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Gephyrin Interacts With The Glutamate Receptor Interacting Protein 1 Isoforms At GABAergic Synapses

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Abstract

We have previously shown that the glutamate receptor interacting protein 1 (GRIP1) splice forms GRIP1a/b and GRIP1c4-7 are present at the GABAergic postsynaptic complex. Nevertheless the role that these GRIP1 protein isoforms play at the GABAergic postsynaptic complex is not known. We are now showing that GRIP1c4-7 and GRIP1a/b interact with gephyrin, the main postsynaptic scaffold protein of GABAergic and glycinergic synapses. Gephyrin coprecipitates with GRIP1c4-7 or GRIP1a/b from rat brain extracts and from extracts of HEK293 cells that have been cotransfected with gephyrin and one of the GRIP1 protein isoforms. Moreover, purified gephyrin binds to purified GRIP1c4-7 or GRIP1a/b, indicating that gephyrin directly interacts with the common region of these GRIP1 proteins, which includes PDZ domains 4–7. An engineered deletion construct of GRIP1a/b (GRIP1a4–7), which both contains the aforementioned common region and binds to gephyrin, targets to the postsynaptic GABAergic complex of transfected cultured hippocampal neurons. In these hippocampal cultures, endogenous gephyrin colocalizes with endogenous GRIP1c4-7 and GRIP1a/b in over 90% of the GABAergic synapses. Double-labeling electron microscopy immunogold reveals that in the rat brain GRIP1c4-7 and GRIP1a/b colocalize with gephyrin at the postsynaptic complex of individual synapses. These results indicate that GRIP1c4-7 and GRIP1a/b colocalize and interact with gephyrin at the GABAergic postsynaptic complex and suggest that this interaction plays a role in GABAergic synaptic function.

Keywords

$GABA_A$ receptor; Clustering; GABA synapse; Gephyrin; GRIP1; PDZ domain

Most of the previous studies on the functional roles that glutamate receptor interacting proteins (GRIPs) play at synapses have focused on glutamatergic synapses, particularly on the involvement of GRIP proteins in the surface expression and postsynaptic localization of AMPA receptors (Dong et al. 1997; Srivastava et al. 1998; Kulangara et al. 2007), the targeting of AMPA receptors to glutamatergic synapses (Bruckner et al. 1999; Dong et al. 1999; Osten et al. 2000; Wyszynski et al. 2002; Hirbec et al. 2003; Liu and Cull-Candy 2005) and the activity-dependent synaptic turnover of AMPA receptor during long-term depression or LTD (Xia et al. 2000; Kim et al. 2001). In addition to these synaptic functions,

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GRIP1 is involved in other functions in various organs and during embryonic development (Bladt et al. 2002; Swan et al. 2004; Takamiya et al. 2004; Swan et al. 2006). There are several GRIP1 isoforms that result from alternative splicing of the mRNA. Some GRIP1 isoforms are 135,000 Mr and contain 7 PDZ domains (ie. GRIP1a/b) while other isoforms are 75,000 Mr and contain PDZ domains 4–7 but have PDZ domains 1–3 missing (ie. GRIP1c4-7). In addition, these isoforms differ in the N- and/or C-termini (Dong et al. 1999; Yamazaki et el. 2001; DeSouza et al. 2002; Charych et al. 2004a; Charych et al. 2006 and Fig. 1I). Depending on the N-terminus, the isoforms can or cannot associate with the plasma membrane via palmitoylation and perhaps myristoylation.

We have recently shown by electron microscopy (EM) immunogold that several isoforms of GRIP1 are present in GABAergic synapses in the intact brain (Charych et al. 2004a; Charych et al. 2006; Li et al. 2005a). The presence of GRIP1 in GABAergic synapses has also been shown in hippocampal cultures by immunofluorescence (Dong et al. 1997; Li et al. 2005a; Wyszynski et al. 1999). We have also shown that while GRIP1a/b isoforms are abundant in interneurons and highly concentrated in the GABAergic presynaptic terminal, GRIP1c4-7 is much less abundant in interneurons and preferentially concentrates at the postsynaptic GABAergic complex (Charych et al. 2004a; Li et al. 2005a). Nevertheless, GRIP1a/b is also present at the postsynaptic GABAergic complex and GRIP1c4-7 at the presynaptic terminal (Charych et al. 2004a; Charych et al. 2006; Li et al. 2005a).

Gephyrin is a 93,000 Mr scaffold protein that concentrates at the postsynaptic complex of the inhibitory GABAergic and glycinergic synapses. Gephyrin is essential for the postsynaptic clustering of glycine receptors and of many $GABA_A$Rs (Kirsch et al. 1993; Bohlhalter et al. 1994; Essrich et al. 1998; Feng et al. 1998; Kneussel et al. 1999; Sassoe-Pognetto et al. 1999; Luscher and Keller 2004; Li et al. 2005b; Yu et al. 2007; Yu and De Blas 2007; Lardi-Studler and Fritschy 2007). In this communication we show that GRIP1c4-7 and GRIP1a/b associate and interact directly with gephyrin and that both GRIP1 isoforms colocalize with gephyrin at the GABAergic postsynaptic complex, suggesting that the interaction between gephyrin and GRIP1 plays a role in GABAergic synaptic function.

MATERIALS AND METHODS

All the animal protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) in University of Connecticut and followed the National Institutes of Health guidelines.

Antibodies

The rabbit and guinea pig anti-GRIP1c4-7 antibodies to amino acids 12–26 (KPHNFHHASHPPLRK) of rat GRIP1c4-7 were raised and characterized in our laboratory (Charych et al. 2004a; Charych et al. 2006). These antibodies recognize GRIP1c4-7 but not GRIP1a/b. The guinea pig antibody to the C-terminus of GRIP1a/b (to amino acids 1100–1112, GGNLETREPTNTL) was also generated in our laboratory as described elsewhere (Li et al. 2005a). This antibody recognizes GRIP1a/b but not GRIP1c4-7. The rabbit anti-GRIP1a/b (to the C-terminal 19 amino acids) was from Dr. Richard Huganir from the Johns Hopkins University, Baltimore, MD (Dong et al. 1997). The guinea pig and the rabbit anti-GRIP1a/b antibodies recognize both GRIP1a and GRIP1b isoforms, as well as the engineered GRIP1a4–7 truncation, since they all have a common C-terminus. The mouse mAb to GRIP1a/b, which also recognizes GRIP1a4–7 and GRIP1c4-7, was from BD Transduction Laboratory (San Jose, CA). The mouse mAb to gephyrin (clone mAb 7a) used in immunocytochemistry was from Cedarlane (Accurate Chemical & Scientific Corp., Westbury, NY). The rabbit anti-gephyrin C-terminus antibody used in immunoblots was prepared as described elsewhere (Kawasaki et al. 1997). The mouse mAb to the HA epitope

was from Covance (Princeton, NJ). The mouse mAb to the His-tag was from Novagen (Madison, WI). The Sheep anti-GAD was from Dr. Irwin J. Kopin (NINDS, Bethesda, MD). The rabbit antibody to BIG2 (brefeldin A-inhibited guanine nucleotide-exchange protein 2) was generated in our laboratory as reported elsewhere (Charych et al. 2004b). It recognizes the C-terminus of rat BIG2 (Charych et al. 2004b). The colloidal gold-labeled 10 nm diameter goat anti-mouse IgG secondary antibody was from ICN (Irvine, CA) and the 18 nm diameter goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). The fluorophore- conjugated anti-species specific IgG secondary antibodies made in donkey were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Construction of various plasmid vectors

The human gephyrin cDNA clone FJ06168 (Gene name KIAA1385) was kindly provided by Dr. Nobumi Kusuhara, Kazusa DNA Research Institute, Japan. This gephyrin isoform, without the c5 cassette, is involved in the clustering of $GABA_A$Rs (Meier and Grantyn 2004). The full-length gephyrin cDNA coding sequence was amplified by PCR and directionally inserted into the Hind III and Xho I sites of either pcDNA3.1(+) for mammalian expression of gephyrin, or pET32a(+) for bacterial expression of His-tagged gephyrin fusion protein. The bacterial fusion protein has the His tag and a 20 kD fusion protein added to the N-terminus of gephyrin (His-gephyrin, 93kD+20kD=113kD). The construct for the bacterial expression of the 31 kD His-tagged fusion protein of the C-terminal 110 amino acid fragment of BIG2 (His-BIG2CT) was also done in pET32a(+) (Charych et al. 2004b). The full-length GRIP1a cDNA (in pBK) was from Dr. Richard Huganir (Johns Hopkins University, Baltimore, MD). GRIP1a was also inserted into the Xma I and Not I sites of pGEX4T1 for bacterial expressions of glutathione-S-transferase (GST)-GRIP1a fusion protein with the GST tag located at the N-terminus of GRIP1a. The engineered GRIP1a4–7 truncation (corresponding to amino acids 417–1112 of GRIP1a), which is homologous to GRIP1c4-7, was inserted into the EcoR I and Not I sites of pGEX4T1 for GST-GRIP1a4–7 fusion protein expression in bacteria. The GRIP1a4–7 with a influenza hemagglutinin (HA) tag at the N-terminus (HA-GRIP1a4–7) was also inserted into the Bam HI and Sal I sites of pcDNA3.1(+) for mammalian expression. The full-length GRIP1c4-7 cDNA was directionally inserted into the Bam HI and Sal I sites of pcDNA3.1(+) for mammalian expression or the EcoR I and Not I sites of pGEX4T1 for GST-GRIP1c4-7 fusion protein expression in bacteria. The GST-tagged β3 intracellular loop of $GABA_A$R (GST-β3IL) was constructed as reported elsewhere (Fernando et al. 1995). The quality of the constructs was confirmed by DNA sequencing and the protein expression was detected by both immunobloting and immunofluorescence.

Transfection of HEK293 cells

For immunofluorescence experiments, the human embryonic kidney cell line 293 (HEK293) was cultured on 18-mm diameter glass coverslips coated with poly-L-lysine and transfected with 2 μg of each plasmid DNA. For immunoprecipitation experiments, HEK293 cells were cultured on 100-mm plastic plates (Becton Dickinson and Company, Franklin Lakes, NJ) and transfected with 10 μg of each plasmid DNA. Transfection was done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA).

