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The Proteoglycan NG2 Is Complexed with α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors by the PDZ Glutamate Receptor Interaction Protein (GRIP) in Glial Progenitor Cells

IMPLICATIONS FOR GLIAL-NEURONAL SIGNALING*

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The proteoglycan NG2 is expressed by immature glial cells in the developing and adult central nervous system. Using the COOH-terminal region of NG2 as bait in a yeast two-hybrid screen, we identified the glutamate receptor interaction protein GRIP1, a multi-PDZ domain protein, as an interacting partner. NG2 exhibits a PDZ binding motif at the extreme COOH terminus which binds to the seventh PDZ domain of GRIP1. In addition to the published expression in neurons, GRIP1 is expressed by immature glial cells. GRIP1 is known to bind to the GluRB subunit of the AMPA glutamate receptor expressed by subpopulations of neurons and immature glial cells. In cultures of primary oligodendrocytes, cells coexpress GluRB and NG2. A complex of NG2, GRIP1, and GluRB can be precipitated from transfected mammalian cells and from cultures of primary oligodendrocytes. Furthermore, NG2 and GRIP can be coprecipitated from developing brain tissue. These data suggest that GRIP1 acts as a scaffolding molecule clustering NG2 and AMPA receptors in immature glia. In view of the presence of synaptic contacts between neurons and NG2-positive glial cells in the hippocampus and the close association of NG2-expressing glial cells with axons, we suggest a role for the NG2·AMPA receptor complex in glial-neuronal recognition and signaling.

The NG2 proteoglycan is as a large transmembrane glycoprotein expressed by oligodendrocyte progenitor cells but down-regulated upon differentiation into mature oligodendrocytes (1, 2). The AN2 proteoglycan (3) is the mouse homolog of NG2 (4). In the developing and adult nervous system many NG2-positive cells abound in both white and gray matter whose classification as oligodendrocyte or astrocyte lineage cells remains unclear but whose electrophysiological properties are typical of immature glial cells (5). Recently, Bergles and colleagues (6) identified a novel type of synapse in the developing and adult rat hippocampus where pyramidal neurons of the CA3 area form morphologically identifiable synaptic boutons on NG2-positive cells. Furthermore, stimulation of the neurons resulted in a Ca^{2+} signal in the NG2-positive glial cells which was dependent on the activity of the AMPA[1] class of glutamate receptors. AMPA receptors are known to be expressed by subclasses of glial cells including oligodendrocyte precursor cells (7) where their function is thought to include an inhibitory influence on proliferation and lineage progression of oligodendrocyte progenitors (8).

NG2 is a single pass transmembrane proteoglycan with a large extracellular domain and two LAM G (LNS) domains near the NH_2 terminus, suggesting a role as an adhesion molecule. NG2 has a short intracellular tail of 76 amino acids. The PDZ protein MUPP1 was identified recently by yeast two-hybrid screening of a library from E9.5/10.5 mouse embryo as an intracellular binding partner of rat NG2 (9), although the biological significance of this finding remains unclear. Because the COOH terminus of NG2 exhibits a putative PDZ binding motif and PDZ domain proteins are adaptor proteins that target and cluster protein complexes including neurotransmitter receptors to the cell surface (10), we sought to identify intracellular partners of NG2 in glial progenitor cells. The cytoplasmic domain of murine NG2 was used as bait in yeast two-hybrid analysis to screen a library from early postnatal mouse brain. This screen revealed the glutamate receptor interaction protein (GRIP) 1 as a binding partner. We show that GRIP1 acts as a direct molecular link between AMPA receptors and the glycoprotein NG2 in immature glial cells. This protein complex, together with as yet to be identified additional members, may contribute to a postsynaptic microdomain in immature glial cells and contribute to glial-neuronal signaling.

EXPERIMENTAL PROCEDURES

Animals—NMRI mice were obtained from the central animal facilities of the University of Heidelberg.

Antibodies—The following primary antibodies were used: monoclonal (mc) GRIP1 (Becton Dickinson), polyclonal (pc) antibodies

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[1] The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GRIP, glutamate receptor interaction protein; GST, glutathione *S*-transferase; mc, monoclonal; MOG, myelin oligodendrocyte glycoprotein; NCAM, neural cell adhesion molecule; PBS, phosphate-buffered saline; pc, polyclonal; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PDZ, postsynaptic density 95/discs large/zona occludens 1; LNS, laminin/neurexin/sex hormone binding globulin.

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against GRIP1 (kind gift from Dr. R. Klein), and pc GluRB[2] (Chemicon, Hofheim, Germany). The antibodies recognize specifically the short GluRB form (COOH-terminal peptide: SVKI*), which binds to GRIP1. We also used pc AN2 antibodies (3), anti-GST880S16 (pc AN2 against a GST fusion protein consisting of amino acids 1255–1545, mc AN2 antibodies (3), pc antibodies against NCAM (11), mc anti-Myc (Sigma), and mc 8-18-C5 against MOG (a kind gift from Dr. C. Linington).

Isolation of GRIP1 by Yeast Two-hybrid Screen—The COOH-terminal region of mouse NG2 (NH_2-RKRNKT . . . NGQYWV-COOH, GenBank accession number AF352400) was fused to the GAL4 binding domain by cloning it into the pGBT9 vector (Clontech) with *Eco*RI/*Bam*HI. The resulting bait construct was designated pGBT9cyto. Using the lithium acetate method, the yeast strain CG1945 was transformed sequentially with pGBT9cyto and a postnatal mouse brain MATCHMAKER cDNA library in pACT2 (Clontech). 33×10^6 transformants were screened. Transformants were grown on SD medium-Leu-Trp-His plates; 5 mM 3-amino-1,2,4-triazole was added to the medium to suppress leaky HIS3 reporter gene expression. Positive clones were tested for β-galactosidase gene activity: yeast colonies were grown on SD-Leu-Trp-His, transferred onto reinforced nitrocellulose membrane, submerged in liquid nitrogen, and placed on a Z-buffer/X-gal-soaked Whatman (Z-buffer: 16.1 g/liter Na_2HPO_4*7H2O, 5.5 g/liter NaH_2PO_4*H2O, 0.75 g/liter KCl, 0.246 g/liter $MgSO_4$*7H2O, pH 7; Z-buffer/X-gal solution: 100 ml of Z-buffer, 0.27 ml of β-mercaptoethanol, 1.67 ml of 20 mg/ml X-gal stock solution). Blue color was allowed to develop for 30 min-3 h. DNA-sequencing results revealed 48 isolates and 10 independent clones of GRIP1. All clones included the seventh PDZ domain and the COOH terminus of GRIP1. The specificity of the NG2-GRIP1 interaction was confirmed by a β-galactosidase test and growth selection of cotransformed yeast cells with pGBT9cyto and isolated library plasmids. Unspecific NG2 interactions were excluded by contransformation of yeast cells with pGBT9cyto and GRIP1 constructs in pGADGH lacking the seventh PDZ domain (kind gift from Dr. R. Klein). Unspecific interactions of the seventh PDZ domain of GRIP1 were excluded by cotransformation of yeast with a pGBT9 construct encoding the cytoplasmic COOH terminus of an unrelated transmembrane protein M6A.[3]

