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Cdk5 regulates EphA4-mediated dendritic spine retraction through an ephexin1-dependent mechanism

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The development of dendritic spines is thought to be crucial for synaptic plasticity. Dendritic spines are retracted upon Eph receptor A4 (EphA4) activation, but the mechanisms that control this process are not well understood. Here we report an important function of cyclin-dependent kinase 5 (Cdk5) in EphA4-dependent spine retraction in mice. We found that blocking Cdk5 activity inhibits ephrin-A1–triggered spine retraction and reduction of mEPSC frequency at hippocampal synapses. The activation of EphA4 resulted in the recruitment of Cdk5 to EphA4, leading to the tyrosine phosphorylation and activation of Cdk5. EphA4 and Cdk5 then enhanced the activation of ephexin1, a guanine-nucleotide exchange factor that regulates activation of the small Rho GTPase RhoA. The association between EphA4 and ephexin1 was significantly reduced in *Cdk5*$^{-/-}$ brains and Cdk5-dependent phosphorylation of ephexin1 was required for the ephrin-A1–mediated regulation of spine density. These findings suggest that ephrin-A1 promotes EphA4-dependent spine retraction through the activation of Cdk5 and ephexin1, which in turn modulates actin cytoskeletal dynamics.

Dendritic spines, highly specialized protrusions on neuronal dendrites, are important sites of excitatory synapses in the brain. The density and morphology of these spines in mature neurons change in ways that are believed to be crucial for synaptic plasticity. These changes, which occur in response to extracellular factors, are mediated by cell surface proteins, such as postsynaptic ion channels that are regulated by neurotransmitters and cell adhesion molecules at pre- and postsynaptic terminals[1]. Recent evidence suggests that synapse formation and plasticity are regulated by the family of Eph receptor tyrosine kinases (Ephs) and their ligands, ephrins[2–4].

Ephs, the largest family of receptor tyrosine kinases (RTKs), regulate a variety of biological functions in the developing and adult nervous systems. Based on their extracellular sequence similarities and binding preferences to their cognate ephrin ligands, these receptors are divided into two subclasses: EphA1–10 and EphB1–6. Ephrin-Eph signaling acts as a repulsive cue in mediating axon pathfinding[5] and in organizing topographical maps during neural development[6]. Furthermore, ephrin-Eph regulates not only long-term synaptic plasticity and memory[3,4,7,8], but also various aspects of synaptic development, including the formation, maintenance and remodeling of synapse geometry[2,9–11]. With regard to synaptic development, various Ephs expressed in dendritic spines of adult brain, including EphA4, EphB1, EphB2 and EphB3, are implicated in regulating spine morphogenesis[2,9]. Signaling mediated by EphBs seems to coordinate the shaping of dendritic spines, as hippocampal neurons from EphB1, B2 and B3 triple-mutant mice do not develop mature dendritic spines, but spine morphogenesis in single-mutant mice is not obviously affected[2]. By contrast, disrupting EphA4-forward signaling in hippocampal neurons leads to severe defects in spine morphogenesis, revealing an essential role for EphA4 in maintaining or eliminating dendritic spines[9].

Spine morphology is regulated by reorganizing the cytoskeleton, a mechanism that is modulated by Ephs through binding to guanine-nucleotide exchange factors (GEFs) that activate small Ras-homologous (Rho) GTPases[12]. Ephs bind distinct GEFs and regulate specific Rho GTPases[12]. Different GTPases affect actin rearrangement and spine development in distinct ways. Rac1 and Cdc42 promote the development and maintenance of dendritic spines, whereas RhoA activation inhibits spine formation[13]. The role of EphBs in modulating spine morphology is well characterized[2,11,14], but the signaling events that link EphA activation to spine retraction remain largely unknown.

Another molecular player increasingly implicated in regulating cytoskeletal dynamics is Cdk5, a proline-directed serine and threonine kinase that is abundantly expressed in postsynaptic densities (PSD)[15]. Cdk5 activity is regulated by neural-specific activators, p35 and p39, and is enhanced by phosphorylation at Tyr15 residue[16,17]. Cdk5 activity is also stimulated by activating RTKs, such as ErbB receptors[18]. Thus, it is tempting to speculate that Cdk5 may transduce extracellular stimuli from RTKs to the cytoskeletal network, thereby affecting spine morphogenesis.

Here we report that EphA4 modulates spine morphogenesis in a sequential mechanism that involves Cdk5-dependent regulation

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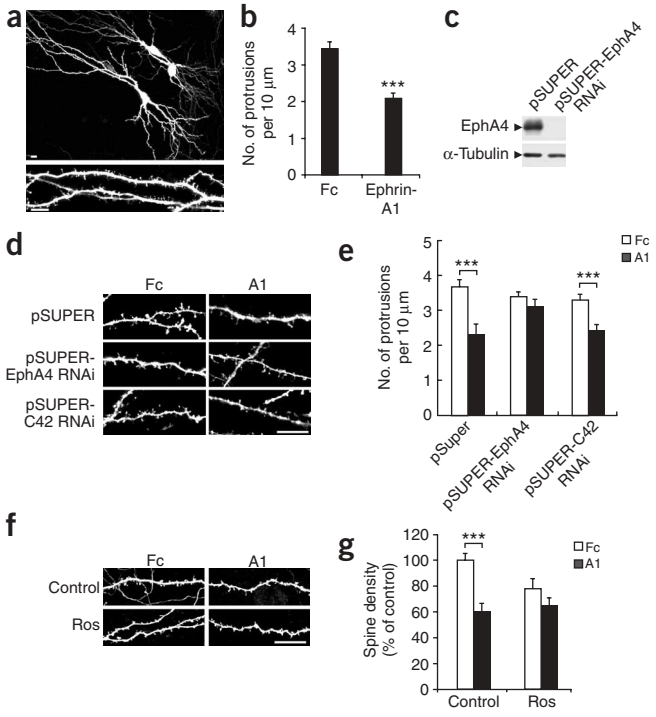

Figure 1 Inhibition of Cdk5 activity attenuates the ephrin-A1–stimulated dendritic spine retraction of pyramidal neurons in organotypic hippocampal slices. (**a**) Above, typical morphology of a biolistically transfected hippocampal pyramidal neuron expressing YFP. Below, a typical example of the dendritic spines observed. (**b**) Ephrin-A1 reduces the number of spine protrusions of YFP-expressing pyramidal neurons in hippocampal slices prepared from P7 mice. (**c**) Knockdown of EphA4 expression by pSUPER-EphA4 RNAi. HEK 293T cells were transfected with the EphA4 expression construct, together with either the pSUPER-EphA4 RNAi construct or the pSUPER vector. α-tubulin acts as control for equal protein loading. (**d**,**e**) EphA4 forward signaling is involved in ephrin-A1 stimulated spine retraction. Hippocampal slices were transfected with pSUPER vector, pSUPER-EphA4 RNAi or pSUPER-C42 RNAi. Images in **d** show dendritic spines of neurons transfected with various RNAi constructs and treated with ephrin-A1 as indicated. The dendritic spines in pyramidal neurons are quantified in **e**. In **b** and **e**, data are presented as number of spines (protrusions) per 10 μm (mean ± s.e.m.; *** $P < 0.005$, ephrin-A1 versus Fc; unpaired Student's *t*-test). (**f**,**g**) Inhibiting Cdk5 activity with roscovitine (Ros) in pyramidal neurons of hippocampal slices prepared from P7 mice attenuates ephrin-A1–mediated spine retraction. Images in **f** show dendritic spines after Ros and ephrin-A1 treatment. The percentage reduction in spine protrusions in DMSO (Control) or Ros-treated neurons after ephrin-A1 treatment is shown in **f** (mean ± s.e.m.; *** $P < 0.005$, ephrin-A1 versus Fc; unpaired Student's *t*-test). Scale bars, 10 μm (**a**,**d**,**f**).

of ephexin1 GEF activity towards RhoA. We demonstrate that ephrin-A1 induces the recruitment of Cdk5-p35 complexes to activated EphA4, with a concomitant increase in Cdk5 activity. We also demonstrate that Cdk5 is important in recruiting ephexin1 to EphA4 and in phosphorylating ephexin1. This Cdk5-mediated phosphorylation of ephexin1 regulates its EphA4-mediated tyrosine phosphorylation, thus stimulating ephexin1's activity towards RhoA, which results in reduced dendritic spine density.

