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A dual role for the RhoGEF Ephexin5 in regulation of dendritic spine outgrowth

A.M. Hamilton [a], J.T. Lambert [a], L.K. Parajuli [a], O. Vivas [a], D.K. Park [a], I.S. Stein [a], J.N. Jahncke [a], M.E. Greenberg [b], S.S. Margolis [c], K. Zito [a,*]

[a] Center for Neuroscience, University of California Davis, Davis, CA 95618, USA
[b] Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA
[c] Department of Biological Chemistry, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA

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ABSTRACT

The outgrowth of new dendritic spines is closely linked to the formation of new synapses, and is thought to be a vital component of the experience-dependent circuit plasticity that supports learning. Here, we examined the role of the RhoGEF Ephexin5 in driving activity-dependent spine outgrowth. We found that reducing Ephexin5 levels increased spine outgrowth, and increasing Ephexin5 levels decreased spine outgrowth in a GEF-dependent manner, suggesting that Ephexin5 acts as an inhibitor of spine outgrowth. Notably, we found that increased neural activity led to a proteasome-dependent reduction in the levels of Ephexin5 in neuronal dendrites, which could facilitate the enhanced spine outgrowth observed following increased neural activity. Surprisingly, we also found that Ephexin5-GFP levels were elevated on the dendrite at sites of future new spines, prior to new spine outgrowth. Moreover, lowering neuronal Ephexin5 levels inhibited new spine outgrowth in response to both global increases in neural activity and local glutamatergic stimulation of the dendrite, suggesting that Ephexin5 is necessary for activity-dependent spine outgrowth. Our data support a model in which Ephexin5 serves a dual role in spinogenesis, acting both as a brake on overall spine outgrowth and as a necessary component in the site-specific formation of new spines.

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1. Introduction

Enhanced growth and stabilization of new spines has been closely linked with the acquisition of new skills (Xu et al., 2009; Yang et al., 2009; Roberts et al., 2010) and adaptation to changes in sensory input (Trachtenberg et al., 2002; Holtmaat et al., 2006; Hofer et al., 2009), supporting that new spine growth and stabilization is vital for experience-dependent plasticity. Indeed, nascent spines rapidly mature functionally (Zito et al., 2009; Kwon and Sabatini, 2011) and stabilize in response to defined patterns of neural activity (Hill and Zito, 2013), consistent with new spines serving as a major source of synaptic plasticity. Furthermore, targeted disruption of spines that grow during skill learning leads to loss of the newly acquired skill (Hayashi-Takagi et al., 2015), demonstrating that spine growth is necessary for learning. Thus, understanding the signaling mechanisms which induce the growth and stabilization of new dendritic spines is a vital step towards understanding how neural circuits are modified during experience-dependent plasticity.

Several studies have convincingly demonstrated that elevating synaptic or network activity leads to enhanced spine outgrowth (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Jourdain et al., 2003; Kato-Negishi et al., 2003; Hamilton et al., 2012). The signaling pathways linking enhanced neural activity to new spine growth are currently being elucidated; many converge upon Rho GTPases as critical regulators of spinogenesis (Tolias et al., 2011; Penzes and Cahill, 2012; Saneyoshi and Hayashi, 2012; Lai and Ip, 2013; Um et al., 2014; Kim et al., 2015; Nishiyama and Yasuda, 2015; Lee et al., 2016). In addition, we recently identified the proteasome as a key regulator of activity-dependent spine outgrowth (Hamilton et al., 2012). To define the signaling mechanisms that connect proteasome activation to new spine outgrowth, we searched for known targets of the proteasome that serve as negative regulators of spine density. Here, we focus on the guanine nucleotide exchange factor (GEF), Ephexin5. Like the other members of the Ephexin family, Ephexin5 functions as an activator of Rho family GTPases; specifically, Ephexin5 has been shown to activate RhoA in neurons (Margolis et al., 2010). Notably, Ephexin5 is ubiquitinated and degraded by the ubiquitin proteasome system (UPS) in response to stimulation of the EphB2 receptor tyrosine kinase (Margolis et al., 2010). Furthermore, Ephexin5 acts as a negative regulator of spine and synapse density in neuronal cultures (Margolis et al.,

* Corresponding author.
E-mail address: kzito@ucdavis.edu (K. Zito).

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2010); however, whether Ephexin5 acts to inhibit spine outgrowth and synapse formation or to enhance spine and synapse loss remained unresolved. We hypothesized that Ephexin5 inhibits new spine outgrowth, and thus that proteasome-mediated degradation of Ephexin5 serves to connect activity-dependent proteasome activation with new spine outgrowth.

Using two-photon time-lapse imaging of neurons in cultured hippocampal slices combined with genetic manipulations of Ephexin5, we show that Ephexin5 inhibits spine outgrowth in a GEF-dependent manner. Furthermore, elevated neural activity leads to rapid degradation of dendritic Ephexin5-GFP. Surprisingly, we also found that dendritic Ephexin5-GFP levels are locally elevated at sites of spinogenesis, where it accumulates in the dendritic shaft prior to new spine outgrowth. Moreover, reducing Ephexin5 levels inhibits new spine outgrowth in response to both global enhancement of neural activity and local stimulation through glutamate uncaging. These data support a dual role for Ephexin5 in activity-dependent spinogenesis, where it operates both as a check on exuberant spinogenesis, and also as a necessary factor in driving new spine outgrowth.

2. Results

2.1. Ephexin5 inhibits spine outgrowth

In order to determine whether Ephexin5 (E5) negatively regulates spinogenesis, we first tested the consequences of reducing E5 levels on the rate of new spine outgrowth. Hippocampal pyramidal neurons in slice culture (5–6 DIV) were co-transfected with EGFP and E5 shRNA or scrambled control shRNA, which we previously validated in dissociated hippocampal neuronal cultures (Margolis et al., 2010). Following 3–4 days of expression, we used two-photon time-lapse imaging at 15 min intervals to compare rates of new spine outgrowth in knockdown versus control (Fig. 1A). We found that shRNA-mediated knockdown of E5 led to increased spine outgrowth (0.62 ± 0.02 spines/10 μm/15 min) relative to EGFP alone (0.43 ± 0.04 spines/10 μm/15 min; $p < 0.001$) or scrambled shRNA (0.42 ± 0.06 spines/10 μm/15 min; $p < 0.05$; Fig. 1B). Scrambled shRNA did not alter spine outgrowth relative to EGFP alone ($p = 0.9$). Thus, decreasing levels of E5 leads to enhanced spine outgrowth, suggesting that E5 acts to negatively regulate spine outgrowth.