GRIP1 Deletion Analysis—The yeast two-hybrid system was used to map the interaction site of GRIP1 with NG2. Results from the sequencing of GRIP1 revealed the seventh PDZ domain as putative interaction site. Mouse GRIP1 deletion constructs in pACT2 were used as resulting from the screen: mouse GRIP1 4p-7 (LEIEFD . . . EPTNTL), mouse GRIP1 5–7 (KHSVEL . . . EPTNTL), mouse GRIP1, 7 (ATIMSG . . . EPTNTL). Further control constructs of rat GRIP1 were kind gifts from Dr. R. Klein: rat GRIP1 4–6 (TSPRGT . . . KLSDVY), rat GRIP1, 6 (EDNSDE . . . KQTDAQ), rat GRIP1 6–7 (GAIIYT . . . EPTNTL). The NH_2- and COOH-terminal sequences above correspond to sequences from mouse GRIP1 library plasmids or to the published rat GRIP1 sequence (12). All constructs were verified by DNA sequencing. Cotransformed yeast cells were grown on double dropout medium and assayed for β-galactosidase gene activity and additionally selected for growth on triple dropout medium.

GRIP2 Interaction Analysis—The yeast two-hybrid system was used to test the interaction of NG2 and the seventh PDZ domain of GRIP2. The GRIP2 cDNA sequence was generated by reverse transcription PCR, which introduced *Eco*RI/*Bam*HI sites and then cloned into the pACT2 vector: rat GRIP2, 7 (RSREVGT . . . SSPQMI); the sequence corresponds to the published rat sequence (13) and was verified by sequencing.

Identification of PDZ Binding Motif—The yeast two-hybrid system was used to map the PDZ binding motif at the extreme COOH terminus of NG2. Individual mutations of the 0, −1, −2, and −3 positions of the COOH-terminal peptide QYWV* were introduced by PCR, cloned into pGBT9, and designated as NG2 0G (Val mutated to Gly), NG2 −1G (Trp mutated to Gly), NG2 −2G (Tyr mutated to Gly), NG2 −2F (Tyr mutated to Phe), NG2 −3G (Gln mutated to Gly). Mutant NG2 constructs were contransformed with mouse GRIP1 PDZ7 and rat GRIP2 PDZ7. Yeast cells were grown on double dropout medium and assayed for β-galactosidase gene activity and additionally selected for growth on triple dropout medium.

Transfection and Expression Constructs—COS7 cells were transfected by electroporation (0.25 kV, 250 microfarads). Plasmids were used at 15 μg/300 μl of cell suspension (4×10^6 cells/ml, Dulbecco's modified Eagle's medium and 10% fetal calf serum). The NG2 deletion mutant was generated by trimolecular ligation of two PCR-amplified regions of NG2 with artificially introduced restriction sites in the pRK5 vector (14). The deletion mutant consists of the signal sequence, one-fourth of the very NH_2-terminal extracellular portion (including both LNS domains), the transmembrane domain, and the complete intracellular region. Mouse GRIP1/PDZ7 and mouse GRIP1/PDZ5–7 expression constructs were generated by cloning murine sequences into pRK5 vector with *Eco*RI/*Bam*HI and *Eco*RI/*Hin*dIII sites introduced by PCR, respectively. The constructs contain a translation initiation sequence (15) and encode an NH_2-terminal Myc tag. GluRB full-length, flop, short form was a kind gift from Dr. H. Monyer. Constructs were verified by sequencing.

Coimmunoprecipitation—Transfected COS7 cells were washed with phosphate-buffered saline (PBS) 24 h after transfection, starved for 1 h in methionine/cysteine-free medium, then metabolically labeled with 100 μCi/ml [^{35}S]Met/Cys for 4 h. Cells were washed twice with PBS and lysed (1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, protease inhibitors), and the lysates were chilled for 30 min and centrifuged at 3,000 rpm for 5 min to remove nuclei. For immunoprecipitation the following antibodies were used: mouse mc Myc (Sigma), rabbit pc AN2 (3), pc GluRB (Chemicon). Lysates were preabsorbed with protein A-Sepharose (Amersham Biosciences) for 1 h at 4 °C, then subjected to immunoprecipitation overnight at 4 °C. Precipitation was performed with protein A-Sepharose. Precipitates were washed three times with radioimmune precipitation assay buffer (0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 7) and once with PBS before adding sample buffer and resolving the proteins by SDS-PAGE. Gels were dried, exposed to screens, and evaluated with a PhosphorImager.

Total mouse brains (P7) were homogenized in buffer (50 mM Tris, pH 7.8, 3 mM $MgCl_2$, 320 mM sucrose, protease inhibitors) using an Ultra Turrax. The homogenate was centrifuged for 10 min at 1,000 rpm, 4 °C, the resulting supernatant was centrifuged for 1 h at $100{,}000 \times g$, 4 °C. The pellet was extracted in buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.05% sodium deoxycholate, 1% Triton X-100, protease inhibitors) for 1 h at 4 °C and centrifuged afterward for 1 h, $100{,}000 \times g$, 4 °C. The supernatant was preabsorbed with protein A-Sepharose and subjected to immunoprecipitation as described above. Precipitates were washed three times with 1% Triton X-100 buffer and once with PBS and analyzed by SDS-PAGE and Western blotting.

Primary oligodendrocytes were lysed in 1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, protease inhibitors, and the lysates were chilled on ice, centrifuged, and subjected to immunoprecipitation. Precipitates were washed three times with lysis buffer and once with PBS and analyzed by SDS-PAGE and Western blotting.

Western Blot Analysis—SDS-PAGE was performed according to Laemmli (16). Proteins were blotted onto polyvinylidene difluoride membrane (Hybond P, Amersham Biosciences), blocked with 4% milk powder in PBS and 0.1% Tween 20, and incubated with primary antibodies for 1 h. The blots were washed with PBST and incubated with appropriate secondary horseradish peroxidase-conjugated anti-mouse immunoglobulin (Dianova, Hamburg, Germany), horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Dianova). They were washed twice with PBST and once with PBS and subsequently developed by enhanced chemiluminescence (Amersham Biosciences).

Lipid Raft Preparation—12×10^6 cells (primary oligodendrocytes) were extracted in 750 μl of 1% Triton X-100 buffer, chilled for 30 min, and centrifuged (see "Coimmunoprecipitation"). 750 μl of extract was adjusted to 40% sucrose with 750 μl of 80% sucrose. The extract was overlaid with 1.75 ml of 30% sucrose and 1.5 ml of 5% sucrose in an SW60 tube (Beckman). After centrifugation (4 h at $218{,}000 \times g$, 4 °C) eight fractions were collected (17) and analyzed by 4–10% SDS-PAGE and Western blotting.

