The Rab5 guanylate exchange factor Rin1 regulates endocytosis of the EphA4 receptor in mature excitatory neurons

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Ephrin signaling through Eph receptor tyrosine kinases regulates important morphogenetic events during development and synaptic plasticity in the adult brain. Although Eph-ephrin endocytosis is required for repulsive axon guidance, its role in postnatal brain and synaptic plasticity is unknown. Here, we show that Rin1, a postnatal brain-specific Rab5-GEF, is coexpressed with EphA4 in excitatory neurons and interacts with EphA4 in synaptosomal fractions. The interaction of Rin1 and EphA4 requires Rin1's SH2 domain, consistent with the view that Rin1 targets tyrosine phosphorylated receptors to Rab5 compartments. We find that Rin1 mediates EphA4 endocytosis in postnatal amygdala neurons after engagement of EphA4 with its cognate ligand ephrinB3. Rin1 was shown to suppress synaptic plasticity in the amygdala, a forebrain structure important for fear learning, possibly by internalizing synaptic receptors. We find that the EphA4 receptor is required for synaptic plasticity in the amygdala, raising the possibility that an underlying mechanism of Rin1 function in amygdala is to down-regulate EphA4 signaling by promoting its endocytosis.

Eph | receptor tyrosine kinase | synaptic plasticity | amygdala LTP

Interactions between two opposing cells through surface-associated ephrin ligands and their Eph receptors control a large variety of cellular responses during development, including cell adhesion, migration, and axon guidance (1). In the adult brain, the Eph-ephrin system modulates structural and synaptic plasticity by regulating spine morphogenesis and glutamate receptor clustering (2–5). Although ephrins bind to Eph receptors with high affinity, the cellular response to Eph-ephrin engagement is often repulsion between the cells. Mechanisms that turn Eph-ephrin-mediated adhesion into repulsion include ectodomain cleavage and endocytosis, as reviewed by Egea and Klein (1). The intracellular pathways by which Eph-ephrin complexes are endocytosed are not well characterized. During axon guidance, the Rho family guanine nucleotide exchange factor (GEF) Vav2 promotes endocytosis of the Eph-ephrin complex and $Vav2^{-/-};Vav3^{-/-}$ mice display defects in axonal projections (6), suggesting that Vav proteins function downstream of Ephs in guiding retinal axons, as reviewed by Flanagan (7).

In the adult brain, several different Ephs and ephrins were shown to be required for activity-dependent synaptic plasticity (5, 8, 9). EphA4 is required for early phases of hippocampal LTP and long-term depression (LTD), but the mechanism is not understood (5). Moreover, a role of endocytosis of Eph-ephrin complexes for neuronal plasticity has not been addressed. In our search for a candidate molecule for regulating Eph endocytosis in the adult brain, we focused our attention on Rin1 (Ras/Rab interactor 1) (10), a Rab5 GEF that promotes epidermal growth factor receptor (EGFR) internalization and actin cytoskeleton remodeling (11–13). Rin1 appeared to be a candidate for Eph endocytosis during neuronal plasticity, because Rin1 expression was highest in the postnatal brain (10) and was restricted to the dendrites of mature neurons (14). Moreover, $Rin1^{-/-}$ mice showed increased LTP in the amygdala (14). The amygdala is a brain structure known to mediate emotional learning, and amygdala LTP is a cellular model for acquisition of fear memory (15). Consistent with this, $Rin1^{-/-}$ mice display enhanced fear conditioning (14).

Here, we show that Rin1 mediates EphA4 endocytosis in amygdala neurons. We further show that $EphA4^{-/-}$ mice displayed reduced amygdala LTP and that inhibition of Eph signaling reduced the elevated LTP in $Rin1^{-/-}$ mice. Together, the findings suggest that one of the underlying mechanisms of Rin1 function in the amygdala is to antagonize EphA4 signaling by regulating its endocytosis.

Results

Rin1 and EphA4 Are Endogenously Coexpressed in Glutamatergic Neurons. To explore a potential relationship between Rin1 and EphA4, we performed *in situ* hybridization analyses with ephA4 and rin1 riboprobes on adjacent sections of adult mouse forebrain. The expression patterns of rin1 and ephA4 were remarkably similar, including all regions of the hippocampus, cingulate cortex, and thalamus [Fig. 1*A*; supporting information (SI) Fig. S1]. In the amygdala, rin1 expression was widespread and comparably high in most substructures, whereas ephA4 expression was predominant in the lateral nucleus and somewhat weaker in the basolateral nucleus (Fig. 1*A*; Fig. S1). In contrast, coexpression of the related Rin2 and Rin3 transcripts with ephA4 was limited to the hippocampus (Fig. S1). To obtain evidence that Rin1 and EphA4 were expressed in the same cells, we performed laser microdissection of single cells followed by RT-PCR on hippocampus and amygdala from wild-type adult brains (Fig. 1 *B* and *C*). We used CamKII expression as a marker for glutamatergic neurons, GAD67 and GAD65 for inhibitory interneurons, and GFAP for glial cells. For quantification, we considered only samples that were positive for CamKII and negative for GAD67, GAD65, and GFAP, indicating that the

