

# Activity-Dependent Stabilization of Nascent Dendritic Spines Requires Nonenzymatic CaMKII $\alpha$ Function

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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 CaMKII $\alpha$  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 CaMKII $\alpha$ '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 CaMKII $\alpha$  functional and structural interactions were altered. Surprisingly, we found that inhibiting CaMKII $\alpha$  kinase activity either genetically or pharmacologically did not impair activity-dependent new spine stabilization. In contrast, shRNA knockdown of CaMKII $\alpha$  abolished activity-dependent new spine stabilization, which was rescued by co-expressing shRNA-resistant full-length CaMKII $\alpha$ , but not by a truncated monomeric CaMKII $\alpha$ . Notably, overexpression of phospho-mimetic CaMKII $\alpha$ -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 support a model in which nascent dendritic spine stabilization requires structural and scaffolding interactions mediated by dodecameric CaMKII $\alpha$  that are independent of its enzymatic activities.

**Key words:** CaMKII; dendritic spine; glutamate uncaging; two-photon imaging

## Significance Statement

The stabilization of nascent dendritic spines is thought to support lasting memory of learned experiences. Here, we show that scaffolding and structural interactions, but not the enzymatic activities, of the kinase CaMKII $\alpha$  are required for activity-dependent new spine stabilization. This study furthers our understanding of the cellular and molecular processes that facilitate learning and memory in the mammalian brain. Understanding the cellular and molecular mechanisms of learning and memory is crucial for our ability to develop therapeutics for memory impairments associated with neurological and neurodegenerative disorders.

## 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 (Xu et al., 2009; Yang et al., 2009; Roberts et al., 2010; Hayashi-Takagi et al., 2015). Interestingly, most new spines are transient (Holtmaat et al., 2005; Berry and Nedivi, 2017) 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

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spine volume (Matsuzaki et al., 2004) known as long-term potentiation (LTP; De Roo et al., 2008a; 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<sup>2+</sup>/calmodulin-activated kinase CaMKII $\alpha$  and the GluN2B subunit of the NMDAR prevents activity-dependent new spine stabilization (Hill and Zito, 2013). Notably, CaMKII $\alpha$ -GluN2B binding facilitates a number of CaMKII $\alpha$  enzymatic and structural functions that promote LTP induction and maintenance, including binding to densin-180 and  $\alpha$ -actinin, activation of signaling molecules, and phosphorylation of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors (Sanhueza and Lisman, 2013; Bayer and Schulman, 2019). Whether these enzymatic and structural activities of CaMKII $\alpha$  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 CaMKII $\alpha$  in activity-dependent new spine stabilization. We found that CaMKII $\alpha$  is present and enriched at mature levels in new spines shortly after outgrowth on CA1 neurons in hippocampal slice cultures, supporting that CaMKII $\alpha$  could play an important role in nascent spine stabilization. Surprisingly, high-frequency glutamate uncaging (HFU) enhanced new spine survivorship even when CaMKII $\alpha$  kinase activity was genetically or pharmacologically inhibited. In contrast, shRNA-mediated knockdown of CaMKII $\alpha$  blocked activity-dependent new spine stabilization, indicating that CaMKII $\alpha$  expression is required for new spine stabilization. Finally, we found that phospho-mimetic CaMKII $\alpha$ -T286D, which generates increased autonomous CaMKII $\alpha$  interactions, enhanced new spine stabilization even when kinase activity was disrupted with the K42R mutation. Together, our results support a model whereby strong glutamatergic transmission facilitates new spine stabilization through structural and scaffolding functions of CaMKII $\alpha$ .

## Materials and Methods

**Preparation and transfection of organotypic slice cultures.** Organotypic hippocampal slice cultures were prepared from postnatal day (P) 6–8 C57BL/6J wild-type (WT) mice of both sexes, as described (Stoppini et al., 1991; Opitz-Araya and Barria, 2011). Neurons were transfected 2–3 d prior to imaging using particle-mediated gene transfer, as described in Woods and Zito (2008), except 6–8  $\mu$ g of DsRed-Express (Clontech), and 6  $\mu$ g of green fluorescent protein (GFP)-tagged constructs or 5–10  $\mu$ g of GFP was coated onto 6–7 mg of 1.6  $\mu$ m gold beads. GFP-tagged constructs included GFP-CaMKII $\alpha$ , GFP-CaMKII $\alpha$ -T286D, GFP-CaMKII $\alpha$ -K42R/T286D (Pi et al., 2010), or GFP-CaMKII $\alpha$ -K42R (Tullis et al., 2020). CaMKII $\alpha$  knockdown used 25  $\mu$ g CaMKII $\alpha$ -shRNA and rescue also contained 6  $\mu$ g shRNA-resistant full-length GFP-CaMKII $\alpha$ \* or a truncated (residues 1–325) monomeric GFP-mCaMKII $\alpha$ \* (Lemieux et al., 2012).

**Two-photon imaging.** Image stacks (512  $\times$  512 pixels, 1  $\mu$ m 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). Data acquisition was controlled by ScanImage (Pologruto et al., 2003) written in MATLAB (MathWorks). The first time point was acquired in slice culture medium at room temperature after which the slice was maintained in the incubator (35°C). To maximize success rate, two cells were interleaved; therefore, if the first cell had no new spines, the second cell could be pursued instead. After 60 min, new spine identification was performed at the second time

point, which was acquired in a bath of recirculating, oxygenated artificial cerebrospinal fluid (ACSF; in mM, 127 NaCl, 25 NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 2.5 KCl, 25 D-glucose,  $\sim$ 310 mOsm, pH 7.2) with 2 mM Ca<sup>2+</sup>, 0–0.1 mM Mg<sup>2+</sup>, and 1  $\mu$ M tetrodotoxin at 31°C. A total of 2.5–3.5 mM of 4-methoxy-7-nitroindolyl-caged L-glutamate (MNI-glutamate) was added for uncaging experiments. Staurosporine (1  $\mu$ M) or an equivalent volume vehicle were added to the bath after new spine identification and 30 min prior to uncaging. Thus, the timing between new spine identification and the HFU stimulation was 30 min on average. All images shown are maximum projections of 3D image stacks after applying a median filter (3  $\times$  3).

**Identification of new spines.** 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 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.

**High-frequency glutamate uncaging.** 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  $\sim$ 0.5  $\mu$ m from the spine head away from the dendrite.

**Image analysis.** 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). Spine length was measured from the tip of the spine to the base of the spine neck where it meets the dendrite.

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 the 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  $<3.23$  a.u., (2) the value of the mean neighbor spine G/R was  $<0.01$ , 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  $<0.05$ . 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  $>20\%$  compared with the first time point) in either the red or green channels were excluded.