Cell Culture and Immunofluorescence Staining—Primary oligodendrocytes were cultured according to Trotter and colleagues (11, 18), shaken-off oligodendrocytes were cultured on poly-L-lysine-coated glass coverslips for 2 days in SATO medium containing 1% horse serum, 10 ng/ml platelet-derived growth factor, and 5 ng/ml basic fibroblast growth factor. Cells were washed with PBS, fixed for 10 min with 4% paraformaldehyde, washed with PBS, permeabilized for 5 min with 0.05% Triton X-100, washed with PBS, and blocked with β-mercaptoethanol and 10% horse serum. Primary antibodies were diluted in blocking buffer and incubated for 30 min. Cells were washed three times with β-mercaptoethanol and 10% horse serum and incubated for 30 min with appropriate secondary Cy2- and Cy3-conjugated pc antibodies (Dianova), diluted in blocking buffer. The washed coverslips were mounted in Moviol and analyzed by confocal microscopy (Leica, Bensheim, Germany).

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[2] The AMPA receptor subunits GluRA, B, C, and D are sometimes referred to as GluR1, 2, 3, and 4, respectively.

[3] H. Werner and K. Nave, unpublished data.

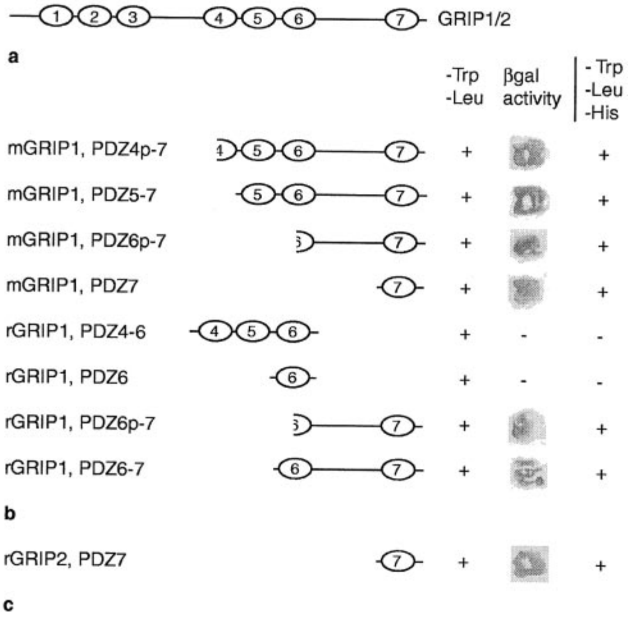

FIG. 1. **NG2 interacts with the PDZ domain 7 of GRIP1 and GRIP2.** *a* depicts the protein structure of GRIP1 and GRIP2; the seven PDZ domains are represented by *ovals*. *b*, different GRIP1 constructs were used to verify the interaction with the cytoplasmic region of NG2. Transformed yeast were grown on SD-Trp-Leu plates, assayed for β-galactosidase (*βgal*) activity, and selected for growth on SD-Trp-Leu-His. *c* shows the interaction of GRIP2 (PDZ7) with NG2. *m*, mouse; *r*, rat; partial PDZ domain are indicated by *p*, + indicates growth on selection plates, and − indicates no growth. The resulting color reaction of β-galactosidase activity from growing yeast is shown.

	mGRIP1, PDZ7			rGRIP2, PDZ7		
	-Trp -Leu	βgal activity	- Trp -Leu -His	-Trp -Leu	βgal activity	- Trp -Leu -His
NG2 wt (QYWV*)	+	+	+	+	+	+
NG2 0G (QYW**G***)	+	-	-	+	-	-
NG2 -1G (QY**G**V*)	+	-	-	+	-	-
NG2 -2G (Q**G**WV*)	+	-	-	+	-	-
NG2 -3G (**G**YWV*)	+	+	+	+	+	+
NG2 -2F (Q**F**WV*)	+	+	+	+	+	+

FIG. 2. **The COOH-terminal peptide of NG2 binds to the PDZ domain of GRIP.** The cytoplasmic region of NG2 was mutated at the indicated positions; the resulting COOH-terminal sequence is shown in *parentheses*. Wild type and mutated COOH termini were tested for interaction with PDZ7 of GRIP1 and GRIP2. Transformants were grown on double dropout selection plates, assayed for β-galactosidase (*βgal*) activity, and selected for growth on triple dropout selection plates.

RESULTS

NG2 Binds to the Seventh PDZ Domain of GRIP1 and GRIP2—The complete mouse NG2 cytoplasmic region consisting of 76 amino acids (RKRN . . . QYWV*, * = translation stop codon) was used as a bait in a yeast two-hybrid analysis to screen a postnatal mouse brain cDNA library. 33 × 10^6 transformants were screened. Ten independent library plasmids represented fragments of the same sequence, which was identical to murine GRIP1, a multi-PDZ domain protein (Fig. 1*a*). GRIP1 was originally identified as an interacting protein of AMPA receptor subunits GluRB and GluRC (12) The GRIP1 clones isolated in the screen were grouped according to their PDZ domain composition: group1 encodes a partial PDZ domain 4 through PDZ domain 7 (GRIP1/4p-7), group2 PDZ domain 5 through 7 (GRIP1/5–7), group3 a partial PDZ domain 6 through 7 (GRIP1/6p-7), group4 encodes PDZ domain 7 only (GRIP1/7). This analysis already identified the seventh PDZ domain as the putative interaction site.

Yeast two-hybrid technology was used to verify the interaction of GRIP1 with the cytoplasmic region of NG2 and to confirm the interaction site by excluding binding of NG2 to GRIP1 PDZ domains other than PDZ7. Yeast was cotransformed with isolated GRIP1 plasmids and pGBT9cyto, grown on double dropout medium, and subsequently assayed for β-galactosidase activity. The yeast colonies were additionally selected for growth on triple dropout medium. As shown in Fig. 1*b*, yeast two-hybrid analysis and subsequent β-galactosidase assay confirmed the binding of NG2 to GRIP1/PDZ4p-7, GRIP1/PDZ5–7, GRIP1/PDZp6–7, and GRIP1/PDZ7. When rat GRIP1 constructs consisting of PDZ domains 4–6 and the sixth domain only were tested for interaction, the yeast cells were negative for β-galactosidase activity, and failed to grow on triple dropout medium. Rat constructs containing the seventh PDZ domain such as GRIP1/PDZ6p-7 and GRIP1/PDZ6–7 restored the interaction of NG2 and GRIP1 and clearly identified the seventh PDZ domain of GRIP as the interaction site.

GRIP2, a GRIP1 homolog, also consists of seven PDZ domains and is expressed in the central nervous system (13, 19). Because the sequences of the corresponding PDZ domains of GRIP1 and GRIP2 are very well conserved, we tested whether the interaction with NG2 was similarly conserved. The results in Fig. 1*c* demonstrate that NG2 also interacts with the seventh PDZ domain of GRIP2, even though no clone for GRIP2 was isolated in the original screen.

