKIIα kinase activity limits new spine volume changes.

Mature spine volume and new spine volume changes on cells expressing DsRed alone, GFP-CaMKIIα, GFP-CaMKIIα-T286D or GFP-CaMKIIα-K42R/T286D were calculated from the bleed -through-corrected and background-subtracted red fluorescent intensity of dendritic spines in images captured during the -30, +1, and +70 min timepoints in the time-lapses series collected for the experiments described in Chapter 2. As described in Chapter 2, I did not expose new spines on these cells to my HFU stimulation so that I could observe the basal changes to spine volume and stability effected by these CaMKIIα phospho-mutants.

I estimated basal mature spine volume using images of dendrites captured during the -30 min timepoint in the time-lapse series (**Fig. 9A**). The -90 min baseline images were captured in culture media rather than ACSF, which scatters light and results in underestimation of mature spine volume. When compared to spines on dendrites expressing either dsRed Express alone or with GFP-WT- CaMKIIα, I found that expression of GFP-CaMKIIα-T286D increased estimated basal spine volume by roughly 30% (T286D: 20949.2 ± 1253.5; dsRed: 16650.9 ± 881.5; *T286D vs dsRed: p=0.013*; WT: 15870 ± 2718.1; *T286D vs WT: p=0.039*) (**Fig. 9B**). Overexpression of GFP-WT- CaMKIIα did not have any significant effect on basal spine volume (*p=0.77)* (**Fig. 9B**), therefore I can attribute the changes in basal spine volume specifically to the activity of autonomous CaMKIIα and not to an overabundance of CaMKIIα itself. I also found that GFP-CaMKIIα-T286D enhanced basal spine volume even when paired with the kinase-dead K42R mutation (T286D/K42R: 22967.1 ± 2236.4; *vs dsRed p=0.027; vs WT: p=0.052*) (**Fig 9B**). Importantly, there was no difference in the range of depths of the dendrites used to measure basal spine volume, as spines on dendrites deeper in the tissue may appear smaller and dimmer due to reduced penetration of the laser into the tissue (**Fig 9C**). For the range of dendrite depths used in my experiments, dendrite depth did not correlate with estimated spine volume (**Fig 9D)**.

Basal new spine volume was calculated from the bleed-through-corrected and background- subtracted red fluorescent intensity of new spines that first appeared in the time lapse series after a 60 min incubation in standard culture media. I found no significant difference in baseline new spine volume between any of my conditions (**Fig 9A**). GFP-CaMKIIα-T286D expression induced spontaneous new spine volume changes at stable new spines, even when kinase activity was inhibited with the K42R mutation (T286D: 145.7 ± 10.9%, *p=0.0001*; T286D/K42R: 174.2 ± 16.4, *p=0.0006*) (**Fig 9B, C**). Stable new spines expressing dsRed Express alone or with GFP-WT CaMKIIα did not undergo any significant change from their baseline volume by the 70 min time point (dsRed: 106.2 ± 11.2%, *p=0.98*; WT: 114.5 ± 9.5%,

p=0.60) (**Fig. 10B, C)**. New spines on GFP-CaMKIIα-T286D/K42R also grew to be significantly larger by the 70 min time point than new spines on cells expressing GFP-CaMKIIα-T286D (T286D: 20610.7 ± 1548; T286D/K42R: 29363.6 ± 3110.9; *p=0.008*) (**Fig. 10B)**. I plotted the cumulative frequency of spine volume changes and found that more new spines on cells expressing GFP-CaMKIIα-T286D, with or without CaMKIIα kinase activity, underwent larger volume changes than spines on cells in control conditions (**Fig. 10C, D).**

New spines are known to be significantly smaller than their mature counterparts (Zito, et al, 2009. I found this to be true for new spines on cells expressing DsRed express alone (*p=0.02*) or with GFP-CaMKIIα-T286D (*p=0.002*). I observed no statistical difference in the average estimated baseline volume of new spines compared to the average estimated volume of mature spines (**Fig. 11**) on the same cells expressing GFP-WT CaMKIIα (*p=0.3*) or GFP-CaMKIIα-T286D/K42R (*p=0.2*). I believe a small "n" could contribute to the lack of any statistical differences between basal new and mature spine volume in the GFP-WT CaMKIIα and GFP-CaMKIIα-T286D/K42R conditions.

These findings further corroborate my conclusion that new spine volume changes are not required for new spine stabilization, as stable new spines expressing dsRed Express alone or with GFP-WT CaMKIIα did not grow larger, although manipulations that enhance new spine stability also tend to increase new spine volume. I can also conclude that at new spines specifically, CaMKIIα promotes volume changes in a manner that requires its autonomous, structural or scaffolding functions, but may be negatively regulated by its enzymatic activity.

DISCUSSION

Activity-dependent new spine stabilization does not require new spine volume changes

Non-enzymatic CaMKIIα is required for activity-dependent new spine stabilization, however the precise structural and scaffolding interactions that CaMKIIα facilitates to enhance new spine survivorship remain unexplored. Interestingly, previous work has demonstrated a link

between spine volume and stability and shows that stabilizing HFU also induces new spine enlargement (Hill and Zito, 2013). CaMKIIα plays a role in regulating dendritic spine size, and likely engages in several concurrent structural and enzymatic interactions to regulate spine volume changes downstream of glutamatergic stimulation (Araki, et al., 2015; Matsuzaki, et al., 2004; Pi, et al., 2010a; Saneyoshi, et al., 2019; Yamagata, et al., 2009). Some of these interactions might also be necessary for activity-dependent new spine stabilization. I therefore hypothesized that new spine volume changes are necessary for activity-dependent new spine stabilization and that these volume changes are mediated by non-enzymatic CaMKIIα activities. I predicted that investigating the discrete roles that CaMKIIα structural and enzymatic activities play in regulating new spine volume changes could provide insight into other molecular interactions that may be necessary for new spine stabilization.

CaMKIIα knock-down impaired activity-dependent new spine stabilization and, very promisingly, also blocked new spine volume changes, both of which were rescued by over-expression of shRNA-resistant CaMKIIα. Remarkably, I also found that activity-dependent new spine enlargement occurred even when CaMKIIα was genetically or pharmacologically blocked, which is also consistent with the retention of enhanced survivorship described in Chapter 2. These results initially supported my hypothesis; however, I was surprised to find that HFU-induced new spine enlargement was blocked on vehicle (control condition) treated cells. DMSO was used as the vehicle in these experiments, and it is known to reduce both NMDAR and AMPAR currents (Lu and Mattson, 2001) which are crucial for structural growth (Hill and Zito, 2013; Lang, et al., 2004; Matsuzaki, et al., 2004; Murakoshi, et al., 2011). Consistent with this, I did observe reduced sLTP at mature spines incubated in the vehicle condition (Chapter 2). New spines are known to already have weakened NMDA-receptor mediated currents as well as greater diffusional coupling to the dendritic shaft (Zito, et al., 2009) and therefore may be more susceptible to the effects of vehicle incubation and lead to an impairment of activity-dependent new spine volume changes. Despite this, stimulated new spine were more stable than their

unstimulated counterparts following incubation in vehicle, indicating that new spine volume changes are not necessary to enhance new spine survivorship.

Interestingly, spine size and volume changes are typically reliable predictors of dendritic spine stability (Araki, et al., 2015; De Roo, et al., 2008a; Hill and Zito, 2013; Kasai, et al., 2003). It is possible that the relationship between spine size and stability at more mature spines are regulated by synchronous but discrete molecular mechanisms and that, similar to new spines, spine volume changes do not confer stability, but simply co-occur with other stabilizing molecular processes. Alternatively, as new spines mature, the changing biochemical landscape (De Roo, et al., 2008b; Lambert, et al., 2017) might introduce new molecules and interactions that make volume changes necessary for stabilization. More work is needed to elucidate the role spine volume changes may play at new spines as they mature.

Role of CaMKIIα structural/scaffolding activity in new spine volume changes

There is substantial evidence that CaMKIIα regulates spine volume at mature spines via both structural/ scaffolding interactions and kinase activity. KN93/KN62 incubation blocks GluN2B binding by preventing necessary $Ca^{2+/}$CaM binding (Bayer, et al., 2001), which also blocks sLTP (Matsuzaki, et al., 2004), implicating both structural/ scaffolding and kinase roles in new spine volume changes. The interaction between CaMKIIα and GluN2B is crucial for activity-dependent spine volume changes and functional enhancement, as tatCN21 application at concentrations that prevent activity-dependent GluN2B binding but do not reverse it (Vest, et al., 2007) block both structural (Stein, et al., 2021) and functional LTP (Buard, et al., 2010) at mature spines. My results suggest that CaMKIIα is similarly required for spine volume changes at new spines. Furthermore, I show that these volume changes are not dependent on CaMKIIα kinase activity. While new spine volume changes are not necessary to enhance new spine survivorship following glutamatergic stimulation, I believe it is worth investigating the role

CaMKIIα in new spine volume changes further to enhance our knowledge of new spines and identify the ways they are molecularly distinct from mature spines.

It is still unclear what proteins interact with CaMKIIα to assist it's structural and scaffolding roles in new spine volume changes. Obvious candidates include those that interact with both the actin cytoskeleton and CaMKIIα in a non-enzymatic manner, such as α-actin and densin-180 (Hell, 2014). CaMKIIα, α-actinin, and densin-180 form a complex that may serve to stabilize the actin cytoskeleton following activity-induced restructuring of actin filaments (Walikonis, et al., 2001) Other putative non-enzymatic interacting partners are the 26S proteasome and synGAP-1α, which I also proposed as potential regulators of new spine stability in Chapter 2 and further explore in Chapter 4. While no role for the proteasome in dendritic spine volume changes has been identified, synGAP-1α is known to regulate dendritic spine maturation, AMPAR content, and spine volume changes (Clement, et al., 2012; Vazquez, et al., 2004). Future experiments should evaluate the role of actin binding proteins, synGAP-1α, and the 26S proteasome to expand our knowledge of the molecular composition of new spines. In Chapter 4, I explore the role of the 26S proteasome and synGAP-1α in activity-dependent new spine stabilization, and I believe that my preliminary findings yield promising new research avenues that will allow us to better understand new spines and the molecular mechanisms that govern their stability.

Role of CaMKIIα enzymatic activity in new spine volume changes

I made the surprising discovery that CaMKIIα is required for activity-dependent spine enlargement at new spines, but that only structural/scaffolding activities are required. In fact, my results suggest that CaMKIIα kinase activity may restrict activity-dependent new spine volume changes. CaMKIIα kinase inhibition by overexpression of the dominant-negative GFP-CaMKIIα-K42R mutant permits activity-dependent new spine enlargement, however the magnitude of volume change is significantly larger than on cells overexpressing GFP-WT-CaMKIIα. More compellingly, I found that while incubation in DMSO prevented new spine enlargement following

HFU, blocking CaMKIIα kinase activity by incubating cells in staurosporine restored activity-dependent new spine volume changes. I also observed a trend towards larger spontaneous spine volume changes when the T286D mutation was overexpressed with the additional K42R mutation (T286D/K42R) than when T286D is overexpressed alone. Together, these findings support a role for CaMKIIα kinase activity that curbs activity-dependent volume changes at new spines.

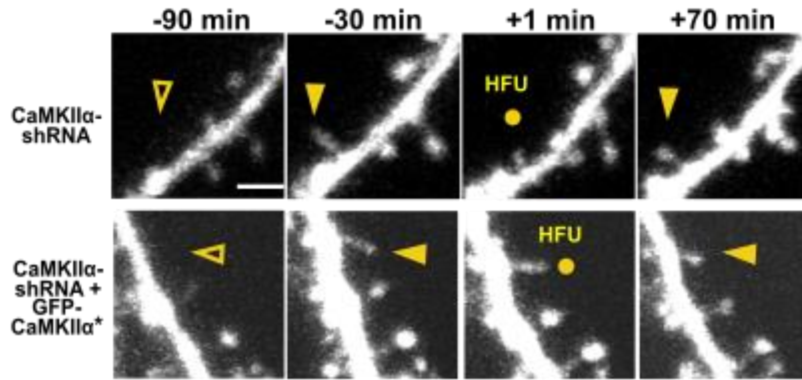
How and why might new spines require CaMKIIα kinase activity to limit the magnitude of activity-dependent volume changes? CaMKIIα kinase activity is required for the activation of the small GTPase Rap1 (Koeberle, et al., 2017; Lim, et al., 2011). Rap1 regulates dendritic spine morphology, and it has been shown that constitutively activity Rap1 increases dendritic spine length while inactive Rap1 increases spine volume (Xie, et al., 2005). Koeberle and colleagues (2017) found that inhibiting CaMKIIα kinase activity by either knocking in the K42R mutation or incubation with KN93 decreased Rap1 activity in dendritic spines, therefore, CaMKIIα kinase activity may curtail new spine enlargement by activating Rap1. Intriguingly, that same study reported increases in spine density and spine outgrowth when CaMKIIα kinase activity was inhibited for at least 24 hours (Koeberle, et al., 2017). I did not observe these changes, which I attribute to differences in the expression time of my kinase-dead genetic interventions and in the incubation time of my pharmacological approach. It was also noted that dispersion of synGAP-1α, a Ras and Rap GAP, might contribute to enhanced Rap1 activity following stimulation. CaMKIIα also phosphorylates synGAP-1α, which biases its GAP activity towards Rap1 (Walkup, et al., 2015), so the interplay between activity-dependent synGAP-1α dispersion and phosphorylation should be further examined in the context of new spine stabilization and enlargement to provide further insight into the role of CaMKIIα kinase activity.

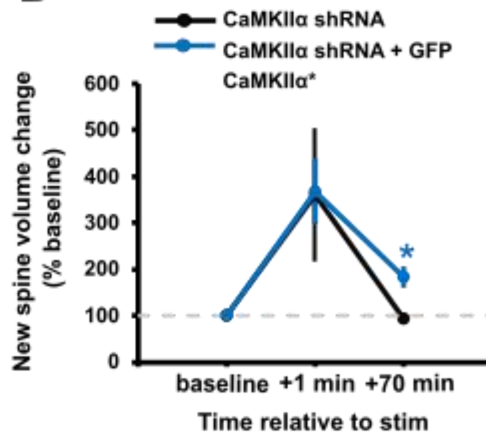
FIGURE LEGENDS and FIGURES

Figure 7: CaMKIIα knock-down blocks activity-dependent new spine enlargement.