Immunoprecipitation

Immunoprecipitations from HEK293 cell extracts were done according to Wyszynski and Sheng (1999). Briefly, 24–48 hours after transfection, HEK293 cells were solubilized in extraction buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 0.3 mM phenylmethylsulphonyl fluoride, PMSF) and a cocktail protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and the supernatant extract was collected after centrifugation at 60,000g for 30 min at 4°C.

Aliquots of 50 μl bed volume of protein A-Sepharose beads, suspended in 100 mM Na-borate buffer, pH 8.0, were incubated with 50 μl of antiserum or PIS for 2 hour at RT followed by washing. A 50 μl aliquot of the detergent extract, containing approximately 200 μg of protein, was added to the antibody-coated beads and incubated overnight at 4°C. After a brief centrifugation in a microcentrifuge, the immuno-beads were washed five times with ice-cold 1×RIPA buffer (5 mM Tris-HCl, pH7.4, 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS). The adsorbed proteins were eluted from the immuno-beads by incubation with sample dissociation buffer (10 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 2% SDS) at 85°C for 15 min and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting and detection by chemiluminescence.

Immunoprecipitation from a rat forebrain extract of a crude synaptosomal P2 fraction was performed as described elsewhere (Luo et al. 1997; Charych et al. 2004a; Charych et al. 2004b). Briefly, the P2 fraction (5 mg of protein) was suspended in 1ml of TE buffer (10 mM Tris-HCl and 5 mM EDTA, pH 7.4) and 0.1 ml of ice-cold DOC buffer (10% DOC in 500 mM Tris-HCl, pH 9.0) was added followed by incubation at 36 °C for 30 min and by the addition of 0.11ml of Triton X-100 buffer (1% Triton X-100, 500 mM Tris-HCl, pH 9.0). The sample was dialyzed in binding buffer (50 mM Tris-HCl, pH7.4, 5 mM EDTA, 0.1% Triton X-100, 0.3 mM PMSF) at 4°C overnight and centrifuged at 37,000 × *g* for 40 min at 4°C. The supernatant was used for the immunoprecipitations with antibody-coated protein A-sepharose beads, as described above.

Pull down from brain extracts

GST and GST-GRIP1c4-7 fusion proteins were expressed in bacteria and absorbed to glutathione coated beads by overnight incubation at 4°C, according to the manufacturer's instructions (Amersham Bioscience, Uppsala, Sweden). Pull downs from brain extracts were done as described before (Wyszynski and Sheng 1999; Charych et al. 2004b). Briefly, 13 μg of GST and 50 μg of GST-GRIP1c4-7 (equal moles), absorbed to 50 μl of bead bed volume, were incubated with the brain extract (200 μg protein) in binding buffer containing the aforementioned cocktail of protease inhibitors, overnight at 4°C. After centrifugation, the beads were washed three times with binding buffer, followed by elution with sample dissociation buffer, SDS-PAGE, immunoblotting and detection by chemiluminescence as described above.

Direct protein interaction binding assay

His-gephyrin fusion protein was expressed in bacteria and purified after adsorption to TALON Metal Affinity Resin (BD Biosciences Clontech, Palo Alto, CA) by incubation for 20 min at RT. Purified His-gephyrin was eluted using elution buffer (150 mM imidazole, 20 mM sodium phosphate, pH 7.4, 500 mM NaCl). The eluted protein was dialyzed in binding buffer overnight at 4°C. Equal moles of bacterially expressed GST (13 μg), GST-GRIP1c4-7 (50 μg), GST-GRIP1a (78 μg), GST-GRIP1a4-7 (50 μg), or GST-β3IL (20 μg) fusion proteins, adsorbed to 50 μl bed volume of glutathione beads, were incubated with 20 μg of purified His-gephyrin or His-BIG2CT in extraction buffer and the aforementioned cocktail of protease inhibitors, overnight at 4°C. After centrifugation, the beads were washed three times with extraction buffer, followed by elution with sample dissociation buffer, SDS-PAGE, immunoblotting and detection by chemiluminescence as described above.

Low-density hippocampal cultures and transfection

Primary hippocampal cultures were prepared from embryonic day 18 (E18) Sprague-Dawley rats according to Goslin et al., (1998) as described elsewhere (Christie et al. 2002a; Christie et al. 2002b; Christie and De Blas 2003). Cultured hippocampal neurons (11 days in culture)

were transfected with 4 μg of the HA-tagged GRIP1a4-7 in pcDNA3.1(+) vector using the CalPhos Mammalian Transfection Kit (BD Biosciences, San Jose, CA). Immunofluorescence was performed five days after transfection.

Immunofluorescence

Doble- or triple-label immunofluorescence of neurons (16–18 days in culture) or HEK293 cells was done as described elsewhere (Christie et al. 2002a; Christie and De Blas 2003; Christie et al. 2006; Yu et al. 2007; Yu and De Blas 2007). Briefly, fixed and Triton X-100-permeabilized cells were incubated with a mixture of primary antibodies followed by incubation with a mixture of secondary antibodies (anti-species specific IgG all made in donkey, Jackson Immunochemicals, West Grove, PA) conjugated to fluorescein isothiocyanate (FITC), Texas Red, or aminomethylcoumarin (AMCA) fluorophores. For HEK293 cells, the nuclei were counterstained with DAPI (4′, 6-diamidino-2-phenylindole). Coverslips were washed with PBS and mounted using Prolong Gold anti-fade mounting solution (Molecular Probes, Eugene, OR).

Image acquisition and analysis

This was done as described before (Christie and De Blas 2003; Christie et al. 2006). Briefly, digital images for hippocampal cultures were collected using a 60 ×pan-fluor objective on a Nikon Eclipse T300 microscope with a Sensys KAF 1401E CCD camera driven by IPLab 3.0 acquisition software (Scanalytics, Fairfax, VA). Images obtained from different fluorescence channels were processed and merged with PhotoShop 7.0 (Adobe) for analysis. Brightness and contrast were adjusted and the image was changed from 16 bits/channel to 8 bits/channel (1315×1035 pixel resolution), sharpened using the unsharp mask tool (setting: amount =125%, radius= 1.5 pixel, threshold = 0 level). Color was added to each channel and the images were merged for color colocalization, which was considered when >25% of the surface of one of the two clusters overlapped with the cluster in the other channel. Clusters present in 25 μm long and 4 μm wide dendrite windows (2–3/neuron) from 6–12 cells from three experiments were analyzed for quantification. Quantitative data are given as mean ± standard error of the mean (SEM). The number of clusters used for each quantification was 386–510.

Electron microscopy immunogold labeling

Postembedding electron microscopy (EM) immunogold labeling was done as reported elsewhere (Riquelme et al. 2002). Briefly, 10 week old male Sprague-Dawley rats were anesthetized and perfused with 800 ml of 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Vibratome sections, 300–500 μm thick, were cryoprotected with 2 M sucrose in PB and plunge-frozen in liquid propane cooled by liquid nitrogen (−186°C). Samples were stained with 1.5% uranyl acetate in anhydrous methanol at −90°C for 30 hours before infiltration with Lowicryl HM20 resin (Polysciences, Warrington, PA, USA). The resin was polymerized with UV light for 72 h in a freeze substitution instrument (Leica AFS, Vienna, Austria) in a temperature gradient (−45°C to 0°C).

Tissue sections (70–80 nm-thick) were subjected to double-label immunogold by incubation with a mixture of two affinity-purified primary antibodies raised in mouse and rabbit followed by incubation with a mixture of two species-specific anti-mouse and anti-rabbit IgG secondary antibodies labeled with colloidal gold particles of 10 and 18 nm diameter respectively. The tissue sections were counterstained with 2% uranyl acetate and then with 2% lead citrate. No immunolabeling was observed when the primary antibodies were omitted. The samples were observed with a Philips 300 transmission EM (Philips,

Eindhoven, The Netherlands). The EM images were stored in Adobe Photoshop 7.0 and contrast and brightness was adjusted.

RESULTS

GRIP1c4-7 associates with gephyrin in transfected HEK293cells and in brain extracts

We have previously shown that the 4-PDZ domain splice form of GRIP1 (GRIP1c4-7) is present in the postsynaptic complex of GABAergic synapses (Charych et al. 2004a). Now we have investigated whether GRIP1c4-7 and gephyrin (a scaffold protein that is present in the postsynaptic complex of many GABAergic synapses) are associated in cotransfected HEK293 cells and in brain extracts. Double-label immunofluorescence shows that when HEK293 cells were cotransfected with gephyrin and GRIP1c4-7, both formed large cytoplasmic aggregates that colocalized with each other (Fig 1A–C, white arrowheads). These large cytoplasmic aggregates were absent in HEK293 cells transfected only with GRIP1c4-7 (Fig 1D, also see Charych et al. 2004a), which exhibited a diffuse distribution of GRIP1c4-7 in the cytoplasm and also in association with the plasma membrane (Figure 1D, black arrowheads). In addition to the large aggregates, double-transfected cells also showed diffuse, membrane-associated and small aggregates of GRIP1c4-7 fluorescence that were not associated with gephyrin (Fig 1A and B). The HEK293 cells transfected only with gephyrin formed large gephyrin aggregates (Fig 2L, red color) similar to the gephyrin aggregates observed in the double-transfected cells (as shown in Fig 1B, white arrowheads). Some of these gephyrin aggregates might localize at the microtubule-organizing center (Maas et al. 2006). Non-transfected cells showed neither gephyrin nor GRIP1c4-7 immunofluorescence labeling (Fig 1A–C, the arrow points to the nucleus of a non-transfected cell). Thus, in double-transfected cells, GRIP1c4-7 is recruited into the gephyrin aggregates. These results are consistent with the notion that GRIP1c4-7 and gephyrin can form molecular associations with one another.

