Supplementary methods

Antibodies and cDNA constructs
Anti-NR1 (Chemicon, 54.1, 1:1000), anti-NR2A/B (Chemicon, 1:200), anti-NR3A (generous gift from Dr. Jim Trimmer), anti-Xpress (Invitrogen, 1:1000), anti-dynamin (gift from Dr. Sandra Schmid), anti-PACSIN 1 (1:10,000, characterized in\(^4\))\(^i\), anti-clathrin heavy chain (ABR, 1:500), anti-14-3-3 β (Santa Cruz Biotechnology, 1:750) and anti-synaptophysin (Chemicon, 1:1000) were used for Western blots or immunoprecipitations. For immunofluorescence, anti-GFP (Chemicon, 1:1500), anti-PACSIN 1 (1:5000), anti-synapsin I (Chemicon, 1:1000), anti-Shank (a gift from Carlo Sala, 1:500), anti-NR1 (S3C11, 0.5 g/ml) and anti-EEA1 (BD Bioscience, San Diego, CA, 1:200) antibodies were used.

Generation of GFP-NR3A was described previously\(^{23}\). YFP-NR1-1a and GFP-NR2A were kindly provided by Dr. Stefano Vicini. For expression in mammalian cells, Xpress tagged mammalian PACSIN 1 and the C-terminal region of PACSIN1 [aa193-441], and myc-tagged PACSIN1 were subcloned into pRK5. PACSIN 1 deletion mutant lacking the SH3 terminal domain was generated by introducing a stop codon just before the SH3 domain coding region, and NPF mutants were obtained by site-directed mutagenesis. Soluble fragments of PACSIN1 containing the NPF motifs (amino acids 340-391) were subcloned into pmyc-CMV (Clontech). All DNA fragments were verified by automated sequencing.

Cell culture and transfection
Primary hippocampal neurons were cultured as described before\(^{36}\). Briefly, hippocampus and cortex were dissected from E17-18 rat pups and dissociated with trypsin. For biochemistry, neurons were plated onto poly-D-lysine-coated 60 mm dishes at a density of 2 ×10\(^6\) cells/dish, and grown in Neurobasal Medium supplemented with 10% FBS. For immunofluorescence, neurons were plated onto poly-D-lysine-coated cover slips in 12 well plates at a density of 30,000-75,000 cells per well, and
maintained in Neurobasal medium supplemented with B27. Neurons were transfected with Lipofectamine 2000. HEK293 cells were maintained in DMEM medium containing 10% FBS, and transfected using calcium phosphate.

**Biochemical fractionation**

Homogenates for synaptic fractionation were prepared from forebrains of 3-4 week old rats. After dissection, the forebrain was homogenized in cold HEPES-buffered sucrose using 10 strokes with a motor driven Dounce homogenizer. The homogenate was spun at 1,000 x g for 15 min at 4°C, and the resulting supernatant (S1) was spun at 10,000 x g for 15 minutes to yield the crude synaptosomal pellet (P2). The supernatant (S2) was centrifuged at 100,000 x g for 15 minutes to separate the cytosolic fraction (S2') from the light membrane pellet (LM). The crude synaptosomal pellet (P2) was resuspended in homogenization buffer and centrifuged at 10,000 x g for 15 minutes at 4°C to yield the washed crude synaptosomal pellet (P2'). Nine volumes of ice cold H2O plus protease/phosphatase inhibitors were added to lyse P2' membranes, and the osmolarity was rapidly readjusted by adding 1 M HEPES, pH 7.4 to a final concentration of 4 mM HEPES. Membranes were then mixed constantly at 4°C for 30 min to ensure complete lysis. The lysate was spun at 25,000 x g for 20 min to yield the lysed synaptosomal membrane fraction (P3). The P3 pellet was resuspended and layered on a discontinuous sucrose gradient containing 1.2 M, 1 M and 0.8 M sucrose. The gradient was centrifuged at 150,000 x g for 2 hours at 4°C, and upon completion, material at the 1.2 M and 1 M interface was recovered, diluted to 0.32 M sucrose and spun at 150,000 x g for 30 min to yield the synaptic plasma membrane fraction (SPM). The SPM pellet was resuspended in 3-5 ml of ice-cold 50 mM HEPES pH 7.4, 2 mM EDTA, plus protease/phosphatase inhibitors. To obtain the PSDI pellet, 0.5 % Triton X-100 was added to the SPM fraction, and the solubilized membranes were centrifuged at 32,000 x g for 20 min. The resulting supernatant constitutes the SPM-TX soluble fraction. An aliquot of the resuspended PSDI pellet was solubilized in 0.5% Triton X-100 to yield the PSDII pellet. To a second PSDI aliquot, 3% N-lauroyl
sarcosyl was added to produce the PSDIII pellet. The PSDII and PSDIII pellets were resuspended in 50 mM HEPES pH 7.4, 2 mM EDTA plus protease/phosphatase inhibitors. Protein concentrations were measured using the Pierce BCA kit (Pierce). Fractions were separated by SDS-PAGE, transferred to PVDF membranes, and NMDAR subunits were detected by immunoblot (ECL Plus, Amersham). Quantification of band intensities was performed on a phosphorimager (Storm 860, Amersham Biosciences) using Image-Quant 5.0 software.

