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DISTINCT DISTRIBUTIONS OF α-AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONATE (AMPA) RECEPTOR SUBUNITS AND A RELATED 53,000 M_R ANTIGEN (*GR53*) IN BRAIN TISSUE

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Abstract—Polyclonal antibodies against specific carboxy-terminal sequences of known α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits (GluR1–4) were used to screen regional homogenates and subcellular fractions from rat brain. Affinity purified anti-GluR1 (against amino acids 877–889), anti-GluR2/3 (850–862), and anti-GluR4a and anti-GluR4b (868–881) labeled distinct subunits with the expected molecular weight of ~105,000. These antigens were shown to have distinct distributions in the brain. While GluR2/3 epitopes had a distribution profile similar to that of the presynaptic marker synaptophysin, GluR1 was notable for its abundance in the hippocampus and its relatively low density in neocortical areas, and GluR4 was highly enriched in cerebellar tissue. An additional antigen (glutamate receptor-related, *GR53*) of lower molecular weight (50,000–59,000) was recognized in rat, human, frog, chick and goldfish brain samples by anti-GluR4a as well as by anti-GluR1*at*, an antibody that specifically recognizes the extracellular aminoterminal domain of GluR1 (amino acids 163–188). Both antibodies also labeled antigens of ~105,000 mol. wt in brain tissue from all species tested. The ~53,000 mol. wt antigen was concentrated 10–20-fold in synaptic membranes vs homogenates across rat brain regions. Both the 105,000 and the 53,000 mol. wt proteins were also concentrated in postsynaptic densities, and neither of the two antigens were evident in seven non-brain tissue samples.

These data indicate that AMPA receptors have regionally different subunit combinations and that some AMPA receptor composites include proteins other than the conventional 105,000 mol. wt GluR subunits.
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Key words: GluR antibodies, AMPA-binding protein, synaptosomes, excitatory amino acids.

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, a subclass of glutamate receptors, are the primary mediators of excitatory neurotransmission in the mammalian CNS. A cDNA encoding an AMPA receptor subunit (GluR1) was originally isolated from a rat brain cDNA library[25] and three additional subunits (GluR2, GluR3 and GluR4) with amino acid sequences of approximately 70% homology were subsequently cloned.[10,29,40] Expression of single GluR subunits or different subunit combinations in oocytes and cultured cells produces receptors that respond to AMPA, kainate and glutamate, and are blocked by 6-cyano-7-nitroquinoxaline-2,3-dione,[29,40,49,50] but may otherwise show broad physiological diversity. All subunits bind [^{3}H]AMPA[29] with an affinity similar to that of AMPA receptors solubilized from mammalian brain.[20,28] Antibodies developed against peptide domains of GluR1, GluR2/3 and GluR4[3,45,55] recognize proteins of similar molecular weight (~105,000) in rat,[6,9,45,55] mouse[3] and human brains.[8] Immunocytochemical analyses utilizing these antibodies have been performed on rat brain;[35,36,42] the distribution of antibody staining in these studies accords with autoradiographic analyses of [^{3}H]AMPA bind-

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; BSA, bovine serum albumin; E, embryonic day; EDTA, ethylenediaminetetra-acetate; EGTA, ethyleneglycolbis(2-aminoethyl ether)tetra-acetate; GluR, AMPA-type glutamate receptor subunit; *GR53*, glutamate receptor-related antigen of 53,000 mol. wt; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; KBP, kainate-binding protein; P, postnatal day; PAGE, polyacrylamide gel electrophoresis; PB, phosphate buffer; PSD, postsynaptic density; SDS, sodium dodecyl sulfate; SPM, synaptic plasma membrane. Single letter notation for amino acids is as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

ing,[37,41,44] thus strengthening the link between the GluR subunits and *in vivo* AMPA receptors.

Other work indicates that the density of GluR subunits varies greatly between brain regions and that the different subunits have different distributions. For example, immunostaining for GluR1 is much more intense in the hippocampus than is the case for the neocortex or thalamus; GluR4 appears to be present in high concentrations only in the cerebellum.[9,35,45,55] Antibodies that distinguish between GluR2 and GluR3 have been difficult to develop, but it is apparent from published data that combined levels of the two subunits are not greatly different in cortex vs hippocampus and are somewhat lower in the brainstem than in the telencephalon.[35,42,55] These regional variations presumably reflect the following factors: (i) density of synapses, (ii) percentage of synapses within a region that are glutamatergic, (iii) number of AMPA-type glutamate receptors per synapse and (iv) the subunit composition of the AMPA receptors. The last of these points is of particular interest because of evidence that the functional properties of the receptors are significantly influenced by subunit stoichiometry.[52]

The present studies first examined the distributions of AMPA receptor subunit densities across brain regions. Comparisons between subunit densities were then made to estimate the degree to which the balance of subunits varied between regions. In addition, evidence was sought as to whether the regional variations in subunits were due to differences in synaptic density. Secondly, antibodies directed against particular AMPA receptor domains also recognize an antigen of approximately 53,000 mol. wt. While this element may be unrelated to glutamate receptors, it is noteworthy that a similar sized glutamate- binding protein has been cloned from frog and chick brains.[14,18,22,53] The present studies tested if the 53,000 mol. wt antigen is brain specific and concentrated in synapses, as expected for a protein related to glutamatergic transmission. Regional distributions of the antigen were then used to determine if it has a particular relationship with any of the GluR subunits.

EXPERIMENTAL PROCEDURES

Materials

Rabbit antibodies (anti-GluR1*at*) developed with a fusion technique toward the sequence TEEGYRMLFQDLE-KKKERLVVVDCES (amino acids 163–188) located in the extracellular amino-terminal domain of the GluR1 subunit were generously provided by Dr K. Sumikawa (Department of Psychobiology, University of California, Irvine, CA, U.S.A.). Protease inhibitors and a monoclonal antibody to synaptophysin were purchased from Boehringer Mannheim Corporation (Indianapolis, IN, U.S.A.). Other protease inhibitors and protein A-Sepharose were from Chemicon International, Inc. (Temecula, CA, U.S.A.) and Sigma Chemical Company (St Louis, MO, U.S.A.). The cross-linker dimethyl pimelimidate dihydrochloride was obtained from Pierce (Rockford, IL, U.S.A.). Nitrocellulose paper and alkaline phosphatase-conjugated antibodies against rabbit and mouse immunoglobulin Gs were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Alkaline phosphatase substrate kits were purchased from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.). [^{3}H]AMPA (50–70 Ci/mmol) was purchased from NEN/Du Pont Co. (Boston, MA, U.S.A.). All other materials were from usual commercial sources.

Antibody synthesis

Antibodies to AMPA receptor subunits (e.g., anti-GluR1) were prepared using methods similar to those described by Wenthold *et al.*[55] The carboxy-terminal peptide SHSSGMPLGATGL (amino acids 877–889) was used for GluR1, EGYNVYGIESVKI (850–862) for GluR2/3 and RQSSGLAVIASDLP (868–881) for GluR4; all peptides were conjugated to bovine serum albumin (BSA) via glutaraldehyde. Antibodies against the peptide domains were raised by injecting the respective conjugates intradermally into female New Zealand rabbits; they were purified by affinity chromatography. Antibodies raised to carboxy-terminal GluR1 and GluR4 sequences were purified by coupling the antigen to AH-Sepharose, while the purification of anti-GluR2/3 used antigen coupled to CH-Sepharose. Antibody purification was carried out using ~2 ml of bead matrix per 5 mg of peptide. Antiserum was incubated overnight with the matrix-bound peptides at 4°C and then set up into vertical columns for antibody collection. The columns were washed with 20 ml phosphate-buffered saline and antibodies were eluted with 50 mM maleic acid, pH 2.5. Phosphate-buffered saline washes were repeated to prepare for the basic elution, which was carried out with 100 mM triethylamine, pH 11.5. In either case, the collected antibodies were neutralized with Tris base or Tris–HCl, and stabilized with 0.1% BSA.

Tissue preparation

Male Sprague–Dawley rats (60–90 days old), in good condition, were killed by metofane anesthesia and decapitation. Each brain was rapidly cooled, removed from the skull and placed in ice-cold homogenization buffer consisting of 0.32 M sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA and the following protease inhibitors: 4 μg/ml antipain, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin A. Each brain was then immediately dissected in a chilled dish. The brainstem and cerebellum were first separated from the endbrain. The latter, after removal of the olfactory bulbs, was dissected into neocortex, hippocampus, striatal-rich material and thalamic-rich material as follows. The frontal third of the endbrain was separated by a coronal cut and the tissue underneath the callosal ring was collected as ''striatal-rich tissue''. The loosely attached core of the caudal two-thirds of the endbrain containing mostly thalamus and hypothalamus was termed ''thalamic-rich tissue''. The neocortical tissue remaining after folding out and separating the hippocampus was combined with the non-striatal tissue from the frontal third of the endbrain. The regions were quickly homogenized in fresh homogenization buffer. Other tissues from rat were homogenized similarly. Locally obtained young adult frogs (*Xenopus laevis*), adult goldfish (*Carassius auratus*) and pre-hatched chicks (*Gallus domesticus*) were also used for brain tissue preparation.