RESULTS

Cdk5 activity regulates ephrin-A1–reduced spine density

Because EphA4 induces spine retraction in the hippocampus[9,19], we first examined the effect of ephrin-A1, the ephrin ligand with the highest affinity to EphA4 (ref. 20), on the dendritic spine density of CA1 hippocampal pyramidal neurons. We biolistically transfected organotypic hippocampal slices with a yellow fluorescent protein (YFP) construct and then treated them with ephrin-A1. These YFP-expressing neurons had normal dendritic morphology and the majority of the spines had well-defined head and neck structures (**Fig. 1a**). Ephrin-A1 treatment for 16 h reduced the spine density in pyramidal neurons in hippocampal slices prepared from postnatal day 7 (P7) mice (~40%; **Fig. 1b**), as seen previously[9]. To determine whether EphA4 was involved in these processes, we silenced the expression of EphA4 by transfecting hippocampal slices with pSUPER-EphA4 RNAi. We confirmed that pSUPER-EphA4 RNAi specifically suppressed the expression of EphA4 (**Fig. 1c**). Control experiments using a RNAi plasmid that targeted a p35-interacting protein, C42 (ref. 21), did not reduce expression of EphA4 (data not shown). We analyzed the spine morphology in these EphA4-knockdown neurons, in which the EphA4 forward signaling was disrupted. Ephrin-A1 reduced the spine density in pSUPER and pSUPER-C42 RNAi-transfected neurons, but it did not reduce the spine density in EphA4-knockdown neurons (**Fig. 1d**,**e**).

Given our previous observation that Cdk5 modulates ErbB signaling[18] and that Cdk5 is localized to postsynaptic regions[18,22], we were interested in assessing the effect of Cdk5 on ephrin-A1–regulated spine morphology. We treated the hippocampal slices with ephrin-A1 and roscovitine, a Cdk5 inhibitor, and found that ephrin-A1 stimulation did not reduce the spine density of these neurons (**Fig. 1f**,**g**). Consistent with this observation, ephrin-A1 also did not reduce the spine density of $p35^{-/-}$ ($Cdk5r1^{-/-}$) CA1 pyramidal neurons of hippocampal slices (data not shown).

Cdk5-p35 interacts with EphA4

We next explored the mechanisms of EphA4-mediated spine retraction *in vivo* by examining the possibility that EphA4 forms a complex with Cdk5-p35. Coimmunoprecipitation experiments revealed that EphA4 associated with Cdk5 or its activator, p35, in HEK 293T human embryonic kidney cells (**Supplementary Fig. 1** online). To address whether EphA4 binds directly to p35 or Cdk5, we performed direct binding assays. EphA4 associated strongly with Cdk5 but not with the p25 fragment (**Supplementary Fig. 1**). Our findings therefore suggest that the interaction between EphA4 and p35 in HEK 293T cells occurs either directly through the p10 region of p35 or indirectly through their mutual association with endogenous Cdk5.

Because EphA4, Cdk5 and p35 are localized to synapses[9,15], we examined whether endogenous EphA4 interacts with Cdk5 and p35. We showed that EphA4 coimmunoprecipitated with Cdk5-p35 in E18 and adult rat brains (**Fig. 2a**), suggesting that these three proteins exist as a complex *in vivo*. Under basal conditions, small amounts of EphA4 phosphorylation, as well as association between EphA4 and Cdk5-p35, could be detected in cortical neurons (**Fig. 2b**). These observations prompted us to investigate whether the association could be enhanced by ligand stimulation, as was previously reported for EphA-mediated signaling[23]. Prominent recruitment of the Cdk5-p35 complex to activated-EphA4 could be detected in a ligand-dependent manner. When neurons were treated with ephrin-A1, significant amounts of tyrosine-phosphorylated EphA4 were readily coimmunoprecipitated by antibodies to p35 (**Fig. 2b**,**c**). The EphA4-Cdk5-p35 complex was relatively stable, as the association was detected for up to 60 min after ligand stimulation (**Fig. 2c**).

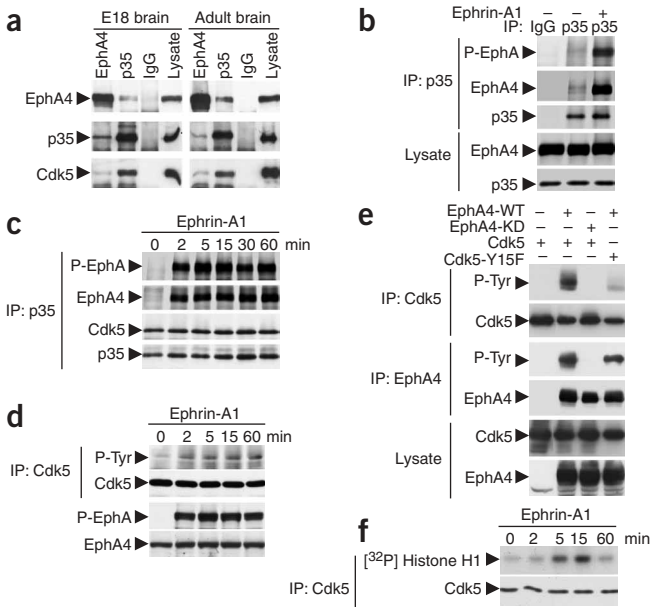

Figure 2 EphA4 activation recruits the Cdk5-p35 complex and stimulates tyrosine phosphorylation of Cdk5. (**a**) Cdk5-p35 complex associates with EphA4 in E18 and adult rat brains. (**b**) Basal interaction between p35 and EphA4 in cortical neurons and augmentation by ephrin-A1 treatment. Cortical neurons were treated with or without ephrin-A1 for 5 min. Lysates were collected, coimmunoprecipitated with antibody to p35 or with IgG and analyzed by western blotting. Ephrin-A1 stimulated tyrosine phosphorylation of EphA4 (P-EphA) and enhanced the interaction between Cdk5, p35 and EphA4. (**c**) Ephrin-A1–stimulated association of activated-EphA4 and Cdk5-p35 is relatively stable. Cortical neurons were treated with ephrin-A1 for 0–60 min as indicated. (**d**) Ephrin-A1 stimulates tyrosine phosphorylation of Cdk5 in cortical neurons. (**e**) Activated EphA4 induced Cdk5 tyrosine phosphorylation at Tyr15. HEK 293T cells were transfected with EphA4 or its kinase-dead mutant together with Cdk5 or its tyrosine phosphorylation mutant (Cdk5-Y15F). Cell lysates were immunoprecipitated with antibody to Cdk5 or EphA4 and then immunoblotted with antibody to phosphotyrosine (P-Tyr). (**f**) Ephrin-A1 increases Cdk5 activity in neurons. Cortical neurons were treated with ephrin-A1 and cell lysates were subjected to kinase assay using histone H1 protein as the substrate. Control loading for Cdk5 is shown below.

Activated EphA4 increases Cdk5 kinase activity

Given that EphA4 associates with Cdk5-p35 and that Cdk5 can be phosphorylated at Tyr15, we tested whether activated EphA4 phosphorylates Cdk5. We found that stimulating cortical neurons with ephrin-A1 led to a rapid increase in tyrosine phosphorylation of Cdk5, which remained elevated for up to 60 min (**Fig. 2d**). Furthermore, we showed that overexpression of EphA4 (EphA4-WT), but not its kinase-dead mutant (EphA4-KD), induced the phosphorylation of Cdk5 at Tyr15 in HEK 293T cells (**Fig. 2e**). This Cdk5 phosphorylation was specific for Tyr15, as it was not observed with the Cdk5-Y15F mutant (**Fig. 2e**). These observations indicate that EphA4 may directly phosphorylate Cdk5 at Tyr15 upon ephrin-A1 treatment, although we cannot rule out that ephrin-A1 might also stimulate tyrosine phosphorylation of Cdk5 through Src kinase that is recruited to the receptor complex[24].

Cdk5 activity increases upon Tyr15 phosphorylation of Cdk5 (refs. 16,17). Consistent with this, we found that ephrin-A1 increased Cdk5 activity in neurons (**Fig. 2f**). The specificity of Cdk5 activity was confirmed, as a similar increase was not observed when the lysate was immunoprecipitated by normal rabbit IgG (data not shown). In addition, *in vitro* kinase assays showed that the recombinant EphA4 protein directly phosphorylated Cdk5 at Tyr15 in a dose-dependent manner (**Supplementary Fig. 2** online). Similarly, EphA4 increased Cdk5 kinase activity in a dose-dependent manner by *in vitro* kinase assays (**Supplementary Fig. 2**). These results suggest that the ligand-dependent recruitment of Cdk5 to activated EphA4 results in Cdk5 phosphorylation at Tyr15 and elevated Cdk5 activity.

