Pergamon

0306-4522(95)00350-9

Neuroscience Vol. 69, No. 4, pp. 1031–1055, 1995
Elsevier Science Ltd

0306-4522/95 $9.50 + 0.00

HIGH-RESOLUTION IMMUNOGOLD LOCALIZATION OF AMPA TYPE GLUTAMATE RECEPTOR SUBUNITS AT SYNAPTIC AND NON-SYNAPTIC SITES IN RAT HIPPOCAMPUS

A. BAUDE,*†‡ Z. NUSSER,* E. MOLNÁR,* R. A. J. McILHINNEY* and P. SOMOGYI*§

*Medical Research Council, Anatomical Neuropharmacology Unit, Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3TH, U.K.

†CNRS, Laboratoire de Neurobiologie, 31 chemin Joseph Aiguier, 13402 Marseille Cx20, France

Abstract—The cellular and subcellular localization of the GluRA, GluRB/C and GluRD subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) type glutamate receptor was determined in the rat hippocampus using polyclonal antipeptide antibodies in immunoperoxidase and immunogold procedures. For the localization of the GluRD subunit a new polyclonal antiserum was developed using the C-terminal sequence of the protein (residues 869–881), conjugated to carrier protein and adsorbed to colloidal gold for immunization. The purified antibodies immunoprecipitated about 25% of ^{3}[H]AMPA binding activity from the hippocampus, cerebellum or whole brain, but very little from neocortex. These antibodies did not precipitate a significant amount of ^{3}[H]kainate binding activity. The antibodies also recognize the GluRD subunit, but not the other AMPA receptor subunits, when expressed in transfected COS-7 cells and only when permeabilized with detergent, indicating an intracellular epitope.

All subunits were enriched in the neuropil of the dendritic layers of the hippocampus and in the molecular layer of the dentate gyrus. The cellular distribution of the GluRD subunit was studied more extensively. The strata radiatum, oriens and the dentate molecular layer were more strongly immunoreactive than the stratum lacunosum moleculare, the stratum lucidum and the hilus. However, in the stratum lucidum of the CA3 area and in the hilus the weakly reacting dendrites were surrounded by immunopositive rosettes, shown in subsequent electron microscopic studies to correspond to complex dendritic spines. In the stratum radiatum, the weakly reacting apical dendrites contrasted with the surrounding intensely stained neuropil. The cell bodies of pyramidal and granule cells were moderately reactive. Some non-principal cells and their dendrites in the pyramidal cell layer and in the alveus also reacted very strongly for the GluRD subunit.

At the subcellular level, silver intensified immunogold particles for the GluRA, GluRB/C and GluRD subunits were present at type 1 synaptic membrane specializations on dendritic spines of pyramidal cells throughout all layers of the CA1 and CA3 areas. The most densely labelled synapses tended to be on the largest spines and many smaller spines remained unlabelled. Immunoparticle density at type 1 synapses on dendritic shafts of some non-principal cells was consistently higher than at labelled synapses of dendritic spines of pyramidal cells. Synapses established between dendritic spines and mossy fibre terminals, were immunoreactive for all studied subunits in stratum lucidum of the CA3 area. The postembedding immunogold method revealed that the AMPA type receptors are concentrated within the main body of the anatomically defined type 1 (asymmetrical) synaptic junction. Often only a part of the membrane specialization showed clustered immunoparticles. There was a sharp decrease in immunoreactive receptor density at the edge of the synaptic specialization. Immunolabelling was consistently demonstrated at extrasynaptic sites on dendrites, dendritic spines and somata.

The results demonstrate that the GluRA, B/C and D subunits of the AMPA type glutamate receptor are present in many of the glutamatergic synapses formed by the entorhinal, CA3 pyramidal and mossy fibre terminals. Some interneurons have a higher density of AMPA type receptors in their asymmetrical afferent synapses than pyramidal cells. This may contribute to a lower activation threshold of interneurons as compared to principal cells by the same afferents in the hippocampal formation.

Key words: neuron, synapse, excitation, ion channel, immunocytochemistry.

‡Present address: CNRS, Laboratoire de Neurobiologie, 31 chemin Joseph Aiguier, 13402 Marseille Cx20, France.

