Journal of Neurochemistry
Raven Press, Ltd., New York

Membrane Topology of the GluR1 Glutamate Receptor Subunit: Epitope Mapping by Site-Directed Antipeptide Antibodies

Elek Molnár, R. A. Jeffrey McIlhinney, Agnès Baude, Zoltán Nusser, and Péter Somogyi

Medical Research Council, Anatomical Neuropharmacology Unit, University of Oxford, Oxford, England

Abstract: In order to define the membrane topology of the GluR1 glutamate receptor subunit, we have examined the location of epitopes. Antibodies were produced against peptides corresponding to putative extracellular and intracellular segments of the rat brain GluR1 glutamate receptor subunit. Immunocytochemistry at the electron microscopic level in the dentate gyrus of the hippocampal formation showed that epitopes for the antiserum to the N-terminal part of the subunit are located at the extracellular face of the plasma membrane, whereas the antigenic determinants for the antiserum to the C-terminal part are found at the intracellular face of the postsynaptic membrane. Furthermore, antibodies to the N-terminal residues 253–267 reacted similarly with both intact and permeabilized synaptosomes, whereas the binding of antibodies to the C-terminal residues 877–889 increased about 1.6-fold following permeabilization. Our data suggest that the N- and C-terminal regions are located on the opposite side of the membrane and, therefore, the GluR1 subunit probably has an odd number of membrane spanning segments. The antibody cross-reactivities in different species and their effect on ligand binding activity were also established. **Key Words:** α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate—Antipeptide antibodies—Topology—Hippocampus—Dentate gyrus—Immunocytochemistry.
J. Neurochem. **63,** 683–693 (1994).

The vast majority of synapses in the central nervous system use glutamate as a neurotransmitter to produce rapid neuronal excitation (Monaghan et al., 1989). By cDNA cloning, a number of different glutamate receptor subunits have been identified (for reviews, see Barnes and Henley, 1992; Gasic and Hollmann, 1992; Nakanishi, 1992; Sommer and Seeburg, 1992; Sprengel and Seeburg, 1993; Wisden and Seeburg, 1993). Glutamate receptors have been classified into two major categories: ionotropic receptors, which function as ion channels, and metabotropic receptors, which are coupled to second-messenger systems. The ionotropic glutamate receptors have been named according to their respective agonist, e.g., the *N*-methyl-D-aspartate (NMDA), quisqualate, kainate (KA), and 2-amino-4-phosphobutyrate receptors (Monaghan et al., 1989). Quisqualate receptors are also activated by the more selective agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). The polypeptide chains of the non-NMDA (AMPA- and KA-sensitive) glutamate receptor subunits vary in length between 862 (GluR2) and 962 (KA-2) amino acid residues, and have sufficient amino acid homology (about 81–36% identity) to indicate that their tertiary folding may be similar.

Hydropathy analysis of the deduced amino acid sequences of the rat brain ionotropic glutamate receptor subunits has been used to suggest that they have an extracellular N terminus, followed by three hydrophobic stretches (transmembrane domains TM1–3), a hydrophilic cytosolic domain, and a further hydrophobic region (TM4) preceding the putative extracellular C terminus (Hollmann et al., 1989, 1990). As such theoretical approaches to protein structure can result in several alternative models, this four-transmembrane domain-containing model of the glutamate receptor subunits must be considered hypothetical in the absence of supporting biochemical data.

Site-directed mutagenesis studies have defined functionally important amino acid residues that control ion flow through AMPA-receptor channels, in what now seems genuinely to be a transmembrane domain termed TM2 (Köhler et al., 1993; Seeburg, 1993; Wisden and Seeburg, 1993). Furthermore, data showing that the non-NMDA glutamate receptor subunits are phosphor-

Received October 5, 1993; revised manuscript received December 23, 1993; accepted December 23, 1993.

Address correspondence and reprint requests to Dr. E. Molnár at Medical Research Council, Anatomical Neuropharmacology Unit, University of Oxford, Mansfield Rd., Oxford, OX1 3TH, U.K.

Abbreviations used: ABC, avidin biotinylated complex; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; ELISA, enzyme-linked immunosorbent assay; g_{av}, average g; KA, kainate; MAP, multiple antigen peptide; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PEG, polyethylene glycol 6000; PKC, protein kinase C; SDS, sodium dodecyl sulphate; TBS, Tris-buffered saline.

ylated in their predicted major intracellular loop between the putative third and fourth transmembrane domains (TM3 and TM4) would seem to confirm the intracellular location of this region (Moss et al., 1993; Raymond et al., 1993; Wright et al., 1993). More recently, Tingley et al. (1993) have suggested that the C-terminal region of the NMDAR1 subunit is intracellular, because it is accessible to protein kinase C (PKC), a known intracellular enzyme. This latter observation is in agreement with our previous high-resolution electron microscopic study on tissue sections that suggested that the N and C termini of the GluR1 subunit are on opposite sides of the plasma membrane (Molnár et al., 1993*a*).

Antipeptide antibodies raised to synthetic sequences of membrane proteins have been used to determine the topology, functional domains, and cellular localization of receptors. Sequence-specific antibodies can be used directly in membrane orientation studies to determine on which side of a membrane preparation a particular antibody will bind (Wang et al., 1989; Bahouth et al., 1991; Schroder et al., 1991; Traxler et al., 1993). High-resolution electron microscopic methods can also be used to determine whether particular epitopes are on the extra- or intracellular face of the plasma membrane (Somogyi et al., 1989; Baude et al., 1992, 1993; Molnár et al., 1993*a*).

In our present study, we have used antipeptide antibodies to investigate the transmembrane topography of the GluR1 glutamate receptor subunit by combining a detailed study at the light and electron microscopic levels of the regional, cellular, and subcellular distributions of the GluR1 subunit in rat hippocampus with in vitro studies on synaptosomes. The results support the idea that the C-terminal domain of the GluR1 subunit is located intracellularly, suggesting that the proposed transmembrane topology model for glutamate receptors may be incorrect. In addition, the cross-reactivity of these antibodies with similar subunits from other species was determined, and their effects on ligand binding were tested. Some of these results have been presented in preliminary form (Molnár et al., 1993*b*).

MATERIALS AND METHODS

Materials

[^{3}H]AMPA (60.0 Ci/mmol) and [*vinylidene*-^{3}H]KA (58.0 Ci/mmol) were purchased from DuPont–New England Nuclear (Boston, MA, U.S.A.). AMPA was obtained from Tocris Neuramin (Bristol, U.K.). Triton X-100, KA, and high molecular weight markers for electrophoresis were purchased from Sigma Chemical Company Ltd. (Dorset, U.K.). Lyophilized human γ-globulin was supplied by Calbiochem (La Jolla, CA, U.S.A.). Polyethylene glycol 6000 (PEG) and 6-amino-*n*-hexanoic acid were from BDH Chemicals Ltd. (Poole, U.K.). Peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins and alkaline phosphatase-conjugated affinity-purified immunoglobulins to rabbit immunoglobulins came from Dako (Glastrup, Denmark).

Animal and human tissue sources

Tissue from a total of 32 Wistar rats (Charles River Ltd., Margate, Kent, U.K.; 100–400 g), one ferret, and one adult homing pigeon (University Farm, Wytham, U.K.) was utilized for this study. All procedures relating to the care and treatment of animals conformed to institutional and Home Office guidelines. Bovine brain was obtained from Reading Abattoir (Reading, U.K.).

Human hippocampal tissue was obtained at autopsy (Department of Neuropathology, Radcliffe Infirmary, Oxford, U.K.) from a 56-year-old man who had died from mechanical injuries caused by an accident. On neuropathological examination, the brain was normal without significant neuropathological finding.

From human hippocampus and rat, ferret, pigeon, and bovine brains, crude synaptic membranes were prepared as described previously (Molnár et al., 1993*a*).