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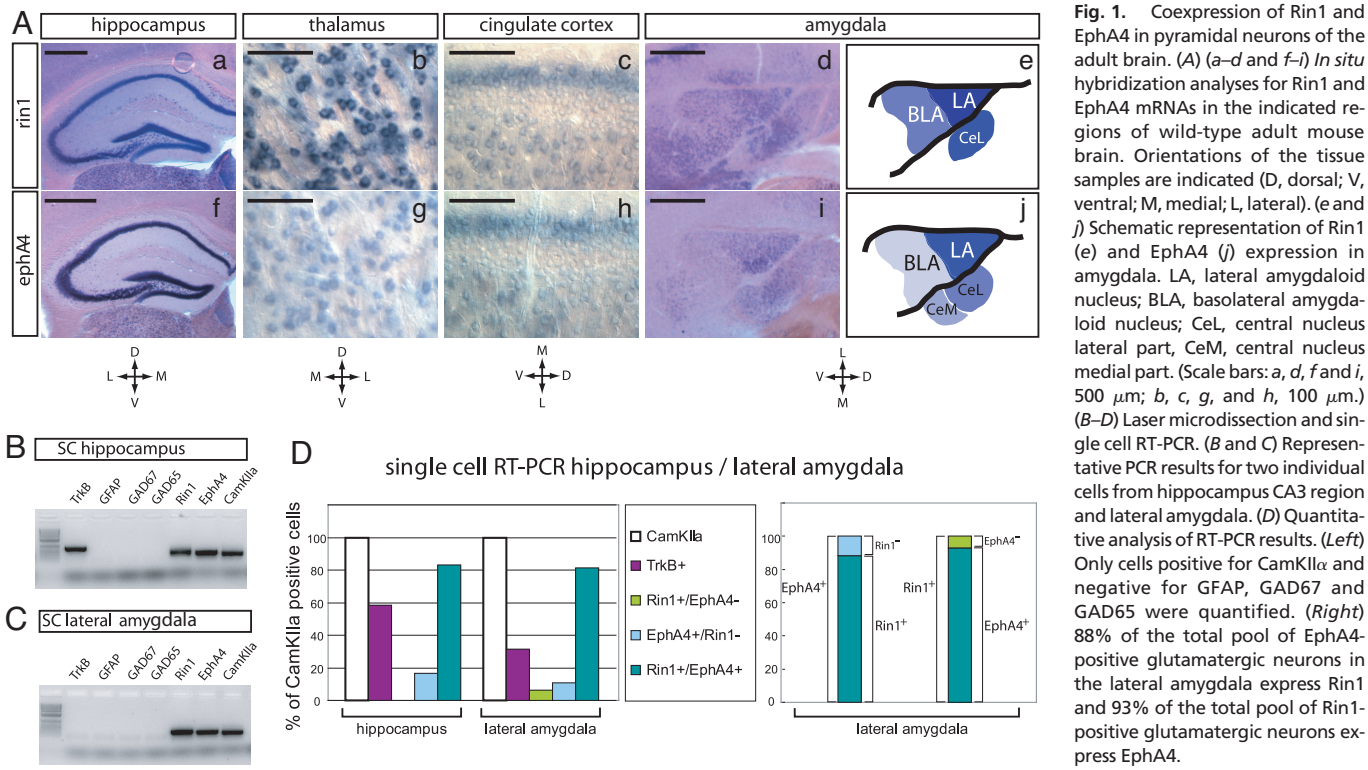

Fig. 1. Coexpression of Rin1 and EphA4 in pyramidal neurons of the adult brain. (*A*) (*a–d* and *f–i*) *In situ* hybridization analyses for Rin1 and EphA4 mRNAs in the indicated regions of wild-type adult mouse brain. Orientations of the tissue samples are indicated (D, dorsal; V, ventral; M, medial; L, lateral). (*e* and *j*) Schematic representation of Rin1 (*e*) and EphA4 (*j*) expression in amygdala. LA, lateral amygdaloid nucleus; BLA, basolateral amygdaloid nucleus; CeL, central nucleus lateral part, CeM, central nucleus medial part. (Scale bars: *a*, *d*, *f* and *i*, 500 μm; *b*, *c*, *g*, and *h*, 100 μm.) (*B–D*) Laser microdissection and single cell RT-PCR. (*B* and *C*) Representative PCR results for two individual cells from hippocampus CA3 region and lateral amygdala. (*D*) Quantitative analysis of RT-PCR results. (*Left*) Only cells positive for CamKIIα and negative for GFAP, GAD67 and GAD65 were quantified. (*Right*) 88% of the total pool of EphA4-positive glutamatergic neurons in the lateral amygdala express Rin1 and 93% of the total pool of Rin1-positive glutamatergic neurons express EphA4.

sample consisted of a single excitatory neuron. Both in hippocampus and lateral amygdala, ≈80% of the glutamatergic neurons coexpressed Rin1 and EphA4 (Fig. 1*D*). Among this population of cells, 88% of the EphA4-positive samples from amygdala were also positive for Rin1, and 93% of the Rin1-positive samples were also positive for EphA4 (Fig. 1*D*). This provided good evidence that Rin1 and EphA4 were endogenously expressed in the same amygdala neurons.

Rin1 Protein Associates with EphA4 in Synaptosome Fractions of Adult Brain. We next studied Rin1 protein expression and found Rin1 levels to be extremely low during development and increasing from 1 week postnatal to adult (Fig. S2). We were unable to obtain more precise localization data for Rin1 protein, because none of the newly generated Rin1 antibodies (Fig. S2) detected endogenous Rin1 in brain tissue (data not shown). EphA4 protein was shown to localize to the presynaptic and postsynaptic sides of excitatory synapses (16). We prepared nonsynaptosome, synaptosome, and postsynaptic density (PSD) fractions from brains of adult mice and found both proteins in synaptosome and PSD fractions (Fig. 2*A*). Importantly, Rin1 coimmunoprecipitated with EphA4 mainly from synaptosome, but not nonsynaptosome fractions, whereas no Rin1 was present in immunoprecipitates from synaptosome when pre-

