A four PDZ Domain-Containing Splice Variant Form of GRIP1 is Localized in GABAergic and Glutamatergic Synapses in the Brain

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SUMMARY

We have isolated, from a rat brain cDNA library, a clone corresponding to a 2779 bp cDNA encoding a novel splice form of the glutamate receptor interacting protein-1 (GRIP1). We call this 696 amino acid splice form GRIP1c 4-7 to differentiate it from longer splice forms of GRIP1a/b containing 7 PDZ domains. The 4 PDZ domains of GRIP1c 4-7 are identical to PDZ domains 4-7 of GRIP1a/b. GRIP1c 4-7 also contains 35 amino acids at the N-terminus and 12 amino acids at the C-terminus that are different from GRIP1a/b. In transfected HEK293 cells, a majority of GRIP1c 4-7 associated with the plasma membrane. GRIP1c 4-7 interacted with GluR2/3 subunits of the AMPA receptor. In low-density hippocampal cultures, GRIP1c 4-7 clusters colocalized with GABAergic and glutamatergic synapses although a higher percentage of GRIP1c 4-7 clusters colocalized with GABA_A receptor (GABA_A) clusters than with AMPA receptor clusters. Transfection of hippocampal neurons with HA-tagged GRIP1c 4-7 showed that it could target to the postsynaptic complex of GABAergic synapses colocalizing with GABA_A clusters. GRIP1c 4-7-specific antibodies, that did not recognize previously described splice forms of GRIP1, recognized a 75 kDa M_r protein that is enriched in a postsynaptic density (PSD) fraction isolated from rat brain. EM immunocytochemistry experiments showed that in intact brain, GRIP1c 4-7 concentrates at postsynaptic complexes of both type I glutamatergic and type II GABAergic synapses although it is also presynaptically localized. These results indicate that GRIP1c 4-7 plays a role not only in glutamatergic synapses, but also in GABAergic synapses.
INTRODUCTION

GRIP1 (Glutamate Receptor Interacting Protein 1) is a 7 PDZ domain-containing protein (1, 2) belonging to a family of highly homologous proteins that includes GRIP2 (2, 3, 4) and the AMPA receptor binding protein ABP (5, 6). GRIP2 is also a 7 PDZ domain-containing protein while ABP is a shorter splice variant of GRIP2, lacking the seventh PDZ domain of GRIP2. Both GRIP2 and ABP derive from the same gene, whereas GRIP1 is encoded by a separate gene. GRIP1, GRIP2 and ABP interact with the C-terminal tail of AMPA receptor subunits GluR2/3/4c through PDZ domains 3-6 (1, 3, 5).

GRIP1 may play a role in the postsynaptic localization of AMPA receptors (7, 8) and in targeting AMPA receptors to the synapse (2, 4, 9). GRIP1 has been implicated in activity-dependent synaptic reorganization of AMPA receptors (10) during LTD (8, 11). GRIP1 also binds to the microtubule-based motor protein kinesin 5 through the region located between PDZ domains 6 and 7, thus being involved in vesicular trafficking of AMPA receptors along dendritic microtubules (12), targeting AMPA receptors to synapses (2, 4). Furthermore, PDZ6 of both GRIP1 and GRIP2 interacts with the C-termini of EphB2/EphA7 receptors and EphrinB1 ligands, recruiting cytoplasmic GRIPs to membrane lipid rafts (4, 13). PDZ6 of both GRIP1 and GRIP2 also interact with members of the liprin-α family, and disruption of this interaction prevents AMPA receptor surface expression and clustering (14). AMPA receptors and GRIP1 as well as other components of glutamatergic synapses are present in lipid rafts (15). GRIP−/− mouse mutants have shown that GRIP1 is important for embryonic development (16, 17).

Two splice forms of GRIP1 (GRIP1a and GRIP1b) differ in a short N-terminal peptide sequence such that GRIP1b can be palmitoylated whereas GRIP1a cannot (18). Similar
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Palmitoylated and non-palmitoylated forms have been described for ABP/GRIP2 (19). Palmitoylation of GRIP1/2 allows the anchoring of GRIP1/2 to the membrane and accumulation of AMPA receptors to the synaptic membrane (19).

In this communication, we report the identification and characterization of GRIP1c 4-7, a novel splice form of GRIP1 that lacks PDZ domains 1-3 of GRIP1a/b but contains PDZ domains 4-7 of GRIP1a/b. GRIP1c 4-7 is not just a short form of GRIP1a/b. GRIP1c 4-7 also contains specific 35 amino acid N-terminal and 12 amino acid C-terminal peptide sequences that differ from GRIP1a/b. GRIP1c 4-7 readily distributes to the plasma membrane and concentrates not only in glutamatergic synapses but also in GABAergic synapses both in cultured neurons and in the intact brain. Thus some forms of GRIP1 might play a more significant role in GABAergic synapses than previously recognized.

EXPERIMENTAL PROCEDURES

All the animal protocols have been approved by the Institutional Animal Care and Use Committee and follow the National Institutes of Health guidelines.

Antibodies

All the anti-GABA$_A$R, anti-GRIP1c 4-7 antibodies were raised in our laboratory. Rabbit and guinea pig antibodies specific for GRIP1c 4-7 were raised to amino acids 12-26 (KPHNFHHASHPPLRK) of rat GRIP1c 4-7. This sequence is not present in GRIP1a/b (Fig. 1).

For anti-GRIP1c 4-7 antibody production, this peptide was covalently linked, via a C-terminal cysteine, to diptheria toxoid (Mimotopes, San Diego, CA). A guinea pig antibody specific for GRIP1a/b was raised to the C-terminal amino acids 1100-1112 of rat GRIP1a/b.
splice variant of GRIP1 (GGNLETREPTNTL). This amino acid sequence is not present in GRIP1c 4-7 (Fig. 1). For anti-GRIP1a/b antibody production, this peptide sequence was coupled via an N-terminal cysteine to keyhole limpet hemocyanin (KLH). A guinea pig and/or New Zealand rabbit were injected subcutaneously with a 1:1 emulsion of either dipheria toxoid- or KLH-coupled peptide in complete Freund’s adjuvant (for the first immunization) and with incomplete Freund’s adjuvant (for all subsequent immunizations) once per month. The antibody titer in the sera was monitored by ELISA. Sera were collected after four months of immunizations and were affinity-purified on immobilized peptide. The anti-GRIP1c 4-7 antibodies showed specificity for GRIP1c 4-7 but not for GRIP1a/b as shown by immunofluorescence of transfected HEK293 cells and immunoblots of brain membranes (see results section). Likewise, the anti-GRIP1a/b antibodies showed specificity for GRIP1a/b but not for GRIP1c 4-7 as shown by immunofluorescence of HEK293 cells (not shown) and immunoblots of brain membranes (see results section). The guinea pig anti-rat α1 subunit antibody was raised to amino acids 1-15 and the rabbit anti-rat γ2 subunit antibody was raised to amino acids 1-15 (20, 21). The monoclonal mouse anti-β2/3 (62-3G1) was raised in our laboratory to affinity-purified bovine GABAA Rs (22, 23). This antibody recognizes an extracellular epitope that is common to β2 and β3 subunits (β2/3) of the rat GABAARs but is not present in β1 (24). All the rabbit and guinea pig antibodies used in light microscopy and EM immunocytochemistry were affinity-purified on immobilized peptide. The generation, affinity-purification, specificity and characterization of these GABAAR antibodies have been described elsewhere (20-23, 25-27). The mouse monoclonal anti-PSD-95, the rabbit anti-GluR1 antibody and the rabbit anti-HA antibody were from Upstate Biotechnologies (Lake Placid, NY) and the anti-HA mouse monoclonal antibody was from Covance (Princeton, NJ). Rabbit anti-GluR2/3 was a gift from Dr. Robert Wenthold (National Institutes of Health, Bethesda, MD), mouse
monoclonal anti-SV2 was a gift from Dr. Kathleen M. Buckley (Harvard Medical School), sheep anti-GAD was a gift from Dr. I. Kopin (National Institute of Health) and guinea pig anti-GABA was from Chemicon (Temecula, CA). Rabbit and guinea pig anti-vesicular glutamate transporter-1 (vGlut-1) were from Synaptic Systems (Goettingen, Germany) and Chemicon (Temecula, CA), respectively. Mouse monoclonal anti-GRIP1 antibody, to a region of GRIP1 that includes part of the linker region between PDZ domains 6 and 7 and all of PDZ domain 7, was from BD Transduction Laboratories (San Diego, CA). Fluorophore-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Colloidal gold-labeled (10 nm) goat anti-mouse secondary antibody was from ICN (Irvine, CA). All other colloidal gold-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories.

Yeast two-hybrid

All vectors and yeast strains for bait analysis and yeast two-hybrid (Y2H) screening were from Dr. Roger Brent (University of California, San Francisco) or Origene Technologies (Rockville, MD). Sense and antisense oligonucleotide primers were designed to amplify the C-terminal 50 amino acids of the AMPA receptor subunit GluR3 including its natural stop codon and the PCR product was directionally cloned into the polylinker of pEG202.

We confirmed that the bait fusion protein could be expressed in yeast by immunoblotting the cell lysate of yeast transformants with mouse anti-LexA monoclonal antibody (Origene Technologies, Rockville, MD). We also tested that this fusion protein did not activate the LacZ reporter by itself. For this purpose, S. Cerevisiae EGY48 was transformed with pSH18-34 and pEG202 (containing the bait insert) and the LacZ reporter activity was tested by replica-plating the transformants on the appropriate X-GAL-containing media. For the positive control, the yeast
was transformed with pSH18-34 and pSH17-4, the latter of which contains the LexA DNA binding domain fused to the B42 transcriptional activator domain. For a negative control, the yeast was transformed with pSH18-34 and pRHFM1 or with pSH18-34 and pEG202, the empty bait vector. We also confirmed that the bait did not activate the genomic LEU2 reporter gene, since the transformants containing the bait did not grow in the absence of leucine.