Preparation of antipeptide polyclonal antibodies

Antipeptide antibodies against residues 253–267, 757–771, and 877–889 of the GluR1 glutamate receptor subunit were obtained as previously described (Molnár et al., 1993*a*). Peptides RTSDSRDHTRVDWKR, KLKNKWWYDKGECGT, and SHSSGMPLGATGL (corresponding residues: 253–267, 757–771, and 877–889, respectively; Hollmann et al., 1989) were synthesized as both multiple copies on a branching lysyl matrix [multiple antigenic peptides (MAPs)] and conventional linear peptides using solid-phase synthesis. Antibodies were raised by immunizing rabbits with the respective MAPs. All rabbits immunized produced antibodies recognizing the corresponding peptide as measured by an enzyme-linked immunosorbent assay (ELISA), using the peptides as the antigen. In this report, we used the bleed-out sera of rabbit 012 and 017 immunized with MAPs 253–267 and 877–889, respectively.

Preparation of synaptosomes

Rats were killed by decapitation, and the brains of the animals were rapidly removed. The brains were cleaned of white matter and dropped into ice-cold 0.32 *M* sucrose, 10 m*M* Tris-citrate (pH 7.4) and chopped into small pieces with scissors. Blood and other debris were washed from the brain tissue by adding more medium and decanting the supernatant from the top of the minced tissue several times. The chopped tissue was then homogenized in 10 volumes of 0.32 *M* sucrose, 10 m*M* Tris-citrate (pH 7.4) in a Potter–Elvehjem-type glass-Teflon homogenizer by 15 up-and-down strokes with a Teflon pestle (total clearance 0.15–0.25 mm). This homogenate was spun at 1,500 g_{av} for 10 min at 2°C. The pellet was rehomogenized and spun in 10 volumes of 0.32 *M* sucrose, 10 m*M* Tris-citrate (pH 7.4) again to increase the yield of the preparation. The supernatants from these two spins were combined and centrifuged at 9,000 g_{av} for 20 min at 2°C. The pellet was dispersed in 0.32 *M* sucrose, 10 m*M* Tris-citrate (pH 7.4) and layered over 0.8 *M* sucrose, 10 m*M* Tris-citrate (pH 7.4), followed by centrifugation at 9,000 g_{av} for 25 min at 2°C. Particles were resolved into three fractions: (a) a thick white band at the 0.32–0.8 *M* sucrose interface, (b) particles dispersed in 0.8 *M* sucrose solution, and (c) pellet. The second fraction, which contained mainly synaptosomes, was separated, and ice-cold deionized water was added dropwise during constant stirring to dilute the sucrose concentration to 0.32 *M*, followed by centrifugation at 20,000 g_{av} for 30 min at 2°C. The resulting pellet was

suspended in 50 m*M* Tris-HCl (pH 7.4), 0.9% NaCl [Tris-buffered saline (TBS)] (Hajós, 1975).

Protein concentrations were determined according to Lowry et al. (1951).

Immunoblot analysis of brain membranes

Membranes prepared from rat, ferret, bovine, and pigeon brains and human hippocampus were subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (50 μg of protein/lane, using 10% polyacrylamide gels) and immunoblotting using affinity-purified antipeptide antibodies GluR1 253–267 and 877–889 (final concentration 5 μg/ml; incubated at 2°C for 14 h). The bound antibodies were detected by reaction with alkaline phosphatase-conjugated anti-rabbit IgG (Dako, Glastrup, Denmark; 1:1,000 dilution; incubated at room temperature for 2 h). The bound conjugated IgG was visualized by reaction with a solution containing 20 ml of 0.1 *M* Tris-HCl (pH 9.5), 0.1 *M* NaCl, 0.05 *M* $MgCl_2$, 50 μl of 150 mg/ml nitroblue tetrazolium in 70% *N,N*-dimethylformamide, and 35 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidine salt in *N,N*-dimethylformamide.

Preparation of synaptosomal fractions for electron microscopy

Synaptosomal fractions were diluted (10-fold) in fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and ~0.2% picric acid in 0.1 *M* sodium phosphate buffer (pH 7.4) and incubated for 1 h at room temperature (Somogyi and Takagi, 1982). The fixed synaptosomes were washed several times by resuspension and centrifugation (at 15,000 g_{av}, for 5 min, at room temperature) in 0.1 *M* sodium phosphate buffer (pH 7.4), followed by resuspension in 1% osmium tetroxide, 0.1 *M* sodium phosphate buffer (pH 7.4), and incubation for 1 h at room temperature. After several washes with 0.1 *M* sodium phosphate buffer (pH 7.4), samples were centrifuged at 15,000 g_{av} for 10 min, and then the pellets were stained with 1% uranyl acetate in 70% ethanol for 1 h. Dehydration and embedding (Durcupan ACM, Fluka, Buchs, Switzerland) were carried out using standard procedures (Somogyi et al., 1989).

ELISA

The accessibility of the antigenic determinants in intact and disrupted synaptosomes was investigated using a quantitative ELISA technique (Molnár et al., 1990). Peptides (20 μg/ml) were dissolved in coating buffer (13 m*M* sodium carbonate, 35 m*M* sodium bicarbonate, pH 9.6) and 50 μl/well used to coat microtitre plates (Falcon 3912 assay plates, Becton Dickinson and Co., Oxnard, CA, U.S.A.) at 4°C for 12 h. The plates were washed three times for 3 min each with 200 μl of washing buffer containing 0.05% Tween 20 in phosphate-buffered saline (PBS; pH 7.2). Blocking of nonspecific protein binding was done with 100-μl aliquots of 1% bovine serum albumin in PBS for 1 h at room temperature.

The reaction with different antibody-containing media was performed for 1 h at 23°C. The plates were then washed three times with 200 μl of PBS containing 0.05% Tween 20 before incubation with 100 μl of anti-rabbit IgG conjugated to horseradish peroxidase (1:1,000 dilution in PBS) for 1 h. After washing as before, the plates were developed by the addition of 100 μl of *o*-phenylenediamine (1 mg/ml) and hydrogen peroxide (0.003%) substrate in 35 m*M* citric acid and 125 m*M* Na_2HPO_4 (pH 4.0). After 15 min, the reaction was stopped with 50 μl of 4 *M* H_2SO_4. The plates were then read at 492 nm on a Titertek Multiskan microtitre plate spectrophotometer (Flow Laboratories, Inc., McLean, VA, U.S.A.).

Radioligand binding

The membrane fraction from rat brain was prepared, and AMPA and KA binding sites were solubilized with Triton X-100 as previously described (Hunter et al., 1990; Molnár et al., 1993*a*). Radioligand binding studies were carried out by a centrifugation assay, using PEG precipitation. Ligand binding assays were performed using either 0.2 mg (for [^{3}H]-AMPA) or 0.4 mg (for [^{3}H]KA) of membranes or solubilized membrane proteins. The reagents were incubated in 50 m*M* Tris-citrate (pH 7.4) for 30 min at 2°C, using either 20 n*M* [^{3}H]AMPA in the presence of 100 m*M* KSCN or 10 n*M* [^{3}H]KA. Nonspecific binding was determined by adding 100 μ*M* AMPA or KA to parallel incubations. Following incubation, 2 mg/ml human γ-globulins and 15% (wt/vol) PEG were added and the samples centrifuged at 10,000 g_{av} in an Eppendorf microfuge for 2 min. The supernatant was aspirated and the pellet rinsed twice superficially using 0.4 ml of ice-cold 15% PEG. The pellets were suspended in 0.5 ml of 1% SDS, then transferred to scintillation vials, and counted after the addition of 4 ml of Liquiscint (National Diagnostics, Manville, NJ, U.S.A.).