We next tested whether enhancing the levels of E5 would be sufficient to decrease the rate of new spine outgrowth. CA1 neurons were co-transfected with EGFP and myc-tagged variants of Ephexin5 and imaged 3–4 days later to monitor rates of spine outgrowth (Fig. 1C). We found that overexpression of the wild-type E5 reduced spine outgrowth (0.18 ± 0.02 spines/10 μm/15 min, $p < 0.005$) relative to cells transfected with EGFP alone (0.31 ± 0.03 spines/10 μm/15 min; Fig. 1D). As E5 has been found to regulate spine density through activation of RhoA, we also examined the effect of the GEF-disabled Ephexin5-LQR (E5-LQR) mutant (Margolis et al., 2010) on spine outgrowth. We found that overexpression of E5-LQR had no significant effect on spine outgrowth (0.36 ± 0.07 spines/10 μm/15 min; $p = 0.5$) compared to

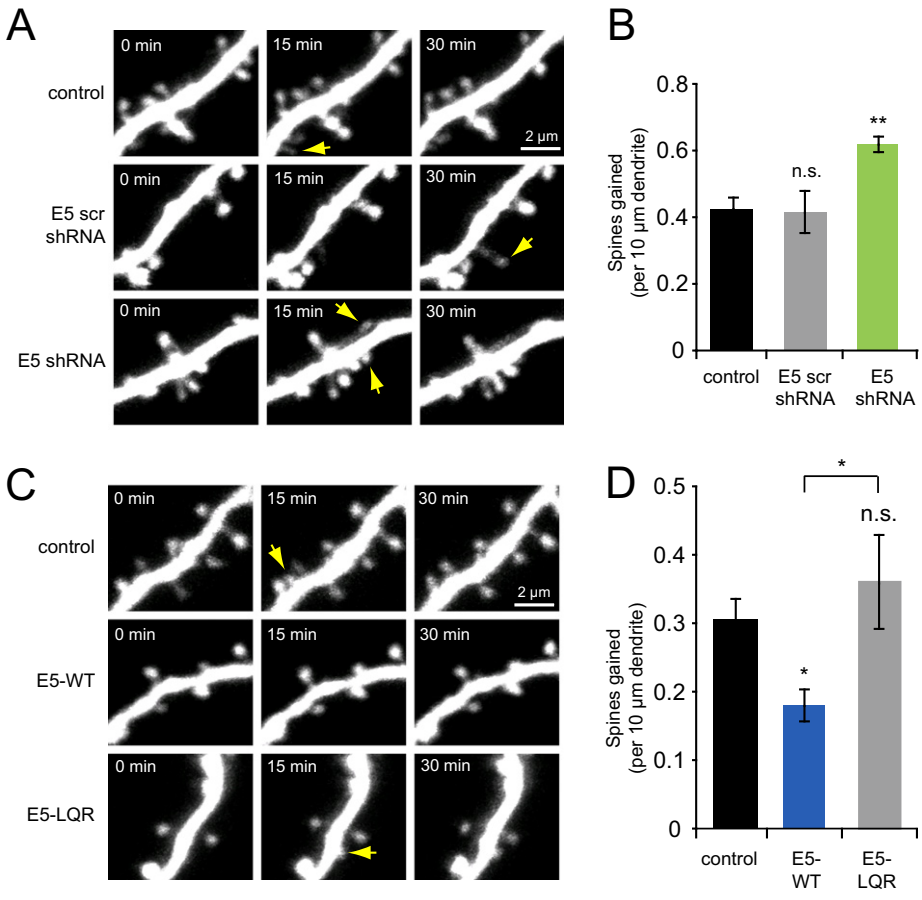

Fig. 1. Ephexin5 inhibits spine outgrowth in a GEF-dependent manner. (A) Images of dendrites from hippocampal pyramidal neurons (DIV 9–10) transfected at DIV 5–6 with EGFP or co-transfected with EGFP + E5 shRNA scramble or E5 shRNA. Yellow arrows indicate sites of new spine outgrowth. (B) Transfection with E5 shRNA increased spine outgrowth (green bar; 7 cells, 101 spines) relative to EGFP controls (black bar; 9 cells, 88 spines; $p < 0.05$), while E5 scrambled shRNA did not (gray bar; 9 cells, 86 spines; $p = 0.9$). **(C)** Images of dendrites from neurons (DIV 9–10) transfected at DIV 6–7 with EGFP or co-transfected with EGFP + E5-WT or E5-LQR. (D) Spine outgrowth was reduced by expression of E5-WT (blue bar; 11 cells, 48 spines) as compared to EGFP controls (black bar; 13 cells, 94 spines, $p < 0.01$) or E5-LQR (gray bar; 8 cells, 99 spines; $p < 0.05$). Overexpression of the GEF-inactive E5-LQR mutant did not alter spine outgrowth relative to EGFP controls ($p = 0.5$). *$p < 0.05$, **$p < 0.001$.

EGFP-only controls (Fig. 1D), indicating that Ephexin5 inhibits spine outgrowth through its activity as a RhoGEF. As a control, we confirmed that the Ephexin5 constructs were expressing at comparable levels using immunostaining against the myc epitope (E5-WT: 84.7 ± 28.6 a.u., $n = 6$ cells; E5-LQR: 116.6 ± 20.9 a.u., $n = 5$ cells; $p = 0.4$; data not shown). Thus, Ephexin5 acts to negatively regulate spine outgrowth through its RhoGEF activity.