For library screening, the yeast strain EGY48, previously transformed with pEG202 (containing the bait) and pSH18-34, was transformed with pJG4-5 containing the oligo-dT primed rat brain cDNA library (Origene Technologies). An aliquot of the pooled transformants was then diluted 1:10 in liquid YNB medium containing galactose (to activate the GAL1 promoter) and allowed to incubate for 4 hours at 30 °C to induce cDNA library expression. Only clones with an activated leucine reporter grew on the medium lacking leucine. After allowing 4-6 days of growth, the fastest growing colonies were replica-plated onto solid YNB galactose growth medium containing X-GAL. Plasmids from yeast clones showing LacZ reporter activity (presumably expressing bait-interactors) were rescued by mechanical disruption and detergent lysis. The DNA was extracted with phenol/chloroform and was used to transform the trp− E.Coli KC8 strain. Growth medium lacking tryptophan was used to select KC8 cells containing pJG4-5. Plasmid preparations from KC8 transformants were subjected to restriction analysis with EcoRI and XhoI, the enzymes used for directional cloning of the cDNA library into pJG4-5. The coding and non-coding strands of the cDNA clones were independently sequenced using the BigDye Terminator DNA sequencing kit (Applied Biosystems, Foster City, CA) and read using the ABI377 Prism DNA sequencer, model 377XL (Applied Biosystems).
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Isolation and sequencing of the 5' UTR of GRIP1c 4-7 cDNA

Using the adaptor-ligated Marathon-ready rat brain cDNA library (BD Clontech, Palo Alto, CA) as template in a PCR reaction, we completed the full cDNA sequence of the GRIP1c 4-7 clone that was isolated by yeast two-hybrid screening. For this purpose, we used an antisense, gene-specific primer that corresponds to part of the GRIP1c 4-7 5' UTR (5' TTCTCTAGGGAAGGGGTGGTACT 3') and a sense primer corresponding to the 5' end adaptor of the cDNA library. For PCR amplification, the Advantage 2 Polymerase Mix (Clontech) was used under the following thermocycling conditions: 94 °C for 30 seconds followed by 20 cycles, each consisting of 94°C for 5 seconds and 68°C for 2 minutes. After obtaining a PCR product of about 500 bp, the specificity of the reaction was confirmed by using nested primers to the 5' adaptor sequence and the GRIP1c 4-7 5' UTR (5' TATAAGACCGCTACGGAGGACCAGGAT 3'). The 500 bp PCR product obtained from the initial 5' RACE was used as the template. The nested 5' RACE product was then cloned, by the T/A cloning method, into pT-Adv plasmid (BD Clontech, Palo Alto, CA) for DNA sequencing. We have submitted the rat GRIP1c 4-7 cDNA full-length sequence to the GenBank (accession number AY437398).

Preparation of tissue fractions

For crude synaptosomal fraction, forebrains (cerebral cortex and hippocampus) from two 6-8 week old Sprague-Dawley rats were homogenized with a glass/Teflon homogenizer in 10 ml solution A (0.32 M Sucrose, 1 mM NaHCO3, 1 mM MgCl2, 0.5 mM CaCl2, 1 mg/L leupeptin, 0.1 mM PMSF) at 4°C. The homogenate was diluted to 20 ml with solution A and centrifuged for 10 minutes at 1400 x g at 4°C and the supernatant was saved. The pellet was suspended in 20 ml
solution A and centrifuged as above. The two supernatants were pooled and centrifuged at 13,800 x g at 4 °C for 10 min, and the pellet was suspended in 16 ml solution B (0.32 M sucrose and 1 mM NaHCO₃) and homogenized with a glass Dounce homogenizer at 4°C. This crude synaptosomal fraction, containing membranes and cytosol, was stored in aliquots at -70°C. Synaptosomes were prepared from homogenates by the method of Carlin et al. (28). A fraction enriched in post synaptic densities (PSDs) was prepared by treating synaptosomes with 0.5% Triton X-100 for 15 min at 4 °C followed by centrifugation at 100,000 x g for 1 hr at 4°C. The pellet containing PSDs was resuspended in 50 mM Tris-HCl, pH 7.4 (29). Samples were subjected to SDS-PAGE and immunoblotted with specific antibodies as described elsewhere (30). Homogenates from various tissues (3 month old male rats) or from forebrain from rats of different ages were prepared as above except that the non-neural tissue was first ground with a PowerGen 125 (Fisher Scientific, Hampton, NH) and then homogenized in a glass/teflon homogenizer as with brain in buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, and protease inhibitors 1 mM EDTA, 1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml aprotonin A and 10 µg/ml leupeptin. The homogenates were used directly for SDS-PAGE and immunoblotting.

**Immunoprecipitations**

Immunoprecipitation from sodium deoxycholate (DOC) extracts of rat cortical/hippocampal membranes was done according to the method of Luo et al. (31). Briefly, a rat brain cortical/hippocampal crude, unlysed synaptosomal fraction, containing 5 mg/ml total protein in solution B (see above), was centrifuged at 13,800 x g at 4°C for 10 min and the pellet was suspended in a volume of TE buffer (10 mM Tris-HCl and 5 mM EDTA, pH 7.4) equal to that of the original suspension. One-tenth volume ice-cold DOC buffer (10% sodium
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deoxycholate in 500 mM Tris-HCl, pH 9.0) was added and the sample was incubated at 36°C for 30 min, followed by the addition of one-tenth volume of Triton X-100 buffer (1% Triton X-100 and 500 mM Tris-HCl, pH 9.0). The sample was dialyzed against solution C (50 mM Tris-HCl, pH 7.4, 5 mM EDTA and 0.1% Triton X-100) at 4°C overnight. Detergent-insoluble material was pelleted by centrifugation at 37,000 x g for 40 min at 4°C and the supernatant was used for immunoprecipitation with an anti-GRIP1c 4-7 antibody. 40 μl of protein A-Sepharose beads, suspended in 450 μl of 50 mM Tris-HCl, pH 7.4, were incubated with 50 μl of guinea pig anti-GRIP1c 4-7 antiserum, or the pre-immune serum, overnight at 4°C with rotation. A volume of the extract, containing 200 μg of protein, was added to the antibody-coated beads and incubated overnight at 4°C. Beads, washed with solution C, were incubated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.01 M Tris-HCl pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 2.3% SDS, 0.005% bromophenol blue) for 10 min at 85°C. The beads were pelleted by centrifugation and the supernatant was subjected to SDS-PAGE and immunoblotted with a rabbit anti-GluR2/3 antibody. The immunoblotting procedure has been described elsewhere (30).

Low density hippocampal cultures and transfection

Hippocampal cultures were prepared by the method of Banker and Goslin (32) as described elsewhere (21). Briefly, dissociated neurons from embryonic day 18 Sprague Dawley rat hippocampi were plated at a density of 3,000-8,000 cells per 18mm diameter circular coverslip and maintained in glial cell conditioned culture medium for up to 21 days. Cultured hippocampal neurons were transfected with 3 μg pcDNA3.1(+) containing GRIP1c 4-7 (with the hemagglutinin (HA) tag at its N-terminus) at 9 days in culture using the Calphos transfection kit (BD
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Biosciences, San Jose, CA) according to the manufacturer’s instructions. Transfected cells were cultured for 7 additional days and processed for immunofluorescence as described below.

**HEK293 cell culture and transfection**

HEK293 cells were maintained in high glucose DMEM medium (Gibco-BRL, Life Technologies) with 5% fetal bovine serum (FBS, Gibco-BRL, Life Technologies) in a 5% CO₂ atmosphere. HEK293 cells were cultured on poly-L-lysine coated 18 mm cover slips and transfected with 1 μg of plasmid DNA, all in pcDNA3.1(+), encoding GRIP1c 4-7, GRIP1a or GRIP1a 4-7 or combinations of GRIP1c 4-7 and AMPA receptor subunits using the Lipofectamine 2000 method following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Cells were then processed for immunofluorescence as described below.

**Immunofluorescence of hippocampal cultures and HEK293 cells**

Double or triple label immunofluorescence detection of various antigens with specific antibodies raised in various species was done as described elsewhere (21, 26, 27). Hippocampal neurons or transfected HEK293 cells were fixed by immersion of coverslips in 4% paraformaldehyde and 4% sucrose in phosphate buffered saline (PBS) for 12 min at RT followed by permeabilization with 0.25% Triton X-100 in PBS for 5 minutes. The cultures were incubated with a mixture of the primary antibodies (defined in the legends for Figs. 3, 5 and 6 and 7), diluted in 0.25% Triton X-100 PBS for 2 hrs at room temperature. Coverslips then were washed and incubated for 1 hr at RT with a mixture of species-specific secondary antibodies all raised in donkey and conjugated to either Texas Red, FITC, and/or AMCA fluorophores (1:200 dilution in 0.25% Triton X-100 PBS, Jackson Immunoochemicals). Optimal primary antibody dilutions were
determined by dilution series. In HEK293 cells, the cell nuclei were labeled with DAPI and cell surface localization was determined with FITC-conjugated phalloidin. The coverslips were washed with PBS, and mounted using Prolong anti-fade mounting solution (Molecular Probes; Eugene, Oregon). Specificity of the immunolabeling by the anti-GRIP1c 4-7 antibody was demonstrated by blocking the binding of the primary antibody with 20 µg/ml of the antigenic peptide. Moreover, no immunolabeling was obtained when the primary antibody was omitted. Images were collected using a 60X pan-fluor objective on a Nikon Eclipse T300 microscope with a Sensys KAF 1401E CCD camera, driven by IPLab 3.0 (Scanalytics, Fairfax, VA) acquisition software. Image files were then processed and merged for color colocalization figures using Adobe PhotoShop 4.01.

Quantification of cluster density and colocalization was performed by normalizing intensity data between fluorophore channels followed by the subtraction of background fluorescence signal seen in the dendrites. To determine cluster density, three experiments were done for each antigen from which 40-50 measurements were made of randomly selected dendrites from a total of 20-30 pyramidal neurons (6-10 neurons per experiment). Each measurement was taken from a 50 µm section of dendritic shaft (with an average width of 2 µm). Cluster density was expressed as the number of clusters per 100 µm² dendrite. For colocalization, color images were merged and the position of the GRIP1c 4-7 clusters were compared with that of the receptor clusters or presynaptic markers. Colocalization was expressed as the percent of the total clusters analyzed.