§To whom correspondence should be addressed.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; BSA, bovine serum albumin; CaMKII, calcium calmodulin-dependent protein kinase II; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; ELISA, enzyme-linked immunosorbent assay; GluRA, GluRB, GluRC, GluRD, subunits of the AMPA type glutamate receptor; mEPSCs, miniature excitatory postsynaptic currents; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

Glutamate produces fast excitation in the CNS through ligand-gated ion channels which are differentiated by binding with high-affinity either α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) or kainate or *N*-methyl-D-aspartate (NMDA, for review see Refs 46, 102). The AMPA-activated channels consist of several transmembrane subunits, which have been named GluR1-4[18,47] or GluRA-D.[57,109] Electrophysiological evidence from *in vitro* co-expression experiments of different subunits strongly suggest that native AMPA type receptors are hetero-oligomeric complexes.[21,45,57,82,102] There are indications that functional diversity of ionotropic AMPA type receptors in the CNS is generated by combining different subunits into heteromeric receptors.[27,43,45,63,70,124,126] However, the subunit composition, localization, and the relative density of glutamate receptors at the postsynaptic membrane of specific cell types, particularly in relation to the different glutamatergic afferents is still undefined.

Glutamate is the major excitatory neurotransmitter in the hippocampal network (for review see Refs 64, 87, 101). The glutamatergic inputs to pyramidal cells of the CA3 and CA1 areas and to granule cells of the dentate gyrus are well characterized physiologically and are easily identifiable due to their segregated laminar distribution on the dendritic trees.[19,34,62,87,95] Therefore, the hippocampus provides a good opportunity to study glutamate receptor distribution in relation to specific synaptic inputs to a relatively homogeneous population of cells. Pyramidal and granule cells express mRNA for all four subunits of the AMPA type receptor.[18,57,100,109] Non-principal cells have also been shown to express mRNAs for several subunits,[100,109] but because they are heterogeneous the co-expression of particular subunits in specific subpopulations remains to be determined. Immunohistochemical studies showed that the GluRA and GluRB/C subunits are present in all layers of the hippocampus, mainly associated with layers that contain the dendritic arborization of principal cells.[14,68,75,76,90,98] These studies employed immunoperoxidase methods which reveal immunoreactive sites using a diffusible marker with high sensitivity but with limited spatial resolution. The diffusible reaction product was found both at synaptic and extrasynaptic sites, confirming results from electrophysiological studies which demonstrated glutamate gated channels activated by synaptic inputs[10,44,52,70,94,118,125] or by agonist application to non-synpatic membrane.[27,54,63,114] However, the precise distribution of the receptors, in relation to the presynaptic release sites and to the anatomically defined synaptic membrane specialization, remains to be determined.

Recently, using high-resolution immunoparticle markers we demonstrated that the 1α form of the metabotropic glutamate receptor is concentrated at the edge of postsynaptic densities of GABA/somatostatin-containing interneurons in hippocampus and of Purkinje cells in cerebellum[8] whereas the AMPA type ionotropic glutamate receptor was found to be concentrated in the main body of the postsynaptic membrane specialization in synapses of Purkinje and granule cells.[84] Thus, the postsynaptic membrane seems to be parcelled according to the type of glutamate receptors. In order to determine the precise location of glutamate receptors on hippocampal cells we re-examined the distribution of the AMPA type receptor subunits in relation to specific afferents and postsynaptic cells. Immunoperoxidase and both pre- and postembedding immunogold methods have been compared for the electron-microscopic localization of receptors. The limitations of each technique have been critically evaluated, and it is shown that the three methods provide complementary information and are best used in combination for defining the precise distribution of receptors. Preliminary accounts of these results have been published in abstract form.[7,83]

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]AMPA (46.1 Ci/mmol, DL-α-[5-methyl-^{3}H]-;) and (vinilidene-[^{3}H])-kainic acid (58.0 Ci/mmol) were purchased from Du Pont-New England Nuclear, Boston, Massachusetts, U.S.A.; AMPA was obtained from Tocris Neuramin, Bristol, U.K.; Triton X-100, protein A-sepharose CL 4B, porcine thyroglobulin, adjuvant peptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine), Freund's complete and incomplete adjuvant, gold chloride, kainic acid, nitroblue tetrazolium, high molecular weight markers for electrophoresis and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., Dorset, U.K.; Affi-Gel 15 came from Bio-Rad Laboratories, Richmond, CA, U.S.A.; lyophilized human γ-globulin was supplied by Calbiochem, La Jolla, CA, U.S.A.; polyethylene glycol 6000 and 6-amino-*n*-hexonoic 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 were from Dakopatts, Denmark. For solid-phase peptide synthesis, all reagents were purchased from Novabiochem, Nottingham, U.K.; 5-brom-4-chlor-3-indolyl-phosphate toluidine salt came from Boehringer Mannheim Gmbh, Germany.

