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

In 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 still not well characterized. During axon guidance, the Rho family guanine nucleotide exchange factor (GEF) Vav2 promotes endocytosis of the Eph-ephrin complex and 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. 1A: 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. 1A). 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. 1B 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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sample consisted of a single excitatory neuron. Both in hippocampus and lateral amygdala, ~80% of the glutamatergic neurons coexpressed Rin1 and EphA4 (Fig. 1D). 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. 1D). 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. 2A). Importantly, Rin1 coimmunoprecipitated with EphA4 mainly from synaptosome, but not nonsynaptosome fractions, whereas no Rin1 was present in immunoprecipitates from nonsynaptosome when pre-
immune serum for EphA4 was used (Fig. 2B). These results indicate that Rin1 interacts with EphA4 predominantly in synapticosome fractions.

Rin1 Is Tyrosine Phosphorylated in Response to Eph Forward Signaling.

Previously, Rin1 was shown to be a substrate and regulator of the Abelson (Ab1) tyrosine kinase (12) and Ab1 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. 2C). 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. 2D). 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. 2E). 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 internalized 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 J). 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. 3C). For quantification, surface clusters were first identified and marked in the monochrome images of the surface staining (no detergent, anti-FcεCy5). Next, a mask of these marked clusters was imported onto the total staining (permeabilized, anti-FcεCy5). 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. 3D). 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 Rin1Δ GEF and Rin1-spllice, respectively (Fig. S4A). Rin1-spllice 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. S4B). 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). EphinB3-induced internalization of EphA4 in the presence of the catalytically inactive Rin1 mutants was lower than in eGFp-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 ephinB3-Fc-induced EphA4 internalization, but not the residual, ligand-independent form of internalization.
Lack of Rin1 Reduces EphA4 Internalization in Primary Neurons. We next asked whether EphA4 internalization was reduced in cells derived from Rin1−/− mice that had defects in amygdala physiology (14). After having confirmed that EphA4 and Rin1 protein were expressed in P15 amygdala (Fig. 4A), 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 prepared, and the protein expression and localization of EphA4 were evaluated in control and experimental conditions. Importantly, we found that EphA4 endocytosis in Rin1−/− mice was significantly reduced compared with wild-type mice, indicating that Rin1 plays a critical role in EphA4 internalization in amygdala neurons. These results suggest that Rin1 may be an essential regulator of EphA4 signaling in the amygdala, and they provide a potential mechanism for the amygdala phenotype of Rin1−/− mice.

EphA4 Is Required for Amygdala LTP in a Manner Opposite to Rin1. We next asked whether the increased LTP in Rin1−/− mice could 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. 5A), 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−/− mice (Fig. 5B). FP control amplitudes also did not differ between wild-type and EphA4−/− mice (Fig. 5B). Using high frequency stimulation (HFS) of presynaptic fibers of the external capsule, we observed LTP in wild-type, but not EphA4−/− brains (Fig. 5C).

Next, we used the tyrosine kinase inhibitor Dasatinib (21) to investigate the contribution of EphA4 in LTP in Rin1−/− mice. This inhibitor will block Eph activity (Fig. 5D) 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. 5C). The enhancement of LTP was less than originally reported (14) most likely due to the use of a C57/Bl6 rather than 129s/C57/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. 5C). 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...
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. 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).

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) Stimulus intensity 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 cell contact-dependent repulsive guidance. Vps 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 Rin1 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/Rob5-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 (Cell Signaling Technology); polyclonal rabbit anti-EphA4 (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 poly-D-lysine and laminin coated coverslips in MEM (GIBCO), 25% horse serum (GIBCO), 25% HBSS (GIBCO), Glutamine 2%, 30 mGluC for 3–4 days in 37°C/5% CO2. Explants were stimulated with 5 μg/ml preclustered ephrinB3-Fc. Additional materials and methods can be found in SI Text.

