The EphA4 receptor regulates dendritic spine remodeling by affecting β1-integrin signaling pathways

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Remodeling of dendritic spines is believed to modulate the function of excitatory synapses. We previously reported that the EphA4 receptor tyrosine kinase regulates spine morphology in hippocampal pyramidal neurons, but the signaling pathways involved were not characterized (Murai, K.K., L.N. Nguyen, F. Irie, Y. Yamaguchi, and E.B. Pasquale. 2003. *Nat. Neurosci.* 6:153–160). In this study, we show that EphA4 activation by ephrin-A3 in hippocampal slices inhibits integrin downstream signaling pathways. EphA4 activation decreases tyrosine phosphorylation of the scaffolding protein Crk-associated substrate (Cas) and the tyrosine kinases focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) and also reduces the association of Cas with the Src family kinase Fyn and the adaptor Crk. Consistent with this, EphA4 inhibits β1-integrin activity in neuronal cells. Supporting a functional role for β1 integrin and Cas inactivation downstream of EphA4, the inhibition of integrin or Cas function induces spine morphological changes similar to those associated with EphA4 activation. Furthermore, preventing β1-integrin inactivation blocks the effects of EphA4 on spines. Our results support a model in which EphA4 interferes with integrin signaling pathways that stabilize dendritic spines, thus modulating synaptic interactions with the extracellular environment.

Introduction

Dendritic spines are highly specialized microscopic protrusions on dendrites that typically have an enlarged head connected to the dendritic shaft by a narrow neck (Hering and Sheng, 2001; Ethell and Pasquale, 2005). Dendritic spines are the primary site where the postsynaptic components of excitatory synapses are located in the mammalian central nervous system. The spine neck serves to compartmentalize Ca^{2+} in the spine head away from the dendritic shaft, thus modulating excitatory synaptic transmission (Nimchinsky et al., 2002; Korkotian et al., 2004; Noguchi et al., 2005).

The formation and maintenance of dendritic spines require the coordinated activity of structural and signaling molecules (Ethell and Pasquale, 2005; Kennedy et al., 2005). Particularly important are proteins involved in the regulation of actin filaments, which are the main cytoskeletal component of spines that help define their shape (Matus, 2000). Time-lapse imaging has revealed that dendritic spines are dynamic structures that can undergo actin-based shape modifications and, in some cases, can form and disassemble within minutes (Matus, 2000; Hering and Sheng, 2001; Bonhoeffer and Yuste, 2002; Segal, 2005). Spine remodeling also occurs during the physiological stimulation of synapses and is correlated with changes in the strength of synaptic transmission. Structural reorganization of spines could be important for cognitive processes such as learning and memory (Yuste and Bonhoeffer, 2001; Nimchinsky et al., 2002; Segal, 2005). Indeed, several cognitive disorders are associated with spine malformations and changes in spine density (Fiala et al., 2002; Ethell and Pasquale, 2005). Thus, molecular dissection of the mechanisms involved in regulating dendritic spine morphology could be critical for understanding cognitive function and pathological conditions of the brain.

The Eph family of receptor tyrosine kinases and their ligands, the ephrins, play critical roles in both the developing and mature nervous system (Kullander and Klein, 2002; Poliakov et al., 2004; Yamaguchi and Pasquale, 2004; Pasquale, 2005). Eph receptors and ephrins interact at sites of cell–cell contact, where both are anchored to the cell surface. Ligand–receptor
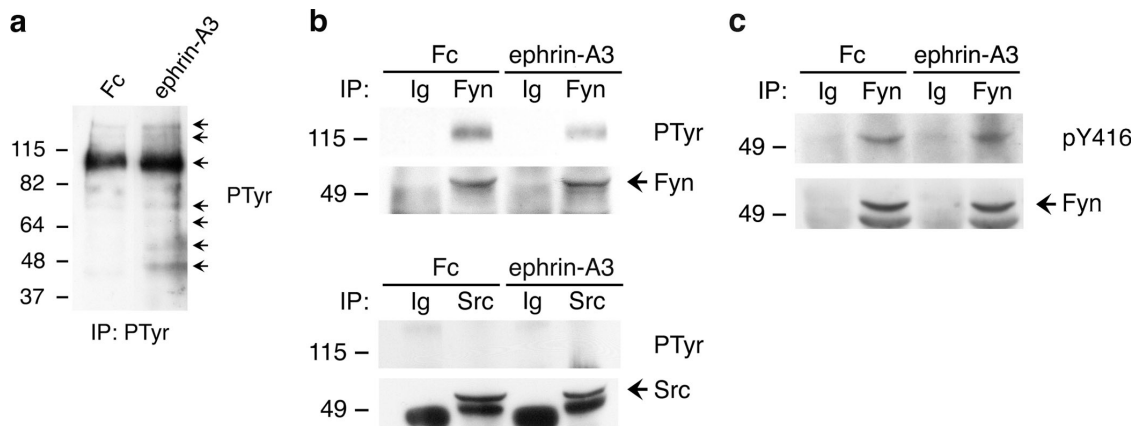
Figure 1. **Ephrin-A3 Fc treatment of hippocampal slices decreases the association of Fyn with a 120-kD tyrosine-phosphorylated protein.** (a) Ephrin-A3 Fc increases the tyrosine phosphorylation of several proteins in P6 mouse hippocampal slices, as indicated by the arrows. (b) A tyrosine-phosphorylated ~120-kD band is detected in Fyn immunoprecipitates from postnatal day 10 hippocampal slices and is less prominent after ephrin-A3 Fc stimulation. The 120-kD band is not detectable in Src immunoprecipitates under the same experimental conditions. Control immunoprecipitates were with nonimmune antibodies (Ig). (c) Ephrin-A3 Fc stimulation of hippocampal slices does not affect Fyn activity. Fyn immunoprecipitates (IP) from Fc- and ephrin-A3 Fc–treated P12 hippocampal slices were probed with phosphospecific antibodies that recognize activated Fyn (pY416) and were reprobed with antibodies to Fyn. Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1) shows quantification and statistical analysis of the data shown in panels b and c.

engagement elicits bidirectional signals: forward signals are generated through the Eph cytoplasmic region, including the tyrosine kinase domain, and reverse signals are communicated by the ephrin through cytoplasmic signaling molecules. Eph receptors and ephrins modify cell shape and movement by reorganizing the actin cytoskeleton. Several recent studies have shown that signaling by EphB receptors regulates dendritic spine morphogenesis, a process whereby the long and thin dendritic filopodial protrusions of immature neurons are replaced by dendritic spines in more mature neurons (Ethell et al., 2001; Henkemeyer et al., 2003; Penzes et al., 2003; Yamaguchi and Pasquale, 2004). Among the Eph receptors of the A class, EphA4 has been shown to regulate dendritic spine morphological plasticity in the adult hippocampus (Murai et al., 2003).

Signaling by EphA4, which is expressed on dendritic spines of pyramidal neurons, reduces spine length and density in acute hippocampal slices. A ligand for EphA4 in the hippocampus is ephrin-A3, which is glycosyl-phosphatidylinositol linked and localized on the surface of the astrocytic processes that surround spines. The molecular interplay between EphA4 and its ligand ephrin-A3 likely mediates a form of cell contact–dependent communication between astrocytes and neurons that helps to maintain the organization and architecture of dendritic spines by restricting their ability to grow and change shape (Murai et al., 2003). Indeed, EphA4 knockout mice have disorganized, longer, and more numerous spines than wild-type mice. Spine defects also accompany the introduction of a kinase-inactive form of EphA4 in pyramidal neurons, suggesting that kinase-dependent signals are involved in spine regulation. Therefore, identifying the signaling pathways that are activated downstream of EphA4 is important for understanding how dendritic spines can be structurally modified by cell surface receptors as well as for elucidating aspects of neuron–glia communication. In this study, we show that EphA4 regulates the morphological plasticity of dendritic spines by modulating integrin activity and downstream signaling molecules.

Results

Ephrin-A3 stimulation inhibits Fyn association with a 120-kD tyrosine-phosphorylated protein

To investigate EphA4 signaling pathways involved in the regulation of dendritic spine morphology, we stimulated acute slices from postnatal mouse hippocampus with the ephrin-A3 ligand. Ephrin-A3 Fc, a soluble dimeric form of ephrin-A3, increased the tyrosine phosphorylation of EphA4 (Murai et al., 2003) and several other proteins (Fig. 1 a).

Src and Fyn are nonreceptor tyrosine kinases of the Src family that function downstream of EphA receptors and regulate the actin cytoskeleton (Ellis et al., 1996; Zisch et al., 1998; Bromann et al., 2004; Knoll and Drescher, 2004). Fyn in particular is present in mature synapses, plays a role in synaptic plasticity, is required for spatial learning and memory, and is needed for maintenance of dendritic spines (Grant et al., 1992; Morita et al., 2006). Interestingly, we found that an ~120-kD tyrosine-phosphorylated protein (or proteins) coprecipitated with Fyn but not Src from hippocampal slices (Fig. 1 b), and ephrin-A3 Fc stimulation decreased the phosphotyrosine signal (Fig. 1 b). This suggests that one or more Fyn-associated proteins are dephosphorylated or become uncoupled from Fyn upon EphA receptor activation by ephrin in hippocampal slices. On the other hand, we did not detect changes in Fyn activation after ephrin-A3 Fc stimulation (Fig. 1 c).