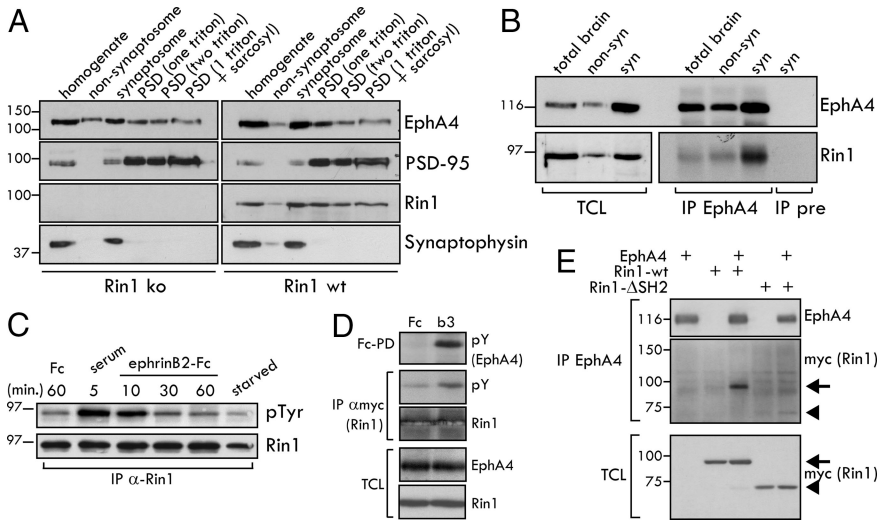

Fig. 2. Rin1 and EphA4 interact in synaptosome fractions and Rin1 is tyrosine phosphorylated in response to Eph signaling. (*A*) Rin1 and EphA4 are present in synaptosome and postsynaptic density (PSD) fractions. The indicated fractions from wild-type and *Rin1*$^{-/-}$ forebrain lysates were immunoblotted with the indicated antibodies. PSD95 is highly enriched in PSD fractions. Synaptophysin, a presynaptic protein, is absent from PSDs (30 μg total protein per lane). (*B*) Rin1 predominantly interacts with EphA4 in synaptosome fractions. EphA4 was immunoprecipitated (IP) from equal amounts (100 μg of protein) of total forebrain lysate, nonsynaptosome (non-syn) and synaptosome (syn) fractions with serum 1383 (rabbit polyclonal anti-mEphA4) followed by blotting with Rin1 (1203) and EphA4 antibodies. Total cell lysates (TCL) of the same fractions (10 μg per lane) show protein expression levels. For Rin1, a different exposure of the same membrane was chosen for the lysates. Preimmune serum for the anti-EphA4 antibody (rabbit 1383) was used in a control IP with 100 μg protein of the same synaptosome fraction used for the anti-EphA4 IP. (*C*) Human SK-N-BE2 cells were stimulated with ephrinB2-Fc, control Fc, or serum for the indicated times or left unstimulated (starved). Endogenous Rin1 was immunoprecipitated with an anti-human Rin1 antibody and blotted with phosphotyrosine (pTyr) or anti-human Rin1 antibodies. (*D*) HeLa cells were transfected with low amounts of mouse EphA4 and mouse Rin1 constructs (2 and 5 μg of DNA per 10-cm dish, respectively), starved for 12 h, and stimulated with ephrinB3-Fc for 10 min. EphA4 and Rin1 tyrosine phosphorylation was increased after ephrinB3-Fc stimulation. (*E*) Rin1 lacking its SH2 domain shows reduced interaction with EphA4. HeLa cells were transiently transfected with EphA4 and myc epitope-tagged full-length WT or Rin1 lacking the SH2 domain (Rin1-wt, Rin1-ΔSH2). EphA4 was immunoprecipitated with anti-EphA4 antibody and coimmunoprecipitated Rin1 was detected by anti-myc antibody. Arrows indicate full-length Rin1; arrowheads, Rin1-ΔSH2.

immune serum for EphA4 was used (Fig. 2*B*). These results indicate that Rin1 interacts with EphA4 predominantly in synaptosome fractions.

Rin1 Is Tyrosine Phosphorylated in Response to Eph Forward Signaling. Previously, Rin1 was shown to be a substrate and regulator of the Abelson (Abl) tyrosine kinase (12) and Abl has been implicated in Eph signaling (17). We therefore asked whether Rin1 could serve as a substrate for activated EphA4. Because embryonic neurons express very little Rin1 and down-regulate EphA4 expression during culture (data not shown), we turned to a neuroblastoma cell line (SKN-BE2). This cell line expresses endogenous Rin1 and EphB2, a related Eph receptor that responds to the same group of ligands as EphA4, namely ephrinB2 and ephrinB3. Stimulation of SKN-BE2 cells with preclustered ephrinB2-Fc induced transient tyrosine phosphorylation of endogenous Rin1 to a level comparable to serum stimulation for 5 min (Fig. 2*C*). To obtain evidence that also EphA4 mediates tyrosine phosphorylation of Rin1, we transfected HeLa cells with epitope-tagged Rin1 together with EphA4 and stimulated the cells with ephrinB3-Fc. We found that under control conditions (Fc-stimulation) Rin1 showed baseline tyrosine phosphorylation which was increased by short-term (10 min) stimulation with ephrinB3 (Fig. 2*D*). These results indicate that Rin1 is transiently tyrosine phosphorylated in response to Eph signaling.

Rin1 Interacts with EphA4 via Its SH2 Domain. Previous work had shown that Rin1 is recruited to the EGFR via its SH2 domain and mediates EGFR trafficking and degradation (11, 18). To disrupt potential SH2-dependent interactions between Rin1 and EphA4, we created a mutant Rin1 protein lacking the first 169 aa including the SH2 domain (Rin1Δ SH2) and coexpressed it with EphA4 in HeLa cells. The Rin1Δ SH2 mutant protein contained the same epitope tag as wild-type Rin1 and was expressed at comparable levels. Whereas wild-type Rin1 was readily detected in EphA4 immunoprecipitates, the amount of coimmunoprecipitated Rin1Δ SH2 mutant protein was near the detection limit (Fig. 2*E*). These results suggest that Rin1 interacts with EphA4 primarily via its N-terminal SH2 domain, however, we cannot exclude that this interaction occurs indirectly (via an adapter molecule).