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Supporting Information

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SI Materials and Methods

Additional Antibodies. Class III β-tubulin (clone TuJ1); mouse monoclonal anti α-tubulin (Sigma); polyclonal rabbit anti GFAP (DAKO); goat anti-human IgG Fc fragment for clustering of Fc proteins (R&D, used 1:10); Cy5-, and TexasRed-conjugated anti-Fc antibodies for immunofluorescence stainings (Jackson ImmunoResearch); mouse monoclonal anti Synaptophasin (Chemicon); mouse monoclonal anti-PSD-95 clone 7E3-1B8 (Sigma); anti GFP antibody clone JL-8 (Clontech); mouse monoclonal anti-myc-tag antibody clone 9E10 (ascites, Sigma).

Surface Biotinylations. Surface biotinylations were essentially carried out as described in ref. 31. Thirty-six hours after transfection, cells were labeled with EZlink-NHS-SS-Biotin (Pierce) for 2 min at 37°C/5% CO2, rinsed and stimulated with Fc or ephrinB3-Fc. Internalization was halted by rapid cooling on ice and remaining biotin was stripped from cell surfaces by incubation with reduced L-Glutathione (Sigma) 2.3g/50 ml 150 mM NaCl, pH8.75, and neutralized by subsequent incubation with Iodoacetamide (0.46g/50 ml PBS-GaM, Sigma). Cells were lysed and avidin pulldowns were carried out with Neutravidin Sepharose (Pierce).

Primary Neuron Culture. Hippocampal cultures were prepared from E18.5 rat embryos and cultured in Neural basal media plus B27 (GIBCO) for 3 days before and 2 days after transfection. Cultures consisted of a mixture of neurons and glia cells and were transfected with pCR2.1TOPO (Invitrogen), linearized with EcoNI at the 5' end. This construct was amplified in a second PCR step using NEBiolabs TAQ polymerase and nested primers for each gene: Rin1F2 5' -taagtggtctgctgctccag-3', Rin1R1 5'-aggtggattgtgcgctgca-3'; EphA4F1 5'-gcacagcgtgcctgagct-3', EphA4R1 5'-ctctgattcgctgtatctgactc-3'; CamKII F1 5'-aagcctcaaaagactg-3', CamKII R1 5'-atctgcagcgtgctgca-3'. Procedure and primer sequences for TrkB, GFAP, GAD65, and CamKII were used instead of random primers: Rin1F1 5'-ggtcaggaaggctgcaacag-3', Rin1R1 5'-gtctctcagcagctgca-3'; EphA4F1 5'-gcagcgtgcctgagctgca-3', EphA4R1 5'-ctctgattcgctgtatctgactc-3'; CamKII F1 5'-aagcctcaaaagactg-3', CamKII R1 5'-atctgcagcgtgctgca-3'. Procedure and primer sequences for TrkB, GFAP, GAD65, and GAD67 have been previously published (5). Specific products were further amplified in a second PCR step using NEBiolabs TAQ polymerase and nested primers for each gene: Rin1F2 5'-acccgctgtgctgctgcag-3', Rin1R2 5'-ttctctcagcagctgca-3'; EphA4F2 5'-aggtggattgtgcgctgca-3'; EphA4R2 5'-ctctgattcgctgtatctgactc-3'; CamKII F2 5'-atctgcagcgtgctgca-3', CamKII R2 5'-atctgcagcgtgctgca-3'.

Surface biotinylations were essentially carried out as described in ref. 31. Thirty-six hours after transfection, cells were labeled with EZlink-NHS-SS-Biotin (Pierce) for 2 min at 37°C/5% CO2, rinsed and stimulated with Fc or ephrinB3-Fc. Internalization was halted by rapid cooling on ice and remaining biotin was stripped from cell surfaces by incubation with reduced L-Glutathione (Sigma) 2.3g/50 ml 150 mM NaCl, pH8.75, and neutralized by subsequent incubation with Iodoacetamide (0.46g/50 ml PBS-GaM, Sigma). Cells were lysed and avidin pulldowns were carried out with Neutravidin Sepharose (Pierce).

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Only cells were included in the quantification that were positive for CamKIIα (excitatory, glutamatergic neurons) and negative for GFAP (astrocyte marker), GAD67, and GAD65 (inhibitory, GABAergic neurons).