Immunocytochemistry

Fourteen male or female Wistar rats (Charles River Ltd.; 100–400 g) were deeply anaesthetized with sodium pentobarbital (150 mg/kg i.p.) and perfused for 1 min through the aorta with a 0.9% NaCl solution, followed by 100–300 ml of ice-cold fixative containing 4% paraformaldehyde, 0.025–0.1% glutaraldehyde, and ~0.2% picric acid made up in 0.1 *M* phosphate buffer, at pH 7.2 (Somogyi and Takagi, 1982). Frontal sections of hippocampus were obtained either with a Vibratome (70–100-μm thick) or with a cryostat (40–60-μm thick). Immunocytochemistry was carried out using the avidin biotinylated horseradish peroxidase complex method (ABC; Vector Laboratories) as previously reported (Molnár et al., 1993*a*). Briefly, sections were incubated in 20% normal goat serum (Vector), and dissolved in TBS for 1 h at room temperature, and then in primary antibody overnight at 4°C. The final protein concentrations for both primary antibodies were 0.5–1 μg of protein/ml for antibody GluR1 253–267 and 2–4 μg of protein/ml for antibody GluR1 877–889. The sections were then incubated for 2 h in biotinylated goat anti-rabbit IgG diluted 1:50 (Vector) and then for 2 h in ABC diluted 1:100, both dissolved in TBS. Peroxidase activity was detected with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 50 m*M* Tris-HCl (pH 7.6). For light microscopic studies (in particular, for cryostat sections), Triton X-100 (0.3%) was used in all incubation and washing solutions, and the peroxidase reaction end-product was enhanced by treatment with ~0.04% osmium tetroxide in phosphate buffer for 10 min. For electron microscopic studies, sections were processed routinely as described earlier (Molnár et al., 1993*a*). Areas of interest were cut out from sections flat-embedded between slide and coverslip in epoxy resin and reembedded for ultrathin sectioning. No lead staining was used.

In control experiments for immunocytochemistry, the omission of primary antiserum during the immunocytochemical procedure resulted in the removal of all staining. There was no reaction with the preimmune sera (1:1,000 dilution)

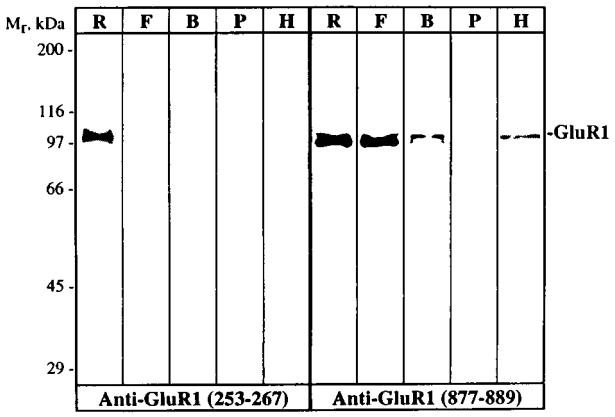

FIG. 1. Immunolabelling of GluR1 subunit in brains of different species by antipeptide antibodies raised against residues 253–267 and 877–889. Crude membrane samples prepared from rat (R), ferret (F), bovine (B), pigeon (P), and human (H) brains were immunolabelled with purified anti-GluR1 253–267 and 877–889 antibodies. Fifty micrograms of membrane protein were applied to each lane of 10% SDS-polyacrylamide gels. For immunoblot analysis, 5 μg/ml affinity-purified antibody was used. The bound antibody was visualized by alkaline phosphatase-conjugated anti-rabbit IgG (1:1,000 dilution). The positions of the molecular mass standards are indicated on the left.

that resembled the reaction obtained with the purified antibodies. Preabsorption of the primary antisera with the corresponding linear synthetic peptide (20 μg of peptide/ml, 5 h of incubation at room temperature) before application to the sections resulted in the disappearance of specific labelling. These experiments confirmed the specificity of the immunocytochemical method (Molnár et al., 1993*a*).

RESULTS

Characterization and cross-reactivity of antibodies with related subunits in different species

To study the rat GluR1 glutamate receptor protein within the nervous system, polyclonal antipeptide antiserum was raised in rabbits against synthetic peptides, corresponding to residues 253–267, 757–771, and 877–889. The binding specificity of the antibodies was determined on immunoblots. Antibodies to residues 253–267 and 877–889 recognized a major band corresponding to the predicted molecular mass of the GluR1 subunit (105 kDa) in rat brain, and specifically immunoprecipitated solubilized [^{3}H]AMPA binding sites. In addition, the staining of the 105-kDa band was abolished by inclusion of the appropriate peptide in the incubation with the primary antibody (Molnár et al., 1993*a*). Similar immunolabelling of western blots was obtained with ferret, bovine, and human membranes using antiserum raised against the C terminus (residues 877–889), suggesting a similar structure of this region in different species (Fig. 1). Antibody against N-terminal residues (253–267) did not react with membrane samples from other species under the same conditions. None of the antibodies recognized glutamate receptor subunits in membranes prepared from pigeon brain (Fig. 1).

Immunocytochemical localization of GluR1 subunit in the rat hippocampus

The pattern of immunoreactivity was identical whether sections were cut on a Vibratome, without freezing, or on a cryostat. However, the cryostat sections, which were also treated with detergent, showed stronger immunoreactivity presumably due to better penetration of antibodies. Therefore, the light microscopic distribution of immunoreactivity was demonstrated on cryostat sections (Fig. 2A–C). Vibratome sections showed better, fine structural detail; therefore, the subcellular distribution of immunoreactivity was demonstrated from this material (Fig. 2D–G).

The distribution of the immunoreactivity for GluR1 in the hippocampus was very similar with both antibodies (Fig. 2A and B). Immunoreactivity obtained with antibody to residues 877–889 was generally weaker than that obtained with antibody GluR1 253–267. The CA1 area and the molecular layer of the dentate gyrus were more strongly immunoreactive than the CA3 area and the subiculum (Fig. 2A and B). The neuropil of the hilus was very weakly labelled, but contained several large neurones immunoreactive for GluR1 as previously reported (Molnár et al., 1993*a*).

The dentate gyrus was chosen for a detailed study, because it is known to receive several well defined and segregated glutamatergic inputs, which are the subjects of extensive pharmacological and physiological investigations (Errington et al., 1987; Lambert and Jones, 1990; Staley and Mody, 1992). The granule cell layer was weakly labelled (Fig. 2C). Immunoreactivity for GluR1 was very rarely observed to be associated with the somatic membrane of the granule cells under electron microscopic examination. The molecular layer of the dentate gyrus that corresponds to the dendritic field of the granule cells was very strongly labelled for both antibodies. The reaction intensity varied systematically throughout the molecular layer. Three bands, with clear boundaries, can be described, with the intensity of labelling increasing from the inner part to the outer part of the molecular layer (Fig. 2C). High-resolution electron microscopic examination of the molecular layer revealed that numerous spines receiving type 1 synapses from boutons were immunoreactive for GluR1 with both antibodies (Fig. 2D–G). Immunoreactivity for GluR1 was also observed at nonsynaptic membranes along the main dendritic shafts of granule cells (data not shown), as described for pyramidal cells in the CA1 area (Molnár et al., 1993*a*).