Cdk5 is involved in ephrin-A1–mediated spine retraction

To further explore the involvement of Cdk5 in EphA4-mediated spine retraction, we examined the subcellular localization of EphA4 in cultured hippocampal neurons. EphA4 clusters were induced along dendrites and axons in hippocampal neurons on ephrin-A1 treatment (**Supplementary Fig. 3** online). Because binding of ephrin to EphA4 triggers the endocytosis of ligand-receptor complex, we examined whether ephrinA1-induced EphA4 clusters represented endocytosed receptors[25]. Antibodies specific for the extracellular region of EphA4 did not detect the EphA4 clusters, suggesting that the receptor clusters were not localized on the cell surface but were internalized, and the newly internalized EphA4 was found to be colocalized with early endosome antigen 1 in the early endosomes (**Supplementary Fig. 3**). Furthermore, the EphA4 immunoreactivity of hippocampal neurons at 21 days *in vitro* (DIV) was detected as punctate signals associated with cell bodies, axons and dendrites (**Fig. 3a**). These EphA4 puncta were colocalized with a postsynaptic marker, postsynaptic density protein 95 (PSD-95), on dendrites (**Fig. 3a**), consistent with an earlier report[9]. The colocalization of EphA4 with PSD-95, together with the detection of EphA4, p35 and Cdk5 in PSD fractions (**Supplementary Fig. 4** online), suggests a potential function of the EphA4–Cdk5-p35 signaling complex at PSD. To confirm that the activation of EphA4 by ephrin occurs at the postsynaptic regions, we examined the localization of phosphorylated EphA4 in neurons on ephrin-A1 stimulation. We occasionally detected phosphorylated EphA4 clusters on the dendrites that were not localized with PSD-95; however, the number of phosphorylated EphA4 clusters was markedly increased after ephrin-A1 treatment, and some clusters were localized with PSD-95 (**Fig. 3b**).

We next examined whether ephrin-A1 regulated the spine morphogenesis in cultured hippocampal neurons as it did in hippocampal slices. We treated hippocampal neurons (20 DIV) with ephrin-A1 and examining the dendritic spine density. At this stage, the neurons were well differentiated, with branching of the dendritic tree and development of mushroom-shaped spines. Treating neurons with ephrin-A1 for 24 h reduced the density of the dendritic spines by ~20% (**Supplementary Fig. 5** online), although the dendritic branching patterns were not affected (**Supplementary Fig. 5**). Treating neurons with ephrin-A5 produced similar effects (data not shown). The decrease in spine density was first detected after 5 h of ephrin-A1 treatment and the effect was maintained for up to 24 h (**Supplementary Fig. 5**).

To examine whether ephrin-A1 elicited its effect on dendritic spine retraction via Cdk5 activation, RNAi was used to knock down Cdk5 expression in neurons. Neurons at 9 DIV were transfected with the pSUPER-Cdk5 RNAi and EGFP construct, and then at 20 DIV they were treated with ephrin-A1 for 24 h. The efficiency of knockdown of endogenous Cdk5 was confirmed by western blotting (**Fig. 3c**). We found that ephrin-A1–mediated spine retraction was abolished in Cdk5 knockdown neurons (**Fig. 3d**). To determine whether Cdk5 phosphorylation is involved in EphA4-dependent spine retraction, we transfected hippocampal neurons with different Cdk5 constructs (WT, DN or Y15F). Neurons overexpressing Cdk5-DN and Cdk5-Y15F had

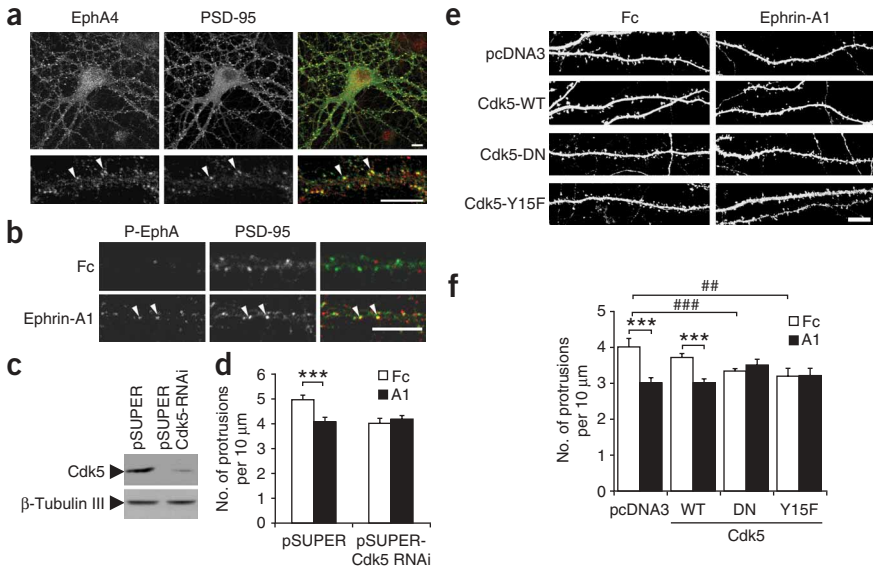

Figure 3 Cdk5 activity regulates dendritic spine retraction in hippocampal neurons. (**a**) EphA4 expression is colocalized with a postsynaptic marker, PSD-95, at dendritic spines in hippocampal neurons (21 DIV). Arrowheads indicate colocalization of EphA4 (red) and PSD-95 (green). (**b**) Activated EphA protein (P-EphA) is detected at the postsynaptic sites in hippocampal neurons (15 DIV) after ephrin-A1 treatment. Arrowheads indicate colocalization of activated EphA protein (P-EphA; red) and PSD-95 (green). (**c**) Western blot analysis showed the knockdown of endogenous Cdk5 by pSUPER-Cdk5 RNAi in hippocampal neurons. Rat hippocampal neurons were transfected with pSUPER-Cdk5 RNAi construct or pSUPER vector. β-tubulin III acts as control for equal protein loading. (**d**) Ephrin-A1 does not reduce spine density in Cdk5-deficient hippocampal neurons after transfection with pSUPER-Cdk5 RNAi. Data are presented as number of spines (protrusions) per 10 μm in hippocampal neurons (mean ± s.e.m., ephrin-A1 versus Fc treatment in pSUPER control, *** $P < 0.005$). (**e**) Inhibition of Cdk5 activity attenuates ephrin-A1–stimulated reduction of dendritic spine density. Hippocampal neurons were transfected with wild-type Cdk5 (Cdk5-WT), dominant-negative Cdk5 (Cdk5-DN), a tyrosine mutant of Cdk5 (Cdk5-Y15F) or vector (pcDNA3) and EGFP, and then treated with ephrin-A1. Representative images showing dendritic spines of neurons overexpressing Cdk5-WT or its mutants after ephrin-A1 treatment. (**f**) Data are presented as number of spines (protrusions) per 10 μm in hippocampal neurons (mean ± s.e.m.; *** $P < 0.005$, ephrin-A1 versus Fc; ### $P < 0.005$, Cdk5-DN versus pcDNA3; ## $P < 0.01$, Cdk5-Y15F versus pcDNA3; unpaired Student's *t*-test). Scale bars, 10 μm (**a**,**b**,**e**).

substantially reduced dendritic trees with fewer branch points compared with pcDNA3 or wild-type Cdk5 transfected neurons (data not shown), suggesting that Cdk5 activity is partially required for dendritic development. Moreover, in hippocampal neurons overexpressing different Cdk5 constructs, the density of dendritic spines was slightly less than that of controls. In response to ephrin-A1 treatment, the neurons overexpressing Cdk5-WT retracted their spines, similar to control neurons (**Fig. 3e**,**f**), but the neurons overexpressing Cdk5-DN and Cdk5-Y15F did not show any change in spine density (**Fig. 3e**,**f**). These results indicate that tyrosine phosphorylation of Cdk5 and its kinase activity are required for ephrin-A1–mediated spine retraction.

Because our results indicated that Cdk5 activity was crucial in ephrin-A1–triggered spine retraction, we sought to further explain the mechanism for this process by analyzing the spine density of $Cdk5^{-/-}$ hippocampal neurons. Similar to neurons transfected with pSUPER-Cdk5 RNAi, Cdk5-DN or Cdk5-Y15F, the $Cdk5^{-/-}$ neurons had markedly defective dendritic development (data not shown) with reduced dendritic spine density (**Fig. 4a**,**b**). Furthermore, the $Cdk5^{-/-}$ neurons did not respond to ephrin-A1 with a reduction in spine density (**Fig. 4a**,**b**). To confirm that the lack of response was due to Cdk5 deficiency, the rescue experiment was performed by transfecting $Cdk5^{-/-}$ neurons with Cdk5. Re-expression of Cdk5 in $Cdk5^{-/-}$ neurons allowed the neurons to show normal dendritic morphology and