Direct evidence of the molecular association between GRIP1c4-7 and gephyrin was obtained in coimmunoprecipitation experiments of extracts from HEK293 cells cotransfected with GRIP1c4-7 and gephyrin. The guinea pig anti-GRIP1c4-7 antibody, which does not recognize GRIP1a/b (Charych et al. 2004a; Li et al. 2005a), coprecipitated gephyrin as revealed in immunoblots with a rabbit anti-gephyrin antibody, which recognized the gephyrin 93 and 96 kD protein doublet (Fig 1E, fourth lane from the left, arrowhead). No gephyrin protein coprecipitated with the corresponding guinea pig pre-immune serum (PIS, third lane from left). Others have shown that in the brain there are several gephyrin isoforms which after SDS-PAGE often show as a 93–96 kD double protein band (Kawasaki et al. 1997; Paarmann et al. 2006). In a reciprocal coimmunoprecipitation experiment, the rabbit anti-gephyrin antibody, but not the corresponding PIS, coprecipitated the 75 kD GRIP1c4-7 (Fig 1F, fourth and third lanes from the left, respectively, arrowhead) as revealed with a mouse mAb that recognizes GRIP1c4-7. These results indicate that GRIP1c4-7 associates with gephyrin when they are co-expressed in heterologous mammalian cells. Immunoblots show that HEK293 cells express low levels of endogenous gephyrin since in non-transfected cells, the rabbit anti-gephyrin antibody reveals the expression of the upper gephyrin protein band of the doublet (Fig 1E, first lane from left). However, no gephyrin immunofluorescence was detected in non-transfected HEK293 cells (Fig 1A–C, arrow), indicating that these cells have a low level of gephyrin expression. Note that the overexpressed gephyrin in HEK293 cells corresponds to the lower band of the doublet, which is not observed in non-transfected cells (Fig 1E lane 2 *vs*. lane 1 from the left).

We also investigated whether GRIP1c4-7 and gephyrin coimmunoprecipitated from brain extracts. The results showed that the guinea pig anti-GRIP1c4-7 antibody, but not the corresponding PIS, coimmunoprecipitated the 93 and 96 kD gephyrin doublet band from

brain extracts (Fig 1G, third and second lane from the left, respectively, arrowhead). Moreover the purified GST-GRIP1c4-7 fusion protein, but not GST, adsorbed to glutathione beads, pulled down the gephyrin doublet from the rat brain extract (Fig 1H, third and second lane from the left, respectively, arrowhead). These results strongly indicate that GRIP1c4-7 and gephyrin associate both in transfected host cells and in the intact brain. Fig 1I shows a schematic representation and alignment of various GRIP1 constructs used in this study.

GRIP1 associates with gephyrin in transfected HEK293 cells and in brain extracts

We have previously shown that although GRIP1a/b, which are 7-PDZ domain isoforms of GRIP1, are very abundant in the presynaptic GABAergic terminals (Li et al. 2005a), they are also present in the postsynaptic GABAergic complex (Li et al. 2005a). We have investigated whether GRIP1a/b associates with gephyrin, as GRIP1c4-7 does. Immunofluorescence of HEK293 cells double-transfected with GRIP1a and gephyrin (Fig 2A–C), formed cytoplasmic aggregates of GRIP1a (Fig 2A, white arrowheads) and gephyrin (Fig 2B, black arrowheads). However, in these cells, the GRIP1 aggregates did not colocalize with the gephyrin aggregates (Fig 2C). The GRIP1 aggregates and the gephyrin aggregates in the double-transfected cells were similar to the corresponding ones in HEK293 cells that were single-transfected with GRIP1a (Fig 2M green color and Charych et al. 2004a) or gephyrin (Fig 2L red color) respectively. We have not identified the organelles where GRIP1 accumulates. These aggregates don't colocalize with endosomal or lysosomal markers (Fu et al. 2003). These results suggest that the interaction of gephyrin with GRIP1a might not be as strong as the interaction with GRIP1c4-7 shown above. Nevertheless, the strong tendency of GRIP1a to form aggregates could prevent the interaction and colocalization of GRIP1a with the gephyrin aggregates. Non-transfected cells did not show immunofluorescence of either GRIP1a or gephyrin (Fig 2A–C, the arrow points to the nucleus of a non-transfected cell), although gephyrin was detected in immunoblots of non-transfected cells as discussed above (Fig 1E and 2D, first lane from the left).

Immunoprecipitations of extracts of HEK293 cells cotransfected with GRIP1a and gephyrin showed that the guinea pig anti-GRIP1a/b but not the corresponding PIS, coprecipitated gephyrin (Fig 2D, arrowhead, fourth and third lanes from the left respectively) as shown in immunoblots with a rabbit antibody to gephyrin. On the other hand, the rabbit anti-gephyrin antibody only coprecipitated a small amount of the 130kD GRIP1a, as shown as a very faint band in the immunoblot with the mouse anti-GRIP1a/b mAb (Fig 2E, arrowhead, fourth lane from the left). Compare the intensity of this band with that of the input (second lane from the left). We also tested whether gephyrin associates with GRIP1a/b in rat brain extracts. The guinea pig anti-GRIP1a/b antibody, but not the corresponding PIS, coprecipitated brain gephyrin as revealed in immunoblots with the rabbit anti-gephyrin antibody (Fig 2F, arrowhead). The rabbit anti-gephyrin antibody, but not the corresponding PIS, coprecipitated a small amount of GRIP1a/b, revealed as a faint protein band with a mouse mAb to GRIP1a/b (Fig 2G, arrowhead). The immunoprecipitation and immunofluorescence results indicate that GRIP1a/b and gephyrin associate in transfected HEK293 cells and in the brain although the association might not be as strong as the association of between GRIP1c4-7 and gephyrin shown in Fig 1.

We also constructed GRIP1a4-7, an engineered truncated form of GRIP1a that contains the fragment that includes PDZ domains 4-7, but does not contain the fragment that includes PDZ domains 1–3. This non-natural truncated form of GRIP1a/b is homologous to and has the same length (696 amino acids) as the natural GRIP1c4-7 splice form. GRIP1a4-7 and GRIP1c4-7 differ in the N-terminal 35 amino acids and the C-terminal 12 amino acids. The other parts of the two molecules are identical including the region containing PDZ domains 4–7. Antibodies that recognize the C-terminus of GRIP1a also recognize GRIP1a4-7 (Fig 2H), since both have the same the C-terminal peptide. When GRIP1a4-7 was coexpressed

with gephyrin in HEK293 cells, it showed a combination of diffuse distribution in the cytoplasm and cytoplasmic aggregates, some of which colocalized with gephyrin aggregates (Fig 2H–J, arrowhead), suggesting that GRIP1a4-7 associates with gephyrin. Cells transfected only with GRIP1a4-7 also showed both aggregates and a diffuse distribution of GRIP1a4-7 (Fig 2N green color). Non-transfected cells showed no immunofluorescence with any of the antibodies (in Fig 2J, the arrow points to the nucleus of a non-transfected cell).

Immunoblots show that the rabbit anti-gephyrin antibody, but not the corresponding PIS, coprecipitated the 75 kD GRIP1a4-7 truncation from extracts of cotransfected HEK 293 cells (Fig 2K, arrowhead, first and second lanes from the right, respectively). Moreover, the intensity of the band corresponding to GRIP1a4-7 protein coprecipitated by the anti-gephyrin antibody was considerably stronger than that of the band corresponding to the full-length GRIP1a protein (Fig 2K first lane from the right *vs*. Fig 2E first lane from the right, arrowheads, respectively). These results indicate that the domains of the GRIP1 isoforms that interact with gephyrin are in the 649 amino acid region common to GRIP1c4-7 and GRIP1a4-7, which includes PDZ domain 4–7. These results indicate that the interaction of gephyrin with GRIP1a4-7, or with GRIP1c4-7, is stronger than the interaction of gephyrin with the full-length GRIP1a. The N-terminal moiety of GRIP1a, which includes PDZ domains 1–3 and is absent in GRIP1a4-7 and GRIP1c4-7, might interfere with the interaction of GRIP1a with gephyrin. However, we can not rule out that instead, the N-terminal moiety of GRIP1a interferes with the binding of the anti-gephyrin antibody to gephyrin, perhaps by occluding the gephyrin epitope recognized by the antibody, thus interfering with the immunoprecipitations of the anti-gephyrin antibody. Nevertheless, it is clear that GRIP1a and gephyrin interact because: I) anti-GRIP1a coprecipitates gephyrin (Fig 2D and F) and II) purified GRIP1a interacts with purified gephyrin as shown below.

Transfection of cultured hippocampal neurons shows that GRIP1a4-7 targets to the postsynaptic GABAergic complex

We have shown elsewhere that the naturally existing GRIP1c4-7 splice form, when transfected to hippocampal neurons, targets to the postsynaptic complex of GABAergic synapses (Charych et al. 2004a). We have also shown that GRIP1c4-7 and GRIP1a/b are present in the GABAergic postsynaptic complex (Li et al. 2005a). We have now tested whether the engineered GRIP1a4-7 truncated form of GRIP1a also targets to the GABAergic synapses. Our prediction is that because GRIP1a4-7 associates with gephyrin, as we have shown above, it would target to the postsynaptic GABAergic complex. To test this hypothesis, we have transfected cultured hippocampal neurons with GRIP1a4-7 tagged at the N-terminal with the HA epitope (HA-GRIP1a4-7). The results showed that indeed HA-GRIP1a4-7 targeted to the GABAergic postsynaptic complex (Fig 3). Triple–label immunofluorescence with a mouse mAb to the HA epitope (green) showed that HA-GRIP1a4-7 formed clusters that frequently colocalized with postsynaptic γ2-$GABA_A$R clusters (red) in apposition to GAD-containing (blue) presynaptic terminals(Fig 3, arrows). The γ2-$GABA_A$R clusters were localized in the neurons transfected with HA-GRIP1a4-7 while the GAD^+ immunofluorescence corresponded to a non-transfected nearby interneuron that had no HA immunoreactivity. Therefore, the HA-GRIP1a4-7 clusters are localized postsynaptically, frequently colocalizing with postsynaptic γ2-$GABA_A$R clusters. As we have shown elsewhere, these GABAergic postsynaptic clusters in these cultures also contain clustered gephyrin (Li et al. 2005a; Christie et al. 2002a; Christie et al. 2002b)

In addition, we observed some HA-GRIP1a4-7 clusters colocalizing with γ2-$GABA_A$R clusters in the absence of presynaptic GAD-containing terminals (Fig 3, arrowheads) indicating that HA-GRIP1a4-7 also forms clusters, outside the GABAergic synapses, that colocalize with γ2-$GABA_A$R clusters. We have previously shown that 94.7% of the γ2-

$GABA_AR$ clusters that are located outside GABAergic synapses are also associated with gephyrin clusters (Christie et al. 2002b). Therefore, the association of gephyrin, γ2-$GABA_ARs$ and HA-GRIP1a4-7 in clusters can also occur in the absence of presynaptic GABAergic terminals.