*Light microscopy immunocytochemistry of rat brain sections*

This procedure has been described elsewhere (22, 25, 33). Briefly, 60 day old male Sprague-
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Dawley rats were anesthetized (80 mg/kg ketamine-HCl, 8 mg/kg xylazine, 2 mg/kg acepromacine maleate) and perfused through the ascending aorta with PLP fixative consisting of 4% paraformaldehyde, 1.37% Lysine and 0.21% sodium periodate in 0.1 M phosphate buffer (PB), pH 7.4. The frozen brain was sliced in parasagittal sections with a freezing microtome. Free-floating sections were incubated at 4 °C for 24 hours with an affinity-purified rabbit anti-GRIP1c 4-7 antibody in 0.3% Triton X-100, 0.1 M PB, pH 7.4. The washed tissue sections were incubated with a biotin-labeled anti-rabbit IgG and avidin-biotin-horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). The reaction product was visualized by incubation with 3-3’ dianimobenzidine tetrahydrochloride (DAB) in the presence of cobalt chloride and nickel ammonium sulfate. Sections were washed and mounted on gelatin-coated glass slides. No tissue immunolabeling was detected when anti-GRIP1c 4-7 antibodies were incubated with 20 μg/ml antigenic peptide or when the primary antibody was omitted.

*Preembedding electron microscopy (EM) immunocytochemistry*

For panels A, B and J of Fig. 9, 34 day old male Sprague Dawley rats were anesthetized as described above and perfused through the ascending aorta with 100 ml 0.12 M PB, pH 7.2 at RT, followed by perfusion with 250 ml of fixative (4% paraformaldehyde, 0.5% glutaraldehyde in 0.12 M PB, pH 7.2). Parasagittal vibratome sections (50 μm-thick) were cut in PBS at 4°C. Fixed brain sections were incubated with 3% normal goat serum in PBS at RT for 1 hour followed by incubation with affinity-purified rabbit anti-GRIP1c 4-7 in PBS overnight at 4°C. The antibody signal was detected using the ABC procedure (Vectastain Elite ABC kit) as instructed by the manufacturer. Sections were washed 3 times with PBS for 20 min each at RT followed by incubation with DAB/H2O2 solution for 5 minutes and washing in PBS for 20 minutes 3 times at
RT followed by post-fixation with 1% osmium tetroxide in 0.12 M PB, pH 7.2 for 45 min at RT. Then, sections were washed with 0.12 M PB, pH 7.2 for 15 min 4 times with 0.12 M PB, RT and dehydrated with the following series of cold ethanol solutions: 50%, 70%, 85%, 95%, each 3 times for 5 min each, and in 100% ethanol 3 times for 15 minutes each. Dehydration was followed by infiltration in Polybed 812 resin with propylene oxide. Sections were embedded with Polybed 812 using aclar strips and polymerized at 60 °C for 2 days.

Postembedding EM immunocytochemistry

For panels A-K of Fig. 8 and C-I and K of Fig. 9, the tissue preparation, freeze substitution, and post-embedding immunogold labeling were done as reported previously (34). Briefly, 35-70 day old male Sprague Dawley rats were anesthetized as described above, and perfused with 60 ml Ringers solution pH 6.9 at RT for 1 min followed by 800 ml fixative (4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PB, pH 7.4). Sections 300-500 μm-thick were cut with a vibratome and cryoprotected with 2 M sucrose and plunge-frozen in liquid propane cooled by liquid nitrogen (-190 °C). Samples were immersed in 1.5% uranyl acetate in anhydrous methanol at –90 °C for 30 hours before they were infiltrated with Lowicryl HM20 resin (Polysciences, Warrington, PA) and polymerized with UV light (-45 to 0 °C) during 72 hours in a Leica (Vienna, Austria) AFS freeze substitution instrument.

70-80 nm-thick sections were collected from the embedded tissue blocks on 400-mesh gold-gilded nickel grids, coated previously with a Coat-Quick “G” pen (Electron Microscopy Sciences, Fort Washington, PA), and a double sided immunoreaction procedure (35) was performed as described elsewhere (34). After sequential incubations with the affinity-purified primary antibody (or the mixture of two antibodies for double-label experiments) and with
colloidal gold-coupled species-specific secondary antibodies (or the mixture of two labeled species-specific anti-IgG secondary antibodies raised in the same species), the tissue sections were counterstained with 2% uranyl acetate for 1 min and with Sato’s lead solution (36) for 1 min, both at RT. No immunolabeling was observed when the primary antibody was omitted.

For panels 8A and 9L, the Lowicryl-emmbedded tissue block was kindly provided by Drs. Zoltan Nusser and Peter Somogyi, who have described the embedding method elsewhere (37). Briefly, rats were anesthetized with 220 mg/kg of pentobarbitone sodium (Sagatal). Rats were then perfused with 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in 0.1 M PB, pH 7.4. 500 μm-thick vibratome sections were cryoprotected in 2 M sucrose in PB followed by slam-freezing, freeze-substitution with methanol at -80°C, and embedding in Lowicryl HM20 at -50°C. A tissue block was sectioned in our laboratory and the ultrathin sections were subjected to the same immunogold procedure described above.

For quantitative analysis, we used the method of Valtschanoff and Weinberg (38). Briefly, to quantify gold particle axodendritic distance from the synapse, a line was drawn along the midline of the synaptic cleft (the zero position), equidistant from both the pre- and postsynaptic membrane. The axodendritic distance was measured as the length of a perpendicular line between the center of the gold particle and the midline of the synaptic cleft. To plot the particle density, the position of the particles were sorted into 5 nm bins. A negative or positive number indicates that the particle was located on the presynaptic or postsynaptic side of the midline, respectively. Binned data were smoothed with a five-point weighted running average using SigmaPlot (Rockware, Inc., Golden, CO).
RESULTS

GRIP1c 4-7 is a 4-PDZ domain-containing splice variant of GRIP1 that interacts with the C-terminus of GluR3

The cDNA clone GS8 was isolated from an adult rat brain cDNA library by Y2H assay using various baits when the stop codon from the pEG202 vector was used to terminate the translation of the fusion protein. We determined that the interaction of GRIP1c 4-7 with the various baits was due to the C-terminal peptide added by the pEG202 vector (S-Y-D-L) which was responsible for the binding of the baits to the class II PDZ domain(s) of GRIP1c 4-7 (for review, see ref. 39). GS8 is a 2,536 bp cDNA fragment with an ORF encoding 696 amino acids and a 3’ UTR containing a poly (A) tail. The GS8 cDNA aligned with PDZ domains 4 to 7 of the glutamate receptor interacting protein 1 (GRIP1, Fig. 1). We therefore named this protein GRIP1c 4-7 to differentiate it from the longer splice forms of GRIP1a/b (1, 2, 18). We determined by Y2H that the 50 amino acid cytoplasmic C-terminus region of GluR3 also bound to GRIP1c 4-7 even when the bait constructs had the natural stop codon of GluR3, indicating that GRIP1c 4-7 binds to the C-terminus of GluR3. Because of the interaction with GluR3 and because this is a novel splice form of GRIP1, we proceeded with its characterization.

The 5’ rapid amplification of cDNA ends (5’ RACE) using an adult rat brain Marathon-Ready cDNA library of full-length cDNAs (Clontech, Palo Alto, CA) revealed that the full-length cDNA of GRIP1c 4-7 was only 235 bases longer than GS8 and all of these bases were at the 5’ UTR. Therefore, the full-length of GRIP1c 4-7 cDNA is 2779 bases long, of which 421 bases are the 5’ UTR, 270 bases the 3’ UTR and 2,088 bases are the ORF. The GRIP1c 4-7 differs from GRIP1a/b in the N-terminal 35 amino acid residues and C-terminal 12 amino acid residues of the
deduced amino acid sequences of GRIP1c 4-7 (Fig. 1). Sequence comparison of GRIP1c 4-7 and GRIP1a/b to the rat genome (National Center for Biotechnology Information) revealed that the GRIP1c 4-7 splice form is made of 15 exons localized on chromosome 7, region q22, the same locus as that of GRIP1a/b. Exons 2-13 of GRIP1c 4-7 are shared with GRIP1a/b. Exon 14 of GRIP1c 4-7 matched only partially with an exon of GRIP1a/b because this exon also contains the 3’ UTR of GRIP1a/b. The first and fifteenth exons of GRIP1c 4-7 did not match any exons of GRIP1a/b. The first exon of GRIP1c 4-7 contains the 5’ UTR, the translational start site and the coding sequence for the GRIP1c 4-7-specific N-terminal 35 amino acids. The fifteenth exon of GRIP1c 4-7, located downstream of the last exon of GRIP1a/b, contains the coding sequence for the GRIP1c 4-7-specific C-terminal 12 amino acids, the translational stop codon and the 3’ UTR.