Internalized EphA4 Traffics Through Rab5 Compartments. Rin1 is a member of a larger protein family of VPS9 domain containing GEFs which show exchange activity for the small GTPase Rab5, involved in the early steps of endocytosis (13). If Rin1 were to regulate endocytosis of EphA4, one would expect to find internalised EphA4 in Rab5 endosomes. To facilitate the detection of such structures, we transfected primary neurons with a constitutively active Rab5-GFP fusion protein (GFP-Rab5Q79L) that allows visualization of characteristically enlarged early endosomes (19). EphA4 was detected directly with specific antibodies or after stimulation with ephrinB3-Fc with antibodies against Fc. Virtually all clusters labeled for ephrinB3-Fc were also positive for EphA4 (data not shown, see also (20). To visualize internalized EphA4, we used a staining procedure based on the distinctive recognition of surface (prepermeabilization) and total (postpermeabilization) EphA4 clusters. Cells were fixed in the absence of detergents and immunolabeled for ephrinB3-Fc on the cell surface (Fig. S3 *A*, *E*, and *I*). Cells were then permeabilized and stained for total ephrinB3-Fc using a secondary antibody coupled to a different fluorophore (see *SI Methods* and Fig. S3 *B*, *F*, and *J*). EphA4 clusters that were exclusively labeled after permeabilization represent the internalized pool of ephrinB3-EphA4 complexes (see Fig. S3 *E* and *I* compared with *F* and *J*, respectively). After stimulation with ephrinB3-Fc, endogenous EphA4 in primary hippocampal neurons (4–5 DIV) localized to Rab5-positive endosomes visualized by GFP-Rab5Q79L (Fig. S3 *F–H* and *J–L*). After stimulation with control Fc, no Eph-ephrin complexes were found in Rab5 endosomes (data not shown). The sizes of EphA4 receptor clusters were not significantly altered by the expression of GFP-Rab5Q79L compared with GFP only (data not shown). We conclude that upon ligand-induced endocytosis, EphA4 traffics through Rab5 endosomes.

Rin1 Enhances Internalization of EphA4. Next, we asked whether Rin1 regulates the internalization of EphA4 by using surface biotinylation. HeLa cells stably expressing EphA4 were transfected with either eGFP or full-length Rin1 and starved for 24 h. Surface proteins were labeled with biotin and cells stimulated with preclustered ephrinB3-Fc to induce EphA4 clustering and internalization. After 20 or 60 min incubation, biotin was stripped from the surface, so that only internalized proteins retained the biotinylation. To visualize internalized EphA4, cell lysates were subjected to avidin pull-downs followed by immunoblotting for EphA4. In the absence of overexpressed Rin1, ephrinB3-Fc stimulation led to a 2-fold increase in biotinylated, internalized EphA4 (Fig. 3 *A* and *B*). Expression of Rin1 under control condition (Fc) did not significantly increase EphA4 internalization. In contrast, ephrinB3 treatment of cells overexpressing Rin1-wt increased EphA4 internalization 4.4-fold compared with Fc-treated control cells (Fig. 3 *A* and *B*). We found no significant effect of overexpressed wt Rin1 on transferrin receptor internalization (Fig. S4 *F* and *G*).

We also used the pre-/postpermeabilization paradigm to quantify the effect of Rin1 overexpression on the numbers of endocytosed EphA4 clusters. HeLa-EphA4 cells were transfected with either eGFP alone (control) or full-length Rin1 and eGFP, starved for 24 h, and then stimulated with preclustered control Fc or ephrinB3-Fc to induce EphA4 internalization (Fig. 3*C*).For quantification, surface clusters were first identified and marked in the monochrome images of the surface staining (no detergent, anti-Fc*Cy5). Next, a mask of these marked clusters was imported onto the total staining (permeabilized, anti-Fc*TR). Only clusters identified in the monochrome images of the total staining that were not marked in the first step were counted as internalized. Cells transfected with eGFP and stimulated with ephrinB3-Fc showed an average internalisation rate of 20% (after 30 min of stimulation), whereas cells transfected with Rin1 showed a significantly enhanced internalisation rate of 28% (Fig. 3*D*). If surface epitopes were not fully saturated in the first step (prepermeabilization staining), they appear yellow or orange in the overlay images.

Catalytically Inactive Rin1 Interferes with Ligand-Induced EphA4 Internalization. To investigate whether Rin1 is required for EphA4 internalization, we expressed catalytically inactive Rin1 proteins in cells to dominantly interfere with the function of endogenous Rin1. We designed two putative dominant negative Rin1 constructs, in which either the entire VPS9-like GEF domain or only the first 48 aa of the GEF domain were deleted, termed Rin1-Δ GEF and Rin1-splice, respectively (Fig. S4*A*). Rin1-splice corresponds to a naturally occurring splice variant of Rin1 (13). Our anti-mouse Rin1 antibodies failed to visualize the endogenous human Rin1 in HeLa cells (Fig. S4*B*). Both Rin1 mutants retained their ability to bind EphA4 via their intact SH2 domains (Fig. S4 *B* and *C*). Surface biotinylation revealed a 2-fold increase in EphA4 internalization after 60 min ephrinB3 stimulation in eGFP transfected samples which was enhanced in the presence of full-length Rin1 (Fig. S4 *D* and *E*). EphrinB3-induced internalization of EphA4 in the presence of the catalytically inactive Rin1 mutants was lower than in GFP-transfected cells (Fig. S4 *D* and *E*). These results indicate that the specific increase observed in wild-type Rin1-transfected samples was due to the catalytic activity of Rin1. They further suggest that catalytically inactive Rin1 dominantly interferes with endogenous Rin1 in HeLa cells to block the ephrinB3-Fc-induced EphA4 internalization, but not the residual, ligand-independent form of internalization.