(A) Images of dendrites (red channel) on CA1 hippocampal neurons in slice culture (DIV 7-9) expressing a dsRed-Express cell fill and either CaMKIIα shRNA (top) or CaMKIIα shRNA + shRNA-resistant GFP-CaMKIIα* (bottom). Representative images presented are different cells from the same data set described in Chapter 2, which were included to visualize new spine volume changes. Spontaneous new spine outgrowth (filled arrowheads) was observed in both conditions. HFU (yellow circle) was used to stimulate a single new spine per cell. ***(B)*** HFU induced transient new spine enlargement returned to baseline in the CaMKIIα knock-down condition (filled black circles; n= 10 spines/ 10 cells). Transient and sustained new spine enlargement occurred following the GFP-CaMKIIα* rescue (filled blue circles; n=11 spines/11 cells). ***(C)*** Activity-dependent new spine enlargement at 70 min (filled bars) was blocked at stimulated new spines following knockdown of CaMKIIα (black) but is enhanced when CaMKIIα is rescued with shRNA-resistant CaMKIIα* (blue). Two-way ANOVA with Dunnett's test to baseline used in ***B*** and unpaired two-tailed t-test ***C***. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 7





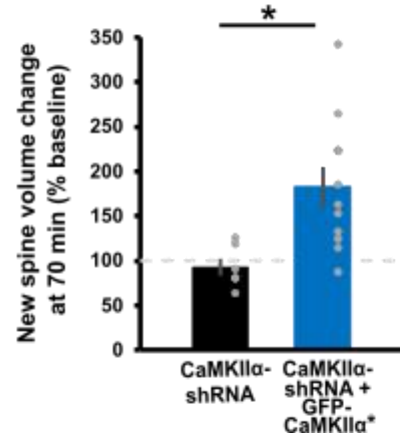


Figure 8: Genetic and pharmacological inhibition of CaMKIIα kinase activity enhances activity-dependent new spine enlargement.

(A) Images (red channel) of a spontaneous new spine outgrowth (filled yellow arrowhead) on DIV 7-9 hippocampal CA1 neurons expressing dsRed-Express and either GFP-WT-CaMKIIα (top row) or GFP-CaMKIIα-K42R (bottom row) from the same date set described in Chapter 2. One new spine per neuron was stimulated with HFU (filled yellow circle). ***(B)*** HFU induced significant transient and sustained enlargement at stimulated new spines on cells expressing either GFP-WT-CaMKIIα (filled black circles; n= 16 spines/16 cells) or GFP-CaMKIIα-K42R (filled blue circles; n=14 spines/14 cells). ***(C)*** Stimulated new spines on cells expressing GFP-CaMKIIα-K42R grew significantly larger than stimulated new spines on cells expressing GFP-WT-CaMKIIα at the 70-minute time point. (***D)*** Images (green channel) from the same data set shown in Chapter 2 showing spontaneous new spine outgrowth (filled arrowhead at 0 min) on dendrites of GFP-transfected hippocampal CA1 neurons (DIV 7-9). One new spine per neuron was stimulated with HFU following 30 min pre-incubation in either vehicle (top row) or 1 μm staurosporine (Sta; bottom row). ***(E)*** HFU failed to induce either a transient or sustained volume change at stimulated new spines following incubation in vehicle (filled black circles; n=9 spines/ 9 cells). HFU at new spines on cells incubated in staurosporine induced a transient increase in spine volume followed by a much larger sustained volume change. ***(F)*** Stimulated new spines on cells incubated in staurosporine had significantly larger new spine volume changes at the 70-minute time point following HFU than new spines on cells incubated in vehicle. Two-way ANOVA with Dunnet's test to baseline used in ***B*** and ***E****.* Unpaired two-tailed t-test ***C and F****.* *p <0.05, **p < 0.01, ***p < 0.001.

Figure 8

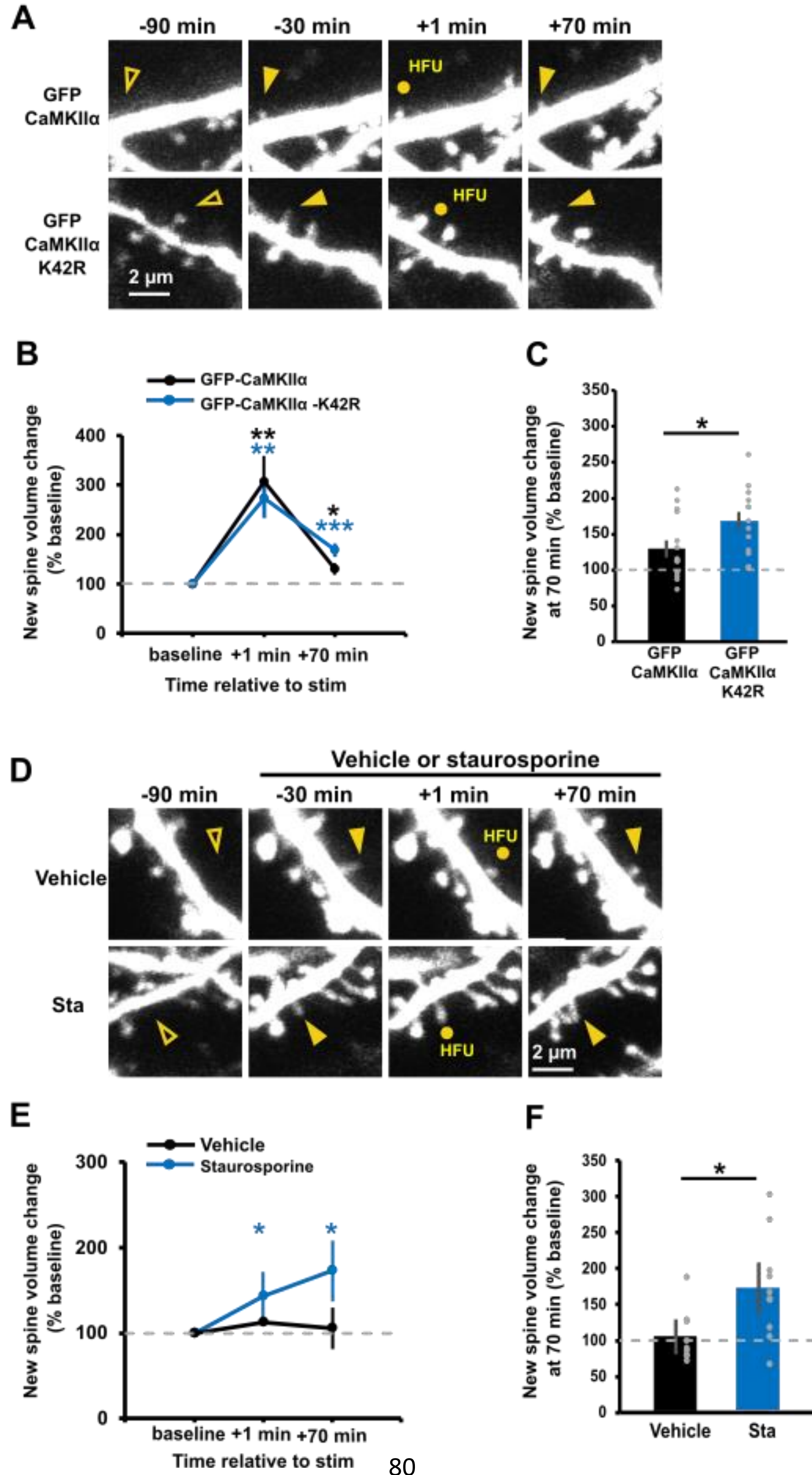


Figure 9: Overexpression of constitutively autonomous CaMKIIα enhances basal spine volume.

(A) Representative images (red channel) of dendrites on organotypic hippocampal CA1 neurons (DIV 7-9) expressing dsRed Express alone (far left) or with either GFP-WT-CaMKIIα, GFP-CaMKIIα-T286D, or GFP-CaMKIIα-T286D/K42R (left to right). Representative images were selected from images acquired for experiments described in Chapter 2. ***(B)*** Overexpression of GFP-WT-CaMKIIα (gray bar, n= average of all spines on 28 dendrites/ 10 cells) did not have any effect on basal spine volume compared to spine volumes on cells expressing dsRed express alone (black bar; average of all spines on 35 dendrites/ 10 cells). Overexpression of GFP- CaMKIIα-T286D increased basal new spine volume (blue bar; average of all spines on 45 dendrites/ 12 cells). CaMKIIα kinase activity is not required to enhance basal new spine volume, as overexpression of GFP-CaMKIIα-T286D/K42R (light blue bar; average of all spines on 30 dendrites/ 8 cells) also increased basal new spine volume. ***(C)*** There was no difference in the depths of the dendrites used to determine basal spine volume in B. ***(D)*** Dendrite depth and the average estimated spine volume were not correlated. One-way ANOVA with Bonferroni correction for multiple comparisons used in ***B*** and ***C*** Pearson's correlation coefficient in ***D***. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 9

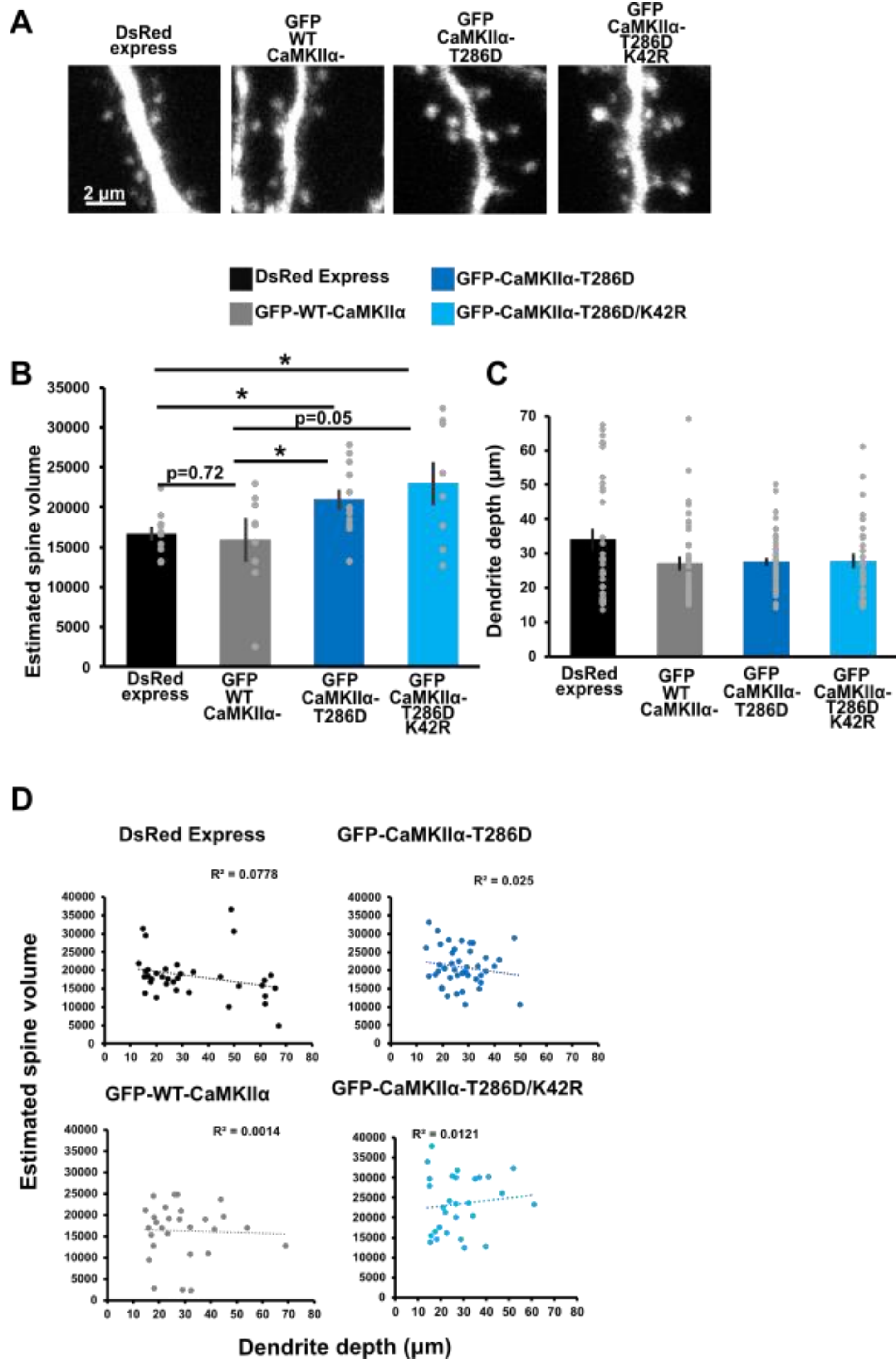


Figure 10: Overexpression of constitutively autonomous CaMKIIα promotes spontaneous new spine enlargement.

(A) There was no significant difference in the volume of newly formed spines identified during the second image in the time-lapse series between cells expressing either dsRed alone or with either GFP-WT-CaMKIIα, GFP- CaMKIIα-T286D, or GFP-CaMKIIα-T286D/K42R. ***(B)*** Stable spines on cells expressing dsRed alone or with GFP-WT-CaMKIIα did not undergo volume changes that were observable at the final 70-minute time point. Overexpression of GFP-CaMKIIα-T286D or GFP-CaMKIIα-T286D/K42R induced spontaneous new spine enlargement at stable new spines. ***(C)*** Magnitude of spontaneous new spine volume changes represented as % increase from basal spine volume. ***(D)*** Cumulative probability distribution of new spine volume changes at the 70-minute time point. One-way ANOVA was used in ***A*** and paired two-tailed t-tests were used in ***B***. Kruskal-Wallis test with Dunn's multiple comparisons test used in ***D***. *p <0.05, **p < 0.01, ***p < 0.001.

