**Figure S1. The GluR1 CTD and the cGKII catalytic domain interact in a mammalian recruitment assay.**

(A) To confirm the results of the yeast two hybrid screen in mammalian cells, the GluR1 CTD fused to GFP (GluR1C-GFP) was coexpressed by transfection in 293T cells with either the catalytic region of cGKII (cGKII aa 400-762) or as a control, with the peptide JA (c-Jun transcriptional activation domain), the latter two peptides both fused to the Fyn protein membrane localization signal. In this mammalian recruitment system, the GluR1 CTD is fused to GFP (GluR1C-GFP) and is diffuse in the cytoplasm when expressed alone. The library peptide cGKII aa 400-762 is fused to a membrane-localization signal (MLS) from the protein Fyn (pFyn-cGKII), and is localized in the cellular membrane. Only upon interaction between the GluR1 CTD and cGKII aa 400-762, cells display membrane-bound GFP distribution.

(B) Upper panels, the GFP signal is localized in the plasma membrane when GluR1C-GFP is coexpressed with pFyn-cGKII aa 400-762, but not when GluR1C-GFP is coexpressed with a control protein (pFyn-JA), or when GFP alone is coexpressed with pFyn-cGKII aa 400-762. Lower panels, localization of GluR1C-GFP when cotransfected with the cGKII deletion mutants cGKII aa 400-682 and cGKII aa 589-762. Deleting 180 aa at the C terminus of cGKII (cGKII aa 400-682) prevented the interaction, as reflected by a diffuse distribution of GluR1-GFP. However, cGKII aa 589-762 still localized GluR1C-GFP on the plasma membrane. These results suggest that the C-terminal 173 aa of cGKII, which contain a segment of the catalytic domain, are required for interaction with GluR1.
Figure S2. Mapping the region of the GluR1 CTD important for interaction with cGKII

(A) GluR1 CTD deletion mutants and interaction with cGKII in pulldown assay shown in (B).

(B) Lysates from 293T cells expressing cGKII were incubated with GST alone or with GST-GluR1C deletion mutants. 293T cells were treated with 8-Br-cGMP (50 μM) for 10 min before lysis. Bound proteins were detected by IB. Lane numbers correspond to the numbers on (A). Neither the GluR1 CTD PDZ ligand nor the juxtamembrane region were necessary for interaction with cGKII (see mutants containing aa 809-879 and aa 838-889). Surprisingly, a GluR1 CTD mutant containing only aa 850-889 and a mutant containing aa 828-873, but not the mutant containing aa 828-868, still bound cGKII. Certain GluR1 CTD mutants had intermediate abilities to bind cGKII, and the regions between aa 833-838 and aa 873-878 in the GluR1 CTD increased binding to cGKII (compare mutants containing aa 850-889, aa 838-889 and aa 828-873 with the mutant containing aa 833-878).
Supplemental Discussion

Contact sites in the cGKII-GluR1 interaction and comparison with CaMKII-NR2B

GluR1 interaction with cGKII requires CTD aa 850-873, an apparent core contact region in GluR1, and also involves aa 833-838, an apparent auxiliary region that strengthens the cGKII-GluR1 interaction but is not strictly required. The intervening region contains S845, the phosphorylation site. In the absence of the auxiliary, this substrate region destabilizes cGKII-CTD interaction.