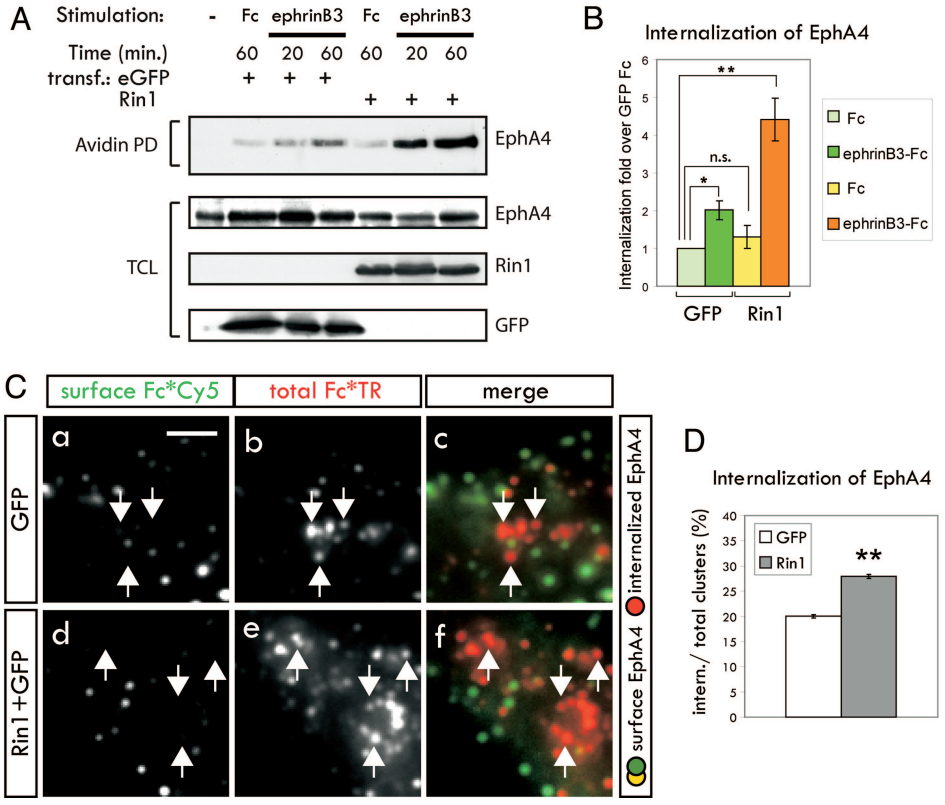

Fig. 3. Rin1 enhances endocytosis of EphA4 in transfected cells. (*A*) HeLa cells stably expressing EphA4 were transfected with GFP (control) or wild-type Rin1 and subjected to surface biotinylation. Biotinylated surface molecules were internalized by stimulation with either preclustered Fc (control) or ephrinB3-Fc. After the indicated time points, biotin was stripped from remaining surface molecules. The first lane (-) is an unstimulated, biotinylated and stripped control that shows no EphA4 after Avidin pulldown (PD). Internalized proteins remained biotinylated and were pulled down with Avidin beads followed by immunoblotting with EphA4 antibody. Total cell lysates were immunoblotted with EphA4, Rin1, and GFP antibodies. (*B*) Quantification of surface biotinylation experiments. Five independent experiments were used to determine the intensity of the internalized EphA4 signal (biotinylated EphA4 divided by total EphA4) and the mean values were normalized to the amount of EphA4 in GFP-transfected, Fc-treated control cells. EphrinB3 treatment for 60 min increased EphA4 internalization 2-fold in GFP-transfected samples ($P < 0.05$, *t* test, two-tailed, equal variance). Expression of Rin1-wt alone did not significantly increase internalization of EphA4 in Fc-controls ($P = 0.35$). EphrinB3 treatment in cells overexpressing Rin1-wt increased EphA4 internalization 4.4-fold ($P < 0.001$). (*C*) Pre- and postpermeabilization staining procedure on stable HeLa-EphA4 cells, either transfected with GFP only (*a*–*c*) or with Rin1 and GFP (*d*–*f*), both stimulated with ephrinB3-Fc for 30 min. Surface ephrinB3-EphA4 clusters (*a* and *d*) appear green in the merged images c and f; Total Fc staining (*b* and *e*) artificially colored red. Internalized ephrinB3-EphA4 clusters appear in red in the merged images (*c* and *f*). (Scale bars: *a* and *b*, 2 μm.) (*D*) For quantification of the number of internalized ephrinB3-EphA4 clusters, surface clusters were first identified and marked in the monochrome images of the anti-Fc-Cy5 samples (surface staining). Only the clusters which were identified in the total staining that were not marked in the surface monochrome images were counted as internalized. Error bars represent SEM. $P < 0.001$, Student's *t* test, two-tailed, equal variance, n = three independent experiments, 8–10 cells quantified per condition and experiment).