Figure 10

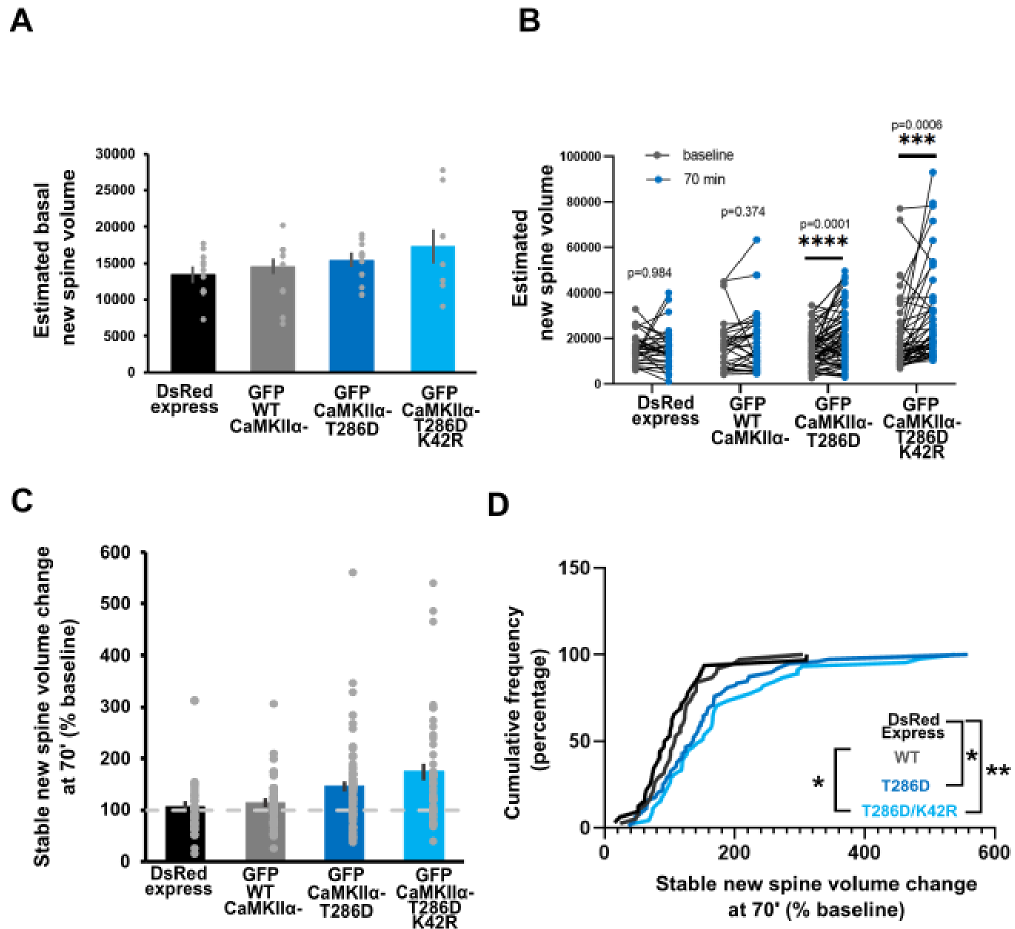


Figure 11: Mature spines were significantly larger than new spines on cells expressing DsRed Express alone or with GFP-CaMKIIα-T286D

(A) There was no significant difference in the volume of newly formed spines compared to mature spines on cells expressing dsRed with either GFP-WT-CaMKIIα or GFP-CaMKIIα-T286D/K42R. Mature spines were significantly larger on cells expressing dsRed alone or with GFP-CaMKIIα-T286D. Student's unpaired t-test. *p <0.05, **p < 0.01, ***p < 0.001.

Figure 11

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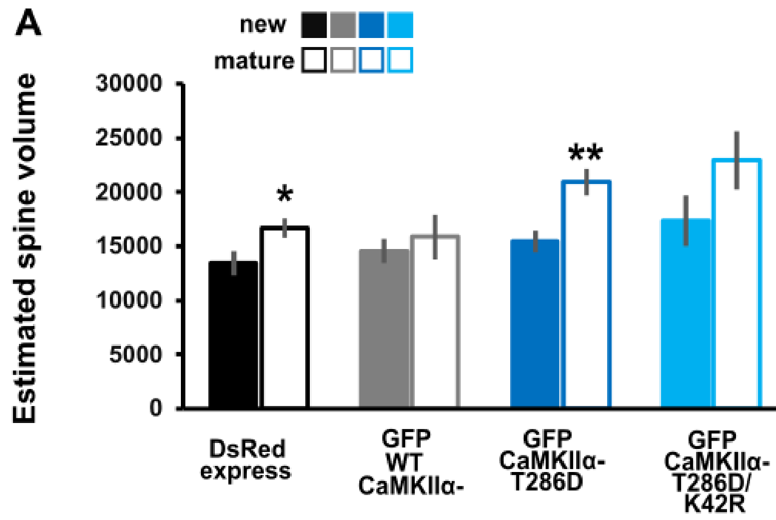


CHAPTER 4: INVESTIGATION OF PUTATIVE CaMKIIα SCAFFOLDING MOLECULES REQUIRED FOR ACTIVITY-DEPENDENT NEW SPINE STABILIZATION

PREFACE

The following chapter contains unpublished work aimed investigating potential molecules that may be involved in CaMKIIα's non-enzymatic roles in new spine stabilization. First, I investigate the role of the 26S proteasome in activity dependent new spine stabilization. I then explore the role of synGAP-1α in activity-dependent new spine stabilization, focusing on its enrichment levels at new spines as well as its activity-dependent dispersion. Finally, I investigated a putative mechanism for synGAP-1α accumulation and dispersion at new spines. I acknowledge the intellectual contributions of my dissertation advisor, Dr Karen Zito, and my dissertation committee: Drs. Elva Diaz, John Gray, Kim McAllister, Johannes Hell, and Michael Ferns. I would also like to acknowledge the input of my peers in the lab, specifically Sam Petshow and Juan Flores. I would like to thank Drs. Richard Huganir and Yoichi Araki for the generous gift of the GFP-tagged synGAP-1α construct used in the following experiments. Finally, I would like to thank myself for conceiving of and conducting all the following experiments and doing all of the intellectual work that went into the figures, analyses, discussion and rationale. This is truly the only bit of my dissertation work that is completely intellectually my own and I believe that deserves acknowledgement.

INTRODUCTION

Learning and memory require the dynamic restructuring of synapses, which can include the modification of existing synaptic weights as well as the addition of new dendritic spines to form new synaptic connections, and the pruning of redundant synapses. The addition of new dendritic spines to form novel connections is particularly important, and learning has been shown to induce the formation of new dendritic spines (Hayashi-Takagi, et al., 2015; Roberts, et al., 2010; Xu, et al., 2009; Yang, et al., 2009). Importantly, the persistence of these new spines predicts memory of learned experiences (Xu, et al., 2009; Yang, et al., 2009), although the mechanisms that determine their persistence are still poorly understood. Recent studies have identified the role strong, glutamatergic stimulation as one molecular mechanism that enhances the stability of new dendritic spines (Hill and Zito, 2013). Downstream of glutamatergic stimulation, the interaction between CaMKII and the GluN2B subunit of the NMDAR are required to facilitate further molecular interactions between CaMKIIα and other molecules that enhance new spine survivorship (Hill and Zito, 2013). In Chapter 2, I discovered that CaMKIIα plays a non-enzymatic role in activity-dependent new spine stabilization, however, the particular structural and scaffolding interactions made by CaMKIIα to promote new spine survivorship have yet to be identified.

There is little understanding of the non-enzymatic roles that CaMKIIα plays at dendritic spines, making it difficult to identify which non-enzymatic interactions may be involved in activity-dependent new spine stabilization. Several molecules are known to interact with CaMKIIα, both directly and indirectly, and a handful of these interactions merit further investigation to determine if they are involved in CaMKIIα's non-enzymatic roles in new spine stabilization. I chose to focus on two molecules that interact with CaMKIIα, the 26S proteasome and synGAP-1α, and my work exploring their roles in activity-dependent new spine stabilization will be discussed in this chapter.

The 26S proteasome is a critical component of the ubiquitin-proteasome system and several studies have implicated its role in protein degradation as a necessary step in activity-dependent synaptic restructuring (Bingol and Sheng, 2011; Türker, et al., 2021). Recent evidence has also suggested that the 26S proteasome may serve a function at nascent dendritic spines, as it has been shown that inhibition of the proteasome inhibits activity-dependent new spine outgrowth (Hamilton, et al., 2012; Hamilton, et al., 2017) While the question of whether the 26S proteasome is required for stabilizing these new spines remains, some of the existing literature suggests it may indeed have a role that intertwined with CaMKIIα's non-enzymatic functions at new spines. Promisingly, CaMKIIα directly binds the proteasome, thereby facilitating the activity-dependent accumulation of the proteasome to dendritic spines in a manner that is both independent of CaMKIIα enzymatic activity and enhanced by the pseudo-autophosphorylated T286D mutant (Bingol, et al., 2010). This led me to hypothesize that HFU at a new spine recruits the proteasome to stimulated new spines via interactions with CaMKIIα that lead to the degradation of negative-regulators of spine stability; consequently, inhibiting the proteasome should block activity-dependent new spine stabilization.

The Ras-Rap GAP synGAP-1α is another promising molecule that might contribute to the non-enzymatic role CaMKIIα performs in activity-dependent new spine stabilization. SynGAP-1α is a known negative regulator of dendritic spine outgrowth, maturation, and plasticity, as evidenced by the precocious accumulation of PSD-family MAGUKs, increased spine volume, and increased synaptic strength observed when synGAP-1α is knocked down or knocked out (Aceti, et al., 2015; Araki, et al., 2015; Clement, et al., 2012; Vazquez, et al., 2004). Interestingly, synGAP-1α does not bind CaMKIIα directly, but through interactions with either PSD-family MAGUKs or the multi-PDZ-domain protein 1, MUPP1 (Kim, et al., 1998; Krapivinsky, et al., 2004). The low expression levels of PSD-family MAGUKS at new spines makes the CaMKII-MUPP1-synGAP- 1α complex a more likely candidate for any potential role synGAP-1α may be playing at new spines (De Roo, et al., 2008b; Lambert, et al., 2017).

Because synGAP-1α negatively regulates dendritic spine stability and maturation, I predicted that it may also be required in activity-dependent new spine stabilization. Specifically, new spine stabilization may require the removal of or exclusion of negative regulators of spine maturation and stability. Structural and functional synaptic plasticity requires the removal of synGAP-1α from stimulated spines (Araki, et al., 2015), and changes at new spines that promote stabilization may also require synGAP-1α evacuation. While the dispersion of synGAP-1α that is anchored to CaMKIIα via interactions with PSD95 requires CaMKIIα kinase activity (Araki, et al., 2015), dissociation of the CaMKIIα -MUPP1-synGAP-1α complex does not. I predicted that the CaMKIIα -MUPP1-synGAP-1α interaction facilitated synGAP-1α accumulation at new spines, and that inhibition of CaMKIIα kinase activity would not disrupt activity-dependent synGAP-1α dispersion, thereby providing a mechanism for activity-dependent stabilization that relies on the non-enzymatic roles of CaMKIIα.

Activity-dependent new spine stabilization requires non-enzymatic CaMKIIα functions, but the specific structural and scaffolding interactions that CaMKIIα makes to support this non-enzymatic role are yet to be determined. Here, I explored the role of CaMKIIα's interactions with the 26S proteasome and synGAP-1α in activity-dependent new spine stabilization. I found that the 26S proteasome plays an unexpected role in basal new spine survivorship that made it difficult to draw conclusions about its involvement in activity-dependent new spine stabilization. I also show that synGAP-1α is less enriched at new spines compared to their mature counterparts, but still undergoes activity-dependent dispersion in a manner that does not appear to require CaMKIIα kinase activity. My results provide novel insights into the molecular mechanisms that govern nascent dendritic spine stability, activity-dependent or otherwise, and opens promising new research avenues that can be built upon to further expand our understanding of learning and memory.

METHODS

Preparation and transfection of organotypic slice cultures

Organotypic hippocampal slice cultures were prepared from postnatal day (P) 6-8 C57BL/6J wild-type mice of both sexes, as described (Opitz-Araya and Barria, 2010; Stoppini, et al., 1991). Neurons were transfected 2-3 days prior to imaging using particle-mediated gene transfer, as described (Woods and Zito, 2008), except 6-8 µg of DsRed-Express (Clontech) and 6 µg of mEGFP-tagged synGAP-1α (Araki, et al., 2015) or 5-10 µg of mEGFP were coated onto 6-7 mg of 1.6 µm gold beads.

Two-photon imaging

Image stacks (512 X 512 pixels, 1 µm z-steps) of 4-6 secondary and tertiary, apical and basal dendritic segments from CA1 pyramidal neurons (6-10 DIV) were acquired on a custom two-photon microscope with a pulsed Ti::Sapphire laser (930 nm, 0.5-3 mW at the sample; Spectra Physics, Newport). Data acquisition was controlled by ScanImage (Pologruto, et al., 2003) written in MATLAB (MathWorks). All images shown are maximum projections of 3D image stacks after applying a median filter (3 X 3). The first time point was acquired in slice culture medium at room temperature (RT) and the slice was maintained in the incubator (35°C) for 1 h between first and subsequent acquisitions. After 1 h, the slice was placed in a bath of recirculating, oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 127 NaCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 2.5 KCl, 25 D-glucose, ~310 mOsm, pH 7.2) with 2 mM Ca^{2+}, 0-0.1 mM Mg^{2+}, and 1 µM tetrodotoxin at 31°C for 30 min prior to uncaging. 2.5-3.5 mM of 4-methoxy-7-nitroindolinyl-caged L-glutamate (MNI-glutamate) was added for uncaging experiments. When needed, lactacystin (10 µM), MG-132 (10 µM), staurosporine (1 µM) or an equivalent volume of vehicle were added to the bath 30 min prior to uncaging.

Identification new spines and estimation of spine size

I defined new spines as any protrusion emanating from the dendrite that was present in the second and/or third images in the time-lapse series (60-90 min later) but not detectable in either

the red or green channels in the first image. Spines of ambiguous persistence or presence due to fluctuations in dendrite swelling, spine motility, or spine drift in the z-axis were excluded. Spine size was estimated from bleed-through-corrected and background-subtracted red (DsRed-Express) fluorescence intensity. Spine brightness measurements give an accurate estimate of relative spine size when compared with electron microscopy (Holtmaat, et al., 2005).

High frequency uncaging (HFU) stimulus

The HFU stimulus consisted of 60 pulses (720 nm, 8-10 mW at the sample) of 2 ms duration delivered at 2 Hz in the presence of 2.5-3.5 mM MNI-glutamate by parking the beam at a point ~0.5 μm from the spine head away from the dendrite.

Quantification of relative enrichment of GFP-tagged proteins

Relative enrichment of GFP-tagged proteins in dendritic spines was calculated using bleed-through-corrected and background-subtracted green (GFP) and red (DsRed-Express) fluorescence intensities from spines and dendrites, as described (Woods, et al., 2011). Briefly, the ratio of green fluorescence intensity to red fluorescence intensity (G/R) was calculated for each new spine, size-matched neighboring persistent spines (6-10), and three representative regions on the dendritic shaft (excluding regions dendrite swelling and GFP-puncta, which were indicative of the presence of a z spine). To quantify spine fluorescence intensities, boxes were drawn around whole spines and spine necks using custom software written in MATLAB. Background subtraction was done by drawing a box next to a target spine that was equal on the axis perpendicular to the dendrite as the box drawn around the spine head and neck. The average intensity of that box was multiplied by the number of pixels in the target spine box and subtracted from the integrated intensity from the target spine box. Relative enrichment of spines was calculated by normalizing the G/R ratio of the target spine to the mean G/R ratio of three locations on the adjacent dendrite. Mature neighbor enrichment levels were represented by the average relative enrichment of all size-matched neighbors on a dendrite that grew a new spine.