In the dentate hilus, electron microscopic analysis showed that intense immunoreactivity associated with the cisternae of the endoplasmic reticulum in the soma of immunoreactive neurones, but no immunoreactivity could be detected at the surface of their somatic membrane. Interestingly, immunoreactivity was very rarely

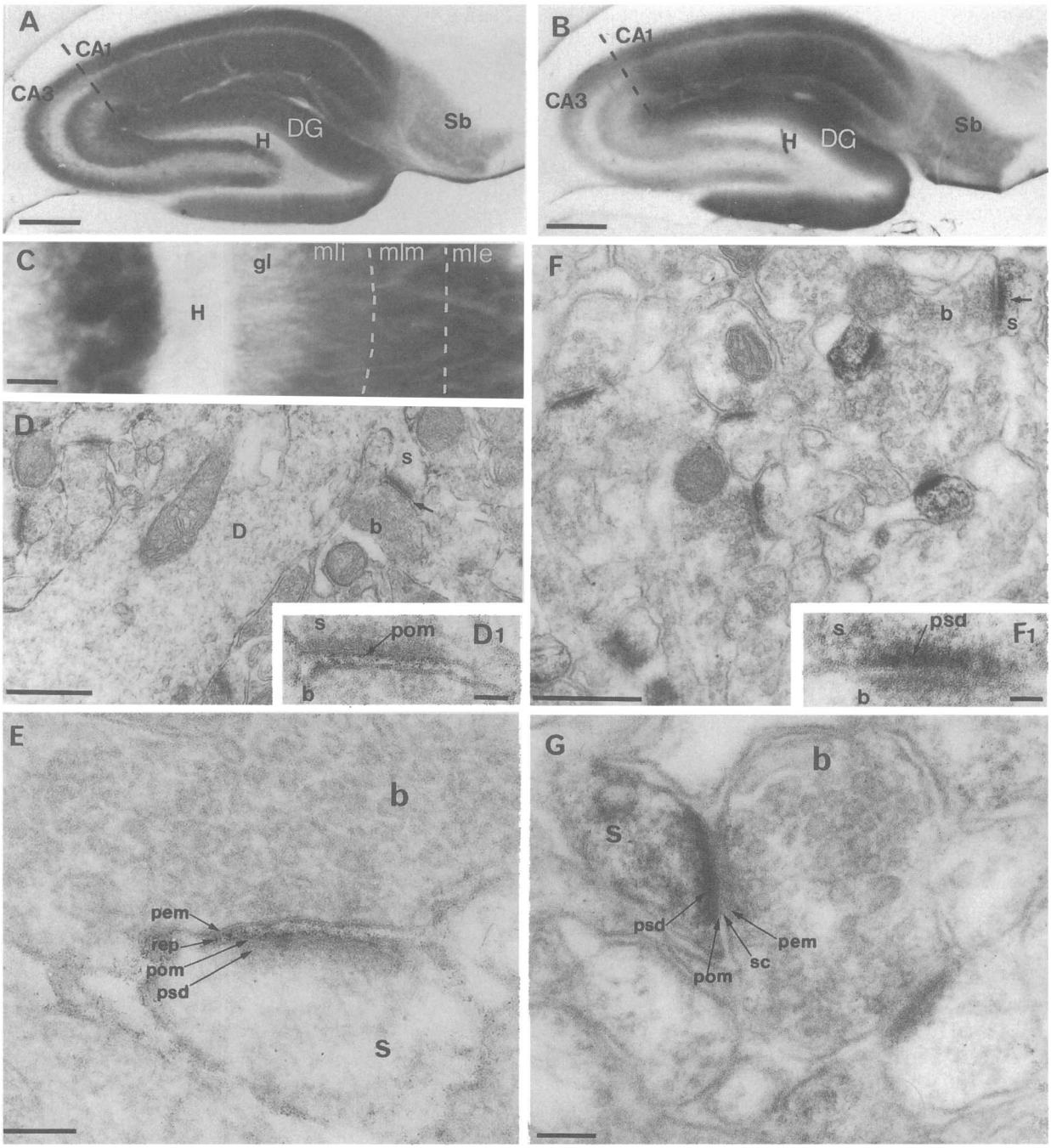

FIG. 2. Immunocytochemical localization of the GluR1 subunit in the rat hippocampus using antibodies against residues 253–267 (**left column**) or 877–889 (**right column**). **A** and **B:** Two serial cryostat sections of rat dorsal hippocampus reacted with anti-GluR1 253–267 (A) or anti-GluR1 877–889 (B), showing similar distribution of immunoreactivity with a clear boundary in the degree of immunoreactivity between the CA1 and CA3 areas, and the CA1 area and the subiculum (Sb). DG, dentate gyrus. **C:** The dentate gyrus in a cryostat section exhibits increasing intensity of immunoreaction from the hilus (H) through the granular layer (gl), and the internal (mli), medial (mlm), and external (mle) parts of the molecular layer. **D** and **E:** Electron micrographs of GluR1 immunoreactivity in the molecular layer of the dentate gyrus as demonstrated with antibody against residues 253–267. Several spines, one of them (s) emanating from a large dendrite (D), establish immunopositive type 1 synapses (e.g., arrow; see also at high magnification in D1) with boutons (e.g., b). The reaction end-product (rep) is concentrated at the extracellular face of the postsynaptic membrane (pom) demonstrating extracellular epitope(s). Note that the postsynaptic density (psd) is devoid of peroxidase reaction product. **F** and **G:** Electron micrographs of GluR1 immunoreactivity in the molecular layer of the dentate gyrus as demonstrated with antibody against residues 877–889. Several spines (e.g., s) making type 1 synapses with boutons (e.g., b; see also at high magnification in F1) are strongly immunoreactive (e.g., arrow). The reaction end-product is located intracellularly at the postsynaptic membrane, on the postsynaptic density (psd), and not in the synaptic cleft (sc) between the pre- (pem) and postsynaptic membranes (pom). This demonstrates intracellular epitope(s). All electron micrographs are taken from Vibratome-cut material. Bars = 0.5 mm (A and B); 50 μm (C); 0.5 μm (D and F); 0.05 μm (D1 and F1); 0.1 μm (E and G).

seen in association with the endoplasmic reticulum in granule cell somas, probably reflecting a lower rate of synthesis and turnover of the receptor protein GluR1 in granule cells as compared with hilar cells. In the hilar neuropil, immunoreactivity was observed along the membrane of small and medium-sized dendritic shafts at synaptic and extrasynaptic locations. Few immunopositive synapses were seen between spines and axon terminals. Synaptic immunoreactivity was always associated with type 1 (asymmetric) synapses. Immunoreactivity for GluR1 could not be detected at the synapses between identifiable mossy fibre terminals originating from granule cells and hilar elements. However, examination of the CA3c area (closest to the hilus) revealed that numerous spines, probably emanating from the CA3 pyramidal neurones, established immunoreactive synapses with mossy fibres as previously reported in the stratum lucidum of the CA3 area (Petralia and Wenthold, 1992). Immunoreactivity for GluR1 was never found in axons, axon terminals, or glia in the dentate gyrus and hilus.

The peroxidase end-product was localized differently after immunostaining with either antibody GluR1 253–267 or 877–889. The use of antibody against residues 253–267 resulted in an immunoreaction located at the extracellular face of the plasma membrane, including the postsynaptic membrane of immunoreactive spines (Fig. 2D1 and E). In contrast, with the antibody to residues 877–889, immunoreaction was obtained at the intracellular face of the plasma membrane, including postsynaptic densities of dendritic spine (Fig. 2F1 and G). Such a location suggests that the antibody to residues 877–889 reveals epitope(s) located at the intracellular face of the membrane.

Determination of the membrane topography of residues 253–267 and 877–889 in synaptosomes

To investigate further the membrane orientation of amino- and carboxy-terminal domains of the GluR1 subunit, intact synaptosomes were separated by centrifugation using a sucrose gradient following the method of Hajós (1975).

The purity and the structure of the synaptosome fraction were judged using electron microscopy. Electron micrographs of the synaptosomal preparations isolated from rat brains showed mainly sealed membrane-delineated presynaptic structures (synaptosomes) and two different postsynaptic profiles. Some of the postsynaptic membranes were completely closed (Fig. 3, filled arrows), whereas others were found to be open with postsynaptic densities exposed to the incubation solution without an intervening membrane (Fig. 3, open arrows). Both types of synapses had an extensive postsynaptic density, which would result in their in situ classification as type 1 (Gray, 1959), or asymmetrical synapses. In addition to the synaptosomes, the preparation contained some free mitochondria, myelin, and undefined cellular fragments.

The transmembrane location of residues 253–267 and 877–889, the binding of the antipeptide antibodies to native or disrupted synaptosomes produced by hypotonic lysis, was examined using a competitive ELISA (Table 1). The 253–267 antigenic site was fully accessible for antibody reaction in the native structure, and disruption of synaptosomes did not change the binding of this antibody. Anti-GluR1 877–889 antibody did bind to native synaptosomes, but permeabilization of vesicles by hypotonic medium increased its binding significantly, suggesting that this epitope is not exposed completely in intact synaptosomes. Some binding of this antibody to the unlysed preparation would be expected, because a significant fraction of the postsynaptic structures is broken during their isolation and the postsynaptic density is thereby exposed to the incubation medium.