Antibodies

The production, purification, characterization and immunocytochemical application of anti-GluRA (253–267) antibodies (code GluRA-N-12) were described previously by Molnár *et al.*[75,76] and Baude *et al.*[6] These antibodies recognize an extracellular domain of the receptor. Affinity-purified antibodies recognize a single band in immunoblots of rat brain membranes of 105,000 mol. wt, and they precipitate AMPA binding.[75] In this study subunit specificity of GluRA-N-14 was tested on transfected COS-7 cells expressing one of the subunits of the AMPA receptor. As shown below only the GluRA subunit was recognized by these antibodies.

Polyclonal antibodies to a C-terminal peptide (850–862) common to the GluRB and GluRC subunits were bought from Chemicon International Inc., U.S.A. The characterization of these antibodies (Ab25) showed that they immunoprecipitate [^{3}H]AMPA binding activity of solubilized membrane samples and labelled a 108,000 mol. wt band in immunoblots from solubilized rat brain membranes.[120] They

also labelled a similar band from COS-7 cells transfected with either GluRB or GluRC cDNA.[120]

A new polyclonal antiserum was raised against C-terminal residues (869–881) of the GluRD subunit of the AMPA type receptor. The preparation of the antigen, the immunization and the characterization of the antibodies from two rabbits (code Ab-GluRD-CT13/7, Ab-GluRD-CT13/8) are described below.

Solid-phase peptide synthesis and peptide–carrier conjugation

Peptide RQSSGLAVIASDL, which corresponds to residues 869–881 of the GluRD glutamate receptor subunit was synthesized as described earlier[75] using fluorenylmethoxycarbonyl chemistry and either pentafluorophenoxy or 3,4-dihydro-4-oxobenzotriazine-3-oxy-activated amino acids. The final yield of pure peptide was routinely between 5 and 8 mg/0.1 g resin. The purity of the final products was assessed by high-performance liquid chromatography and mass spectrometry amino acid analysis.

Synthetic GluRD peptide (4 mg) and 10 mg of carrier protein (thyroglobulin for immunization or bovine serum albumin (BSA) for coating enzyme-linked immunosorbent assay (ELISA) plates and for the preparation of immunoaffinity columns) were dissolved in 1.67 ml phosphate-buffered saline (PBS, pH 7.2) to which 0.33 ml of 5% glutaraldehyde was added slowly with constant agitation. The mixture was allowed to react overnight at 4°C. The reaction was terminated by addition of a molar excess of ammonium chloride (50 μl of 200 mg/ml). The peptide–thyroglobulin conjugate was dialysed either against 1000 ml PBS (four changes overnight at 4°C), or against 0.1 M 4-morpholinepropanesulfonic acid (pH 7.5) when the peptide–BSA conjugate was to be coupled to Affi-Gel 15.

Generation of antisera

Polyclonal antibodies against the GluRD subunit were produced by immunizing three-month-old New Zealand white rabbits (Charles River, U.K. Ltd) with colloidal gold-conjugated immunogens as described by Pow and Crook.[93] Monodisperse colloidal gold spheres (80–100 nm) were produced by reduction of gold chloride with sodium citrate.[9,32]

Gold–immunogen conjugate (0.5 mg of carrier protein and coupled peptide) was injected into the rear marginal ear vein. The initial i.v. injection was accompanied by subcutaneous (s.c.) injections of 0.5 mg of conjugate emulsified in Freund's complete adjuvant. A boosting i.v. injection of the gold-conjugate was administered three weeks later and was accompanied by a s.c. injection of 0.25 mg conjugate in Freund's incomplete adjuvant. Serum antibody titres were monitored seven days after the second and subsequent injections by ELISA using the BSA-conjugated peptide bound to the microtitration plates. Boosts were repeated four additional times at five-weekly intervals.

Immunoaffinity purification of anti-peptide antibodies on antigen columns

Peptide was coupled to BSA with glutaraldehyde as described above, and 10 mg conjugated peptide was immobilized on Affi-Gel 15 (Bio-Rad), following the manufacturer's instructions. After coupling and washing the agar gel beads with the covalently attached antigen, 5 ml of immune serum was added. After 12–16 h incubation at 4°C beads were transferred to a column, washed with 20 bed-volumes of 10 mM Tris-HCl (pH 7.5), and then with 20 bed volumes of 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5) at room temperature. Antibody was eluted by passing 10 × 1 ml of 100 mM glycine-HCl (pH 2.5) through the column. The eluate was collected in Eppendorf tubes containing 100 μl of 1 M Tris-HCl (pH 8.0) and OD_{280} was determined. The antibody containing fractions were combined and dialysed against 1000 ml PBS (four changes overnight at 4°C). The antibody solution was supplemented with 1% BSA and 0.02% sodium azide.