The binding of cGKII to GluR1 resembles the interaction of the CaMKII catalytic domain with NR2B. In the inactive state of the kinase, the CaMKII AI domain binds and inhibits the kinase catalytic domain (Lisman and McIntyre, 2001). Following binding of Ca++-calmodulin, the kinase phosphorylates and releases the AI domain, activating the kinase and allowing NR2B to bind the catalytic domain (Bayer et al., 2001). CaMKII then phosphorylates NR2B (Omkumar et al., 1996). Similarly, following binding of cGMP to cGKII, cGKII phosphorylates the AI domain, displacing it from the catalytic domain, allowing GluR1 to bind proximal to the cGKII catalytic site. cGKII then phosphorylates GluR1. In the CaMKII case, a region of NR2B and the CaMKII AI domain both interact with the CaMKII catalytic domain and notably, display homology with each other (Lisman and McIntyre, 2001). Interestingly, the GluR1 region that interacts with cGKII shows an analogous homology with the cGKII AI domain. In cGKII, although the major autophosphorylation site is S114, only phosphorylation of S126 converts cGKII into a constitutively active kinase (Vaandrager et al., 2003). Alignment of GluR1 S845 with cGKII S126 reveals that in both cases 8 residues away from the Ser in the N terminal direction, an Arg is located, R118 in cGKII and R837 in GluR1 (Fig. 8iii). R118 in cGKII corresponds to a conserved basic residue found in other pseudosubstrate sequences that interacts with conserved Glu residues in the catalytic cleft (Taylor et al., 2002). Interestingly, mutation of R118 to Ala in cGKII decreases the AI domain interaction with the catalytic region, confirming the importance of R118 for AI region affinity for the catalytic site (Taylor et al., 2002). The GluR1 aa 833-838 auxiliary binding sequence contains an Arg, R837, which corresponds by sequence alignment to R118 in cGKII. Strikingly, when we mutated R837 to Ala, the interaction of the GluR1 CTD with cGKII was decreased, demonstrating its functional similarity to cGKII R118.

Homology of GluR1 core contact region with the cGKII AI domain

A sequence in the GluR1 CTD 9 aa C terminal to the phosphorylation site at S845 (SGENGRV) (Fig. 8iii) is related to a cGKII sequence (SFEKARV) that is involved in autoinhibition (Taylor et al., 2002) and is found 16 aa C terminal to the autophosphorylation site, S126. Just as the AI domain binds the cGKII catalytic domain, this GluR1 region may anchor GluR1 to the catalytic region of cGKII. Flexibility of a Gly and Ala rich region between S845 and the sequence homology in GluR1 may compensate for the difference in spacing of the regions. Thus, GluR1 may anchor itself to cGKII through two regions, one involving GluR1 aa 850-873, and the other involving GluR1 aa 833-838. By employing this mode of anchoring, GluR1 appears to mimic the mechanism the kinase AI employs to anchor to the kinase catalytic domain. cGKII phosphorylation of GluR1 may be restricted to S845, and not be sufficient to disrupt the GluR1-cGKII interaction. In contrast, the autophosphorylation of multiple Ser in the cGKII AI domain following cGMP binding will exert a greater electrostatic repulsion that fully disrupts the AI domain interaction with the catalytic site (Vaandrager et al., 2003). Since in GluR1 only one Ser may be phosphorylated, GluR1 may remain bound to cGKII.
References for Supplemental Discussion


Supplemental Experimental Procedures

Antibodies
To generate the anti-cGKII serum, rabbits were immunized with a synthetic peptide corresponding to aa 410-421 of cGKII. Peptide synthesis, Ab generation and affinity purification were done by Covance. Other antibodies were: mouse anti-SV2, developed by K.M. Buckley (Developmental Studies Hybridoma Bank, University of Iowa); rabbit anti-GluR1 CTD and rabbit anti-GluR2/3 (Chemicom); rabbit anti-GluR1 N terminus (Oncogene); rabbit anti-S845-PO4 GluR1 and mouse anti-MAP2 (Upstate); rabbit anti-synaptophysin (Zymed); mouse anti-HA (Covance); goat anti-HA (Bethyl Laboratories); goat anti-cGKII (Santa Cruz), rabbit anti-GFP (Invitrogen).

Expression Vectors
Plasmids and viral vectors expressing HA-GluR1, GST-R2C and GST-R1C have been described (Greger et al., 2003; Osten et al., 2000; Srivastava et al., 1998). The wt cGKII cDNA was expressed from pcDNA1 (Invitrogen). GST-R1C mutants were cloned by PCR and ligated into pGEX-4T1 (Pharmacia). The HAGluR1-S845A, HAGluR1Δ7, HAGluR1-R837A and HAGluR1-R837E mutants were generated by the QuickChange mutagenesis kit (Stratagene). All mutants were confirmed by sequencing. For construction of GFP-cGKII-i, the 416 aa cGKII N terminus was cloned into the EcoRI/Smal site of pEGFP-N vector (Clontech), and then recloned into the viral vector as described (DeSouza et al., 2002).