Aliquots of brain homogenate samples were used to isolate P2 and synaptic plasma membranes as described previously.[5,13] Briefly, this involved differential centrifugative pelleting, buoyant density centrifugation in Percoll gradients and hypo-osmotic lysis. Similar methods were used to separate crude synaptosomal preparations on sucrose density gradients and to isolate postsynaptic densities.[11] Isolated synaptic elements were suspended at 1–4 mg protein/ml in 35 mM Tris, pH 7.4, with 0.05 mM EDTA and broad spectrum protease inhibitors (20 μg/ml antipain, 2 μg/ml aprotinin, 40 μg/ml calpain inhibitor I, 2 μg/ml

leupeptin, 2 μg/ml pepstatin A, 35 μg/ml fresh phenylmethylsulfonyl fluoride and 20 μg/ml *N*-tosyl-L-phenylalanine chloromethylketone), divided into aliquots, and stored at −80°C. Protein content was determined using the Bio-Rad Protein Assay with a BSA standard.

Receptor binding assay

[^{3}H]AMPA binding was studied by using a modification of the filtration method described previously.[51] Aliquots of column fractions were equilibrated with 50 nM [^{3}H]AMPA in the presence of 60 mM Tris–HCl (pH 7.5), 5 mM EDTA, 50 mM KSCN and 0–100 mM NaCl for 60 min in an ice-water bath. Bound ligand was determined by rapidly diluting each sample with 4 ml ice-cold 50 mM Tris–acetate (pH 7.2) and 80 mM KSCN, and immediately filtering it through a polyethyleneimine-coated glass-fiber filter followed by two sequential 4-ml washes. The filters were assayed for ^{3}H content by liquid scintillation spectroscopy with a counting efficiency of 0.40.

AMPA receptor preparation

Forebrain membranes were prepared from homogenate and solubilized with Triton X-100 as described previously.[6,20] AMPA receptors were isolated from the solubilized membranes with the use of sequential chromatographic steps and by following the [^{3}H]AMPA binding activity.[6] Briefly, the soluble extract was applied to a DEAE–Sepharose column and the receptors were eluted with a linear KSCN gradient. AMPA receptor-containing fractions were pooled and applied to a wheat-germ lectin affinity column, from which the receptors were eluted with the addition of *N*-acetyl-D-glucosamine to the column buffer. Pooled fractions containing [^{3}H]AMPA-binding activity were then injected into a high-performance liquid chromatography size exclusion column or a polyethyleneimine anion-exchange column; receptors were eluted from the latter with a linear gradient of KSCN. Eluted protein was measured with the nanogram-sensitive Quantigold assay (Diversified Biotech, Newton Centre, MA, U.S.A.).

Immunoprecipitation

Homomeric AMPA receptors were expressed in HEK-293 cells from cDNAs encoding $GluR1_{flop}$, $GluR2_{flip}$ and $GluR3_{flop}$ as described previously,[24] and cerebellar P2 preparations were used as a source for GluR4 and the glutamate receptor-related antigen *GR53*. In all cases, lysed membranes were solubilized with 1% (w/v) sodium dodecyl sulphate (SDS) in 30 mM Tris/100 mM NaCl at 100°C for 5 min, after which the samples were cooled, the SDS diluted 10-fold and Triton X-100 added to 0.8% (w/v). Appropriate GluR antibodies, permanently attached to protein A-Sepharose CL-4B with the cross-linker dimethyl pimelimidate dihydrochloride and pre-washed in 50 mM diethylamine/0.5% deoxycholate (pH 11.5), were then incubated with the respective receptors for 6–8 h at 4°C. The immobilized receptors were washed thoroughly in 30 mM Tris/0.5 M NaCl/0.2% Triton X-100, extracted from the immuno-support with diethylamine/deoxycholate at pH 11.5, and immediately neutralized with 0.5 M Tris, pH 6.

Immunoblot analysis

The samples were subjected to one freeze–thaw cycle and homogenized by mild tip sonication in 30–50 μl hypo-osmotic buffer containing 8 mM HEPES (pH 7.4), 1 mM EDTA, 0.3 mM EGTA and the protease inhibitors mentioned above. Homogenate samples (100 μg protein) were treated with 2.5% (w/v) SDS in the presence of 3% (v/v) 2-mercaptoethanol and incubated at 100°C for 5 min. The denatured samples were then subjected to electrophoresis[33] on polyacrylamide gradient gels (3–17%; PAGE) and transferred to nitrocellulose (0.2 μm pore size) for 1 h with the Trans-Blot system from Bio-Rad Laboratories. Antibodies used for blot staining were as follows: anti-GluR1 (2 μg/ml), anti-GluR2/3 (2 μg/ml), anti-GluR4a (6 μg/ml), anti-GluR4b (6 μg/ml), anti-GluR1*at* (6 μg/ml) and anti-synaptophysin (50 ng/ml). Incubation of the nitrocellulose with diluted antibody was carried out at 4°C with agitation for 12–16 h. Secondary antibody incubation utilized anti-immunoglobulin G–alkaline phosphatase conjugates, and color development used either the 5-bromo-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system or the Vector Substrate Kit II. Calibration of immunoblots using pre-stained protein molecular weight standards allowed the determination of the molecular weight for pertinent species.

Color development of immunoreactive bands was terminated well before maximal intensity was reached in order to avoid saturation and allow comparative studies within each blot. The relative optical densities and image areas of the labeled antigens were quantitatively compared within single immunoblots using a computerized image analysis system or by scanning laser densitometry using a Zeineh SLR-504-XL densitometer (BioMed Instruments, Inc., Fullerton, CA, U.S.A.) and a Varian 4270 integrator (Palo Alto, CA, U.S.A.). The specific immunoreactivity ((density − background) × area)) for each blot lane was determined from these values. The immunostaining of GluR subunits on blots was routinely tested for linearity within the optical density range exhibited by samples from different brain regions or species. Tests were conducted by immunoblotting sets of 14–18 samples containing 2–100 μg protein from a single preparation of adult telencephalon tissue and staining with different antibodies; specific optical density × area was measured and plotted vs protein. The graphs were consistently linear between approximately 8 and 100 μg for anti-GluR1 (r = 0.94–0.98) and anti-GluR2/3 (r = 0.97–0.99); for anti-GluR4a (100,000 and 53,000 mol. wt bands tested separately), anti-GluR4b and the monoclonal anti-synaptophysin antibody used in this study, staining was linear for protein values between ∼15 and 100 μg (r = 0.91–0.99). Subsequent blots using the same antibody preparations, the same color development stock solutions and similar development times were compared to the linearity test blots with regard to optical density range.

Immunocytochemistry

Adult male Sprague–Dawley rats were killed by overdose of sodium pentobarbital and transcardial perfusion with 0.9% saline in 0.1 M phosphate buffer, pH 7.2 (PB), followed by 4% paraformaldehyde in PB. Brains were removed, incubated for 24 h in paraformaldehyde–PB and 24 h in paraformaldehyde–PB with 20% sucrose, and subsequently sectioned in the coronal plane (20 μm thickness) with a freezing microtome. Sections were treated with a BSA solution then immunostained with anti-GluR antibodies (5–10 μg/ml). The avidin–biotin–peroxidase technique was used with the enzymes, substrate and method recommended by Vector Laboratories, Inc. Finally, the slices were mounted on gelatin-coated slides, rapidly dehydrated through ascending concentrations of ethanol, cleared in AmeriClear (Baxter, McGaw Park, IL, U.S.A.) and coverslipped with Permount.