The results on the transfection of cultured hippocampal neurons with GRIP1a4-7, shown in this communication, or with GRIP1c4-7, shown elsewhere (Charych et al. 2004a), indicate that the domain of the GRIP1 protein isoforms involved in both their targeting to the postsynaptic GABAergic complex and in their targeting to $GABA_AR$ and gephyrin clusters outside GABAergic synapses is localized in the region common to GRIP1a4-7 and GRIP1c4-7, which includes PDZ-domains 4–7. This is also the region involved in the association of the GRIP1 protein isoforms with gephyrin, as shown by the coimmunoprecipitation experiments described above. These results are consistent with the notion that the binding of GRIP1 to gephyrin and the co-targeting of GRIP1 isoforms and gephyrin to the GABAergic synapses are related events.

GRIP1c4-7 can associate with GRIP1a/b in transfected HEK293 cells and in brain extracts

Previous studies have shown that GRIP1a can interact with itself forming homomultimers (Dong et al. 1999; Im et al. 2003). These homophilic interactions occur through the PDZ domains 4–6 as shown by yeast two-hybrid and protein coprecipitation experiments. Moreover, X-ray crystallography has also shown that PDZ domain 6 can form antiparallel dimers through a site distal to the ligand-binding groove (Im et al. 2003). Since GRIP1c4-7 contains the PDZ domain 4–6, we hypothesized that GRIP1c4-7 interacts with GRIP1a/b.

We have shown above that when HEK293 cells were transfected only with GRIP1c4-7, the latter showed a diffuse distribution in the cytoplasm and also an association with the plasma membrane, as shown in Fig 1D (see also Charych et al. 2004a). In contrast, when HEK293 cells were cotransfected with GRIP1c4-7 and GRIP1a (Fig 4A–C), the GRIPc4-7 isoform formed aggregates in the cytoplasm that colocalized with the GRIP1a aggregates (Fig 4A–C, arrowheads). Similar aggregates were also present in HEK293 cells transfected only with GRIP1a (Fig. 2M green color). Non-transfected cells showed no immunofluorescence (Fig 4A–C, the arrow points to the nucleus of a non-transfected cell).

The guinea pig anti-GRIP1a/b antibody, but not the corresponding PIS, coprecipitated the 75 kD GRIP1c4-7 from extracts of HEK293 cells cotransfected with GRIP1a and GRIP1c4-7, as shown in immunoblots with the rabbit anti-GRIPc4-7 antibody (Fig 4D, arrowhead, fourth and third lanes from the left respectively). The guinea pig anti-GRIP1c4-7 antibody, but not the PIS, coprecipitated the 130 kD GRIP1a from the same HEK293 cell extract, as shown in immunoblots with the rabbit anti-GRIP1a/b antibody (Fig 4E, arrowhead, fourth and third lanes from the left respectively). As indicated above, the guinea pig anti-GRIP1a/b does not recognize GRIP1c4-7 (Charych et al. 2004a;Li et al. 2005a) and the rabbit anti-GRIP1a/b does not recognize GRIP1c4-7 (Charych et al. 2004a;Li et al. 2005a;Charych et al. 2006).

We also tested whether GRIPc4-7 associates with GRIP1a/b in the rat brain. The guinea pig anti-GRIP1c4-7 antibody, but not the PIS, coprecipitated GRIP1a/b from the rat brain extract (Fig 4F, arrowhead, third and second lanes from the left respectively). Moreover the purified GST-GRIP1c4-7 fusion protein adsorbed to glutathione beads, pulled down GRIP1a/b from a rat brain extract while GST adsorbed to the beads did not (Fig 4G, third and second lanes from the left respectively). These experiments indicate that GRIP1a/b and GRIP1c4-7 can form heteromultimers. Since the interaction of gephyrin with GRIP1c4-7 seems to be stronger than the interaction of gephyrin with GRIP1a/b, as shown above and

below, the formation of heteromultimers might have a functional relevance by facilitating the accumulation of GRIP1a/b at the GABAergic postsynaptic complex.

Purified GRIP1c4-7, GRIP1a and GRIP1a4-7 directly interact with purified gephyrin

Coimmunoprecipitations and pull-down experiments indicate that GRIP1c4-7, GRIP1a and GRIP1a4-7 can molecularly associate with gephyrin, however they don't reveal if this association occurs via direct molecular interactions or is mediated by other proteins, thus forming molecular complexes. Moreover, the interaction among GRIP1 proteins, forming homo-and heteromultimeric complexes, making it more difficult to determine whether gephyrin directly interacts with each or some of the GRIP1 isoforms. Thus we have tested whether gephyrin directly interacts with each of the GRIP1 protein isoforms in bimolecular reactions. For this purpose, fusion proteins GST-GRIP1c4-7, GST-GRIP1a4-7, GST-GRIP1a and GST-β3IL were expressed in bacteria and immobilized on glutathione beads. These beads were incubated with purified and solubilized His-tagged gephyrin or His-tagged C-terminal 110 amino acid fragment of BIG2 (His-BIG2CT) as a control. BIG2 is the brefeldin A-inhibited guanine nucleotide-exchange protein 2. The His-BIG2CT interacts with the large intracellular loop (IL) of the β subunits (including β3) of the $GABA_A$Rs as we have shown elsewhere (Charych et al. 2004b). The results in Fig 5A and B show that GST-GRIP1c4-7, GST-GRIP1a4-7 and GST-GRIP1a, but not GST, bound to His-gephyrin (113 kD, arrowhead) as revealed by immunoblotting with the rabbit anti-gephyrin antibody. However, none of the GRIP1 isoforms or GST bound the 31 kD His-BIG2CT negative control, as revealed with a mouse anti-His antibody (Fig 5C, arrowhead, lanes 2–5 from the left) or anti-BIG2 antibody (not shown). In contrast, the positive control GST-β3IL bound His-BIG2CT (Fig 5C, first lane from the left) as expected. These results indicate that gephyrin directly binds to each of the GRIP1 protein isoforms but not to GST or the His-tag or BIG2CT. Note the lower intensity of the protein band corresponding to His-gephyrin pulled down by GST-GRIP1a (Fig 5B lane on the right side) when compared with the intensity of the His-gephyrin protein band pulled down by GST-GRIP1c4-7 (Fig 5A lane on the right side) or GST-GRIP1a4-7 (Fig 5B, third lane from the left side). This is probably a reflection of a weaker interaction of gephyrin with GRIP1a than with GRIP1c4-7 or with GRIP1a4-7, since we used equal number of moles of the GST fusion protein for the binding.

Gephyrin clusters frequently colocalize with GRIP1c4-7 and GRIP1a/b clusters in cultured hippocampal neurons

Immunofluorescence of cultured hippocampal neurons shows that the majority of the synaptic gephyrin clusters (Fig 6A and E, arrows), identified by their apposition to GAD-containing presynaptic boutons (Fig 6C and G, respectively, arrows), colocalize with GRIP1c4-7 clusters (Fig 6B and D, arrows) or with GRIP1a/b clusters (Fig 6E and H, arrows). The results show that the majority of the synaptic gephyrin clusters colocalize with GRIP1c4-7 or GRIP1a/b clusters (91±3% and 93±2% respectively) in individual GABAergic synapses. In the case of GRIP1a/b, much of the immunofluorescence in GABAergic synapses might correspond to presynaptically localized GRIP1a/b, where it concentrates, as shown elsewhere (Li et al. 2005a), while GRIP1c4-7 is mostly postsynaptically localized, as shown elsewhere (Charych et al. 2004a). This would explain the larger size GRIP1a/b clusters corresponding to the larger presynaptic GABAergic terminals (Fig 6F and G respectively, arrows).

In addition, many (60±3% and 65±2%) of the non-synaptic gephyrin clusters, which are not apposed to GAD^+ presynaptic boutons, colocalize with GRIP1c4-7 (Fig 6A–D, white arrowheads) or with GRIP1a/b (Fig 6E-H, white arrowheads) respectively, indicating that gephyrin can colocalize with GRIP1c4-7 or GRIP1a/b in the absence of presynaptic GABAergic terminals. Some gephyrin clusters did not colocalize with either GRIP1c4-7

(Fig 6A–D, crossed arrows) or with GRIP1a/b (Fig 6E–H, crossed arrows). There are also many GRIP1c4-7 (Fig 6A-D, black arrowheads) and GRIP1a/b clusters (Fig 6E–H, black arrowheads) that do not colocalize with gephyrin clusters. We have also shown elsewhere that many of the GRIP1a/b and GRIP1c4-7 clusters that are not associated with gephyrin or $GABA_AR$ clusters are associated with glutamatergic synapses (Charych et al. 2004a;Li et al. 2005a). Preliminary data suggest that $GABA_AR$ clusters lacking gephyrin do not contain GRIP1a/b or GRIP1c4-7.

The colocalization of gephyrin with GRIP1c4-7 or GRIP1a/b in both synaptic and non-synaptic gephyrin clusters, supports the notion that the molecular association of gephyrin and GRIP1 proteins that we have detected in brain and HEK293 extracts also occurs in intact neurons and in individual GABAergic synapses.

