were harvested in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% or 1% Triton-X-100) containing complete protease inhibitors (Roche), 4.4 mg NaN3 and 0.8 mg/ml NaF. Protein concentrations were estimated using the DC Protein Assay (Bio-Rad). Immunoprecipitations were performed by incubating samples with anti EphA4 antibodies and 40 µl of a 50% slurry protein A Sepharose (GE Healthcare) for 2 h at 4°C. Proteins were eluted by the addition of loading buffer and analyzed by Western blot.

**Synaptosomal and Postsynaptic Density Preparations.** Synaptosomal and postsynaptic density preparations were performed as described (1) using 6 forebrains from adult C57Bl6 mice per preparation. For coimmunoprecipitations, Triton-X100 was added to fractions to a final concentration of 0.5%.

**Stimulations.** Fc- and ephrin-Fc fusion proteins (R&D Systems) were preclustered with goat-anti-human Fc (R&D) at a ratio of 10:1 for 1 h at RT. Clustered Fc and Ephrin-Fc proteins were used at a concentration of 1 µg/ml of medium.

**Immunofluorescence and Image Acquisition.** All cell images were obtained with an Axioplan-2 imaging fluorescent microscope (Carl Zeiss MicroImaging) equipped with a RT Slider 2.3.1 digital color camera (Diagnostic Instruments).

**Laser Capture Single Cell RT-PCR.** For tissue preparation, adult wt C57Bl6 males were killed by cervical dislocation and brains were quickly dissected out. They were placed on dry ice for 1 h and transferred to −80°C. Brains were cut with a cryostat into 10-µm coronal sections which were placed on Palm Membrane Slides (1-mm PET, PALM microlaser, Bernried). Every fourth section was placed on a separate slide (SuperFrost Plus, Menzel) for hematoxylin and eosiin staining to facilitate identification of the regions of interest.

For laser microdissection, were dried in a vacuum desiccator for 3 min, placed on the platform of a Microbeam-Z microscope (PALM), and cells were isolated by laser microbeam microdissection and pressure catapulting. To establish the PCR conditions, we collected pieces that contained ~20 cells from the hippocampus and the lateral amygdala. Single cells in CA1 and CA3 regions in the hippocampus were isolated as positive controls since in situ hybridization indicated strong coexpression of Rin1 and EphA4. The lateral amygdala was identified and marked with an outline at ≥5 magnification, whereas cells were isolated at ≥40 magnification. Tissue samples and single cells were stored at −80°C. To amplify the RNA from single cells, the Qiagen OneStep RT-PCR Kit was used according to manufacturer's instructions. For the RT reaction, specific primers for TrkB, GFAP, GAD67, GAD65, Rin1, EphA4 and CamKII were used instead of random primers: Rin1F1 5'-tgtacctacagggctacagccagcc-3', Rin1R1 5'-aggttgtaggtccagaggaaggc-3'; EphA4F1 5'-gcatggaagaaactgtgaacagggc-3'; EphA4R1 5'-ctctgactgctcgattcatctccgc-3'; CamKIIaF1 5'-aagcctcccaacagctgtcagcagcc-3'; CamKIIaR1 5'-gaagctcccaacagctgtcagcagc-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'-accctggcttggttctgatcagccagcc-3'; Rin1R2 5'-tggtgtagathtggtcagaggaaggc-3'; EphA4F2 5'-aggttgtaggtcagaggaaggaacagggc-3'; EphA4R2 5'-tccctgactgctcgattcatctccgc-3'; CamKIIaF2 5'-atcctccacacaggagtgtcagcagcc-3', CamKIIaR2 5'-tcctggtcagacaggttgttcagcagcc-3').
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 biotinylations (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–L 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, Rin<sup>+/−</sup> and Rin<sup>−/−</sup> 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. In serum 1204 a particularly strong, unspecific band was detected close to full-length Rin1, which made it less useful (asterisk). (B) (a) Serum 1203 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. (b) Nuclei of cells were revealed with Hoechst stain. (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.