GRIP1c 4-7-specific antibodies recognize a 75 kDa brain-specific protein that is enriched in PSDs and co-precipitate AMPA receptors containing the GluR2/3 subunits

We raised a rabbit and a guinea pig antibody to the synthetic peptide corresponding to amino acid residues 12-26 of the N-terminus of GRIP1c 4-7. This amino acid sequence is present in GRIP1c 4-7 but not in GRIP1a/b (Fig. 1) or GRIP2. Moreover, the anti-GRIP1c 4-7 antibodies recognized GRIP1c 4-7 protein expressed in transfected HEK293 cells but did not recognize GRIP1 (see below) or GRIP2 (not shown) expressed in these transfected cells. Based on the deduced amino acid sequence of the GRIP1c 4-7 open reading frame, the calculated molecular weight of GRIP1c 4-7 is 75,420 Da. Immunoblots of rat forebrain synaptosomal and postsynaptic density (PSD) fractions (Fig. 2A lane 1, Fig. 2B lanes 1 and 3, Fig. 2C lanes 1-3 and Fig. 2D lane 1) showed that both the rabbit and guinea pig antibodies recognize a 75 kDa Mr protein. The immunoreactivity of the antibodies with the 75 kDa protein was displaced by the antigenic
splice variant of GRIP1 peptide (Fig. 2A lane 2 and Fig. 2B lanes 2 and 4). The 75 kDa GRIP1c 4-7 peptide was enriched in a brain PSD fraction (Fig. 2C lane 3) over synaptosomes (Fig. 2C lane 2) and forebrain homogenate (Fig. 2C lane 1). In PSD immunoblots, the anti-GRIP1c 4-7 did not recognize the 135 kDa Mr GRIP1a/b protein (Fig. 2D lane 1) that was recognized by an antibody to the C-terminal 12 amino acid peptide of GRIP1a/b (Fig. 2D, lane 2). In contrast, the 75 kDa GRIP1c 4-7 protein was recognized by the anti-GRIP1c 4-7 antibody (Fig. 2D lane 1) but not by the anti-GRIP1a/b antibody (Fig. 2D lane 2). It has been shown that the C-termini of GluR2 and GluR3 bind to PDZ domains 4 and 5 of GRIP1a/b (1, 3, 5) and we have shown by Y2H, as indicated above, that GRIP1c 4-7 interacts with the C-terminus of GluR3, which was expected since GRIP1c 4-7 has the same PDZ domains 4 and 5 as GRIP1a/b. The interaction of GRIP1c 4-7 with AMPA receptors also occurred in detergent extracts from brain. An antibody to GRIP1c 4-7 co-immunoprecipitated the AMPA receptors (GluR2/3) from detergent extracts of cortical/hippocampal membranes, as shown by immunoblotting with a rabbit anti-GluR2/3 antibody (Fig. 2E, lane 1) but not by the pre-immune serum (Fig. 2E lane 2). The immunoprecipitated protein comigrated with the 105 kDa Mr protein recognized by the anti-GluR2/3 antibody in the total detergent extract (Fig. 2E, lane 3).

Several experiments were carried out to test whether GRIP1c 4-7 interacted with GABAARs. Various GRIP1c 4-7 fusion proteins were used in pull-down experiments from rat brain extracts prepared with several different detergents and buffers. Likewise, we used both the rabbit and guinea pig antibodies to GRIP1c 4-7 in combination with antibodies to several GABAAR subunits aiming to demonstrate coprecipitation of GRIP1c 4-7 and GABAARs from brain extracts prepared with several detergents and buffers. We did precipitation with the anti-GRIP1c 4-7 antibodies followed by immunoblotting with GABAAR subunit antibodies or
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radioligand binding to GABA\(_A\)Rs. We also did precipitations with anti-GABA\(_A\)R antibodies followed by immunoblotting with anti-GRIP1c 4-7 antibodies. Contrary to the aforementioned results with GRIP1c 4-7 and GluR2/3, where we could easily detect coprecipitation of GRIP1c 4-7 and AMPA receptors, we could not demonstrate coprecipitation of GRIP1c 4-7 and the GABA\(_A\)Rs from brain extracts.

Immunoblots of forebrain homogenates (Fig. 2F) show that GRIP1c 4-7 is undetectable at embryonic day 16 (E16) and postnatal day 0 (P0), but show a very faint band corresponding to GRIP1c 4-7 at P3 and P8, suggesting that GRIP1c 4-7 is expressed at low levels at these time points. GRIP1c 4-7 is expressed at higher levels at P13, P28, P45 and P90. This time course of GRIP1c 4-7 expression during development contrasts with the developmental profile of GRIP1a/b expression (2), where it is robustly expressed early in development and increases until P6-8 after which expression levels decrease slightly. Immunoblots of homogenates from various tissues in the adult rat (Fig. 2G) shows that the 75 kDa GRIP1c 4-7 protein is expressed in the forebrain (Br) but not in heart (Ha), lung (Lu), liver (Li), spleen (Sp) or testes (Te). However, the antibody recognized a 58 kDa protein in heart and liver and a 55 kDa protein in heart, lung, liver, spleen and testes, suggesting that other splice forms of GRIP1c 4-7 might be expressed in other tissues.

**Expression of GRIP1c 4-7 in host HEK293 cells**

When GRIP1c 4-7 was expressed in HEK293 cells after transfection, a significant proportion of the immunofluorescence localized to the plasma membrane (Fig. 3A and 3C, arrows), as shown by colocalization with phalloidin immunofluorescence (Fig. 3B and 3C, arrows). Significant GRIP1c 4-7 immunofluorescence also localized in the cell cytoplasm and in the perinuclear region, which did not colocalize with phalloidin (Fig. 3A and B, arrowheads).
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contrast, when these cells were transfected with GRIP1a, immunofluorescence for GRIP1a localized in the cytoplasm (Fig. 3D and 3F, arrowheads), frequently forming intracellular aggregates showing no colocalization with phalloidin (Fig. 3E and 3F, arrows), indicating that GRIP1a does not translocate to the plasma membrane. No immunofluorescence was detected when either anti-GRIP1c 4-7 or anti-GRIP1a/b antibodies were used to label non-transfected human cells (not shown).

In order to determine whether the surface distribution of GRIP1c 4-7 resulted from the absence of PDZ domains 1-3 and the associated linker regions of GRIP1a or from the presence of the N-terminal 35 amino acid and C-terminal 12 amino acid unique regions of GRIP1c 4-7, we constructed a truncated form of GRIP1a equivalent to GRIP1c 4-7 where the PDZ domains 1-3 of GRIP1a and the associated linker regions were deleted. We call this engineered truncation GRIP1a 4-7. Transfection of HEK293 cells with GRIP1a 4-7 indicated that this form exhibited a predominantly intracellular and perinuclear distribution (Fig. 3G, arrowheads), although it also showed some surface distribution (Fig. 3G and 3H, arrows) as seen with GRIP1c 4-7. Note, however, that the surface immunofluorescence of GRIP1a 4-7 was considerably less intense than that of GRIP1c 4-7. A GRIP1c 4-7 chimera construct where the C-terminal 12 amino acids of GRIP1c 4-7 were replaced with those of GRIP1a/b (GRIP1c 4-7ΔC12) showed a distribution (not shown) similar to the wild-type GRIP1c 4-7 (Fig. 3A). Conversely, when the C-terminal 12 amino acids of the truncation construct GRIP1a 4-7 was replaced with those of GRIP1c 4-7 (GRIP1a 4-7ΔC12), this chimeric construct showed a distribution (not shown) similar to the GRIP1a 4-7 (Fig. 3G). These results suggest that the N-terminal 35 amino acid region of GRIP1c 4-7 specifies its surface localization.
**GRIP1c 4-7 interacts with AMPA receptor GluR2/3 subunits in HEK293 cells**

We also tested the interaction of GRIP1c 4-7 with AMPA receptor subunits GluR1, GluR2 or GluR3. As described above, when GRIP1c 4-7 was expressed alone, it distributed on the plasma membrane and diffusely in the cytoplasm and the perinuclear region. When co-expressed with GluR1 (Fig. 3J-L), GRIP1c 4-7 maintained its normal distribution in the cell surface (Fig. 3J and L, arrows) and in the cytoplasm (Fig. 3J and L, arrowheads), while GluR1 was retained in intracellular membranes, most likely the endoplasmic reticulum (ER, Fig. 3J and L, arrowheads). Therefore, GluR1 showed no colocalization with GRIP1c 4-7, indicating that GRIP1c 4-7 does not interact with GluR1. In contrast, when GRIP1c 4-7 was cotransfected with either GluR2 (Fig. 3M-O) or GluR3 (Fig. 3P-R), GRIP1c 4-7 colocalized with both GluR2 and GluR3. However, GRIP1c 4-7 and GluR2 colocalized in the perinuclear region and in intracellular aggregates (Fig. 3M-O, arrowheads) as well as on the plasma membrane (Fig. 3M-O, arrows), while GRIP1c 4-7 and GluR3 colocalized in intracellular compartments and the perinuclear region (Fig. 3P-R, arrowheads). In these cells, GRIP1c 4-7 did not accumulate on the plasma membrane. These experiments, the Y2H experiments and the co-precipitation experiments shown above indicated that GRIP1c 4-7 interacts with both GluR2 and GluR3.

Parallel experiments with the GRIP1a 4-7 engineered truncation indicated that GRIP1a 4-7 also interacted with GluR2 and GluR3 but not with GluR1 (not shown). Therefore, the specificity of the interaction of GRIP1c 4-7 and GRIP1a 4-7 with AMPA receptor subunits is similar to that of full-length GRIP1a/b (1, 3).
Light microscopy immunocytochemical localization of GRIP1c 4-7 in the rat brain

Immunocytochemistry of rat brain sections (Fig. 4) indicated that the GRIP1c 4-7 antibody showed strong reactivity throughout the brain, including the olfactory bulb, cerebral cortex, corpus striatum, thalamus, hippocampus, substantia nigra and the cerebellum (Fig. 4A). At higher magnification, immunoreactivity concentrated in the cell soma and neuropil, particularly in areas enriched in synapses such as stratum lucidum and pyramidale of the hippocampus (Fig. 4B and C). This immunoreactivity in the hippocampus is consistent with the enrichment of GRIP1c 4-7 in the synapses made by mossy fiber terminals originating from the granule cells onto the proximal dendrites of the pyramidal cells. The strong immunoreactivity in the mossy fiber synapses was obtained with both the rabbit and guinea pig antibodies to GRIP1c 4-7, although the immunoreactivity in these synapses was very strong with the rabbit antibody as shown in figure 4B and C. Strong immunoreactivity was also seen in the glomeruli of the olfactory bulb (Fig. 4D), the corpus striatum (Fig. 4E), the molecular layer of the cerebellum (Fig. 4F) and in various layers of the cerebral cortex (Fig. 4G).