Gephyrin colocalizes with GRIP1c4-7 and GRIP1a/b in the postsynaptic complex of rat brain synapses

To test whether synaptic gephyrin and GRIP1c4-7 or GRIP1a/b colocalize at the postsynaptic side of individual synapses in the intact brain, double-label postembedding EM immunogold experiments were performed using a mouse anti-gephyrin mAb in combination with either a rabbit anti-GRIP1c4-7 antibody or a rabbit anti-GRIP1a/b antibody. The results show that the small gold particles corresponding to gephyrin (arrowheads) and the large gold particles (arrows) corresponding to either GRIP1c4-7 (Fig 7A, B arrows) or GRIP1a/b (Fig 7C and D) colocalized at the postsynaptic side of brain synapses with Gray's type II (symmetric) morphology, which is characteristic of GABAergic synapses. The postsynaptic side of the synapse is identified by its apposition to the presynaptic terminal containing synaptic vesicles (Fig 7A–D). Some of the gold particles corresponding to anti-GRIP1a/b were associated to the postsynaptic membrane (Fig 7C, D, arrows), probably corresponding to the palmitoylated GRIP1b isoform, which has been shown to be associated to membranes (Yamazaki et al. 2001; DeSouza et al. 2002). Gold particles corresponding to GRIP1a/b were also frequently found at the presynaptic terminal (Fig 7C, D, crossed arrows). The localization of GRIP1c4-7 or GRIP1a/b agrees with what we have reported in other studies on the localization of these GRIP1 isoforms in GABAergic synapses at the EM level (Charych et al. 2004; Li et al. 2005a). In the present communication we are presenting the first direct evidence that GRIP1c4-7 and GRIP1a/b colocalize with gephyrin at the postsynaptic side of individual type II GABAergic synapses. These EM studies and the results from the coprecipitations, pull downs and direct binding experiments described above support the notion that gephyrin and GRIP1 protein isoforms directly interact with each other in the postsynaptic GABAergic complex of brain synapses and that this interaction is functionally relevant.

DISCUSSION

The main findings presented in this communication are: I) that there is molecular association of gephyrin with GRIP1 proteins. This association was demonstrated by coimmunoprecipitation and pull down of gephyrin with the GRIP1 protein isoforms from extracts of cotransfected HEK293 cells or from the brain; II) that purified gephyrin directly binds to purified GRIP1 proteins; and III) that there is morphological association of gephyrin and GRIP1 proteins at the postsynaptic complex of individual GABAergic synapses. These results suggest that the postsynaptic interaction of gephyrin and GRIP1 proteins is functionally relevant.

One possible functional explanation for this interaction is that GRIP1 proteins are directly involved in the trafficking and/or postsynaptic localization of gephyrin at GABAergic synapses in a way similar to their involvement in the trafficking and localization of AMPA

receptor at glutamatergic synapses (Dong et al. 1997; Setou et al. 2002; Steiner et al. 2005; Gindhart 2006; Kulangara et al. 2007). There is a direct interaction of the C-terminal tetrapeptide of the AMPA receptor subunits GluR2, GluR3 and GluR4c with the PDZ domains 4 and 5 of GRIP1a/b and GRIP1c4-7 (Dong et al. 2007; Dong et al. 1999). Thus AMPA receptors coimmunoprecipitate from brain extracts with either GRIP1a/b (Dong et al. 1997; Wyszynski 1999) or GRIP1c4-7, as we have shown elsewhere (Charych et al. 2004a). In contrast, we have not been able to coimmunoprecipitate $GABA_A$Rs with either anti-GRIP1c4-7 (Charych et al. 2004a) or anti-GRIP1a/b antibodies (not shown) from the same brain extracts. Thus, it is unlikely that GRIP1 isoforms are directly involved with the trafficking of $GABA_A$Rs at the GABAergic synapses. Nevertheless, GRIP1a/b and GRIP1c4-7 could indirectly affect the retention of postsynaptic $GABA_A$Rs by being involved in the postsynaptic accumulation of gephyrin at GABAergic synapses, since it has been shown that the postsynaptic clustering of gephyrin is important for the stabilization and retention of several types of $GABA_A$Rs at GABAergic synapses (Essrich et al. 1998; Kneussel et al. 1999; Levi et al. 2004; Jacob et al. 2005; Yu et al. 2007; Yu and De Blas 2007). The mechanism by which postsynaptic gephyrin stabilizes postsynaptic $GABA_A$Rs is not understood since there is no evidence of direct biochemical interaction between $GABA_A$Rs and gephyrin (Luscher and Keller 2004; Meyer et al. 1995; Chen and Olsen 2007). Moreover, the postsynaptic association between $GABA_A$R and gephyrin does not seem to be mediated by GRIP1 isoforms since $GABA_A$Rs do not bind to GRIP1 proteins.

It has been proposed that the postsynaptic association of gephyrin and $GABA_A$Rs is mediated by the $GABA_A$R associated protein (GABARAP), since GABARAP interacts with both gephyrin (Kneussel et al. 2000) and with $GABA_A$Rs (Wang et al. 1999). Nevertheless, GABARAP does not seem to mediate the anchoring of the postsynaptic $GABA_A$Rs to gephyrin since, contrary to $GABA_A$Rs, gephyrin or GRIP1c4-7, GABARAP does not accumulate at the postsynaptic GABAergic complex (Wang et al. 1999; O'Sullivan 2005). It has also been shown that GABARAP is not essential for $GABA_A$R targeting to the synapses (Kneussel 2000; O'Sullivan 2005). Instead GABARAP is localized in intracellular compartments, particularly the Golgi-apparatus, and is involved in the trafficking of $GABA_A$Rs from intracellular organelles to the plasma membrane (Kneussel 2000; O'Sullivan 2005; Kittler et al. 2001). For the same reason, although GABARAP associates with GRIP1 (Kittler et al. 2004), this association might not play a significant role at the GABAergic postsynaptic complex, since as indicated above, GABARAP does not concentrate at GABAergic synapses. Nevertheless, the interaction of GABARAP with GRIP1 could be important for the transport and trafficking of GRIP1 to the GABAergic postsynaptic complex.

Our coimmunoprecipitation and pull down experiments on brain extracts indicate that there is a molecular association between gephyrin and GRIP1 proteins. Moreover, the coimmunoprecipitation experiments in cotransfected HEK293 cells and particularly the affinity binding experiments using purified gephyrin and GRIP1 proteins indicate that gephyrin and GRIP1 proteins directly interact with each other. This interaction might be relevant not only at the postsynaptic complex, where both proteins concentrate, likely acting as scaffold proteins, but also during microtubule-associated transport of GRIP1 proteins and gephyrin to the GABAergic postsynaptic complex, since the motor protein kinesin-5 binds to GRIP1 proteins through the peptide linking PDZ domains 6 and 7 of GRIP1 (Setou et al. 2002; Gindhart 2006), which is also present in GRIP1c4-7 (Charych et al. 2004a). Thus GRIP1 proteins could act as motor adaptors in the microtubule-associated transport of gephyrin and other synaptic proteins (Hoogenraad et al. 2005).

Our results show that gephyrin binds to the fragment of the GRIP1 protein that includes PDZ domains 4–7. Thus gephyrin is one of the several proteins that have been shown to

interact with various regions of this fragment including: GluR2/3 (Dong et al., 1997), GABARAP (Kittler et al. 2001), GRASP-1 (Ye et al. 2000), kinesin-5 heavy chain (Setou et al. 2002), EphB2/EphA7 receptors and EphrinB1 ligand (Torres et al. 1998; Bruckner et al. 1999), liprin α(Wyszynski et al. 2002), and Drosophila Frizzled-2 (DFz2) receptors (Ataman et al. 2006) among others. These interacting domains of GRIP1a/b are also present in GRIP1c4-7 and GRIP1a4-7. We have also shown that the same fragment, which includes PDZ domains 4–7, is also involved in the targeting of HA-GRIP1a4-7 (in this study) and HA-GRIP1c4-7 (Charych et al. 2004a) to the postsynaptic GABAergic complex.

The immunofluorescence in hippocampal cultures shows that there is colocalization of gephyrin and GRIP proteins at individual GABAergic synapses (Fig 6). Nevertheless, this technique does not have the resolution to show whether gephyrin and GRIP1 proteins colocalize at the postsynaptic complex. Particularly, because GRIP1 proteins are also localized at the presynaptic GABAergic terminal. Nevertheless, the aforementioned results obtained after transfection of hippocampal pyramidal cells with HA-GRIP1c4-7 or HA-GRIP1a4-7 suggest that some GRIP1 proteins are located at the postsynaptic GABAergic complex, at least in these cultures. We have used postembedding double-label EM immunogold to ascertain that in the intact brain, there is also colocalization of GRIP1 proteins and gephyrin in the postsynaptic GABAergic complex. We have shown elsewhere by EM immunogold that GRIP1c4-7 concentrates at the postsynaptic complex of both glutamatergic and GABAergic synapses (Charych et al. 2004a). Nevertheless, there was no direct evidence for postsynaptic colocalization of gephyrin and GRIP proteins in the same synapse. We are now showing by double-label EM immunogold that indeed gephyrin colocalizes with GRIP1c4-7 or GRIP1a/b at the postsynaptic GABAergic complex of individual synapses. These experiments together with our coimmunoprecipitation and protein-protein binding results support the notion that gephyrin, directly interacts with GRIP1c4-7 and GRIP1a/b at the postsynaptic GABAergic complex and that this interaction is functionally relevant.

We have also shown that GRIP1a/b is abundant at the presynaptic GABAergic terminals (Li et al. 2005a). Thus the pre- and postsynaptic localization of GRIP1 molecules at GABAergic synapses could conceivably be involved in the pre- and postsynaptic accumulation and recycling of several synaptic components including postsynaptic gephyrin at GABAergic synapses and postsynaptic AMPA receptors at glutamatergic synapses, and of transynaptic cell recognition molecules such as the presynaptic EphrinB1 ligand and the postsynaptic Eph B2/A7 ephrin receptors, which are known to interact with GRIP1 and are involved in synaptic structure and function (Kittler et al. 2004; Murai and Pasquale 2004). Moreover, presynaptic GRIP1a/b interacts with presynaptic liprins (Wyszynski et al. 2002), which are organizers of the presynaptic dense projections (Kaufmann et al. 2002). Thus pre- and postsynaptic GRIP1 proteins seem to be involved in the transport, trafficking and synaptic turnover of various synaptic proteins including gephyrin.