Enzyme-linked immunosorbent assay

Titres of antipeptide antisera were determined using an immunoperoxidase ELISA. Wells of microtitre plates (Falcon 3912 assay plates, Becton Dickinson and Co. Oxnard, CA, U.S.A.) were coated with 1 μg/well of BSA-conjugated peptide (dissolved in 50 μl 13 mM Na_2CO_3, 35 mM $NaHCO_3$ buffer, pH 9.6) by incubation at 4°C overnight. Wells were then washed three times with PBS containing 0.05% Tween 20. After any unoccupied sites of the wells were blocked with 1% BSA in PBS (blocking buffer) for 1 h at room temperature, the wells were washed with PBS containing 0.05% Tween 20. Several dilutions of antiserum (10^{-2}–10^{-6}) in blocking buffer (100 μl) were added to the wells and then the plate was incubated for 2 h at room temperature. After washing, antibodies bound to the immobilized antigen were reacted with 100 μl of 1:1000 dilution of horseradish peroxidase-conjugated swine anti-rabbit IgG in blocking buffer for 2 h at room temperature. The resultant immune-complex was detected by addition of 100 μl of substrate solution; 1 mg/ml *O*-phenylenediamine and 0.003% H_2O_2 in 35 mM citric acid; and 125 mM Na_2HPO_4, (pH 4.0). Colour development was allowed to proceed for 10 min at room temperature and stopped by addition of 50 μl of 4 M H_2SO_4. Absorbance at 492 nm was measured by a Labsystems Multiskan microlitre plate spectrophotometer.

Cell culture and membrane preparation

COS-7 cells were grown in Dulbecco's modified Eagle's medium containing fetal calf serum (10%) supplemented with 100 μg/ml penicillin and streptomycin and 1 mM sodium pyruvate. Cells were transfected with DNA for one of GluRA-D at 0.9–1.5 μg DNA/25 cm^2 flask using DEAE-dextran as described by Seed and Aruffo.[103] The mammalian expression vectors containing the genes coding for the receptor GluRA-D used in this study were the gift of Dr Hannah Monyer (Center for Molecular Biology, University of Heidelberg, Im Neuenheimer Field 282, Heidelberg, Germany) and were prepared for transfection studies using the Wizard Maxiprep plasmid kit (Promega). Cells were harvested for membrane preparation or for immunofluorescence 48 h after transfection. Cell membranes were prepared by shearing cells in hypotonic medium as previously described.[8]

Immunofluorescence labelling of expressed AMPA receptor subunits

Cells for immunofluorescence were cultured on No. 1 glass coverslips (22 × 22 mm) which had been washed with 1 M HCl, followed by ethanol and flame sterilization. All subsequent procedures were performed on the coverslip grown monolayer cells. Following transfection (48 h) with DNA at 0.3–0.5 μg/coverslip culture the coverslips were washed twice with PBS (2 ml). The cells were fixed for 15 min in 4% paraformaldehyde in PBS at room temperature. Cells for permeabilization were then incubated in TBS (0.1 M NaCl, 50 mM Tris-HCl pH 7.2) containing 0.2% Triton X-100 for 5 min at room temperature. All coverslips were washed twice with 2 ml TBS before incubation with TBS containing 1.0% BSA and 5% normal goat serum for 15 min. The coverslips were transferred to a moist chamber and carefully overlaid with the antibody (100 μl). The antibodies were all affinity-purified and were used at a concentration of 1 μg/ml. The incubation was performed at room temperature for 2 h following which the coverslips were washed three times for 5 min at room temperature using 2 ml TBS–BSA before being overlaid for 1 h with affinity-purified fluorescein isothyocyanate labelled goat anti-rabbit IgG (Sigma) at a dilution of 1:50 in TBS–BSA. The coverslips were washed with TBS and mounted for

fluorescence microscopy in 50% glycerol/PBS. The coverslips were sealed with nail varnish and viewed using a Leitz Dialux 20 epifluorescence microscope. Photography was performed using Kodak TMAX 400 film using a standard 45-s exposure time.