2.2. Elevated neural activity rapidly induces proteasomal degradation of Ephexin5

E5 is ubiquitinated and degraded by the UPS in response to stimulation of the EphB2 receptor tyrosine kinase (Margolis et al., 2010). Because activity-dependent spine outgrowth is proteasome-dependent (Hamilton et al., 2012), we hypothesized that E5 might also be degraded

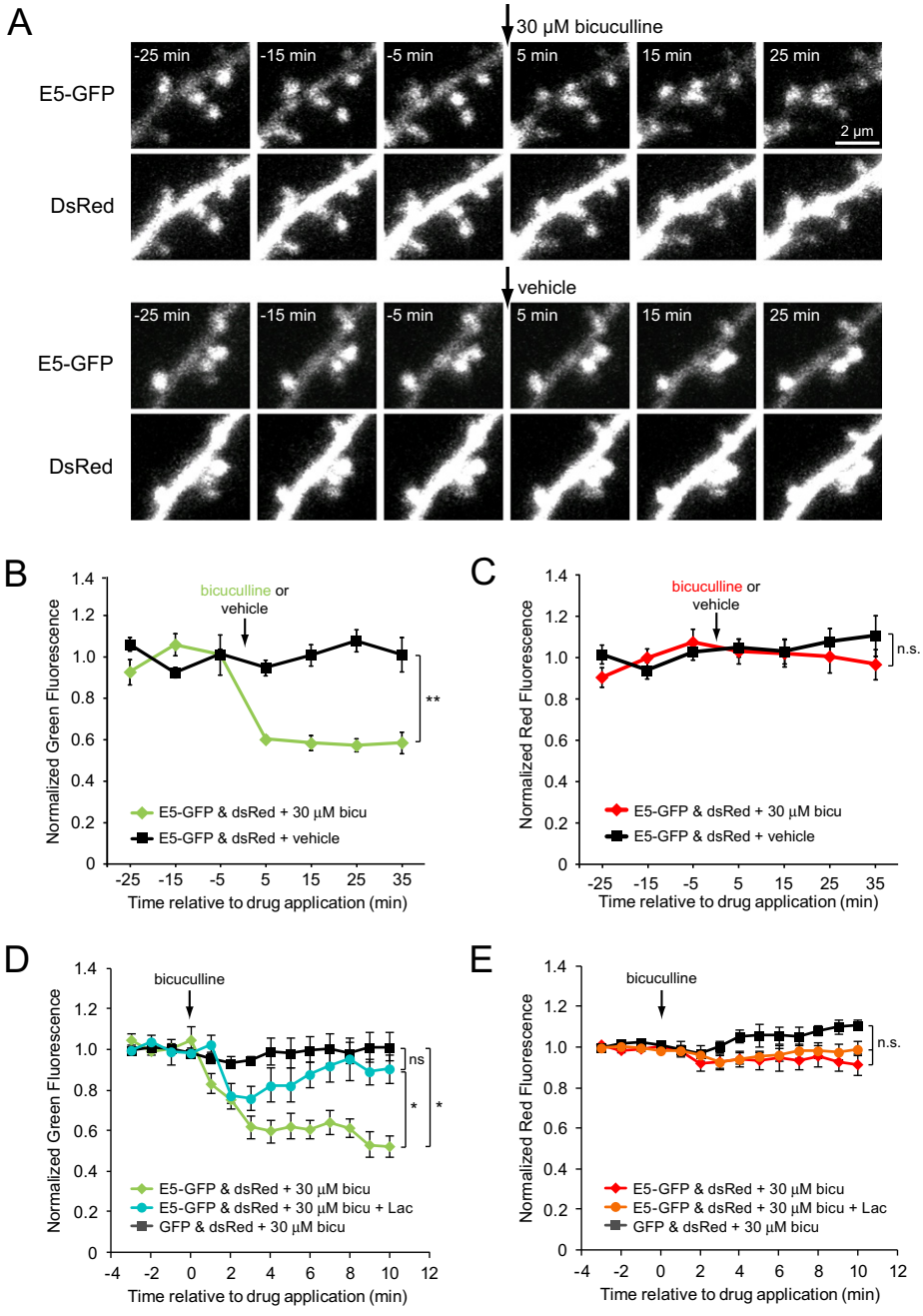

Fig. 2. Dendritic Ephexin5 levels are reduced in response to increased neural activity. (A) Images of dendrites from neurons (DIV 9–12) co-transfected with E5-GFP + DsRedExpress (DsRed) and treated with bicuculline or vehicle at $t = 0$ min. (B) Enhanced neural activity through application of 30 μM bicuculline (arrow) reduced E5-GFP (green fluorescence) levels in the dendrite (green line; 12 dendrites, 5 cells) relative to vehicle-treated controls (black line; 12 dendrites, 6 cells). (C) Application of 30 μM bicuculline (arrow) did not alter DsRed cell fill (red fluorescence) levels in the dendrite (red line) relative to vehicle-treated controls (black line; $p > 0.1$ for all post-treatment time-points). (D) Proteasome inhibition with lactacystin (10 μM) blocked the persistent decrease in E5-GFP fluorescence following bicuculline application (cyan line; 8 dendrites, 5 cells) compared to E5-GFP-expressing cells treated with bicuculline in the absence of lactacystin (green line; 8 dendrites, 7 cells; $p < 0.01$). Cells expressing GFP showed no significant decrease in green fluorescence in response to bicuculline (black line; 3 dendrites, 3 cells). (E) Application of bicuculline and lactacystin did not alter DsRed cell fill (red fluorescence) levels in the dendrites of cells expressing E5-GFP (red line, bicuculline alone; orange line, bicuculline + lactacystin) or GFP (black line, $p = 0.18$). Significance for panels B-E was determined using a one-way ANOVA of the average of the last 5 min for each cell followed by Bonferroni's multiple comparison test, except for control cells expressing GFP, where a paired t-test was used to determine the significance of fluorescence changes at each time-point compared to baseline. $^{*}p < 0.05$, $^{**}p < 0.001$.

in response to neural activity, thus releasing its brake on spinogenesis and leading to enhanced new spine outgrowth.

To address the regulation of E5 levels by neural activity, we co-transfected hippocampal slice cultures (DIV 9–12) with Ephexin5-GFP (E5-GFP) and DsRedExpress (DsRed) as a cell fill. After 2–3 days of expression, we found that E5-GFP was present in the dendrite and concentrated in dendritic spines (Fig. 2A). To address whether enhanced neural activity would decrease E5 levels, we measured E5-GFP fluorescence in both the dendrite and in dendritic spines that were present across all images, before and after treatment with vehicle or the $GABA_A$ receptor blocker bicuculline (Fig. 2A), which rapidly increases neural activity in our cultured slices (Hamilton et al., 2012). While dendritic E5-GFP signal remained constant in vehicle-treated controls, treatment with 30 μM bicuculline resulted in a rapid, persistent decrease in dendritic green signal within 5 min of drug application ($p < 0.001$; heteroscedastic *t*-test of the average of all post drug time-points; Fig. 2B). In contrast, DsRedExpress signal did not change significantly between vehicle and bicuculline-treated conditions in the dendrite ($p = 0.48$; heteroscedastic *t*-test of the average of post-drug time-points; Fig. 2C), demonstrating that the drop in E5-GFP signal was not due to shrinkage of the dendrite. A reduction in green (but not red) signal was also observed in mature spine heads ($p < 0.05$, Fig. S1), suggesting systemic degradation of E5 following enhanced neural activity.