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Abbreviations

ABP	AMPA receptor binding protein
AMCA	aminomethylcoumarin

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BIG2	Brefeldin A (BFA)-inhibited guanine nucleotide-exchange protein 2
DMEM	Dulbecco's Modified Eagle's Medium
DOC	sodium deoxycholate
EGFP	enhanced green fluorescent protein
EM	electron microscope(y)
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
GABARAP	GABA receptor associated protein
GAD	glutamic acid decarboxylase
GRIP1	Glutamate receptor interact protein 1; Hanks' Balanced Salt Solution
HEK293	human embryonic kidney 293 cells
PBS	phosphate-buffered saline
PDZ	postsynaptic density PSD-95/SAP90, the Drosophila septate junction protein disc large, and the tight junction zonula occludens-1
PIS	pre-immune serum
PSD	postsynaptic density
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
shRNA	small hairpin RNA

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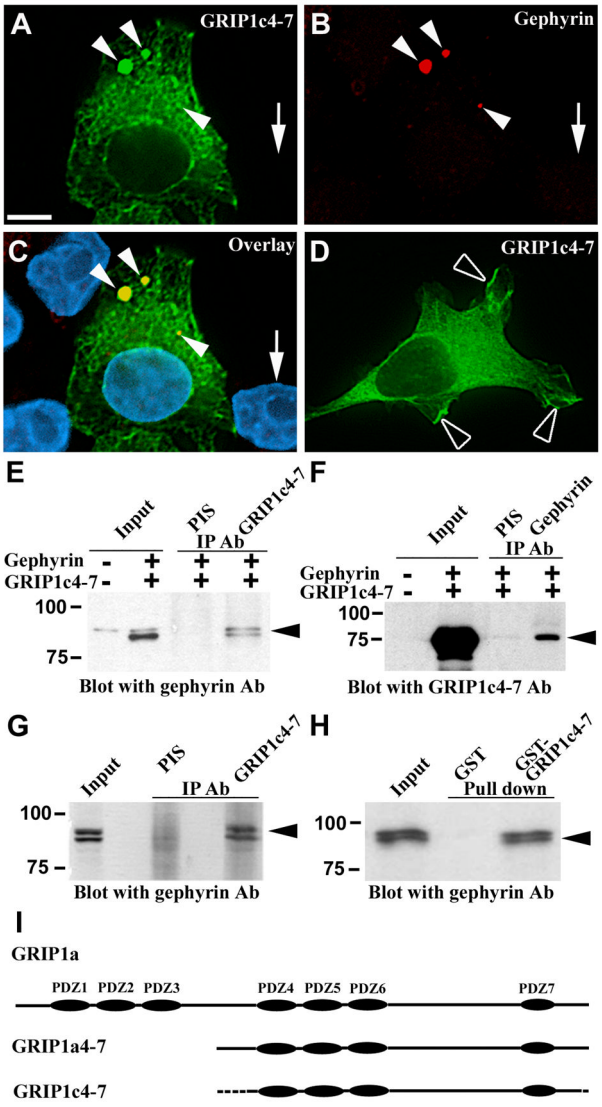

Fig. 1. GRIP1c4-7 associates with gephyrin in transfected HEK293 cells and in brain extracts

A–C, HEK293 cells were cotransfected with GRIP1c4-7 and gephyrin. Double immunofluorescence labeling was performed with the guinea pig anti-GRIP1c4-7 (green) and the mouse anti-gephyrin antibody (red). Some of the GRIP1c4-7 (A) is recruited at intracellular compartments (white arrowheads) where gephyrin accumulates (B). The overlay (C) also shows cell nuclei that were counter-stained with DAPI. The arrow shows the nucleus of a non-transfected cell. Note that these cells show no immunofluorescence of either GRIP1c4-7 or gephyrin. **D,** When HEK293 cells were transfected with GRIP1c4-7 only, GRIP1c4-7 showed a diffuse distribution in the cytoplasm and did not form intracellular aggregates. GRIP1c4-7 also associated with plasma membrane (black arrowheads). When HEK293 cells were transfected only with gephyrin, the latter formed aggregates (Fig 2L) similar to the ones shown in panel B. The illustrated expression patterns in A–C are representative of 39 out of 42 cells and in D of 66 out of 73 cells (from 3 and 4 transfection experiments respectively). **E,** The guinea pig anti-GRIP1c4-7 antibody, but not the corresponding PIS, coprecipitated associated gephyrin (arrowhead, 93 and 96 kD doublet) from extracts of HEK293 cotransfected with gephyrin and GRIP1c4-7, as shown in the immunoblot with the rabbit anti-gephyrin antibody. **F,** The rabbit anti-gephyrin

antibody, but not the corresponding PIS, coprecipitated GRIP1c4-7 (arrowhead, 75 kD) from extracts of the cotransfected HEK293 cells, as shown in immunoblots with a mouse mAb that recognizes GRIP1c4-7. **G,** The guinea pig anti-GRIP1c4-7 antibody, but not the corresponding PIS, coprecipitated the gephyrin doublet (arrowhead) from a rat brain extract, as shown with the rabbit anti-gephyrin antibody. **H,** Beads coated with the GST-GRIP1c4-7 fusion protein pulled down gephyrin (arrowhead) from a rat brain extract, but beads coated with GST did not, as shown with the rabbit anti-gephyrin antibody. **I,** Schematic representation and alignment of various GRIP1 constructs used in this study. GRIP1a is a 135 kD protein with 7 PDZ domains. GRIP1a4-7 is an engineered truncated form of GRIP1a that is identical to amino acids 417–1112 of GRIP1a. GRIP1a4-7 has the same length as GRIP1c4-7. GRIP1c4-7 is a naturally existing short splice variant of GRIP1. Amino acids 36–684 of GRIP1c4-7 are identical to amino acids 452–1100 of GRIP1a. Broken lines at the N and C termini of GRIP1c4-7 represent the 35 and 12 amino acid peptides respectively, which are not present in GRIP1a, GRIP1b or GRIP1a4-7. GRIP1b is identical to GRIP1a except for the N-terminal 18 and 19 amino acid peptides of GRIP1a and GRIP1b respectively. All immunoprecipitation and pull-down experiments were done three times with similar results. Scale bar, 5 μm.

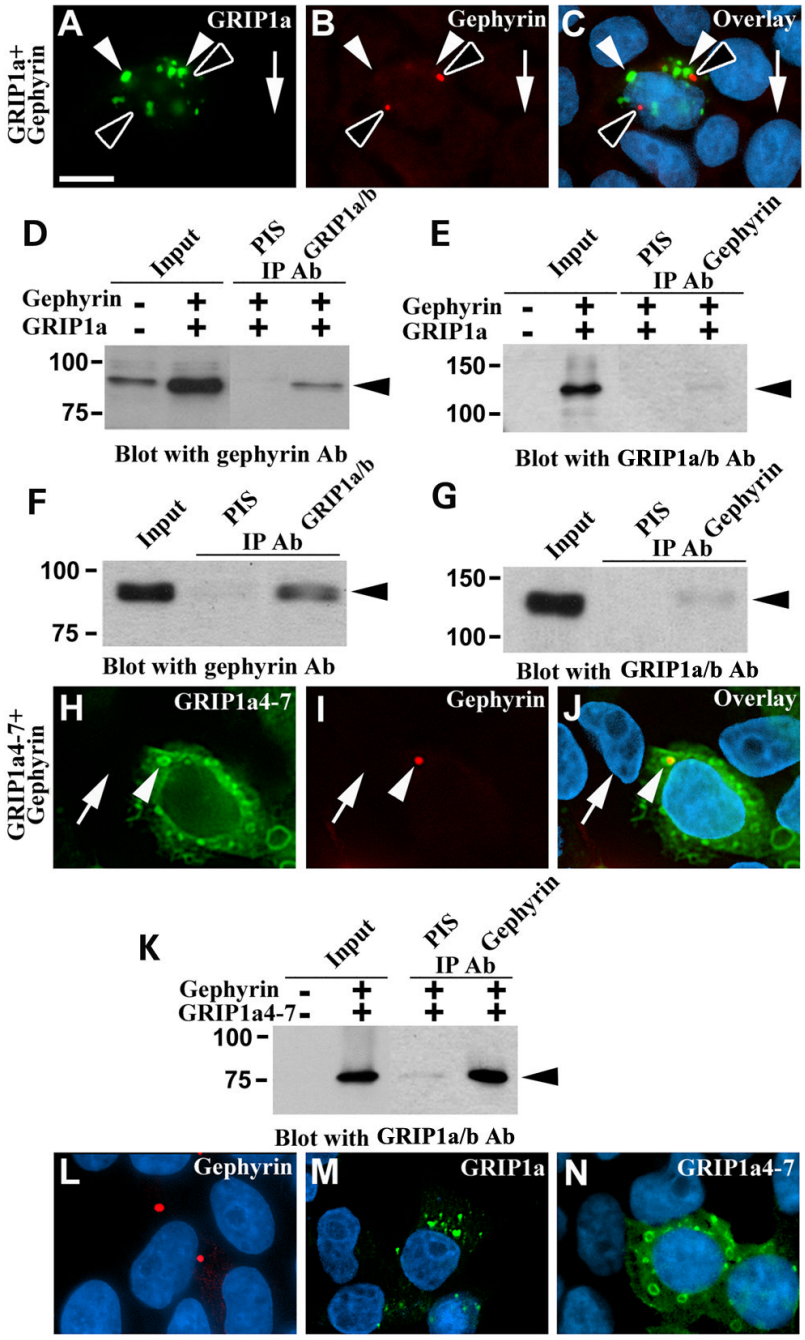

Fig 2. GRIP1a/b and GRIP1a4-7 associate with gephyrin in transfected HEK293 cells and in brain extracts