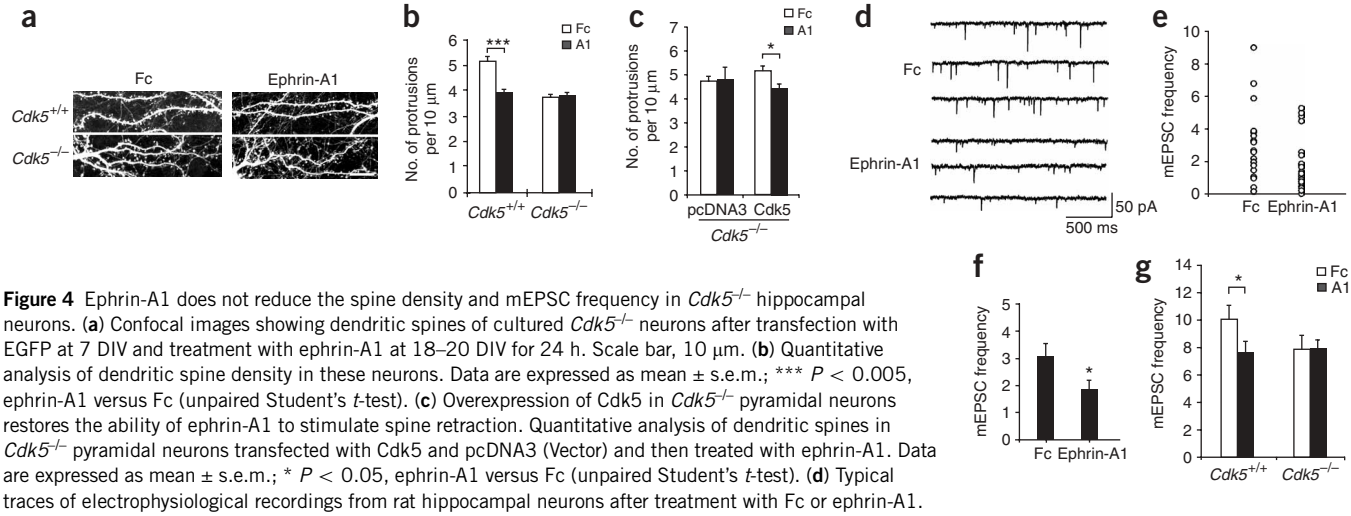

Figure 4 Ephrin-A1 does not reduce the spine density and mEPSC frequency in $Cdk5^{-/-}$ hippocampal neurons. (**a**) Confocal images showing dendritic spines of cultured $Cdk5^{-/-}$ neurons after transfection with EGFP at 7 DIV and treatment with ephrin-A1 at 18–20 DIV for 24 h. Scale bar, 10 μm. (**b**) Quantitative analysis of dendritic spine density in these neurons. Data are expressed as mean ± s.e.m.; *** $P < 0.005$, ephrin-A1 versus Fc (unpaired Student's *t*-test). (**c**) Overexpression of Cdk5 in $Cdk5^{-/-}$ pyramidal neurons restores the ability of ephrin-A1 to stimulate spine retraction. Quantitative analysis of dendritic spines in $Cdk5^{-/-}$ pyramidal neurons transfected with Cdk5 and pcDNA3 (Vector) and then treated with ephrin-A1. Data are expressed as mean ± s.e.m.; * $P < 0.05$, ephrin-A1 versus Fc (unpaired Student's *t*-test). (**d**) Typical traces of electrophysiological recordings from rat hippocampal neurons after treatment with Fc or ephrin-A1. (**e**,**f**) Distribution (**e**) and quantification (**f**) of mEPSC frequency of these results are depicted; mean ± s.e.m.; * $P < 0.05$, ephrin-A1 versus Fc. Ephrin-A1–mediated reduction of mEPSC frequency is abolished in $Cdk5^{-/-}$ hippocampal neurons. (**g**) Quantification of mEPSC frequency of hippocampal neurons from $Cdk5^{+/+}$ and $Cdk5^{-/-}$ mice; mean ± s.e.m.; * $P < 0.05$, ephrin-A1 versus Fc in $Cdk5^{-/-}$ hippocampal neurons. The difference in the basal mEPSC frequency between **f** and **g** may be due to the different neurotransmission properties of rat and mouse neurons or the number of neurons plated.

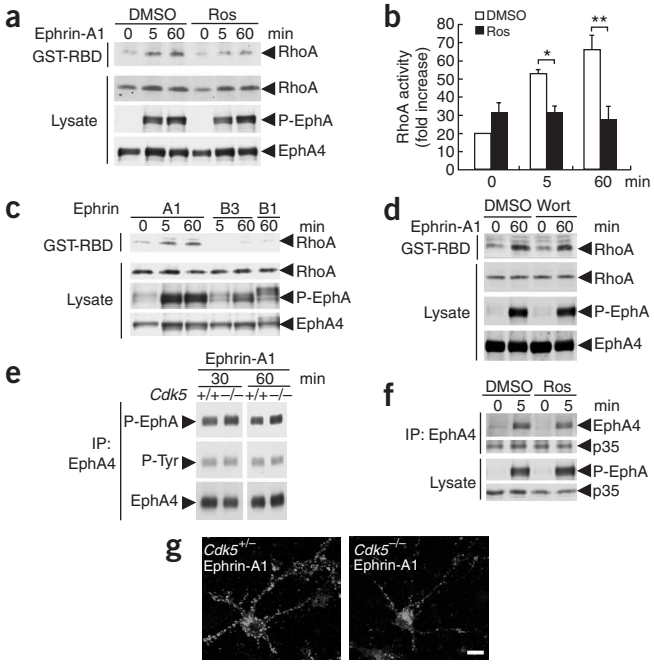

Figure 5 Cdk5 activity regulates ephrin-A1–stimulated EphA4 activation and RhoA activity in neurons. (**a**) Cdk5 activity regulates ephrin-A1–stimulated RhoA activity. Cortical neurons were pretreated with 25 μM roscovitine (Ros) for 1 h before ephrin-A1 stimulation. Lysates were collected, incubated with GST-RBD and immunoblotted with antibody to RhoA. (**b**) Quantification of fold increase of RhoA activity ($n = 3$); data are expressed as mean ± s.e.m.; * $P < 0.05$, ** $P < 0.01$; Ros versus DMSO control. (**c**) The stimulation of RhoA activity is specific to ephrin-A1 treatment. Cortical neurons were treated with different ephrins and assayed for RhoA activity. (**d**) Wortmannin did not inhibit ephrin-A1–stimulated RhoA activation. Cortical neurons were pretreated with 10 nM wortmannin (Wort) for 1 h before ephrin-A1 stimulation. (**e**) Ephrin-A1 induces similar tyrosine phosphorylation of EphA4 in *Cdk5*$^{+/+}$ and *Cdk5*$^{-/-}$ cortical neurons. Cortical neurons from *Cdk5*$^{+/+}$ and *Cdk5*$^{-/-}$ mice were stimulated with ephrin-A1. Cell lysates were immunoprecipitated with antibody to EphA4 and analyzed by western blotting for P-EphA, phosphotyrosine (P-Tyr) and EphA4. (**f**) Inhibition of Cdk5 activity did not affect the association between p35 and EphA4. Cortical neurons were pretreated with 25 μM Ros for 1 h before ephrin-A1 stimulation. (**g**) Attenuation of EphA4 clustering in Cdk5-deficient neurons. Cultured *Cdk5*$^{+/-}$ and *Cdk5*$^{-/-}$ hippocampal neurons (2 DIV) were treated with ephrin-A1 for 1 h and immunostained with antibody to EphA4. EphA4 clusters were detected in cell bodies and neurites. Scale bar, 10 μm.

restored the response to ephrin-A1 (**Fig. 4c** and data not shown). Notably, a reduction in the basal level of spine density was observed following the loss of Cdk5 activity. We speculate that such a decrease might be attributable, at least in part, to the defective dendritic morphology in Cdk5-deficient neurons. Given the ability of Cdk5 to phosphorylate multiple substrates, it is possible that the loss of Cdk5 activity perturbs critical processes during neuronal development, leading to abnormal dendritic branching and affecting spine density. Unlike *Cdk5*$^{-/-}$ hippocampal neurons, *p35*$^{-/-}$ neurons showed overall normal dendritic morphology, and their synaptic density was similar to that of wild type, perhaps owing to the compensatory effect of p39. Nonetheless, ephrin-A1 treatment also did not reduce the spine density in *p35*$^{-/-}$ neurons (data not shown), suggesting that spine retraction is due to p35-associated Cdk5 activity.

Most spines are postsynaptic sites of excitatory synapses and receive synaptic inputs. To examine the physiological significance of the ephrin-A1–mediated decrease in spine density, we recorded spontaneous miniature excitatory postsynaptic currents (mEPSCs) from neurons treated with ephrin-A1. mEPSCs are caused by the quantal release of neurotransmitters from presynaptic terminals[26] and thus changes in mEPSC frequency could reflect changes in synapse number. In cultured rat hippocampal neurons treated with ephrin-A1, the frequencies of mEPSCs decreased significantly, to 61.6 ± 10.1% (mean ± s.e.m.) of control (**Fig. 4d–f**, $n = 29$; $P < 0.05$ compared to controls, $n = 19$). The mEPSC amplitudes were not affected by ephrin-A1 treatment (data not shown). Ephrin-A1 treatment of *Cdk5*$^{+/+}$ neurons similarly decreased the frequencies of mEPSCs (73.0 ± 7.5% of control, $n = 25$; $P < 0.05$ compared to controls, $n = 26$, **Fig. 4g**), but ephrin-A1–triggered reduction in mEPSC frequency was abolished in *Cdk5*$^{-/-}$ neurons (100.3 ± 8.5% of control, $n = 19$; $P > 0.05$ compared to controls, $n = 19$, **Fig. 4g**). Together, these observations show that Cdk5 is important in the ephrin-A1–mediated reduction of synapse density in cultured hippocampal neurons.