Effect of bound antibodies on the GluR1 ligand binding activity

The effect of the antibodies on the [^{3}H]AMPA and [^{3}H]KA binding activities was studied using membrane-bound and solubilized samples (Table 2). After preincubation (16 h at 4°C) with 5 μg/ml affinity-purified anti-GluR1 antibodies, [^{3}H]AMPA and [^{3}H]KA binding activities were determined by centrifugation assay using PEG precipitation. The binding of both ligands remained essentially unchanged in the presence of antibodies with either membrane-associated or solubilized forms of the receptor, although a small inhibition of ligand binding was observed with both [^{3}H]-AMPA and [^{3}H]KA. However, as earlier experiments had shown that these antibodies specifically immunoprecipitate AMPA binding sites and not KA binding (Molnár et al., 1993*a*), this small effect, which was not ligand-specific, is probably due to interference of ligand binding by other components of the antibody-containing medium.

DISCUSSION

Specificity of antibodies

Molecular cloning has revealed the primary structure of a number of glutamate receptor subunits. The organization and topography of these proteins have been predicted to have four hydrophobic membrane-spanning domains. For membrane proteins displaying multiple hydrophobic domains, the interpretation of the hydropathy analysis becomes critical to the development of a first-approximation model of the topography of the molecule (Wisden and Seeburg, 1993). Antibodies directed against defined epitopes provide immunological tools with which the topography of membrane proteins can be probed by direct means (Wang et al., 1989; Molnár et al., 1990; Bahouth et al., 1991; Schroder et al., 1991; Baude et al., 1993; Traxler et al., 1993). To study the topography of the GluR1 glutamate receptor subunit, antibodies were prepared against three synthetic peptides corresponding to hydrophilic sequences. Two of the antipeptide

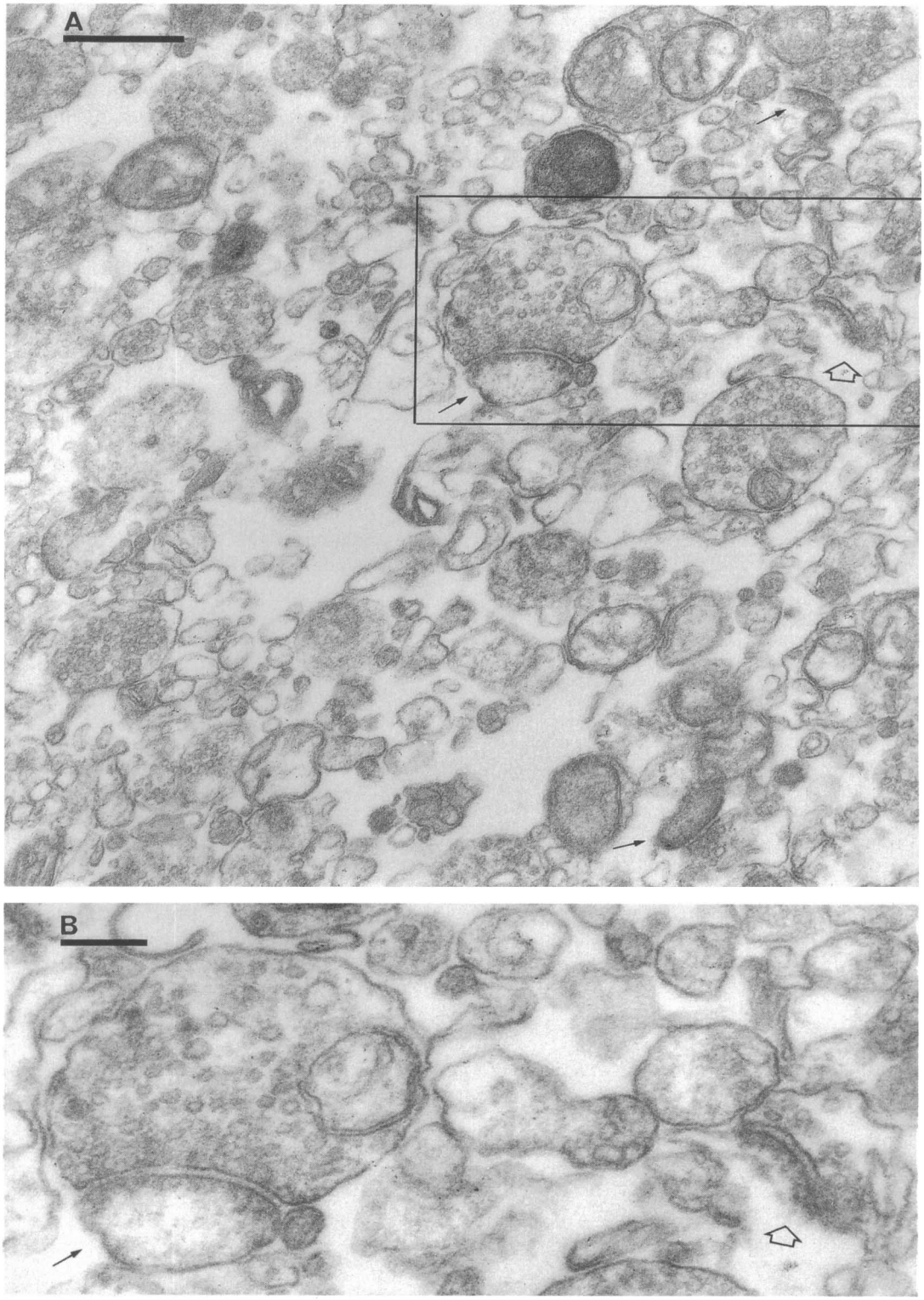

FIG. 3. Electron micrographs of the synaptosomal fraction. The synaptosomal fraction was separated on sucrose gradient as described by Hajós (1975). Filled arrows point to synaptosomes showing both pre- and postsynaptic elements after incubation in isotonic medium. The majority of the presynaptic membranes were closed. The majority of the postsynaptic bags were also closed (filled arrows in **A** and **B**), but some were found to be open (open arrows in A and B). B is a higher magnification view of the framed area in A. Bars = 0.5 μm (A) and 0.2 μm (B).

TABLE 1. *Determination of antibody binding to GluR1 subunit in native and osmotically disrupted synaptosomes*

	Anti-GluR1 253–267		Anti-GluR1 877–889	
Medium	% of antibody removed	Relative amount of bound antibody (%)	% of antibody removed	Relative amount of bound antibody (%)
Isotonic	59.4 ± 3.1	100	37.2 ± 4.1	100
Hypotonic	60.4 ± 4.7	102.7 ± 4.9	73.7 ± 2.8	164.3 ± 7.4

Synaptosome fractions (Fig. 3) were preincubated with antibodies (1 μg/ml) for 16 h at 4°C, in a medium containing 50 m*M* Tris-citrate (pH 7.4), and 1% bovine serum albumin, and 0.9% NaCl (Isotonic) or without 0.9% NaCl (Hypotonic). In a parallel series of control experiments, the antibodies were incubated under identical conditions in the absence of synaptosomes. The unbound antibody in the medium was determined by centrifuging the samples at 10,000 g_{av} for 5 min and analysing the supernatant solutions by ELISA, using synthetic peptides as fixed antigens in amounts of 1 μg/well. Absorbance of the peroxidase reaction product was determined at 492 nm. The difference in absorbance between the control samples and the samples obtained after preincubation with synaptosomes was expressed as a percentage of the control absorbance to illustrate the antibody binding to the synaptosome samples.