A–C, HEK293 cells were cotransfected with gephyrin and full-length GRIP1a. Double immunofluorescence labeling was performed with the guinea pig anti-GRIP1a/b (green) and the mouse mAb to gephyrin (red). Full-length GRIP1a (white arrowheads) and gephyrin (black arrowheads) formed cytoplasmic aggregates in HEK293. The overlay (C) also shows cell nuclei that were counter-stained with DAPI. The arrow in C shows the nucleus of a non-transfected cell. The illustrated expression patterns in A–C are representative of 36 out of 49 cells from 3 transfection experiments. **D,** In HEK293 cells cotransfected with GRIP1a and gephyrin, the guinea pig anti-GRIP1a/b antibody, but not the corresponding PIS,

coprecipitated gephyrin (93–96 kD, arrowhead) as revealed in the immunoblot with the rabbit anti-gephyrin antibody. **E,** The rabbit anti-gephyrin antibody, but not the corresponding PIS, coimmunoprecipitated a very small amount of the full-length GRIP1a (130 kD, arrowhead, faint protein band, first lane from the right) when compared with the input (arrowhead, second lane from the left) from extracts of HEK293 cells cotransfected with gephyrin and GRIP1a, as shown with a mAb to GRIP1a/b, which also recognizes GRIP1a4-7 (see panel K). **F,** The guinea pig anti-GRIP1a/b antibody but not the corresponding PIS, coprecipitated gephyrin (93–96 kD, arrowhead) from a rat brain extract. **G,** The rabbit anti-gephyrin antibody but not the corresponding PIS, coprecipitated GRIP1a/b (130 kD, arrowhead) from a rat brain extract. Note that the protein band corresponding to GRIP1a/b is very faint (first lane from the right). **H–J,** HEK293 cells were cotransfected with engineered GRIP1a4-7 and gephyrin. Double immunofluorescence labeling reveals that GRIP1a4-7 (green) shows both diffuse distribution in the cytoplasm and concentration in cytoplasmic compartments. Some GRIP1a4-7 was recruited to the cytoplasmic compartments where gephyrin (red) accumulates (white arrowhead). The overlay (J) also shows cell nuclei that were counter-stained with DAPI. The arrow in J shows the nucleus of a non-transfected cell. The antibodies used were the same as in panels A–C. The illustrated expression patterns in H–J are representative of 38 out of 47 cells from 3 transfection experiments. **K,** The anti-gephyrin antibody coprecipitated GRIP1a4-7 (75 kD, arrowhead, first lane from the right) when compared with the input (arrowhead, second lane from the left) from extracts of HEK293 cells cotransfected with gephyrin and GRIP1a 4-7. Note that the density of the band corresponding to the coprecipitated truncated GRIP1a4-7 form (75 kD, arrowhead, first lane from the right) is considerably stronger than that of the co-precipitated full-length GRIP1a protein in panel E (130 kD, arrowhead, first lane from the right). The same mAb was used in panels E and K to identify GRIP1a and GRIP1a4-7 in the immunoblots. **L–N,** HEK293 cells were single-transfected with gephyrin (L, red), GRIP1a (M, green), or GRIP1a4-7 (N, green). Cell nuclei were counter-stained with DAPI. The illustrated expression patterns in L–N are representative of 66 out of 72 (L), 83 out of 99 (M) and 85 out of 101 (N) cells from 5 transfection experiments. All immunoprecipitation experiments were done three times with similar results. Scale bar, 5 μm.

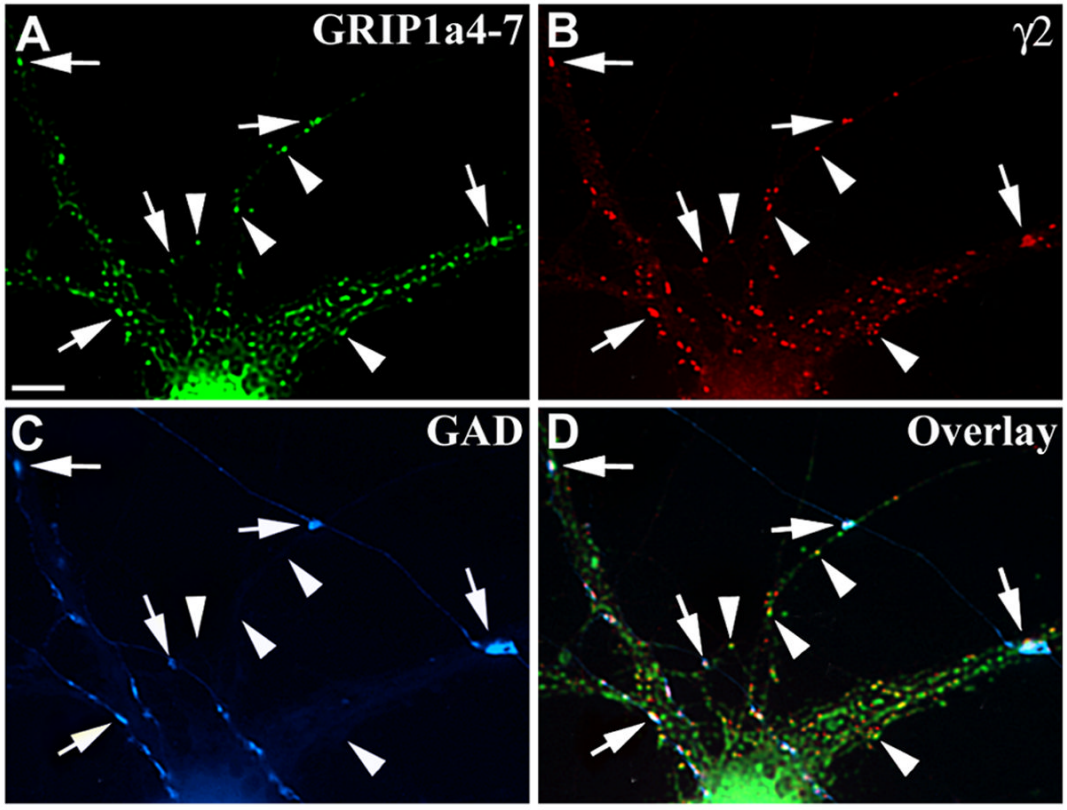

Fig 3. HA-GRIP1a4-7 colocalizes with postsynaptic $GABA_A$Rs and extrasynaptic $GABA_A$R clusters in transfected hippocampal pyramidal neurons

A–C, Triple-label immunofluorescence of hippocampal neurons transfected with HA-GRIP1a4-7 cDNA using a combination of mouse mAb to the HA epitope (green, A), rabbit anti-γ2 $GABA_A$R subunit (red, B), and sheep anti-GAD (blue C). Clusters of HA-GRIP1a4-7, in a transfected pyramidal cell, colocalize with γ2-$GABA_A$R clusters at GABAergic synapses (arrows) as shown by their apposition to presynaptic GAD^+ terminals. The interneuron, from which the axon containing GAD^+ presynaptic terminals originates, has no HA immunoreactivity. Thus the localization of the HA-GRIP1a4-7 in these GABAergic synapses is postsynaptic. Clusters of HA-GRIP1a 4-7 immunoreactivity also colocalize with some non-synaptic γ2-$GABA_A$R clusters (arrowheads), since they are not apposed to presynaptic GAD^+ terminals. **D,** Overlay of A, B and C. Scale bar, 20 μm.

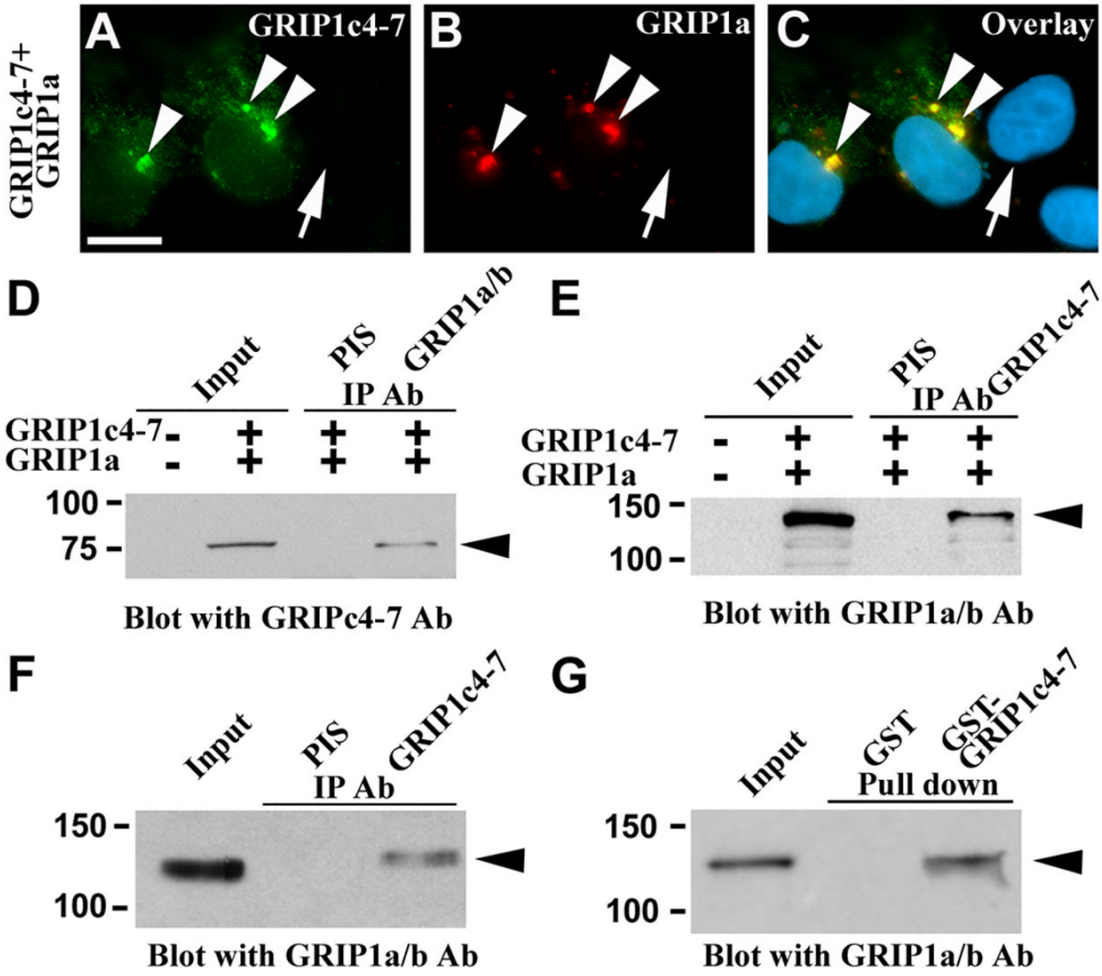

Fig 4. GRIP1c4-7 can associate with GRIP1a in transfected HEK293 cells and in brain extracts