Cdk5 regulates EphA4-mediated induction of RhoA activity

To determine the mechanism by which Cdk5 regulates EphA4-mediated spine retraction, we examined whether Cdk5 activity is involved in transducing EphA4 forward signaling, such as RhoA GTPase activity[24,27]. Within 5 min of treating cortical neurons with ephrin-A1, RhoA activity increased markedly and remained elevated for up to 60 min (**Fig. 5a**,**b**). This increase in RhoA activity was specific for ephrin-A1, as neither ephrin-B1 nor ephrin-B3 elicited similar effects (**Fig. 5c**). We found that treatment of these neurons with roscovitine suppressed ephrin-A1–induced RhoA activity (**Fig. 5a**,**b**). On the other hand, inhibition of PI3K by wortmannin did not attenuate the ephrin-A1–triggered activation of RhoA (**Fig. 5d**).

Maximal EphA4 function has been proposed to require both kinase activation and receptor clustering[25]. Rather than promoting further increases in activity, EphA4 clustering is important for recruiting or activating cytoplasmic effectors such as ephexin1 (ref. 28). We therefore examined whether Cdk5 regulates EphA4-mediated signaling by modulating the phosphorylation status or clustering of EphA4 upon activation. We found that stimulating *Cdk5*$^{-/-}$ cortical neurons with ephrin-A1 led to a robust increase in the tyrosine phosphorylation of EphA4, similar to that observed in *Cdk5*$^{+/+}$ neurons (**Fig. 5e**), and that inhibiting Cdk5 activity with roscovitine did not attenuate the association of EphA4 and p35 (**Fig. 5f**). These results collectively suggested that Cdk5 neither affects EphA4 phosphorylation nor regulates the recruitment of Cdk5-p35 to activated EphA4. Nonetheless, the soma and neurites of ephrin-A1–treated *Cdk5*$^{-/-}$ neurons still showed EphA4 clusters, whose sizes were significantly lower than those observed in *Cdk5*$^{+/-}$ neurons (~30% reduction; $P < 0.05$) (**Fig. 5g** and data not shown). These results suggest that Cdk5 may contribute to regulating EphA4 clustering, thereby affecting the recruitment of signaling molecules other than Cdk5-p35 to activated EphA4.

Cdk5 phosphorylates ephexin1 and regulates its activity

Because our results showed that Cdk5 activity is important for EphA4-mediated RhoA activation and spine retraction, and EphA4-stimulated RhoA activity in axon guidance of retinal ganglion cells has been shown to involve GEF ephexin1 (ref. 24), it is tempting to speculate that Cdk5 may regulate EphA4-triggered RhoA activation and spine retraction by modulating the activity or recruitment of ephexin1. To examine this possibility, we first characterized the subcellular localization of

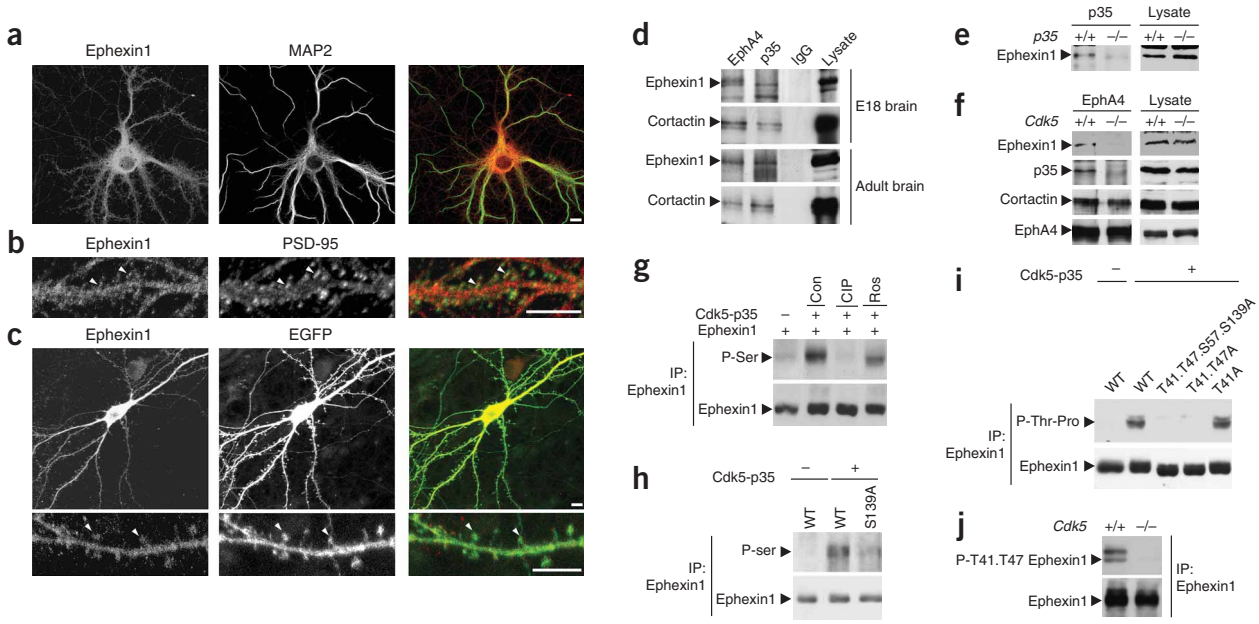

Figure 6 Ephexin1 is phosphorylated by Cdk5-p35. (**a**–**c**) Subcellular localization of endogenous (**a** and **b**) and ectopically expressed (**c**) ephexin1 in neurons. Hippocampal neurons (21 DIV) were stained with antibodies to ephexin1 (red) and MAP2 or PSD-95 (green). Arrowheads indicate the colocalization of ephexin1 and PSD-95. In **c**, hippocampal neurons were transfected with ephexin1 and EGFP constructs. A higher-magnification image of the dendrites (below) shows that the ephexin1 is expressed at the spine protrusions. Arrowheads indicate the localization of ectopically expressed ephexin1 (red) to spine protrusions. (**d**) Association of p35 and ephexin1 in E18 and adult rat brains. (**e**) Antibody to p35 did not pull down ephexin1 from *p35*$^{-/-}$ brain lysate. (**f**) The association between EphA4, ephexin1 and p35 is abolished in *Cdk5*$^{-/-}$ brain. Interaction between EphA4 and ephexin1 or p35 was not observed in *Cdk5*$^{-/-}$ brain. Interaction of EphA4 and cortactin in Cdk5-deficient brain served as a control. (**g**) Phosphorylation of ephexin1 by Cdk5. HEK 293T cells were transfected with ephexin1, whose phosphorylation by Cdk5 was examined in the presence of roscovitine (Ros) or calf intestinal phosphatase (CIP). (**h**,**i**) Cdk5 phosphorylates ephexin1 at multiple serine and threonine residues. HEK 293T cells were transfected with ephexin1 (WT) or its mutants, together with Cdk5 and p35 expression constructs. Lysates were immunoprecipitated using antibody to ephexin1 and subjected to western blot analysis using antibodies to P-Ser (**h**) and proline-directed P-Thr (P-Thr-Pro) (**i**). Expression of ephexin1 served as control. (**j**) Phosphorylation of ephexin1 at Thr41.47 was abolished in E18 *Cdk5*$^{-/-}$ brain.

ephexin1 in hippocampal neurons by determining the distribution of endogenous and ectopically expressed ephexin1 in neurons at 21 DIV. We found that endogenous ephexin1 was expressed in cell bodies and along axons, dendrites and dendritic spines (**Fig. 6a**,**b**). Similar subcellular localization was also observed for ectopically expressed ephexin1, especially in spine protrusions (**Fig. 6c**). Together with the observation that ephexin1 can be extracted with Sarkosyl, but not Triton X-100, our findings suggest that ephexin1 is tightly associated with PSD[29] (**Supplementary Fig. 4**). We then investigated whether Cdk5 regulates ephrin-A1–induced RhoA activity via ephexin1. We found that p35 interacted with ephexin1 in HEK 293T cells when both were overexpressed (data not shown). In addition, p35 associated with ephexin1 in both embryonic and adult rat brains (**Fig. 6d**), but not in embryonic *p35*$^{-/-}$ brains (**Fig. 6e**), confirming the specificity of the interaction between p35 and ephexin1 *in vivo*. The interactions of EphA4 with ephexin1 or p35 were both abolished in *Cdk5*$^{-/-}$ brains, but the association between EphA4 and cortactin[30] remained intact (**Fig. 6f**). This observation suggests that the association of ephexin1 with both p35 and EphA4 requires Cdk5.