RESULTS

Antibodies to AMPA receptor subunits

Rabbit antibodies were developed against the carboxy-terminal domains of AMPA receptor subunits GluR1, GluR2/3 and GluR4, and affinity purified on columns containing respective peptide

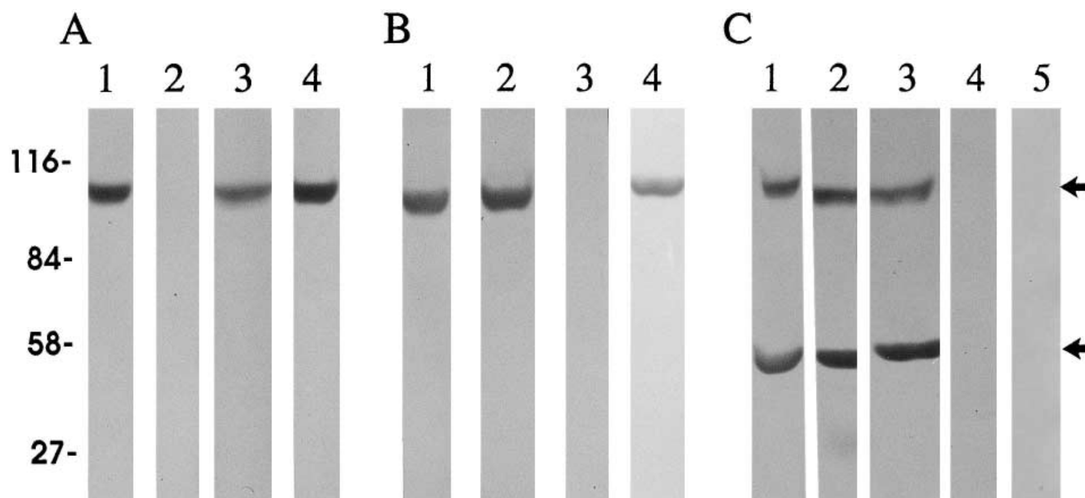

Fig. 1. Affinity-purified antibodies developed against carboxy-terminal domains of GluR subunits. SDS–PAGE blot strips each containing hippocampal homogenate (40 μg protein) and cerebellar SPMs (40 μg) from rat were labeled with anti-GluR1 (A), anti-GluR2/3 (B) and anti-GluR4a (C). In each case, the concentrated antibodies were pre-incubated (before dilution) for 2 h at room temperature with no addition (lane 1), with 400 μM GluR1 peptide antigen (lane 2), with 400 μM GluR2/3 antigen (lane 3), or with 400 μM GluR4 antigen (lane 4). Lane 5 in C was stained with pre-immune serum taken earlier from the GluR4 rabbit. All blot strips received equal amounts of secondary antibodies, substrates for color development and development time. GluR bands of ~105,000 mol. wt and *GR53* are marked with arrows. The positions of 116,000, 84,000, 58,000 and 27,000 mol. wt standards are shown.

sequences immobilized on activated Sepharose. Bound antibodies were eluted with acidic buffer first (e.g., anti-GluR4a), after which each column was thoroughly washed and more antibodies eluted with basic buffer (e.g., anti-GluR4b). Fig. 1 shows that all of the purified antibodies recognized rat brain GluR subunits with the expected molecular weight (~105,000) as deduced from cloned cDNAs.[25] The GluR1 and GluR2/3 epitopes were highly concentrated in preparations of partially purified AMPA receptors;[6] the GluR4 epitope was weakly recognized in the partially purified receptors (Fig. 2). Antigenic specificity was demonstrated by pre-absorption of the corresponding synthetic peptide to each antibody preparation. The ability of the antibodies to label the ~105,000 mol. wt GluR protein was blocked only by the respective antigenic peptide sequence (10–400 μM) obtained from the carboxy terminus of each subunit (Fig. 1).

Both acid- (Fig. 1) and base-eluted (not shown) anti-GluR1 and anti-GluR2/3 antibodies labeled single ~105,000 mol. wt bands on immunoblots. However, whereas the base-eluted anti-GluR4b antibodies labeled a single ~105,000 mol. wt band in brain samples (see below), the acid-eluted anti-GluR4a also recognized a smaller species with a molecular weight of ~53,000 (Fig. 1C). Higher concentrations of anti-GluR4b faintly labeled the ~53,000 mol. wt band, suggesting that the majority of GluR4 antibodies that recognize the smaller protein were eluted from the peptide affinity column with the initial acid wash. It has been reported previously that antibodies (named anti-GluR1*at*) raised against the extracellular amino-terminal domain of GluR1 (amino acids 163–188) specifically recognized two proteins of ~105,000 and ~58,000 mol. wt.[3,6] Fig. 3 illustrates that the two antigens labeled by anti-GluR1*at* and anti-GluR4a have similar electrophoretic mobilities. The 50,000–59,000 mol. wt antigen was termed *GR53* in accord with its size and antigenic similarity with GluR

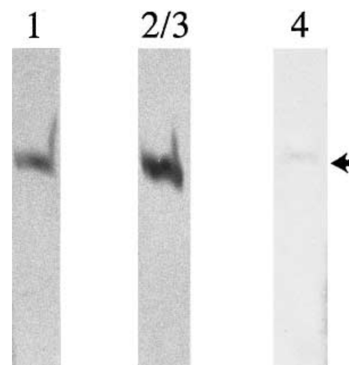

Fig. 2. GluR immunoreactivity in partially purified AMPA receptors. (^{3}H)AMPA binding activity was isolated from solubilized rat forebrain membranes by DEAE anion-exchange, wheatgerm affinity and polyethyleneimine anion-exchange chromatographies. SDS–PAGE blot strips each containing 5 μg protein from the AMPA receptor preparation were labeled with anti-GluR1 (lane 1), anti-GluR2/3 (lane 2/3) and anti-GluR4a (lane 4). The strips received equal amounts of secondary antibodies, substrates for color development and development time. GluR bands of ~105,000 mol. wt are marked with an arrow. The bottom of the strips correspond to the position of the 27,000 mol. wt standard.

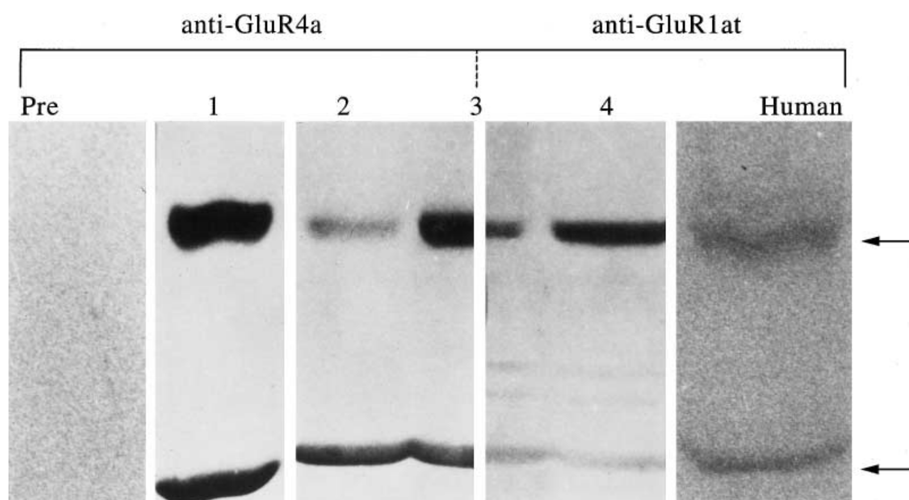

Fig. 3. Anti-GluR4a (against GluR4 amino acids 868–881) and anti-GluR1*at* (to GluR1 amino acids 163–188) recognize *GR53* in rat and human brain tissue. SDS–PAGE blot strips were prepared with 100-µg protein samples of rat cerebellar SPMs (lanes Pre, 1 and 3), rat hippocampal SPMs (lanes 2 and 4), or lysed P2 membranes from human hippocampus (lane human). Strips were labeled with anti-GluR4a and anti-GluR1*at* as shown; note that lane 3 was split down the middle so that the lane could be labeled by both antibodies. The lane labeled ''Pre'' was incubated with pre-immune serum from the GluR4 rabbit instead of with the affinity purified antibodies. All blot strips received equal amounts of secondary antibodies, substrates for color development and development time. Antigens of ~105,000 and ~53,000 mol. wt are marked with arrows.

subunits. Both antibodies were unable to label either band when pre-treated with antigenic sequences (see Fig. 1C). Pre-immune serum taken earlier from the rabbit which developed the anti-GluR4a antibodies did not label any bands in rat brain samples (Fig. 1C, lane 5; Fig. 3).

To test the specificity of the antibodies that recognize *GR53*, individually expressed GluR subunits 1–3 (Fig. 4) and denatured proteins precipitated by anti-GluR4a (Fig. 5) were analysed by immunoblot; with regard to the latter, cerebellar membranes were denatured with SDS prior to immunoprecipitation in order to detach the GluR4 subunit from hetero-oligomeric AMPA receptors. As expected, GluR1 was labeled by anti-GluR1 and anti-GluR1*at* but not by antibodies to GluR4 (Fig. 4A) nor by those to GluR2/3 (not shown; see Ref. 24). Similarly, recombinantly expressed GluR2 and GluR3 were only labeled by anti-GluR2/3 (Fig. 4B, C); they were not recognized by the other GluR antibodies, including anti-GluR1 (not shown; see Ref. 24). It should be noted that multiple forms of GluR1 and GluR2 were evident, with two to three bands of slightly different electrophoretic mobility likely stemming from post-translational modifications (Fig. 4A, B); the expression of heterogeneous GluR1 antigens appears to involve the removal of a small domain(s) from the carboxy terminus.