GRIP1c 4-7 forms clusters that colocalize with GABA\textsubscript{A}R and AMPA receptors in GABAergic and glutamatergic synapses in cultured hippocampal neurons

This was demonstrated with both the rabbit (Fig. 5A-C) and the guinea pig (Fig. 5D-O) anti-GRIP1c 4-7 antibodies, which gave similar results. In these studies, we preferentially used the guinea pig antibody because we could do more antibody combinations for triple-label immunofluorescence experiments. Therefore, quantifications were done using the guinea pig antibody. GRIP1c 4-7 formed clusters that colocalized with GABAergic synapses (Fig. 5A-F and J-L) and glutamatergic synapses (Fig. 5G-I and M-O). GRIP1c 4-7 clusters (Fig. 5A and D)
colocalized with GABA_A receptor clusters (Fig. 5B and E) at both GABAergic synapses (colocalizing with GAD-containing boutons; Fig. 5C and F, filled arrows) and non-GABAergic synapses (not colocalizing with GAD, Fig. 5C and F, filled arrowheads). Some GRIP1c 4-7 clusters neither colocalized with GABA_AR nor GAD (Fig. 5A-F, empty arrowheads). A significant percentage (68 ± 3%, mean ± S.E.M.) of all the GABA_AR clusters colocalized with GRIP1c 4-7 clusters. Moreover, 91± 2% of the GAD^+ synapses colocalized with GRIP1c 4-7 clusters. Thus, GRIP1c 4-7 was present in the majority of GABAergic synapses and a high proportion of GABA_AR clusters colocalized with GRIP1c 4-7. Note the larger size of the GABA_AR clusters and GRIP1c 4-7 clusters (filled arrows) when they are associated with GABAergic terminals (therefore at GABAergic synapses) over the size of the clusters not associated with GAD (filled arrowheads). GRIP1c 4-7 clusters (Fig. 5G) also colocalized with PSD95 (Fig. 5H) and vGlut1 (Fig. 5I), as shown by filled arrows. PSD-95 and vGlut1 are glutamatergic post-synaptic and pre-synaptic markers, respectively. Similarly, GRIP1c 4-7 clusters (Fig. 5M) colocalized with GluR2/3 (Fig. 5N) and SV2 (Fig. 5O) as shown by filled arrows. A significant percentage (62 ± 3%) of PSD-95 clusters (Fig. 5H), or 59 ± 3% of GluR2/3 clusters (Fig. 5N), and 71 ± 2% of vGlut1-containing terminals colocalized with GRIP1c 4-7 clusters. Filled arrowheads show PSD-95 clusters associated with GRIP1C 4-7 but not with vGlut1 and empty arrowheads show GRIP1C 4-7 clusters neither associated with PSD-95 clusters nor vGlut1-containing terminals (Fig. 5G-I).

The experiments also showed that I) a significant percentage of GRIP1c 4-7 clusters (59 ± 2%) colocalized with GABA_AR (β2/3) clusters; II) 40 ± 3% of GRIP1c 4-7 clusters colocalized with AMPA receptor (GluR2/3) clusters; and III) 44 ± 3% of GRIP1c 4-7 clusters colocalized with PSD-95 clusters. Immunofluorescence with antibodies to GRIP1c 4-7, GABA_ARs and
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AMPAs showed that 90% of the GRIP1c 4-7 clusters colocalized with GABAAR or AMPA receptor clusters, indicating that the majority of GRIP1c 4-7 clusters are associated with these neurotransmitter receptors and/or GABAergic and glutamatergic synapses.

In mature hippocampal cultures, some GRIP1c 4-7 clusters associate with GABAARs in the absence of presynaptic contacts

These experiments did not indicate whether the GRIP1c 4-7 clusters that associated with GABAARs or PSD-95 were pre or post-synaptically localized, since we have previously shown that, in these low density hippocampal cultures with limited GABAergic innervation, a significant amount of GABAAR clusters that did not localize at GABAergic synapses were associated with glutamatergic terminals, forming mismatched synapses (21, 27, 40). Therefore, the GABAAR clusters not associated with GAD were not necessarily devoid of a presynaptic terminal. They could be mismatched to a presynaptic glutamatergic terminal. Moreover, vGlut1 might not have labeled all glutamatergic terminals (ie. the ones containing vGlut2 or vGlut3 but not vGlut1). Therefore, we did triple label experiments to determine whether the association of GRIP1c 4-7 with GABAAR (Fig. 5J-L) and glutamatergic receptors (Fig. 5M-O) could occur in the absence of pre-synaptic innervation (i.e. in the absence of the presynaptic vesicle marker SV2, that labels both GABAergic and glutamatergic presynaptic terminals). Consistent with the previous results, a significant percentage (81 ± 2%) of synapses (containing SV2) had colocalizing GRIP1c 4-7. Significant percentages of synaptic (SV2+) GABAAR clusters (79 ± 2%) and AMPA receptor clusters (83 ± 2%) colocalized with GRIP1c 4-7 (Fig. 5J-O, arrows). Nevertheless, significant proportions of non-synaptic (SV2−) GABAAR (46 ± 4%) and AMPA receptor clusters (33 ± 4%) also colocalized with GRIP1c 4-7 (Fig. 5J-O, filled arrowheads). A few GRIP1c 4-7 clusters
were neither associated with GABA\(_A\)R, AMPA receptors or synapses (Fig. 5J-O, empty arrowheads). These experiments showed that, in the absence of presynaptic elements, GRIP1c 4-7 clusters could colocalize with GABA\(_A\)R clusters or AMPA receptor clusters. Therefore, GRIP1c 4-7 immunoreactivity is also associated with non-synaptic GABA\(_A\)R and AMPA receptor clusters. This result suggested the possibility that some of the synaptic GRIP1c 4-7 immunoreactivity might be postsynaptically localized. The postsynaptic colocalization of GRIP1c 4-7 with GABA\(_A\)R clusters was confirmed in experiments with transfected hippocampal neurons and by EM immunogold of intact brain, as shown below.

**In hippocampal cultures, the association of GRIP1c 4-7 and GABA\(_A\)Rs precedes the onset of GABAergic innervation**

We studied the time course development of GRIP1c 4-7 clusters in relation to the development of GABA\(_A\)R clusters and GABAergic innervation in hippocampal cultures (Fig. 6). At 3 and 5 days in culture (Fig. 6A and B, respectively) GRIP1c 4-7 (arrowheads, Fig. 6A1 and B1) and the GABA\(_A\)Rs (arrowheads, Fig. 6A2 and B2) formed clusters, some of the larger and brighter of which colocalize with one another (arrowheads, Fig. 6A4 and B4). This colocalization occurred in the absence of GABAergic innervation of the pyramidal cells, as determined by the absence of presynaptic GAD immunofluorescence (Fig. 6A3 and B3). No GAD\(^+\) neurons were detected at these ages. No clusters of GRIP1c 4-7 or GABA\(_A\)R were observed before day 3. At 10 and 15 days in culture (Fig. 6C and D, respectively), after the onset of GABAergic innervation as determined by the presence of GAD immunofluorescent puncta (Fig. 6C3 and D3), clusters of GRIP1c 4-7 (Fig. 6C1 and D1) and the GABA\(_A\)Rs (Fig. 6C2 and D2) colocalize both at GABAergic synapses (arrows, Fig. 6C4 and D4) and outside GABAergic synapses (arrowheads,
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Fig. 6C4 and D4). These results suggest that I) the appearance and colocalization of GRIP1c 4-7 with GABA\(_A\)R clusters is simultaneous and independent of GABAergic innervation and that II) GABAergic innervation induces the accumulation of GABA\(_A\)R (21) and GRIP1c 4-7 at GABAergic synapses.

Transfection of Cultured Hippocampal Neurons shows that GRIP1c 4-7 can colocalize with postsynaptic GABA\(_A\)Rs

We transfected hippocampal neurons with GRIP1c 4-7 tagged with the hemagglutinin (HA) epitope at the N-terminus. In order to distinguish the exogenously expressed HA-GRIP1c 4-7 from the endogenous GRIP1c 4-7, the HA-GRIP1c 4-7 was visualized with a mouse mAb to the HA epitope. Fig. 7 shows that HA-GRIP1c 4-7 clusters (green) colocalized (arrows) with GABA\(_A\)R \(\gamma_2\) subunit clusters (red) at GABAergic synapses as revealed by their colocalization with the vesicular GABA transporter (VGAT, blue), which labeled the presynaptic GABAergic terminals. The VGAT immunofluorescence in Fig. 7 originated from a nearby interneuron that was not transfected with HA-GRIP1c 4-7 since it showed no HA immunoreactivity (not shown), indicating that the HA-GRIP1c 4-7 present in these GABAergic synapses was postsynaptic. In addition, we observed instances where the HA-GRIP1c 4-7 colocalized with the GABA\(_A\)R \(\gamma_2\) subunit in the absence of VGAT (arrowheads) indicating that HA-GRIP1c 4-7 and the GABA\(_A\)R also colocalized in clusters outside the GABAergic synapses. Interestingly, the expression of HA-GRIP1c 4-7 in transfected neurons did not significantly affect GABA\(_A\)R cluster density (13.9 ± 0.3, \(n=38\) dendrites in transfected neurons vs. 14.5 ± 0.6, \(n=42\) dendrites in non-transfected neurons, \(p = 0.17\); cluster density values are the number of clusters per 100 \(\mu\)m\(^2\) of dendrite surface). Although the aforementioned studies with cultured hippocampal neurons indicate that
GRIP1c 4-7 and GABA\(_A\)R clusters can colocalize with each other postsynaptically and in the absence of GABAergic innervation, they do not rule out the presence of GRIP1c 4-7 in the presynaptic GABAergic terminals. The EM experiments shown below revealed that GRIP1c 4-7 is localized both pre and postsynaptically in GABAergic and glutamatergic synapses.

**Electron Microscopy Immunocytochemistry**

Single-label postembedding immunogold with both rabbit and guinea pig anti-GRIP1c 4-7 antibodies showed that GRIP1c 4-7 localized to ER (Fig. 8A, arrows), Golgi apparatus (Fig. 8B, arrows) and along microtubules (Fig. 8C, arrows), consistent with the notion that GRIP1c 4-7, as other GRIP family proteins, plays a role in protein trafficking to the synapse (12, 41). GRIP1c 4-7 was also frequently found at synapses. We often found GRIP1c 4-7 localized on the pre- and postsynaptic membranes (Fig. 8D, H and I, arrow), on the post synaptic density (Fig. 8E), in the postsynaptic cytoplasm (Fig. 8F and I filled arrowhead, and J) and presynaptically, sometimes close to the active zone (Fig. 8I, empty arrowhead) or more distally in the presynaptic bouton (Fig. 8G and K).