Several criteria were used to ensure that analyzed data were of high quality. Cells that exhibited lower green fluorescence intensity than the background ROI were excluded. Cells with extremely high levels of GFP-tagged protein expression such that synaptic enrichment was lost were excluded. Cells were also excluded if after background and bleed-through subtraction, the following criteria were met: (1) the value of the mean green pixel intensity (G) from neighbor spines was less than 3.23 a.u., (2) the value of the mean neighbor spine G/R was less than 0.01, or (3) the ratio of the square of the mean persistent spine G/R to the absolute value of the mean dendrite G/R was less than 0.05. These criteria allowed unbiased exclusion of cells that returned negative pixel intensity values after background and bleed-through subtraction. Cells that exhibited significant photobleaching (a decline in average integrated fluorescence intensity in the dendrite greater than 20% compared to the first time point) in either the red or green channels were excluded.

Statistical Analysis

To compare survivorship at individual time points, I used Fisher's exact test. For comparisons of spine enrichment between two time points (i.e., -30 min vs +1 min) or two conditions at a single time point (+1 min, new vs neighbors), I used a two-tailed unpaired heteroscedastic Student's *t* test. Comparisons of new spine enrichment levels for more than two time points, such as that conducted in **Fig 3C**, required fitting the data to a mixed-effects model with appropriate *post-hoc* tests for multiple comparisons to account for the loss of unstimulated new spines. Error bars represent standard error of the mean (SEM).

RESULTS

Pharmacological inhibition of the 26S proteasome increases basal new spine survivorship

CaMKIIα acts as a scaffold for the 26s proteasome in a manner that does not require CaMKIIα kinase activity. I tested whether the 26S proteasome might be necessary for activity-dependent new spine stabilization by pharmacologically inhibiting the 26S proteasome and

exposing new spines to our stabilizing HFU protocol. New spines on GFP-expressing hippocampal CA1 neurons were identified using 2-p time lapse imaging (**Fig 12A**). Cells were incubated in a saturating concentration (10 μM), Hamilton, et al., 2012), of the selective 26S proteasome inhibitor, lactacystin or an equivalent volume of vehicle, for 30-35 minutes, after which a single new spine per cell was stimulated with HFU. Stability was monitored using time lapse imaging of identified new spines at 10 min intervals for a total of 70 min. Surprisingly, I found that incubation in lactacystin enhanced basal new spine survivorship even in the absence of HFU (**Fig 12B, C;** unstim: 85%). HFU did not induce any statistically significant increase in new spine stability at the 70 min time point on cells incubated with lactacystin (stim: 90%; p>0.99). This makes it difficult to determine if inhibition of the 26S proteasome impairs activity-dependent new spine stabilization, since basal stabilization rates appear to be enhanced by the loss of 26S proteasome activity. I observed normal basal spine survivorship rates and activity-dependent enhancement of stability on cells incubated in the vehicle condition (**Fig 1B, C**; stim: 92%; unstim: 68%; p=0.104). Unstimulated new spines were significantly more stable on cells incubated in lactacystin than unstimulated new spines that were incubated in the vehicle control (**Fig 12B, C**; p=0.024). The data from this experiment make it difficult to determine the role of the 21S proteasome on activity-dependent new spine stabilization since rates of basal survivorship were high enough to mask any deficits in activity-dependent survivorship.

I attempted to corroborate my findings that inhibition of the 26S proteasome enhances basal new spine survivorship by incubating cells in a different 26S proteasome inhibitor, MG-132. New spine identification and stimulation were conducted using the methods described above, except cells were incubated in 10 μM MG-132 or an equivalent volume of vehicle for 30 min prior to stimulation (**Fig 12D**). While I decided not to continue experiments with MG-132 due to issues with cell health and concerns regarding drug specificity, my preliminary data differs from what I observed with lactacystin incubation, although caution should be exercised when drawing any conclusions from these data due to the small sample size. Survivorship rates were

statistically indistinguishable between stimulated and unstimulated new spines following 30 min incubation in MG-132 (**Fig 12E, F**; stim: 75%; unstim: 81%; p>0.999). Stimulated spines appeared to be more stable than unstimulated spines on cells incubated only in vehicle (**Fig 12E, F**; stim: 100%; unstim: 73%; p=0.268), although I was unable to reach significance due to the small sample size.

Ultimately, it was decided that the role of the 26S proteasome in activity-dependent new spine stabilization should not be further explored since the high basal stabilization rate of unstimulated spines makes it difficult to determine if activity-dependent survivorship is affected.

GFP-synGAP-1α enrichment in new spines is lower than that in size-matched neighboring spines

Investigating a putative role for synGAP-1α in activity-dependent new spine stabilization first required me to determine the expression patterns of synGAP-1α at new spines. While synGAP-1α is enriched in the postsynaptic density (PSD) (Gamache, et al., 2020), it is known that the enrichment levels of PSD-family MAGUKs, key components of the postsynaptic density, are remarkably low at new spines (De Roo, et al., 2008a; Lambert, et al., 2017), meaning other proteins normally found at the PSD might be similarly unenriched at new spines. Additionally, PSD-family MAGUKs facilitate the synaptic accumulation of synGAP-1α (Araki, et al., 2015; Kim, et al., 1998), therefore their low expression at new spines might also result in low expression levels of synGAP-1α.

I used biolistic transfection to sparsely express GFP-tagged synGAP-1α and dsRed Express in organotypic hippocampal CA1 neurons. Spontaneous new spine outgrowth was observed using 2-photon time lapse imaging (**Fig. 13A**). I found that the synaptic enrichment levels of GFP- synGAP-1α were significantly lower than the levels at size-matched neighboring spines on the same cells (**Fig 13B, C**; new: 1.56 ± 0.17; neighbors: 3.72 ± 0.24; $p<0.001$). Despite these low enrichment levels, I wondered if synGAP-1α could still negatively regulate activity-dependent changes at new spines.

HFU induces GFP-synGAP-1α dispersion at new spines

SynGAP-1α negatively regulates dendritic spine morphology, maturation, and function, and synaptic activity has been identified as a mechanism that induces the removal of synGAP-1α from spines, thus permitting structural and functional changes at these spines. These changes may be necessary for activity-dependent new spine stabilization, and it may therefore be necessary that synGAP-1α must be removed from new spines, despite low enrichment levels. I hypothesized that synaptic activity would cause the dispersion of GFP-synGAP- 1α from stimulated new spines.

Time-lapse imaging of dendrites on neurons expressing dsRed-Express GFP-synGAP-1α was used to observe multiple new spines that spontaneously grew on each cell (**Fig. 14A**). One new spine per cell was exposed to HFU stimulation. As synGAP-1α is a negative regulator of dendritic spine maturation and plasticity, I was concerned that overexpression of GFP-synGAP-1α may alter spine both basal and activity-dependent spine survivorship. To determine if new spine stability was affected by overexpression effects, I monitored the survivorship of stimulated and unstimulated new spines on the same cells using time-lapse imaging. The survivorship rates of unstimulated new spines were comparable to those observed in my other previously conducted experiments, and I am confident that overexpression of GFP-synGAP-1α did not interfere with my ability to investigate the role of synGAP-1α in new spine stabilization. I also found that activity-dependent new spine stabilization was unaffected, as stimulated new spines on cells overexpressing GFP-synGAP-1α were significantly more stable than unstimulated new spines on the same cells (**Fig 14B**; stim: 100%; unstim: 63%; $p=0.04$).

To determine if activity-dependent synGAP-1α dispersion occurs at new spines, I monitored the GFP-synGAP-1α enrichment levels at stimulated and unstimulated spines -30 min, +1 min, and +30 min following stimulation of a single new spine per cell with the same stabilizing HFU stimulus as described in my previous experiments. There was a trend towards higher basal (-30 min) enrichment levels at stimulated new spines compared to unstimulated

new spines (stimulated: 2.1 ± 0.40; unstimulated: 1.35 ± 0.24; *p=0.11*), which I attribute to unintentional bias in the selection criteria for spine stimulation, which are criteria I have used for all experiments. Briefly, new and mature spines selected for stimulation were typically on dendrites with lower spine densities compared to others on the cell and I favored medium-sized spines that were ~2μm from any nearby neighbors. While I did not investigate other factors that may have contributed to the discrepancies in enrichment levels between stimulated and unstimulated new spines, I was careful to avoid this selection bias in subsequent uncaging experiments. I found that enrichment levels plummeted significantly at stimulated spines +1 min following exposure to HFU (**Fig 14Ai, C-D;** -30 min: 2.1 ± 0.4; +1 min: 1.3 ± 0.31; percent change: 65 ± 0.71%; *p=0.01*). GFP-synGAP-1α levels at unstimulated new spines did not decrease and in fact increased between the -30 min and + 1 min time points (**Fig 14Aii, C-D;** -30 min: 1.35 ± 0.24; +1 min: 1.93 ± 0.20; percent change: 227 ± 446%; *p=0.09*). GFP-synGAP-1α enrichment levels did not significantly change at mature, neighboring spines, although I noticed a trend towards decreasing enrichment levels (**Fig 14D**; -30 min: 3.67 ± 0.25; +1 min: 3.29 ± 0.23; percent change: 94 ± 0.065%; *p=0.09*). SynGAP-1α regulates spontaneous structural changes at dendritic spines at developmental time points comparable to those used in my experiments (Clement, et al., 2012) and the decline in GFP-synGAP-1α enrichment levels at mature spines may correspond with developmentally regulated spine dynamics, which require fluctuations in synGAP-1α levels in spines (Araki, et al., 2015).

Persistent structural changes at dendritic spines require initial activity-dependent dispersion and sustained exclusion of synGAP-1α (Araki, et al., 2015). I predicted that these structural changes may have a role in activity-dependent new spine stabilization and would therefore also require persistent synGAP-1α dispersion following HFU at new spines. To determine if activity-dependent new spine stabilization coincided with persistent synGAP-1α exclusion, I analyzed new spine enrichment changes +70 min following exposure to HFU. GFP-synGAP-1α levels remained at the low levels observed at the +1 min time point 70 min following

stimulation (**Fig 14C;** +70 min: 1.2 ± 0.2; -30 min vs +70 min; *p=0.06*; +1 min vs + 70 min: *p=0.9*). Comparatively, GFP-synGAP-1α enrichment levels at unstimulated new spines returned to the baseline levels I observed at the -30 min time point (**Fig 14C;** +70 min: 1.4 ± 0.3; -30 min vs +70 min; *p=0.9*; +1 min vs + 70 min: *p=0.01*). GFP-synGAP-1α enrichment levels were not different between stimulated and unstimulated new spines at the final 70 min time point (*p=0.7*). These results support my hypothesis that activity-dependent synGAP-1α evacuation and exclusion from new spines may enhance new spine survivorship.

Blocking synGAP-1α dispersion can convert stable spines into unstable spines (Araki, et al., 2015), and I wondered if the increase in GFP-synGAP-1α enrichment levels at unstimulated spines ~31 min following outgrowth could contribute to their impermanence. Unstimulated spines were separated based on whether they persisted and were present at the final +70 min (**Fig. 14C**, gray filled circles) time point or not (**Fig 14C**, red filled circles), and I looked to see if there were statistical differences in their enrichment changes. I observed no statistical differences between stable and unstable unstimulated new spines in the magnitude of enrichment change between -30 min and +1 min (*p=0.3)*, however, I did note that GFP-synGAP-1α enrichment levels increased by at least 150% or more at all but one of the eliminated spines (**Fig 15A**; eliminated: 237 ± 40%). Conversely, stable unstimulated spines had smaller enrichment changes on average (**Fig 15A**; stable: 173 ± 33%), and, importantly, 44% of all stable unstimulated spines decreased their enrichment levels between the -30 and +1 min time points. None of the eliminated spines showed a decrease in enrichment. While strong conclusions cannot be drawn from these data, they suggest that even at unstimulated spines, lower GFP-synGAP-1α enrichment levels may predict basal stability.

Investigating the mechanism for GFP-synGAP-1α accumulation and dispersion at new spines

SynGAP-1α does not interact directly with CaMKIIα, therefore understanding its role in activity-dependent new spine stabilization and its relationship to CaMKIIα's non-enzymatic

activities at new spines requires identification of the molecules that facilitate the synGAP-1α-CaMKIIα interaction. PSD-family MAGUKs, particularly PSD95 and SAP102, are known scaffolding intermediaries between synGAP-1α and CaMKIIα (Araki, et al., 2015; Kim, et al., 1998). Although PSD-family MAGUKs are enriched at low levels at new spines (De Roo, et al., 2008b; Lambert, et al., 2017), the possibility remains that these low levels are sufficient for synGAP-1α accumulation at new spines, as I show that enrichment levels of synGAP-1α at new spines are similarly low. The multi-PDZ domain protein 1, MUPP1, is a large scaffolding protein with thirteen PDZ domains (Ullmer, et al., 1998), that has also been identified as a scaffolding partner for CaMKIIα and synGAP-1α (Krapivinsky, et al., 2004). I investigated whether synGAP-1α accumulation and dispersion at new spines was disrupted by CaMKIIα kinase inhibition to gain insight into the possible mechanisms facilitating the interaction between and synGAP-1α and CaMKIIα.

SynGAP-1α that interacts with CaMKIIα via structural interactions with PSD-family MAGUKs requires CaMKIIα kinase activity, which triggers dissociation of synGAP-1α from PSD95 (Araki, et al., 2015). This is not the case with synGAP-1α that is bound to MUPP1. *In vitro* studies show that occupancy of the Ca^{2+}/CaM binding pocket of CaMKIIα by either Ca^{2+}/CaM or the kinase inhibitor KN93 is sufficient to trigger dissociation of the synGAP-1α-MUPP1 complex from CaMKIIα. It is unclear if activity-dependent, kinase-independent separation of the CaMKIIα-MUPP1-synGAP-1α complex occurs in dendritic spines. I predicted that I could gain insight into the mechanism for synGAP-1α accumulation and dispersion at new spines by observing whether GFP-synGAP-1α dispersion is preserved when CaMKIIα kinase activity is inhibited by staurosporine.