**Electrophysiology: Slice Preparation.** Adult (2–6-month-old) EphA4−/− and Rin−/− mice and wild-type littermate controls of mixed SV129/C57Bl6 (EphA4) and C57/Bl6 (Rin1) background were used for the investigation. The brains were removed rapidly and placed in ice-cold, artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO3, 2 CaCl2, 1 MgCl2, 25 D-glucose, 1.25 NaH2PO4 (pH 7.4), bubbled with carbogen (95% O2/5% CO2). Coronal slices of the amygdala (400-μm thick) were prepared using a vibroslicer. After incubation in a holding chamber with ACSF (22–25°C) for at least 60 min, the slices were placed in the recording chamber of the setup and superfused with ACSF at a flow rate of 3 mL/min.

**Electrophysiological Recordings.** Square pulse stimuli (0.066 Hz, 6–15 V, 200 μs) were delivered via a bipolar tungsten electrode (insulated to the tip, 50-μm diameter) and all recordings were performed at room temperature (22–25°C). Stimulation electrodes were positioned on the border between the LA and the external capsule (EphA4 slices) or within the LA (Rin1 slices). Recordings were performed in the LA (EphA4 slices) or the basolateral nucleus (Rin1 slices). All FPs were recorded using glass microelectrodes (1–2 MΩ open tip resistance) filled with ACSF. The stimulus intensities were adjusted in a manner to produce a FP of ~50% of the maximum amplitude. The voltage differences between the sharp negative onset and the negative peak (a), and between the negative peak and succeeding positive peak (b), were measured, and the amplitudes of the FPs were calculated as (a+b)/2. Slices were preincubated for 2 h with Dasatinib at a final concentration of 500 nM final DMSO concentration of 0.005%.

**Transferrin Uptake Assay.** Uptake assays were performed as described by Irie et al. (4) with the following changes. A 5-μg quantity of biotinylated transferrin was used for stimulations. Experiments were performed with the same cells used for surface biotinylation (stable HeLa-EphA4) transfected either with GFP as a control or Rin1. Three independent experiments were quantified.