In contrast to the above results, immunoprecipitated GluR4 and *GR53* proteins, which were included on the same set of blots in Fig. 4, displayed significant labeling only by GluR4 antibodies (Fig. 5). While equal aliquots of the precipitated GluR4 and *GR53* were applied to multiple gel lanes, no antigen labeling or only trace staining was detected in those lanes incubated with anti-GluR1 and anti-GluR2/3; the trace immunostaining probably stems from incomplete denaturation of AMPA receptor complexes or from similarities in the antigenic domains used to prepare the different GluR antibodies (e.g., the GluR1 domain is ~40% identical to that of GluR4). Anti-GluR1*at*, on the other hand, strongly recognized the precipitated *GR53* protein while having no detection of GluR4 (Fig. 5, last lane); anti-GluR1*at* also labeled a non-specific protein of ~70,000 mol. wt which was present in mock samples extracted from untreated protein A-Sepharose. Similar staining results as those in Fig. 5 were produced with material precipitated by anti-GluR4b antibodies (data not shown). In additional immunoblots, no bands were detected by anti-GluR1, anti-GluR2/3, anti-GluR4a, anti-GluR4b or anti-GluR1*at* antibodies in non-brain tissue samples from kidney, liver, lung, spleen, smooth muscle, heart and adrenal gland (see Fig. 6 and Fig. 7). Thus, distinct GluR proteins and the *GR53* antigen are selectively labeled by subunit-specific antibodies and appear to be brain specific.

Distribution of AMPA receptor-related proteins in rat brain

The regional distributions of AMPA receptor subunits and *GR53* were determined across seven rat brain areas. Immunoblots in Fig. 6 show that GluR1, GluR2/3 and GluR4 (the latter labeled by anti-GluR4b) have distinct profiles across brain region homogenates. Mean immunoreactivity levels for

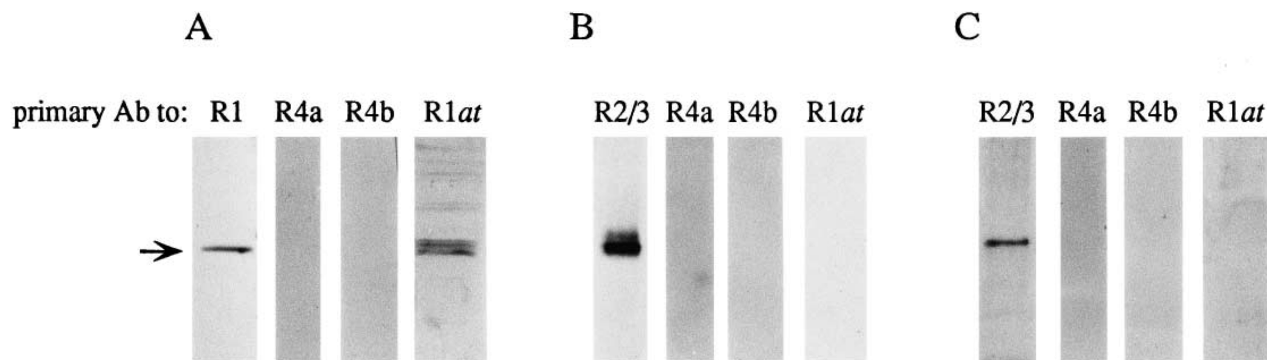

Fig. 4. Specificity of GluR antibodies determined by immunoblot analysis. Full-length cDNAs for GluR1 (A), GluR2 (B) and GluR3 (C) were individually expressed in HEK-293 cell lines and the resulting membranous subunits were subjected to solubilization and selective immunoprecipitation as described in Experimental Procedures. Each subunit preparation was equally apportioned to four SDS–PAGE blot strips which were immunostained with different GluR antibodies. All strips were treated with the same secondary antibody and color development solutions, for the same development time. GluR bands of ~105,000 mol. wt are marked with an arrow.

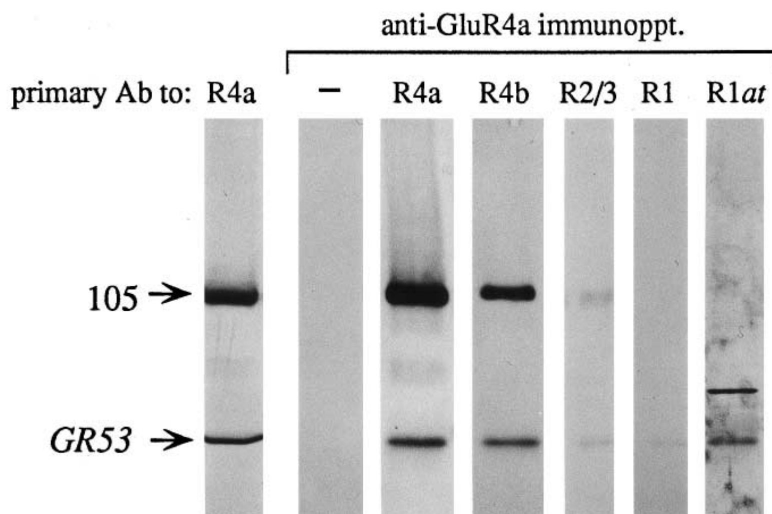

Fig. 5. Specific immunodetection of the GluR4 subunit and *GR53*. Cerebellar AMPA receptors were denatured in SDS at 100°C, after which they were diluted into Triton X-100-containing buffer to allow for immunoprecipitation with anti-GluR4a antibodies, as described in Experimental Procedures. The precipitated material was equally apportioned to six blot strips which were immunostained as and alongside those in Fig. 4; one strip was treated without primary antibodies as a control for the immunostaining produced by secondary antibodies alone. The first blot strip of the figure contains cerebellar P2 membranes (70 μg protein) in order to provide the typical electrophoretic migration pattern of GluR4 and *GR53* for comparisons (see arrows).

each antigen are shown in the upper four panels in Fig. 8; the densitometric values ((density − background) × area) were normalized within each immunoblot and then averaged across three to four blots. Also, the optical density values for the antigens were confirmed to be within the linear range for each respective antibody (see Experimental Procedures); however, values for non-cerebellar GluR4 bands occasionally fell below the linearity curve; thus, the actual GluR4 content in these samples may be slightly lower than the measurements shown in Fig. 8. Analyses of variance (one-way ANOVA) revealed that GluR1 and GluR4 have statistically different concentrations across homogenate samples from the seven brain regions tested ($P < 0.0001$; $F = 18.9$ and 19.1, respectively), while GluR2/3 does not. Also, there is a striking difference between GluR1 and GluR2/3 in that the neocortical area had less GluR1 than most other areas but had one of the highest concentrations of GluR2/3. On the other hand, hippocampal GluR1 stood out as being two to 10 times more abundant ($P < 0.01$, two-tailed t-test) than the other six regions. GluR4 had the most distinctive profile, with intense staining in cerebellar samples and light staining in all other brain areas (see Fig. 8).

The two antigens recognized by anti-GluR4a antibodies had dissimilar profiles across brain regions