Preembedding immunoperoxidase experiments showed that GRIP1c 4-7 can be localized postsynaptically at symmetric type II synapses (Fig. 9A and B, arrows) and in asymmetric type I synapses (Fig. 9J, arrow). However not all synapses showed immunolabeling (Fig. 9J, asterisk). In addition, double-label immunogold experiments with GRIP1c 4-7 (Fig. 9C-I, K and L, arrows) and anti-GABA (Fig. 9G and H, arrowheads) or either GABA\(_A\)R \(\alpha1\) (Fig. 9C-E, arrowheads) or \(\beta2/3\) (Fig. 9F, arrowheads) subunits showed that GRIP1c 4-7 is localized at GABAergic synapses with type II morphology. Likewise, GRIP1c 4-7 (Fig. 9K and L, arrows) localized at glutamatergic asymmetric synapses as determined by double-label immunogold experiments with
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the glutamatergic presynaptic marker vGlut1 (Fig. 9K and L, arrowheads). Note that in the
double-label experiments of Fig. 9, gold particles corresponding to GRIP1c 4-7 immunoreactivity
in GABAergic synapses were located at the synapse (Fig. 9C, arrows), both at the presynaptic
terminal and at the synapse (Fig. 9D and G), at the presynaptic terminal (Fig. 9E and F), pre and
postsynaptically (Fig. 9I) or postsynaptically (Fig. 9H). A similar distribution was observed in
type I glutamatergic synapses as shown above in Fig. 8 and in Fig. 9 K and L. Nevertheless, the
localization of an individual gold particle might not correspond to the exact localization of the
antigen due to the size of the primary and secondary antibodies and the size of the gold particle.
Thus, for 12 and 18 nm gold particles, the antigen could be located up to 29 and 32 nm,
respectively, from the center of the gold particle (42). Therefore, quantitative analysis of the
distribution of gold particles is necessary to ascertain the presynaptic or postsynaptic localization
of GRIP1c 4-7. We quantified separately the axodendritic distribution of GRIP1c 4-7 gold
particle density in GABAergic synapses and in glutamatergic synapses. GABAergic synapses
were identified by double label immunogold experiments with anti-GRIP1c 4-7 and anti-GABA
or anti-GABA\(_\text{A}\)R antibodies. Glutamatergic synapses were identified by double label experiments
with anti-GRIP1c 4-7 and anti-vGlut1 or anti-GluR2/3 AMPA receptor subunit antibodies. The
distribution of gold particles in GABAergic and glutamatergic synapses is shown in Fig. 9M and
9N, respectively. In both GABAergic and glutamatergic synapses, the particle density
corresponding to GRIP1c 4-7 immunoreactivity was highest in the postsynaptic membrane and
postsynaptic density. Within 75 nm on each side of the synaptic cleft midline of GABAergic
synapses, 51% of the gold particles were postsynaptic and 49% were presynaptic. The
corresponding proportion of gold particles in the equivalent window of glutamatergic synapses
was 55% postsynaptic and 45% presynaptic. It should be noted that the high density of gold
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particles in the glutamatergic synapses extended further in the postsynaptic cytoplasm than in the GABAergic synapses. This is attributed to the thicker postsynaptic density of glutamatergic type I synapses (≥ 40 nm thick) compared to the much thinner GABAergic type II postsynaptic density (≤ 20 nm thick). Thus, in the intact brain, GRIP1c 4-7 localized pre- and postsynaptically in both GABAergic and glutamatergic synapses.

DISCUSSION

We have shown that GRIP1c 4-7 is an isoform of GRIP1 that concentrates in GABAergic and glutamatergic synapses in the intact brain and cultured hippocampal neurons. It has been reported that GRIP1a/b localizes to GABAergic and glutamatergic synapses in hippocampal cultures (2, 41) but that in the intact adult brain GRIP1a/b might not be present at GABAergic synapses (41) since EM immunocytochemistry methods have shown that GRIP1a/b and GRIP2/ABP are mainly present in type I asymmetric (excitatory) synapses, concentrating postsynaptically, with very limited (less than 2%) presence in inhibitory type II symmetric synapses (2, 5, 41). We have found that GRIP1c 4-7, as GRIP1a/b, is present in asymmetric type I synapses both pre and postsynaptically. However, contrary to other forms of GRIP, we have found GRIP1c 4-7 to be present in type II GABAergic synapses, which brings attention to a possible role of GRIP proteins in GABAergic transmission. In randomly selected fields 29.7% of the GABAergic synapses and 30.8% of the glutamatergic synapses were labeled with gold particles corresponding to GRIP1c 4-7 immunoreactivity. The combination of our results and the results from the literature suggests that in the intact brain there is differential postsynaptic localization in the GABAergic synapses of GRIP1c 4-7 and GRIP1a/b, the former being present and the latter being absent from the postsynaptic GABAergic complex. The localization of
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GRIP1c 4-7 in both GABAergic and glutamatergic synapses also occurred in hippocampal cultures although more GRIP1c 4-7 clusters colocalized with GABA_ARs than with AMPA receptor clusters (59% and 40%, respectively). Moreover, we have shown that in transfected hippocampal cells, GRIP1c 4-7 and GABA_ARs colocalized within the same neuron postsynaptically at GABAergic synapses and outside GABAergic synapses. The postsynaptic localization of GRIP1c 4-7 was also supported by the observed GRIP1c 4-7 enrichment in a brain PSD fraction. The results with hippocampal cultures also show the existence of colocalizing GRIP1c 4-7 and GABA_ARs clusters in the absence of GABAergic innervation. Nevertheless, the results with hippocampal cultures do not exclude the presence of GRIP1c 4-7 in the presynaptic GABAergic terminals. In fact, the EM immunogold of intact brain shows that GRIP1c 4-7 is also localized presynaptically in GABAergic and glutamatergic terminals.

Differences in localization and function between GRIP1c 4-7 and GRIP1a/b could derive from the different amino acid sequences of the N- and C- termini of GRIP1c 4-7 and the equivalent regions of GRIP1a/b and from the absence of PDZ domains 1-3 in GRIP1c 4-7, since GRIP1a/b interacts with some proteins through these domains such as GRASP-2, -3 and -4 and Fras1, which interact with PDZ domains 1-3 of GRIP1a/b (17, 64). The development of antibodies specific for each splice form will allow to shed light on the relationship between localization and function of the various splice forms.

We have shown that GRIP1c 4-7 interacts with the C-terminus of AMPA receptor GluR2/3 subunits and that GRIP1c 4-7 and AMPA receptors coprecipitate from brain extracts. This was expected since others have shown that the interaction of GRIP1a/b with the C-terminus of GluR2/3 occurs via PDZ domains 4 and 5 of GRIP1a/b (1, 2), which are identical to those of GRIP1c 4-7. We have also shown that GRIP1c 4-7 is partly localized postsynaptically in
glutamatergic synapses where AMPA receptors concentrate. It has been shown that GRIP1a/b binds to the microtubule-based motor protein kinesin-5 through the linker region between PDZ6 and 7 (12), which is also present in GRIP1c 4-7. Therefore, it is expected that GRIP1c 4-7 will bind to kinesin-5. This is supported by the association of GRIP1c 4-7 with microtubules as shown by EM immunocytochemistry. Thus, the interaction of GRIP1c 4-7 with AMPA receptors, its postsynaptic localization in glutamatergic synapses and its association with microtubules support the notion that GRIP1c 4-7, like GRIP1a/b, is involved in both vesicular trafficking of AMPA receptors along dendritic microtubules and synaptic targeting of AMPA receptors to the synapse (2, 4, 7, 12).

We have also shown that GRIP1c 4-7 is localized postsynaptically in GABAergic synapses, colocalizing with GABAARs in cultured neurons and intact brain. Nevertheless, GRIP1c 4-7 does not seem to directly interact with GABAARs since I) while we could co-precipitate GRIP1c 4-7 and AMPA receptors from brain extracts, we could not convincingly demonstrate co-precipitation of GRIP1c 4-7 and GABAARs after preparing brain extracts with various detergents and buffers and II) the C-termini of GABAAR subunits are extracellular and do not have PDZ binding sequences, although this notion does not preclude the interaction of GABAARs with non-PDZ domains of GRIP1c 4-7. Therefore, GRIP1c 4-7 might be a component of the postsynaptic complex in some GABAergic synapses as gephyrin (43-46) and dystrophin (47-50) are. Gephyrin and dystrophin are present in GABAergic postsynaptic complexes but don’t directly interact with GABAARs (51). Some proteins that have been shown to directly interact with GABAARs such as GABARAP (52-54), Plic-1 (55), GRIF-1 (56), AP2 (57), calcineurin (58) and BIG2 (59) are involved in trafficking and/or turnover of GABAARs, although GABARAP might also be involved in anchoring GABAARs at the synapse (52) and calcineurin is
involved in plasticity of some GABAergic synapses (58). Other proteins that interact with the GABA\(_A\)Rs (60-63) do not seem to concentrate at GABAergic postsynaptic dendrites. In addition, GRIP1c 4-7 is present in the presynaptic boutons of both GABAergic and glutamatergic synapses. Both GRIP1a/b and GRIP2/ABP are also present in presynaptic terminals (2, 41). Therefore, GRIP1c 4-7 must play a complex role in both glutamatergic and GABAergic synaptic function.

GRIP1a/b and GRIP2/ABP bind to EphB2/EphA7 receptors, Ephrin B1 ligand, and liprin-\(\alpha\) via PDZ domain 6 (13, 14) and to the Ras GEF, GRIP-associated protein-1 (GRASP-1) via PDZ domain 7 (64). Therefore, it is likely that GRIP1c 4-7, containing the same PDZ domains, also interacts with these molecules, therefore being involved in coupling AMPA receptors and other molecules to ephrinB1 signaling (4), Ras signaling (64) and liprin-\(\alpha\) (14). Some of these signaling mechanisms could also operate in GABAergic synapses. GRIP1a/b has been implicated in synaptic turnover of AMPA receptors during LTD (10, 11, 65-67). Thus, GRIP1c 4-7 might be involved in LTD since these studies did not distinguish between the various GRIP1 splice forms including GRIP1c 4-7.