EphA4 activation by ephrin-A3 decreases Cas phosphorylation and association with Fyn and Crk

To identify the tyrosine-phosphorylated proteins associated with Fyn, we examined the interaction of Fyn with ~120-kD proteins known to bind Src family kinases, including the two closely related nonreceptor tyrosine kinases focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2), and the
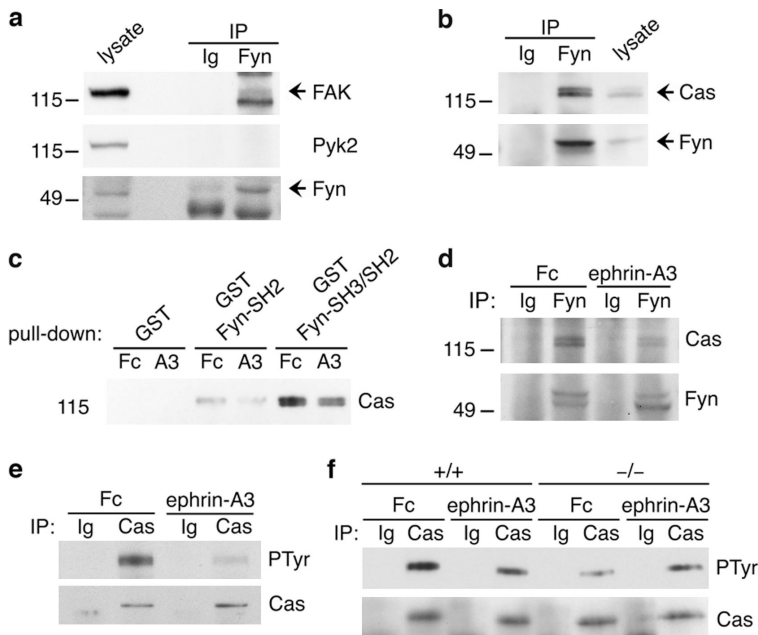
a

lysate | IP
Ig | Fyn

115— ← FAK

115— Pyk2

49— ← Fyn

b

IP | lysate
Ig | Fyn

115— ← Cas

49— ← Fyn

c

pull-down: | GST | GST Fyn-SH2 | GST Fyn-SH3/SH2

Fc A3 | Fc A3 | Fc A3

115 — Cas

d

Fc | ephrin-A3
IP: Ig Fyn | Ig Fyn

115— Cas

49— Fyn

e

Fc | ephrin-A3
IP: Ig Cas | Ig Cas

PTyr

Cas

f

+/+ | −/−
Fc ephrin-A3 | Fc ephrin-A3
IP: Ig Cas Ig Cas | Ig Cas Ig Cas

PTyr

Cas

Figure 2. **Ephrin-A3 stimulation regulates the association of Fyn with Cas.** (a and b) Lysates from P12 acute hippocampal slices were used for immunoprecipitation with Fyn antibodies. The immunoprecipitates were then probed with Fyn and FAK, Pyk2, or Cas antibodies as indicated. (c) Ephrin-A3 Fc reduces Cas binding to the SH2 and SH3 domains of Fyn. Lysates were incubated with equal amounts of purified GST fusion proteins containing the Fyn SH2 or SH2 and SH3 domains or only GST as a control. Bound proteins were probed with Cas antibodies. (d) Ephrin-A3 Fc decreases Fyn–Cas association, as shown by probing Fyn immunoprecipitates with Cas and Fyn antibodies. (e) Ephrin-A3 Fc decreases Cas tyrosine phosphorylation, as shown by probing Cas immunoprecipitates with phosphotyrosine antibodies (PTyr) and Cas antibodies. (f) Ephrin-A3 Fc decreases Cas tyrosine phosphorylation in hippocampal slices from EphA4$^{+/+}$ mice but not in slices from EphA4$^{−/−}$ mice. Lysates from EphA4$^{+/+}$ or EphA4$^{−/−}$ hippocampal slices were stimulated with ephrin-A3 Fc or Fc as a control. Cas immunoprecipitates were probed with phosphotyrosine antibodies (PTyr) and reprobed with Cas antibodies. Fig. S1 shows quantification and statistical analysis of the data shown in panels c–e. Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1) shows quantification of the data in panel f and an additional experiment similar to the one shown in panel f.

scaffolding protein Crk-associated substrate (Cas). Probing Fyn immunoprecipitates from hippocampal lysates for FAK or Pyk2 did not reveal a reproducible association (Fig. 2 a), although in some experiments, weak signals for FAK and Pyk2 were detectable (Fig. 2 a and not depicted). In contrast, substantial amounts of Cas coprecipitated with Fyn (Fig. 2 b). Both the SH2 and SH3 domains of Src family kinases can associate with the Src-binding domain of Cas (O'Neill et al., 2000; Bouton et al., 2001). Treatment of hippocampal slices with ephrin-A3 Fc decreased Cas binding to the SH2 domain of Fyn alone or fused with the SH3 domain in pull-down experiments (Fig. 2 c) and inhibited Cas association with full-length Fyn in coimmunoprecipitation experiments (Fig. 2 d).

As expected from the reduced Cas–Fyn association (Nakamoto et al., 1996; Ruest et al., 2001), ephrin-A3 Fc also decreased Cas tyrosine phosphorylation (Fig. 2 e). This inhibition of Cas phosphorylation was not observed in hippocampal slices from EphA4 knockout mice (Fig. 2 f and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1), suggesting that EphA4 is the main EphA receptor responsible for the effects of ephrin-A3 Fc in hippocampal slices. The reason for the lower basal levels of Cas phosphorylation in the EphA4 knockout slices remains to be determined.

Using a phosphospecific antibody, we found that ephrin-A3 Fc stimulation decreased phosphorylation of the substrate domain of Cas (Fig. 3 a), which contains multiple YxxP motifs.
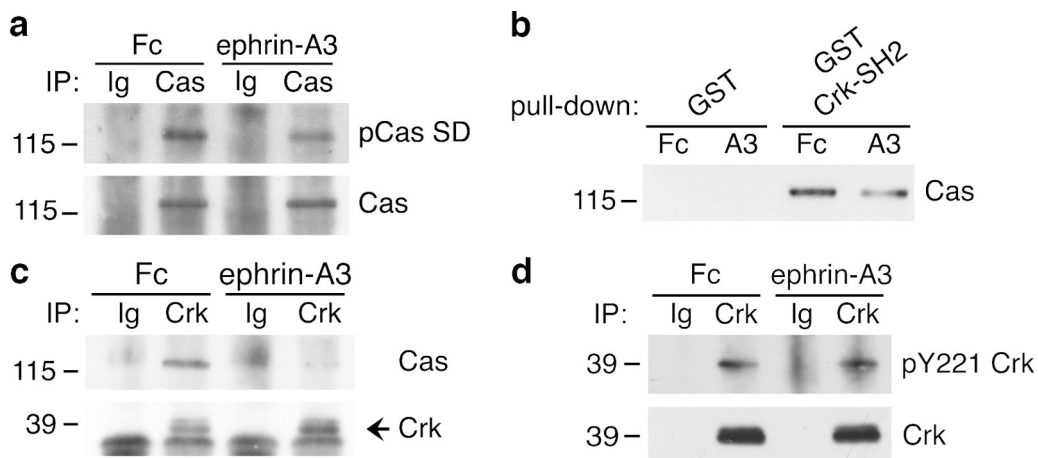

a

Fc | ephrin-A3
IP: Ig Cas | Ig Cas

115 — pCas SD

115 — Cas

b

pull-down: | GST | GST Crk-SH2

Fc A3 | Fc A3

115 — Cas

c

Fc | ephrin-A3
IP: Ig Crk | Ig Crk

115 — Cas

39 — ← Crk

d

Fc | ephrin-A3
IP: Ig Crk | Ig Crk

39 — pY221 Crk

39 — Crk

Figure 3. **Ephrin-A3 regulates Cas phosphorylation in the substrate domain and association with Crk.** (a) Ephrin-A3 Fc decreases the phosphorylation of Cas in the substrate domain. Cas immunoprecipitates from Fc- or ephrin-A3 Fc–treated hippocampal slices were probed with antibodies recognizing several tyrosine phosphorylation sites in the Cas substrate domain (Fonseca et al., 2004) and reprobed with antibodies to Cas. (b) Ephrin-A3 Fc decreases Cas binding to the SH2 domain of Crk. Equal amounts of GST-Crk SH2 domain and GST (as a control) were incubated with lysates from Fc- or ephrin-A3 Fc–treated hippocampal slices and probed with Cas antibodies. (c) Ephrin-A3 Fc reduces Crk–Cas association, as shown by probing Crk immunoprecipitates with Cas antibodies. (d) Ephrin-A3 Fc does not affect Crk phosphorylation on tyrosine 221, as shown by probing Crk immunoprecipitates with phospho-specific antibodies (pY221). Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1) shows quantification and statistical analysis of the data shown in panels a–d.

a

	synaptosome	synaptic cytosol	synaptic membrane	soluble membrane	PSD	
115 –						EphA4
115 –						Cas
84 –						PSD-95
39 –						Syntaxin
64 –						SHP2

b

	brain	hippocampal neuron	glial cells	
115 –				Cas
47 –				β actin
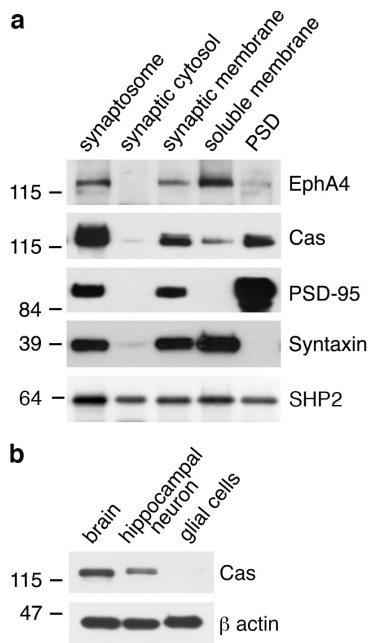
Figure 4. **Cas is expressed in hippocampal neurons and enriched in the PSD.** (a) Subcellular localization of EphA4 and Cas in a synaptosomal preparation from adult mouse brain. Equal amounts of protein from the indicated fractions were probed for EphA4, Cas, PSD-95 (postsynaptic marker), syntaxin (presynaptic marker), and the tyrosine phosphatase SHP2 (control present in all fractions). PSD, postsynaptic density fraction. (b) Cas is expressed in cultured hippocampal neurons but not in glial cells. Cas expression was detected by immunoblotting lysates from adult mouse brain and primary hippocampal neurons and glial cells.