Lack of Rin1 Reduces EphA4 Internalization in Primary Neurons. We next asked whether EphA4 internalization was reduced in cells derived from *Rin1*$^{-/-}$ mice. The only described *in vivo* phenotypes for *Rin1*$^{-/-}$ mice had been defects in amygdala physiology (14). After having confirmed that EphA4 and Rin1 protein were expressed in P15 amygdala (Fig. 4*A*), we investigated ephrinB3-induced EphA4 internalization in amygdala neurons. To assay the contribution of Rin1 to EphA4 endocytosis in the amygdala, small explants from either wild-type or *Rin1*$^{-/-}$ lateral amygdala were cultured for 3–4 DIV. GFAP-positive astrocytes migrated radially from the explant and provided a feeder layer for Tuj1-positive neurons (Fig. 4*B*). The cultures were stimulated with ephrinB3-Fc and then stained for surface and total ephrinB3 clusters using the pre-/postpermeabilization procedure. Most of the ephrinB3 clusters were positive for EphA4 and stimulation with control Fc did not induce any clusters (data not shown). EphrinB3-Fc induced endocytosis of ligand-receptor complexes in both wild-type and *Rin1*$^{-/-}$ neurons; however, the internalization rate in *Rin1*$^{-/-}$ (15% ± 0.39 SEM) was significantly reduced compared with wt explants (21% ± 0.87 SEM; Fig. 4*D*). These results indicate that ligand-induced internalization of neuronal EphA4 receptors is positively modulated by Rin1.

EphA4 Is Required for Amygdala LTP in a Manner Opposite to Rin1. We next asked whether the increased LTP in *Rin1*$^{-/-}$ mice could in part be attributed to impaired Eph receptor internalization. We hypothesized that the role of Rin1 in the amygdala may include targeting Eph receptors for degradation. If this were true, then EphA4 should play an important role in amygdala physiology. The prediction would be that the amygdala phenotype of *EphA4*$^{-/-}$ mice would be opposite of that of *Rin1*$^{-/-}$ mice (reduced LTP). In previous work we showed that lack of EphA4 did not change basic synaptic neurotransmission in the hippocampus (5). To assay lateral amygdala LTP in acute slices (Fig. 5*A*), we recorded field potentials (FPs), which are composed of excitatory postsynaptic potentials and neuronal spikes. Stimulus intensities, which were adjusted to produce half-maximal FP amplitude, did not differ significantly between wild-type and *EphA4*$^{-/-}$ slices (Fig. 5*B*). FP control amplitudes also did not differ between wild-type and *EphA4*$^{-/-}$ slices (Fig. 5*B*). Using high frequency stimulation (HFS) of presynaptic fibers of the external capsule, we observed LTP in wild-type, but not *EphA4*$^{-/-}$ brains (Fig. 5*A*).

Next, we used the tyrosine kinase inhibitor Dasatinib (21) to investigate the contribution of Eph signaling to the elevated LTP in *Rin1*$^{-/-}$ mice. This inhibitor will block Eph activity (Fig. 5*D*) and (downstream) Src/Abl signaling (22), but should not affect Ras-Mapk signaling or general synaptic receptor trafficking. With HFS, we elicited basolateral amygdala LTP and confirmed that this LTP was significantly higher than the LTP observed in control littermate slices (Fig. 5*C*). The enhancement of LTP was less than originally reported (14) most likely due to the use of a C57/Bl6 rather than 129xC57/Bl6 background and a modified stimulation protocol. Dasatinib markedly reduced the LTP in *Rin1*$^{-/-}$ slices, suggesting that this reduction might represent the contribution of (the now inhibited) EphA4 signaling (Fig. 5*C*). These results indicate an important role for EphA4 in amygdala LTP and raise the possibility that an underlying mechanism of Rin1 function in amygdala LTP is to antagonize EphA4 signaling.

Discussion

Here, we have shown that Rin1 coexpresses with EphA4 in excitatory neurons of the postnatal forebrain and that Rin1 interacts

