SUPPLEMENTARY METHODS

Constructs and antibodies

To generate expression constructs for overexpression studies, the tyrosine phosphorylation mutant of Cdk5 (Y15F), and expression constructs encoding different serine or threonine mutants of GST-ephexin1 (1-200 aa) were generated by PCR using complementary primers containing the mutations and were subcloned into corresponding vectors. The pSUPER-EphA4, ephexin1, Cdk5 and C42 siRNAs were designed as described. The RNAi target sequence for mouse EphA4 is 5’ GCAATTGCGTATCGTAAAT 3’, for mouse ephexin1 is 5’ GATGAGCCGCACAGAACAG 3’, for rat Cdk5 is 5’ TGCCACCGGGAGAGACCTG 3’. The complementary oligonucleotides for the corresponding sequence were annealed and subcloned into the pSUPER vector. Subsequently, the pSUPER-EphA4, Cdk5 and ephexin1 RNAi constructs were expressed either in HEK 293T cells or cortical neurons by LipofectAMINE PLUS reagent or nucleofection, and their knockdown efficiencies were confirmed by Western blot analysis.

Antibodies specific for Cdk5 (C-8), p35 (C-19), Cdk5 (DC-17), RhoA (26C4), EphA4 (S-20), and His antibodies were purchased from Santa Cruz Biotechnology. Antibodies specific for phosphoserine and synaptophysin were purchased from Chemicon, antibodies specific for EEA1 and GST were purchased from BD Biosciences Pharmingen, phospho-Tyr15 Cdc2, and phosphothreonine from Cell Signaling Technology, antibodies specific for MAP2, α-tubulin and β-tubulin III from Sigma and PSD-95, phosphotyrosine (4G10) and cortactin antibodies from Upstate. Histone H1 peptide (PKTPKKAKKL), extracellular domain of EphA4, phospho-Tyr596,602 EphA3 (P-EphA), phospho-Tyr87 ephexin1, and ephexin1 antibodies have been previously described. Phospho-specific antibodies against phosphorylated ephexin1 at Thr41,47 was raised in rabbit using a synthetic phospho-peptide against the corresponding amino acid sequence. The resulting serum was first purified using nonphosphorylated peptide coupled to a Sulfolink column (Pierce Biotechnology), followed by an affinity-purification over the phosphorylated peptide linked to a Sulfolink column. Recombinant proteins encoding histone H1 and the kinase domain of EphA4 used in the in vitro kinase assay, Roscovitine and wortmannin were purchased from Upstate.

Preparation of organotypic hippocampal slices

Organotypic hippocampal slices were prepared from P5-P7 normal mice or ephexin1−/− mice and grown on Millicell inserts (Millipore) as previously described. On 2 DIV, the
slices were transfected with YFP expression construct together with pSUPER RNAi constructs targeting for EphA4, C42 or ephexin1, or DNA constructs encoding ephexin1 or its phosphorylation mutant (T41.A7.S57.S139.A). Transfection with a Biorad Helios GeneGun using 1.6 μm gold particles coated with the expression plasmids was performed as described

### Cell cultures and Transfection

HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics. Primary cortical and hippocampal neurons were prepared from embryonic day 18–19 rat embryos. Cortical neurons (5×10⁶ per plate) were plated on 100 mm culture dishes coated with poly-D-lysine (12.5 μg ml⁻¹; Sigma). Cortical neurons were fed with Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen). Rat hippocampal neurons were seeded on 18 mm coverslips coated with poly-D-lysine (50 μg ml⁻¹; Sigma) at two densities: high and low density (2×10⁵ and 0.5×10⁵ per 18 mm coverslip) for calcium phosphate transfection and immunocytochemical analysis, respectively. Hippocampal neurons from day 18 embryos of Cdk5⁻/⁻ mice were plated on 12 mm coverslips (1 hippocampus; ~5x10⁵ cells per coverslip). The hippocampal neuron cultures were then grown in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM glutamine. Hippocampal neurons at 7–9 DIV were transfected with different plasmids plus EGFP using calcium phosphate precipitation

### Fusion protein generation, protein extraction, immunoprecipitation, and Western blot analysis

To perform in vitro phosphorylation assays, recombinant proteins encoding for GST-Cdk5 and GST-ephexin1 were expressed in Escherichia coli (BL21 strain) and purified using a glutathione-sepharose 4B column according to the manufacturer’s instructions (GE). Proteins were extracted using different lysis buffers supplemented with various protease inhibitors. HEK 293T cells and cultured neurons were lysed in lysis buffer A (in mM) (Tris, pH 8.5, 100; NaCl, 100; EDTA, 1) with 0.5% Nonidet P-40 and various protease inhibitors. Brain tissues were homogenized in lysis buffer B (in mM) (Tris, pH 8, 50; NaCl, 150; EGTA, 2; DTT, 1; NaF, 5) with 1% Nonidet P-40 and 0.25% sodium deoxycholate.