Preparation and solubilization of rat brain membranes

Membranes from whole brains or dissected cortical, hippocampal, cerebellar and spinal cord areas of adult Wistar rats (250–300 g) were prepared as described by Zukin *et al.*[127] In addition, the resulting pellets were washed a further three times by resuspension and centrifugation in 50 mM Tris-citrate (pH 7.4) at 4°C for 20 min at 54,000 g_{av}, and the resulting membrane pellet was stored in aliquots at −70°C until use.

Solubilization was performed according to Hunter *et al.*[49] and Hampson *et al.*[40] Membranes were thawed and then washed twice by resuspension and centrifugation in 50 mM Tris-citrate buffer (pH 7.4). The membrane pellet was then resuspended in 0.5 M potassium phosphate (pH 7.0), containing 20% glycerol to give a final protein concentration of 5 mg/ml. Under the conditions employed,[75] 70–74% of the proteins became soluble. Protein concentrations were determined according to Lowry *et al.*[65]

Immunoprecipitation of solubilized AMPA binding subunits

Immunoprecipitation of GluRD receptor subunit was carried out using antibody bound to protein A-Sepharose. Briefly, 400 μl of antiserum was incubated with 400 μl of packed protein-A beads for 3 h at 2°C. After washing four times with 50 mM Tris-citrate (pH 7.4), 10% glycerol, 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, the antibody-protein A-sepharose complex was incubated overnight with 2 ml of Triton X-100 solubilized membrane preparations from rat brain. Beads were removed by centrifugation (1000 g_{av} for 2 min), and the supernatants were used for [^{3}H]AMPA and [^{3}H]kainate binding assay.

Receptor binding assay

[^{3}H]AMPA and [^{3}H]kainate binding was studied using a centrifugation assay. Solubilized rat brain membrane samples (100–200 μg of protein in a final volume of 200–400 μl) were incubated with either 20 nM [^{3}H]AMPA or 10 nM [^{3}H]kainate for 40 min at 0°C. All experiments involving [^{3}H]AMPA were performed with 100 mM potassium thiocyanate present during the incubation. To produce protein aggregation, 50–100 μl of 8 mg/ml γ-globulin and 200–400 μl of 30% polyethylene glycol (6000) dissolved in assay buffer were added to each sample at the end of the incubation period. Following centrifugation at 10,000 *g* for 2 min the supernatants were aspirated and the pellets superficially rinsed with 0.4 ml of ice-cold 15% polyethylene glycol and dissolved in 0.5 ml 1% sodium dodecyl sulphate. Radiolabelled content was assayed by liquid scintillation spectroscopy in 4 ml of Liquiscint (National Diagnostics, Manville, NJ, U.S.A.). Results were expressed as specifically bound [^{3}H]AMPA and [^{3}H]kainate, which equals the difference between the total bound and nonspecifically bound ligand. Nonspecific binding was defined as the binding in the presence of 100 μM AMPA or kainate. All determinations were made in triplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis, electrophoretic transfer of proteins and immunoblot analysis

Samples taken for electrophoresis were diluted with equal volume of 2% sodium dodecyl-sulphate, 50 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol and 0.1% Bromophenol Blue, and incubated for 5 min at 100°C. Electrophoresis, transfer of proteins and immunodetection of subunits was performed as described earlier[75,76] using membrane samples (50–100 μg protein) and affinity-purified antibodies (2–7 μg/ml). The bound antibodies were visualized by the enzyme reaction of alkaline phosphatase conjugated anti-rabbit IgG diluted 1:1000 in blocking solution. The substrate solution contained 0.46 mM nitroblue tetrazolium, 0.2 mM 5-brom-4-chlor-3-indolyl-phosphate toluidine salt, 0.1 M Tris (pH 9.5), 0.1 M NaCl and 0.05 M $MgCl_2$.

Preparation of animals and tissues for immunocytochemistry

Eleven adult female Wistar rats (100–400 g; Charles River, U.K. Ltd) were deeply anaesthetized with sodium pentobarbital (220 mg/kg, i.p.) and perfused through the aorta with saline (1 min) followed by ice-cold fixative (6–9 min) containing 4% paraformaldehyde, 0.025–0.1% glutaraldehyde and approximately 0.2% picric acid,[110] made up in 0.1 M phosphate buffer (PB, pH 7.2–7.4). Nine animals were used for pre-embedding experiments, the two others were used for post embedding experiments. Blocks of tissue were frozen in liquid nitrogen following cryoprotection in sucrose, then thawed in PB. Vibratome sections were cut at 70 μm thickness.