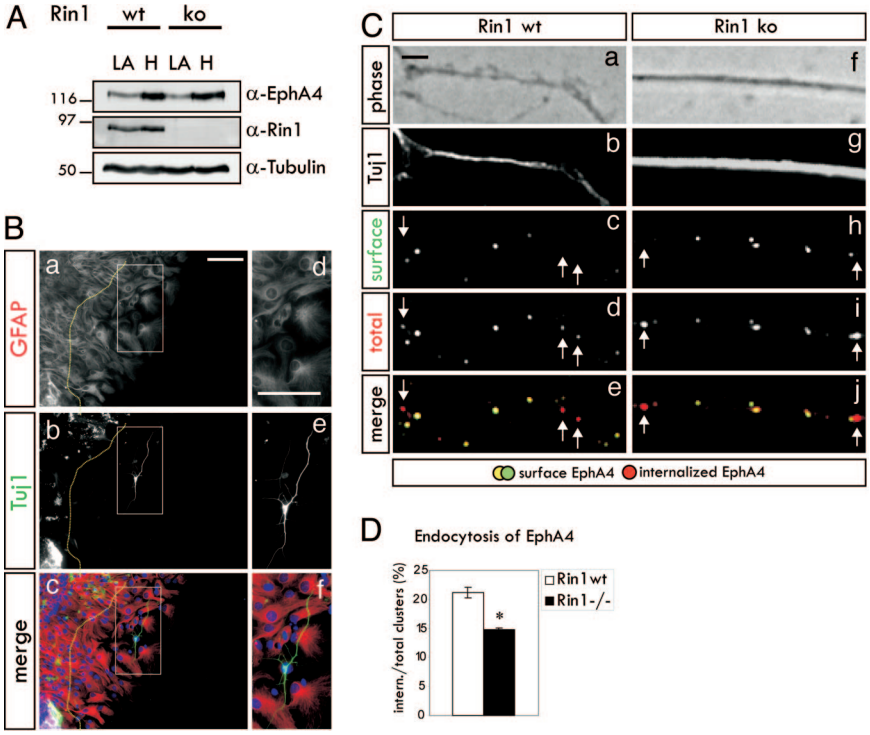

Fig. 4. Reduced endocytosis of EphA4 in explants from lateral amygdala of *Rin1*$^{-/-}$ mice. (*A*) Levels of Rin1 and EphA4 proteins in lateral amygdala (LA) and hippocampus (*H*) at P15. Protein lysates (40 μg/lane) of wild-type and *Rin1*$^{-/-}$ (ko) littermates were immunoblotted for EphA4, Rin1 and Tubulin, respectively. (*B*) Tissue explant from P15 wild-type amygdala cultured for 5 DIV and stained for the glia marker GFAP and the neuronal marker Tuj1. The merged images in c and f also contain HOECHST as a nuclear stain. (Scale bars: *a* and *d*, 100 μm.) Yellow dotted lines indicate explant boundaries. (*C*) EphA4 internalization in amygdala neurons. Phase-contrast (*a* and *f*) and Tuj1-stained images (*b* and *g*) of neurites from LA neurons derived from wild-type and *Rin1*$^{-/-}$ mice. Examples of surface (*c* and *h*, artificially colored green/yellow in the merge) and total (*d* and *i*, artificially colored red in the merge) Fc staining after 45 min of ephrinB3-Fc stimulation. Arrows indicate internalized Eph-ephrin complexes. (Scale bar: *a*, 2 μm.) (*D*) Quantification of internalization ratios in wt and *Rin1*$^{-/-}$ LA explants, stimulated with ephrinB3-Fc. Only clusters identified in the total Fc staining monochrome images that were absent from surface monochrome images were quantified as internalized ($P < 0.01$, *t* test, two-tailed, equal variance, $n = 3$ animals per genotype, 5–15 cells or stretches of neurites per animal). We observed no statistically significant differences in fluorescence intensity between internalized clusters in wt and *Rin1*$^{-/-}$ neurons (data not shown).

with EphA4 in synaptosomal fractions. This interaction is mediated by Rin1's SH2 domain and leads to Rin1 tyrosine phosphorylation in transfected cells. In cultured neurons, ephrinB3 stimulation causes internalized EphA4 to sort to Rab5-positive endosomes. Lack of Rin1 reduces ephrin-induced internalization of EphA4 in cultured amygdala neurons. Conversely, ectopic expression of Rin1 enhances EphA4 internalization in HeLa cells. These results demonstrate that Rin1 is required and sufficient to mediate EphA4 internalization. Our results further suggest that Rin1's interaction with EphA4 is physiologically relevant for neuronal plasticity in the amygdala. Rin1 is one of few genes the genetic ablation of which specifically enhanced amygdala LTP (14, 23), suggesting that one of Rin1's functions may be to internalize and suppress the action of a required synaptic receptor. Here, we have shown that EphA4 could be such a candidate receptor required for amygdala LTP.