After separation from the supernatant, pellets (containing membranes and the bound antibodies) were washed four times in either isotonic or hypotonic medium and incubated with peroxidase-conjugated anti-rabbit antibodies (1:5,000 dilution), followed by washing. The optical density of the coloured peroxidase reaction product was determined after centrifugation of the samples. The reaction obtained in isotonic samples (100%) served as a reference for samples preincubated in hypotonic medium. The values are the means ± SD from three independent determinations.

antibodies (GluR1 253–267 and 877–889) displayed immunoreactivity for both the synthetic peptide antigen and the GluR1 subunit. These antibodies recognized a single protein (105 kDa) in immunoblots of rat brain synaptosomal membranes. Antibodies raised against residues 757–771 failed to detect the GluR1 subunit under the same conditions.

In the present study, we have shown that affinity-purified rabbit polyclonal antibodies raised against an N-terminal epitope (residues 253–267) of the rat brain GluR1 glutamate receptor subunit did not cross-react with analogous subunits in ferret, bovine, pigeon, and human brain. In contrast, antibody to the C-terminal part of the GluR1 subunit (residues 877–889) reacted freely with membranes from ferret, bovine, and human brains, reflecting the level of sequence identity that exists between these subtypes. The sequence of the analogous human subunit is identical in the C terminus (SHSSGMPLGATGL), but somewhat different in the N-terminal region in the human GluR1 subunit (KNS-OAROHTRVOWK) compared with the corresponding rat sequence (RTSDSROHTRVOWK), which could explain the differences in antibody reaction (Hollmann et al., 1989; Puckett et al., 1991). The amino acid sequences of homologous receptor subunits in other examined species are not available yet, but based on the observed antibody cross-reactions, similar proteins are present in different species, with the C-terminal part of the molecules being highly conserved between species compared with the N-terminal regions of the proteins.

Immunoblotting of different dissected human brain regions with similar antibodies against the C-terminal parts of the GluR1–4 and mGluR1 glutamate receptors yielded results similar to those described here (Blackstone et al., 1992*a*).

The GluR1 subunit in the dentate gyrus

The molecular layer of the dentate gyrus that corresponds to the dendritic field of the granule cells was strongly immunoreactive for GluR1, as previously described (Blackstone et al., 1992*b*; Martin et al., 1992, 1993; Petralia and Wenthold, 1992; Molnár et al., 1993*a*). The pattern of staining correlates well with autoradiographic studies that described a high density of [^{3}H]AMPA binding sites in the dentate gyrus (Monaghan et al., 1984; Olsen et al., 1987). Indeed the gran-

TABLE 2. *Effect of anti-GluR1 253–267 and 877–889 on the [^{3}H]AMPA and [^{3}H]KA binding activity of membrane-bound and solubilized GluR1 subunit*

		[^{3}H]AMPA binding activity		[^{3}H]KA binding activity	
Antibody	Receptor	pmol/mg	% of control	fmol/mg	% of control
Control	Membrane-bound	0.57 ± 0.02	100	67.2 ± 2.8	100
GluR1 253–267		0.49 ± 0.04	86 ± 8	59.7 ± 3.3	89 ± 4
GluR1 877–889		0.50 ± 0.01	87 ± 2	62.0 ± 1.9	92 ± 3
Control	Solubilized	1.13 ± 0.07	100	111.3 ± 6.2	100
GluR1 253–267		1.03 ± 0.14	91 ± 14	88.6 ± 1.8	80 ± 2
GluR1 877–889		1.30 ± 0.15	114 ± 12	105.6 ± 8.9	95 ± 8

The crude membrane fraction was prepared from rat brain and solubilized by the addition of 1% Triton X-100 detergent as described by Hunter et al. (1990). Membrane-bound and solubilized samples were incubated for 16 h at 4°C in the presence of anti-GluR1 antibodies (5 μg/ml). Affinity-purified antibodies were dialysed against 50 m*M* Tris-citrate (pH 7.4) before application. Specific [^{3}H]AMPA and [^{3}H]KA-binding activities were determined by centrifugation assay using PEG precipitation. The binding assays were performed in triplicate, and the values are the means ± SD from three independent determinations.

ule cells have been shown to highly express mRNAs coding for GluR1, GluR2, and GluR3 and, to a lesser extent, the mRNA coding for GluR4 (Keinänen et al., 1990; Sommer et al., 1990). In the present study, we have demonstrated that the GluR1 subunit is present all along the dendritic surface of the granule cell membrane and is concentrated at the membrane of the dendritic spines. Granule cells receive at least three major excitatory inputs. In the inner third of the molecular layer, the commissural and association pathways terminate; in the medial part and the outer third of the molecular layer for the medial and lateral entorhinal cortex, afferents provide glutamatergic terminals. These three pathways establish asymmetrical synapses mainly on the dendritic spines of the granule cells. Axon terminals involved in asymmetrical synapses throughout the molecular layer have been found to contain a high concentration of glutamate, which consequently is thought to be used as neurotransmitter at these synapses (Bramham et al., 1990). Moreover, lesion of the perforant pathway induces a dramatic decrease of immunoreactivity for glutamate in the outer two-thirds of the molecular layer (Grandes and Streit, 1991). In addition, all electrophysiological experiments have demonstrated that synaptic responses to perforant path stimuli are mediated through AMPA and NMDA receptors (Lambert and Jones, 1990; Keller et al., 1991; Staley and Mody, 1992). In the present study, we have demonstrated that GluR1 immunoreactivity is associated with type 1 synapses on dendritic spines throughout the different parts of the molecular layer. These results strongly suggest that the three major excitatory inputs to the granule cells are mediated, at least partially, through AMPA receptors, including the GluR1 subunit. The increasing intensity of GluR1 immunoreactivity from the inner third to the outer third of the molecular layer may reflect the differing degrees of expression of the protein GluR1, depending on the origins of the excitatory glutamatergic inputs. The dentate hilus contains a large variety of cell types, probably all of which receive glutamatergic synapses from granule cell terminals (for review, see Leranth et al., 1992), which are not immunopositive for GluR1, in contrast to the mossy synapses in the CA3 region (Petralia and Wenthold, 1992; present results). However, some small axospinous synapses in the hilus are immunopositive for GluR1, which may reflect the ability of hilar cells to concentrate different receptors at different synapses. Glutamatergic input to hilar spines may originate from the collaterals of mossy cells (Buckmaster et al., 1992) or from a recurrent projection from pyramidal cells of the CA3 area.

Membrane topology of the glutamate receptor subunits

The transmembrane conformation of the glutamate receptor is a matter of controversy (Hollmann et al., 1989, 1990; Nakanishi, 1992; O'Hara et al., 1993; Seeburg, 1993; Tingley et al., 1993; Wisden and Seeburg, 1993). The N and C termini have been predicted to be on the extracellular side of the membrane, according to one model for the transmembrane topology of the GluR1 subunit (Hollmann et al., 1989; Gasic and Hollmann, 1992). This model was based on simple hydropathy plots of the amino-acid sequence of the cloned GluR1 subunit and by analogy with the proposed transmembrane topology of subunits for other ligand-gated ion channels, such as the nicotinic acetylcholine receptor and the $GABA_A$ receptor. Very little independent evidence is available to support this model (Wisden and Seeburg, 1993). Key amino-acid residues controlling ion flow through AMPA-receptor channels have been identified by mutagenesis experiments in the putative second transmembrane domain (TM2). These data would support the view that this is a genuine transmembrane segment. Recent data have also shown that the non-NMDA glutamate receptor subunit GluR6 is phosphorylated by cyclic AMP-dependent protein kinase on Ser^{684}, which is contained in the predicted major intracellular loop between the putative third and fourth transmembrane domains, confirming the intracellular location of this region (Raymond et al., 1993). The presence of consensus phosphorylation sites in this intracellular loop of GluR1–4 for calcium/calmodulin-dependent protein kinase II and PKC has also been established (Boulter et al., 1990; Keinänen et al., 1990; McGlade-McCulloh et al., 1993; Wright et al., 1993). Tingley et al. (1993) have suggested that the C-terminal region of the NMDAR1 subunit is intracellular, because it is accessible to PKC and it seems unlikely that PKC phosphorylation occurs on an extracellular domain of the protein. An intracellular location of the NMDAR2 subunit carboxy terminus has also been proposed (Seeburg, 1993).