NG2 Has a PDZ Binding Motif at the Extreme COOH Terminus—PDZ domains bind to COOH-terminal peptides of the interacting protein. The COOH-terminal tetrapeptide of NG2 (QYWV*) is conserved among rat, mouse, human, and *Drosophila*. It is similar to the PDZ binding motifs of ephrin B1 (YYKV*), which binds to PDZ6 of GRIP1/2 (19) and neurexins (EYYV*), which bind to CASK (20), with a valine at position 0 and a tyrosine at the −2 position. To test whether QYWV* of NG2 is indeed a PDZ binding motif, positions 0, −1, −2, and −3 were mutated individually to glycine (Fig. 2). The mutations 0G, −1G, and −2G eliminated the interaction of NG2 and GRIP1/2, confirming that there is a PDZ binding motif at the extreme COOH terminus of NG2. Mutating the −3 position was without effect. The −2 position has been reported to be critical for the interaction. To characterize further the relevance of the amino acid at the −2 position, tyrosine was mutated to phenylalanine (Fig. 2), thus testing whether the hydroxyl group was essential for the interaction as is the case of PSD95 binding to the third PDZ domain of CRIPT (21), where a hydrogen bond forms with the residue of the binding groove. However, this mutation (−2F) did not interfere with the interaction of NG2 and GRIP, showing that it is not the OH group that is essential but rather the hydrophobic nature of the aromatic side chain.

PDZ7 of GRIP1/2 thus belongs to class II PDZ domains, which bind to motifs with hydrophobic amino acids at positions 0 and −2: $\Phi X \Phi$* (where Φ is a hydrophobic amino acid, and X is any amino acid (10, 22)). NG2 associates with GRIP1 or GRIP2 in mammalian cells.

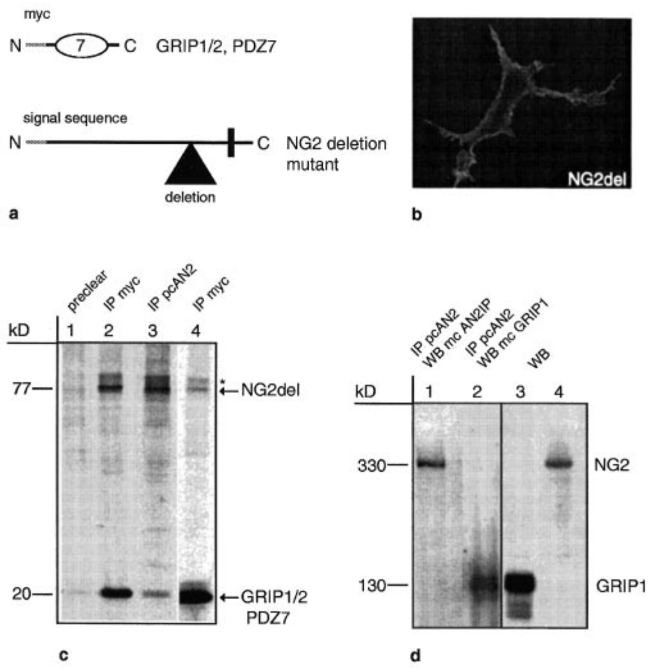

FIG. 3. **NG2 associates with GRIP1 and GRIP2 in transfected COS7 cells and with GRIP1 in the brain.** *a* shows the Myc-tagged GRIP1/2 construct and the NG2del construct giving rise to a 77-kDa protein and a larger glycosylated form. *b*, to control for correct membrane insertion of NG2del, COS7 cells were transfected with the NG2del construct, cultured for 24 h, followed by immunofluorescence staining of the live cells with pc AN2 antibodies and fixation. Confocal analysis shows NG2del staining at the cell surface. *c*, COS7 cells were cotransfected with NG2del and GRIP1 or GRIP2, respectively. The cells were cultured for 24 h, incubated for 1 h in Met/Cys-free medium, metabolically labeled for 4 h with [^{35}S]Met/Cys, lysed in 1% Nonidet P-40 buffer, and subjected to immunoprecipitation (*IP*). *Lane 1*, protein A-Sepharose preclear; *lane 2*, immunoprecipitated GRIP1 associates with NG2del; *lane 3*, immunoprecipitated NG2del associates with GRIP1; *lane 4*, immunoprecipitated GRIP2 associates with NG2del. The *asterisk* indicates glycosylated NG2del. *d*, total mouse brain (P7) was homogenized using an Ultra Turrax, nuclei were removed by centrifugation, and the homogenate was centrifuged at 100,000 × *g*. The pellet was solubilized in detergent-containing buffer, and insoluble material was removed by centrifugation. Lysates were subjected to immunoprecipitation using pc AN2 antibodies, resolved by SDS-PAGE on a 4–10% gel, and analyzed by Western blotting (*WB*). *Lane 1* shows an immunoprecipitation with pc AN2 antibodies followed by Western blotting with mc AN2 antibodies. *Lane 2* shows immunoprecipitation with pc AN2 followed by Western blotting with mc GRIP1. *Lanes 3* and *4* are control lanes that confirm the presence of GRIP1 and NG2 in the lysate used for immunoprecipitation.

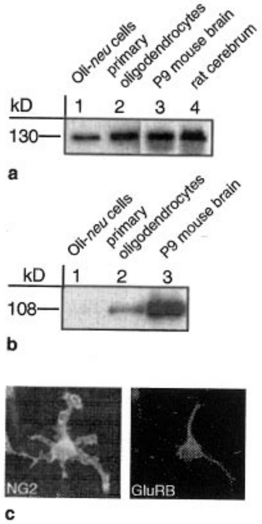

FIG. 4. **Cultures of mixed primary oligodendrocytes and the Oli-*neu* cell line express GRIP1 and NG2-positive oligodendrocyte lineage cells express the AMPA receptor subunit GluRB.** *a*, lysates from Oli-*neu* (*lane 1*), primary oligodendrocytes (*lane 2*), P9 mouse brain (*lane 3*, control), and rat cerebrum (*lane 4*, control) were resolved by 10% SDS-PAGE, blotted onto membrane, probed with mc GRIP1 antibodies, and developed with enhanced chemiluminescence. A 130 kDa band is visible in *lanes 1–4*. *b*, lysates from Oli-*neu* (*lane 1*), primary oligodendrocytes (*lane 2*), and P9 mouse brain (*lane 3*, control) were resolved by 10% SDS-PAGE, blotted onto membrane, probed with pc GluRB antibodies, and developed with ECL. There is no GluRB expression in Oli-*neu*, but cultures of primary oligodendrocytes and the control (mouse brain, P6) demonstrate the presence of GluRB. *c*, shaken off primary oligodendrocytes were grown on poly-L-lysine-coated coverslips for 2 days, fixed, permeabilized, and costained with mc AN2 antibodies and pc GluRB antibodies.

NG2-GRIP1 and NG2-GRIP2 associations were further confirmed by coimmunoprecipitation of NG2 together with GRIP1 or GRIP2 expressed in COS7 cells. First, cells were analyzed after transfection with an NG2 construct (NG2del, Fig. 3*a*), comprising the signal sequence, approximately one-fourth of the extracellular portion, the transmembrane domain, and the complete intracellular region. The protein was incorporated into the membrane and recognized by pc AN2 antibodies (Fig. 3*b*). Subsequently the cells were transfected with the NG2del construct together with Myc-tagged GRIP1/PDZ7 or GRIP2/PDZ7, respectively (Fig. 3*a*). Anti-Myc antibodies precipitated GRIP1 and associated NG2del from cotransfected COS7 cells (Fig. 3*c*, *lane 2*) and pc AN2 antibodies recognizing mouse NG2 precipitate NG2del and associated GRIP1 (Fig. 3*c*, *lane 3*). Anti-Myc antibodies also precipitate GRIP2 and associated NG2del from transfected COS7 cells (Fig. 3*c*, *lane 4*) expressing both these constructs.