Organotypic hippocampal slice cultures were sparsely transfected with GFP-synGAP-1α and dsRed Express. Spontaneous new spine outgrowth was observed on transfected hippocampal CA1 neurons using 2-photon time-lapse imaging (**Fig 16Ai-ii**). Typically, I observed multiple new spines grow on a single cell; one new spine was selected for stimulation

and the rest were monitored as unstimulated control spines. Prior to HFU, slices were incubated in either 1 μM staurosporine or an equivalent volume of vehicle for 30 minutes. Staurosporine is a non-specific inhibitor of CaMKII, PKA, and PKC. Despite this, I chose to use staurosporine to inhibit CaMKIIα kinase activity because I know it does not inhibit activity-dependent new spine stabilization (Chapter 2), allowing me to determine if GFP-synGAP-1α dispersion might be one mechanism contributing to CaMKIIα's non-enzymatic role in enhancing new spine stability. Additionally, staurosporine inhibits CaMKIIα kinase activity without disrupting normal Ca^{2+}/CaM binding, which is crucial for understanding the role of normal, activity-dependent mechanisms in synGAP-1α dispersion. KN93/KN62 would also permit dissociation of MUPP1-synGAP-1α from CaMKIIα but would block activity-dependent new spine stabilization due to disruption of the interaction between CaMKIIα and the GluN2B subunit of the NMDA- type glutamate receptor (Barcomb, et al., 2013).

My preliminary findings show that, under the vehicle condition, HFU induced normal GFP-synGAP-1α dispersion (**Fig 16B-C**; -30 min: 1.5 ± 0.5; +1 min: 0.8 ± 0.7; enrichment change: 85%; $p=0.7$). I was unable to observe any significant changes in enrichment due to the low sample size (n=4). Unstimulated new spines in the vehicle condition underwent no changes in GFP-synGAP-1α enrichment (**Fig 16B-C**; -30 min: 1.6 ± 0.2; +1 min: 1.3 ± 0.2; enrichment change: 123%; $p=0.6$). I did not see an increase in GFP-synGAP-1α enrichment at unstimulated new spines, as I observed in **Fig 14**, which I attribute to the use of DMSO as a vehicle. Remarkably, exposure to HFU triggered GFP-synGAP-1α evacuation from stimulated new spines on cells incubated in the CaMKIIα kinase inhibitor (**Fig 16B-C**; -30 min: 1.8 ± 0.3; +1 min: 0.9 ± 0.3; enrichment change: 60%; $p=0.1$). Again, I was unable to see a significant decrease in GFP-synGAP-1α enrichment levels at stimulated new spines due to the low sample size (n=4). GFP-synGAP-1α enrichment levels showed no trend towards decreasing or increasing at unstimulated new spines on cells incubated in staurosporine (**Fig 16B-C**; -30 min: 1.6 ± 0.2; +1 min: 1.5 ± 0.2; enrichment change: 103%; $p=0.6$). Mature neighboring spines also showed no

change in GFP-synGAP-1α enrichment levels (**Fig 16D**; vehicle: 90 ± 0.11%; *p= 0.2*; staurosporine: 90 ± 0.1; *p=0.8*). These preliminary results suggest that synGAP-1α dispersion at new spines does not require CaMKIIα kinase activity, implicating MUPP1 as a possible mechanism for synGAP-1α accumulation and dispersion at new spines. Dissociation of the MUPP1-synGAP-1α complex from CaMKIIα may be one of the ways in which CaMKIIα regulates new spine survivorship in a non-enzymatic manner.

DISCUSSION

Investigating the role of the 26S proteasome in new spine stabilization

I initially predicted that the proteasome may have a role in activity-dependent new spine stabilization due to its role in activity-dependent new spine outgrowth (Hamilton, et al., 2012) and evidence of CaMKIIα kinase-independent recruitment of the 26S proteasome to new spines that is consistent with my observation of CaMKIIα's kinase-independent role in regulating new spine stabilization. Based on this hypothesis, glutamatergic stimulation would recruit the proteasome to new spines via non-enzymatic CaMKIIα activity, where the proteasome would degrade negative regulators of synaptic plasticity. In fact, there is evidence that the proteasome is involved in the degradation of molecules such as synGAP-1α (Zhang, et al., 2020), which, as I have discussed, is known to regulate dendritic spine maturation (Aceti, et al., 2015; Clement, et al., 2012; Vazquez, et al., 2004) as well as Ephexin-5, an inhibitor of new spine outgrowth (Hamilton, et al., 2017)

I hypothesized that inhibiting the proteasome with the inhibitor lactacystin would block activity-dependent new spine stabilization, however, I found that unstimulated new spines were significantly more stable when the proteasome was inhibited than unstimulated new spines in the vehicle condition. This suggests that, under normal conditions, the proteasome may be negatively regulating basal new spine stability. Additionally, HFU did not enhance stability of spines on cells incubated in lactacystin beyond the enhanced rates of basal stability. Together, this evidence suggests that, at new spines, normal proteasomal degradation may be targeted

towards proteins that promote stabilization, but that loss of the proteasome may also interfere with some of the molecular mechanisms involved in activity-dependent new spine stabilization. Because the molecular composition of new spines is poorly understood, it is difficult to speculate which molecules the proteasome might be constitutively degrading to regulate new spine stability. Putative targets of the proteasome at new spines might be found by examining the literature to identify synaptic proteins that are known to be degraded by the proteasome. One such protein that I identified as a promising target is Shank3, a scaffolding protein that indirectly associates with a number of PSD proteins, including mGLuRs and NMDARs, as well as a number of actin binding proteins (Sala, et al., 2015; Sheng and Kim, 2000) to regulate synaptic strength and the stabilization of actin polymers. Overexpression of Shank3 increases dendritic spine density and decreases filopodial density, both of which are thought to represent changes in dendritic spine maturity (Durand, et al., 2012). It is also a known target of the proteasome (Campbell and Sheng, 2018; Ehlers, 2003), and blocking the degradation of Shank3 by overexpressing a deubiquitinase that targets Shank3 increases dendritic spine stability (Campbell and Sheng, 2018). Going forward, future studies to investigate the role that the proteasome plays in basal spine stability should explore its functions degrading proteins such as Shank3.

Mechanisms of GFP-synGAP-1α accumulation and dispersion at new spines.

My results suggest that synGAP-1α accumulates at new spines and undergoes activity-dependent dispersion. I found that incubating hippocampal slice cultures in staurosporine did not prevent GFP-synGAP-1α dispersion following HFU at new spines, implicating that the synGAP-1α located at new spines is not bound to PSD95, as CaMKII SynGAP-1α dissociation from the CaMKIIα-PSD95 complex requires CaMKIIα kinase activity (Araki, et al., 2015). MUPP1 expression at dendritic spines, particularly at new spines, has not yet been experimentally verified, however, my observation that synGAP-1α dispersion occurs in the absence of CaMKIIα kinase activity suggests that PSD-family MAGUKs are not the primary

mechanism for anchoring the population of synGAP-1α that is sensitive to glutamatergic stimulation at new spines.

It is likely that interactions with PSD-family MAGUKs regulate synGAP-1α accumulation and dispersion at spines at later time points. PSD-family MAGUK enrichment levels take 9-24 hours to reach mature levels (De Roo, et al., 2008b; Lambert, et al., 2017). Importantly, inhibiting CaMKIIα kinase activity prevented synGAP-1α dispersion, dissociation from PSD95, and sLTP at what can be considered “mature spines” (Araki, et al., 2015). Conversely, disrupting the synGAP-1α-MUPP1 complex by injection with inhibitory peptide fragments increased basal AMPAR EPSCs, consistent with synGAP-1α’s role as a negative regulator of synaptic strength (Clement, et al., 2012; Vazquez, et al., 2004) however did not interfere with activity-dependent LTP (Rama, et al., 2008). I show that, even at the +70 min time point, the enrichment levels of GFP-synGAP-1α were lower than their mature neighbors. I predict that the arrival time of GFP-synGAP-1α would follow the arrival of PSD-family MAGUKS, particularly PSD95 and SAP102 (Araki, et al., 2015; Kim, et al., 1998). As new spines mature, PSD-family MAGUK accumulation might take over as the primary mechanism for synGAP-1α accumulation and, therefore, dispersion at new spines. This prediction would be consistent with the role for CaMKIIα kinase activity in synapse stability at later time points (Araki, et al., 2015; Koeberle, et al., 2017). It is unknown what role MUPP1 might be playing once spines mature and the CaMKIIα-PSD95-synGAP-1α interaction dominates, and the role of MUPP1 in both new and mature spines merits further investigation based on the preliminary data I’ve collected.

I found that unstimulated new spines increase their GFP- synGAP-1α enrichment levels during the ~30 min window between the -30 min and +1 min time points. It is unclear why this occurs, since this time scale does not appear to line up with the recruitment of PSD-family MAGUK arrival at new spines, even for the earliest arriving members (Lambert, et al., 2017). The enrichment pattern of MUPP1 at new spines has not yet been investigated, but it is worth determining the expression of MUPP1 to further characterize the molecular composition of new

spines and the role it plays in synGAP-1α levels and new spine stability. Interestingly, I found that unstimulated spines that were ultimately eliminated underwent larger increases in GFP-synGAP-1α levels over the ~30 min time frame than unstimulated new spines that were stable at the +70 min time point. This suggests that a larger increase in synGAP-1α levels at new spines may predict new spine elimination, and it is critical that we determine the mechanisms that contribute to the disparities in synGAP-1α at new spines to better understand the molecular mechanisms governing new spine survivorship.

The Ras and Rap GAP activity of synGAP-1α may be involved in the role it plays in activity-dependent new spine stabilization. The Rap1 and Rap2 GTPases regulate the stability of actin polymers, and its activation promotes destabilization of the cytoskeleton, resulting in decreased dendritic spine densities, increased the presence of filopodial spines, and impaired learning (Fu, et al., 2007; Ryu, et al., 2008; Xie, et al., 2005). Activation of Rap GTPases is regulated in part by synGAP-1α, and synaptic activity induces two seemingly opposing processes: activation of CaMKII results in synGAP-1α phosphorylation that, in turn, increases its GAP activity towards Rap1 (Walkup, et al., 2015), but this same phosphorylation by CaMKII also triggers evacuation of synGAP- 1α from stimulated spines (Araki, et al., 2015) and increases Rap1 activation (Xie, et al., 2005). Curiously, basal Rap1 activity is increased at new spines (Koeberle, et al., 2017), which may be a direct result of the low levels of synGAP-1α at new spines. Koeberle and colleagues (2017) also showed that inhibiting CaMKIIα kinase activity both genetically and pharmacologically increased dendritic spine density and decreased Rap1 activity. Considering these findings in the context of my own, it is likely that the low levels of synGAP-1α at new spines may contribute to the increased Rap1 activation, contributing to new spine impermanence. It is still unclear what effects activity-dependent synGAP-1α dispersion may have on Rap1 activity at new spines. Future studies should investigate the interplay between CaMKIIα, MUPP1, synGAP-1α, and Rap1 activity to fully understand the role that these molecules play in new spine stabilization.

FIGURE LEGENDS and FIGURES

Figure 12. Pharmacological inhibition of the 26S proteasome increases basal new spine survivorship.

(A) Images (green channel) of spontaneous new spine outgrowth (filled arrowhead at 0 min) on dendrites of GFP-transfected hippocampal CA1 neurons (DIV 7-9). One new spine per neuron was stimulated with HFU (yellow circle) following 30 min pre-incubation in either vehicle (top row) or 1 µm lactacystin (lacta; bottom row). ***(B)*** Survivorship of stimulated new spines in vehicle (filled green circles; 13 spines/13 cells) was not different from unstimulated new spines on the same cells (open green circles; 106 spines/13 cells). Basal new spine survivorship rates were increased by lactacystin incubation such that the survivorship rates of stimulated new spines (filled blue circles; 10 spines/10 cells) were indistinguishable from those of unstimulated new spines (open blue circles; 61 spines/10 cells). ***(C)*** Survivorship of HFU-stimulated new spines (filled bars) at 70 min was increased compared to unstimulated new spines (open bars) on the same cells in the vehicle condition (green bars). The survivorship of HFU-stimulated new spines on cells incubated in lactacystin (lacta; blue bars) was not significantly different from that of unstimulated new spines at the 70 min timepoint. Unstimulated new spines on cells incubated in lactacystin were significantly more stable than those on cells incubated in vehicle. ***(D)*** Same as ***A***, except a single new spine per cell was stimulated with HFU following 30 min pre-incubation in either vehicle (top row) or 10 µm MG-132 (MG-132; bottom row). ***(E)*** HFU uncaging enhanced new spine survivorship for stimulated new spines incubated in vehicle (filled light green circles; 4 spines/4 cells) compared to unstimulated new spines on the same cells (open light green circles; 37 spines/4 cells). Survivorship curves of stimulated (filled light green and light blue circles; vehicle: 2 spines/2 cells; MG-132: 4 spines/4 cells) spines and unstimulated spines (open light green and light blue circles, vehicle: 21 spines/ 2 cells; MG-132: 37 spines/4 cells) after incubation in either vehicle (light green) or MG-132 (light blue). (F) Survivorship of HFU-stimulated new spines (filled bars) and unstimulated new spines (open bars) for on the

same cells for both vehicle (light green bars) or lactacystin (light blue bars). Log-rank task was used in ***B*** and ***E*** and Fisher's exact test was used in ***C*** and ***E****.* *p <0.05, **p < 0.01, ***p < 0.001.