Several immunocytochemical studies have demonstrated that site-directed antibodies are useful tools to determine the location of epitopes on transmembrane receptor proteins, such as the subunits of the $GABA_A$ receptor (Somogyi et al., 1989; Ewert et al., 1990; Baude et al., 1992), the nicotinic acetylcholine receptor (Sargent and Pang, 1989), the glycine receptor (Triller et al., 1985; Schroder et al., 1991), the muscarinic acetylcholine receptor (Mrzljak et al., 1993), and the metabotropic glutamate receptor (Baude et al., 1993). Our immunocytochemical results in the CA1 region (Molnár et al., 1993*a*) demonstrated that epitopes detected with antibodies against an amino-terminal peptide of GluR1 are located at the extracellular face of synaptic and extrasynaptic neuronal membranes. These results confirm the extracellular location of the N-terminal part of the GluR1 subunit of the AMPA receptor, as suggested by all the proposed models drawn from molecular biology (Hollmann et al., 1989, 1990; Seeburg, 1993). Conversely, using an antibody directed to the C-terminal part of GluR1, we have obtained intracellular labelling, demonstrating the intracellular location of the epitope(s) (Molnár et al., 1993*a*; present results) and, consequently, of the C-terminal part of

the GluR1 subunit. Similar intracellular labelling was also observed by other groups using antibodies directed to the carboxy terminus of GluR1 (Martin et al., 1992, 1993; Craig et al., 1993) or GluR2/3 and GluR4 subunits (Petralia and Wenthold, 1992).

The antibody against residues 253–267 reacted freely with the GluR1 subunit in both native and disrupted membranes, confirming the extracellular location of the N-terminal domain. In contrast, antibody binding to the epitope contained in the region 877–889 increased about 1.6-fold after permeabilization of vesicles by hypotonic medium. These data suggest that a significant portion of the epitopes is hidden in native synaptosomes, which is in agreement with our electron microscopic findings of a significant proportion of closed postsynaptic membranes in isotonic medium during the incubation with antibodies. In other studies of the postsynaptic membrane sac in similar preparations (Verheul et al., 1993), it was estimated that about 24% of the vesicles are closed. Further experimental data for an intracellular C terminus come from immunolabelling performed on cultured hippocampal neurons with a similar antiserum that labels only permeabilized cells, supporting our findings (Craig et al., 1993).

The intracellular immunoreactivity obtained with antibody to the C-terminal peptide (residues 877–889) suggests an intracellular position of this part of the protein. One possible explanation to reconcile these results is that the C-terminal domain crosses the membrane via a fifth transmembrane region or, alternatively, the fourth transmembrane domain may not cross the membrane, leaving the C terminus on the intracellular side of the membrane. In the study of Unwin (1993*a,b*) on the nicotinic receptor by cryoelectron microscopy, which used helical diffraction methods on tubular crystals of postsynaptic membranes, the secondary structure of this membrane protein was resolved. In this study, only one membrane-spanning α-helix (presumed to be TM2) was found per subunit. The other three membrane-spanning regions of each subunit were found to be β-pleated sheets, which contrasts with current ideas that transmembrane segments are likely to be α-helices.

Acknowledgment: We wish to thank Saul A. Richmond and Dr. Adam L. Smith for supplying the human hippocampal and ferret brain membrane fractions. The authors are grateful to Miss Diana Latawiec and Mr. J. David B. Roberts for technical assistance and to Mr. Frank Kennedy and Mr. Paul Jays for photographic assistance. Agnès Baude was supported by a Commission of European Communities fellowship (ERB CHB ICT 920036). Zoltán Nusser was partly supported by a grant from the Merck Sharp and Dohme Research Laboratories.

REFERENCES

Bahouth S. W., Wang H., and Malbon C. C. (1991) Immunological approaches for probing receptor structure and function. *Trends Pharmacol. Sci.* **12,** 338–343.

Barnes J. M. and Henley J. M. (1992) Molecular characteristics of excitatory amino acid receptors. *Prog. Neurobiol.* **39,** 113–133.

Baude A., Sequier J.-M., McKernan R. M., Olivier K. R., and Somogyi P. (1992) Differential subcellular distribution of the α6 subunit versus the α1 and β2/3 subunits of the $GABA_A$/benzodiazepine receptor complex in granule cells of the cerebellar cortex. *Neuroscience* **51,** 739–748.

Baude A., Nusser Z., Roberts J. D. B., Mulvihill E., McIlhinney R. A. J., and Somogyi P. (1993) The metabotropic glutamate receptor (mGluR1α) is concentrated at perisynaptic membrane of neuronal sub-populations as detected by immunogold reaction. *Neuron* **11,** 771–787.

Blackstone C. D., Levey A. I., Martin L. J., Price D. L., and Huganir R. L. (1992*a*) Immunological detection of glutamate receptor subtypes in human central nervous system. *Ann. Neurol.* **31,** 680–683.

Blackstone C. D., Moss S. J., Martin L. J., Levey A. I., Price D. L., and Huganir R. L. (1992*b*) Biochemical characterization and localization of a non-*N*-methyl-D-aspartate glutamate receptor in rat brain. *J. Neurochem.* **58,** 1118–1126.

Boulter J., Hollmann M., O'Shea-Greenfield A., Hartley M., Deneris E., Maron C., and Heinemann S. (1990) Molecular cloning and functional expression of glutamate receptor subunit genes. *Science* **249,** 1033–1037.

Bramham C. R., Torp R., Zhang N., Storm-Mathisen J., and Ottersen O. P. (1990) Distribution of glutamate-like immunoreactivity in excitatory hippocampal pathways: a semiquantitative electron microscopic study in rats. *Neuroscience* **39,** 405–417.

Buckmaster P. S., Strowbridge B. W., Kunkel D. D., Schmiege D. L., and Schwartzkroin P. A. (1992) Mossy cell axonal projections to the dentate gyrus molecular layer in the rat hippocampal slice. *Hippocampus* **2,** 349–362.

Craig A. M., Blackstone C. D., Huganir R. L., and Banker G. (1993) The distribution of glutamate receptors in cultured rat hippocampal neurons—postsynaptic clustering of AMPA-selective subunits. *Neuron* **10,** 1055–1068.

Errington M. L., Lynch M. A., and Bliss T. V. P. (1987) Long-term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D(−)aminophosphonovalerate. *Neuroscience* **20,** 279–284.

Ewert M., Shivers B. D., Luddens H., Möhler H., and Seeburg P. H. (1990) Subunit selectivity and epitope characterization of mAbs directed against the $GABA_A$/benzodiazepine receptor. *J. Cell Biol.* **110,** 2043–2048.

Gasic G. P. and Hollmann M. (1992) Molecular neurobiology of glutamate receptors. *Annu. Rev. Physiol.* **54,** 507–536.

Grandes P. and Streit P. (1991) Effect of perforant path lesion on pattern of glutamate-like immunoreactivity in rat dentate gyrus. *Neuroscience* **41,** 391–400.

Gray E. G. (1959) Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.* **93,** 420–433.

Hajós F. (1975) An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.* **93,** 485–489.

Hollmann M., O'Shea-Greenfield A., Rogers S. W., and Heinemann S. (1989) Cloning by functional expression of a member of the glutamate receptor family. *Nature* **342,** 643–648.

Hollmann M., Rogers S. W., O'Shea-Greenfield A., Deneris E. S., Hughes T. E., Gasic G. P., and Heinemann S. (1990) Glutamate receptor GluR1: structure, function, and expression in the brain. *Cold Spring Harbour Symp. Quant. Biol.* **55,** 41–55.

Hunter C., Wheaton K. D., and Wenthold R. J. (1990) Solubilization and partial purification of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid binding sites from rat brain. *J. Neurochem.* **54,** 118–125.