**Statistical analysis.** Survivorship curves were compared using the log-rank test. 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, two-way ANOVAs with Bonferroni's post hoc tests for multiple comparisons were used. Between-group comparisons of spine baseline volumes, lengths, and rates of new spine outgrowth were performed using a two-tailed unpaired heteroscedastic Student's *t* test (in the case of two groups), or one-way ANOVA with Tukey's multiple comparisons (in the case of three groups), unless otherwise noted. Error bars represent standard error of the mean (SEM). Statistical tests were performed using GraphPad Prism 9.2 software.

## Results

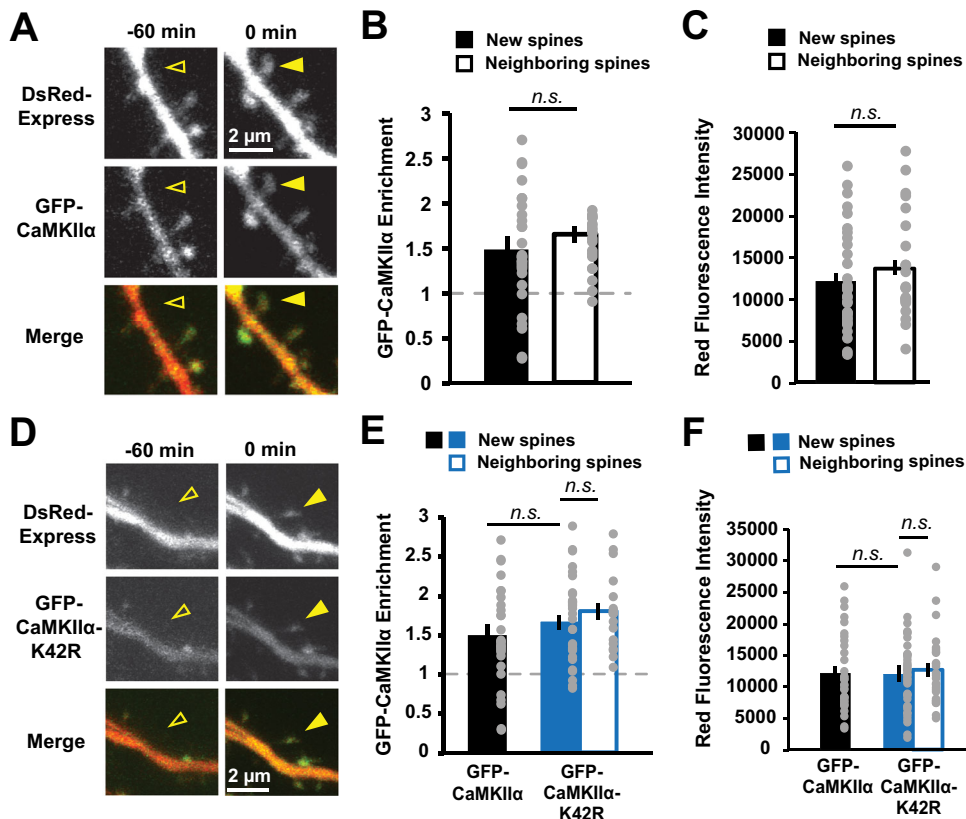
### GFP-CaMKII $\alpha$ enrichment in new spines is comparable to that in size-matched neighboring spines

To understand the role of CaMKII $\alpha$  in activity-induced new spine stabilization, we first needed to determine whether CaMKII $\alpha$  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 postsynaptic density-membrane-associated guanylate kinase (PSD-MAGUK) family of postsynaptic scaffolding molecules are present at very low levels in new spines and can take up to 24 h to accumulate to mature enrichment levels (De Roo et al., 2008a; 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,

two-photon imaging to observe spontaneous new spine outgrowth on the dendrites of hippocampal CA1 neurons in slice culture biolistically transfected with mEGFP-tagged CaMKII $\alpha$  (GFP-CaMKII $\alpha$ ) and a DsRed-Express cell fill (Fig. 1A). We found no difference in the enrichment of GFP-CaMKII $\alpha$  in new spines as compared with size-matched neighboring control spines (new,  $1.5 \pm 0.2$ ; neighbor,  $1.7 \pm 0.1$ ;  $p = 0.14$ ; Fig. 1B,C). We conclude that CaMKII $\alpha$  is able to rapidly accumulate 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 CaMKII $\alpha$ kinase activity does not impair activity-dependent new spine stabilization

To investigate the role of CaMKII $\alpha$  in activity-dependent new spine stabilization, we tested whether interfering with CaMKII $\alpha$  kinase activity would disrupt the robust activity-dependent stabilization of new spines induced by 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-CaMKII $\alpha$  containing the K42R point mutation that inhibits CaMKII $\alpha$  kinase activity (Yamagata et al., 2009; Pi et al., 2010; Tullis et al., 2020). This CaMKII $\alpha$ -K42R mutant has been shown to act in a dominant-negative manner (Pi et al., 2010; Rossetti



**Figure 1.** GFP-CaMKII $\alpha$  enrichment in new spines is comparable to that in size-matched neighboring spines. **A**, Images of a dendrite from a hippocampal CA1 neurons in slice culture (DIVs 7–9) expressing DsRed-Express (red) and GFP-CaMKII $\alpha$  (green) before (open arrowhead) and after (filled arrowhead) spontaneous new spine outgrowth. **B**, Enrichment (spine:dendrite ratio) of GFP-CaMKII $\alpha$  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-CaMKII $\alpha$ -K42R (green) and DsRed-Express (red) before (open arrowhead) and after (filled arrowhead) spontaneous new spine outgrowth. **E**, Enrichment of GFP-CaMKII $\alpha$ -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,  $p = 0.4$ ; neighbors,  $p = 0.99$ ). Data for GFP-CaMKII $\alpha$ -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 was found between WT (black) and K42R (blue;  $p = 0.99$ ). Data for GFP-CaMKII $\alpha$  new spine size is from **C**. Two-way ANOVA with Bonferroni's multiple-comparisons test.



et al., 2017). Importantly, enrichment of GFP-CaMKII $\alpha$ -K42R in new spines was comparable to that in size-matched mature neighboring spines (new,  $1.7 \pm 0.1$ ; neighbor,  $1.8 \pm 0.1$ ;  $p = 0.39$ ), basal spine enrichment levels of GFP-CaMKII $\alpha$ -K42R in new spines were comparable to those of GFP-CaMKII $\alpha$  ( $p = 0.37$ ; Fig. 1D–F), and enrichment levels after HFU did not change compared with baseline for either GFP-CaMKII $\alpha$  (+1 min,  $1.3 \pm 0.3$ ; +70 min,  $1.0 \pm 0.1$ ;  $p > 0.99$ ) or GFP-CaMKII $\alpha$ -K42R (+1 min,  $0.9 \pm 0.1$ ; +70 min,  $0.9 \pm 0.1$ ;  $p > 0.99$ ). Furthermore, cells expressing GFP-CaMKII $\alpha$ -K42R and GFP-CaMKII $\alpha$  exhibited comparable target new spine sizes, lengths, and rates of new spine outgrowth (Table 1).