To confirm the NG2-GRIP1 interaction *in vivo*, extracts from P7 total mouse brain were subjected to immunoprecipitation using AN2 antibodies followed by Western blot analysis of coprecipitated GRIP1. The results (Fig. 3*d*) demonstrate that GRIP1 associates with NG2 *in vivo*. Pc AN2 antibodies precipitate NG2 (*lane 1*) and associated GRIP1 as revealed by Western blotting with antibodies against GRIP1 (*lane 2*). *Lanes 3* and *4* are controls showing the presence of GRIP1 and NG2 in the brain extracts before precipitation.

NG2-positive Cultured Oligodendrocyte Progenitors Express GRIP and AMPA Receptors—Biochemical studies of GRIP1 have largely focused on neuronal expression, where GRIP1 is enriched in the postsynaptic density and at synaptic plasma membranes (13, 23). Because NG2 is expressed by immature glial cells including oligodendrocyte progenitors (3), it was important to demonstrate that the interaction partner GRIP1 is also expressed in these cells. A Western blot analysis of lysates of the murine oligodendrocyte progenitor cell line Oli-*neu* (24) and cultures of mouse primary oligodendrocytes consisting of a range of differentiation stages with antibodies against GRIP1 demonstrated a band of 130 kDa (Fig. 4*a*, *lanes 1* and *2*). A total lysate from P9 mouse brain exhibited a band at the same molecular mass (*lane 3*) as in the control blot with rat cerebrum (*lane 4*). These results demonstrate that GRIP1 is indeed expressed in oligodendrocyte lineage cells.

Because GRIP also binds to the GluRB subunit of AMPA receptors (12) and AMPA receptors are expressed by glial cells (7), we examined whether NG2-positive cells also express GluRB. First, a Western blot analysis was performed using antibodies to GluRB (Fig. 4*b*). A band of 108 kDa was detected in lysates of primary oligodendrocytes (*lane 2*) and in the control lysate (P9 total mouse brain, *lane 3*). No expression was detected in the Oli-*neu* cell line (*lane 1*). To define GluRB expression in individual cells, mixed cultures of primary oligodendrocytes were costained with AN2 antibodies and GluRB

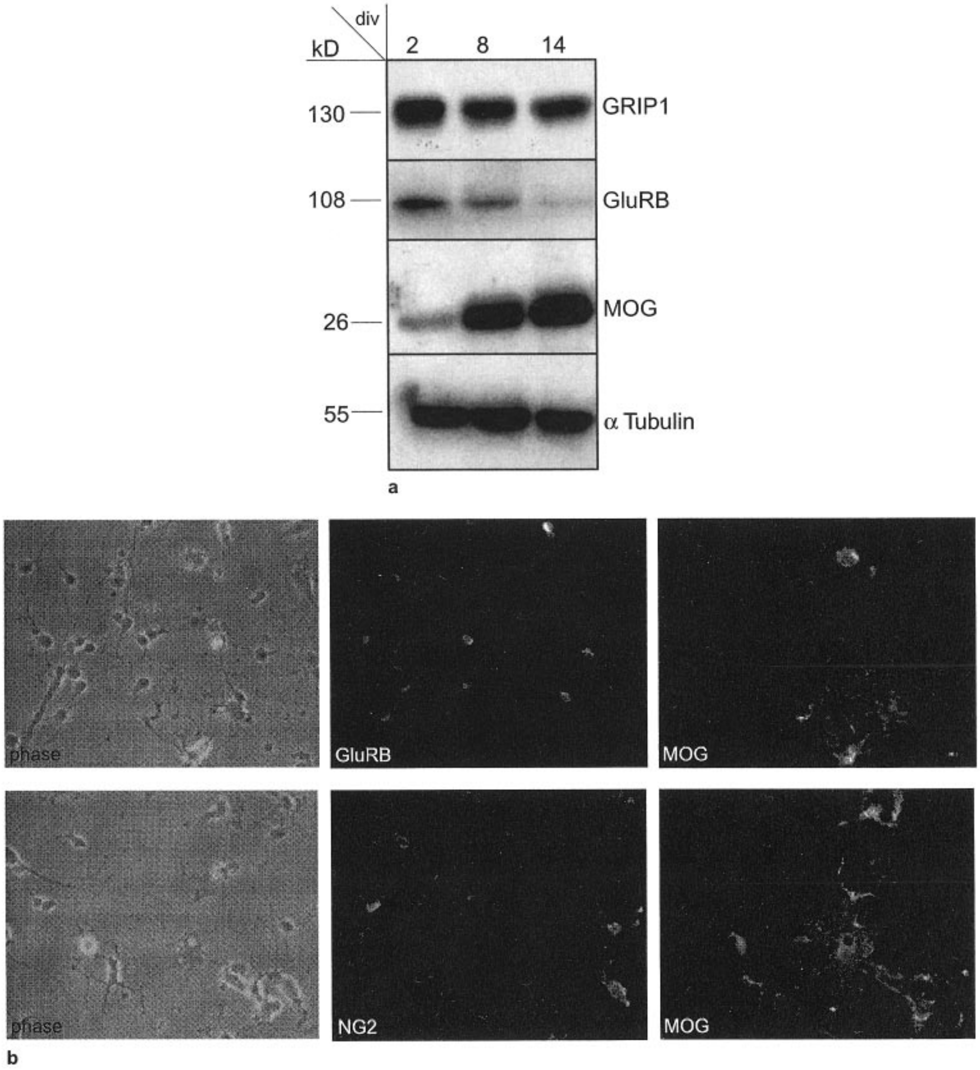

FIG. 5. **Comparison of GRIP1 and GluRB expression in developing oligodendrocytes in culture.** *a*, cultures of primary oligodendrocytes (*div2*, *8*, *14*) were lysed, and equal amounts of protein were resolved by 7.5% SDS-PAGE and immunoblotted with mc GRIP. Membranes were stripped and reprobed with pc GluRB antibodies, mc MOG antibodies (marker for mature oligodendrocytes), and α-tubulin antibodies. *b*, primary oligodendrocytes stained with either anti-GluRB and anti-MOG antibodies, or anti-AN2 and anti-MOG antibodies, show mutually exclusive immunofluorescent signals.

antibodies (Fig. 4*c*). All oligodendrocyte lineage cells staining with antibodies to GluRB coexpressed NG2; however, not all NG2-positive cells expressed GluRB. GluRB staining was restricted to more immature cells with a few processes and revealed a punctate distribution of the protein along the major processes in addition to an intense intracellular staining. More mature oligodendrocytes characterized by a more complex morphology no longer expressed GluRB (see also Fig. 6*d*, *white star*).