**Yeast two-hybrid screen and biochemical binding assays**

The cDNA encoding the complete carboxy-terminal domain of NR3A (amino acids 952-1115) was amplified by PCR using specific primers and fused in frame with the GAL4 DNA-binding domain of pAS2-1 (Clontech). This construct was used to screen 2 x 10⁶ clones of a rat hippocampal cDNA library fused to the GAL4 DNA-activating domain of pACT2 (Clontech). After transformation onto Y190 yeast cells, the transformation mixture was plated on agar plates lacking leucine, tryptophan and histidine, and positive clones were identified by growth in histidine-deficient plates and confirmed by a β-galactosidase colorimetric assay. The carboxy-termini of NR1 (NR1-1a, NR1-2a, NR1-3a, NR1-4a splice variants), NR2A and NR2B subunits, and the GluR5, -6, -7, and KA1 subunits were fused to the GAL4 binding domain in pAS2-1 and used as specificity controls.

For GST pull down assays, the carboxy-terminus of NR3A was subcloned into pGEX-2T (Pharmacia), expressed in *E. coli* and purified using GST-Sepharose beads. Extracts of HEK293 transfected with Xpress-tagged PACSIN 1 were incubated with control GST beads, or beads bound to GST fused to the carboxyl-terminus of NR3A, washed with buffer containing 150 mM NaCl and 0.1% TX-100, and eluted with SDS. Bound proteins were detected by Western blotting using an anti-Xpress antibody. Coimmunoprecipitation experiments in forebrain extracts from P12-P16 rats and wild-type or NR3A knockout mice (kind gift from Dr. Stuart Lipton) were performed as described¹⁰.

**Histological procedures**
In situ hybridization was performed with $^{32}$P-labeled RNA probes (rat NR3A-probe: nucleotides 3330-3821, U29873, rat PACSIN1-probe: nt 745-1600, AF104402). For immunohistochemistry, mouse anti-NR3A and rabbit anti-PACSIN1 antibodies were used as primary antibodies. The specificity of the monoclonal NR3A antibody was tested in NR3A knockout mice, which showed no detectable labeling.

**Electron microscopy**

Adult Wistar rats (250–270 g, approximately 60 days-old) were transcardially perfused with saline, followed by 4% paraformaldehyde and 0.6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed and post-fixed in paraformaldehyde overnight at 4°C.

*Pre-embedding immunogold method.* Brain sections (50-70 μm thick) were cut on a Vibratome and then processed for immunocytochemical detection of NR3A by using silver-enhanced immunogold techniques. Briefly, sections were incubated for 1 hour in 0.1 M phosphate buffer containing 3–5% normal goat serum at room temperature, and then incubated with anti-NR3A antibody$^{22}$ (1:1,000–1:2,000) for 48-72 hours at 4°C. After thorough washing in phosphate buffer, selected sections were incubated for 1.5 hours in a solution of goat anti-mouse IgG coupled to 10 nm gold particles (BB International). Sections were washed, placed in 2% glutaraldehyde in phosphate buffer for 10 min and then rinsed with deionized water. Sections were reacted in silver enhancing solution (BB International) for 5-16 min at room temperature and washed thoroughly in phosphate buffer. All immunostained sections were examined under the light microscope, and sections containing somatosensory cortex and motor cortex, which displayed dense immunostaining, were selected for electron microscopy. The sections were post-fixed in 1% OsO$_4$ for 10-20 min, dehydrated in increasing concentrations of ethanol and in acetone, and flat-embedded in Araldite. Serial thin sections were cut on a Reichert-Jung ultramicrotome at 60-70 nm and collected on Formvar-coated, single slot nickel grids. The grids were lightly stained with lead citrate and examined in a Philips CM-10 electron microscope. Electron micrographs were taken at X15, 500. The EM negatives were scanned in a Epson Twain scanner at
2,000 d.p.i., images were imported to Photoshop 6.0 (Adobe System), and the brightness and contrast of the images were adjusted.