For direct binding assay, GST fusion proteins of p25 or Cdk5 were incubated with 50 or 100 ng of recombinant His-EphA4 fusion proteins. The protein complexes were
immunoprecipitated using glutathione-conjugated beads (GE) and EphA4 protein was detected using Western blot analysis for His epitope. For immunoprecipitation analysis, HEK 293T and neuronal cell lysates were incubated with the corresponding antibodies (2 µg) at 4 °C for 2 hours and incubated with 40 µl of protein G sepharose (GE) at 4 °C for 1 hour. The samples were washed with buffer A and resuspended in SDS sample buffer. Proteins were co-immunoprecipitated and detected using Western blot analysis. Co-immunoprecipitation studies using brain lysates were performed as described.

**Electrophysiology**

Whole cell recordings were obtained from hippocampal neurons at 21 DIV using the MultiClamp 700A amplifier (Axon Instruments, Foster City, CA). The pipettes used typically had a resistance of 3-5 MΩ when filled with an internal solution consisting of (in mM): K-glucuronate 130, KCl 10, HEPES 10, EGTA 1, MgCl₂ 2, Na₂ATP 2, Tris GTP 0.4, and pH was adjusted to 7.3. The cells were continuously superfused with an external solution of the following composition (in mM): NaCl 125, KCl 4.0, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, glucose 11, NaHCO₃ 26, at a flow rate of 1.5-2 ml min⁻¹. The external solution was bubbled with carbogen and maintained at 34 ± 1 °C. Tetrodotoxin (1 µM) and bicuculline (10 µM) were included to block action potentials and GABA transmission respectively. Once a whole-cell recording was achieved, the cell was held at -70 mV to record the miniature excitatory postsynaptic currents (mEPSCs), filtered at 3 kHz, for 5 to 15 min. Involvement of AMPA receptors in mediating the mEPSCs was confirmed at the conclusion of experiments by adding CNQX (20 µM), which eliminated all events. The mEPSCs were analyzed by a commercial software MiniAnalysis (Synaptosoft, Decatur, GA).

**In vitro phosphorylation assay and GTPase activation assay**

To identify Cdk5 phosphorylation sites on ephexin1, recombinant ephexin1 and its mutant proteins (200 ng) were used as substrates for reconstituted Cdk5.p35 in the *in vitro* phosphorylation assay. The kinase assay was performed at 30 °C for 30 min in kinase buffer (20 mM MOPS, pH 7.4, 15 mM MgCl₂, 100 µM ATP) containing 1 µCi of [γ⁻³²P] ATP. Phosphorylated proteins were separated by 15% SDS-PAGE and visualized by autoradiography. The band intensity was quantified using IMAGE J software (National Institutes of Health). Tyrosine phosphorylation of Cdk5 by EphA4 was assayed *in vitro* by incubating GST-Cdk5 with His-EphA4 at 30 °C for 10 min. Phosphorylated proteins were
separated by SDS-PAGE and immunoblotted by phospho-Tyr\textsuperscript{15} Cdc2 antibody. The Cdk5 kinase assay in ephrin-A1 treated cortical neurons was performed as previously described\textsuperscript{7}.

RhoA GTPase activity was measured using pull-down analysis. Briefly, cultured cortical neurons were treated with clustered ephrin-A1, washed twice with ice-cold TBS, and lysed with lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl\textsubscript{2}, 1 mM PMSF, 10 µg ml\textsuperscript{-1} leupeptin and aprotinin). Lysates were incubated with agarose beads conjugated with GST-RBD, a Rhotekin domain that specifically binds the GTP-bound form of RhoA, on ice for 90 min. The beads were washed four times with Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl\textsubscript{2}, 1 mM PMSF, 10 µg ml\textsuperscript{-1} leupeptin and aprotinin. The beads were resuspended with sample buffer, and bound proteins were separated by SDS-PAGE. The active GTP-bound RhoA was detected using RhoA antibody.

**Fibroblast morphology assay**

Briefly, REF-52 cells were maintained in DMEM supplemented with 10% newborn calf serum, 2 mM glutamine, and antibiotics. The cells were transfected with different DNA expression constructs using Fugene (Roche). After 24 hours of transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rhodamine-conjugated phalloidin (Molecular Probes). Cells with stress fiber, filopodia and lamellopodia phenotypes were scored in a blinded manner\textsuperscript{3}.

**REFERENCES**