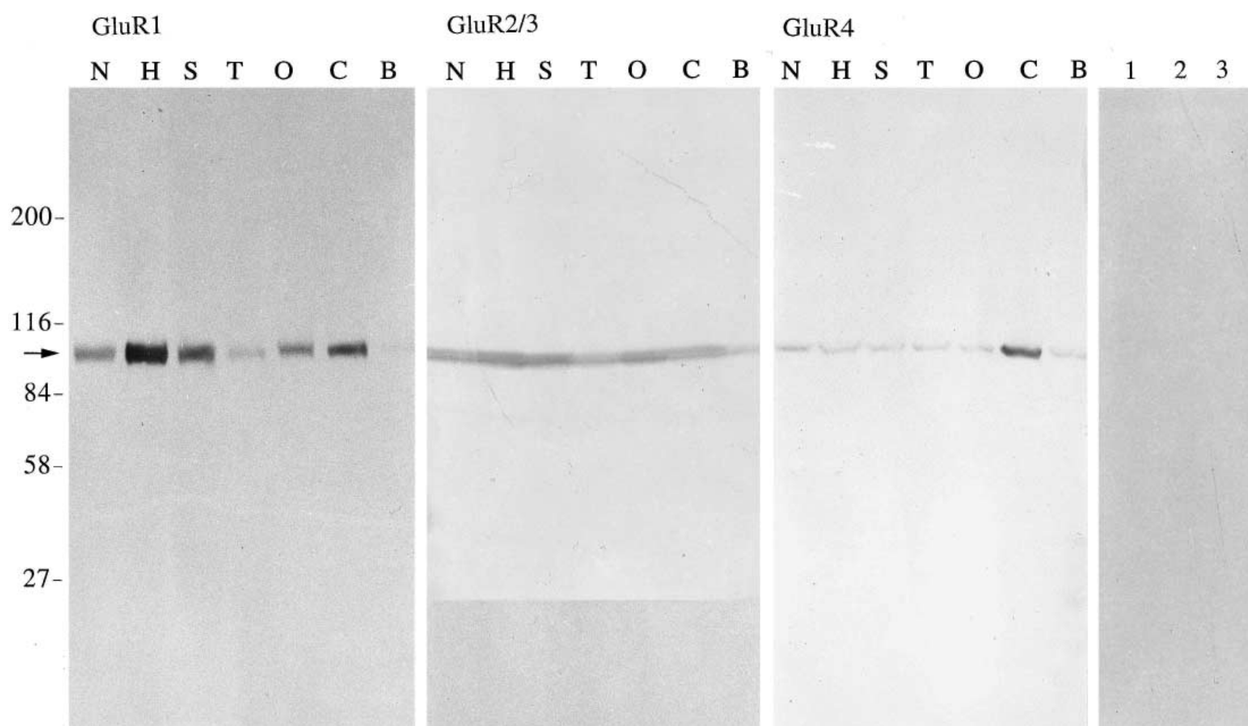

Fig. 6. Distribution of GluR subunits across different brain regions and non-brain tissues. Aliquots of homogenate protein (70 μg/lane) from isolated rat brain regions were subjected to SDS–PAGE and immunoblotted with antibodies to GluR1, GluR2/3 and GluR4; the latter used anti-GluR4b. Regions include: N, neocortex; H, hippocampus; S, striatal-rich tissue; T, thalamic-rich tissue; O, olfactory bulb; C, cerebellum; B, brainstem. The three antibodies did not label any antigens in seven non-brain tissues from rat (also see Fig. 7). As an example, homogenate samples (70 μg protein) from kidney (lane 1), lung (lane 2) and spleen (lane 3) were stained with anti-GluR2/3 antibodies and allowed to develop for an extended period of time. GluR bands of ~105,000 mol. wt are marked with an arrow. The positions of 200,000, 116,000, 84,000, 58,000 and 27,000 mol. wt standards are shown.

(Fig. 7). The *GR53* antigen expressed a relatively stable profile across brain regions compared to that of the 105,000 mol. wt GluR4; a similar *GR53* profile was obtained with the anti-GluR1*at* antibodies. It is of interest that anti-GluR4a, which recognizes both GluR4 and *GR53*, produced moderate immunolabeling in the dendritic fields of the hippocampus and other cortical areas; similar staining was generated by the antibodies to GluR1 and GluR2/3, as shown previously.[4] In contrast, anti-GluR4b, which labels GluR4 alone, produced little if any staining in these regions, while both GluR4 antibodies strongly labeled distinct areas in the cerebellum (not shown; see Ref. 42).

The regional distributions for each of the GluR-related antigens were compared with that for synaptophysin, a presynaptic vesicular marker for most nerve terminals.[15,54] The data summarized in Table 1

Table 1. Glutamate receptor-related antigens normalized to synaptophysin content in different brain regions

Brain region	GluR1	GluR2/3	GluR4	*GR53*
Neocortex	0.27 ± 0.06	1.0 ± 0.21	0.09 ± 0.05	0.67 ± 0.19
Hippocampus	1.6 ± 0.15	1.4 ± 0.47	0.08 ± 0.03	0.97 ± 0.32
Striatal-rich	0.76 ± 0.09	1.1 ± 0.44	0.04 ± 0.02	0.63 ± 0.04
Thalamic-rich	0.27 ± 0.14	0.91 ± 0.33	0.04 ± 0.03	1.0 ± 0.04
Olfactory bulb	0.93 ± 0.53	1.0 ± 0.21	0.17 ± 0.07	0.93 ± 0.08
Cerebellum	0.99 ± 0.12	1.6 ± 0.32	3.3 ± 0.62	2.8 ± 0.86
Brainstem	0.81 ± 0.54	2.2 ± 0.96	0.65 ± 0.13	3.5 ± 0.71

A monoclonal antibody to synaptophysin was used to label the synaptic marker on all GluR and *GR53* blots from Fig. 6 and Fig. 7. The regional distribution data for GluR-related antigens in Fig. 8 was normalized to the synaptophysin content in each brain region as determined by image analysis of immunoblots. Each value represents the normalized mean ± S.E.M. ($n = 3$–4).

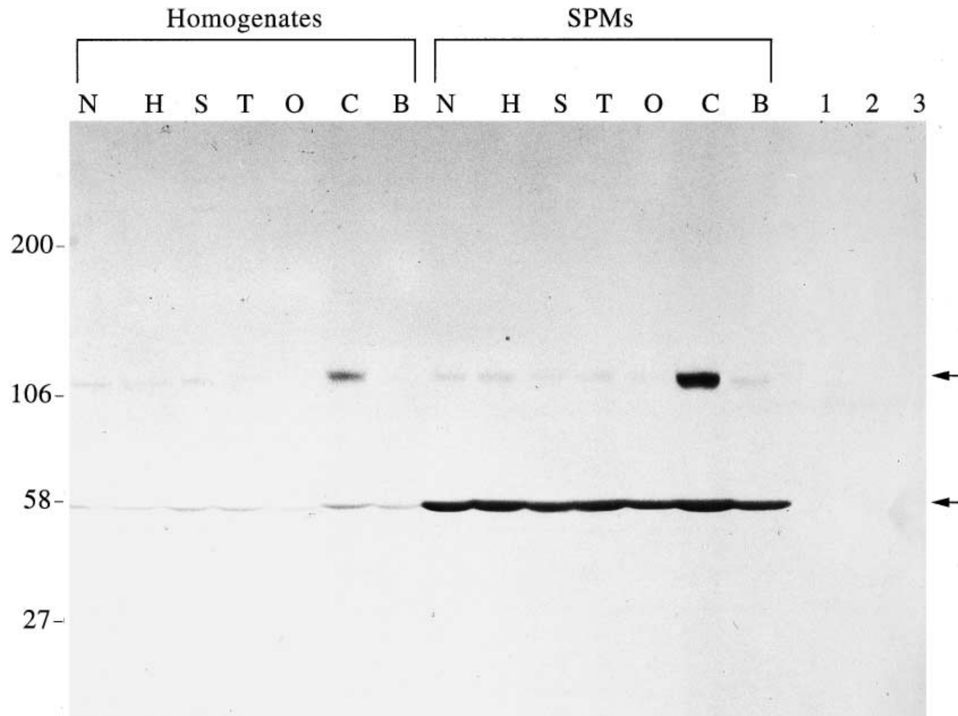

Fig. 7. GluR4 and *GR53* are differentially distributed in brain homogenate and SPM samples. Protein samples (70 μg/lane) of homogenates and SPMs from different rat brain regions were subjected to SDS–PAGE and immunoblotted with anti-GluR4a antibodies. Regions include: N, neocortex; H, hippocampus; S, striatal-rich tissue; T, thalamic-rich tissue; O, olfactory bulb; C, cerebellum; B, brainstem. The GluR band of ~105,000 mol. wt and *GR53* are marked with arrows. The two antigens were not labeled in seven non-brain tissues from rat (also see Fig. 6). As an example, homogenate samples (70 μg protein) from smooth muscle (lane 1), heart (lane 2) and adrenal gland (lane 3) were also stained with anti-GluR4a and allowed to develop for an extended period of time. The positions of 200,000, 106,000, 58,000 and 27,000 mol. wt standards are shown.

show that, for GluR1, GluR2/3 and *GR53*, immunoreactivity levels across brain regions increased similarly after normalization to the synaptophysin content for each respective region. The hippocampal content remained above other regions with regard to GluR1 ($P < 0.04$, $F = 3.12$), whereas *GR53* content was most prevalent in cerebellar and brainstem samples after normalization ($P < 0.01$, $F = 5.54$). While the relative GluR4 content increased in brainstem samples after normalization, the profile remained dominated by the cerebellar region. GluR2/3 concentrations were distinct in that they normalized to values near one for all brain regions (Table 1), suggesting that the GluR2/3 content is directly related to synaptic density. To test this, immunoreactivity values within each animal were normalized to the hippocampus value in order to reduce animal to animal variance, and then the mean values were plotted versus the synaptophysin concentrations (see Fig. 9); the GluR4 data were plotted without prior normalization due to their low concentration in the hippocampus. GluR2/3 was significantly correlated with synaptophysin across the seven brain regions, resulting in a linear correlation coefficient of 0.90 ($P < 0.005$). The two lower panels in Fig. 8 also illustrate the similarity between GluR2/3 and synaptophysin distribution profiles. Such a strong correlation between the variations in an AMPA receptor subunit and a universal marker for synapses suggests that certain GluR distributions are related to the regional synaptic densities, a point supported by the fact that an estimated 80–90% of telencephalic synapses are glutamatergic.[3,12,46] Interestingly, however, none of the other GluR-related antigens showed a comparable correlation with the synaptic marker, although GluR1 appears to have a high correlation with synaptophysin across four of the seven brain regions (Fig. 9, top panel). Thalamic and neocortical samples have GluR1 levels that fall below the linear relationship, while the hippocampal GluR1 concentration is well above the line. It may well be the case that GluR2/3 subunit epitopes are general components of most synaptic AMPA receptors, at least in the telencephalon, whereas the other GluR-related proteins are selectively concentrated in brain regions requiring their particular functional characteristics.