GRIP Expression Is Down-regulated Later in Maturing Oligodendrocytes Than GluRB—NG2 expression shows a partial overlap with the expression of the O4 marker (3), which characterizes a late stage of oligodendrocyte progenitor cells (25) and is retained on more mature oligodendrocytes (26). In contrast, there is no overlap of NG2 and proteins expressed by more mature oligodendrocytes (3). To study the timing of GRIP1 and GluRB expression in oligodendrocyte lineage cells (Fig. 5), mixed cultures of primary oligodendrocytes were used as an *in vitro* model for oligodendrocyte maturation (27) and analyzed after 2, 8, and 14 days in culture by Western blotting. These cultures represent a dynamic population of different developmental stages that initially consists (div2) of predominantly immature cells. GRIP1 expression is high in immature cultures and decreases slowly as oligodendrocytes mature. In contrast, GluRB expression drops dramatically as oligodendrocytes mature. Maturation of oligodendrocytes is shown by increasing expression of MOG, a marker of mature oligodendrocytes. Staining of such cultures demonstrates that NG2 and MOG are never coexpressed and that GluRB-expressing cells are always MOG-negative. GRIP1 expression persists over a longer period of maturation than GluRB expression, implying that GRIP1 may have multiple binding partners during the course of oligodendroglial development.

NG2 Forms a Trimeric Complex with GRIP1 and the AMPA Receptor Subunit GluRB—Transfected COS7 cells were subjected to radiolabeling and immunoprecipitation to investigate whether a complex consisting of NG2, GRIP1, and GluRB can be isolated. Cells were transfected with the NG2del, Myc-tagged GRIP1 and the AMPA subunit GluRB (flop, short form). NG2 as well as the GluRB subunit were incorporated into the plasma membrane. The GRIP1 construct encodes PDZ domains 5–7 (Fig. 6*a*). GluRB was reported to bind to PDZ4–5 of GRIP (12), and we have shown that NG2 binds to GRIP/PDZ7. Fig. 6*b* shows immunoprecipitation of the complex: pc AN2 antibodies precipitate NG2del together with associated GRIP1/PDZ5–7 and GluRB (*third lane* from *left*), pc GluRB antibodies precip-

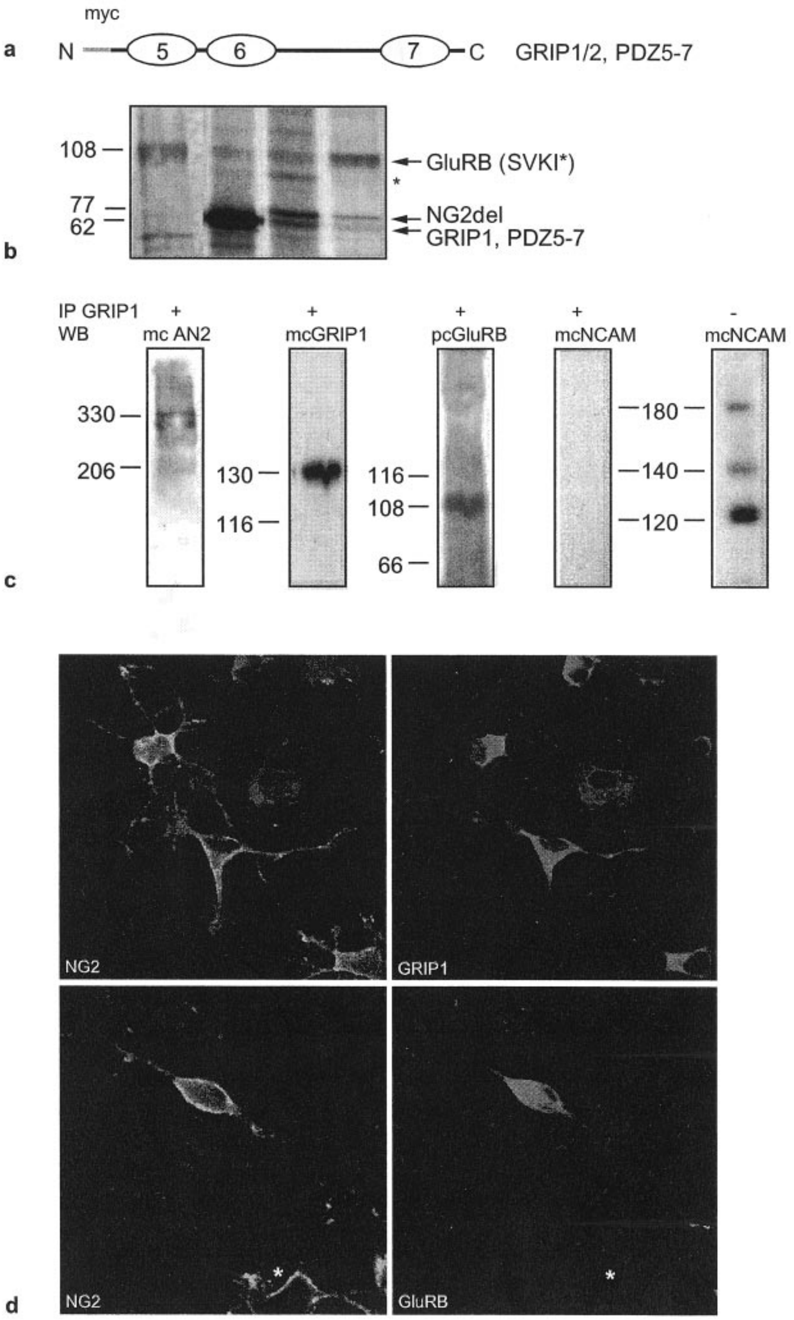

FIG. 6. **NG2 associates with GRIP1 and GluRB in transfected COS7 cells and primary cultures of oligodendrocytes.** *a* shows an NH_2-terminally Myc-tagged GRIP1 construct consisting of PDZ 5–7. *b*, COS7 cells were triple transfected with NG2del, GRIP1 (PDZ, m, 5–7) and full-length GluRB (flop, short form, COOH-terminal peptide SVKI*). Cells were grown for 24 h, starved for 1 h in Met/Cys-free medium, metabolically labeled with [^{35}S]Met/Cys, lysed in 1% Nonidet P-40 buffer, and subjected to immunoprecipitation. *First lane* from *left* (control), pc GluRB antibodies precipitate only GluRB from COS7 cells transfected with GluRB and NG2del, but without GRIP1 (PDZ5–7). *Second lane*, Myc antibodies precipitate GRIP1 and associated NG2del and GluRB from cells transfected with all three constructs. *Third lane*, pc AN2 antibodies precipitate NG2del and associated GRIP (PDZ5–7) and GluRB. *Fourth lane*, pc GluRB antibodies precipitate GluRB and associated NG2del and GRIP1 (PDZ5–7). The *asterisk* indicates glycosylated NG2del. *c*, cultured primary oligodendrocytes were subjected to immunoprecipitation using pc GRIP1 antibodies followed by Western blot analysis. pc GRIP1 antibodies precipitate GRIP1 with associated NG2 (*first lane*), GRIP1 (*second lane*), and GluRB (*third lane*). No NCAM could be detected in the GRIP1 precipitate (*fourth lane*), although it is present in the total lysate (*fifth lane*). *d*, primary oligodendrocytes were cultured for 2 days, fixed with paraformaldehyde, permeabilized with 0.05% Triton X-100, and costained with mc AN2, pc GRIP1 antibodies, and pc GluRB antibodies, respectively. Cells were analyzed by confocal microscopy.

itate GluRB with associated NG2del and GRIP1 (*fourth lane*), and anti-Myc antibodies precipitate GRIP1 with associated NG2 and GluRB (*second lane*). Although it was reported that PDZ4 and 5 and the NH_2-terminal residues of GRIP1 are required for interaction in the yeast two-hybrid system, the GRIP1 (PDZ5–7) protein was sufficient to bind to GluRB in mammalian cells. In cells transfected with NG2del and GluRB but without GRIP1, pc GluRB antibodies precipitate only GluRB with no associated NG2del (*first lane*, control).