When phosphorylated, these motifs mediate binding of the SH2 domain of the adaptor protein Crk, which is an important effector of Cas (Feller, 2001; Chodniewicz and Klemke, 2004). Indeed, ephrin-A3 Fc treatment inhibited Cas binding to Crk in pull-down (Fig. 3 b) and coimmunoprecipitation experiments (Fig. 3 c). Phosphorylation of Crk on tyrosine 221 is also known to negatively regulate Crk interaction with Cas (Feller, 2001), but ephrin stimulation did not affect the phosphorylation of this site (Fig. 3 d). Thus, EphA4-induced dephosphorylation of Cas in the hippocampus disrupts Cas complexes.

Cas regulates dendritic spine morphology

Cas is a well-known regulator of cell shape and motility (O'Neill et al., 2000; Bouton et al., 2001) and, therefore, may play a role in dendritic spine regulation downstream of EphA4. Indeed, both EphA4 and Cas are present in brain synaptosomal preparations, including the postsynaptic density (PSD) fraction (Fig. 4 a). Furthermore, Cas expression in primary hippocampal neurons but not in glial cells (Fig. 4 b) supports the idea that Cas is involved in EphA4 forward signals that control dendritic spine morphology.

Biolistic transfection of a pool of four Cas siRNAs or individual Cas siRNAs in cultured hippocampal slices caused a substantial decrease in dendritic spine density (30–45%; Fig. 5) and a small but significant decrease in spine length (13–16%; P < 0.01) compared with transfection with control siRNAs that do not knock down Cas expression (Fig. 5 and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1).

Cas siRNA transfection also decreased the number of mushroom-shaped spines, which represent the most common category of spines in hippocampal pyramidal neurons, and increased the number of stubby spines (Fig. 5). These effects were caused by the down-regulation of Cas expression because they were abolished when human Cas was cotransfected with a mouse-specific Cas siRNA (Fig. S4). Expression of the different Cas siRNAs did not detectably affect the overall morphology of the pyramidal neurons (unpublished data). These results implicate Cas as an important regulator of dendritic spines.

To identify the functional domains of Cas that play a role in dendritic spines, we transfected Cas deletion mutants (Fig. 6 a). Spine density was reduced by 48% in pyramidal neurons expressing the CasΔSB mutant (which lacks the Src-binding domain) and by 47% in neurons expressing the CasΔSH3 mutant (Fig. 6, a and b). In contrast, transfection of a Cas mutant that lacks the substrate domain (CasΔSD) and, therefore, cannot bind Crk did not significantly affect spine density (P > 0.05; Fig. 6, a and b). Spine length and width were not significantly affected by any of the Cas mutants (P > 0.05), with the exception of a small increase in spine width in the neurons transfected with CasΔSH3 and CasΔSB. With regard to spine shape, the CasΔSH3 and CasΔSB mutants caused a decrease in mushroom spines and a prominent increase in stubby spines, whereas CasΔSD decreased mushroom spines while increasing thin spines (Fig. 6 c). These results suggest that protein interactions mediated by the Cas SH3 and Src-binding domains contribute to maintaining spine density and play a role in defining spine shape.

Ephrin-A3 inhibits β1-integrin activity in neuronal cells

The Cas SH3 domain is known to be a docking site for the FAK and Pyk2 kinases (O'Neill et al., 2000; Bouton et al., 2001), and we found that treatment of hippocampal slices with ephrin-A3 Fc decreases both FAK and Pyk2 tyrosine phosphorylation (Fig. 7, a and b). Because FAK, Pyk2, and Cas all become tyrosine phosphorylated upon integrin-mediated adhesion (Girault et al., 1999; Playford and Schaller, 2004), the coordinated decrease in their phosphorylation suggested that EphA4 activation may suppress integrin activity. Indeed, ephrin-A3 Fc stimulation of the hippocampal HT22 neuronal cell line, which expresses endogenous EphA4, caused a 35% decrease in cell attachment to surfaces coated with the integrin ligand fibronectin, whereas the integrin-independent attachment to poly-L-lysine was not affected (Fig. 7 c). Similar results were obtained with the neuronal-like B35 cell line, which stably expresses EphA4 (Fig. 7 d). Concurrent with the reduction in cell attachment, ephrin-A3 Fc stimulation reduced the level of Cas tyrosine phosphorylation in both cell lines (Fig. 7, c and d), which is similar to the effect observed in hippocampal slices.

Integrins are heterodimers containing an α and β subunit, and integrins containing the β1 subunit are highly expressed in the brain and influence hippocampal synaptic plasticity (Pinkstaff et al., 1999; Clegg et al., 2003; Chan et al., 2006; Z. Huang et al., 2006). To determine whether EphA4 may inactivate β1 integrins, we used the 9EG7 antibody, which, at low concentrations, detects
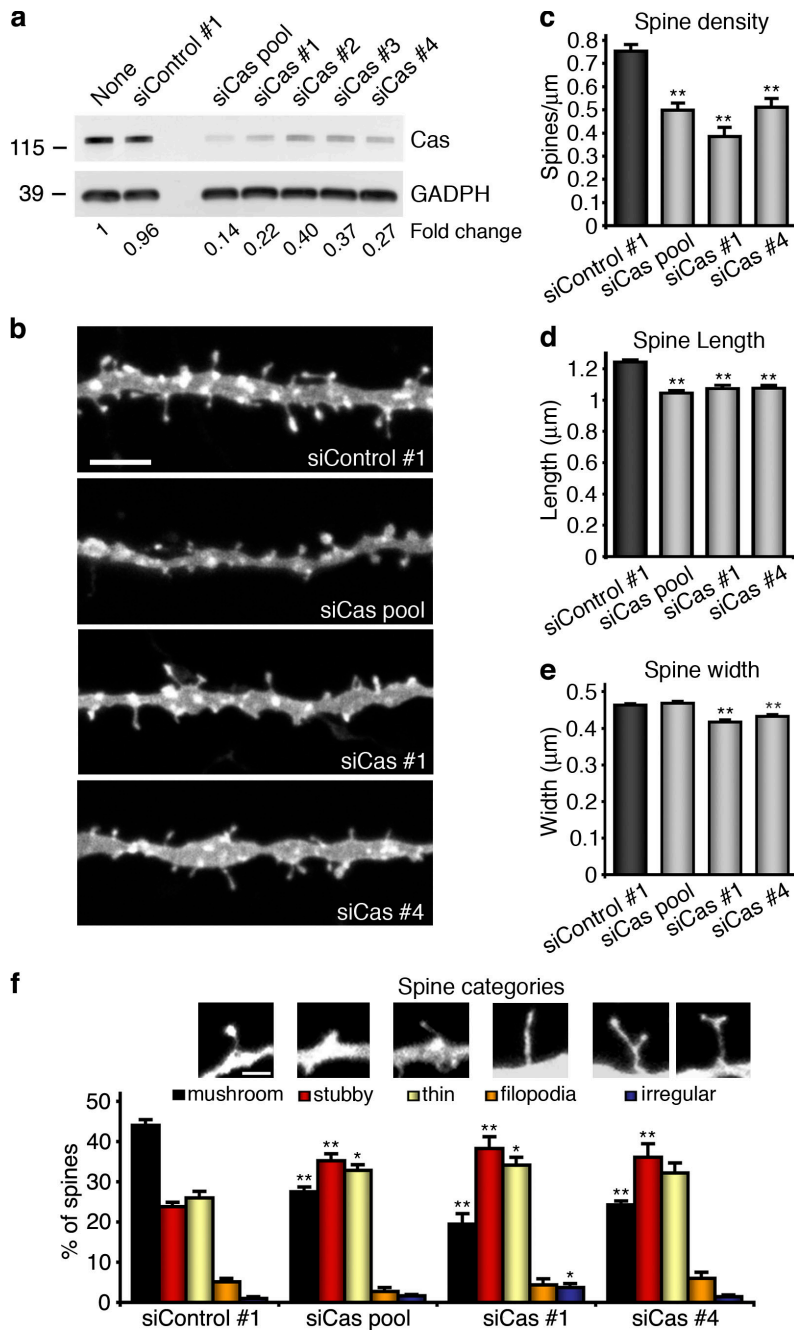
a

115 — Cas
39 — GADPH

None: 1
siControl #1: 0.96
siCas pool: 0.14
siCas #1: 0.22
siCas #2: 0.40
siCas #3: 0.37
siCas #4: 0.27