In order to establish whether this activity-induced reduction in E5-GFP signal was due to proteasomal degradation, we treated E5-GFP cells with 10 μM lactacystin to block proteasome activity (10 min preincubation and during the entire experiment), and found that this prevented the persistent reduction in E5-GFP signal observed with bicuculline alone ($p < 0.05$ for all points after 5 min post-bicuculline; Fig. 2D). We also found that bicuculline had no effect on dendritic green signal in cells expressing GFP ($p > 0.05$ for all post-bicuculline time points; Fig. 2D) nor on dendritic DsRedExpress signal ($p > 0.05$ for all post-treatment time points; Fig. 2E). Taken together, these data demonstrate that elevated neural activity rapidly induces proteasomal degradation of Ephexin5, clearing the way for new spine outgrowth.

2.3. Ephexin5-GFP is enriched at sites of new spine formation

If dendritic E5 must be reduced to allow spinogenesis to occur, we hypothesized that E5 levels would be reduced locally at regions of the dendrite experiencing activity-dependent spine outgrowth. In order to examine local E5 content at sites of new spine outgrowth, we performed time-lapse imaging to identify new spines on pyramidal cells co-expressing E5-GFP and DsRedExpress (Fig. 3A). We then quantified the levels of E5-GFP on open regions of the dendrite and at the base of new and persistent spines. Green fluorescence intensity values were normalized to DsRedExpress to correct for fluctuations in laser intensity

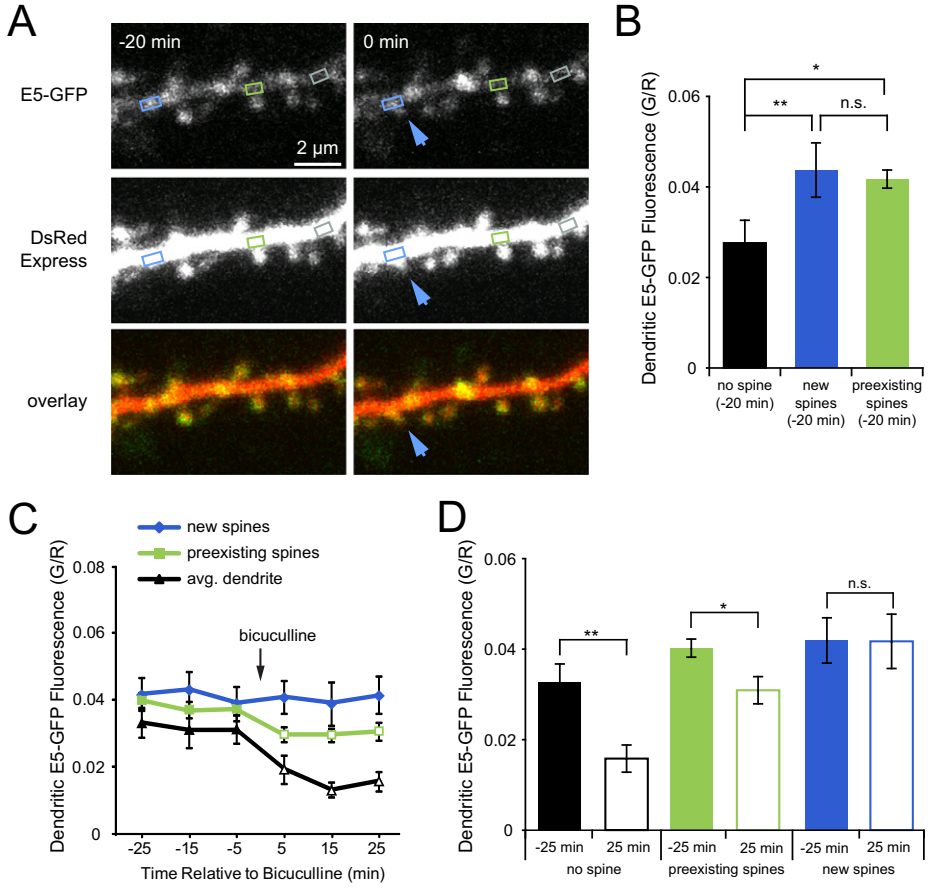

Fig. 3. Ephexin5-GFP is enriched at sites of new spine outgrowth. (A) Images of dendrites from neurons (DIV 9–10) co-transfected at DIV 7–8 with E5-GFP + DsRedExpress (DsRed) and treated with bicuculline to enhance the rate of new spine outgrowth. Blue arrows indicate sites of new spine outgrowth. Time stamps are relative to time of new spine outgrowth. Boxes show representative regions of dendrite at the base of a new spine (blue), preexisting spine (green), or intervening dendrite (gray). (B) Twenty minutes prior to new spine outgrowth, E5-GFP is already concentrated in the dendritic shaft at sites of new spine growth (blue bar; 12 cells, 21 spines) above levels measured on dendritic regions between spines (black bar; 12 cells, 17 segments), but similar to levels observed at the base of preexisting spines (green bar; 12 cells, 75 spines; $p = 0.1$). (C) Within 5 min of treatment with 30 μM bicuculline, E5-GFP levels drop significantly (open symbols, $p < 0.005$) in the dendrite (black line) and at the base of preexisting spines (green line; $p < 0.001$), but not at sites of new spine outgrowth (blue line; $p = 0.8$), compared to average pretreatment levels. (D) In response to bicuculline treatment, E5-GFP is reduced in the dendrite (open black bar; 12 cells, 17 segments; $p < 0.001$) and at the base of preexisting spines (open green bar; 12 cells, 75 spines; $p < 0.001$), but is not changed at the base of new spines (open blue bar; 12 cells, 21 spines; $p = 0.8$). $^*p < 0.05$, $^{**}p < 0.001$, Student's paired *t*-test.

over time. Unexpectedly, we found that E5-GFP was enriched on the dendrite at the base of spines (G/R 0.042 ± 0.002) relative to open areas of the dendritic shaft (G/R 0.028 ± 0.005; $p < 0.005$; Fig. 3B). Remarkably, we also found that E5-GFP was enriched on the dendrite at sites of new spine outgrowth, even 20 min before the appearance of the new spine (G/R 0.044 ± 0.006; $p < 0.001$; Fig. 3A, B). The finding that new spine outgrowth occurs on the dendrite at sites with higher concentrations of Ephexin5 is especially surprising, considering that globally Ephexin5 acts to inhibit spinogenesis (Fig. 1).