Mammalian recruitment system
A PCR fragment containing the GluR1 CTD was inserted into pEGFP-N1 (Clontech). To generate the plasmid expressing the membrane-bound protein, two complementary oligonucleotides encoding a myristoylation signal in Fyn were annealed and inserted into PcDNA3.1 to create pFyn. The cGKII fragment found in the yeast two-hybrid screen and a PCR fragment containing the JA domain (aa 1-223) were inserted into pFyn. Plasmids were transfected into 293T cells with Polyfect Reagent (Qiagen) following the manufacturer’s instructions. At ~36 hr after transfection cells were fixed and images were acquired using a Nikon PCM 2000 confocal microscope.

293T cell culture and GST pulldown
Plasmids were transfected into 293T cells with Polyfect Reagent (Qiagen) following the manufacturer’s instructions. At 36-48 hr after transfection, cells were lysed with 1% NP-40 buffer, centrifuged, and the supernatants were used in GST pulldown experiments. Before lysis, cells were either left untreated or treated with 50 µM 8-Br-cGMP for 10 min. GST and GST-fusion proteins purification, and GST pulldowns, were performed as described (Osten et al., 1998).

Preparation and fractionation of cell extracts and immunoprecipitation
Brain fractions were prepared as described (Cohen et al., 1977; Jordan et al., 2004; Suzuki et al., 2001). The different fractions were subjected to SDS-PAGE/IB analysis. Synaptosomal fractions were prepared as described (Phelan, 1987). Purified synaptosomes were lysed in lysis buffer (10 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100 and 1% CHAPS) with phosphatase inhibitors. For coIP from cortical cell lysates, 7-10 div cultured cortical cells were used. Cells were stimulated with saline (control) or drugs, as indicated in the text, in ECS, then lysed in lysis buffer. For both synaptosomal and cortical cell lysates, approximately 1 mg of the lysate and 1 µg/sample of anti-cGKII Ab or anti-IgG Ab were used for IP, as indicated. Precipitated proteins were detected by IB.
In vitro phosphorylation experiments

In vitro kinase reactions with cGKII were performed as described (Pohler et al., 1995), with some modifications. GST and GST-fusion proteins were incubated with purified recombinant rat cGKII (Alexis Biochemicals) in cGKII kinase buffer (10 mM HEPES pH 7.4, 5 mM MgCl₂, 1 mM DTT, 0.2 mM EDTA, 1 mM Na₃VO₂, 1 mM NaF, 1mM ATP, 5 µM 8-Br-cGMP) for 10 min at 30 °C. For PKA kinase reactions, substrates were incubated with the PKA catalytic subunit (Upstate) and the reaction was assayed following manufacturer’s instructions. For radio labeling in vitro reactions, [γ³²P] ATP (PerkinElmer) was added to the reaction buffer.

GluR1 phosphorylation in cortical cultures

To assess the phosphorylation of GluR1, 7-10 DIV cortical cells were transferred to ECS and pretreated with 1 µM Okadaic Acid (Calbiochem) for 30 min at 37 °C, and then treated for 10 min with saline (control), or with drugs, as indicated in the text. Cells were lysed as described (Rameau et al., 2003). Lysis buffer consisted of 50mM Tris-Hcl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1% Triton X-100 and 0.1% SDS plus protease and phosphatase inhibitors. Proteins were detected by IB. Protein bands were quantified using the gel analyzer from ImageJ software.