Fold change

b

siControl #1
siCas pool
siCas #1
siCas #4

c Spine density

d Spine Length

e Spine width

f Spine categories

mushroom stubby thin filopodia irregular

siControl #1 siCas pool siCas #1 siCas #4

Figure 5. **Cas knockdown decreases dendritic spine density and length.** (a) Cas siRNAs effectively and selectively reduce Cas expression. Immunoblot analysis of Cas levels in NIH3T3 cells transfected with four individual Cas siRNAs (siCas #1–4), a pool of the four siRNAs (siCas pool), a control siRNA (siControl #1) or no siRNA (none). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) levels were not affected. (b) Confocal images of dendrites from CA1 pyramidal neurons in cultured hippocampal slices cotransfected with the indicated siRNAs and EGFP-F as a marker. (c–e) Histograms show the mean spine density, length, and width of neurons transfected with Cas siRNAs. Densities are as follows: 0.75 ± 0.02 spines/μm (control #1), 0.49 ± 0.03 spines/μm (siCas pool), 0.38 ± 0.04 spines/μm (siCas #1), and 0.51 ± 0.04 spines/μm (siCas #4). Lengths are as follows: 1.24 ± 0.01 μm (control #1), 1.04 ± 0.01 μm (siCas pool), 1.07 ± 0.02 μm (siCas #1), and 1.07 ± 0.02 μm (siCas #4). (f) Percentage of spines in the indicated morphological categories. Statistical analysis was performed by ANOVA followed by Dunnett's posthoc test for comparisons of neurons transfected with Cas siRNAs to neurons transfected with a control siRNA. *, $P < 0.05$; **, $P < 0.01$. Error bars represent SEM. A total of $n = 709$–$1,525$ spines from 27–42 dendrites of 8–10 CA1 pyramidal neurons were analyzed per condition in three experiments. Additional experiments using a second control siRNA are shown in Fig. S3 (available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1). Bars (b), 5 μm; (f) 1 μm.

the ligand-occupied (active) conformation of β1 integrins without interfering with the binding of extracellular matrix ligands (Bazzoni et al., 1995). Ephrin-A3 Fc treatment of HT22 cells caused a strong decrease in 9EG7 antibody binding (Fig. 7 e) without affecting the cell surface levels of β1 integrins, as determined by surface protein biotinylation (Fig. 7 f). Thus, EphA4 activation by ephrin-A3 inhibits β1-integrin activity and adhesion to fibronectin in neurons.

Inhibition of integrin activity induces spine retraction

To examine whether integrins regulate dendritic spine morphology, we treated cultured hippocampal slices with an integrin function-blocking peptide (RGDFV) or a control peptide

(RADFV; Fig. 8 a). Peptides containing the RGD (Arg-Gly-Asp) motif competitively antagonize integrin binding to extracellular matrix ligands (Ruoslahti, 1996; Shimaoka and Springer, 2003; Hama et al., 2004). Treatment of slices with the RGDFV peptide significantly decreased Cas tyrosine phosphorylation (P < 0.05; Fig. 8 b). Although RGD peptides have also been shown to promote integrin activation depending on the experimental conditions (Lin et al., 2003; Bernard-Trifilo et al., 2005; Shi and Ethell, 2006), this result is consistent with the expected inhibition of integrin downstream signaling pathways. The dendritic spines in slices treated with RGDFV peptide were 28% shorter than those treated with the control peptide (Fig. 8, a and c), whereas spine width was reduced only slightly, and spine density was not significantly altered (P > 0.05; Fig. 8 d).

Figure 6. **The Cas SH3 and Src-binding domains are important for the regulation of spine morphology.** (a) Confocal images of dendrites from CA1 pyramidal neurons in hippocampal slices cotransfected with the indicated Cas constructs and EGFP-F as a marker. On the right are schematic representations of the constructs used for the transfections, including wild-type Cas, CasΔSD (Cas lacking the substrate domain), CasΔSH3 (Cas lacking the SH3 domain), and CasΔSB (Cas lacking the Src-binding domain). (b) Quantitative analysis of dendritic spine parameters in transfected neurons. Densities are as follows: 0.52 ± 0.02 spines/μm (control), 0.62 ± 0.02 spines/μm (Cas wild type), 0.53 ± 0.03 spines/μm (CasΔSD), 0.27 ± 0.02 spines/μm (CasΔSH3), and 0.27 ± 0.03 spines/μm (CasΔSB). (c) Percentage of spines in the indicated morphological categories. Statistical analysis was performed by ANOVA followed by Dunnett's posthoc test for comparisons of neurons transfected with Cas constructs to control-transfected neurons. *, $P < 0.05$; **, $P < 0.01$. Error bars represent SEM. A total of $n = 478$–2,182 spines from 23–53 dendrites of 6–10 CA1 neurons were analyzed per condition in three experiments. Bar, 5 μm.
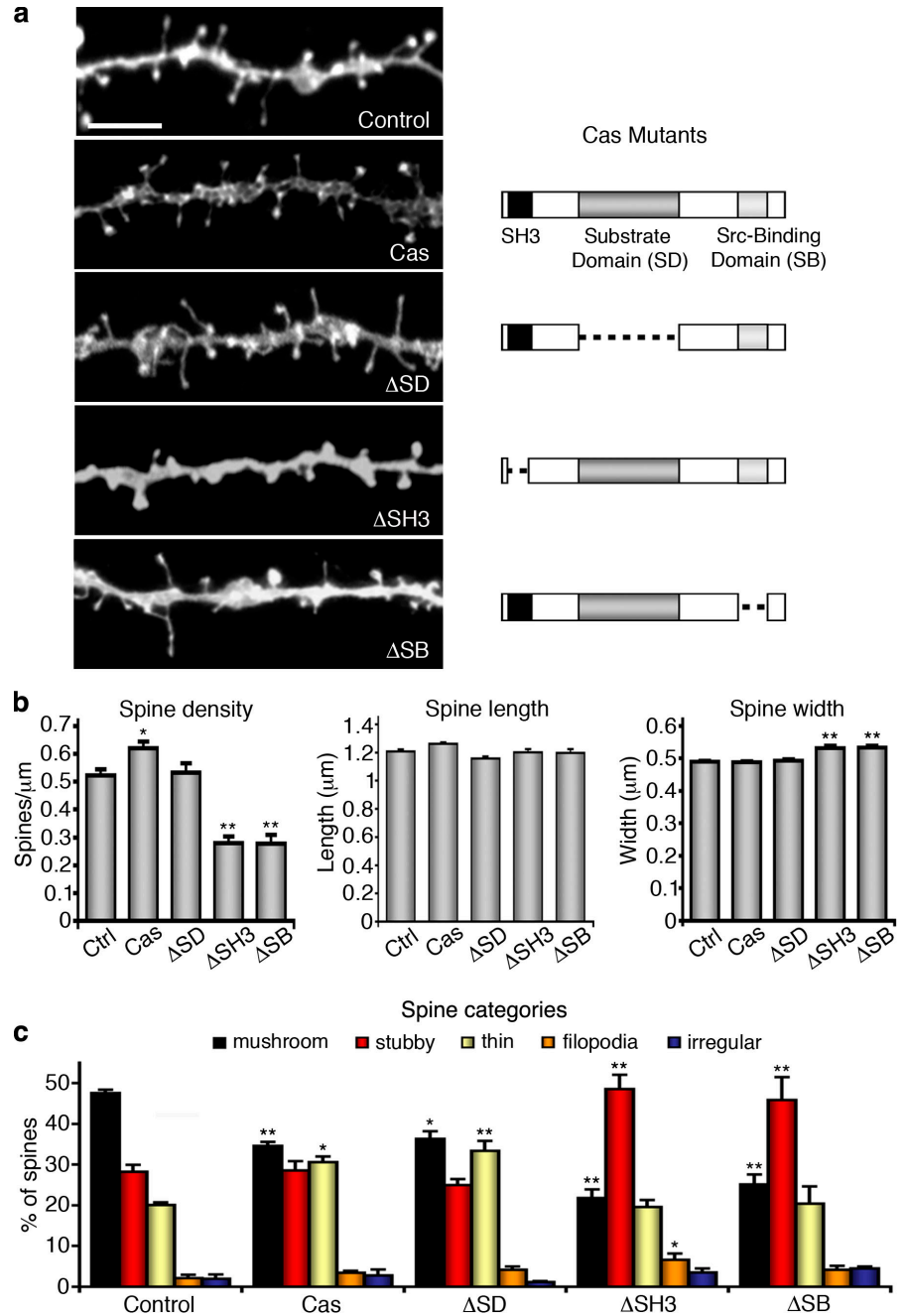
The shape of spines treated with the RGDFV peptide shifted from a mushroom-shaped to a stubby appearance (Fig. 8 e). These results show that integrin activity is required to maintain proper spine length and suggest that suppression of integrin function could play an important role in the effects of EphA4 on dendritic spine morphology.