GRIP1 and GRIP2 exhibit alternate splicing with N-terminal peptide sequences generating differentially palmitoylated isoforms. Thus, GRIP1b (18) as well as a splice form of GRIP2 (pABP-L, ref. 19) contain short N-terminal peptide sequences that are palmitoylated. Although presumably not palmitoylated (i.e. there are no cysteines within the N-terminal 35 amino acid region), GRIP1c 4-7 readily translocates to the plasma membrane in transfected HEK293 cells, while GRIP1a and other non-palmitoylated forms of GRIP form cytoplasmic aggregates (Fig. 3D; ref. 19). We have shown that the membrane association of GRIP1c 4-7 is not due to the absence of PDZ domains 1-3 since an equivalent truncated form of GRIP1a does not translocate to the
plasma membrane as readily as GRIP1c 4-7. Moreover, swapping the C-termini of GRIP1c 4-7 and the truncated GRIP1a construct (GRIP1a 4-7) did not change the targeting of the two isoforms, indicating that translocation of GRIP1c 4-7 to the plasma membrane is related to the N-terminal 35 amino acids of GRIP1c 4-7. These 35 amino acids contain a putative myristylation site (amino acids 27-32). Myristylation may serve to tether GRIP1c 4-7 to the plasma membrane allowing it to interact with synaptic membrane proteins such as AMPA receptors, ephrin receptors, ephrin ligands and liprin-α as the palmitoylated isoforms of the GRIP family do (18, 19).

Other differentially spliced isoforms of GRIP have been described in rat. Thus, GRIP1 and GRIP2 can contain 55 and 42 amino acid insertions, respectively, between PDZ domains 3 and 4 (2). The functional significance of these splice variants are unknown. In addition, a short form of GRIP1, DLX interacting protein-2 (DIP2), is a truncated form of GRIP1a/b lacking PDZ1 and the majority of PDZ2, and there is also a 41 amino acid deletion between PDZ3 and PDZ4 (68). DIP2 co-activates transcription by interacting with DLX homeodomain proteins. Therefore some splice forms of GRIP1 might regulate gene transcription. The high heterogeneity of GRIP1 and GRIP2 isoforms and their modular design for molecular interactions have the potential for specificity and involvement in a wide range of signal events.

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interacts with gephyrin but is not involved in receptor anchoring at the synapse. Proc Natl Acad Sci U S A 97:8594-8599.


**FIGURE LEGENDS**

Figure 1. **Alignment of rat GRIP1c 4-7 with rat GRIP1a amino acid sequence.**  
A, schematic alignment showing that GRIP1c 4-7 is a short splice variant of GRIP1 containing PDZ domains 4-7. Amino acids 36-684 of GRIP1c 4-7 are identical to amino acids 452-1100 of GRIP1a. Broken lines at both the N- and C-termini of GRIP1c 4-7 indicate the two amino acid sequences where GRIP1c 4-7 differs from GRIP1a. The specific N- and C-terminal sequences are shown on top of the diagram.  
B, amino acid sequence alignment of the full length GRIP1c 4-7 with the corresponding region of GRIP1a. Dots represent amino acids of GRIP1 that are identical to those of GRIP1c 4-7 in the common region. PDZ domains 4-7 are underlined. Asterisks in A and B mark the amino acid sequence of the synthetic peptide used to generate anti-GRIP1c 4-7 antibodies.

Figure 2. **Immunoblots of rat brain fractions with anti-GRIP1c 4-7 antibodies.**  
A, in immunoblots of crude rat forebrain synaptosomal fraction a rabbit anti-GRIP1c 4-7 recognizes a protein of 75 KDa Mr (A, lane 1). The interaction of the antibody with the protein was blocked by incubating the antibody with 5 µg/ml of the synthetic antigenic peptide (A, lane 2).  
B, Immunoblots of a fraction enriched in post-synaptic densities (PSD) with two anti-GRIP1c 4-7 antibodies raised in rabbit (B, lanes 1 and 2) or in guinea pig (B, lanes 3 and 4). The immunoreactivity of the 75 kDa protein was blocked with 5 µg/ml of synthetic antigenic peptide (B, lanes 2 and 4).  
C, Immunoblot of rat forebrain homogenate (C, lane 1), synaptosomes (C, lane 2) and a fraction enriched in PSDs (C, lane 3) with a rabbit anti-GRIP1c 4-7 antibody. The same amount of protein (12 µg) was transferred to each lane.  
D, immunoblot of a rat fraction
enriched in PSDs, using a guinea pig antibody to GRIP1c 4-7 (D, lane 1) and a guinea pig antibody to the C-terminal 12 amino acids of GRIP1a/b (D, lane 2). The 135 kDa Mr GRIP1a/b protein was recognized by the anti-GRIP1a/b but not by the anti-GRIP1c 4-7 antibody. Likewise, the 75 kDa Mr GRIP1c 4-7 protein was recognized by the anti-GRIP1c 4-7 antibody but not by the GRIP1a/b antibody. E, a guinea pig anti-GRIP1c 4-7 antibody co-precipitated GluR2/3 from detergent extracts of a rat cortical/hippocampal crude synaptosomal fraction. Immunoblot with rabbit anti-GluR2/3 antibody shows the presence of the 105 kD GluR2/3 protein in the precipitate (lane 1). This protein was absent in the precipitates with the pre-immune serum (lane 2). A protein of the same mobility is revealed in the detergent extracts with anti-GluR2/3 (lane 3). F, immunoblot with the rabbit anti-GRIP1c 4-7 of forebrain homogenates from rats of different ages. GRIP1c 4-7 is undetectable at E16 and P0, is weakly expressed at P3 and P8 and strongly expressed between P13 and P90. G, immunoblot of homogenates with the rabbit anti-GRIP1c 4-7 antibody shows that the 75 kD GRIP1c 4-7 is expressed in the rat forebrain (Br) but not in homogenates of heart (Ha), lung (Lu), liver (Li), spleen (Sp), and testes (Te). However, 55 and 58 kD proteins, which may correspond to shorter GRIP1c 4-7 splice variants, are recognized by the antibody in other tissues.

Figure 3. Expression of GRIP1c 4-7 in transfected HEK293 cells and interaction with AMPA receptors. A-I, double-label immunofluorescence of HEK293 cells transfected with GRIP1c 4-7 (A-C), GRIP1a (D-F) or GRIP1a 4-7 (G-I) revealed with the guinea pig anti-GRIP1c 4-7 (A) and mouse monoclonal anti-GRIP1a/b antibody (D and G). Phalloidin fluorescence (B, E and H) bound to F-actin was used to reveal the plasma membrane (arrows). Cell nuclei were visualized with DAPI fluorescence (C, F and I). These panels also show color overlays. A-C,
transfected GRIP1c 4-7 is distributed in the plasma membrane (A and C, arrows) as well as in the perinuclear region and in cytoplasmic aggregates (A and C, arrowheads). **D-F**, transfected GRIP1a is found in intracellular aggregates (D, arrowheads) but not on the cell surface (D, arrows). **G-I**, GRIP1a 4-7, an engineered form of GRIP1a, equivalent to GRIP1c 4-7, distributed both intracellularly (G, arrowheads) as well as on the cell surface (G, arrows). **J-R**, HEK293 cells were cotransfected with GRIP1c 4-7 (J-R) and GluR1 (J-L) or GluR2 (M-O) or GluR3 (P-R). Double label immunofluorescence using combinations of guinea pig anti-GRIP1c 4-7 (J, M and P) with rabbit anti-GluR1 (K) or rabbit anti-GluR2/3 (N and Q). DAPI fluorescence and color overlays are shown in L, O and R. Arrows show surface localization and arrowheads show intracellular localization. GRIP1c 4-7 interacts with GluR2 and GluR3 but not GluR1 (see results). Secondary antibodies used were FITC-conjugated goat anti-rabbit (K, N and Q), Texas Red-conjugated goat anti-mouse (D and G) and Texas Red conjugated goat anti-guinea pig (A, J, M and P). The illustrated expression patterns were observed in 56 out of 63 cells (56/63) for A-C; 77/90 for D-F; 75/92 for G-I; 19/19 for J-L; 30/41 for M-O and 57/60 for P-R. For each experimental condition, the results were obtained from 2-4 transfection experiments. Scale bar, 10 μm.

**Figure 4. Light microscopy immunocytochemistry of rat brain with anti-GRIP1c 4-7.** A, the rabbit anti-GRIP1c 4-7 immunoreactivity is ubiquitously distributed throughout the brain, including the olfactory bulb (OB), cerebral cortex (CC), corpus striatum (CS), thalamus (TH), hippocampus (HP), substantia nigra (SN), pontine nucleus (PN) and the cerebellum (CB). **B-G**, several regions are shown at higher magnification including hippocampus and dentate gyrus (B), CA2 region of the hippocampus (C), olfactory bulb (D), corpus striatum (E), cerebellum (F) and...
cerebral cortex (G). Note in B and C the very high punctate immunoreactivity corresponding to
the synapses that mossy fibers make onto the proximal dendrites of the pyramidal cells in the
stratum lucidum and stratum pyramidale. Also note strong labeling in the glomeruli of the
olfactory bulb (D), the corpus striatum (E), the molecular layer and Purkinje cell layer of the
cerebellum (F) and throughout various layers of the cerebral cortex (G). Other abbreviations are
CL, claustrum; CO, corpus callosum; EP, external plexiform layer; GL, glomeruli; GR, granule
cell layer; ML, molecular layer; NA, nucleus accumbens; OT, olfactory tubercle; PK, Purkinje
cell layer; SL, stratum lucidum; SO, stratum oriens; SP, stratum pyramidale; SR, stratum
radiatum. Scale bar = 3 mm (panel A) and 100 μm (panels B-G).