Post-embedding immunogold method. Slices of the hippocampus were cut at 500 μm, cryoprotected, slammed onto copper blocks cooled in liquid nitrogen, and embedded in Lowicryl HM 20 (TAAB Laboratories) after freeze substitution with methanol. Ultrathin sections (70-90 nm) from three Lowicryl-embedded blocks were incubated for 45 min on coated nickel grids with drops of blocking solution consisting of 2% albumin in 0.05 M Tris buffered saline (TBS), 0.9% NaCl, and 0.03% Triton X-100. The grids were transferred to solutions of NR1, NR2A/B, or NR3A antibodies at concentrations of 10 μg/ml overnight at room temperature, followed by incubation with goat anti-rabbit or anti-mouse IgG conjugated to 10 nm colloidal gold particles, or a mixture of goat anti-rabbit IgG conjugated to 10 nm colloidal gold particles and goat anti-guinea pig IgG conjugated to 20 nm colloidal gold particles (Nanoprobes). Grids were then washed in TBS for 30 min and counterstained for electron microscopy with saturated aqueous uranyl acetate and lead citrate. Ultrastructural analyses were performed with a Jeol (Peabody, MA) 1010 electron microscope. Electron photomicrographs were captured with a CCD camera (MegaView III; Soft Imaging System).

Immunofluorescence analysis of synaptic localization

Hippocampal neurons were transfected at DIV10 with GFP-tagged NR3A alone or together with myc-tagged PACSIN1 constructs, and stained at DIV 21-23 using anti-Shank or synapsin I antibodies as synaptic markers. Only neurons showing low to moderate levels of overexpression were accepted for analysis. Briefly, neurons were fixed in 4% paraformaldehyde with 4% sucrose, and permeabilized in phosphate buffer saline containing 0.1% Triton X-100 (for synapsin I) or in methanol at -20°C (for Shank), and incubated for 2 hours at room temperature in guinea pig anti-Shank or mouse anti-synapsin I antibodies, followed by incubation with Cy3-conjugated secondary antibodies. For NR1 staining, neurons were fixed in sequential paraformaldehyde and methanol and stained with a monoclonal
antibody to NR1 (3SC11) followed by a Cy3-conjugated anti-mouse antibody (Jackson
Immunoresearch).

Data analysis. Image stacks were acquired in a Nikon-inverted microscope using a spinning-
disk confocal (CSU10, Yokugawa) and a 12-bit cooled CCD camera (Hamamatsu), and the
colocalization of GFP fluorescence with Shank or synapsin I immunostaining (red, Cy3) was assessed
in maximum projection images using Metamorph Imaging software (Universal Imaging Co.). Briefly,
GFP-NR3A or GFP-NR2A clusters were selected as discrete puncta of intensity > 1.5-fold brighter than
background fluorescence. Fluorescence clusters that satisfied the criteria were marked, their locations
transferred into the red channel and the image was analyzed for fluorescent signal at the marked position.
Synaptic clusters were defined by either visual inspection or fluorescence intensity 2-fold higher than
background. The total number of clusters and the number of synaptic clusters per neuron were counted.
The proportion of synaptic clusters was calculated for individual neurons as the number of clusters that
colocalized with the postsynaptic marker shank divided by the total number of clusters. Colocalization of
GFP-NR3A and endogenous NR1 was measured by the pixel-by-pixel correlation of GFP-fluorescence
and immunostained NR1 fluorescence intensities. Fluorescence intensities for both fluorophores were
determined for points (five pixels in width) along a line drawn down the length of a dendrite. Values
were background subtracted, and correlation was calculated as a linear regression.

Surface expression and internalization assay
Hippocampal neurons were transfected at DIV10 with GFP-tagged NR3A alone or together with myc-
tagged PACSIN1 constructs. To avoid problems associated with protein overexpression, the transfection
conditions were carefully controlled by titrating the amounts of DNA and Lipofectamine, and only
neurons showing low to moderate levels of overexpression were accepted for analysis. Surface
expression and internalization assays were carried out at DIV12-14. To label surface-tagged receptors,
live neurons were incubated with chicken anti-GFP antibody (1:1000, Chemicon) for 15-30 min at 37°C
(for surface expression) or 4-8°C (internalization experiments). For surface expression, neurons were fixed immediately after with 4% paraformaldehyde, 4% sucrose in phosphate buffer saline pH7.4 (PBS), washed and blocked with 4% normal serum before application of Cy3-conjugated secondary antibody. For endocytosis experiments, cells were washed with cold culture media, and internalization was allowed for 30 min at 37°C in conditioned medium, or in response to 20 M NMDA, 100 M glycine (2.5 min, plus 7.5 min to allow internalization). A parallel set of controls was kept at 4-8°C to stop trafficking. Neurons were then fixed as for surface experiments, blocked with normal serum, remaining surface receptors were blocked or labeled with FITC-secondary antibody (non-permeabilized), and internalized receptors were labeled with Cy3-conjugated secondary antibody after methanol permeabilization. For EEA1 colocalization experiments, transfected neurons were incubated live with anti-GFP antibody for 15 min at 37°C, fixed, washed and permeabilized with 0.2% saponin before incubation with anti-EEA1 and corresponding secondary antibodies. Wide-field fluorescence images were acquired with a Zeiss 40x or 100x objective and a CoolSnap CCD camera. All images were analyzed using Metamorph.