We proceeded to test whether expression of GFP-CaMKII $\alpha$ -K42R would disrupt stabilization of nascent dendritic spines. We used time-lapse imaging of dendrites on neurons expressing dsRed-Express and either GFP-CaMKII $\alpha$  or GFP-CaMKII $\alpha$ -K42R to identify multiple new spines that spontaneously grew on each cell. One new spine per cell was exposed to HFU stimulation (Fig. 2A). 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 enhanced stimulated new spine survivorship compared with unstimulated new spines on cells expressing GFP-CaMKII $\alpha$  (Fig. 2B–D; stim, 94%; unstim, 62%;  $p = 0.03$ ). Surprisingly, we found that HFU also robustly enhanced new spine stabilization on cells expressing GFP-CaMKII $\alpha$ -K42R (Fig. 2B–D; stim, 100%; unstim, 68%;  $p = 0.02$ ), suggesting that CaMKII $\alpha$  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-CaMKII $\alpha$  or GFP-CaMKII $\alpha$ -K42R conditions (stim GFP-CaMKII $\alpha$  vs GFP-CaMKII $\alpha$ -K42R,  $p = 0.99$ ; unstim GFP-CaMKII $\alpha$  or GFP-CaMKII $\alpha$ -K42R,  $p = 0.79$ ). Importantly, we confirmed that GFP-CaMKII $\alpha$ -K42R was acting as a dominant negative, as GFP-CaMKII $\alpha$ -K42R-transfected neurons exhibited impaired HFU-induced long-term growth of mature spines (K42R,  $138 \pm 16\%$ ;  $p = 0.11$ ), which is intact in neurons expressing GFP-CaMKII $\alpha$  (WT,  $188 \pm 23\%$ ;  $p = 0.01$ ; Fig. 2E,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 of CaMKII $\alpha$  enzymatic activity in activity-induced new spine stabilization, we pharmacologically inhibited CaMKII $\alpha$  using staurosporine, a potent, broad-spectrum kinase inhibitor that competitively binds the ATP-binding pocket of CaMKII $\alpha$ . Unlike many of the more widely used CaMKII $\alpha$  kinase inhibitors with higher specificity, staurosporine does not interfere with the interaction between activated CaMKII $\alpha$  and the GluN2B subunit (Barcomb et al., 2013). Using staurosporine to inhibit CaMKII $\alpha$  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.

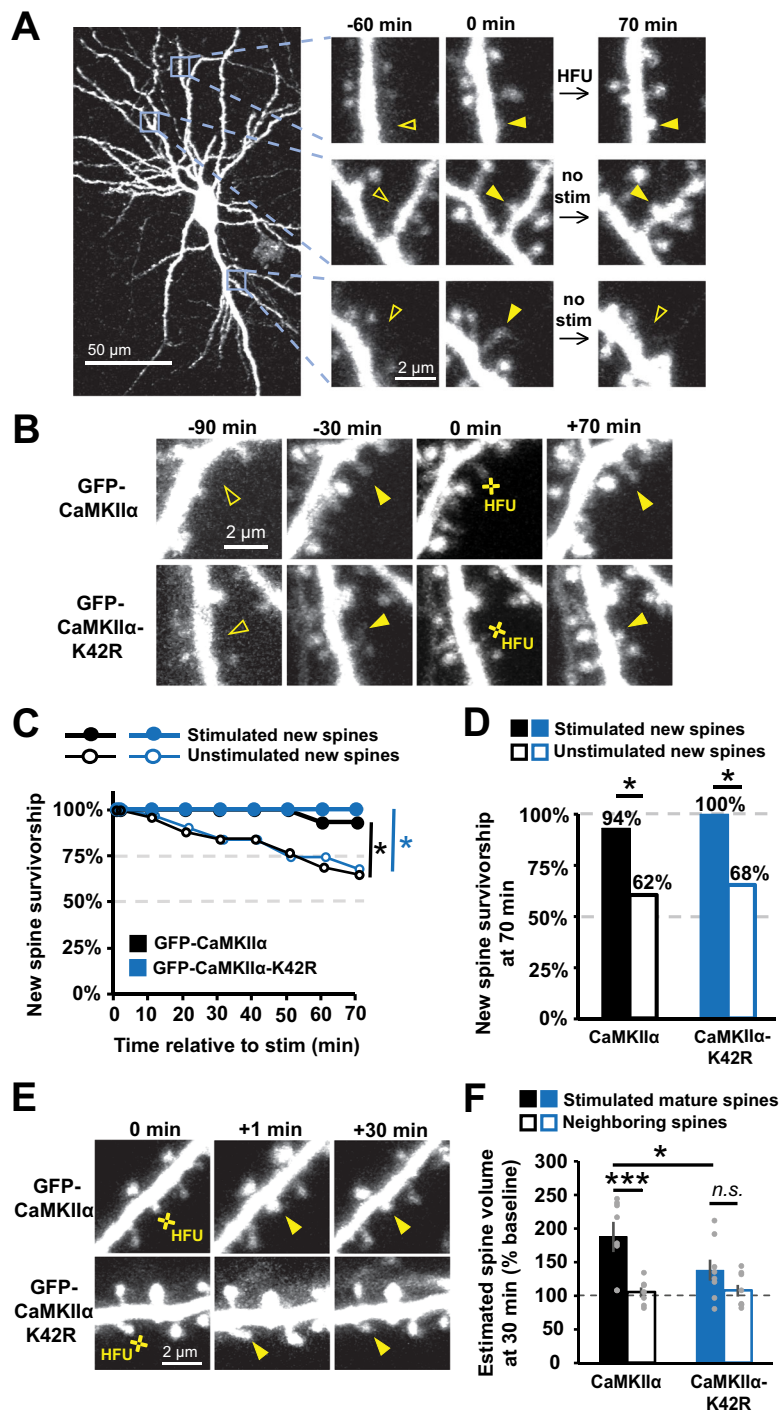
Using time-lapse two-photon imaging of dendrites on hippocampal CA1 neurons expressing GFP, we identified multiple new spines that spontaneously grew on each cell (Fig. 3A). We then added staurosporine (final concentration of  $1 \mu\text{M}$ ) or an equivalent