Ratios between glutamate receptor subunits

Re-analysis of the subunit distribution data was performed to look for regional differences in AMPA receptor composition. Specifically, ratios between the

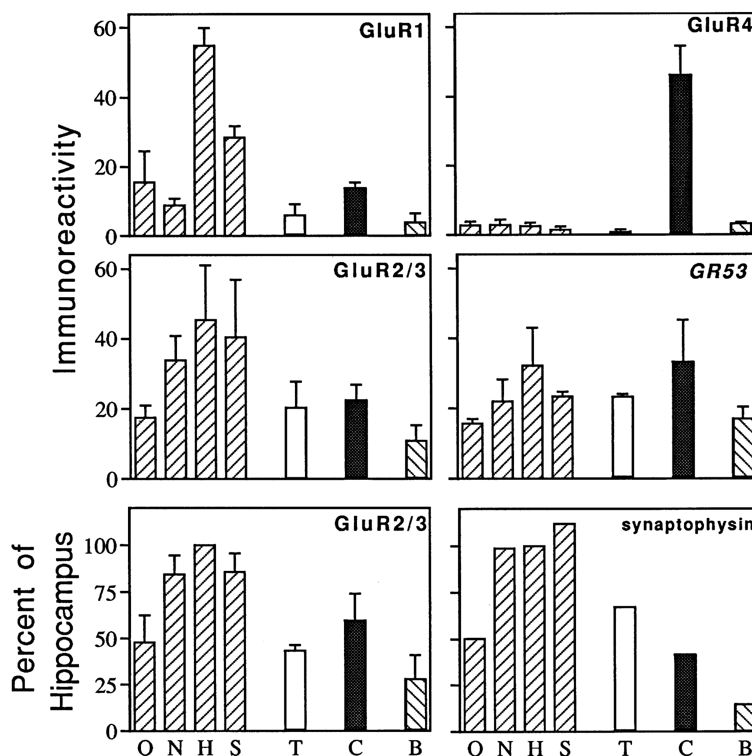

Fig. 8. Relative abundances for GluR-related antigens across different brain regions. Immunoreactivity values for four different antigens across brain regions were determined from Fig. 6, Fig. 7, and similar blots using image analysis as described in Experimental Procedures. Each panel consists of data from seven regions relative to each other inclusively. Each bar in the upper four panels represents the mean immunoreactivity ((density − background) × area)) ± S.E.M. ($n = 3$–4). In the two lower panels, GluR2/3 and synaptophysin profiles were normalized within each animal to their respective concentration in the hippocampus and then averaged across animals ($n = 2$–4). O, olfactory bulb; N, neocortex; H, hippocampus; S, striatal-rich tissue; T, thalamic-rich tissue; C, cerebellum; B, brainstem.

receptor subunits were examined across brain regions with the focus being on GluR1 in particular, since it has been suggested to be an important AMPA receptor element required for hippocampal plasticity.[52] The ratios were determined within each homogenate sample used in the upper panels of Fig. 8 and the means were calculated for each brain region; they are shown in Fig. 10. It is clear that there are regional differences in the ratio between GluR1 and GluR2/3 levels ($P < 0.01$, $F = 5.03$). The hippocampus, in particular, had an R1 : R2/3 ratio of much greater than one, whereas neocortical, thalamic and hindbrain areas had ratios of ≤ 0.5. R1 : R2/3 values in olfactory bulb and striatal-rich tissue were about one. Note that since the antibodies may have different binding affinities, the R1 : R2/3 ratio describes the relative regional distribution of the subunits and not the relative number of subunits. R1 : R4 ratios were not determined due to the extremely low GluR4 concentrations in non-cerebellar samples.

Glutamate receptor-related antigens in synaptic elements

The GluR antibodies were used to confirm that AMPA receptor subunits are concentrated in synaptic elements, and to address the question of whether the GluR-related antigen *GR53* is localized to these domains. Synaptic plasma membrane (SPM) fractions were isolated from aliquots of the seven brain region homogenate samples used above. Immunoblot analyses were conducted to determine GluR immunoreactivity levels in equal protein amounts of homogenate and SPM samples (see Fig. 3 and Fig. 7). Total immunoreactivity as determined by image analysis (and confirmed by laser scanning densitometry) was compared between paired samples for each region. As expected, GluR1 and GluR2/3 were concentrated two- to three-fold in the synaptosomal membranes as compared to homogenates. Cerebellar GluR4 had a similar SPM : homogenate ratio of 2.3; GluR4 values from other regions were too small for reliable ratio measurements. Surprisingly, the anti-GluR4a-labeled *GR53* was concentrated in the synaptic fractions to a greater extent than that typically found for any of the AMPA receptor subunits. Its mean SPM : homogenate ratio across all seven regions was 13 ± 2 (mean ± S.E.M.); the olfactory bulb had the highest ratio of 24. Postsynaptic density (PSD) fractions isolated from five different rat brain regions also displayed more ~105,000 mol. wt

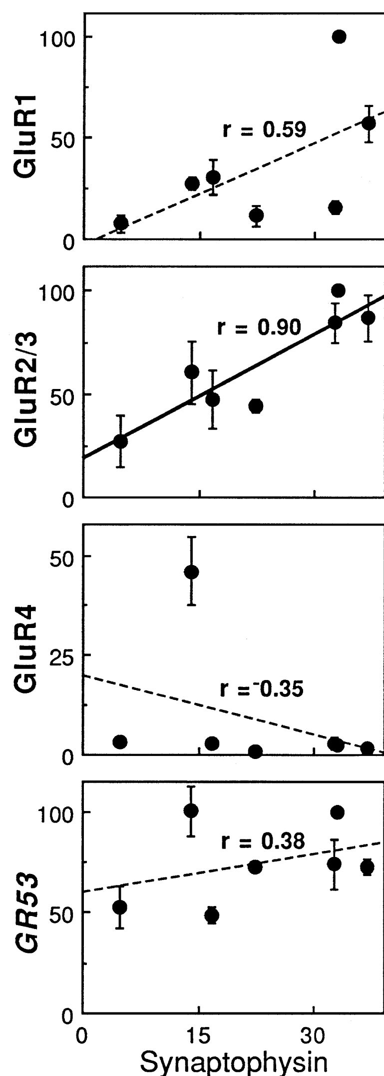

Fig. 9. GluR-related antigens vs synaptophysin. Immunoreactivity levels for GluR1, GluR2/3, *GR53* and synaptophysin were normalized within each animal to the hippocampus, and then the mean ± S.E.M. values for GluR-related antigens were plotted against the mean synaptophysin values; the GluR4 data were plotted without prior normalization. Each data set was subjected to linear regression in order to determine the correlation coefficient (r): GluR1, $r = 0.59$, $P = 0.08$; GluR2/3, $r = 0.90$, $P = 0.003$; GluR4, $r = -0.35$, $P > 0.2$; *GR53*, $r = 0.38$, $P > 0.2$.

bands and *GR53* than their homogenate counterparts, as measured by immunostaining with anti-GluR1*at* (Fig. 11) and other GluR antibodies (not shown). Low amounts of protein were loaded on the immunoblots to avoid reaching saturated immunoreactivity levels in the PSD samples; homogenate lanes were at or below detectability thresholds at these protein levels. Note that some of the PSD preparations exhibited a faintly immunolabeled band of — 130,000 mol. wt which may correspond to the AMPA-binding protein purified by spider toxin affinity chromatography.[48]

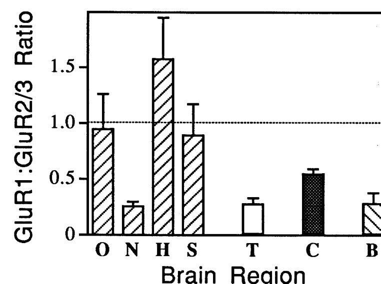

Fig. 10. GluR1 to GluR2/3 ratio in different rat brain regions. The ratios were determined within each sample used in the upper panels of Fig. 8, and the mean ± S.E.M. values were calculated for each brain region. O, olfactory bulb; N, neocortex; H, hippocampus; S, striatal-rich tissue; T, thalamic-rich tissue; C, cerebellum; B, brainstem.