Considering that neural activity causes E5 degradation (Fig. 2), we next examined what happened at sites of new spine outgrowth following treatment with 30 μM bicuculline. As expected, we found that within 5 min of bicuculline application, E5 levels dropped rapidly in open dendritic regions and at the base of preexisting spines (−5 min vs. 5 min, $p < 0.001$; Fig. 3C, D). Unexpectedly, E5 levels at the base of new spines were unaffected by bicuculline (−25 min vs. 25 min, $p = 0.8$; Fig. 3C, D), suggesting an alternate state for Ephexin5 at these sites of new spine outgrowth, in which degradation or dispersal is limited.

2.4. Ephexin5 is necessary for activity-dependent spine outgrowth

We have shown that E5 acts to inhibit spine outgrowth (Fig. 1); however, the finding that E5 is enriched at the base of new spines even prior to new spine outgrowth suggests that E5 may also play a positive role in spinogenesis. To test whether E5 is necessary for activity-

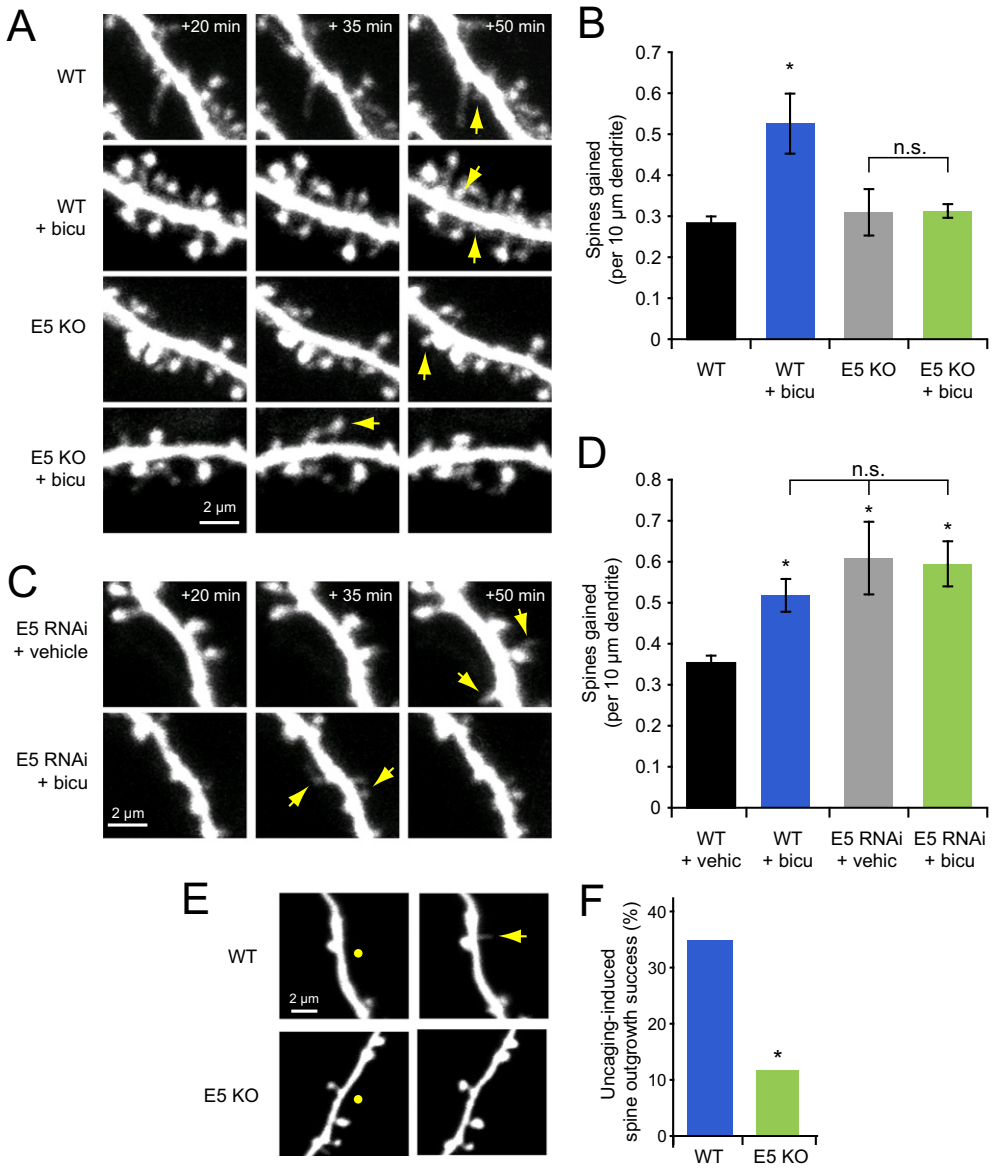

Fig. 4. Ephexin5 is necessary for activity-induced spine outgrowth. (A) Images of dendrites from neurons in slice cultures (DIV 9–10) from E5 KO mice or WT littermates transfected at DIV 6–7 with EGFP and treated with 30 μM bicuculline or vehicle at 0 min. Yellow arrows indicate sites of new spine outgrowth. (B) As expected, treatment with 30 μM bicuculline increased spine outgrowth on cells from WT littermates (blue bar; 4 cells, 68 spines) compared to vehicle-treated WT littermate controls (black bar; 4 cells, 39 spines; $p < 0.05$). In contrast, no increase in spine outgrowth was observed following bicuculline treatment of E5 KO cells (green bar; 4 cells, 28 spines) relative to vehicle-treated E5 KO controls (gray bar; 4 cells, 29 spines; $p = 0.9$). (C) Images of dendrites from neurons in rat slice cultures (DIV 9–10) transfected at DIV 6–7 with EGFP or co-transfected with EGFP + E5 shRNA and treated with 30 μM bicuculline or vehicle at 0 min. Yellow arrows indicate sites of new spine outgrowth. (D) Bicuculline treatment increased spine outgrowth on EGFP-transfected cells (blue bar; 4 cells, 48 spines) relative to vehicle-treated EGFP controls (black bar; 7 cells, 59 spines; $p < 0.05$), but did not on E5 shRNA cells (green bar; 4 cells, 56 spines) relative to vehicle-treated E5 shRNA controls (gray bar; 6 cells, 83 spines; $p = 0.9$). (E) Images of dendrites of CA1 pyramidal cells in slices from WT and E5 KO mutant mice exposed to local uncaging (50 pulses of 4 ms at 5 Hz in 0 Mg^{2+}) of MNI-glutamate (5 mM) at DIV 6–7. Yellow circles represent sites of localized uncaging stimulation. Yellow arrow indicates new spine. (F) Uncaging-induced spine formation success rate was lower on dendrites of E5 KO neurons (green bar; 10 cells, 4 of 34 segments) relative to that observed on dendrites from WT littermates (blue bar; 12 cells, 14 of 40 segments; $p < 0.05$).