Figure 12

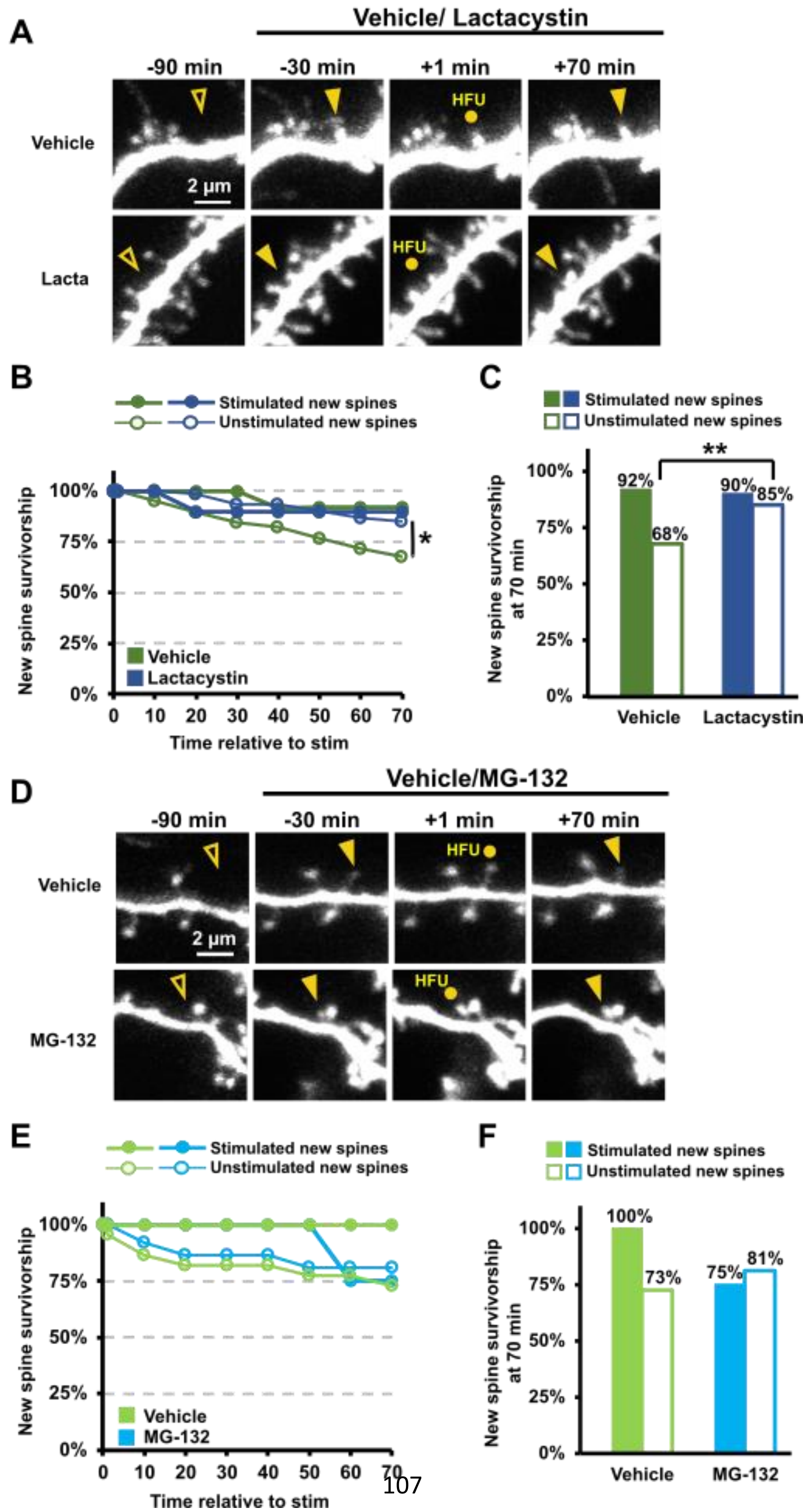
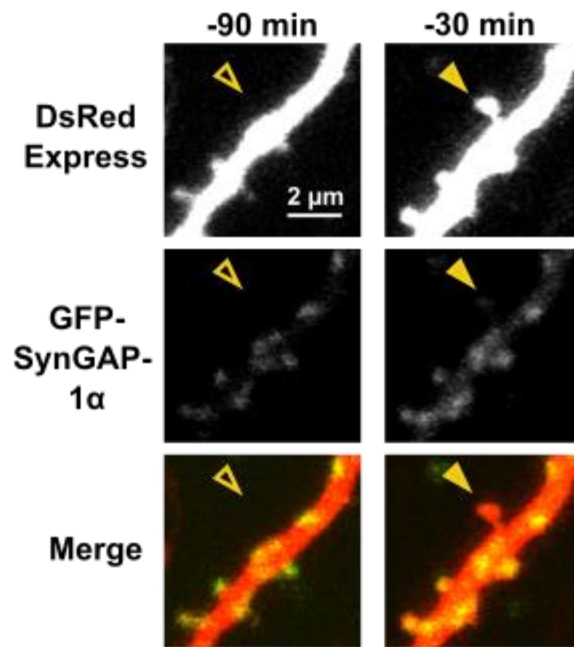


Figure 13. GFP-synGAP-1α enrichment in new spines is lower than that in size-matched neighboring spines.

(A) Images of dendrites from hippocampal CA1 neurons in slice culture (DIV 7-9) expressing GFP-synGAP-1α (green) and DsRed-Express (red) before (open arrowhead) and after (filled arrowhead) spontaneous new spine outgrowth. ***(B)*** The relative enrichment (spine: dendrite ratio) of GFP-synGAP-1α in new spines (n = 36 spines/10 cells) was lower than that of size-matched neighboring spines (n = average of neighbors on 21 dendrites/10 cells). ***(C)*** Neighboring spines used for enrichment calculations in ***B*** were size-matched to new spines (*p = 0.61*). Unpaired two-tailed t-test used in ***B*** and ***C***. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 13

A



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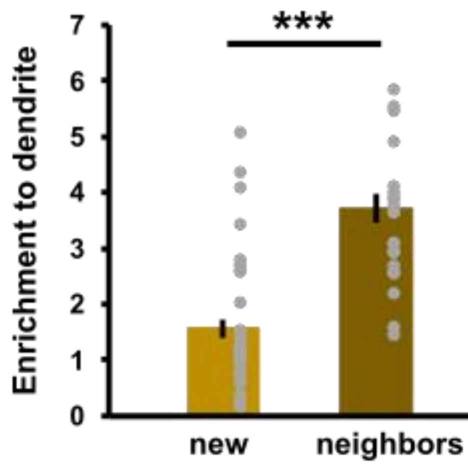


C

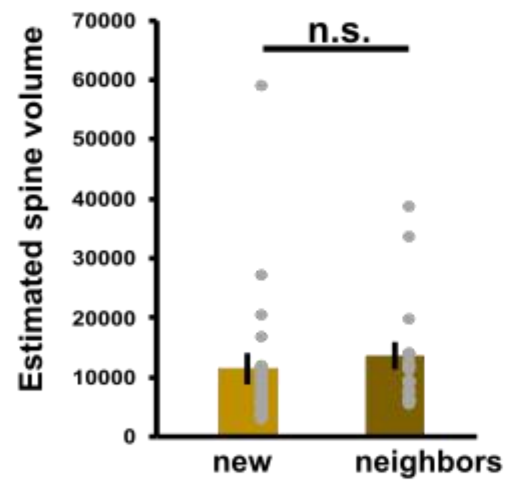


Figure 14. HFU induces GFP-synGAP-1α dispersion at new spines.

(Ai) Representative image of spontaneous new spine outgrowth (filled yellow arrowhead) on a CA1 hippocampal neuron in slice culture (DIV 7-9) expressing a DsRed-Express cell fill (top row) and GFP-synGAP-1α (middle row; dual color image on bottom row). The new spine was exposed to HFU (filled yellow circle). ***(Aii)*** Same as ***Ai***, except two new spines were identified on the same dendrite and neither were exposed to HFU. ***(B)*** Survivorship rates of HFU-stimulated new spines (filled gold bar; n= 10 spines/10 cells) was enhanced compared to unstimulated new spines (open gold bar, 26 spines/ 10 cells) on the same GFP-synGAP-1α transfected cells. ***(C)*** The relative enrichment levels of GFP-synGAP-1α decreased +1 min following exposure to HFU (khaki bar) and remained low at the +70 min time point (yellow bar) compared to baseline levels (-30 min; brown bar) for stimulated new spines (left). Unstimulated spines (right) increased their GFP-synGAP-1α enrichment levels at the +1 min time point (khaki bar) but returned to baseline (brown bar) levels by the +70 min time point (yellow bar). Enrichment changes of individual spines are represented by the gray filled circles. Red filled circles indicate spines that were eliminated by the final +70 min time point. ***(D)*** GFP-synGAP-1α enrichment change of stimulated new (khaki bar), unstimulated new (yellow bar) and unstimulated, size-matched mature neighbors (brown bar) represented as the percent change between the -30 min and +1 min time points. Fisher's exact test used in ***B*** and a mixed-effects model with Bonferroni correction for multiple comparisons used in ***C***. *p <0.05, **p < 0.01, ***p < 0.001.

Figure 14

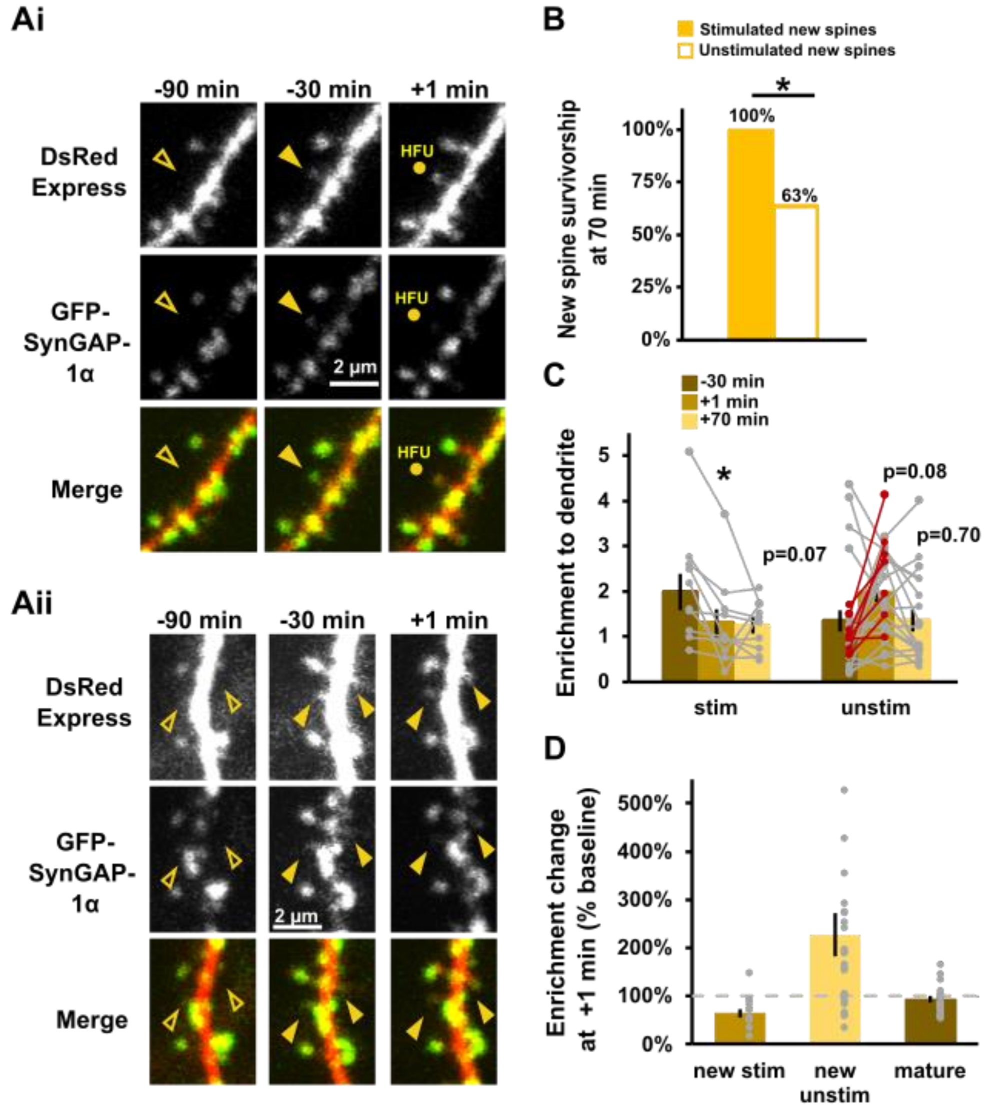


Figure 15. Bidirectional GFP-synGAP-1α enrichment changes at unstimulated new spines may determine survivorship.

(A) GFP-synGAP-1α enrichment change of stable (filled bar) and eliminated (open bar) unstimulated new spines represented as the percent change between the -30 min and +1 min time points. Unaired two-tailed t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 15

A

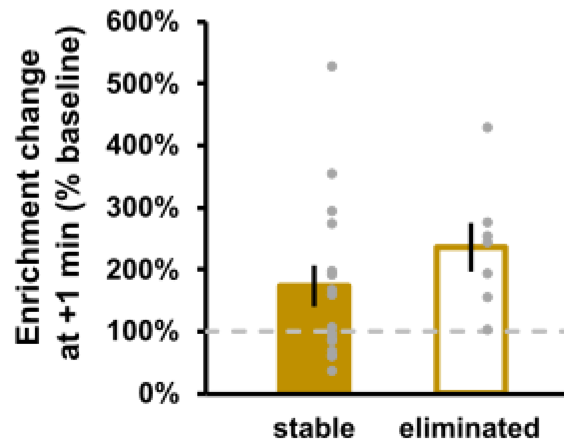
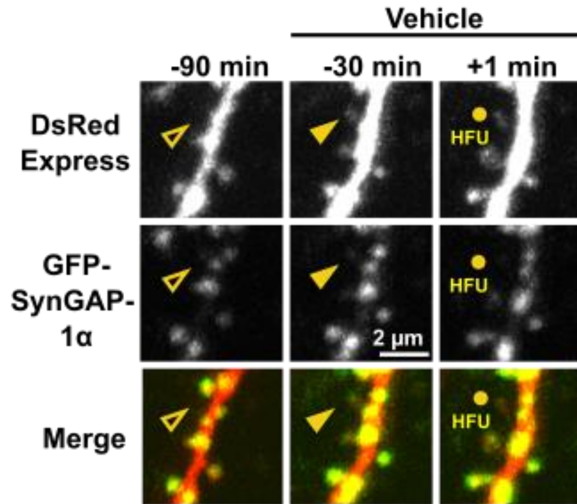
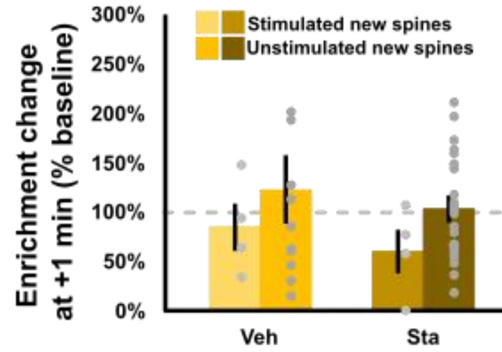


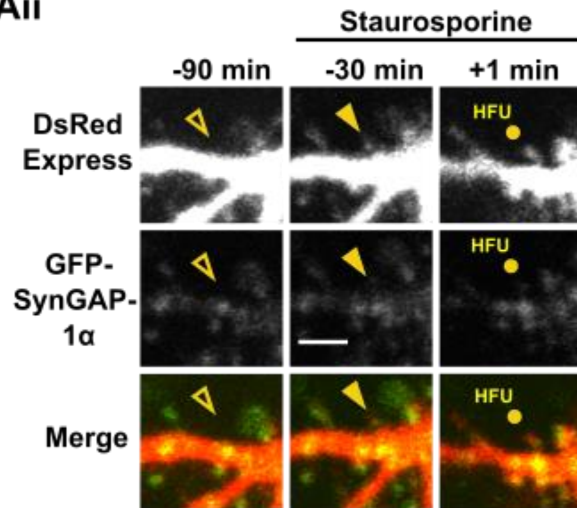
Figure 16. Inhibition of CaMKIIα kinase activity with staurosporine may not impair activity-dependent GFP-synGAP-1α dispersion.