Sustained β1-integrin activation blocks the effects of EphA4 on dendritic spines

To prevent β1-integrin inactivation during ephrin-A3 Fc stimulation, we treated acute hippocampal slices with high concentrations of the 9EG7 antibody. The antibody alone did not affect dendritic spine length, density, or width (Fig. 9, a–d; compare Fc with β1-Ab/Fc). As previously shown (Murai et al., 2003),

treatment of hippocampal slices with ephrin-A3 Fc caused a significant decrease in spine length (P < 0.001; Fig. 9, a and b) and density (Fig. 9 c), with minor effects on spine width (Fig. 9 d, compare Fc with A3-Fc). In addition, as we show here, ephrin-A3 Fc decreased the proportion of mushroom-shaped spines while increasing stubby spines (Fig. 9 f). Remarkably, the 9EG7 antibody completely blocked all of the effects of ephrin-A3 Fc on the spines (Fig. 9, a–d; compare β1-Ab/Fc with β1-Ab/ephrin-A3 Fc). This is consistent with the lack of detectable Cas dephosphorylation when ephrin-A3 Fc was used together with the 9EG7 antibody (Fig. 9 e), although the antibody itself decreased Cas phosphorylation. We obtained very similar results when we treated hippocampal slices with the divalent cation Mn^{2+}, which is known to promote the activation of many integrins, including
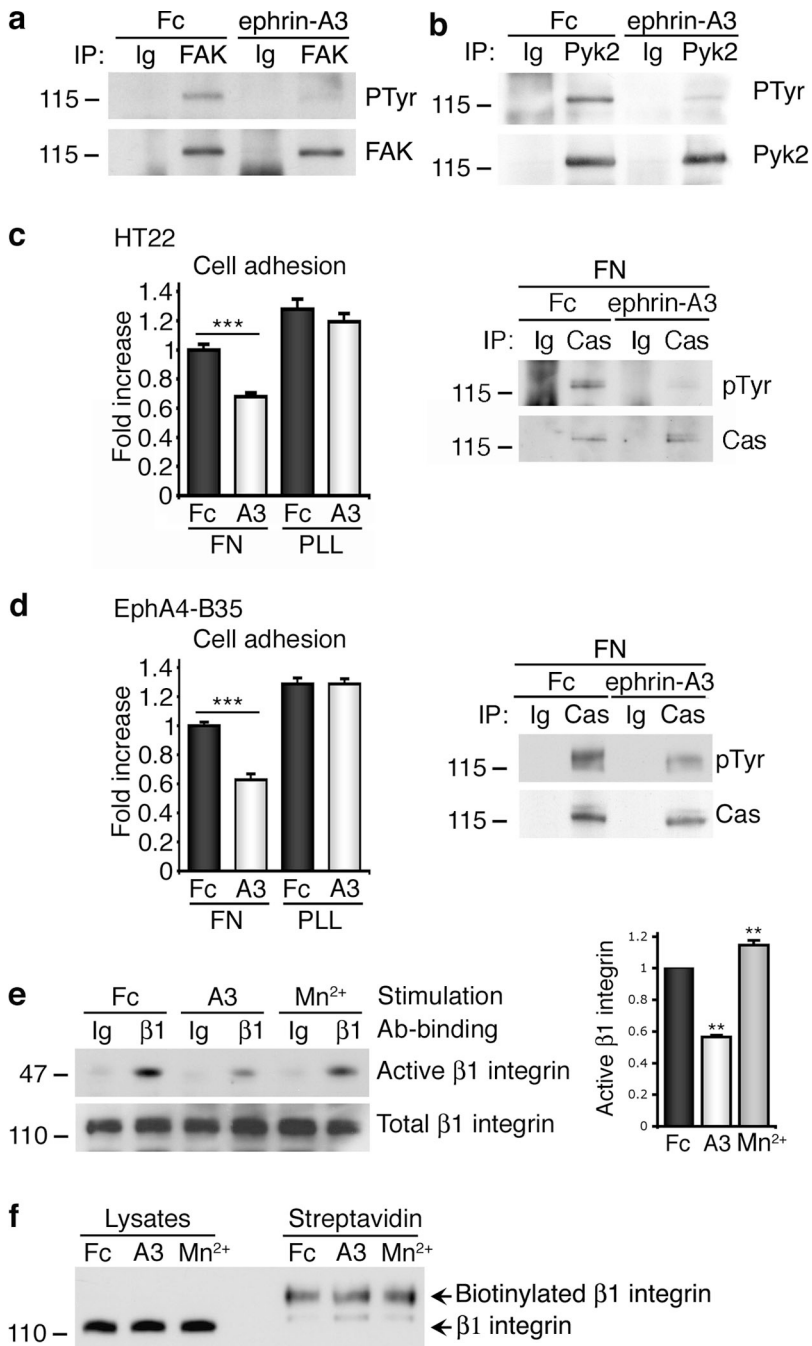
Figure 7. **EphA4 activation by ephrin-A3 Fc inhibits β1 integrin–mediated adhesion.** (a and b) Ephrin-A3 Fc decreases the tyrosine phosphorylation of FAK (a) and Pyk2 (b) in P12 hippocampal slices. Immunoprecipitated FAK and Pyk2 were probed by immunoblotting with an anti-phosphotyrosine (PTyr) antibody and reprobed with FAK or Pyk2 antibodies. (c and d) Ephrin-A3 Fc decreases attachment of the HT22 hippocampal cell line and the neuronal-like EphA4 B35 cell line to fibronectin (FN) but not to poly-L-lysine (PLL). Ephrin-A3 Fc also reduces Cas tyrosine phosphorylation in both cell lines. Immunoprecipitated Cas was probed by immunoblotting with an antiphosphotyrosine (PTyr) antibody and reprobed with a Cas antibody. (e) Ephrin-A3 Fc decreases the levels of active β1 integrin in cultured neuronal cells. HT22 cells plated on fibronectin and stimulated with ephrin-A3 Fc, Fc, or Mn^{2+} (an ion known to promote integrin activation) were incubated with a low concentration (2 µg/ml) of rat 9EG7 antibody or IgG (control) to detect active β1 integrin. The bound antibodies were detected by immunoblotting with an anti–rat antibody. For quantification of active β1 integrin, the signal in the Ig lane (background) was subtracted from the signal in the β1 lane and normalized to total β1 integrin. (f) Ephrin-A3 Fc does not affect the levels of β1 integrins on the cell surface. Cells were treated as described in panel e, and surface proteins were labeled with biotin, isolated with streptavidin beads, and probed by immunoblotting with an anti–β1-integrin antibody. Immunoblotting of the cell lysates shows similar levels of total β1 integrin. Statistical analyses were performed by ANOVA followed by Tukey's posthoc test (c and d) or Dunnett's posthoc test (e). **, $P < 0.01$; ***, $P < 0.001$ for comparison with Fc-treated cells. Error bars indicate SEM from three independent experiments. Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1) shows quantification and statistical analysis of the data shown in panels a–d and f.

β1 integrins (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1; Ivins et al., 2000; Arnaout et al., 2005). Thus, preventing β1-integrin inactivation counteracts the effects of EphA4 on dendritic spines.

Discussion

This study provides the first direct evidence that EphA receptors regulate β1-integrin activity and downstream signaling pathways in neurons, which is crucial for governing the morphological plasticity of dendritic spines. Activation of EphA4 by ephrin-A3 may disrupt critical integrin-mediated attachment to the extracellular matrix and cell surface integrin ligands, thus inhibiting integrin signaling to Cas and other downstream signaling proteins. Inhibition of integrin function with an RGD antagonistic peptide and inhibition of Cas function with siRNAs decreased spine length and the proportion of mushroom-shaped spines while increasing stubby spines in hippocampal slices. These spine morphological changes are strikingly similar to those elicited by EphA4 activation with ephrin-A3 Fc, which is consistent with the idea that the different manipulations affect components of a common signaling pathway. However, the RGD peptide did not decrease spine density, suggesting that integrin inhibition by the peptide down-regulates Cas function less drastically than Cas siRNAs or that it may affect additional Cas-independent pathways.

Figure 8. **Integrin activity regulates spine morphology.** (a) Dendritic spines of pyramidal neurons have shorter spines when treated with RGD peptide than when treated with control RAD (Arg-Ala-Asp) peptide. Cultured hippocampal slices transfected with EGFP-F as a marker were incubated for 24 h with 170 μM RAD or 170 μM RGD peptide. Representative confocal micrographs of dendrites from transfected CA1 pyramidal neurons are shown. (b) Cas is dephosphorylated in RGD-treated hippocampal slices. Lysates from cultured hippocampal slices treated as described in panel a were used to immunoprecipitate Cas, and the immunoprecipitates were probed with phosphotyrosine antibodies (PTyr) and reprobed with Cas antibodies. For quantification, Cas phosphorylation levels were normalized to the total amount of Cas immunoprecipitated and to the RAD control condition. The histogram represents mean ± SEM from three experiments. *, P < 0.05 by t test. (c) Quantification of spine length in RGD- and RAD-treated slices: 1.28 ± 0.02 μm for RAD versus 0.95 ± 0.01 μm for RGD. P < 0.001 (Kolmogorov-Smirnov test and t test). (d) Quantification of spine density and spine width. (e) Morphological categories of spines. Statistical analyses were performed with the t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001 for comparison between RGD- and RAD-treated spines. A total of n = 1,250–1,700 spines from 36–56 dendrites of 8–13 CA1 neurons were analyzed per condition in three experiments. Error bars indicate SEM. Bar, 5 μm.
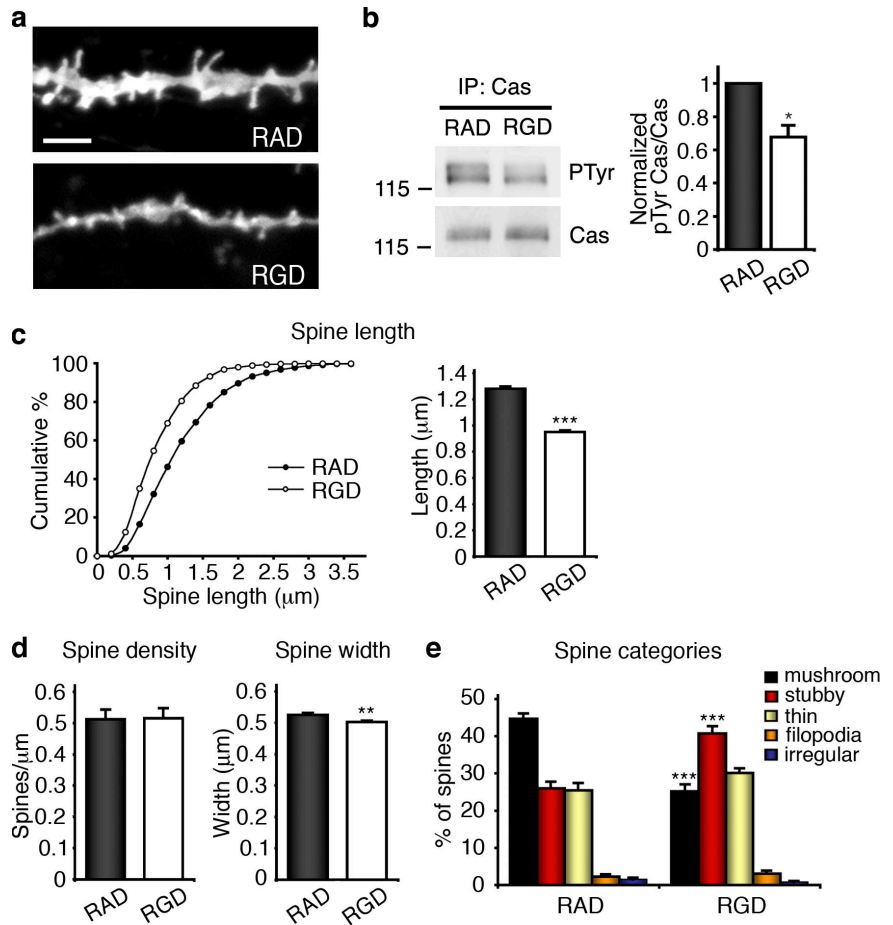
Several studies have shown that Eph receptor forward signaling modulates the adhesion and migration of nonneuronal cells by affecting integrin function (Table S1 in Pasquale, 2005). Both positive and negative effects of Eph receptors on integrin-mediated adhesion have been reported, depending on the receptor involved and the cellular context. The interplay between Eph receptors and integrins may involve a direct physical association, as reported for EphA4 and αIIbβ3 integrin in platelets (Prevost et al., 2005).