volume of vehicle for the remainder of the experiment. After a 30 min incubation in either staurosporine or vehicle, 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 more stable than unstimulated new spines on the same cells after incubation in either vehicle (Veh; stim, 100%; unstim, 65%;  $p = 0.04$ ) or staurosporine (Sta; stim, 100%; unstim, 70%;  $p = 0.04$ ; Fig. 3B,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 ( $101 \pm 9\%$ ;  $p = 0.03$ ) but intact in vehicle ( $140 \pm 11\%$ ;  $p = 0.99$ ), indicating the effectiveness of staurosporine as a kinase inhibitor (Fig. 3D,E). Our results with staurosporine are consistent with our finding that genetic inhibition of CaMKII $\alpha$  kinase activity did not impair activity-induced new spine stabilization. Together, these results strongly support that CaMKII $\alpha$  kinase activity is not necessary for activity-dependent new spine stabilization.

### Knockdown of CaMKII $\alpha$ blocks activity-dependent new spine stabilization

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

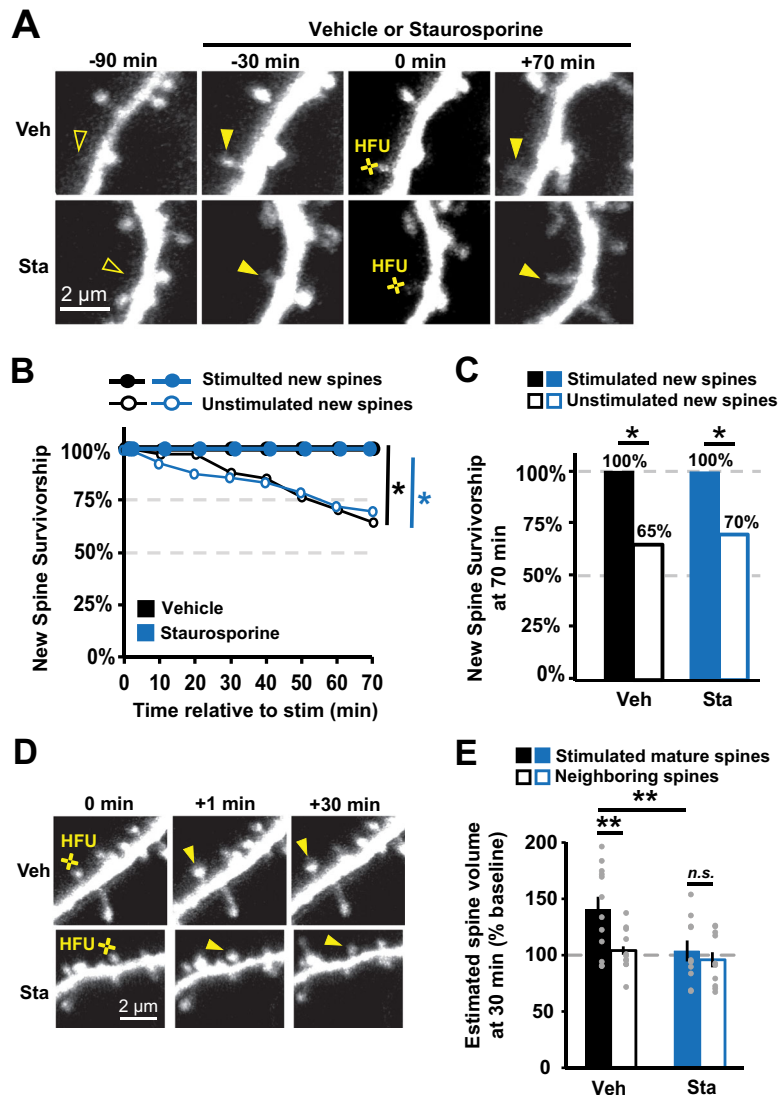
We tested whether structural and/or scaffolding activities of CaMKII $\alpha$  are needed to support activity-dependent nascent spine stabilization using an shRNA-mediated knockdown of endogenous CaMKII $\alpha$  with an shRNA that was designed and validated in previous work (Lemieux et al., 2012). We validated this CaMKII $\alpha$ -shRNA in our preparation by demonstrating that HFU-induced long-term growth of mature spines was blocked by knockdown of CaMKII $\alpha$  ( $98 \pm 10\%$ ;  $p = 0.99$ ) and rescued by co-expression of shRNA-resistant GFP-CaMKII $\alpha^*$  ( $200 \pm 26\%$ ;  $p = 0.04$ ; Fig. 4A,B). We next examined the effect of CaMKII $\alpha$ -shRNA on HFU-induced new spine stabilization. We found that knockdown of CaMKII $\alpha$  disrupted HFU-induced new spine stabilization (Fig. 4C–E; stim, 60%; unstim, 61%;  $p = 0.98$ ). To rule out possible effects of nonspecific shRNA activity, we rescued the knockdown by co-expressing an shRNA-resistant form of GFP-CaMKII $\alpha$  (GFP-CaMKII $\alpha^*$ ). Rescuing CaMKII $\alpha$  levels restored activity-dependent new spine stabilization, as new spines that received the HFU stimulus were again more stable than unstimulated control new spines (Fig. 4C–E; stim, 100%; unstim, 67%;  $p = 0.03$ ). Target new spine sizes, lengths, and outgrowth rates were comparable in cells expressing CaMKII $\alpha$ -shRNA alone and those expressing both the shRNA and the



**Figure 2.** GFP-CaMKII $\alpha$ -K42R does not impair activity-dependent new spine stabilization. **A**, Images of red fluorescence showing dendrites from CA1 neurons in slice culture (DIVs 7–9) expressing DsRed-Express and GFP-CaMKII $\alpha$ . Three new spines appeared (solid arrowheads), one of which was stimulated with HFU. One unstimulated spine was eliminated (open arrowhead at +70 min). **B**, Images (red channel) of dendrites from CA1 neurons expressing DsRed-Express and either GFP-CaMKII $\alpha$  (top row) or GFP-CaMKII $\alpha$ -K42R (bottom row) showing new dendritic spines stimulated with HFU at 0 min. **C**, HFU stimulation enhanced new spine survivorship (filled circles; WT,  $n = 16$  spines/16 cells; K42R,  $n = 14$  spines/14 cells) relative to unstimulated new spines (open circles; WT,  $n = 31$  spines/16 cells; K42R,  $n = 32$  spines/14 cells) on the same cells for both GFP-CaMKII $\alpha$ -WT (black) and GFP-CaMKII $\alpha$ -K42R (blue). **D**, Survivorship of HFU-stimulated new spines (filled bars) at 70 min was increased compared with unstimulated new spines (open bars) on the same cells for both GFP-CaMKII $\alpha$ -WT (black) and GFP-CaMKII $\alpha$ -K42R (blue). **E**, Images of red fluorescence showing dendrites before and after HFU at mature spines at 0 min. **F**, GFP-CaMKII $\alpha$ -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-CaMKII $\alpha$  (filled black;  $n = 8$  spines/8 cells). Log-rank task in **C**, Barnard’s exact test in **D**, and two-way ANOVA with Bonferroni’s multiple-comparisons test in **F**. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

shRNA-resistant GFP-CaMKII $\alpha^*$ , with the exception of new spine size being significantly larger in the CaMKII $\alpha$ -shRNA alone condition relative to the rescue with shRNA-resistant GFP-CaMKII $\alpha^*$  (Table 1), which would be expected to stabilize