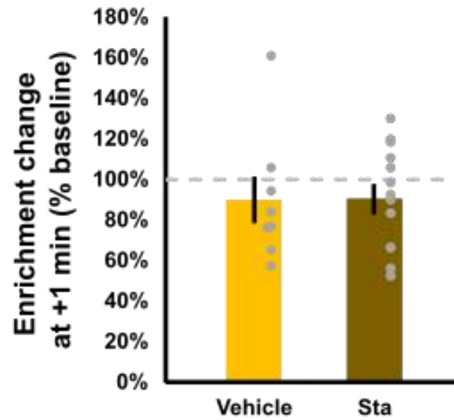
(Ai) Images showing spontaneous new spine outgrowth (filled arrowhead) on the dendrite of a hippocampal CA1 neuron expressing dsRed Express (top row) and GFP-synGAP-1α (middle row; merged image on bottom row). The new spine was stimulated with HFU (filled yellow circle) following a 30 min incubation in vehicle. ***(Aii)*** Same as ***Ai***, except the spine was exposed to HFU following 30 min incubation in 1 μM staurosporine. ***(B)*** Exposure to HFU reduced the relative enrichment of GFP-synGAP-1α between the -30 min (open bars) and +1 min (filled bars) time points at new spines on cells incubated in either vehicle (yellow bars; n=4 spines/4 cells) or staurosporine (khaki bars; n=4 spines/4 cells). GFP-synGAP-1α enrichment levels did not statistically change for unstimulated new spines in either the vehicle (gold bars; n=14 spines; 4 cells) or staurosporine (brown bars; n=23 spines/4 cells) conditions. ***(C)*** GFP-synGAP-1α enrichment change of stimulated new spines (yellow and khaki bars) and unstimulated new spines (gold and brown bars) on cells incubated in either vehicle (left) or staurosporine (right), represented as the percent change between the -30 min and +1 min time points. ***(D)*** Enrichment levels of GFP-synGAP-1α at mature, neighboring spines did not change between the -30 min and +1 min time points on cells incubated in either vehicle (gold bar; n= average of 8 dendrites/ 4 cells) or staurosporine (sta; brown bar; n= average of 14 dendrites/ 4 cells). Paired two-tailed t-test used in ***B*** and unpaired two-tailed t-test used in ***D***. *p <0.05, **p < 0.01, ***p < 0.001.

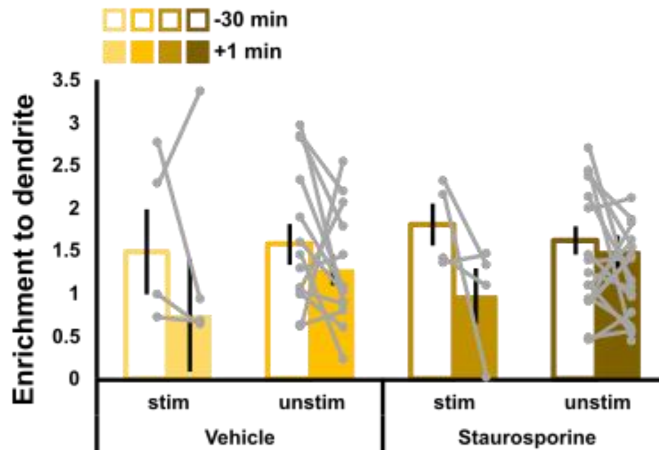
Figure 16











CHAPTER 5: CONCLUDING REMARKS

My contribution to the field of new spine stability

When I began my dissertation work, the Zito lab and others had already done a substantial amount of work to contribute to the current understanding of new spine stabilization. Specifically, their work has helped establish the molecular composition of new spines and some of the mechanisms that contribute to their stabilization. However, there remain gaps in our knowledge of new spine stabilization, with many of these "gaps" being questions raised to follow up on the incredible discoveries already made in the field. What does the CaMKII-GluN2B interaction do downstream of LTP-stimulation to enhance new spine stability? What do the low enrichment levels of PSD-family MAGUKs at new spines mean for other PSD proteins? I set out to answer some of these questions with my dissertation work, which has resulted in further elucidation of the molecular composition of new spines as well as the mechanisms downstream of strong glutamatergic stimulation that contribute to activity-dependent new spine stabilization.

CaMKIIα and new spine stabilization

I first set out to determine the role of CaMKIIα in activity-dependent new spine stabilization. Previous work by Hill and Zito (2013) established a role for the interaction between CaMKII and the GluN2B subunit of the NMDAR in activity-dependent new spine stabilization. I chose to follow up on this finding, hypothesizing a role for CaMKIIα that did not require its enzymatic activities. Using a combination of genetic and pharmacological manipulations *in vitro*, I confirmed that non-enzymatic CaMKIIα is required to enhance new spine stability downstream of high-frequency glutamate uncaging (HFU). GFP-CaMKIIα was as enriched in new spines as in size-matched mature spines, strengthening my prediction that it may indeed be serving a primarily non-enzymatic scaffolding or structural role at new spines. Remarkably, new spine stability was enhanced following exposure to HFU on cells over-expressing the kinase-dead CaMKIIα-K42R mutation or incubated in staurosporine, however, shRNA knock-down of CaMKIIα did impair activity-dependent new spine stabilization. Furthermore, overexpression of

constitutively autonomous CaMKIIα containing the T286D point mutation enhanced basal new spine stabilization without the need for further glutamatergic stimulation or kinase activity, as pairing the T286D mutation with the K42R mutation still enhanced basal new spine survivorship. Initial T286 phosphorylation requires kinase activity CaMKIIα or binding to the GluN2B subunit to retain autonomous activity (Bayer, et al., 2001; Halt, et al., 2012; Barcomb, et al., 2016). These findings therefore confirmed my prediction that sustained CaMKIIα enzymatic activity was not required to enhance new spine stability, and that structural interactions facilitated by GluN2B binding promote new spine survivorship. My work also raised several new questions that I sought answers to. What are the non-enzymatic structural/scaffolding interactions that are required to enhance new spine survivorship? As even the molecular composition of new spines is still largely unknown, answering this question would be no easy feat. I therefore looked to see if new spine structural plasticity could indicate which putative molecular mechanisms may be present in new spines and acting to enhance new spine survivorship.

I tested the hypothesis that CaMKIIα structural and scaffolding activities induced new spine volume changes that were necessary for enhancing survivorship. When I looked to see if there was a relationship between new spine stabilization and both basal and activity-dependent new spine volume changes, I made the surprising discovery that new spine volume changes were not necessary for enhancing stabilization. While a manipulation that impaired activity-dependent new spine survivorship, shRNA knock-down of CaMKIIα, did also block new spine enlargement, another manipulation that maintained normal activity-dependent new spine, incubation in the vehicle DMSO prior to glutamate uncaging, did so while failing to induce new spine volume changes. This finding indicates that new spine volume changes are not necessary to enhance new spine stability, although the mechanisms that determine spine stability may greatly overlap with those that enhance spine volume. Consistent with this, overexpression of the T286D and T286D/K42R mutant CaMKIIα constructs, which both enhance basal new spine stability, also induced large spontaneous volume changes at stable new spines in the absence

of further glutamatergic stimulation. As we know little about the mechanisms involved in activity-dependent new spine stabilization, investigating some of the known targets of CaMKIIα that may regulate spine volume changes may point us towards identifying some of the molecular mechanisms involved in new spine stabilization due to this overlap.

I identified the 26S proteasome and the Ras and RapGAP synGAP-1α as promising candidates for regulating new spine stability downstream of non-enzymatic CaMKIIα function. Surprisingly, I found that inhibition of the 26S proteasome with lactacystin enhanced basal new spine survivorship and likely occluded further activity-dependent stabilization, which is contrary to my predictions. This finding suggests that the proteasome may be constitutively degrading molecules that promote new spine stability, although there are few clues as to what these molecules may be. Glutamatergic stimulation recruits the proteasome to new spines (Bingol, et al., 2010) however CaMKIIα phosphorylation of the 26S proteasome enhances its degradatory activity (Djakovic, et al., 2009), making unclear how the balance of possible constitutive-degradation may be affected by glutamatergic stimulation and downstream CaMKIIα activation. SynGAP-1α proved to be a much more promising molecular mechanism. While I found that GFP-tagged synGAP-1α is less enriched at new spines than their mature neighbors, our HFU was sufficient to induce its dispersion from synapses, which is necessary for mature spine functional and structural plasticity (Araki, et al., 2015) and may contribute to similar changes at new spines that promote stability. Critically, incubation in staurosporine did not block activity-dependent dispersion of GFP- synGAP-1α. This finding enhances both our knowledge of new spine molecular composition as well as the mechanisms underlying new spine survivorship. Importantly, synGAP-1α following incubation in staurosporine rules in the possibility that MUPP-1 regulates new spine accumulation of synGAP-1α and points to a putative mechanism for activity-dependent new spine stabilization that requires kinase-independent functions of CaMKIIα.

The bigger picture

How do my findings, fragments of the whole, intricate processes regulated by CaMKIIα and its effectors, enzymatic or otherwise, fit into the larger body of work aimed at understanding some of the most crucial and elusive aspects of human cognition: learning and memory? I believe that further elucidation of the molecular composition of new spine and the mechanisms that govern their survivorship can have profound implications for better understanding learning and memory as well as the neurological diseases and disorders that affect them.

New spines and plasticity

Previous work on new spines, specifically work done by other members of the Zito lab, made important contributions to our understanding of new spine molecular composition and the mechanisms that occur downstream of glutamatergic stimulation to enhance their stability. As my work expands on those findings, the implications in the larger scope are much the same. My discoveries on the molecular composition of new spines provide new insights that can be used to functionally target new spines not only for therapeutic endeavors, but also for a better understanding of the structural changes that underlie cognition. Many studies have shown the correlation between new spine outgrowth, stability, and learning and memory, however the necessity for new spine outgrowth and stabilization has yet to be experimentally confirmed. Hayashi-Takagi and colleagues (2015) confirmed that photoactivatable constructs that target recently activated dendritic spines also target new spines, consistent with the established roles newly formed spines are thought to play in learning (Xu, et al., 2009; Yang, et al., 2009) as well as their ability to contribute to excitatory glutamatergic transmission shortly after their outgrowth (Zito, et al., 2009). This study used photoactivation to destabilize activity-dependent structural changes at spines and disrupt motor learning but did not report on the effects that photoactivation had on the construct-tagged new spines or speculate on the role that those new spines may have had in the loss of motor learning. It is clear that while new spines have much in common with their mature neighbors, they still differ greatly in their molecular make-up,

stability, volume, and Ca^{2+} handling (De Roo, et al., 2008b; Hill and Zito, 2013; Lambert, et al., 2017; Zito, et al., 2009) Further elucidation of the ways new spines differ from their mature counterparts may allow us to selectively target them in future studies and further investigate the role they play in learning and memory. While my work is just a small step in that direction, I believe that my contributions can be expanded upon in later work towards that goal.

Although it has not yet been determined if new spines undergo LTP, a long-lasting increase in synaptic strength and spine size, in response to our HFU protocol, unpublished work from others in the lab indicate that new spine stabilization on the time scale of ~70 minutes requires neither functional GluR1-containing AMPAR insertion nor spine enlargement. This suggests that that new spines may not undergo LTP in the same manner as mature spines. Why then can only LTP-inducing stimulation protocols enhance new spine stability? The answer to this may lie in earlier work characterizing the functional properties of new spines, which I alluded to in the previous paragraph. New spines have smaller Ca^{2+} transients and greater diffusional coupling to the dendritic shaft (Zito, et al., 2009), making it likely that stronger stimulation protocols are required to meet a certain threshold for Ca^{2+} influx that is required to stabilize new spines. So far, there are no studies correlating HFU strength, Ca^{2+} transients, and new spine stability. As previous work as well as my own work have identified Ca^{2+}/CaM-dependent role for CaMKIIα GluN2B binding, T286 phosphorylation, and synGAP-1α dispersion as putative mechanisms for enhancing new spine stability downstream of LTP-inducing stimulation, I believe these are questions worth pursuing answers for.

While conducting my thesis research, I made the surprising discovery that although activity-dependent new spine volume changes often correlate with enhanced stability, they are not necessary. Enhancing new spine survivorship requires exposure to levels of glutamate that induce structural and functional LTP at mature spines (Hill and Zito, 2013), making it all the more remarkable that these structural changes are not required to enhance stability. It remains to be seen if structural changes are necessary for maturation and stability at later time points,

although I do predict that spine enlargement is a requirement for the long-term stability that the hippocampus and neocortex demand, (which I discuss in the following section). PSD95 enrichment levels strongly correlate with dendritic spine stability (Cane, et al., 2014; Yusifov, et al., 2021) and at mature spines PSD95 enrichment correlates with volume fluctuations (Woods, et al., 2011). Strong glutamatergic stimulation appears to disrupt the tight coupling of spine volume and PSD95 enrichment, as spine volume changes occur first, followed by protracted PSD95 recruitment (Bosch, et al., 2014) that likely relies on the volume changes as some form of synaptic tag. New spines exhibit delayed PSD95 recruitment (De Roo, et al., 2008b; Lambert, et al., 2017), and it is unclear if PSD95 can be recruited to spines that do not undergo volume changes but can still be stabilized acutely, such as stimulated new spines in my DMSO vehicle condition. Alternatively, PSD95 may be recruited to new spines that do not undergo volume changes, however, due to the correlation between PSD95 enrichment and stability (De Roo, et al., 2008a), smaller spines or spines that do not undergo activity-dependent enlargement might have lower levels of PSD95 and may therefore be less stable. I would predict that volume changes at new spines may be necessary as new spines continue to mature and alter their molecular makeup in ways that further support their stability. Spine volume changes may also serve to alter the size and complexity of the PSD (Sun, et al., 2021) or induce cytoskeletal changes to confer more stable morphologies (Bourne and Harris, 2007) during the maturation process. Although my work indicates that new spine volume changes are not required to enhance stability on a relatively short timeframe, it would be intriguing and worthwhile to explore the role of new spine enlargement on stability on timescales that align with the long-term demands of cognition.