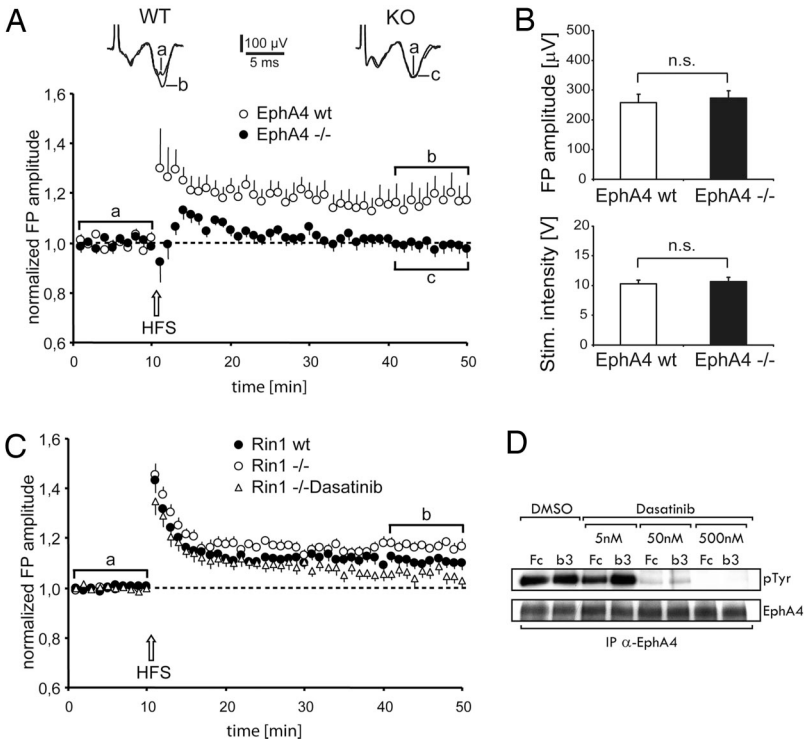

Fig. 5. *EphA4*$^{-/-}$ mice are defective in amygdala LTP. (*A*) (*Upper*) Representative recording traces obtained within time periods a–c indicated in the graph below. (*Lower*) *EphA4*$^{-/-}$ mice (99% of baseline, black circles, $n = 21$ slices from nine animals) failed to display LTP after HFS; wt littermates (117%, 30–40 min after HFS, open circles, $n = 17$ slices from 7 animals) are shown as control. Paired Student's *t* test: *b* vs. *a* ($P = 0.038$); *c* vs. *a* ($P = 0.73$); unpaired Student's *t* test: *b* vs. *c* ($P = 0.018$). Data are presented as mean ± SEM. The reason for the transient decrease in FP amplitude after HFS in *EphA4*$^{-/-}$ mice is currently unknown. (*B*) (*Upper*) FP amplitudes of wt (258 μV ± 28 SEM) and *EphA4*−/− (273 μV ± 25 SEM) littermate samples. (*Lower*) Stimulation intensities adjusted to produce half-maximal FP amplitudes in wt (10.4V ± 0.6 SEM) and *EphA4*$^{-/-}$ littermate samples (10.9V ± 0.8 SEM). There are no significant differences in these parameters between the 2 genotypes (unpaired Student's *t* test). (*C*) LTP in *Rin1*$^{-/-}$ mice (117%, open circles, $n = 16$ slices from seven animals, *b* vs. *a* ($P < 0.001$), paired Student's *t* test) is elevated compared with wt littermates (111%, black circles, $n = 17$ slices from five animals, *b* vs. *a* ($P < 0.001$), paired Student's *t* test) and is partially suppressed by preincubation of the slices with Dasatinib (106%, open triangles, $n = 16$ slices from six animals, *b* vs. *a* ($P = 0.06$), paired Student's *t* test). Unpaired Student's *t* test at b: *Rin1*$^{-/-}$ vs. *Rin1*$^{-/-}$ Dasatinib, $P = 0.003$; Rin1 wt vs. *Rin1*$^{-/-}$, $P = 0.046$; Rin1 wt vs. *Rin1*$^{-/-}$ Dasatinib, $P = 0.178$. (*D*) EphA4 transiently expressed in HeLa cells shows increased phosphorylation upon stimulation with ephrinB3-Fc. EphA4 phosphorylation is inhibited by Dasatinib in a dose-dependent manner.

Ephrin-Eph endocytosis plays an important role in repulsive signaling during axon guidance, in particular for cell detachment after growth cone collapse (reviewed in ref. 1). Endocytosis of Eph receptors may be used by cells/neurons in different contexts to achieve different cellular responses. Vav proteins facilitate Eph endocytosis to potentiate signaling, thus positively regulating Eph-mediated repulsive guidance. The circumstances may be considerably different at mature synapses responding to excitatory stimulation. Rather than mediating cell detachment, ephrin-Eph endocytosis may modulate signaling events that underlie LTP or LTD. In the absence of Rin1, amygdala LTP is increased correlating with reduced EphA4 internalization in amygdala neurons in culture. We believe these changes to happen in a localized, activity-dependent manner, because we could not observe gross changes in levels of EphA4. Genetic ablation of EphA4 produced the opposite phenotype of Rin1 ablation, namely decreased amygdala LTP suggesting that Rin1 is a negative rather than a positive regulator of Eph signaling.

The induction and expression of LTP at amygdala synapses involves both presynaptic and postsynaptic mechanisms (15). Lateral amygdala (LA) neurons receive cortical and thalamic afferents and in turn project to other subnuclei in the amygdala. It is currently not known whether EphA4 is solely required in LA/BLA neurons or also in cortical and thalamic neurons. Moreover, it is not known whether EphA4 kinase signaling is required, or whether EphA4 acts in a signaling-independent manner as shown in the hippocampus (5). Eph forward signaling during axon repulsion primarily relies on the regulation of Rho GTPases and changes in the actin cytoskeleton (6, 24). Rho and Rho-associated kinase, ROCK, are required for fear learning (25) and it would be interesting to explore the possibility that Eph signaling via the Rho/ROCK pathway mediates amygdala LTP. We have not subjected *EphA4*$^{-/-}$ mice to fear conditioning experiments, because any defects in avoidance learning may be confounded by their hindlimb locomotion problems that are caused by defects in spinal cord and limb innervation (26).

Our work also provides the first insights into the molecular mechanism that underlies the increase in amygdala LTP in *Rin1*$^{-/-}$ mice. Previously, it was suggested that Rin1 may compete with Raf proteins for binding to activated Ras (14) and thereby inhibit Ras/Mapk signaling which is known to underlie amygdala LTP and fear conditioning (27, 28). However, there is at present no evidence for this type of effector competition in *Rin1*$^{-/-}$ neurons (14). Alternatively, Rin1 could mediate the internalization and degradation of RTKs by activating Rab5-dependent endocytosis (11, 13, 18). Here, we have shown that Dasatinib suppresses the elevated LTP in the amygdala of *Rin1*$^{-/-}$ mice. In cell based assays, Dasatinib inhibits several tyrosine kinases that are expressed in neurons including Src, Abl, and Ephs, but not other potential mediators of LTP such as TrkB (21). These findings are consistent with the involvement of Eph in amygdala LTP. Src kinases are essential mediators of Eph signaling (22) and Abl kinases have also been placed downstream of Eph receptors (29). This severely complicates further dissection of these pathways until more specific Eph inhibitors become available. Altogether, however, our present work suggests that Rin1/Rab5-mediated endocytosis of EphA4 contributes significantly to the regulation of amygdala LTP.

Materials and Methods

Antibodies. Polyclonal rabbit (1203 and 1204) and goat (113) anti-murine Rin1 antisera were raised against the full-length, hexahistidine-tagged protein; polyclonal rabbit anti-human Rin1 (BD PharMingen); polyclonal rabbit anti EphA4 S20 (Santa Cruz Biotechnology); mouse monoclonal EphA4 clone 35 (BD Transduction Laboratories); polyclonal rabbit anti EphA4 was raised against an intracellular peptide as described in ref. 30.

Amygdala Explant Culture. The amygdala at P12–P14 was identified on coronal sections using a stereomicroscope. The area between the two fiber tracts (external capsule) was dissected out with a microblade, cut into smaller tissue pieces and placed on polyD-lysine and laminin coated coverslips in MEM (GIBCO), 25% horse serum (GIBCO), 25% HBSS (GIBCO), Glutamine 2%, 30 mM Glucose for 3–4 days in 37°C/5% CO_2. Explants were stimulated with 5 μg/ml preclustered ephrinB3-Fc.

Additional materials and methods can be found in *SI Text*.

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