Ephexin1 has four proline-directed serine and threonine residues that can be phosphorylated by Cdk5. To investigate whether the interaction between Cdk5-p35 and ephexin1 results in ephexin1 phosphorylation, we performed *in vitro* kinase assays and found that active Cdk5 phosphorylated ephexin1 (**Supplementary Fig. 6** online). We then examined the major phosphorylation sites on ephexin1 by constructing different phosphorylation mutants (in which Thr41, Thr47, Ser57 or Ser139 were mutated to alanine or combined in mutations as shown in **Supplementary Fig. 6**). Mutating Thr41, Thr47 and Ser139 to alanine in ephexin1 substantially reduced ephexin1 phosphorylation by Cdk5 (by ~80%; **Supplementary Fig. 6**). Consistent with this observation, adding roscovitine reduced the serine phosphorylation of ephexin1 (**Fig. 6g**). Moreover, mutating Thr47 on ephexin1 did not substantially affect its phosphorylation by Cdk5, suggesting that Thr41 and Ser139 represent the principal Cdk5 phosphorylation sites (**Fig. 6h**,**i**). Analysis of proteins extracted from *Cdk5*$^{-/-}$ brains showed a significant reduction of phosphorylation at Thr41 and Thr47 sites, further supporting the notion that ephexin1 is a physiological substrate for Cdk5 (**Fig. 6j** and **Supplementary Fig. 6**).

Because EphA4 activation redirects the exchange activity of ephexin1 towards RhoA through Src phosphorylation of ephexin1 at Tyr87 (ref. 24), we examined whether Cdk5 phosphorylation regulated the tyrosine phosphorylation and activity of ephexin1. Expression of EphA4 in HEK 293T cells induced a robust increase in Tyr87 phosphorylation of ephexin1 (**Fig. 7a**). Tyr87 phosphorylation was reduced in the ephexin1-T41.T47.S57.S139A mutant, whereas in the phosphomimetic ephexin1 (T41.T47.S139E), the degree of EphA4-activated tyrosine phosphorylation was similar to those of wild-type ephexin1 (**Fig. 7a**). The interaction between the ephexin1-T41.T47.S57.S139A mutant and the EphA4 receptor was not different from that of wild-type ephexin1 (**Fig. 7b**), suggesting that Cdk5 regulates the susceptibility of ephexin1 to phosphorylation by Src but not its interaction with EphA4.

To further investigate the involvement of Cdk5-p35 in EphA4-triggered activation of ephexin1 and Rho GTPase, we overexpressed

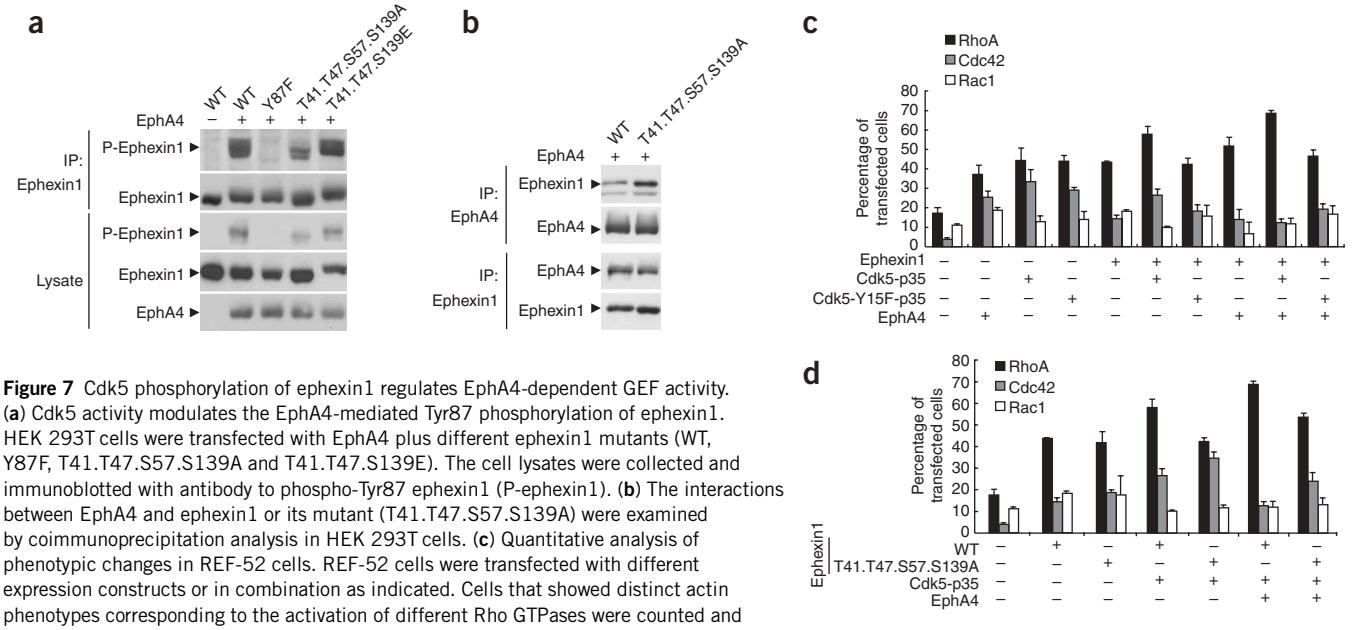

Figure 7 Cdk5 phosphorylation of ephexin1 regulates EphA4-dependent GEF activity. (**a**) Cdk5 activity modulates the EphA4-mediated Tyr87 phosphorylation of ephexin1. HEK 293T cells were transfected with EphA4 plus different ephexin1 mutants (WT, Y87F, T41.T47.S57.S139A and T41.T47.S139E). The cell lysates were collected and immunoblotted with antibody to phospho-Tyr87 ephexin1 (P-ephexin1). (**b**) The interactions between EphA4 and ephexin1 or its mutant (T41.T47.S57.S139A) were examined by coimmunoprecipitation analysis in HEK 293T cells. (**c**) Quantitative analysis of phenotypic changes in REF-52 cells. REF-52 cells were transfected with different expression constructs or in combination as indicated. Cells that showed distinct actin phenotypes corresponding to the activation of different Rho GTPases were counted and expressed as the percentage of transfected cells. Cdk5 activity increases the EphA4-regulated ephexin1 exchange activity towards RhoA. REF-52 cells were transfected with expression constructs encoding EphA4, ephexin1, p35, Cdk5 and its tyrosine mutant Cdk5-Y15F. (**d**) The display of stress fiber phenotype observed in REF-52 cells induced by Cdk5 activity (RhoA activation) is dependent on the phosphorylation of ephexin1. Overexpression of the phosphorylation-deficient mutant of ephexin1 (ephexin1-T41.T47.S57.S139A) abolished the Cdk5-mediated increase of cells expressing the RhoA phenotype.

EphA4, ephexin1, Cdk5 and p35 constructs in REF-52 cells, which have been widely used to examine the activation of small Rho GTPases based on changes in characteristic actin phenotypes[31]. Co-expressing EphA4 with ephexin1 increased the percentage of REF-52 cells expressing stress fibers (52% versus 43% in cells expressing only ephexin1; **Fig. 7c**), as previously reported[24,32], indicative of RhoA activation. We also observed decreases in the percentages of cells with lamellipodia and filopodia, representing Rac1 and Cdc42 phenotypes, respectively. Co-expressing Cdk5 and p35 with EphA4 and ephexin1 further increased the percentage of REF-52 cells that showed stress fiber phenotypes to ~70% (**Fig. 7c**). This RhoA phenotype did not increase, however, when the tyrosine mutant of Cdk5 was overexpressed (**Fig. 7c**), indicating that Cdk5-mediated enhancement of EphA4-stimulated ephexin1 activity towards RhoA required Cdk5 phosphorylation. Furthermore, an increase of the RhoA phenotype was not observed when the Cdk5 phosphorylation-deficient mutant of ephexin1 (ephexin1-T41.T47.S57.S139A) was expressed (**Fig. 7d**), verifying the importance of ephexin1 phosphorylation by Cdk5 for inducing RhoA activation.