GR53 *development in rat telencephalon*

To determine whether *GR53* development parallels that of synaptic markers as shown previously for GluR subunits,[19] telencephalic membranes from rats of different embryonic (embryonic days (E) 16 and 19) and postnatal (postnatal days (P) 2, 7, 14 and adult) ages were assessed for their *GR53* content using anti-GluR4a antibodies. Membrane samples of equal protein content were analysed by immunoblot to quantitate the mean *GR53* immunoreactivity levels in three to six separate samples per age group; the data are shown in Fig. 12. As can be seen, little staining was evident at the two earliest developmental stages tested (E16 and E19). Subsequently, *GR53* staining increased steadily throughout postnatal development and was highest in the adult samples. It is concluded that the *GR53* developmental profile closely follows synaptogenesis and the postsynaptic assembly processes of AMPA receptors.[19]

Glutamate receptor-related antigens in brains of different species

The molecular weight of *GR53* is similar to those of known kainate-binding proteins (KBPs) from the brains of frog and chick, which have approximately 36–40% homology to rat GluR cDNAs.[18,53] Since antibodies to the frog KBP recognize AMPA receptor subunits,[21] it is conceivable that certain GluR antibodies recognize KBP-like polypeptides in rat brain and that one of these proteins may be *GR53*. Accordingly, brain tissue from different species, including those that are known to have sizeable concentrations of KBPs, were tested for the presence of *GR53* and other antigens labeled by the antibodies characterized above. Anti-GluR4a and anti-GluR1*at* antibodies labeled antigens in frog, chick and goldfish brain samples (Fig. 13), as well as in human hippocampal tissue (Fig. 3). As listed in Table 2, the most prominent antigens labeled in frog, chick and goldfish brains have the same molecular weight as the *GR53* antigen in rat brain (see Fig. 13). The *GR53*-like immunoreactivity in brain homogenate from

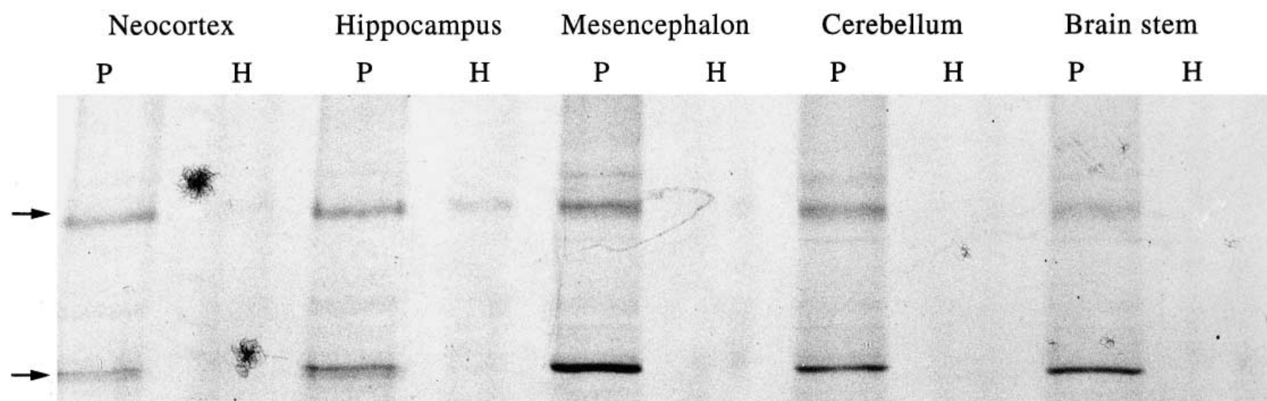

Fig. 11. GluR1 and *GR53* are concentrated in postsynaptic density (PSD) preparations. Samples of PSD material (P) and homogenates (H) from different rat brain regions were subjected to SDS–PAGE and immunoblotted with anti-GluR1*at* antibodies. Samples of mesencephalon contained small amounts of diencephalic material. Low amounts of protein (12 μg/lane) were used so as to not saturate the nitrocellulose support. The GluR band of ~105,000 mol. wt and *GR53* are marked with arrows.

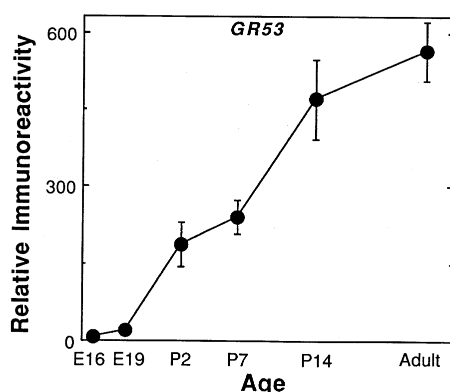

Fig. 12. Developmental regulation of *GR53* in brain. Equal aliquots of telencephalic membrane samples (30 μg protein/lane) from E16 (n = 3), E19 (n = 3), P2 (n = 4), P7 (n = 6), P14 (n = 6) and adult (n = 3) rats were analysed by immunoblot (see Experimental Procedures and Fig. 7). Relative immunoreactivity levels for *GR53* across development were determined with anti-GluR4a antibodies and image analysis. Each datum represents the mean immunoreactivity of the antigen ± S.E.M.

goldfish occasionally appeared as a 52,000–53,000 mol. wt doublet. The related antigen in human hippocampus was slightly larger (~56,000 mol. wt). The *GR53*-like proteins were concentrated four- to 14-fold in lysed P2 membranes as compared to the respective tissue homogenate samples (see Fig. 13); similar localization in P2 and synaptic elements is a distinct feature of rat brain *GR53* (see above). Fig. 13 also shows that each of the P2 membrane preparations from the five species contains concentrated amounts (two- to six-fold) of AMPA receptor-like antigens ranging in molecular weight from 99,000 to 109,000 (Table 2). In addition, the 104,000 mol. wt human and 99,000 mol. wt goldfish antigens were accompanied by faintly stained bands of 114,000 and 106,000 mol. wt, respectively. The amounts of AMPA receptor-like immunoreactivity per milligram protein in frog, chick and goldfish brains were four- to seven-times lower than in rat brain, whereas the level of *GR53*-like immunoreactivity in these samples was equal to or greater than that in the rat (Table 2). Lastly, faint bands between 25,000 and 38,000 mol. wt were evident in frog and goldfish samples labeled by anti-GluR4a antibodies.

DISCUSSION

The development of subunit-specific antibodies has allowed the determination of the relative concentrations of the major AMPA receptor components across rat brain areas. This study has shown that different GluR subunits have distinct distribution profiles and, in addition, has provided evidence indicating that AMPA receptors of different subunit combinations exist throughout the brain. The carboxy-terminal epitopes of the GluR2 and GluR3 subunits are apparently related to the regional amount of presynaptic terminals. This is also partially true for the GluR1 subunit; however, some brain regions have much different GluR1 levels than expected according to synaptic marker density. Specifically, hippocampal receptors appear to be enriched in GluR1, while thalamic and cortical receptors have unexpectedly low levels of the subunit. The immunoreactivity ratio between GluR1 and GluR2/3 was relatively high in three telencephalic structures (olfactory bulb, hippocampus and striatum) compared to the remaining four sample regions. These results provide a quantitative description that agrees with the immunocytochemical findings of Rogers *et al.*[45] and Blackstone *et al.*[9] *In situ* hybridization experiments indicate that mRNA for GluR2 is present at higher concentrations than that for GluR3.[17,29,47] The strong relationship between levels of GluR2/3 protein and synaptic number (as estimated from synaptophysin) may therefore reflect a relatively constant number of GluR2 subunits in AMPA receptors throughout the brain. Immunoprecipitation studies are needed to determine if the

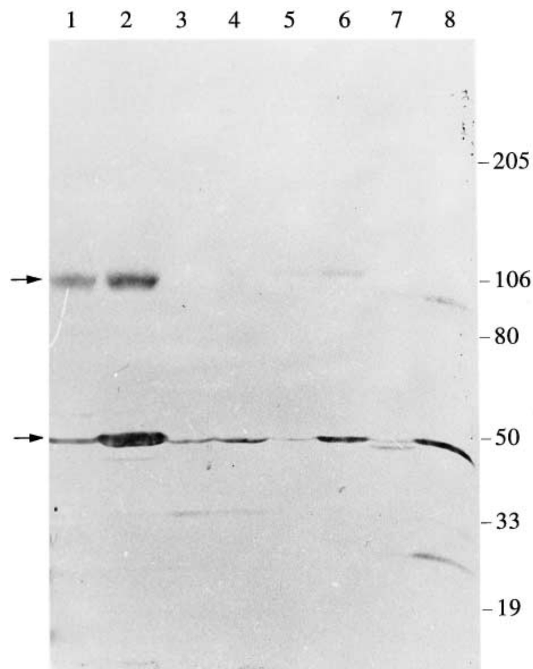

Fig. 13. Anti-GluR4a antibodies recognize ~105,000 and ~53,000 mol. wt brain antigens from different species. Cerebellar homogenate (lane 1) and SPMs (lane 2) from rat (70 μg protein/lane), and whole brain homogenate and lysed P2 membranes from frog (lanes 3 and 4, respectively), chick (lanes 5 and 6) and goldfish (lanes 7 and 8) (60 μg/lane) were subjected to SDS–PAGE and immunoblotting with anti-GluR4a antibodies. The GluR band of ~105,000 mol. wt and *GR53* are marked with arrows. The electrophoretic positions of 205,000, 106,000, 80,000, 50,000, 33,000 and 19,000 mol. wt standards are shown.

variations in the balance of subunits is reflected in receptor stoichiometries. Assuming this to be the case, then it would appear that AMPA receptors in the subcortical telencephalon are distinctly different from those in the remainder of the brain.