dependent spine outgrowth, we treated hippocampal slices from WT and E5 KO (Margolis et al., 2010) mice with vehicle or 30 μM bicuculline. As expected, in WT neurons, spine outgrowth increased following bicuculline treatment (0.53 ± 0.07 spines/10 μm/15 min) relative to vehicle-treated controls (0.28 ± 0.02 spines/10 μm/15 min; $p < 0.05$; Fig. 4A, B). In contrast, there was no increase in spine outgrowth on E5 KO cells treated with bicuculline (0.31 ± 0.02 spines/10 μm/15 min) over that observed on their vehicle-treated counterparts (0.31 ± 0.06 spines/10 μm/15 min; $p = 0.9$; Fig. 4A, B). Thus, surprisingly, Ephexin5 both restricts spine outgrowth and is necessary for activity-dependent increases in spine outgrowth.

The lack of response to bicuculline in the E5 knockout mouse could be caused by altered connectivity, rather than a direct role for E5 in spine outgrowth. In order to determine whether E5 plays a cell autonomous role in activity-induced spine outgrowth, we examined the rate of bicuculline-induced spine outgrowth in neurons transfected with either EGFP alone or EGFP + E5 shRNA. We found that while 30 μM bicuculline increased spine outgrowth in EGFP neurons (0.52 ± 0.04 spines/10 μm/15 min) relative to vehicle-treated EGFP-only controls (0.35 ± 0.02 spines/10 μm/15 min; Fig. 4C, D; $p < 0.05$), this stimulation had no significant effect on E5 shRNA cells (0.60 ± 0.06 spines/10 μm/15 min) relative to vehicle treated E5 shRNA controls (0.61 ± 0.09; $p = 0.9$, Fig. 4C, D).

In addition to global stimulation of synaptic activity, we also examined the necessity for E5 in inducing spine outgrowth through targeted local dendritic stimulation by uncaging with MNI-glutamate. We found that while uncaging on the dendrites of EGFP-transfected CA1 neurons in slices from WT mice resulted in new spines at a frequency similar to previously published rates (Kwon and Sabatini, 2011; Hamilton et al., 2012), the same stimulation applied to E5 KO neurons resulted in dramatically lower rates of outgrowth (35% WT vs 11.8% E5 KO, $p < 0.05$, Fig. 4E, F). Thus, while Ephexin5 acts as a global brake on spine outgrowth, it also is required for spinogenesis in response to both circuit-wide and highly localized increases in neural activation.

2.5. Ephexin5 is not the sole target of the proteasome in regulating spine outgrowth

We previously proposed a model of activity-dependent spine outgrowth in which neural activity led to local proteasome activation that then caused the degradation of inhibitory factors blocking spine outgrowth (Hamilton et al., 2012). If E5 were the sole target of the proteasome in controlling spine outgrowth, the absence of E5 should render the UPS unnecessary for spine outgrowth. To test this hypothesis, we imaged EGFP-transfected hippocampal slices from E5 KO mice and WT littermates before and after the application of vehicle or the proteasome inhibitor lactacystin (Fig. 5A). Similar to previous results (Hamilton et al., 2012), spine outgrowth was decreased on WT neurons treated with 10 μM lactacystin (0.20 ± 0.04 spines/10 μm/15 min) relative to vehicle treated WT controls (0.47 ± 0.07 spines/10 μm/15 min; $p < 0.05$; Fig. 5B). Similar to WT, E5-KO cells treated with lactacystin displayed a reduction in spine outgrowth (0.13 ± 0.04 spines/10 μm/15 min) relative to vehicle-treated E5 KO controls (0.41 ± 0.03 spines/10 μm/15 min; $p < 0.001$). Thus, while E5 may be one of the proteins degraded by the proteasome in order to allow for new spine outgrowth, it is not the only connection between the UPS and spinogenesis.

3. Discussion

3.1. A complex role for Ephexin5 in regulation of activity-dependent spine outgrowth

Here, we demonstrate that reduced Ephexin5 levels increase spine outgrowth, and enhanced Ephexin5 levels decrease spine outgrowth, strongly supporting a role for Ephexin5 as a negative regulator of spinogenesis. We also show that elevated neural activity, which promotes spine outgrowth, is sufficient to rapidly induce Ephexin5 degradation by the ubiquitin proteasome system. However, the classification of Ephexin5 as a simple inhibitor of spinogenesis is not consistent with all of our findings. First, the concentration of GFP-tagged Ephexin5 is locally increased at sites of new spine outgrowth, contrary to our expectation that sites of new spine outgrowth would be associated with lower levels of Ephexin5. Second, while elevated neural activity results in reduced Ephexin5 content in open dendrite and at the base of preexisting spines, the Ephexin5 at the base of new spines is protected from degradation or dispersion, contradicting the hypothesis that Ephexin5 levels must be reduced to permit spine outgrowth. Third, reduced Ephexin5 levels inhibit activity-dependent spine outgrowth. These findings demonstrate that Ephexin5 is not a simple inhibitor that must be removed in order to permit spine outgrowth; instead, these data suggest that Ephexin5 acts both globally as an inhibitor of