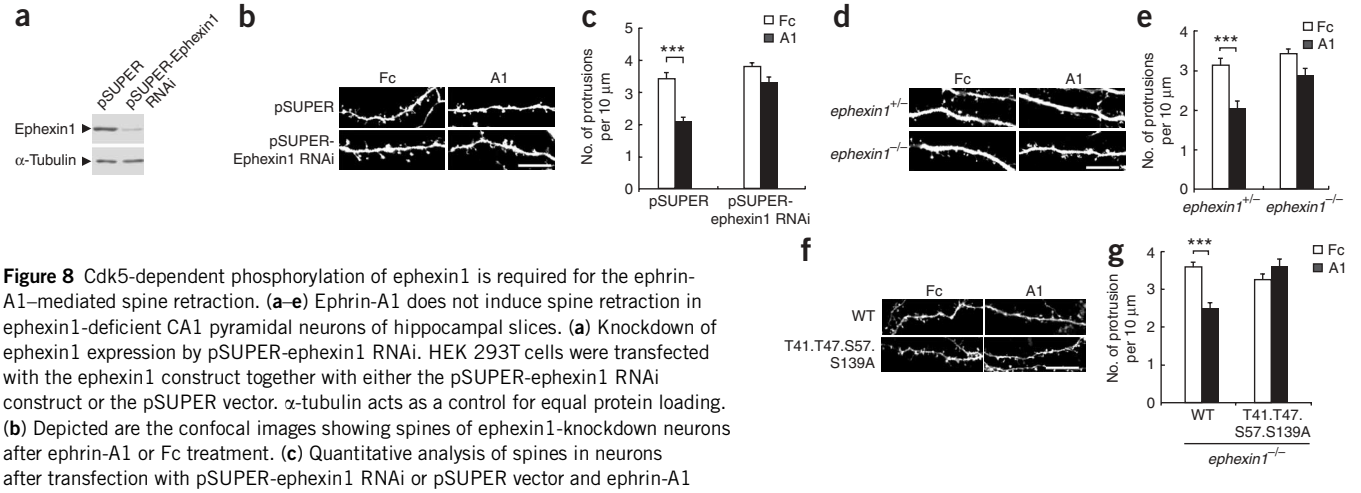

Figure 8 Cdk5-dependent phosphorylation of ephexin1 is required for the ephrin-A1–mediated spine retraction. (**a**–**e**) Ephrin-A1 does not induce spine retraction in ephexin1-deficient CA1 pyramidal neurons of hippocampal slices. (**a**) Knockdown of ephexin1 expression by pSUPER-ephexin1 RNAi. HEK 293T cells were transfected with the ephexin1 construct together with either the pSUPER-ephexin1 RNAi construct or the pSUPER vector. α-tubulin acts as a control for equal protein loading. (**b**) Depicted are the confocal images showing spines of ephexin1-knockdown neurons after ephrin-A1 or Fc treatment. (**c**) Quantitative analysis of spines in neurons after transfection with pSUPER-ephexin1 RNAi or pSUPER vector and ephrin-A1 stimulation. Data is presented as the number of spines (protrusions) per 10 μm (mean ± s.e.m.; *** $P < 0.005$, ephrin-A1 versus Fc; unpaired Student's *t*-test). (**d**) Depicted are the confocal images of dendritic spines of *ephexin1*$^{+/-}$ and *ephexin1*$^{-/-}$ hippocampal slices after treatment with ephrin-A1 or Fc. (**e**) The spine density of these neurons was quantified after treatment with ephrin-A1 (mean ± s.e.m., *** $P < 0.005$, ephrin-A1 versus Fc; unpaired Student's *t*-test). (**f**,**g**) Ephexin1 (WT) but not its phosphorylation mutant (T41.T47.S57.S139A) rescues ephrin-A1–mediated spine retraction in *ephexin1*$^{-/-}$ neurons. Shown are confocal images showing spines (**f**) and quantitative analysis of spines (**g**) of *ephexin1*$^{-/-}$ neurons after transfection with ephexin1 constructs are shown. Data is presented as the number of spines (protrusions) per 10 μm in pyramidal neurons (mean ± s.e.m.; *** $P < 0.005$, ephrin-A1 versus Fc; unpaired Student's *t*-test). Scale bar, 10 μm.

Ephexin1 is required for ephrin-A1–regulated spine density

To further assess the effects of ephexin1 on ephrin-A1–regulated spine morphology, we silenced the expression of ephexin1 in pyramidal neurons by transfecting hippocampal slices with pSUPER-ephexin1 RNAi. The effect of pSUPER-ephexin1 RNAi was confirmed by the reduced expression of ephexin1 in HEK 293T cells (**Fig. 8a**). Hippocampal slices were transfected with pSUPER-ephexin1 RNAi and then treated with ephrin-A1 for 16 h. Consistent with our earlier observations, ephrin-A1 reduced spine density in pSUPER-transfected neurons (by ~40%). In ephexin1 knockdown neurons, however, ephrin-A1 did not reduce spine density (**Fig. 8a–c**). Similarly, ephrin-A1 did not reduce spine density in neurons from genetically ephexin1-deficient (*ephexin1*$^{-/-}$; *Ngef1*$^{-/-}$) neurons (**Fig. 8d,e**).

To investigate the importance of Cdk5-dependent phosphorylation of ephexin1 in ephrin-A1–mediated spine retraction, we expressed ephexin1 and its Cdk5-phosphorylation defective mutant (T41.T37.S57.S139A) in *ephexin1*$^{-/-}$ hippocampal neurons (**Supplementary Fig. 7** online) and compared their ability to rescue the impaired function of spine retraction. Expression of ephexin1 in *ephexin1*$^{-/-}$ neurons rescued the impaired ability of ephrin-A1 to induce spine retraction; however, the phosphorylation mutant (T41.T37.S57.S139A) did not (**Fig. 8f,g**). These results demonstrate that Cdk5 phosphorylation of ephexin1 is required for ephrin-A1–regulated spine retraction.

DISCUSSION

The molecular mechanism by which EphB activation leads to dendritic spine development has been extensively studied. However, relatively little is known about the pathways involved in mediating EphA4-triggered dendritic spine retraction. Our findings reveal a concerted mechanism through which EphA4 activation induces spine retraction in hippocampal pyramidal neurons by recruiting Cdk5 and GEF ephexin1 (**Supplementary Fig. 8** online). We demonstrate that ephrin-A1 activation of EphA4 signaling stimulates the recruitment of Cdk5-p35 complex to EphA4, and promotes Cdk5 activity by phosphorylating Tyr15. Furthermore, we show that knockdown of Cdk5 expression or inhibition of Cdk5 activity blocks ephrin-A1–regulated spine retraction, revealing the essential role of Cdk5 in EphA4-mediated spine morphogenesis. We describe the underlying mechanism of this process by showing that the transduction of EphA4 signaling to the actin cytoskeleton requires the Cdk5 phosphorylation of ephexin1 and its recruitment to activated EphA4. Thus, phosphorylation of ephexin1 by Cdk5 augments the tyrosine phosphorylation of ephexin1 upon ephrin-A stimulation, thereby enhancing its GEF activity towards RhoA, in turn regulating actin reorganization in dendritic spines.

EphA4-induced spine retraction depends on Cdk5

Dendritic spines are enriched with F-actin, whose dynamics are precisely regulated to shape spine heads. Activation of EphBs by ephrinBs transduces signals that cause local changes in small GTPase activity, leading to actin rearrangement and spine remodeling[11,14]. Although activation of EphA4 is known to promote dendritic spine retraction in hippocampal slices[9], little is known about the underlying mechanisms. In this study, we have identified an important role for Cdk5 in mediating EphA4-activated spine retraction. Like Ephs, Cdk5 is important in regulating actin dynamics[33]. Because Cdk5 and its activators are concentrated at PSD[15], and various synaptic proteins have been identified as Cdk5 substrates[22], Cdk5 has emerged as a protein that has an important role at synapses[34]. For example, work in our laboratory has demonstrated that Cdk5 functions in postsynaptic specialization at the neuromuscular junction[18,35] and that Cdk5 activity regulates the cluster size of neurotransmitter receptor clusters *in vivo*[36]. Here we show that Cdk5 functions as a key regulator of EphA4-mediated actin cytoskeletal reorganization and thus contributes to spine retraction.

In this study, we have identified an interaction between EphA4 and Cdk5-p35. Although a small amount of basal interaction between EphA4 and Cdk5-p35 can be detected in cultured neurons, this association is substantially enhanced after ephrin-A1 treatment. Concomitant with the recruitment of Cdk5-p35 complex to the activated receptor, activated EphA4 enhances Cdk5 activity by phosphorylating Tyr15. In addition to promoting the kinase activity, tyrosine phosphorylation of Cdk5 may regulate its substrate specificity or subcellular localization. Although it is likely that active Cdk5 modulates various receptor signaling events at synapses to affect excitatory synapse formation and maintenance, it remains unclear in which subcellular compartment Cdk5 is activated.