In light of the evidence that different AMPA receptor subtypes exist with distinct subunit stoichiometries and, therefore, functions, it is of particular interest that AMPA binding has been shown to be decreased in the aged mouse telencephalon,[3,34] but not in hindbrain regions.[3] Further experiments also revealed that GluR1 and the related antigen *GR53* are both selectively reduced in the aged telencephalon.[3] Moreover, immunocytochemical[1] and *in situ* hybridization[16] analyses found that protein and mRNA levels for AMPA receptor subunits were markedly reduced in cortical areas of Alzheimer brains. The latter studies are of particular interest since they indicate that the subunit reductions arise prior to any evident neuronal death, and this has also been shown to occur in an *in vitro* model using long-term hippocampal slices induced to express prominent features of aging and Alzheimer's disease.[2] Thus, it is suggested that the loss of cognitive ability with aging may reflect, at least in part, regional changes in the balance of GluR subunits and with these a change in functional properties of AMPA receptors.

The subcellular distributions for GluR1 staining in the present study correspond well with previously published work by Rogers *et al.*[45] and Blackstone *et al.*[9] The two reports demonstrated that GluR1 immunoreactivity was enriched in SPM and PSD fractions, and was found at a high density in forebrain and cerebellum, with lower levels of staining in diencephalon and brainstem areas. The current analysis confirms and extends these findings by demonstrating that the immunolabeling of both GluR2/3 and GluR4, as in the case of GluR1, is concentrated in synaptic fractions. These results are consistent with immunocytochemical studies utilizing electron microscopy, which have shown that staining with anti-GluR antibodies is most prominent in postsynaptic domains.[42] In addition, western blot data presented here are consistent with previous *in situ* hybridization and immunocytochemical studies showing that GluR1–3 are distinctly distributed throughout the telencephalon.[17,35,42,47] In the case of GluR4, its level in the telencephalon is equivocal. Keinänen *et al.*[29] and Monyer *et al.*[38] reported that GluR4 mRNA is found only in low levels in forebrain and is mainly restricted to the cerebellum, while Bettler *et al.*[7] and Sato *et al.*[47] found moderate to high levels of the message in many telencephalic areas. Meanwhile, Petralia and Wenthold[42] reported moderate to high levels of GluR4 immunostaining throughout the telencephalon, but Martin *et al.*[35] found that staining for GluR4 was high only in the cerebellum and that the low level of GluR4 staining in the cerebral cortex was restricted to astrocytes. In the present report, immunostaining of GluR4 was quite pronounced in the cerebellum; the antigen was difficult to detect in all other regions tested, including samples from telencephalic areas. The fact that the antibody preparations to GluR4 also recognized a prominent telencephalic antigen (*GR53*) may shed some light on the discrepancies concerning the distribution of GluR4.

Given that all of the currently cloned AMPA receptor subunits have a predicted molecular weight in the range of 105,000, it was surprising to find that a 53,000 mol. wt antigen (*GR53*) is recognized by GluR antibodies in the brains of many animal species. Several groups have previously reported that antigens of smaller size (50,000–60,000 mol. wt) are labeled by anti-GluR antibodies;[36,45,55] in each of these cases, the band was determined to be an artifact. In the Rogers *et al.*[45] study, an anti-GluR1 antiserum recognized a 60,000 mol. wt band which was also recognized by pre-immune serum. *GR53*, however, is not recognized by pre-immune serum. In both the Wenthold *et al.*[55] and Molnar *et al.*[36] studies, a 50,000–60,000 mol. wt band was seen in samples which had been immunopurified on anti-GluR affinity columns; this band was non-specific

Table 2. Glutamate receptor-related antigens in brain tissue samples from different species

	Rat	Human	Frog	Chick	Goldfish
GluR4-105					
Mol. wt	107 ± 3	104 ± 1	109 ± 1	113 ± 3	99 ± 1
Relative content (%)	100	n.d.	17	23	13
GR53					
Mol. wt	53 ± 1.5	56 ± 0.7	53 ± 1.4	53 ± 0.9	53 ± 1.4
Relative content (%)	100	n.d.	147	99	271

Brain homogenate and membrane samples from different species were analysed by immunoblot using anti-GluR4a (see Fig. 13). The electrophoretic migrations of protein molecular weight standards were used to calibrate each immunoblot (correlation coefficients > 0.99), and the mean mol. wt (± SEM, $n = 4$) for the labeled antigens of ~100,000 mol. wt (GluR4-105) and ~53,000 (*GR53*) were determined accordingly. The relative content of each antigen in the brain homogenates was normalized to the value in rat and is expressed as a percentage.

since it was recognized by the secondary antibody in the absence of a primary antibody, and it was determined in both cases to be the heavy chain of immunoglobulin G. This could not be the case for the *GR53* antigen characterized in the present paper, since samples were not immunoabsorbed nor was *GR53* recognized by secondary antibodies alone. Moreover, *GR53* was not evident in any of the non-brain tissues examined and the antigen increased in concentration throughout postnatal development, closely following the production of synapses. Thus, it seems that the *GR53* antigen is not likely to be an artifact of sample preparation. This raises obvious questions concerning the identity of the 53,000 mol. wt species.

It is possible that *GR53* represents a proteolytic fragment of a 105,000 mol. wt GluR subunit. This is not likely, however, since incubations of brain homogenates without protease inhibitors at 35°C (to activate endogenous proteases) failed to increase the proportion of 53,000 mol. wt staining in blot samples (unpublished observations). Moreover, peptide epitopes used to develop anti-GluR4a (the carboxy-terminal domain) and anti-GluR1*at* (an amino-terminal area ~35% similar to that in GluR4) are 680 residues apart in the GluR4 sequence; a breakdown product that is recognized by both antibodies would have to be significantly larger than *GR53* (i.e. ~80,000 mol. wt). Thus, the *GR53* antigen cannot be a GluR fragment containing both epitopes unless it consists of two polypeptides representing the perfect halves of a subunit. Immunoprecipitation studies indicate that the latter is not the case.

A second and more interesting possibility is that the anti-GluR4a and anti-GluR1*at* antibodies in the current study are labeling a 53,000 mol. wt protein which has some degree of homology with AMPA receptor subunits and is perhaps itself an AMPA-binding protein. However, the fact that *GR53* does not co-purify with [^{3}H]AMPA binding activity from solubilized rat forebrain[6] suggests that the 53,000 mol. wt polypeptide is not involved in the ability of AMPA receptors to bind ligand and, alone, represents only a small proportion of the AMPA- binding sites in the brain. None the less, it is interesting that radiation inactivation experiments have estimated that the molecular weight for [^{3}H]AMPA-binding proteins in rat cortex[27] and in chick telencephalon[23] is approximately 56,000. It is also of interest that KBPs from frog[53] and chick[18] are comparable to *GR53* with regard to size, and are similar to rat GluR subunits with respect to cDNA sequence[25,29] and antigenic determinants.[21] Another study of note is that of Kumar *et al.*,[32] which showed that a glutamate-binding protein from rat hippocampus has a molecular weight of 57,000. Although it is unclear as to which of the related proteins mentioned help make up AMPA receptors *in situ*, it is likely that different receptor composites exist, since different cell types have been shown to express functionally distinct AMPA receptor assemblies.[26,35,39,42] Whether *GR53* weakly associates with AMPA receptors or represents a unique [^{3}H]AMPA-binding protein remains to be tested by further experimentation. Notwithstanding, there are examples of proteins that associate with neurotransmitter receptors and, more importantly, share antigenic determinants with particular subunits of the receptor;[30,43] these proteins are thought to be involved in receptor clustering in the brain.[31]

CONCLUSION

The current study has characterized the regional and subcellular distribution of AMPA receptor subunits in rat brain. The developed antibodies to GluR subunits produced the most significant staining in SPM and PSD fractions, consistent with the notion that most AMPA receptors are localized to synapses. Levels of GluR1, GluR2/3 and GluR4 were shown to vary significantly across rat brain areas. Two of the antibodies also recognized a brain-specific 53,000 mol. wt protein named *GR53* which is enriched in SPMs and has a regional distribution distinct from those of the 105,000 mol. wt subunits. Identification of func-

tional roles for *GR53* in the brain will require further characterization.

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