CaMKII $\alpha$ -shRNA new spines, instead of destabilize them, as we observed. These results confirm a role for CaMKII $\alpha$  in activity-induced new spine stabilization. Together with our previous results, we conclude that nonenzymatic CaMKII $\alpha$  function is



**Figure 3.** Inhibition of CaMKII $\alpha$  kinase activity with staurosporine does not impair activity-dependent new spine stabilization. **A**, Images of green fluorescence showing dendrites from GFP-transfected CA1 neurons in slice culture (DIVs 7–9). One new spine (filled arrowhead at 0 min) per neuron was stimulated with HFU at 0 min, following 30 min pre-incubation in either vehicle (Veh, top row) or 1  $\mu$ M 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 with unstimulated new spines (open bars) on the same cells in both vehicle (black) and staurosporine (blue) conditions. **D**, Images of green fluorescence showing dendrites on GFP-transfected CA1 neurons before and after HFU (yellow circle). **E**, Incubation with 1  $\mu$ M staurosporine (Sta; filled blue;  $n = 10$  spines/10 cells) blocked HFU-induced long-term growth of mature spines ( $101 \pm 9\%$ ;  $p = 0.03$ ), which was intact in vehicle conditions (Veh; filled black;  $n = 12$  spines/12 cells;  $140 \pm 11\%$ ;  $p = 0.99$ ). Volume of unstimulated neighbors was unchanged (open bars; Veh,  $104 \pm 3\%$ ;  $p = 0.99$ ; K42R,  $96 \pm 7\%$ ;  $p = 0.99$ ). Log-rank task in **B**, Barnard's exact test in **C**, and two-way ANOVA with Bonferroni's multiple-comparisons test in **E**. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

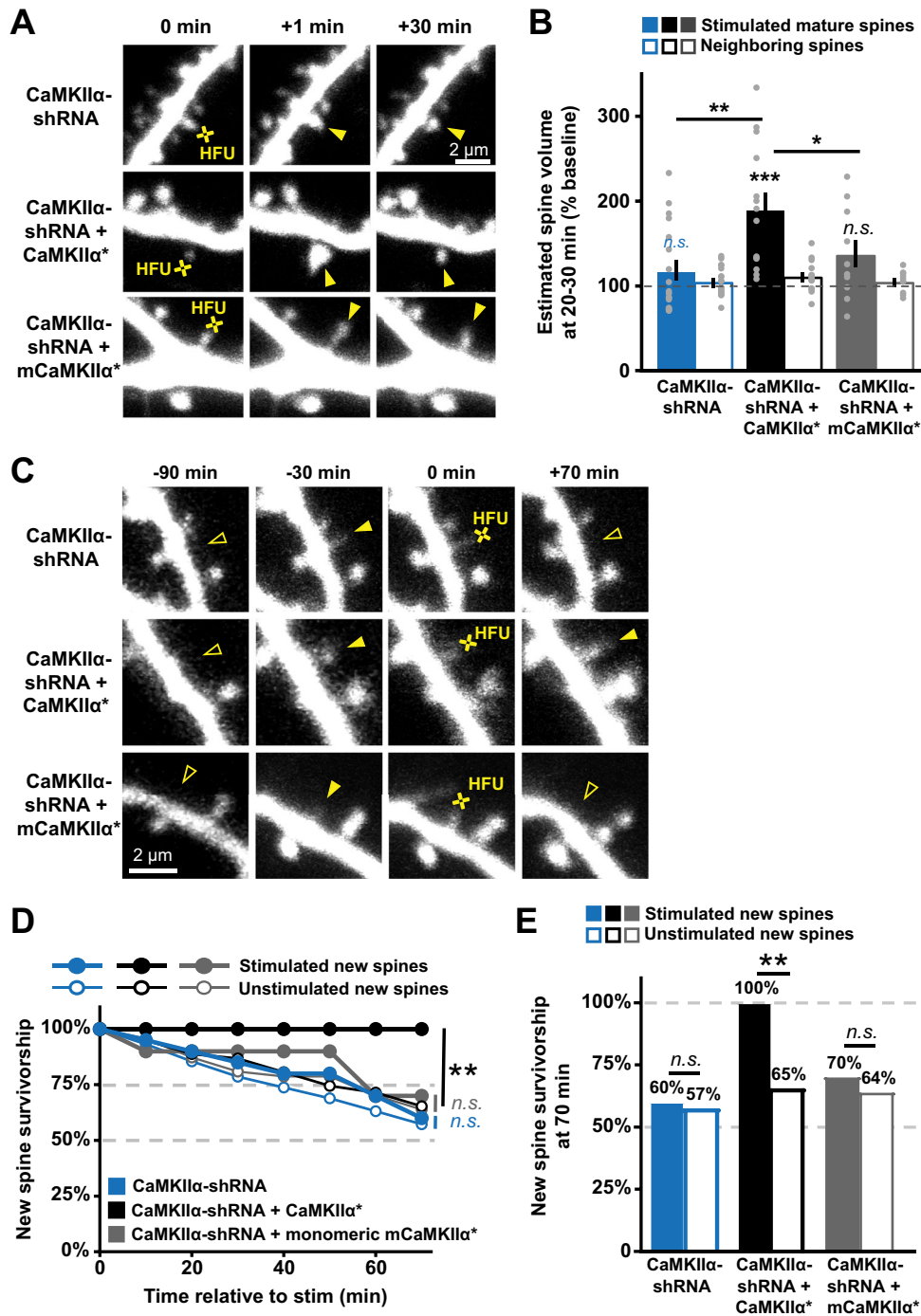
required to enhance new spine stabilization downstream of strong glutamatergic stimulation.