Neuronal immunocytochemistry

Cell-surface recombinant receptors were labeled by incubating live cultures with HA-monoclonal Ab (4 µg/ml) in Neurobasal growth medium for 15 min at 37 °C. Neurons were fixed and stained with secondary Ab under nonpermeant condition. Cells were then permeabilized and total recombinant receptors were labeled with HA-polyclonal Ab (1 µg/ml), followed by polyclonal secondary Ab. For labeling endogenous surface GluR1, live cultures were incubated with an anti-GluR1 N terminus Ab (20 µg/ml) following the same procedure described for the expressed receptors. After permeabilization, cells were incubated with an SV2 Ab (1 µg/ml) or a MAP2 Ab (1 µg/ml), as indicated in each figure. Experiments with glycine were done as described (Lu et al., 2001). Neurons were treated with glycine (200 µM) for 3 min in extracellular solution (ECS) consisting of (in mM): NaCl (140); CaCl₂ (1.3); Kcl (5.0); HEPES (25); glucose (33); TTX (0.0005); strychnine (0.001); and bicuculline methiodide (0.02) (pH 7.4). After treatment, neurons were transferred to ECS without any added glycine for 15-20 min. Labeling was then performed as described above. Treatment with NOR-3 (10 µM) was done in ECS for 10 min. After NOR-3 treatment, surface GluR1 labeling was performed as described above. In all cases, inhibitors were applied to neurons in ECS, before treatment with glycine or NOR-3.

Patch clamp experiments

Cultured rat hippocampal neurons were voltage clamped with the whole cell ruptured patch technique throughout the experiment. The bath solution consisted of (in mM) NaCl (119), KCl (5), HEPES (20), CaCl₂ (2), glucose (30), strychnine (0.001), bicuculline (0.02), pH 7.3, osmolarity adjusted to 330 mOsm with sucrose. Tetrodotoxin (1 µM) was added to the bath solution to suppress action potentials. The solution in the whole cell patch electrode consisted of (in mM) K-gluconate (130), KCl (10), MgCl₂ (5), EGTA (0.6), HEPES (5), CaCl₂ (0.06), Mg-ATP (2), GTP (0.2), leupeptin (0.2), phosphocreatine (20), and creatine-phosphokinasr (50 U/ml). Currents were recorded with a Warner amplifier (model PC-501A) (CT), and filtered at 1 kHz. In order to eliminate artifacts due to variation of the seal properties, the access resistance was monitored for constancy throughout all experiments. The recordings were digitized (Digidata 1322A, Axon Instruments) and analyzed with the minianalysis program (version 4.0) from Synaptosoft, Inc. (GA).

Slice preparation and recording

Mice were maintained for 1-2 months after the purchase on a 12-12h light–dark cycle (with light onset at 06:00 hours) in temperature and humidity controlled rooms of the Columbia University Facility. Animals were sacrificed by cervical dislocation followed by decapitation. Hippocampi were quickly removed. Transverse hippocampal slices (400 µm) were cut and transferred to a recording chamber where they were maintained at 29°C and perfused with artificial cerebrospinal fluid (ACSF) continuously bubbled with 95% O₂ and 5% CO₂. The ACSF composition in mM was: 124.0 NaCl, 4.4 KCl, 1.0 Na₂HPO₄, 25.0 NaHCO₃, 2.0 CaCl₂, 2.0 MgSO₄,
10.0 glucose. Field extracellular recordings were performed by stimulating the Schaeffer collateral fibers through a bipolar tungsten electrode and recording in CA1 stratum radiatum with a glass electrode filled with ACSF, as previously described (Puzzo et al., 2005). A 15-minute baseline was recorded every minute at an intensity that evoked a response approximately 35% of the maximum evoked response. LTP was induced using a theta-burst stimulation (4 pulses at 100 Hz, with the bursts repeated at 5 Hz and each tetanus including 3 ten-burst trains separated by 15 seconds). Responses were recorded for 2 hrs after tetanization and measured as field-excitatory-post-synaptic potential (fEPSP) slope expressed as percentage of baseline. The results were expressed as mean ± Standard Error Mean (SEM). The following drugs were used: 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) from Cayman Chemical; (4S)-N-(4-Amino-5[aminoethyl]aminopentyl)-N'-nitroguanidine, TFA (nNOS inhibitor I), KT5823 from Calbiochem. Before tetanus slices were perfused with nNOS inhibitor (10 µM) for 20 minutes and ODQ (10 µM), KT5823 (2 µM), Rp-8-pCPT-cGMPS (10 µM) for 10 minutes.

References for Supplemental Experimental Procedures