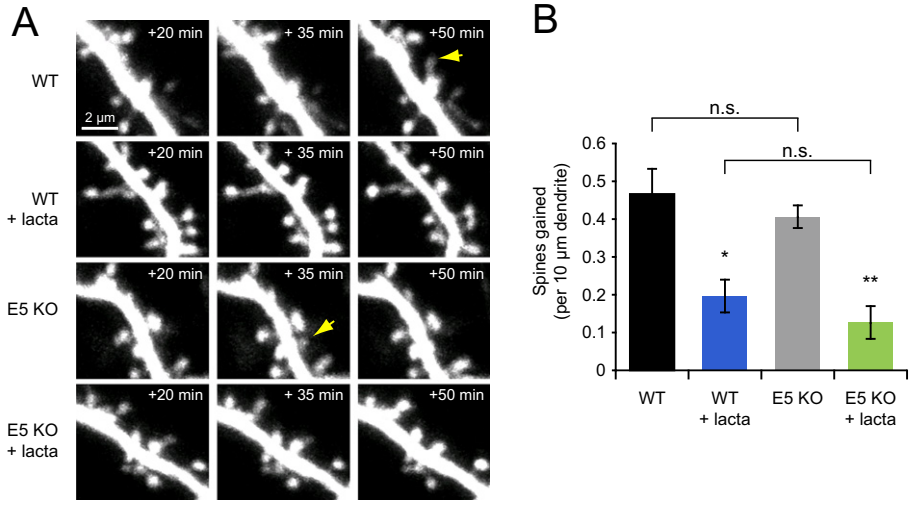

Fig. 5. Ephexin5 is not the sole target of the proteasome during activity-dependent spine outgrowth. (A) Images of dendrites from neurons in slice culture (DIV 9–10) from E5 KO or WT littermates transfected at DIV 6–7 with EGFP and treated with 10 μM lactacystin or vehicle at time 0 min. (B) Treatment of WT mice with 10 μM lactacystin reduced spine outgrowth (blue bar; 5 cells, 21 spines) relative to vehicle-treated WT controls (black bar; 5 cells, 56 spines; $p < 0.05$). A lactacystin-induced decrease in spine outgrowth was also observed in E5 KO cells (green bar; 5 cells, 15 spines) relative to vehicle-treated E5 KO cells (gray bar; 5 cells, 47 spines; $p < 0.001$). WT and KO vehicle treated ($p = 0.4$) and lactacystin treated ($p = 0.3$) groups were not significantly different from each other. *$p < 0.05$, **$p < 0.001$.

spinogenesis and locally as a necessary component in activity-dependent spinogenesis.

The precise mode of action of Ephexin5 in both restricting and permitting spine outgrowth remains unclear, although previous studies of the related GEF Ephexin1 provide an intriguing possible model. A closely related homolog of Ephexin5, Ephexin1 displays GEF specificity that is regulated by its phosphorylation state. Similarly to Ephexin5, Ephexin1 has been shown to target RhoA, but is also capable of activating Cdc42 and Rac1 (Shamah et al., 2001; Sahin et al., 2005), both of which enhance spine density (Tashiro et al., 2000; Wiens et al., 2005; Wegner et al., 2008; Kim et al., 2013; Ueda et al., 2013; Dhar et al., 2014; Raynaud et al., 2014; Evans et al., 2015; Jaudon et al., 2015; Kim et al., 2015; Valdez et al., 2016). Phosphorylation of Ephexin1 by Src kinase, however, causes a shift in Ephexin1 affinity away from Rac1 and Cdc42 towards RhoA, a switch which mediates EphA4-dependent growth cone collapse through RhoA activation (Shamah et al., 2001; Sahin et al., 2005). In a similar manner, spatially restricted phosphorylation of Ephexin5 may locally control a switch between enhancing and inhibiting spinogenesis. Indeed, while neuronal Ephexin5 has been shown to be specific to RhoA in vitro (Margolis et al., 2010), more recent studies have found that Ephexin5 activates Cdc42 in retinal endothelial cells (Kusuhara et al., 2012) and both Rac1 and Cdc42 in cancer cell lines (Fukushima et al., 2016). Perhaps neuronal Ephexin5 can also be induced to activate small GTPases other than RhoA, thus switching from inhibiting to activating spine outgrowth. As a reduction in Ephexin5 is not observed at the base of new spines following bicuculline stimulation, this local Ephexin5 population may also be protected from the forces driving Ephexin5 degradation in the surrounding dendrite. Further research into regulation of Ephexin5 activity and its binding partners will prove vital to understanding Ephexin5's complex role in spine outgrowth.

3.2. A spinogenic complex on the dendrite prior to new spine outgrowth?

The accumulation of Ephexin5 on the dendrite prior to new spine outgrowth, and the necessity of Ephexin5 in activity-dependent spine outgrowth, suggest the presence of a nearby shaft synapse or of a spinogenic complex that assembles prior to new spine outgrowth. Which proteins might contribute to such a complex in addition to Ephexin5? The NMDA receptor (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Tolias et al., 2005; Kwon and Sabatini, 2011; Hamilton et al., 2012; Lin et al., 2013) and the EphB2 receptor tyrosine kinase (Penzes et al., 2003; Tolias et al., 2005; Tolias et al., 2007) both have demonstrated roles in facilitating spine outgrowth and have been shown to physically interact (Dalva et al., 2000; Tolias et al., 2005). Furthermore, Ephexin5 interacts physically with EphB2 (Margolis et al., 2010), and EphB2 stimulation has been shown to induce the recruitment of NMDA receptors and other synaptic proteins to EphB2 puncta (Dalva et al., 2000), suggesting that EphB2 could be a focal point for spinogenesis. Others have postulated such a complex for the control of spine and dendrite morphogenesis based upon the RhoGEFs kalirin-7 (Penzes et al., 2003) and Tiam1 (Tolias et al., 2005), which act as positive regulators of spinogenesis through activation of Rac1. Indeed, recent work has demonstrated that the Rac1 GEF Tiam1 and GAP Bcr form a complex with the EphB2 receptor, and that this complex regulates spine outgrowth in response to ephrinB1 stimulation (Um et al., 2014).

3.3. Molecular mechanisms of activity-dependent spine outgrowth

As an overall inhibitor of spine outgrowth which is degraded in response to increased neural activity, Ephexin5 is an excellent candidate for connecting activity-dependent control of proteolysis with the emergence of new dendritic spines (Hamilton et al., 2012). However, our observation that proteasome-dependent spine outgrowth occurs at similar levels in Ephexin5 knockout mice and their wild-type littermates strongly indicates that another target or targets must be degraded by the proteasome in order for activity-dependent spine outgrowth to occur. While a great many proteins involved in neuronal morphogenesis are known to be regulated by the ubiquitin-proteasome system (Ehlers, 2003; Hamilton and Zito, 2013), the small GTPases, regulators of the actin cytoskeleton, show exceptional promise as acute mediators of spine outgrowth (Tolias et al., 2011; Um et al., 2014).