Keinänen K., Wisden W., Sommer B., Werner P., Herb A., Verdoorn T. A., Sakmann B., and Seeburg P. H. (1990) A family of AMPA-selective glutamate receptors. *Science* **249,** 556–560.

Keller B. U., Konnerth A., and Yaari Y. (1991) Patch-clamp analysis of excitatory synaptic currents in granule cells of rat hippocampus. *J. Physiol. (Lond.)* **435,** 275–293.

Köhler M., Burnashev N., Sakmann B., and Seeburg P. H. (1993)

Determination of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. *Neuron* **10,** 491–500.

Lambert J. D. C. and Jones R. S. G. (1990) A reevaluation of excitatory amino acid-mediated synaptic transmission in rat dentate gyrus. *J. Neurophysiol.* **64,** 119–132.

Leranth C., Nitsch R., Deller T., and Frotscher M. (1992) Synaptic connections of seizure-sensitive neurons in the dentate gyrus, in *The Dentate Gyrus and Its Role in Seizures* (*Epilepsy Res.* Suppl. 7) (Ribak C. E., Gall C. M., and Mody I., eds), pp. 49–64. Elsevier Science Publishers, Amsterdam.

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Martin L. J., Blackstone C. D., Huganir R. L., and Price D. L. (1992) Cellular localization of a metabotropic glutamate receptor in rat brain. *Neuron* **9,** 259–270.

Martin L. J., Blackstone C. D., Levey A. I., Huganir R. L., and Price D. L. (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. *Neuroscience* **53,** 327–358.

McGlade-McCulloh E., Yamamoto H., Tan S.-E., Brickey D. A., and Soderling T. R. (1993) Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. *Nature* **362,** 640–642.

Molnár E., Seidler N. W., Jóna I., and Martonosi A. N. (1990) The binding of monoclonal and polyclonal antibodies to the Ca^{2+}-ATPase of sarcoplasmic reticulum: effects on interactions between ATPase molecules. *Biochim. Biophys. Acta* **1023,** 147–167.

Molnár E., Baude A., Richmond S. A., Patel P. B., Somogyi P., and McIlhinney R. A. J. (1993*a*). Biochemical and immunocytochemical characterization of antipeptide antibodies to a cloned GluR1 glutamate receptor subunit: cellular and subcellular distribution in the rat forebrain. *Neuroscience* **53,** 307–326.

Molnár E., Baude A., McIlhinney R. A. J., and Somogyi P. (1993*b*). Use of anti-peptide antibodies to study the transmembrane topography of the GluR1 glutamate receptor subunit. *Brain Res. Assoc. Abstr.* **10,** 12.

Monaghan D. T., Yao D., and Cotman C. W. (1984) Distribution of [^{3}H]AMPA binding sites in rat brain as determined by quantitative autoradiography. *Brain Res.* **324,** 160–164.

Monaghan D. T., Bridges R. J., and Cotman C. W. (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.* **29,** 365–402.

Moss J. S., Blackstone C. D., and Huganir R. L. (1993) Phosphorylation of recombinant non-NMDA glutamate receptors on serine and tyrosine residues. *Neurochem. Res.* **18,** 105–110.

Mrzljak L., Levey A. I., and Goldman-Rakic P. S. (1993) Association of m1 and m2 muscarinic receptor proteins with asymmetric synapses in the primate cerebral cortex—morphological evidence for cholinergic modulation of excitatory neurotransmission. *Proc. Natl. Acad. Sci. USA* **90,** 5194–5198.

Nakanishi S. (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* **258,** 597–603.

O'Hara P. J., Sheppard P. O., Thøgersen H., Venezia D., Haldeman B. A., McGrane V., Houamed K. M., Thomsen C., Gilbert T. L., and Mulvihill E. R. (1993) The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron* **11,** 41–52.

Olsen R. W., Szamraj O., and Houser C. R. (1987) [^{3}H]AMPA binding to glutamate receptor subpopulations in rat brain. *Brain Res.* **402,** 243–254.

Petralia R. S. and Wenthold R. J. (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J. Comp. Neurol.* **318,** 329–354.

Puckett C., Gomez C. M., Korenberg J. R., Tung H., Meier T. J., Chen X. N., and Hood L. (1991) Molecular cloning and chromosomal localization of one of the human glutamate receptor genes. *Proc. Natl. Acad. Sci. USA* **88,** 7557–7561.

Raymond L. A., Blackstone C. D., and Huganir R. L. (1993) Phosphorylation and modulation of recombinant GluR6 glutamate receptors by cAMP-dependent protein kinase. *Nature* **361,** 637–641.

Sargent P. B. and Pang D. Z. (1989) Acetylcholine receptor-like molecules are found in both synaptic and extrasynaptic clusters on the surface of neurons in the frog cardiac ganglion. *J. Neurosci.* **9,** 1062–1072.

Schroder S., Hoch W., Becker C.-M., Grenningloh G., and Betz H. (1991) Mapping of antigenic epitopes on the α1 subunit of the inhibitory glycine receptor. *Biochemistry* **30,** 42–47.

Seeburg P. H. (1993) The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci.* **16,** 359–365.

Sommer B. and Seeburg P. (1992) Glutamate receptor channels: novel properties and new clones. *Trends Pharmacol. Sci.* **13,** 291–296.

Sommer B., Keinänen K., Verdoorn T. A., Wisden W., Burnashev N., Herb A., Kohler M., Takagi T., Sakmann B., and Seeburg P. H. (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* **249,** 1580–1585.

Somogyi P. and Takagi H. (1982) A note on the use of picric acid-paraformaldehyde-glutaraldehyde fixative for correlated light and electron microscopic immunocytochemistry. *Neuroscience* **7,** 1779–1783.

Somogyi P., Takagi H., Richards J. G., and Möhler H. (1989) Subcellular localization of benzodiazepine/$GABA_A$ receptors in the cerebellum of rat, cat, and monkey using monoclonal antibodies. *J. Neurosci.* **9,** 2197–2209.

Sprengel R. and Seeburg P. H. (1993) The unique properties of glutamate receptor channels. *FEBS Lett.* **325,** 90–94.

Staley K. J. and Mody I. (1992) Shunting of excitatory input to dentate gyrus granule cells by a depolarizing $GABA_A$ receptor-mediated postsynaptic conductance. *J. Neurophysiol.* **68,** 197–212.

Tingley W. G., Roche K. W., Thompson A. K., and Huganir R. L. (1993) Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. *Nature* **364,** 70–73.

Traxler B., Boyd D., and Beckwith J. (1993) The topological analysis of integral cytoplasmic membrane proteins. *J. Membr. Biol.* **132,** 1–11.

Triller A., Cluzeaud F., Pfeiffer F., Betz H., and Korn H. (1985) Distribution of glycine receptors at central synapses: an immunoelectron microscopy study. *J. Cell Biol.* **101,** 683–688.

Unwin N. (1993*a*) The nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.* **229,** 1101–1124.

Unwin N. (1993*b*) Neurotransmitter action: opening of ligand-gated ion channel. *Cell* **10,** 31–41.

Verheul H. B., de Leeuw F.-E., Scholten G., Tulleken C. A., Lopes da Silva F. H., and Ghijsen W. E. J. M. (1993) $GABA_A$ receptor function in the early period after transient forebrain ischaemia in the rat. *Eur. J. Neurosci.* **5,** 955–960.

Wang H., Lipfert L., Malbon C. C., and Bahouth S. (1989) Site-directed anti-peptide antibodies define the topography of the β-adrenergic receptor. *J. Biol. Chem.* **264,** 14424–14431.

Wisden W. and Seeburg P. H. (1993) Mammalian ionotropic glutamate receptors. *Curr. Opin. Neurobiol.* **3,** 291–298.

Wright M. S., Sefland I., and Walaas S. I. (1993) Cloning of the long intracellular loop of the AMPA-selective glutamate receptor for phosphorylation studies. *J. Recept. Res.* **13,** 653–665.