Many integrin α and β subunits are expressed in the brain (Pinkstaff et al., 1999; Clegg et al., 2003). Among them, the β1 subunit, which can associate with various α subunits, is found in brain synaptosomal preparations and in the dendritic spines of cultured hippocampal neurons (Schuster et al., 2001; Clegg et al., 2003; Shi and Ethell, 2006). Signals mediated by integrins, including β1, have been shown to increase the length and density of dendritic protrusions and promote synapse formation and remodeling in cultured hippocampal neurons (Hama et al., 2004; Shi and Ethell, 2006). Furthermore, siRNA knockdown of the α5- or β1-integrin subunits in hippocampal neurons has been shown to decrease the number of dendritic protrusions and synapses (Webb et al., 2007; D.J. Webb, H. Zhang, and A.F. Horwitz. 2005. Society for Neuroscience. Abstr. 501.5). These findings are consistent with the idea that integrin inactivation contributes to the effects of EphA4 on dendritic spine morphology and density. The ability of a β1 integrin–activating antibody to block spine retraction downstream of EphA4 and the observed inhibition of β1-integrin activity by EphA4 suggest that integrins containing the β1 subunit play a particularly important role downstream of EphA4.

It is noteworthy that inhibiting integrin function with RGD peptides through the use of integrin function–blocking antibodies or by targeted gene deletion has been shown to cause defects in long-term potentiation (LTP), a form of synaptic plasticity (Chun et al., 2001; Chan et al., 2006; Z. Huang et al., 2006; Kramar et al., 2006). The effects of integrin inactivation on synaptic plasticity have been associated with decreased N-methyl-D-aspartate (NMDA) receptor function and defects in actin polymerization (Lin et al., 2003; Kramar et al., 2006). It will also be interesting to examine whether the changes in spine shape induced by integrin inactivation may have a functional impact on synaptic transmission and plasticity. Recent findings suggest that the induction of LTP, which is dependent on an increase in cytosolic Ca^{2+} concentration, may be more difficult to achieve in dendritic spines with thick neck regions such as stubby spines as a result of the faster dispersion of Ca^{2+} ions into the dendritic shaft (Nimchinsky et al., 2002; Korkotian et al., 2004; Noguchi et al., 2005). Inhibition of integrin activity may therefore moderate LTP induction and stabilization by EphA4 (Grunwald et al., 2004).

Consistent with a role of integrins in dendritic spine regulation and synaptic physiology, the extracellular matrix has
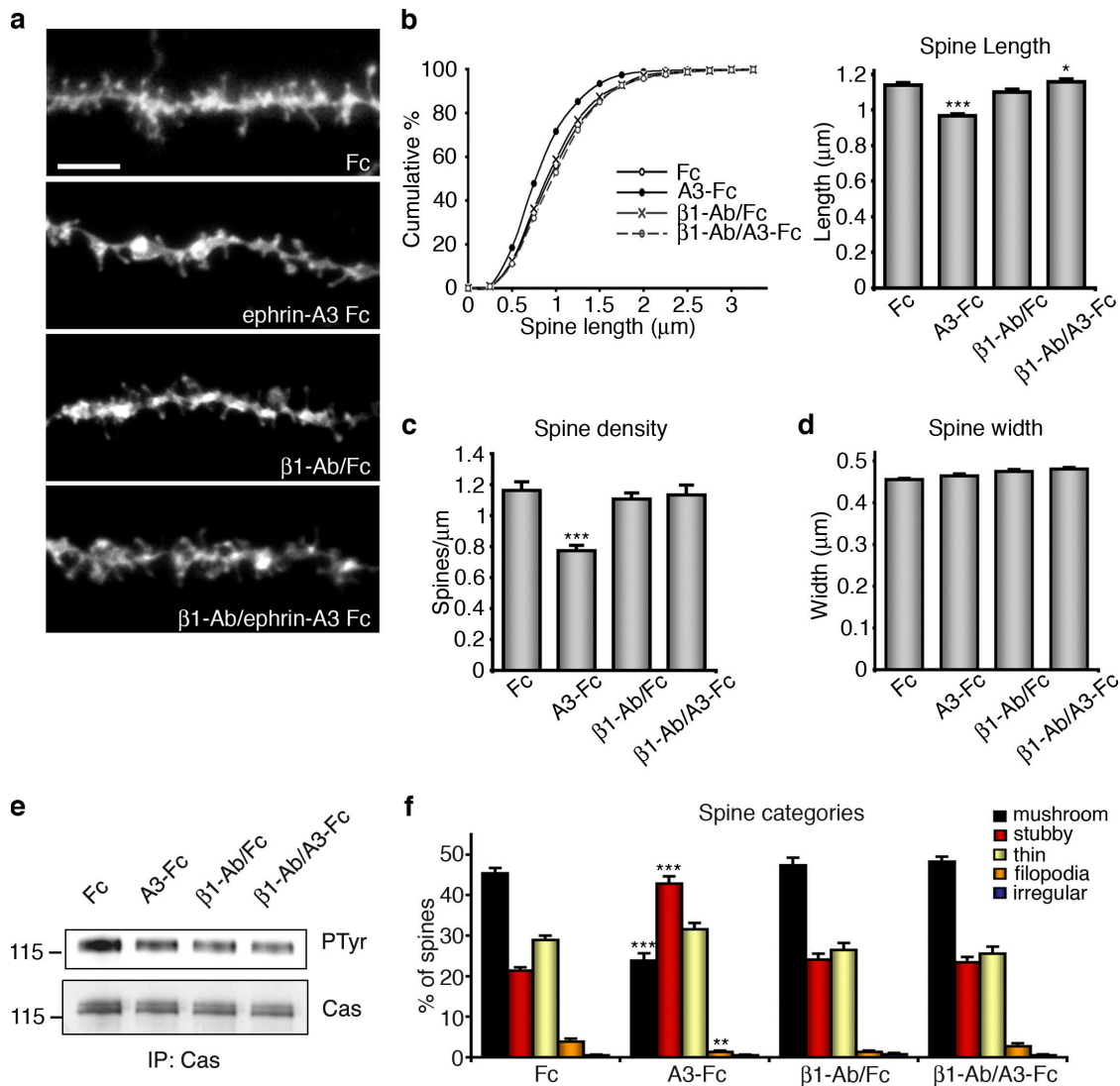
Figure 9. **Preventing β1-integrin inactivation counteracts ephrin-A3 Fc–induced spine retraction.** (a) Postnatal day 23–25 acute hippocampal slices were pretreated with a high concentration (20 μg/ml) of the 9EG7 antibody to promote β1-integrin activation before stimulation with ephrin-A3 Fc or Fc as a control. Representative confocal micrographs of DiI-labeled dendritic spines of CA1 pyramidal neurons are shown. (b) Quantitative analysis of dendritic spine length. Lengths are as follows: 1.14 ± 0.01 μm for Fc versus 0.97 ± 0.01 μm for A3-Fc ($P < 0.0001$ by Kolmogorov-Smirnov test and $P < 0.001$ by ANOVA and Bonferroni's posthoc test) and 1.10 ± 0.01 μm for β1-Ab/Fc versus 1.15 ± 0.01 μm for β1-Ab/ephrin-A3-Fc ($P > 0.05$ by Kolmogorov-Smirnov test, ANOVA, and Bonferroni's posthoc test). (c) Quantitative analysis of dendritic spine density. Densities are as follows: 1.16 ± 0.05 spines/μm for Fc versus 0.77 ± 0.03 spines/μm for A3-Fc and 1.10 ± 0.04 spines/μm for β1-Ab/Fc versus 1.13 ± 0.06 spines/μm for β1-Ab/ephrin-A3-Fc. (d) Quantitative analysis of dendritic spine width. (e) Treatment with the 9EG7 antibody prevents Cas dephosphorylation after ephrin-A3 Fc treatment. Lysates from hippocampal slices treated as described in panel a were used to immunoprecipitate Cas, and the immunoprecipitates were probed with phosphotyrosine antibodies (PTyr) and reprobed with Cas antibodies. (f) Morphological categories of spines. Statistical analyses in panels c, d, and f were performed with multifactorial ANOVA and Tukey's posthoc test (c and f) or Bonferroni's posthoc test (d). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for comparison between Fc versus ephrin-A3 Fc or for β1-Ab/Fc versus β1-Ab/ephrin-A3 Fc. In panel d, $P < 0.01$ for the comparison of Fc versus β1-Ab/Fc. A total of $n = 1{,}071$–$1{,}341$ spines from 19–24 dendrites of 9–10 CA1 neurons were analyzed per condition in three experiments. Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200610139/DC1) shows quantification and statistical analysis of the data shown in panel e. Error bars indicate SEM. Bar, 5 μm.

also been shown to influence spine morphological plasticity. For example, mutant mice that have reduced levels of reelin, a large secreted glycoprotein that binds to the α3β1 integrin, have fewer and shorter spines compared with wild-type mice (Liu et al., 2001). Furthermore, degradation of extracellular matrix proteins by tissue plasminogen activator, which likely reduces integrin activity, has been implicated in the structural remodeling of spines that occurs in the visual cortex during experience-dependent plasticity (Mataga et al., 2004;

Oray et al., 2004). Whether EphA4 may inhibit integrin activity by promoting proteolytic degradation of the extracellular matrix remains to be determined.