Ephexin1-Cdk5 dependent transduction of signals from EphA4

The small GTPases of the Rho family are key integrators of extracellular cues to actin dynamics, which are critical for spine formation as well as for the maintenance and plasticity of mature synapses[37]. Inhibition of Cdk5, as well as RhoA activity, suppresses ephrin-A–induced growth cone collapse, suggesting that these two proteins might be involved in ephrin-EphA signaling[27]. In this study, we show that ephrin-A1 stimulates Cdk5-dependent RhoA activation, leading to spine retraction in hippocampal neurons. The action of RhoA signaling in spine morphogenesis is likely to be mediated by phosphorylating myosin light chains and by actomyosin contractility[13].

Rho GTPase activation is under tight and balanced regulation, mainly by GEFs of the Dbl family. Several GEFs, including kalirin, GEFT, intersectin-1, Tiam-1, PIX and Lfc, are implicated in spine morphogenesis[11,14,26,38–40]. Partly on the basis of our data from ephexin1 knockdown neurons or genetically ephexin1-deficient hippocampal slices, we now add ephexin1 to this list by showing that ephrin-A1 regulates spine morphology in hippocampal neurons via ephexin1-mediated regulation of RhoA activity. Combined with our data indicating that ephexin1 is localized to the dendrites and cell bodies of hippocampal neurons (**Fig. 6a–c**), these findings reveal an important role for ephexin1 in mediating the effect of ephrin-A1 on spine morphology. EphA4 activation has previously been shown to redirect the exchange activity of ephexin1 towards RhoA through the Src phosphorylation of ephexin1 on Tyr87 (ref. 24). We found that inhibiting Cdk5 phosphorylation of ephexin1 substantially reduced phosphorylation at Tyr87, suggesting that Cdk5 activity regulates Src-mediated tyrosine phosphorylation of ephexin1. Moreover, given that the association between EphA4 and ephexin1, as well as EphA4 clustering, is substantially reduced in *Cdk5*$^{-/-}$ brains, Cdk5 may regulate the recruitment of the signaling complex, including ephexin1, to activated EphA4, thus affecting the exchange activity of ephexin1. Finally, given that the major phosphorylation sites are concentrated at the N-terminal domain of ephexin1, modulation might be achieved through reversible steric or allosteric hindrance. Accordingly, ephexin1 is the candidate protein for integrating the signals transduced by EphA4-stimulated activation of Cdk5 and Src, thereby contributing to RhoA activation and spine retraction. Further studies are necessary to decipher the precise mechanism by which Cdk5 phosphorylation of ephexin1 modulates its activity and to explain how Cdk5-mediated signaling coordinates with Src activation to regulate ephexin1 activity. Nonetheless, given the presence of EphA4, ephexin1, Cdk5 and p35 at PSD and the localization of

phosphorylated EphA clusters to dendritic spines, activation of EphA4-Cdk5-ephexin1–mediated signaling events at those sites is probably important *in vivo*.

Functional implications of EphA4-induced spine retraction

Dendritic spines are highly dynamic structures that undergo constant changes in morphology and density, even in adult brains. The Eph family of RTKs regulates spine morphogenesis through different downstream pathways[11,14,26]. Unlike EphBs, which are important for promoting spine morphogenesis[11], EphA4 signaling mediates spine retraction in adult hippocampal slices[9]. Because we observed a similar effect in ephrin-A1–treated brain slices at P7, we propose that EphA4-mediated spine retraction may also occur during postnatal development. The spine retraction induced by EphA4 represents one type of plasticity that is mediated by modulating the geometry of existing spines. In the present study, we report that the reduction in spine density is paralleled by a decrease in functional excitatory synapses, as reflected by the change in mEPSC frequency. Impaired long-term potentiation (LTP) and long-term depression (LTD) have also been reported in *EphA4*$^{-/-}$ mice[4]. It is important to note that LTP and LTD are also impaired in the hippocampus of *p35*$^{-/-}$ mice[41], supporting the notion that like Ephs, Cdk5 is also involved in spatial learning and memory.

What are the functional consequences of EphA4-mediated spine retraction? The retraction of spines reduces the postsynaptic surface area and scaffold capacity, which may then affect the number of receptors localized to the postsynaptic membrane[42]. Moreover, actin dynamics associated with spine retraction may affect the delivery of signaling proteins and surface receptors to postsynaptic membranes. Finally, spine geometry controls the efficiency of postsynaptic calcium signaling, which ultimately might influence LTP and LTD. The number and structure of spines can affect synaptic plasticity and the efficacy of synaptic transmission. Although there are conflicting reports on spine density after LTP induction, new spines have been observed at activated spine synapses[43–45]. By contrast, shrinkage of dendritic spines in the hippocampus has been associated with LTD[46,47]. Differences in spine dynamics after induction of LTP and LTD are reminiscent of the distinct roles of EphA and EphB family receptors in spine morphogenesis. Thus, it is likely that these two families of Eph receptors may cooperatively regulate spine structure to maintain the optimal spine density, such that the generation of new spines might need to be counteracted by the retraction of other spines. Activated EphB signaling may promote spine maturation during development, whereas EphA may regulate the proper balance of dendritic spines by maintaining and eliminating them in response to experience. The dynamics and balance between EphA and EphB signaling might be important in the learning and memory process. Indeed, emerging evidence suggests a function for Eph receptors in mammalian cognitive processes[7,48]. Precise determination of the roles and mechanisms of action of Eph receptors in cognition awaits further studies.

METHODS

Constructs and antibodies. Constructs, siRNAs, antibodies and peptides used in this study are described in the **Supplementary Methods** online.

Preparation of organotypic hippocampal slices. Hippocampal slices were prepared as described (**Supplementary Methods** online). To study the effect of ephrin-mediated spine retraction, the YFP-expressing brain slices were treated with ephrin-A1 for 16 h. Subsequently, the slices were fixed with 4% paraformaldehyde, 0.5% glutaldehyde and 5% sucrose, and the spine density of YFP-expressing CA1 pyramidal neurons was visualized by confocal imaging (Fluoview BX61, Olympus).

Cell cultures and transfection. Primary cortical and hippocampal neurons were prepared and transfected as described (**Supplementary Methods** online). HEK 293T cells were transiently transfected with different combinations of plasmids using LipofectAMINE PLUS reagents (Invitrogen). REF-52 cells were cultured as described[24] and subjected to a fibroblast morphology assay for which a detailed protocol is provided in the **Supplementary Methods** online.

Ephrin-Fc clustering. Ephrin-A1–Fc, B1-Fc, B3-Fc (R&D Systems) and Fc (Jackson Immunoresearch Labs) were preclustered with goat or mouse anti-body to human Fc (Jackson Immunoresearch Labs) in a ratio of 1:4.5 and incubated at room temperature for 60 min before use. The final concentration for ephrin-A1–Fc or Fc was 5 μg ml^{-1} for dissociated neurons and 10 μg ml^{-1} for hippocampal slices.

Fusion protein generation, protein extraction, immunoprecipitation and western blot analysis. Detailed protocols for fusion protein generation, protein extraction, immunoprecipitation and western blotting are described (**Supplementary Methods** online).

Immunocytochemical analysis. To examine the endogenous expression of EphA4, P-EphA and ephexin1, the low-density neurons were fixed with either 4% paraformaldehyde and 5% sucrose at 25 °C or –20 °C methanol for 20 min. Immunostaining was performed as previously described[49]. To examine the effect of EphA4, Cdk5 and ephexin1 on ephrin-A1–mediated changes in dendritic arbors and spine morphology, we treated the transfected hippocampal neurons at 20 DIV with clustered ephrin-A1 or Fc for 24 h. The neurons were fixed with 4% paraformaldehyde and 5% sucrose, and the morphology of their dendritic arbors and spines was visualized by the expression of GFP under confocal microscopy (Fluoview BX61, Olympus).

Electrophysiology. A detailed protocol for electrophysiological study is described (**Supplementary Methods** online).

***In vitro* phosphorylation assay and GTPase activation assay.** Detailed protocols for *in vitro* phosphorylation assay and GTPase activation assay are described (**Supplementary Methods** online).

Quantitative analysis. To quantify dendritic branch segments in cultured hippocampal neurons, a stack of images (*z* step, 0.5 μm) was collected using a 60× objective. The number of dendritic segments was quantified by counting dendritic branch points and dendritic terminal ends[13]. To quantify spine density in organotypic hippocampal slices and cultured hippocampal neurons, a stack of images (*z* step, 0.5 μm) was collected using a 60× and 100× objective, respectively. Images were merged and analyzed using MetaMorph software (Universal Imaging Corp). Dendritic spines were scored and quantified in a double-blinded manner[50]. For dissociated neurons, 20–30 neurons were analyzed from 2–3 independent experiments. Data are presented as mean ± s.e.m. per 10 μm of dendritic segment. For organotypic hippocampal slices, all analyses were performed blind to genotype and each experiment was repeated on three independent days. Data from one of the representative experiments are presented as mean number of spines ± s.e.m. per 10-μm dendritic segment. Statistical significance was determined using unpaired Student's *t*-test. All animal studies were approved by the Animal Care Committee in accordance with institutional guidelines.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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