Data analysis. For measures of surface expression, the average intensity of surface fluorescence staining (red) was determined after cell tracing, and normalized to the total GFP-intensity (green) to correct for differences in expression. For quantifying the spine enrichment of surface-targeted GFP-tagged NMDAR subunits, a region of interest was drawn on a dendritic spine and the adjacent dendritic shaft. Surface ratios were obtained by dividing the background subtracted Cy3 and GFP fluorescence intensities, and the spine/shaft ratio was calculated. For quantification of dendritic internalization, three to four 100 m dendritic segments were collected from each neuron analyzed. Average fluorescence intensities in the green (total) and red (internalized) channels were measured, background subtracted, and the internalized/fluorescence ratio was calculated for each individual segment. Individual measurements were first grouped and averaged per neuron; means from different neurons were then averaged to obtain a final mean ± s.e.m. value. Statistical differences were analyzed by ANOVA followed by Student's t-test.
Transferrin uptake assay

Hippocampal neurons were incubated with Alexa 568-conjugated transferrin (Tf, 50 μg/ml) in serum-free Neurobasal media for 15 min at 37°C. Cells were then washed with serum-free medium at 10°C, and neurons were incubated with holotransferrin (500 μg/ml) in conditioned media for 30 min at 4°C to exchange the surface bound transferrin and selectively monitor the endocytosed fraction. After washing, neurons were fixed and the remaining intracellular Alexa 568-Tf imaged. For quantification, fluorescent intensities of Alexa-Tf within three to four 50 m dendritic segments were measured from each neuron analyzed, averaged, and means from different neurons were averaged to obtain a final mean ± s.e.m. value.

Electrophysiology

HEK 293 cells were transfected with NR1-1a, NR2A and GFP-NR3A using calcium phosphate. Plasmids encoding wild type or mutant forms of PACSIN1 were included as indicated. GFP was used as a transfection marker in cells where GFP-NR3A was omitted. d-2-amino-5-phosphonovaleric acid (APV, 250 μM) was added to the culture medium to prevent excitotoxicity. Twenty-four hours after transfection, cells were visually selected for recording by GFP epifluorescence. Whole-cell recordings were made with an Axoclamp 700A amplifier (Axon Instruments). Patch pipettes (2 to 4 MΩ) contained: 140 mM Cs methanesulfonate, 10 mM HEPES, 5 mM adenosine triphosphate (Na+ salt), 5 mM MgCl2, 0.2 mM CaCl2, and 10 mM BAPTA (pH 7.40). The extracellular solution contained 150 mM NaCl, 5 mM KCl, 2 or 10 mM CaCl2, 10 mM HEPES, 10 mM glucose (pH 7.40) and was adjusted to 330 mOsm with sucrose. The bath was perfused at 1-2 ml/min. Glutamate (1 mM) and glycine (100 μM) were added, and solution exchanges were accomplished through a series of flow pipes controlled by solenoid valves (Warner). HEK293 cells were lifted off the coverslip to speed the solution exchange time. Currents were digitized at 2 kHz and filtered at 0.5-1 kHz with a Digidata 1322A board and
Clampex 9 software (Axon Instruments). Series resistance (90 to 95%) and whole-cell capacitance compensation were employed. Series resistance was monitored throughout the experiments by 10 mV hyperpolarizing jumps prior to each application of glutamate/glycine. Only cells with series resistance < 6 MΩ, and which were stable throughout the recording were included for analysis. All experiments were performed at a holding potential of -80 mV at 20°C. A 300 ms voltage ramp (-80 to 50 mV) protocol was elicited following a 3 sec application of glutamate (1 mM) and glycine (100 µM) to obtain steady-state I-V relationships. Membrane currents elicited by the ramp protocol in the absence of agonists were then subtracted from the glutamate-gated ramp currents to generate the net I-V relationship. This protocol was repeated at least twice to ensure the stability of $E_{rev}$ in each condition. Agonists were applied every 2 min while obtaining the I-V relationships in order to avoid any use-dependent downregulation of the current. Currents were monitored when switching extracellular Ca$^{2+}$ by evoking a 1 sec application of agonists every 30 sec until the current level reached a stable value indicating that exchange of extracellular Ca$^{2+}$ was complete. The shift in reversal potential ($E_{rev}$) was calculated by subtracting the $E_{rev}$ obtained in 2 mM Ca$^{2+}$ from the $E_{rev}$ measured in 10 mM Ca$^{2+}$. The measured junction potentials in 2 and 10 mM Ca$^{2+}$ were 9.2 and 9.4 mV, respectively. Thus the raw $E_{rev}$ was reduced by 0.2 mV to achieve the final corrected $E_{rev}$.  