Mixed cultures of primary oligodendrocytes (div2) were also subjected to coimmunoprecipitation to demonstrate the interaction of endogenously expressed proteins. Two different pc GRIP antibodies precipitated GRIP1 with associated NG2 and GluRB. Fig. 6*c* shows a Western blot of GRIP immunoprecipitates analyzed with mc AN2 antibodies (*first lane*), mc GRIP1 antibodies (*second lane*), and pc GluRB antibodies (*third lane*). The specificity of the coprecipitated proteins was tested by probing the blot with antibodies to NCAM, which is highly expressed by oligodendrocytes. No signal for NCAM could be detected in the GRIP1 precipitates, although this protein is present at a high level in the lysates (Fig. 6*c*, *fourth* and *fifth lanes*, respectively), thus demonstrating the specificity of the coprecipitation.

Confocal analysis of immature oligodendrocytes costained with AN2 antibodies and antibodies to GRIP (Fig. 6*d*) showed that GRIP is highly expressed in the cell body and in the proximal regions of the processes. NG2 staining outlines the cell body plasma membrane into the very tips of the processes. There is an overlap of GRIP and NG2 staining at the somal cell membrane. NG2 also overlaps with GluRB staining at the plasma membrane, even though there are additionally high levels of intracellular GluRB.

GRIP Is Not Localized in Lipid Raft Microdomains in Immature Oligodendroglia—GRIP and other PDZ domain proteins play an important role in targeting to and clustering proteins at the membrane (10, 28–30). Bruckner and colleagues (19)

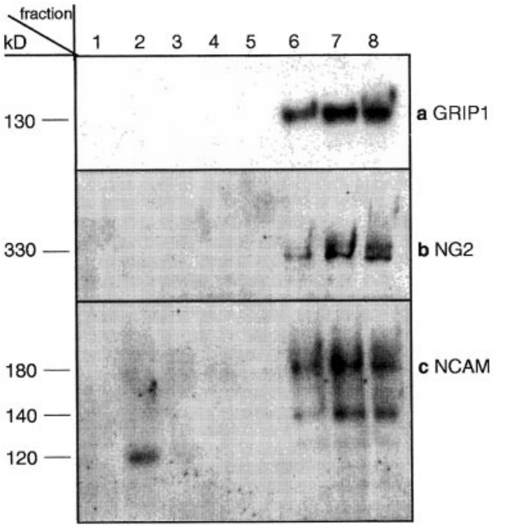

FIG. 7. **GRIP1 is not raft-associated in oligodendrocytes.** Primary oligodendrocytes (div2) were lysed in 1% Triton X-100 buffer and adjusted to 40% sucrose followed by overlayering with 30 and 5% sucrose. The gradient was centrifuged for 4 h at 218,000 × *g*, 4 °C. Eight fractions were collected from the top, resolved by 4–10% SDS-PAGE, and immunoblotted with different antibodies. *a*, mc GRIP antibodies; *b*, pc AN2 antibodies (GST880S16); *c*, pc NCAM antibodies. GRIP, NG2, and NCAM140/180 are located in the bottom gradient fractions (6–8); glycosylphosphatidylinositol-anchored NCAM120 is in the raft fraction (2).

described the association of GRIP proteins with detergent-insoluble membrane microdomains (lipid rafts). Lipid rafts are implicated in cell signaling, and raft-associated proteins can be isolated by their insolubility in Triton X-100 at low temperature and flotation in sucrose density gradients (31, 32). Because lipid rafts were shown to be important for signaling and sorting of proteins in oligodendrocytes (33, 34), we analyzed oligodendroglial rafts for the presence of NG2 and GRIP. Fig. 7 shows that neither GRIP1 (*a*) nor NG2 (*b*) is raft-associated in oligodendroglial cells. Both proteins are located in bottom fractions (6–8) of a density gradient and not in the lipid raft fraction floating in low density gradient fractions. NCAM 120, a lipid raft-associated glycosylphosphatidylinositol-anchored protein, is in fraction 2, whereas NCAM 140 and 180, both transmembrane isoforms, are in the bottom fractions (6–8, Fig. 7*c*). This suggests that NG2 binds to GRIP in a lipid raft-independent membrane domain of oligodendroglial progenitors, whereas in neurons raft-associated proteins recruit GRIP into lipid rafts.

DISCUSSION

NG2 Is a Constituent of a Protein Complex in Immature Glial Cells—Using the yeast two-hybrid approach, we identified GRIP1, a multi-PDZ domain protein, as an interaction partner of NG2 in the central nervous system. GRIP1 was originally identified as binding to the AMPA receptor subunits GluRB and GluRC (12). In addition, it was reported to interact with ephrin B1 ligand (19), Eph receptors (35), GRASP-1, a Ras guanylate exchange factor (36), liprin-α (37), and kinesin (38). PDZ domains function as scaffolding units clustering multiprotein complexes at the cell surface (10) by binding to the COOH termini of transmembrane proteins. NG2, a large transmembrane proteoglycan, has a short cytoplasmic COOH terminus that binds to the seventh PDZ of both GRIP1 and its homolog GRIP2.

Immature Glia Form a Complex of NG2, GRIP, and AMPA Receptors—GRIP1 was originally defined as a neuron-specific protein, but our results show that is also expressed by glial cells, implying that GRIP1 can act as a scaffolding molecule in this cell type and cluster protein complexes at the cell surface. GRIP1 binds to the AMPA receptor subunits GluRB and C (12), and it has been shown that AMPA receptor subunits are expressed not only by neurons but also by glia (6, 39–41), including cells of the oligodendroglial lineage (7) and hippocampal glial cells *in situ* (42–44). In this report we have shown the coexpression of GRIP1 and GluRB in NG2-positive cultured oligodendroglial precursor cells. NG2 and GRIP have several intracellular partners in glial cells (9),[4] and thus visualization of the complex as spots at the cell membrane and a complete overlap of staining of NG2, GRIP, and GluRB would not be expected.