Several small GTPases have been characterized as enhancers of spine density, especially Cdc42 (Tashiro et al., 2000; Wegner et al., 2008; Kim et al., 2013; Jaudon et al., 2015) and Rac1 (Tashiro et al., 2000; Penzes et al., 2001; Penzes et al., 2003; Tolias et al., 2007; Bacon et al., 2013; Ueda et al., 2013; Dhar et al., 2014; Raynaud et al., 2014; Evans et al., 2015; Jaudon et al., 2015; Kim et al., 2015; Valdez et al., 2016); others have been shown to inhibit spine density, in particular Ras (Yang et al., 2013; Lee et al., 2016) and RhoA (Tashiro et al., 2000; Margolis et al., 2010; Alder et al., 2013; Kim et al., 2015). Furthermore, modulators of activity for Ras (Pham and Rotin, 2001), Cdc42 (Hayakawa et al., 2008; Yamaguchi et al., 2008), and RhoA (Margolis et al., 2010; Lin et al., 2011; Papadimitriou et al., 2012) have been shown to be degraded by the proteasome, as well as Rac1 (Torrino et al., 2011; Zhao et al., 2013) and RhoA themselves (Wang et al., 2003; Cheng et al., 2011). Degradation of several of these proteins may be required in order to permit activity-dependent spine outgrowth, making small GTPases and their regulators a very promising avenue of future research into the molecular mechanisms of proteolysis- and activity-dependent spine outgrowth.

4. Experimental methods

4.1. Cultures and transfection

Organotypic hippocampal slice cultures were prepared as described (Stoppini et al., 1991) from postnatal day (P)6–7 Sprague-Dawley rats or wild-type and E5 KO C57BL/6 mice (Margolis et al., 2010) of both sexes. DNA constructs were delivered 3–4 days (EGFP and EGFP + E5 variants) or 2 days (DsRedExpress + E5-GFP) prior to imaging using biolistic gene transfer (130–180 PSI) as described (Woods and Zito, 2008). We coated 1.6 μm gold beads with 10–20 μg of EGFP (Clontech), 10 μg EGFP + 25 μg E5-WT, E5-LQR, E5 shRNA or E5 shRNA scramble (Margolis et al., 2010), or 10 μg DsRedExpress (Clontech) + 5 μg E5-GFP.

4.2. Time-lapse imaging

Fluorescently-labeled pyramidal neurons (9–10 days in vitro [DIV]) were imaged at 15 min intervals for spine dynamics or 10 min or 1 min intervals for E5-GFP quantification, using a custom 2-photon microscope with a pulsed Ti::sapphire laser (Mai Tai: Spectra Physics) tuned to 930 nm. The microscope was controlled with ScanImage (Pologruto et al., 2003). Slices were imaged at 29 °C in artificial cerebrospinal fluid (ACSF) containing in mM: 127 NaCl, 25 $NaHCO_3$, 25 D-glucose, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, and 2 $CaCl_2$, aerated with 95%O_2/5%CO_2. For each neuron, image stacks (512 × 512 pixels; 0.035 μm/pixel) with 1 μm steps were collected from 1 to 6 segments of secondary dendrites (apical and basal). All displayed images are maximum projections of three-dimensional (3D) image stacks.

4.3. Glutamate uncaging

Organotypic hippocampal slices prepared from P6 to P8 wild-type or E5 KO littermates were transfected with 20 μg EGFP at DIV 3–4. CA1 pyramidal cells at 20–40 μm depth were imaged as above at DIV 6–7. Slices were bathed in Mg^{2+}-free ACSF with 5 mM MNI-glutamate for at least 10 min before being exposed to uncaging stimulus, delivered by parking a 720 nm laser beam at a point 0.5 μm from an open segment of a secondary or tertiary dendrite. The uncaging stimulus consisted of 50 pulses of 4 ms at an interstimulus interval of 200 ms (5 Hz). A maximum of 4 dendrites (2 basal, 2 apical) per cell were stimulated in this manner. Spine outgrowth was scored blindly by 3 independent observers

comparing pre- and post-uncaging 3-dimensional z stacks of the target dendrite. Rate of uncaging-induced spine outgrowth was compared between WT and E5 KO littermates.

4.4. Pharmacology

We prepared 1,000× stocks by dissolving bicuculline (Tocris) and lactacystin (EMD Biochemicals) in water. Vehicle controls were matched in identity and volume to the solution in which the inhibitors were dissolved.

4.5. Immunostaining

Immediately following imaging, slices were submerged in ice-cold 4% paraformaldehyde in 4% sucrose PBS and incubated at 4 °C for 1 h, washed and permeabilized in PBS containing 0.3% triton X-100 and 10% goat serum at 4 °C overnight. Slices were blocked 4 h at RT in 10% goat serum PBS, and incubated with anti-myc (9E10 monoclonal; Covance; 30–50 μg/ml working concentration) overnight at 4 °C, and then washed and incubated with goat anti-mouse IgG1-Alexa 594 for 3 to 4 h at RT, and finally washed and mounted in 100% glycerol. Neurons were imaged on a Zeiss LSM 500 META confocal microscope. All images were acquired using the same laser power, set to assure that pixel values were not saturated.

4.6. Image analysis

Spine addition rates were blindly analyzed in 3D using custom software in MATLAB. For quantification of dendritic E5 levels in living neurons, integrated dendritic green (E5-GFP) and red (DsRedExpress) fluorescence intensities were measured from three boxed regions of interest (ROIs; ~1.0–0.5 μm^2) on the dendritic segment in a single Z slice. Bleed-through corrected and background-subtracted (Woods et al., 2011) mean fluorescence intensities from the three ROIs for each dendritic segment were then normalized to the average of the three pre-treatment time-points. Preexisting spine head E5-GFP was quantified from all mature spines with good separation from the dendrite, and which were present for the entire duration of imaging. For quantification of myc-tagged E5 levels in fixed neurons, Alexa594 fluorescence intensity from anti-myc immunostaining was determined by measuring mean pixel intensity in a circular region entirely within the soma and background subtracted against the same circle dragged to a region devoid of target cell.

4.7. Statistics

Error bars represent standard error of the mean and significance was set at $p = 0.05$ (two-tailed, heteroscedastic t-test, unless otherwise noted). All statistics were calculated across cells. $^{*}p < 0.05$ and $^{**}p < 0.001$. Uncaging-induced spine outgrowth was compared using a two-tailed Fisher's exact test.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mcn.2017.02.001.

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