We have found that integrin inactivation by EphA4 results in the disassembly and inactivation of integrin signaling complexes, including the dissociation of Cas–Fyn complexes and the dephosphorylation of Cas, FAK, and Pyk2. The phosphatase mediating these dephosphorylation events remains unknown, although the tyrosine phosphatase SHP2 has been previously

implicated in FAK dephosphorylation downstream of an EphA receptor in nonneuronal cells (Miao et al., 2000).

Even though Cas is most highly expressed in the brain and has been detected in complex with the NMDA receptor (Husi et al., 2000; J. Huang et al., 2006), its role in the nervous system is poorly characterized. Recent data have implicated Cas in axon elongation in cerebellar granule neurons (J. Huang et al., 2006), and our data show that Cas is preferentially expressed in hippocampal neurons rather than glial cells and is enriched in the PSD fraction of synaptosomal preparations. Furthermore, Cas knockdown in hippocampal slices decreased dendritic spine density and caused changes in spine morphology. The observed involvement of the Src-binding and SH3 domains of Cas in spine regulation further suggests that Cas may cooperate with Fyn and kinases of the FAK family, which interact with these two domains, to maintain proper spine morphology.

Interestingly, we detected Cas association with Fyn but not Src in hippocampal tissue. Although both Src and Fyn are components of the PSD and of NMDA receptor complexes (Salter and Kalia, 2004), differences in Src and Fyn protein interactions in the brain have been reported (Husi et al., 2000; Lauri et al., 2000). Thus, the synaptic functions of these two closely related kinases are not completely redundant. Accordingly, Fyn but not Src knockout mice demonstrate defects in the induction of LTP in adult CA1 hippocampal synapses (Grant et al., 1992; Kojima et al., 1997).

The dephosphorylation of FAK and Pyk2 that we observed in hippocampal slices stimulated with ephrin-A3 is consistent with reduced integrin function and suggests that inactivation of these two kinases may also contribute to spine morphological plasticity downstream of EphA4. However, the role of FAK in dendritic spines seems to be complex because FAK activation has been recently shown to be important for spine shortening and morphogenetic changes downstream of EphB receptors in cultured hippocampal neurons (Moeller et al., 2006).

Surprisingly, we did not detect significant changes (P > 0.05) in dendritic spines expressing the CasΔSD mutant, which does not bind Crk, except for a decrease in mushroom-shaped spines. Crk couples phosphorylated Cas to the guanine nucleotide exchange factor DOCK180, which activates Rac1, thus promoting actin polymerization and cell migration (Playford and Schaller, 2004). Therefore, either the Cas–Crk pathway does not play a major role in spine regulation or another signaling pathway can compensate for the disruption of Cas–Crk complexes. Indeed, EphA4 activation does not affect Crk phosphorylation on tyrosine 221, suggesting that the adaptor function of Crk is not impaired by EphA4 and that molecules other than Cas could still recruit Crk to promote Rac1 activation (Chen et al., 2004).

Our results demonstrate that EphA4 function in dendritic spines is closely tied to the regulation of β1-integrin signaling pathways, but additional pathways can also influence spine structure downstream of EphA4. For example, in neurons, EphA4 promotes the activity of at least two exchange factors that activate Rho family GTPases, Ephexin-1, and Vav2 (Shamah et al., 2001; Cowan et al., 2005; Sahin et al., 2005). Recently, a pathway involving the serine-threonine kinase Cdk5 and Ephexin-1 has

been reported to decrease dendritic spine density downstream of EphA4 (Fu et al., 2007). This pathway likely affects dendritic spines through the activation of RhoA, a GTPase that promotes actomyosin contraction (Bonhoeffer and Yuste, 2002; Ethell and Pasquale, 2005). Nevertheless, β1-integrin inactivation appears to be a crucial event because preventing it counteracts all EphA4-dependent spine modifications. Further studies will be necessary to fully elucidate the Eph forward and ephrin reverse signaling pathways that modulate the morphological plasticity of excitatory synapses.

Materials and methods

Antibodies
Monoclonal antibodies to phosphotyrosine (clone 4G10), Src, PSD95, and polyclonal antibody to Pyk2 were obtained from Upstate Biotechnology. Monoclonal antibody to FAK, Cas, Crk, and β1 integrin were purchased from BD Biosciences. The phosphospecific antibodies to Crk tyrosine 221, Cas tyrosine 165, and Src tyrosine 416 were obtained from Cell Signaling. The monoclonal 9EG7 antibody to activated β1 integrin was obtained from BD Biosciences. Monoclonal antibodies to β-actin and syntaxin were purchased from Sigma-Aldrich. Antibodies to Fyn and SHP2 and a Cas polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. The monoclonal antibody to EphA4 was obtained from Zymed Laboratories.

GST pull-down assays and immunoprecipitations
Tissues or cells were lysed in Hepes buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 10% glycerol, and 1% Triton X-100) or modified radioimmunoprecipitation assay buffer with 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, and other protease inhibitors (Sigma-Aldrich). GST-Fyn SH2 and GST-Crk SH2 fusion proteins have been previously described (Zisch et al., 1998, 2000). Recombinant GST fusion proteins were affinity purified from *Escherichia coli* BL21 (Pfizer) by adsorption to glutathione–Sepharose 4B beads (Pfizer). GST-Fyn SH3/SH2 fusion protein was purchased from Santa Cruz Biotechnology, Inc. For GST pull-down assays, cell lysates were incubated with 10 μg GST fusion proteins immobilized on glutathione beads. For immunoprecipitations, cell lysates were incubated with 2–5 μg of antibodies and GammaBind Plus Sepharose beads (GE Healthcare) for 3 h at 4°C. Protein extracts were separated by SDS-PAGE and probed by immunoblotting. Detection of HRP-conjugated secondary antibodies (GE Healthcare) was performed with enhanced chemiluminescence detection systems (GE Healthcare or Pierce Chemical Co.).

Preparation of synaptosomal fractions
All steps were performed at 4°C as described previously (Rogers and Lemaire, 1991). Freshly dissected adult mouse brains were homogenized in 10 vol of ice-cold homogenization buffer (buffer H [50 mM Tris-HCl, pH 7.4, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 1 mM NaHCO$_3$] containing 320 mM sucrose) using a motor-driven glass-fluorinated ethylene polymer homogenizer. The homogenate was centrifuged at 700 g for 10 min to produce supernatant S1. S1 was further centrifuged at 17,000 g for 15 min to produce pellet P2. P2 was resuspended in homogenization buffer, layered on top of sucrose gradient (equal volumes of 1.2, 1.0, 0.8, and 0.65 M sucrose in buffer H), and centrifuged at 100,000 g for 2 h. A turbid fraction was recovered from the 1.0 /1.2 M sucrose interface and resuspended in at least 2 vol of homogenization buffer. The suspension was centrifuged for 30 min at 100,000 g to produce the pellet P2′ (synaptosome fraction). P2′ was resuspended in Tris buffer (50 mM Tris-HCl, pH 7.4, and 1 mM EDTA with protease inhibitors), incubated for 45 min on ice, and centrifuged at 100,000 g for 30 min. The supernatant containing soluble synaptic proteins was collected (synaptic cytosol fraction). The membrane pellet was resuspended in homogenization buffer, applied on top of a five-step discontinuous sucrose gradient (1.2, 1.0, 0.8, and 0.65 M sucrose), and centrifuged at 100,000 g for 2 h. Pure synaptic membranes were recovered from the 1.2/1.0 M sucrose interface and diluted with at least 2 vol of homogenization buffer (synaptic membrane fraction). A pure membrane fraction was pelleted at 100,000 g for 30 min, resuspended in homogenization buffer, and mixed with an equal volume of 3% Triton X-100 in homogenization buffer. Membranes were incubated for 30 min on ice and centrifuged at 100,000 g for 2 h to produce a supernatant

(soluble membrane fraction) and a Triton X-100–insoluble material pellet. Triton X-100–insoluble proteins were resuspended in homogenization buffer containing 1.5% Triton X-100 and applied on top of a 0.83-M sucrose cushion. After centrifugation at 105,000 g for 1 h, PSDs were collected as the resulting pellet and resuspended in Tris buffer containing 0.25% Triton X-100 (PSD fraction). Equal amounts of protein from synaptosome, synaptic cytosol, synaptic membrane, soluble membrane, and PSD fractions were analyzed by immunoblotting.