A–C, HEK293 cells were cotransfected with GRIP1c4-7 and GRIP1a. Double immunofluorescence labeling was performed with the guinea pig anti-GRIP1c4-7 antibody (green), which does not recognize GRIP1a/b, and the rabbit anti-GRIP1a/b antibody (red), which does not recognize GRIP1c4-7. The GRIP1c4-7 and GRIP1a formed large aggregates that frequently colocalized (A–C, arrowheads). GRIP1c4-7 does not form aggregates when HEK293 cells are transfected only with GRIP1c4-7 (Fig 1D). The overlay (C) also shows cell nuclei that were counter-stained with DAPI. The arrow shows the nucleus of a non-transfected cell. The illustrated expression patterns in A–C are representative of 45 out of 49 cells from 3 transfection experiments. **D,** The guinea pig anti-GRIP1a/b antibody but not the corresponding PIS, coprecipitated GRIP1c4-7 (75 kD, arrowhead) from detergent extracts of HEK293 cells cotransfected with GRIP1c4-7 and GRIP1a, as shown by immunoblotting with the rabbit anti-GRIP1c4-7 antibody. **E,** The guinea pig anti-GRIP1c4-7 antibody, but not the corresponding PIS, coprecipitated GRIP1a (130 kD, arrowhead) from a detergent extract of HEK293 cells cotransfected with GRIP1c4-7 and GRIP1a, as shown by immunoblotting with a rabbit antibody to GRIP1a/b. **F,** The guinea pig anti-GRIP1c4-7 antibody, but not the corresponding PIS, coprecipitated GRIP1a/b (arrowhead) from the rat brain extract. **G,** Beads coated with the GST-GRIP1c4-7 fusion protein pulled down GRIP1a/b (arrowhead) from a rat brain extract, while beads coated with GST did not. All the antibodies used in the immunoblots (D–G) were made in rabbit. All immunoprecipitation and pull-down experiments were done three times with similar results. Scale bar, 5 μm.

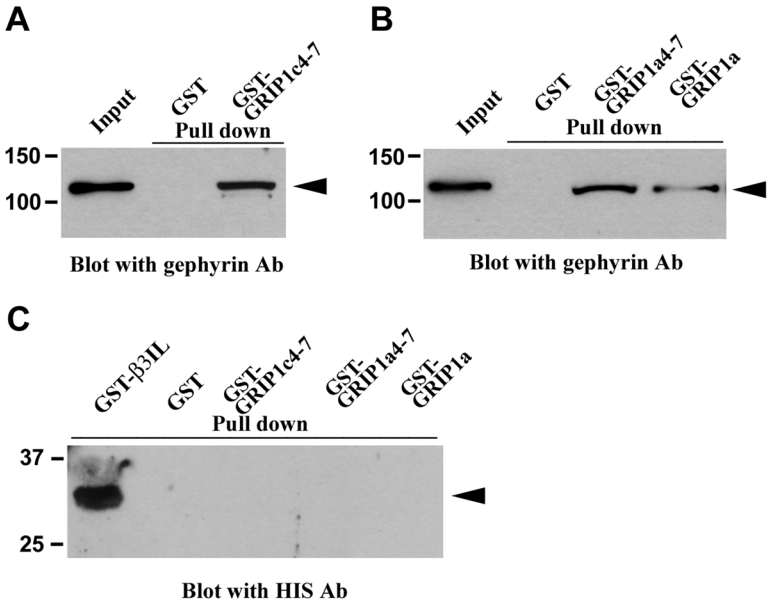

Fig 5. Purified GRIP1c4-7 and GRIP1a bind to purified gephyrin

A, Purified and solubilized His-gephyrin (113 kD, arrowheads) bound to GST-GRIP1c4-7 but not GST immobilized on glutathione beads. **B,** Purified His-gephyrin (arrowhead) bound to GST-GRIP1a4-7 and GST-GRIP1a but not GST immobilized on glutathione beads. In A and B His-gephyrin was detected in immunoblots with the rabbit anti-gephyrin antibody. **C,** Purified His-BIG2CT (31kD, arrow head) bound to GST-β3IL, but not to GST, GST-GRIP1c4-7, GST-GRIP1a4-7, or GST-GRIP1a immobilized on glutathione beads as revealed with an anti-His mAb. The experiments were done three times with similar results. Scale bar, 5 μm.

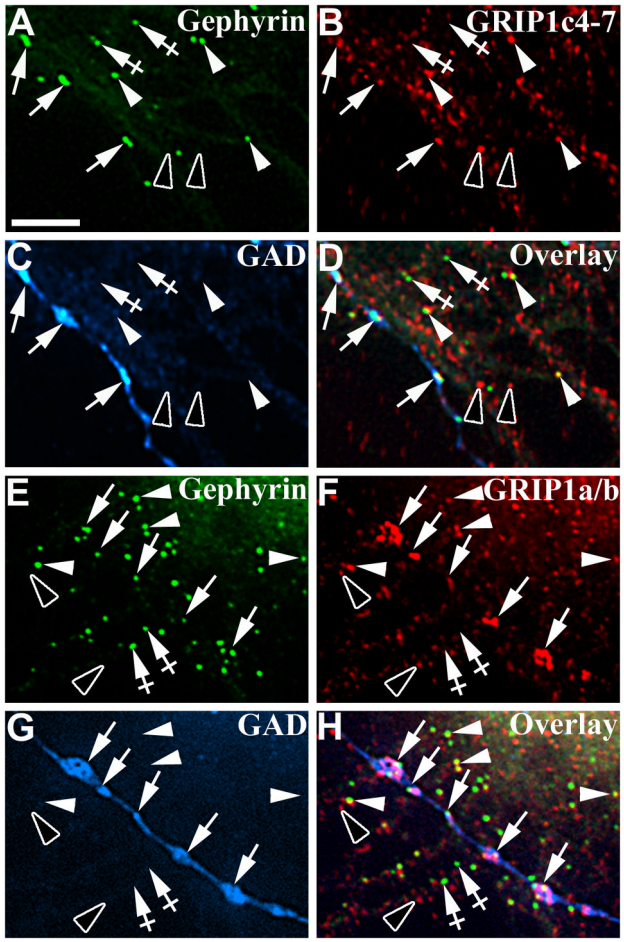

Fig 6. Gephyrin frequently colocalizes with GRIP1c4-7 and GRIP1a/b at GABAergic synapses in cultured hippocampal neurons

A–D, Triple-label immunofluorescence with mouse mAb to gephyrin (green), rabbit anti-GRIP1c4-7 (red) and sheep anti-GAD (blue) antibodies. **E–H,** Triple-label immunofluorescence with mouse mAb to gephyrin (green), rabbit anti-GRIP1a/b (red) and sheep anti-GAD (blue) antibodies. Arrows show the colocalization of gephyrin clusters with GRIP1c4-7 or GRIP1a/b clusters at GABAergic synapses. Synaptic clusters are identified by their apposition to GAD^+ presynaptic terminals. White arrowheads show colocalization of some gephyrin clusters with GRIP1c4-7 or GRIP1a/b clusters outside GABAergic synapses (not apposed to GAD^+ terminals). Crossed arrows show gephyrin clusters that are not colocalized with GRIP1c4-7 or GRIP1a/b clusters and are not apposed to GAD^+ terminals. Black arrowheads show GRIP1c4-7 or GRIP1a/b clusters that are not colocalized with gephyrin clusters and are not apposed to GAD^+ terminals. Scale bar, 5 μm.

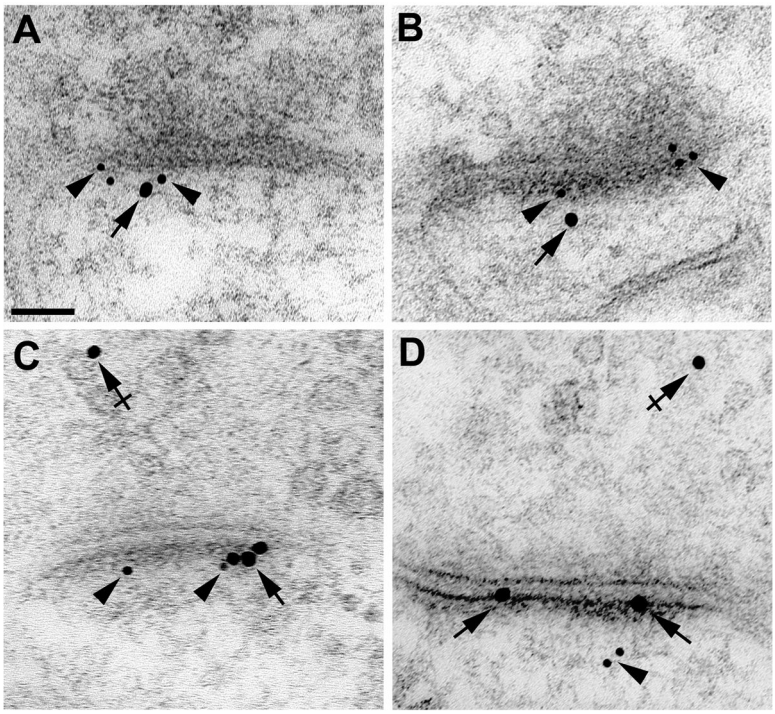

Fig 7. Gephyrin colocalizes with GRIP1c4-7 and GRIP1a/b at the postsynaptic complex of individual synapses in the intact brain

Postembedding EM immunogold labeling of the cerebellum. In all panels, the presynaptic terminal containing synaptic vesicles is located above the synaptic cleft. **A, B,** Double-labeling with the mouse mAb to gephyrin (small particles, arrowheads) and the rabbit anti-GRIP1c4-7 (large particles, arrows) shows that the two types of gold particles, corresponding to gephyrin and GRIP1c4-7, colocalize postsynaptically at the same synapse. **C, D,** Double-labeling with the mouse mAb to gephyrin (small particles, arrowheads) and the rabbit anti-GRIP1a/b antibody (large particles, arrows) shows that gold particles corresponding to gephyrin and GRIP1a/b colocalize postsynaptically in the same synapse. Crossed arrows in C, D show gold particles corresponding to presynaptically localized GRIP1a/b. The images correspond to synapses of stellate cells onto Purkinje cell dendrites. Scale bar, 75 nm.