In order to test whether the dodecameric structure of CaMKII $\alpha$  is critical for activity-dependent nascent spine stabilization, we utilized a truncated monomeric version of CaMKII $\alpha$  (amino acids 1–325) that removes the association domain but retains the regulatory domain (Lemieux et al., 2012). Notably, in conjunction with shRNA-CaMKII $\alpha$  knockdown, rescuing CaMKII $\alpha$  levels with shRNA-resistant monomeric GFP-mCaMKII $\alpha^*$  did not restore activity-dependent new spine stabilization, as new spines that received the HFU stimulus were not more stable than unstimulated control new spines on the same cell (Fig. 4C–E; stim, 70%; unstim, 64%;  $p = 0.7$ ). Importantly, enrichment levels in new spines of GFP-CaMKII $\alpha^*$  ( $1.4 \pm 0.1$ ) and monomeric GFP-mCaMKII $\alpha^*$  ( $1.3 \pm 0.1$ ) were comparable

( $p = 0.8$ ). Furthermore, target new spine sizes, lengths, and outgrowth rates were comparable in cells expressing CaMKII $\alpha$ -shRNA alone and those expressing both the shRNA and the shRNA-resistant monomeric GFP-mCaMKII $\alpha^*$  (Table 1). Thus, we conclude that the dodecameric structure of CaMKII $\alpha$  is critical for activity-dependent nascent spine stabilization.

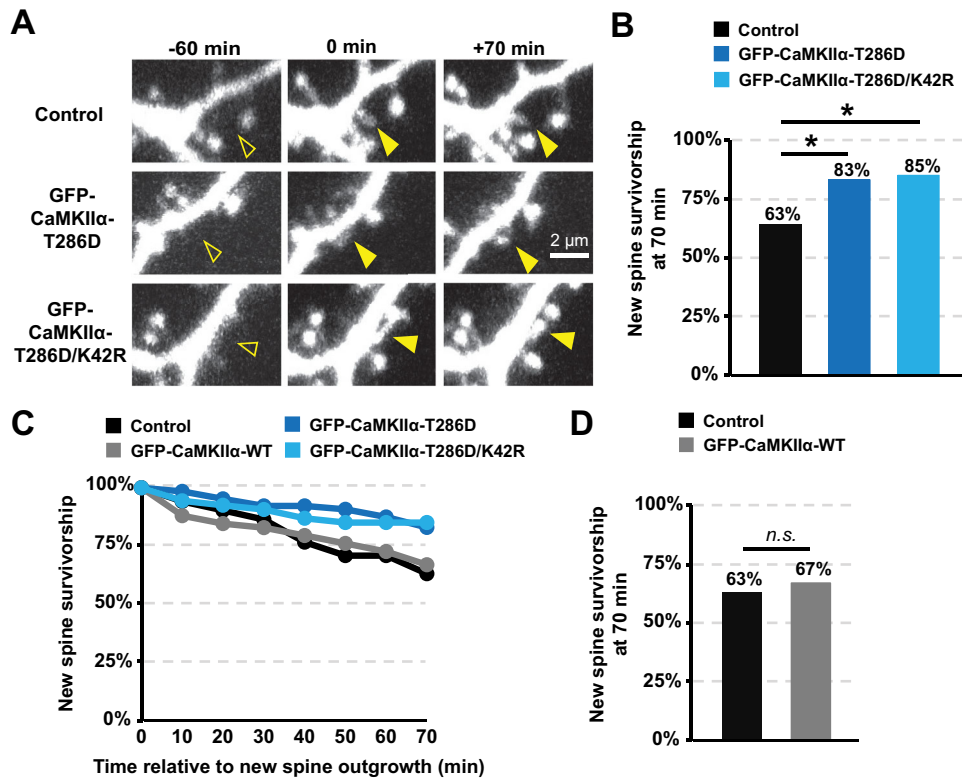
#### Overexpression of pseudo-autophosphorylated CaMKII $\alpha$ enhances basal spine survivorship independent of kinase activity

We next probed whether CaMKII $\alpha$ 's nonenzymatic structural and/or scaffolding activities are not only necessary but sufficient to enhance activity-dependent new spine stabilization. We took advantage of phospho-mimetic CaMKII $\alpha$  mutants that increase basal levels of CaMKII $\alpha$ -GluN2B binding (Barcomb et al.,



**Figure 4.** CaMKII $\alpha$  knockdown blocks activity-dependent new spine stabilization. **A**, Images of red fluorescence showing mature spines on dendrites from hippocampal CA1 neurons in slice culture (DIVs 7–9) transfected with dsRed-Express and CaMKII $\alpha$  shRNA alone (top row) or co-expressed with shRNA-resistant CaMKII $\alpha^*$  variants (middle and bottom rows), showing mature dendritic spines stimulated with HFU at 0 min. **B**, shRNA-mediated knockdown of CaMKII $\alpha$  (filled blue;  $n = 8$  spines/8 cells) impaired HFU-induced long-term growth of mature spines that was restored with co-expression of shRNA-resistant GFP-CaMKII $\alpha^*$  (black;  $n = 14$  spines/14 cells) but not with co-expression of monomeric mGFP-CaMKII $\alpha^*$  (gray;  $n = 10$  spines/10 cells). **C**, Images of red fluorescence showing dendrites on CA1 neurons (DIVs 7–9) expressing a DsRed-Express cell fill and either CaMKII $\alpha$  shRNA (top row) or CaMKII $\alpha$  shRNA + shRNA-resistant GFP-CaMKII $\alpha^*$  variants (bottom two rows). HFU-induced new spine (filled arrowheads) stabilization failed following knockdown of CaMKII $\alpha$  (open arrowhead at 70 min), which was rescued with expression of shRNA-resistant GFP-CaMKII $\alpha^*$ , but not with expression of monomeric shRNA-resistant mGFP-CaMKII $\alpha^*$ . **D**, Knockdown of CaMKII $\alpha$  disrupted the stabilization of new spines (filled circles) as compared with unstimulated new spines (open circles) at times beyond 30–40 min (blue; stimulated, 20 spines/20 cells; unstimulated, 103 spines/20 cells). Rescuing with full-length shRNA-resistant CaMKII $\alpha^*$  restored activity-dependent new spine stabilization (black; stim, 18 spines/18 cells; unstim, 98 spines/18 cells), but not shRNA-resistant monomeric mCaMKII $\alpha^*$  (gray; stim, 10 spines/10 cells; unstim, 47 spines/10 cells). **E**, Activity-dependent new spine stabilization at 70 min (filled bars) was not different from that of unstimulated new spines (open bars) following knockdown of CaMKII $\alpha$  (black), which was restored when CaMKII $\alpha$  is rescued with shRNA-resistant CaMKII $\alpha^*$  (blue), but not with shRNA-resistant monomeric mCaMKII $\alpha^*$ . Two-way ANOVA with Bonferroni’s multiple-comparisons test in **B**, log-rank task in **D**, and Barnard’s exact test in **E**. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .





**Figure 5.** Overexpression of constitutively autonomous CaMKII $\alpha$  enhances basal spine survivorship independent of kinase activity. **A**, Images of red fluorescence showing new spine outgrowth (filled arrowhead at 0 min) and stabilization (filled arrowhead at 70 min) on dendrites from hippocampal neurons (DIVs 7–9) expressing DsRed-Express alone (top row) or co-expressed with GFP-CaMKII $\alpha$ -T286D (middle row) or GFP-CaMKII $\alpha$ -K42R/T286D (bottom row). **B**, Basal spine survivorship rates were higher on cells expressing GFP-CaMKII $\alpha$ -T286D (dark blue; 63 spines/10 cells) and GFP-CaMKII $\alpha$ -K42R/T286D (light blue; 53 spines/7 cells) compared with survivorship rates on cells expressing only DsRed-Express (black; 51 spines/10 cells). Barnard's exact test with Bonferroni's multiple-comparisons correction. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

2014), specifically the replacement of threonine 286 with an aspartic acid, or CaMKII $\alpha$ -T286D (Pi et al., 2010). The T286D mutation renders CaMKII $\alpha$  constitutively active, allowing interactors and substrates access to the kinase and regulatory domains. Pairing this mutation with the K42R point mutation generates increased autonomous CaMKII $\alpha$  interactions, while blocking CaMKII $\alpha$  enzymatic activities.

To determine the effect of autonomous CaMKII $\alpha$  on new spine survivorship with and without kinase activity, we expressed the GFP-CaMKII $\alpha$ -T286D or GFP-CaMKII $\alpha$ -K42R/T286D with a dsRed-Express cell fill in organotypic hippocampal slice cultures. For these experiments where we wanted to determine whether constitutively autonomous CaMKII $\alpha$  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. We found that new spines were more stable on cells expressing either GFP-CaMKII $\alpha$ -T286D or GFP-CaMKII $\alpha$ -K42R/T286D compared with new spines on cells expressing only DsRed-Express (Fig. 5A–C; DsRed, 63%; T286D, 83%;  $p = 0.02$ ; K42R/T286D, 85%;  $p = 0.01$ ). Importantly, expression of WT GFP-CaMKII $\alpha$  did not alter new spine survivorship as compared with dsRed-Express alone (Fig. 5C,D; DsRed, 67%; WT, 65%;  $p = 0.84$ ), so increased survivorship was due to pseudo-autophosphorylated CaMKII $\alpha$ , independent of kinase activity. Furthermore, new spine size, length, and outgrowth rate were comparable between control cells and those expressing GFP-CaMKII $\alpha$ -T286D or GFP-CaMKII $\alpha$ -K42R/T286D (Table 1). When combined with our previous published results (Hill and Zito, 2013), our findings support a model in which CaMKII $\alpha$ -GluN2B binding facilitates

nonenzymatic CaMKII $\alpha$  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 (Xu et al., 2009; Yang et al., 2009; Roberts et al., 2010; Hayashi-Takagi et al., 2015; Albarran et al., 2021). 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 (Zito et al., 2009; Kwon and Sabatini, 2011) and ultrastructural evidence shows that a subset of new spines are found directly apposed to presynaptic boutons (Trachtenberg et al., 2002; Knott et al., 2006; 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, new spines exhibit very low expression levels of the PSD-family MAGUKs (De Roo et al., 2008a; Lambert et al., 2017), key scaffolding molecules that regulate synaptic strength, maturation, and stability (Ehrlich and Malinow, 2004; Boehm et al., 2006; Elias et al., 2008; Cane et al., 2014; Taft and Turrigiano, 2014). NMDAR currents are also smaller in new spines (Zito et al., 2009; Kwon and Sabatini, 2011), where they demonstrate greater diffusional coupling to the



**Table 1. Target new spine morphological characteristics and new spine outgrowth rates**

Target new spine size						
	Groups	<i>n</i> value (spines/cells)	Pixel value/1,000	Groups compared	<i>p</i> value	Test
Fig. 2	CaMKIIa	16/16	10 ± 1	CaMKIIa vs CaMKIIa-K42R	0.2	Unpaired two-tailed <i>t</i> test
	CaMKIIa-K42R	14/14	14 ± 2			
Fig. 4	CaMKIIa-shRNA + CaMKIIa*	18/18	13 ± 2	CaMKIIa-shRNA + CaMKIIa* vs CaMKIIa-shRNA + mCaMKIIa*	0.7	One-way ANOVA with Tukey's multiple comparisons
	CaMKIIa-shRNA + mCaMKIIa*	10/10	16 ± 2			
	CaMKIIa-shRNA	20/20	21 ± 2			
Fig. 5	GFP-CaMKIIa-T286D	60/9	14 ± 2	GFP-CaMKIIa-T286D vs GFP-CaMKIIa-T286D/K42R	0.5	Control vs GFP-CaMKIIa-T286D Control vs GFP-CaMKIIa-T286D/K42R
	GFP-CaMKIIa-T286D/K42R	29/6	17 ± 3			
	Control (dsRed-Express)	51/9	13 ± 1	0.9		
				0.3		
Target new spine length						
	Groups	<i>n</i> value (spines/cells)	μm	Groups compared	<i>p</i> value	Test
Fig. 2	CaMKIIa	16/16	1.2 ± 0.2	CaMKIIa vs CaMKIIa-K42R	0.6	Unpaired two-tailed <i>t</i> test
	CaMKIIa-K42R	14/14	1.3 ± 0.2			
Fig. 4	CaMKIIa-shRNA + CaMKIIa*	18/18	1.2 ± 0.1	CaMKIIa-shRNA + CaMKIIa* vs CaMKIIa-shRNA + mCaMKIIa*	0.1	One-way ANOVA with Tukey's multiple comparisons
	CaMKIIa-shRNA + mCaMKIIa*	10/10	0.8 ± 0.1			
	CaMKIIa-shRNA	20/20	1.1 ± 0.1			
Fig. 5	GFP-CaMKIIa-T286D	60/9	1.1 ± 0.1	GFP-CaMKIIa-T286D vs GFP-CaMKIIa-T286D/K42R	0.1	Control vs GFP-CaMKIIa-T286D Control vs GFP-CaMKIIa-T286D/K42R
	GFP-CaMKIIa-T286D/K42R	29/6	1.5 ± 0.2			
	Control (dsRed-Express)	51/9	1.3 ± 0.1	0.6		
				0.3		
New spine outgrowth rate						
	Groups	<i>n</i> value (spines/cells)	# spines/μm /60 min	Groups compared	<i>p</i> value	Test
Fig. 2	CaMKIIa	76/16	0.07 ± 0.01	CaMKIIa vs CaMKIIa-K42R	0.8	Unpaired two-tailed <i>t</i> test
	CaMKIIa-K42R	86/14	0.07 ± 0.01			
Fig. 4	CaMKIIa-shRNA + CaMKIIa*	104/18	0.07 ± 0.01	CaMKIIa-shRNA + CaMKIIa* vs CaMKIIa-shRNA + mCaMKIIa*	0.3	One-Way ANOVA with Tukey's multiple comparisons
	CaMKIIa-shRNA + mCaMKIIa*	57/10	0.06 ± 0.01			
	CaMKIIa-shRNA	148/20	0.06 ± 0.01			
Fig. 5	GFP-CaMKIIa-T286D	60/9	0.06 ± 0.01	GFP-CaMKIIa-T286D vs GFP-CaMKIIa-T286D/K42R	0.5	Control vs GFP-CaMKIIa-T286D Control vs GFP-CaMKIIa-T286D/K42R
	GFP-CaMKIIa-T286D/K42R	29/6	0.05 ± 0.01			
	Control (dsRed-Express)	51/9	0.05 ± 0.01	0.7		
				0.9		