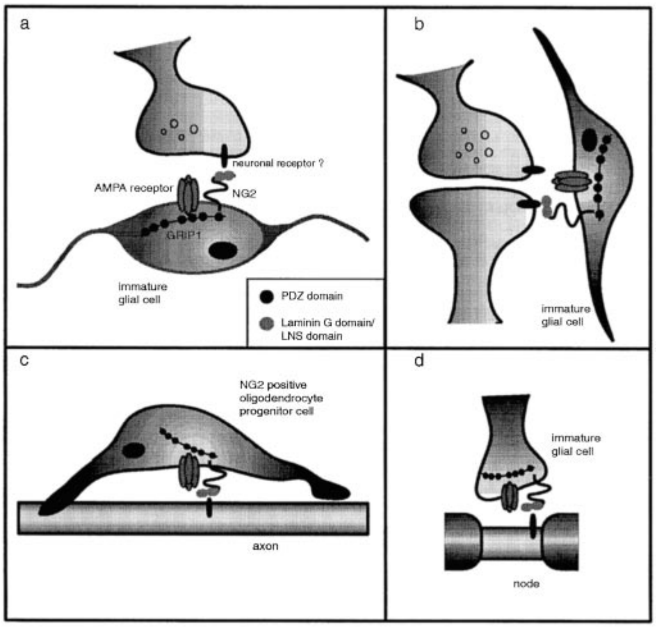

FIG. 8. **Model of interactions between NG2-positive glial cells expressing AMPA receptors and neurons.** Based on the finding of Bergles and colleagues (6), where neuronal boutons make synapses onto NG2 positive glial cells, the NG2·GRIP·AMPA receptor complex could be involved in the alignment and formation of a glial-neuronal synapse (*a*). *b* shows a modified model after Benson and colleagues (55), which focuses on the importance of adhesion molecules in shaping a synapse. This event may involve the NG2·GRIP·AMPA receptor complex expressed on immature glial cells surrounding the synapse. NG2-positive oligodendrocyte progenitor cells also make contact to axons as shown in *c*, and NG2-positive glial cells are present at the nodal region in the CNS (*d*) (51).

A triple complex of these proteins can be precipitated from transfected COS7 cells and can be isolated from immature glial cells in culture. NG2 isolated from brain extracts is bound to GRIP 1, and thus the complex of NG2, GRIP, and AMPA receptor exists *in vivo*. GRIP1 functions as an adaptor molecule and binds to both NG2 and GluRB/C, whereby the PDZ5–7 domains are sufficient for the interaction. In reality the complex may indeed be a lot larger than these three components and may include proteins that have been shown to interact with GRIP in neurons, such as the ephrins. Furthermore there are studies of GRIP1 interacting with itself and GRIP2 (13) via a PDZ-PDZ interaction, thus magnifying the complexity of the associations. Such typical multiprotein clusters determine the pre- and postsynaptic density (for review, see Refs. 10, 30, and 45).

NG2-positive Glial Cells Expressing AMPA Receptors Are Situated at Sites of Glial-Neuronal Contact and Receive Neuronal Signals—NG2-expressing cells are found throughout the developing central nervous system in both white and gray

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[4] J. Stegmüller, H. Werner, K.-A. Nave, and J. Trotter, unpublished results.

matter. The function and lineage assignment of these cells are under intense discussion. NG2 cells include the precursors for oligodendrocytes; NG2-positive cells isolated from P3 brain behave like classic O2A cells, developing into oligodendrocytes or astrocytes according to the composition of the culture medium (5), and NG2 cells from optic nerve and cerebellum have also been proposed to represent O2A progenitor cells (46, 47). However, NG2 cells, especially in the adult central nervous system, most likely represent a heterogeneous population.

The functional role of these AMPA receptors in immature oligodendrocytes is thought to include a regulation of cell proliferation and differentiation (8, 48, 49). The receptors are down-regulated when the glial progenitor cells mature into oligodendrocytes or astrocytes. In the adult rat hippocampus many NG2-positive cells surround synapses (50), and it has been shown recently that in the developing and adult rat hippocampus, neurons of the CA3 area make synapses on NG2-positive glial cells visualized by electron microscopy (6). Stimulation of the neurons results in activation of the glial AMPA receptors via the released glutamate, yielding a change in membrane potential and a Ca^{2+} signal in the glia (6). The NG2·GRIP1·AMPA receptor complex that we have isolated may be part of this glial postsynaptic domain. In the adult central nervous system, NG2-positive cells make close contact to the node of Ranvier (51). Early work by Fulton and co-workers (52) showed that in the adult nervous system cells taking up cobalt in response to quisqualate stimulation (acting on AMPA receptors) are also localized at the nodes and would suggest that these paranodal NG2-positive cells may also have AMPA receptors.

The AMPA GluRC subunit can also bind GRIP, suggesting that NG2 can complex with both calcium-permeable (GluRB-containing) and calcium-impermeable (lacking GluRB) glutamate receptors. Activation of glial AMPA receptors and controlled variation of their calcium permeability may be important regulators of the morphology of glial processes and regulate signaling at neuron-glial synapses or neuron-neuron synapses where glial cells ensheath the synapse (50, 53–55).

The Ménage à Trois Involving GluRB, GRIP, and NG2 May Position Glial AMPA Receptors toward Neurons—The NG2 molecule when first cloned was found to have little homology to known proteins but limited homology to cadherins (56). However, NG2 has been found to possess two LAM G (LNS) domains at the amino terminus (57). These domains are found in the neurexins and are thought to be independently folding domains (58, 59). Neurexins bind protein ligands with high affinity (60) and are thought play a role as cell adhesion molecules; β-neurexins and their binding partners neuroligins have been demonstrated to trigger cell-cell adhesion (61). It is very likely that NG2 is an adhesion molecule. A putative association between adhesion molecules and neurotransmitter receptors has been observed previously: the *N*-methyl-D-aspartate receptor and adhesion molecules N-cadherin and L1 are physically associated in large multiprotein complexes isolated by immunoprecipitation of *N*-methyl-D-aspartate type of glutamate receptors from mouse brain (62). However, a direct link between these molecules has not been demonstrated. Similarly, members of the ephrin family of cell adhesion molecules (ligands and receptors) bind GRIP (19, 35), thus potentially complexing AMPA receptors and ephrins.

The close apposition of NG2-positive glial cells with the nodes of Ranvier in the central nervous system (51), the deposition of NG2 at nodes in the peripheral nervous system (63), and the expression of NG2 by immature oligodendrocytes as well as Schwann cells in close association with neurons (4) support the existence of a neuronal receptor. A neuronal receptor for NG2 has been postulated before in the context of axonal growth inhibition (64). We propose the following functions of the NG2·AMPA receptor complex. First, NG2 may function to position glial AMPA receptors toward glutamergic neurons and may be instructive for the formation of the glial-neuronal synapses (Fig. 8*a*) described by Bergles and colleagues (6). Second, NG2-positive glial cells may surround classical neuronal synapses (Fig. 8*b*) (50) and could thus influence synaptic formation and signaling (53–55, 65, 66). The complex may play a role in sensing neuronal activity. Third, NG2 expressed by oligodendroglial progenitor cells may play a role in axonal recognition during the early phases of myelination: the associated AMPA receptor could thus sense neuronal activity, which would in turn regulate the proliferation and differentiation of the glial cells (Fig. 8*c*). Fourth, NG2-positive glia at the node of Ranvier may play a regulatory role at the node (Fig. 8*d*). It will be important to determine the role of NG2 cells in glial-neuronal signaling and to elucidate the functional implications of the elicited glial signals in this network.

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