Figure 5. GRIP1c 4-7 colocalizes with GABA<sub>A</sub>Rs and AMPA receptor clusters in mature
cultures of hippocampal neurons. Triple-label immunofluorescence using combinations of
rabbit anti-GRIP1c 4-7 (A), guinea pig anti-GRIP1c 4-7 (D, G J and M), mouse mAb to β2/3
subunits of the GABA<sub>A</sub>R (B), sheep anti-GAD (C and F), rabbit anti-γ2 subunit of the GABA<sub>A</sub>R
(E and K), mouse anti-PSD-95 (H), rabbit anti-GluR2/3 subunits of the AMPA receptors (N),
rabbit anti- vGlut1 (I) and mouse mAb anti-SV2 (L and O) antibodies. In A-F, GRIP1c 4-7,
revealed with either the rabbit or the guinea pig antibody, colocalizes with GABA<sub>A</sub>R clusters both
at GABAergic synapses (filled arrows) and outside GABAergic synapses (filled arrowheads) as
determined by the presence or absence of GAD<sup>+</sup> terminals, respectively. Some of the GRIP1c 4-7
clusters do not colocalize with GABA<sub>A</sub> receptor clusters or GAD<sup>+</sup> terminals (empty arrowhead).
In G-I, GRIP1c 4-7 colocalizes with PSD-95 clusters at glutamatergic synapses (vGlut1<sup>+</sup>, filled
arrows). Some GRIP1c 4-7 clusters colocalize with PSD-95 but not with vGlut1 (filled
arrowheads). In J-L, GRIP1c 4-7 colocalizes with GABA<sub>A</sub>R clusters in the presence (filled
arrows) or absence (filled arrowheads) of SV2-containing pre-synaptic terminals. In M-O, GRIP1c 4-7 colocalizes with AMPA receptor clusters in the presence (filled arrows) or absence (filled arrowheads) of SV2-containing pre-synaptic terminals. Empty arrowheads show some GRIP1c 4-7 clusters that do not colocalize with any GABAergic or glutamatergic pre or postsynaptic markers in the combinations used. Secondary antibodies were FITC-conjugated donkey anti-guinea pig (D, G, J and M) and anti-mouse (B); Texas Red-conjugated donkey anti-rabbit (A, E, K, N and I); AMCA-conjugated donkey anti-mouse (H, L and O) and donkey anti-goat (C and F). Neurons were kept in culture for 21 days. Scale bar, 5 μm.

Figure 6. Development of GRIP1c 4-7 clusters in hippocampal cultures. Triple-label immunofluorescence of hippocampal neurons at 3 (A), 5 (B), 10 (C) and 15 (D) days in culture using rabbit anti-GRIP1c 4-7 (red, A1, B1, C1 and D1), mouse mAb anti-GABAAR β2/3 (green, A2, B2, C2 and D2) and sheep anti-GAD (blue, A3, B3, C3 and D3) antibodies. Overlays are shown in A, B, C and D and A4, B4, C4 and D4. At 3 and 5 days in culture, GRIP1c 4-7 (arrowheads, A1 and B1) and GABAAR (arrowheads, A2 and B2) clusters can colocalize in the absence of presynaptic GABAergic innervation (arrowheads, A3 and B3) as shown in the overlays (A4, B4). In these early cultures, the dendritic arborizations of the neurons are not well developed. At 10 and 15 days in culture, GRIP1c 4-7 (C1 and D1) colocalizes with GABAAR at GABAergic synapses (arrows) and outside GABAergic synapses (arrowheads) determined by colocalization with GAD⁺ presynaptic terminals as shown in the overlays (arrows, C3 and D3). Note that the dendritic arborizations are more developed as the cultures mature from A and B to C and D. Secondary antibodies were Texas Red-conjugated donkey anti-rabbit (A1, B1, C1 and
Splice variant of GRIP1

D1); FITC-conjugated donkey anti-mouse (A2, B2, C2 and D2); and AMCA-conjugated donkey anti-goat (A3, B3, C3, and D3). Scale bars: whole cell = 10 μm, insets = 3 μm.

Figure 7. Exogenous HA-GRIP1c 4-7 colocalizes with postsynaptic GABAAR clusters in transfected hippocampal neurons. Triple-label immunofluorescence of hippocampal neurons transfected with HA-GRIP1c 4-7 cDNA using a combination of rabbit anti-GABAAR γ2 subunit (red, A), mouse mAb to the HA epitope (green, B) and guinea pig anti-VGAT (blue, C). A color overlay is shown in D. Clusters of HA-GRIP1c 4-7, of a transfected pyramidal cell colocalize with GABAAR γ2 subunit clusters at GABAergic synapses (arrows) as shown by the presence of presynaptic VGAT. The interneuron from which the axon containing VGAT+ presynaptic terminals originates, has no HA immunoreactivity. Clusters of HA-GRIP1c 4-7 immunoreactivity also colocalize with some GABAAR γ2 subunit clusters that are not localized at GABAergic synapses (arrowheads), since they do not colocalize with presynaptic VGAT. Secondary antibodies were Texas Red-conjugated donkey rabbit (A), FITC-conjugated donkey anti-mouse (B) and AMCA-conjugated donkey anti-guinea pig (C). Scale bar, 20 μm.

Figure 8. GRIP1c 4-7 immunogold is associated with rough endoplasmic reticulum, Golgi apparatus, microtubules and synapses. EM postembedding immunogold labeling with rabbit anti-GRIP1c 4-7 (A-G) or guinea pig anti-GRIP1c 4-7 (H-K) antibodies. GRIP1c 4-7 immunoreactivity was associated with the rough ER (A, arrows), Golgi apparatus (B, arrows), microtubules (C, arrows) and synapses (D-K). In panels D-K, the presynaptic terminal is on top and the postsynaptic element is on the bottom. Gold particles are localized on synaptic membranes (D, E, H and I arrow) and/or the PSD (D and E), the postsynaptic cytoplasm (F, I
Splice variant of GRIP1 filled arrowhead, and J) and the presynaptic bouton (G and K). Sometimes the gold particles are on or close to the presynaptic active zone (I, empty arrowhead). The secondary antibodies, conjugated to 12 nm (A, B and H) or 18 nm (C-G, I-K) diameter colloidal gold particles, were raised in donkey (A-C and H-K) or goat (D-G). Tissue blocks were from cerebral cortex (A), cerebellum (B, C and F-K) or the CA1 region of the hippocampus (D and E). Scale bar = 90 nm for all panels.

Figure 9. GRIP1c 4-7 is present in both symmetric GABAergic (A-I) and asymmetric glutamatergic (J-L) synapses. Preembedding, single-label (A, B and J) or double-label postembedding immunogold (C-I, K and L) experiments using rabbit anti-GRIP1c 4-7 (A, B and J) or combinations of rabbit anti-GRIP1c 4-7 with either guinea pig anti-GABA<sub>A</sub>R α1 subunit (C-E), mouse anti-GABA<sub>A</sub>R β2/3 (F), guinea pig anti-GABA (G, H and I) or guinea pig anti-vGlut1 (K). Panel L shows a combination of guinea pig anti-GRIP1c 4-7 and rabbit anti-vGlut1. In all panels, the presynaptic terminal is on top and the postsynaptic element at the bottom. In the upper synapse in J, the presynaptic element is on the right side. Preembedding immunocytochemistry (A, B and J) showed the peroxidase reaction accumulates postsynaptically in symmetric type II synapses (A and B, arrows) and in asymmetric type I synapses (J, arrow). Note that another asymmetric synapse was not labeled (J, asterisk). GRIP1c 4-7 immunoreaction (arrows, 12 nm diameter gold particles in C, D, E and 18 nm in F) colocalized in the same synapse with the GABA<sub>A</sub>R α1 subunit immunoreactivity (arrowheads, 18 nm gold particles in C, D and E) and GABA<sub>A</sub>R β2/3 (arrowhead, F, 10 nm diameter gold particles) in type II symmetric synapses. GRIP1c 4-7 immunoreactivity (arrows, 12 nm gold particles in G, H, I and K and 18 nm gold particles in L) was also present at type II synapses showing GABA immunoreactivity in
Splice variant of GRIP1

presynaptic terminals (arrowheads, 18 nm gold in G, H and I) and type I synapses, containing vGlut1 immunoreactivity at the presynaptic terminals (arrowheads, 18 nm gold in K and 12 nm in L). M and N show the distribution of gold particle density in the axo-dendritic axis of GABAergic synapses (M) and in glutamatergic type I synapses (N) corresponding to rabbit anti-GRIP1c 4-7 immunoreactivity. 76 (M) and 115 (N) gold particles were distributed in 5 nm bins according to the distance from the center of each gold particle to the midline of the synaptic cleft, defined as the zero point. Double label experiments with rabbit anti-GRIP1c 4-7 and the GABAergic synaptic markers GABA or GABA\(_A\)R (M) or rabbit anti-GRIP1c 4-7 and the glutamatergic synaptic markers vGlut1 or AMPA receptor (N) were used for identifying GRIP1c 4-7 in GABAergic and glutamatergic synapses, respectively. Negative and positive values represent presynaptic and postsynaptic localization, respectively. Particle density represents the number of particles in a 5 nm bin. The graphs were smoothed using a five-point weighted running average. A goat anti-rabbit secondary antibody was used in panels A, B and J. Combinations of a donkey anti-rabbit (conjugated to 12 nm gold particles) and a donkey anti-guinea pig (conjugated to 18 nm gold particles) secondary antibodies were used in panels C-E, G-I, K and L. A combination of goat anti-rabbit (conjugated to 18 nm gold particles) and goat antimouse (conjugated to 10 nm gold particles) secondary antibodies were used in panel F. A donkey anti-rabbit (conjugated to 12 nm gold particles) secondary antibody was used in panels G, H and I. Tissue blocks were from cerebral cortex (A, B, J and L) or from cerebellum (C-I and K). Scale bar = 90 nm for all panels except for panel C (45 nm).
Figure 1
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Figure 7
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Figure 9
A four PDZ domain-containing splice variant form of GRIP1 is localized in GABAergic and glutamatergic synapses in the brain

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