The morphological characteristics of target new spines and outgrowth rates are compared at the time of first appearance (at the end of the first 60 min time-lapse interval).

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 synaptic stability (De Roo et al., 2008a,b; Cane et al., 2014; Taft and Turrigiano, 2014; Lambert et al., 2017), suggesting that the low basal survivorship rates of new spines may be due to their distinct molecular composition and signaling. 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-CaMKIIa at levels comparable to those in mature spine levels, independent of CaMKIIa kinase activity. CaMKIIa's presence in new spines supports that CaMKIIa signaling could play a critical role in new spine function. Indeed, evidence supports a requirement for the CaMKIIa-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 CaMKIIa's functions at new spines may precede any form of synaptic stimulation.

**Role of CaMKIIa kinase activity in new spine stabilization**

Despite our finding that CaMKIIa is present at mature levels in new spines, we were surprised to find that CaMKIIa kinase activity is not required for enhanced new spine stabilization induced either by strong glutamatergic stimulation at single spines or by overexpression of the CaMKIIa-K42R/T286 phospho-mutant. Our results in new spines are in contrast with what is known regarding the important role of CaMKIIa kinase activity in stabilization of the long-term growth of mature spines (Araki et al., 2015; Cornelia 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., 2008a; 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 CaMKIIa 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. Overall, our data demonstrate that CaMKII $\alpha$  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–CaMKII $\alpha$ binding in new spine stabilization

Our finding that CaMKII $\alpha$  kinase activity is not required for activity-dependent new spine stabilization leaves an undefined role for the required CaMKII $\alpha$ –GluN2B interaction (Hill and Zito, 2013). This interaction has long been known to be important in the regulation of basal synaptic transmission and LTP maintenance (Barria and Malinow, 2002; Halt et al., 2012; Barcomb et al., 2014; Incontro et al., 2018), where it is thought to play a role in bringing Ca<sup>2+</sup>/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 nonenzymatic role for CaMKII $\alpha$  in new spine stabilization. Indeed, we show that, although CaMKII $\alpha$  kinase activity is not required, knockdown of CaMKII $\alpha$  disrupts activity-dependent new spine stabilization. Altogether our results suggest that the interaction between GluN2B and CaMKII $\alpha$  is required to support a primarily structural or scaffolding role for CaMKII $\alpha$  in new spine stabilization.

While we found that CaMKII $\alpha$ -T286D, which enhanced the interaction between GluN2B and CaMKII $\alpha$ , also increased basal spine stabilization, survivorship rates for CaMKII $\alpha$ -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 (Lee et al., 2003; Malinow, 2003; Murakoshi et al., 2011; Bosch et al., 2014; Stein et al., 2021) that are not replicated by overexpression of the CaMKII $\alpha$ -T286D phosphomutant. It is likely that at least a subset of these mechanisms acts in conjunction with GluN2B–CaMKII $\alpha$  binding to enhance activity-dependent new spine stabilization. In addition, the GluN2B–CaMKII $\alpha$  interaction may serve to bring CaMKII $\beta$ , which complexes with CaMKII $\alpha$  at a 3:9 ratio in the hippocampus, within optimal proximity to its binding partners in order to regulate cytoskeletal stability (Okamoto et al., 2007; Kim et al., 2015; Kim et al., 2019).

#### Nonenzymatic CaMKII $\alpha$ function in new spine stabilization

Although CaMKII $\beta$  is perhaps more well recognized than CaMKII $\alpha$  for its nonenzymatic functions in regulating the spine actin cytoskeleton, CaMKII $\alpha$  has also been shown to participate in several functionally important scaffolding and structural interactions that are distinct from those made by CaMKII $\beta$  (Hell, 2014; Bayer and Schulman, 2019). Some of these interactions are less likely to be relevant for the earliest stages of new spine stabilization, such as roles with stargazin, TARP  $\gamma$ -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 CaMKII $\alpha$  kinase activity. However, other known CaMKII $\alpha$  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 CaMKII $\alpha$  directly to the 26S proteasome, and indirect interactions of CaMKII $\alpha$  with SynGAP-1 $\alpha$  via the

multi-PDZ domain protein MUPP-1 (Krapivinsky et al., 2004; Bingol et al., 2010).

Indeed, CaMKII $\alpha$ 's nonenzymatic interactions with the proteasome and the MUPP1–SynGAP-1 $\alpha$  complex appear particularly promising in the context of understanding new spine stabilization. SynGAP-1 $\alpha$  is a negative regulator of synapse maturation, and its exclusion from synapses contributes to synaptic strengthening, precocious PSD-95 accumulation, and increased spine volume (Vazquez et al., 2004; Clement et al., 2012; Aceti et al., 2015; Araki et al., 2015). Interestingly, activity-dependent dissociation of the MUPP1–SynGAP-1 $\alpha$  complex from CaMKII $\alpha$  does not require CaMKII $\alpha$  kinase activity (Krapivinsky et al., 2004) and thus may provide a mechanism for SynGAP-1 $\alpha$  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 nonenzymatic functions of CaMKII $\alpha$  downstream of GluN2B–CaMKII $\alpha$  to promote new spine survivorship is an intriguing and compelling avenue for future study.

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