Fig. S1. *In situ* hybridization with antisense probes against EphA4, Rin1, Rin2, and Rin3. (A–D) One-half of a coronal section probed with the indicated antisense probes. (E–H) Higher magnifications images of the hippocampus. (I–L) Higher magnifications images of the amygdala. Scale bars: A–D 1 mm, E–H 0.5 mm. hpc, hippocampus.
Fig. S2. Generation and characterization of anti-mouse-Rin1–specific antibodies. (A) Western blot showing total forebrain lysates (50 μg/lane) from adult wild-type, Rin1−/− and Rin1−− littermates probed for Rin1 expression using 2 different rabbit antisera (1203 and 1204) and 1 goat antiserum (113) raised against full-length His-6-tagged mouse Rin1 protein. Serum 1203 was used in this work for immunoprecipitation and Western blotting because it showed little background signal close to full-length Rin1 protein (arrowhead at 90 kDa). In addition to the full-length protein, antisera 1204 and 113 specifically detected a smaller isoform of Rin1 (arrow at ~70 kDa), possibly representing the naturally occurring splice variant described by Han et al. (2), although the calculated size of the splice variant is expected to be ~80 kDa. Serum 1204 was used at a dilution of 1:100 to detect overexpressed full-length mouse Rin1 in HeLa cells. Rin1 antibody was detected with an anti-rabbit secondary antibody conjugated to Cy2. (c) An overlay of a and b shows that native, untransfected HeLa do not show any signal with serum 1203. In primary neurons in culture or tissue sections we were not able to obtain specific staining for endogenous mouse Rin1 (data not shown). (C) Protein lysates (50 μg/lane) of wild-type (wt) forebrain samples (except E13.5, whole head) of the indicated embryonic (E) and postnatal stages (P) were immunoblotted with antibodies against EphA4, Rin1 and Tubulin.
Fig. S3. Internalized EphA4 traffics through Rab5 compartments. Primary cultures of hippocampal neurons were transfected with constitutively active GFP-Rab5Q79L at 2 DIV and cultured for an additional 48 h before stimulation with preclustered ephrinB3-Fc for 30 min. (A, E, and I) Cells were fixed in the absence of detergent and stained for surface receptor-ligand-complexes with an anti-Fc antibody conjugated to Cy5 (surface Fc*Cy5, artificially colored blue in the merge). (B, F, and J) Cells were then permeabilized and all receptor-ligand-complexes were stained with an anti-Fc antibody conjugated to TexasRed (total Fc*TR, artificially colored red in the merge). (C, G, and K) Fluorescence of GFP-Rab5Q79L-labeled endosomes (artificially colored green in the merge). (D, H, and L) Merge of monochrome images. (A–D) A GFP-Rab5Q79L-transfected neuron. Boxed areas of soma and neurite are enlarged in (E–H) and (I–L), respectively. In the merged images, surface receptor clusters appear in purple, internalized clusters in red, and complexes that have reached Rab5-positive endosomes in yellow or orange, indicated by arrowheads. Scale bars in A, E, and I, 10 μm.
Fig. S4. Catalytically inactive Rin1 interferes with ligand-induced EphA4 internalization. (A) Schematic representation of wt Rin1, Rin1-ΔGEF lacking the entire GEF domain, and Rin1-splice lacking the first 48 aa of the GEF domain. (B and C) Rin1 GEF domain mutants are still able to bind EphA4. HeLa cells were transiently transfected with EphA4, Rin1-wt and Rin1-ΔGEF (B) or Rin1-wt and Rin1-splice (C). Immunoprecipitations were performed for EphA4 and co-IP of Rin1 constructs was assessed by immunoblotting with an anti-myc antibody. (B) IP from 500 μg lysate, TCL 20 μg; (C) IP from 100 μg lysate, TCL 40 μg. (D) HeLa cells stably expressing EphA4 were transfected with GFP, myc-tagged wt Rin1, Rin1-ΔGEF, or Rin1-splice and subjected to surface biotinylation. Biotinylated surface molecules were internalized by stimulation with either preclustered Fc (control) or ephrinB3-Fc. Avidin pull-downs were done as described in (Fig. 4A). Pulled-down proteins were subjected to immunoblotting with EphA4 antibody. Total cell lysates were also immunoblotted with anti-Myc antibodies (Rin1) and GFP antibodies. DN, dominant-negative. (E) Quantifications of surface biotinylation experiments. For each experiment, the internalization of EphA4 was quantified as the ratio of the ephrinB3-stimulated sample over the Fc-stimulated control, transfected with the same construct (i.e., GFP, Rin1-wt, Rin1-ΔGEF, Rin1-splice). In GFP-transfected cells, ephrinB3 stimulation enhanced EphA4 internalization on average 2-fold, in Rin1-wt transfected cells a significant increase to nearly 4-fold was observed. Results for the 2 catalytically inactive, dominant-negative forms of Rin1 both showed a reduction compared to GFP controls. Each data point is the mean of at least 3 independent experiments. Y error bars = SEM, P value from Student’s t test, 2-tailed, equal variance. (GFP 2.02 ± 0.25; Rin1wt 3.89 ± 0.61, P = 0.01; Rin1-ΔGEF 1.39 ± 0.4, P = 0.18 and Rin1-splice 0.96 ± 0.12, P = 0.017). (F) HeLa cells stably expressing EphA4 were transfected with GFP or Rin1-wt, labeled with biotinylated transferrin (Tf) and left to internalize Tf for 2 or 5 min, then rapidly cooled and acid stripped of remaining receptor-bound surface Tf. A 20-μg quantity of lysate per sample was immunoblotted for biotinylated Tf (avidin-horseradish peroxidase). No significant difference in the amount of endocytosed Tf was observed between Rin1- and GFP transfected controls. TfR, Tf receptor. (G) Quantification of 3 independent experiments shown in F. Gray values of biotinylated Tf were normalized against the values of total transferrin receptor to account for slight differences in protein amounts and then set in relation to GFP-transfected cells, 2-min time point. Deininger et al. www.pnas.org/cgi/content/short/0801174105