Neuronal and glial cultures

Primary hippocampal neurons were prepared from embryonic day 17 rat embryos and cultured as described previously (Zafra et al., 1990) with minor modifications. In brief, after trypsinization and mechanical dissociation, hippocampal cells were suspended in DME supplemented with 10% FCS and preattached on uncoated culture plates for 2 h to remove glial cells. Neurons recovered as nonadherent cells were plated on poly-D-lysine– (50 μg/ml) and laminin (5 μg/ml)-coated plates at a density of 3×10^4 cells/cm^2 in neurobasal medium supplemented with B27, L-glutamine, and penicillin/streptomycin. Neurons were cultured for 14 d in the presence of 2 μM AraC to prevent the growth of contaminating glial cells. The glial cells separated from the neurons by attachment on uncoated culture plates were cultured for 14 d in DME supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin.

Hippocampal slice cultures

Hippocampal slices (300 μm in thickness) were prepared and stimulated for 45 min as previously described using the middle one third of the hippocampus (Stoppini et al., 1991; Murai et al., 2003). Slices were kept for 2–5 min in MEM (Invitrogen) supplemented with 25 or 5% horse serum (Invitrogen), 25% HBSS (Irvine Scientific), and 0.65% dextrose before stimulation. In some experiments, 9.5 μg/ml ephrin-A3 Fc (R&D Systems) was preclustered with 1 μg/ml anti-Fc antibodies, which produced similar EphA4 phosphorylation and Cas dephosphorylation as unclustered ephrin-A3 Fc. To promote β1-integrin activation, slices were pretreated with 20 μg/ml 9EG7 antibody for 25 min or 500 μM Mn^{2+} for 20 min before stimulation with Fc proteins. Slices were either lysed and analyzed by immunoblotting or fixed in 4% PFA in 0.1 M PBS for 30 min, labeled with DiI crystals (Invitrogen), and analyzed by confocal z-series imaging as previously described (Murai et al., 2003). EphA4 knockout mice were provided by A. Boyd and M. Dottori (Queensland Institute of Medical Research, Brisbane, Australia).

A gene gun (Helios; Bio-Rad Laboratories) was used to transfect plasmids into pyramidal neurons. Hippocampal slices prepared as described in the previous paragraph were cultured for 6 d on 0.4-μm Millicell-CM organotypic inserts (Millipore) in MEM containing 25% horse serum, 25% HBSS, and 0.65% dextrose. Slices were then transfected (80 pounds per square inch) with pSSRα/farnesylated enhanced GFP (EGFP-F; control), Cas/EGFP-F, CasΔSD/EGFP-F, CasΔSH3/EGFP-F, or CasΔSB/EGFP-F plasmids ((25 μg Cas plasmid mixed with 20 μg EGFP-F plasmid coated onto 25 mg of 1.6-μm gold particles; Nakamoto et al., 1996). Cas plasmids were provided by K. Vuori and A. Motoyama (Burnham Institute for Medical Research, La Jolla, CA). After 48 h, the slices were fixed in 4% PFA in 0.1 M PBS for 30 min before z-series imaging of dendrites from transfected CA1 pyramidal neurons. For integrin inhibition, 170 μM RGDFV or RADFV (control) peptide (BIOMOL Research Laboratories, Inc.) was added to the media of EGFP-F–transfected cultured hippocampal slices. After an additional 24 h in culture, the slices were analyzed either by confocal z-series imaging or immunoblotting.

Cas siRNA knockdown

Four siRNAs for mouse Cas were obtained from Dharmacon, including siCas #1 (GCATAGGGCATGACATCTA; nucleotides 678–696; GenBank/EMBL/DDBJ accession no. NM_009954), siCas #2 (AGACGGAGCAGG-ATGAGTA; nucleotides 804–822), siCas #3 (CCGAGGAAGTCCAGGA-TTC, nucleotides 1,816–1,834), and siCas #4 (GATTCTGGTTGGCATGTA, nucleotides 292–310). Two scrambled siRNAs (nontargeting siRNA #1 and #2; Dharmacon) were used as controls. To obtain the pEGFP-hCas vector, human Cas cDNA (GenBank/EMBL/DDBJ accession no. BC062556; Open Biosystems) was amplified by PCR with the forward primer 5′-GCTAG-CCTCGAGCATGAACCACCTGAACGT-3′ and the reverse primer 5′-GCTAG-CGAATTCTCAGGCGGCTGCCAGACA-3′. The PCR product was digested and inserted into the XhoI and EcoRI sites of pEGFP-C2 (BD Biosciences) and verified by sequencing.

Hippocampal slices were cotransfected with the siRNAs and the indicated plasmid using a gene gun as described previously (Boda et al.,

2004; Govek et al., 2004). 24 μg of duplex siRNA and 12 μg of plasmid were mixed and coated onto 10 mg of 1.6-μm gold particles. Transfected (green fluorescent) dendrites were analyzed 2 d after transfection by confocal z-series imaging. The effectiveness of siRNA transfection into hippocampal pyramidal neurons was verified by using a rhodamine-labeled siRNA transfected together with EGFP-F. The effectiveness of Cas knockdown by the siRNAs and the rescue of Cas expression by EGFP-hCas was verified in NIH3T3 cells. Cells were plated at 80% confluency, cultured overnight in DME and 10% bovine calf serum, and transfected in Opti-MEM with 78 nM siRNAs (with or without plasmids) using LipofectAMINE 2000 (Invitrogen). After 4 h, the medium was replaced by the DME culture medium, and Cas expression was assessed after 48 h by immunoblotting of cell lysates.

Quantification of spine dimensions and shape

Images were collected using a confocal microscope (MRC 1024; Bio-Rad Laboratories) and analyzed using ImageJ 1.62 software (National Institutes of Health [NIH]), and the analysis of spines was confined to secondary and tertiary dendrites from the stratum radiatum. Quantification of spine lengths and widths was performed as described previously (Murai et al., 2003). In brief, the length was measured from the tip of spine head to the interface with the dendritic shaft. The width was taken as a diameter of the spine head perpendicular to the length of the spine. Spines were assigned to morphological categories as described previously (Maruoka et al., 2005) by an investigator blind to the conditions. Mushroom spines are <2 μm in length, >0.5 μm in width, and are connected to the dendritic shaft by a narrower portion (neck). Stubby spines are <2 μm in length, >0.5 μm in width, and lack a defined neck. Thin spines are <2 μm in length, <0.5 μm in width, and have a neck. Filopodia spines are >2 μm in length, <0.5 μm in width, and do not have a distinct spine head. Irregular spines are branched spines (two heads and two necks merged into a single neck at the base) and multilobed spines (many heads fused together on top of a single neck). All measurements were obtained by counting 478–2,182 spines per condition (from 6–13 neurons) from independent experiments. Spine cumulative distributions were compared using the Kolmogorov-Smirnov test, and means were compared using the t test or analysis of variance (ANOVA) test followed by Dunnett's, Tukey's, or Bonferroni's posthoc tests.

Cell attachment assays

To obtain the EphA4-B35 cells, the rat B35 neuronal-like cell line (Schubert et al., 1974) was transfected with the EphA4 cDNA in pcDNA3 using SuperFect (QIAGEN) and selected for 2 wk with 500 μg/ml G418. To perform the cell attachment assays, the HT22 hippocampal neuron cell line (Li et al., 1997) or EphA4-B35 cells were starved overnight in DME with 1% BSA, detached, and kept in suspension for 30 min at 37°C in DME–1% BSA. Cells (7×10^4 cells per well of a 12-well plate) were then allowed to attach to poly-L-lysine– (20 μg/ml; Sigma-Aldrich) or fibronectin-coated coverslips (20 μg/ml fibronectin for EphA4-B35 and 10 μg/ml for HT22; Chemicon) for 10 min at 37°C in the presence of 10 μg/ml Fc or 10 μg/ml ephrin-A3 Fc. Attached cells were fixed in 4% PFA, stained with DAPI (Invitrogen), and counted blind (seven to nine 20× microscope fields were counted per condition). To determine Cas phosphorylation, both adherent and nonadherent cells were pooled, lysed, and probed by immunoblotting. HT22 and B35 cell lines were provided by W. Stallcup and B. Moosmann (Burnham Institute for Medical Research, La Jolla, CA), respectively.

Determination of activated and cell surface β1 integrins

HT22 was starved and plated on tissue culture plates coated with 10 μg/ml fibronectin for 1 h at 37°C in the presence of 10 μg/ml Fc, 10 μg/ml ephrin-A3 Fc, or 1 mM MnCl$_2$. To determine the levels of activated β1 integrin, live cells were rinsed with PBS and incubated with 2 μg/ml 9EG7 rat monoclonal antibody or control rat IgG for 15 min at 37°C. Cells were rinsed with PBS and lysed in SDS sample buffer, and bound antibodies were detected by immunoblotting with biotin-conjugated anti–rat IgG antibody (Vector Laboratories) followed by HRP-streptavidin (Zymed Laboratories). To determine the levels of cell surface β1 integrin, cells were washed with PBS, and surface proteins were labeled with 1 mM sulfosuccinimidyl-6-(biotin-amido)hexanoate (Pierce Chemical Co.) for 30 min at 4°C. Cells were washed with PBS and lysed in Hepes buffer with protease inhibitors, and biotin-labeled proteins were incubated with 50 μl streptavidin-conjugated beads (Biochemika) for 1 h at 4°C. Bound proteins were eluted and analyzed by immunoblotting with anti–β1-integrin antibodies.

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