My work expanded upon the findings that strong glutamatergic stimulation enhances the survivorship of newly formed hippocampal spines in a manner that requires CaMKII-GluN2B binding (Hill and Zito, 2013). likely to facilitate stabilizing non-enzymatic CaMKIIα interactions. In an intact system, this phenomenon would play out as a sub-cellular manifestation of Hebb's

postulate: new spines would be more likely to stabilize upon receiving glutamatergic input from a presynaptic cell, thus increasing the likelihood that later release events from that same presynaptic cell would contribute to firing of the postsynaptic cell. The restructuring and strengthening of neural circuits due to the stabilization of new dendritic spines is a likely candidate for the structural basis of lasting memory, however, recent studies on the longevity of hippocampal spines raises the question of how long these hippocampal spines need to persist to support lasting memory. Super-resolution imaging has greatly improved the spatial resolution of 2-photon microscopy and led to the discovery that most hippocampal CA1 neurons likely persist for only a matter of days, rather than the months and years needed for lasting memory (Attardo, et al., 2015; Pfeiffer, et al., 2018). Current hypotheses suggest that the hippocampus serves as a “short -term” memory repository while the neocortex stores memories on a longer time scale (Attardo, et al., 2015; Kitamura, et al., 2017). It is not yet known if manipulations that acutely (~70 minutes) enhance new spine survivorship support stability on a time scale long enough to support the short-term (5-15 days; Attardo, et al., 2015) storage properties of the hippocampus. Would such manipulations as pharmacological inhibition of CaMKIIα kinase activity with staurosporine stabilize new spines long enough to meet the long-term storage demands of the neocortex? As our knowledge of new spines, their unique molecular composition, and the mechanisms that stabilize them advances, perhaps we may also find answers to other questions that will allow us to better contextualize the role of nascent dendritic spines in learning and memory at the circuit level.

New spines and disease

Many neurological diseases and disorders, particularly those that present with deficits in learning and memory, are also marked by deviations from the typical lifetime trajectory of dendritic spine number. Early post-mortem studies in human tissue found that spine densities in pyramidal layer III of the temporal and frontal cortices were reduced by over 50% compared to tissue from control patients in deceased diagnosed schizophrenia patients (Garey, et al., 1998).

Later work investigating the relationship between dendritic spine densities and schizophrenia have elucidated some of the molecular mechanisms potentially underlying this phenotype. Work on schizophrenia patients and animal models of schizophrenia found many of the genes associated with the disorder alter levels of the Rac1 effector protein kalirin (Glausier and Lewis, 2013). Evidence of reduced D-serine levels associated with schizophrenia (Bendikov, et al., 2007; Hashimoto, et al., 2003) supports a model for NMDA-receptor hypofunction (Hashimoto, et al., 2003) and increased non-ionotropic signaling (Park, et al., 2022) as an underlying cause of dendritic spine elimination. Curiously, the neurodegenerative elimination of dendritic spines resulting from schizophrenia may be biased towards smaller spines (MacDonald, et al., 2017). While I show that new spine stabilization may not require activity-dependent volume changes, the selective elimination of smaller spines in schizophrenia suggests that dendritic spine enlargement may serve to protect new spines from elimination at much later time points. Given the strong link between new spine outgrowth and stabilization and learning and memory (Xu, et al., 2009; Yang, et al., 2009), understanding the molecular pathways that alter new spine volume and further investigating the implications of those volume changes on long-term stability may lead to interventions that can address some of the phenotypes of this disorder and others that are associated with dendritic spine loss.

Determining ways to specifically target and increase new spine stability will likely be crucial for the development of interventions for neurodegenerative disorders. This may be especially important for diseases such as Huntington's Disease (HD), where work in animal models of the disease have revealed that while new spine outgrowth is increased, the survivorship of these newly formed spines is remarkably low compared to controls (Murmu, et al., 2013). Elucidation of the molecular makeup and functional pathways that are unique to new spines may lead to breakthroughs that can precisely target new spines for therapeutic interventions for diseases such as HD. Recent studies have demonstrated that promoting dendritic spine stability *in vivo* by knocking-down the production of PirB protein can improve

learning and alter developmental critical periods (Albarran, et al., 2021; Djurisic, et al., 2013), however the effects of these mutations are widespread and may interfere with the removal of older spines that is also required for the restructuring of neural circuitry during learning and memory (Xu, et al., 2009; Yang, et al., 2009). This highlights the importance of developing tools to selectively promote the stability of newly formed spines. Treatment of schizophrenia and HD are far from the only diseases and disorders that would benefit from expanding research on new dendritic spine stability; disease pathology of autism spectrum disorder (ASD) and Alzheimer's disease (AD) are also linked to changes in dendritic spine density (Ash, et al., 2021; Penzes, et al., 2011) that aberrations in new spine stabilization may contribute to. Further exploration of new spines should prioritize understanding the molecular composition and signaling that occurs to differentiate them from mature spines and aid the development of therapeutic interventions for a number of neurological diseases and disorders.

Future directions

While I have had the incredible opportunity to advance our understanding of learning and memory through my small contribution of studying nascent dendritic spine stability, my work raises more questions than it answers, as all good science does. Here, I will discuss some of the questions I've come up with during my time conducting research and writing the rest of the dissertation. I hope that some of these questions and ideas presented here will help to guide further exploration based on the work I've done.

Putting it all together

I think the most obvious and simplest of follow-up experiments based on my work will be to determine the effects that CaMKIIα kinase inhibition and T286D overexpression have on long-term nascent spine stabilization. I show that genetic and pharmacological inhibition of CaMKIIα kinase activity do not disrupt the activity-dependent enhancement of new spine survivorship on the relatively short timeframe of ~70 minutes, but does this stability persist long enough for further maturation to occur? Hill and Zito (2013) found that survivorship of stimulated

new spines declined by ~50% after 24 hours. Will stimulated new spines have similar or reduced rates of survivorship at later time points if CaMKIIα kinase activity is still inhibited? It would also be interesting to see if PSD-family MAGUK recruitment is in any way disrupted by CaMKIIα kinase inhibition. In a similar vein, I think it would be interesting to explore the implications of CaMKIIα-T286D overexpression, with or without kinase activity, on new spine survivorship at later time points as well as PSD-Family MAGUK recruitment.

Fully connecting the results of my three chapters will likely be the most intriguing future direction and, accordingly, I have the most questions regarding the mechanisms that connect my findings on non-enzymatic CaMKIIα functions in new spine stabilization, new spine volume changes, and synGAP-1α accumulation and dispersion at new spines. I believe that the most likely path to bridge my findings will be to explore signaling molecule activity in new spines using biosensors, particularly biosensors for CaMKIIα and Rap1. Strong glutamatergic stimulation activates CaMKIIα (Lee, et al., 2009) and CaMKIIα activation directly leads to increased phosphorylation or activation of its effector proteins, including signaling molecules (Murakoshi, et al., 2011; Saneyoshi, et al., 2019). I, however, show that the increased enzymatic activities of CaMKIIα following HFU of MNI-caged glutamate are not required to enhance new spine survivorship. The first step to tying together my results will be to investigate CaMKIIα activation before and after glutamatergic stimulation. I also believe that investigating Rap1 activation will be equally as important, as Rap1 is the most likely candidate for bridging my intriguing findings on the effects of CaMKIIα-K42R overexpression on new spine stabilization and volume changes as well as my preliminary synGAP-1α findings. SynGAP-1α is a Ras and RapGAP that is regulated by CaMKIIα and CDK5 phosphorylation (Walkup, et al., 2015). Specifically, CaMKIIα phosphorylation enhances its RapGAP activity while CDK5 phosphorylation increases its activity towards Ras. Rap1 activation destabilizes dendritic spines (Penzes and Rafalovich, 2012) and induces morphological changes that are consistent with reduced stability, including smaller spine volumes and longer neck lengths. CaMKIIα phosphorylation of synGAP-1α may lead to

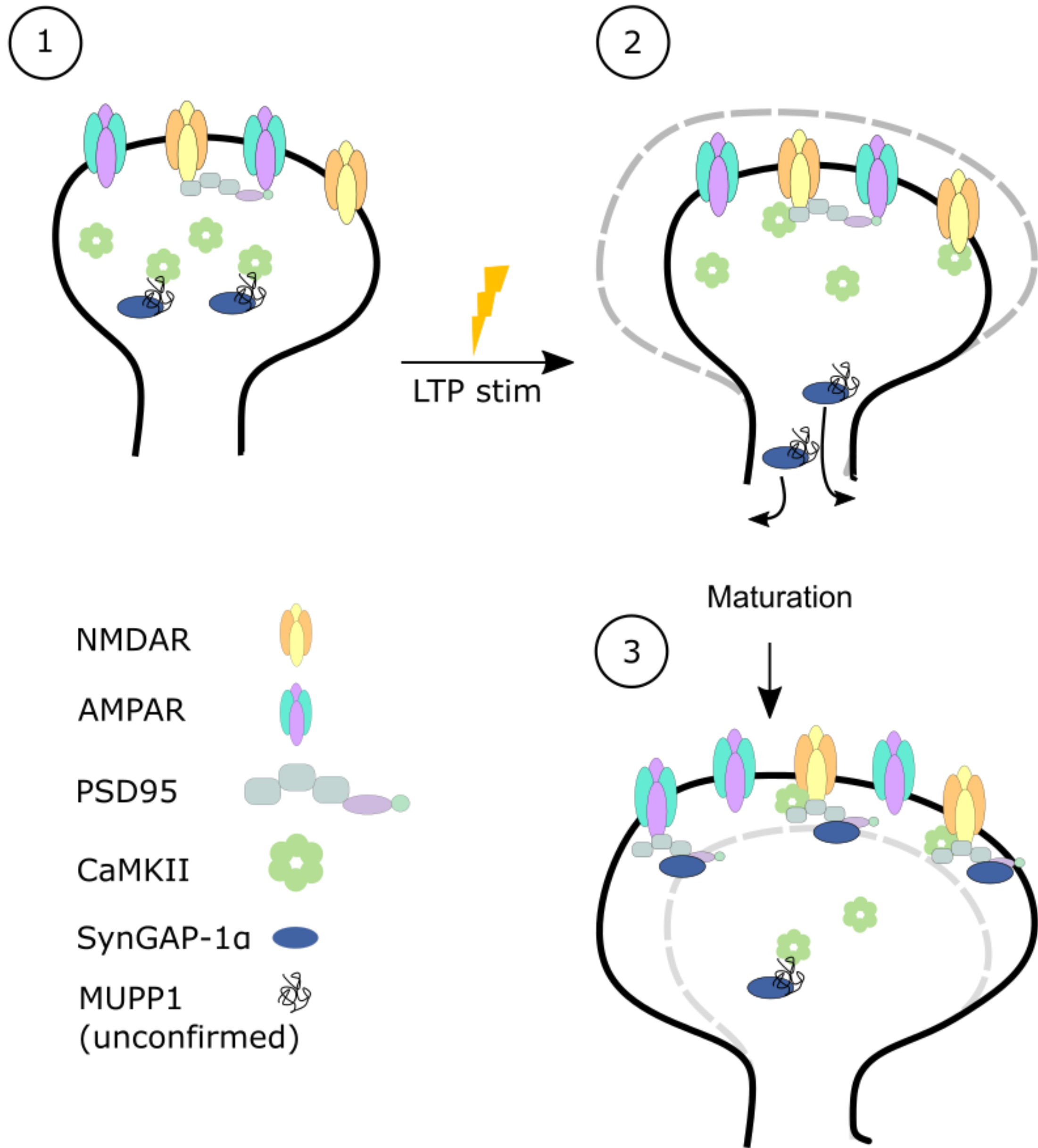
decreased Rap1 activity, which may serve to enhance new spine stability. Conversely, I show that inhibiting CaMKIIα kinase activity does not block activity dependent new spine stabilization and enhances new spine volume changes. Similar manipulations report that blocking CaMKIIα kinase activity actually decreases Rap1 activity (Koeberle, et al., 2017). Furthermore, HFU triggers an evacuation of GFP-synGAP-1α from new spines, which likely reduces the amount of Rap1 GAP activity in new spines. Further investigation of Rap1 activity, particularly under WT-CaMKIIα and kinase-dead conditions before and after glutamatergic stimulation will be key to understanding the roles that CaMKIIα enzymatic and non-enzymatic activities work with synGAP-1α activity and dispersion to regulate new spine stability and volume changes.

FIGURES AND FIGURE LEGENDS

Figure 17: Proposed model for the non-enzymatic role of CaMKII and the interaction with synGAP-1α in nascent spine stabilization.

Newly formed dendritic spines are rapidly functionally mature and equipped with AMPARs and NMDARs (Zito, et al., 2009; Kwon and Sabatini, 2011). CaMKII enrichment levels in new spines are comparable to those in mature spines (Chapter 2), however levels of PSD95 and synGAP-1α are lower in new spines than in mature spines. SynGAP-1α is shown anchored to CaMKII via an unconfirmed interaction with the multi-PDZ domain protein MUPP1 (Krapivinsky, et al., 2004) (**1**). LTP-inducing stimulation at new spines triggers CaMKII translocation (Chapter 2) and synGAP-1α dispersion (Chapter 4), which may be mediated by dissociation of the MUPP-1-synGAP-1α complex from CaMKII due to Ca2+/calmodulin binding (Krapivinsky, et al., 2004). New spines may also enlarge in response to LTP-stim, although activity-dependent new spine enlargement is not required to enhance survivorship (Hill and Zito, 2013; Chapter 3). SynGAP-1α dispersion independent of CaMKII kinase activity may facilitate new spine volume changes as well as later stages of maturation. CaMKII translocation and binding to the GluN2B subunit of the NMDAR is required to enhance new spine stability (Hill and Zito, 2013) and may facilitate other non-enzymatic interactions that are required for new spine survivorship, such as interactions with the 26S proteasome (not shown) (**2**). Stabilized new spines may then become mature spines, a process that involves spine head enlargement, AMPAR recruitment, and PSD95 accumulation (Vazquez, et al., 2004; Lambert, et al., 2017; De Roo, et al., 2008b). SynGAP-1α levels increase during maturation (Chapter 4), likely recruited to maturing spines by the accumulation of PSD95. A population of synGAP-1α anchored to CaMKII via interactions with MUPP1 may be present in mature spines, but the population of PSD95-bound synGAP-1α is likely greater and plays a larger role in regulating plasticity, as CaMKII kinase activity is necessary for LTP induction and maintenance in the mature CNS (Araki, et al., 2015) and the CaMKII-MUPP1-synGAP complex has been shown modulate but not regulate the expression of LTP (Rama, et al., 2008) (**3**).

Figure 17



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