Pre-embedding immunoperoxidase reaction

Floating sections were incubated, as reported,[75,111] first in normal serum diluted in TBS, to reduce non-specific binding of antibodies to the tissue. They were then treated, either with affinity-purified primary antibodies, or with control antibodies overnight at 4°C. Antibodies were used in the following concentrations: GluRA-N-12, 2.5 μg protein/ml; Ab25 for GluRB/C, 4–10 μg protein/ml; Ab-GluRD-CT13/8, 3–7 μg protein/ml. After several washes in TBS the sections were incubated in biotinylated goat anti-rabbit IgG (diluted 1:50, Vector Lab.) in TBS for 2 h, followed by incubation in avidin biotinylated horseradish peroxidase complex (diluted 1:100, Vector Lab.) for 2 h. Peroxidase enzyme reaction was carried out with 3,3′-diaminobenzidine tetrahydrochloride as chromogen and H_2O_2 as substrate. For light microscopy, 0.3% Triton X-100 was added to the TBS throughout the experiment.

Pre-embedding immunogold rections

Sections for both immunogold and immunoperoxidase reactions were incubated together in the blocking and primary antibody solutions and then separated into glass vials. After washing the sections several times, they were incubated in TBS containing anti-rabbit IgG (diluted 1:100), coupled to 1.4-nm gold particles (Nanoprobes Inc., Stony Brook, U.S.A.) and dissolved in 1% normal goat serum. Gold particles were silver enhanced for 10–20 min using the HQ Silver kit (Nanoprobes Inc.). Sections were then routinely processed for electron microscopic examination as described previously.[75]

Postembedding immunogold reaction

After perfusion, blocks of hippocampus were extensively washed in PB and sectioned at 500 μm thickness. The sections were processed for slam freezing, freeze substitution and Lowicryl embedding as previously described.[8,85] Postembedding immunocytochemistry was carried out on ultrathin sections mounted on nickel grids. The grids were incubated for 30 min in blocking solution consisting of 5% fetal calf serum (Sigma), 0.8% bovine serum albumin (Sigma) and 0.1% cold water fish skin gelatine (Sigma) dissolved in PBS. Sections were then transferred to primary antibodies diluted in blocking solution and incubated overnight at room temperature. Antibodies were used in the following concentrations: GluRA-N-12, 5–50 μg protein/ml; Ab25 for GluRB/C, 25–30 μg protein/ml; Ab-GluRD-CT13/8, 15–30 μg protein/ml. After washing, sections were incubated for 90 min in goat anti-rabbit IgG coupled to 1.4-nm gold particles (diluted 1:100) and made up in blocking solution. Sections were washed in PB for 30 min, then put on 2% glutaraldehyde in PB for 5 min, washed in PB again and finally, washed several times in ultra-pure water prior to silver enhancement (4 min) in

the dark with the HQ Silver kit. After several washes in ultra-pure water, sections were contrasted in saturated aqueous uranyl acetate and lead citrate before air drying.

Control incubations

Selective labelling, resembling that obtained with the specific antibodies, could not be detected when the primary antibodies were either omitted or replaced by 5% normal rabbit serum. Antibodies were preincubated for 2 h with the antigen used for immunization, 120 μg/ml for GluRD, 20 μg/ml for GluRA subunit, at a final antibody concentration similar to that used for the reactions. This pretreatment completely abolished immunoreactivity in all methods described above. Using polyclonal antibodies to synapsin,[81] no plasma membrane labelling was observed with our methods. These immunohistochemical controls provide evidence that labelling observed on the plasma membrane is due to the anti-receptor antibodies.

Since Ab-GluRD-CT13/8 labelled a low-molecular-weight band in immunoblots we tried to eliminate this presumed cross-reactivity by incubating the purified antibody (7 μg protein/ml) with fixative treated rat liver sections. These were cut from the liver of an animal perfused for pre-embedding immunocytochemistry. The sections were extensively washed, treated for 1 h with TBS containing 20 mM glycine and 20% normal goat serum, followed by incubation with the anti-receptor antibodies for 2 h at room temperature. The pretreated antibodies were then used for both immunoblotting and pre-embedding immunoreactions as described above. In pre-embedding immunoperoxidase and silver intensified immunogold reaction, the regional, laminar and subcellular pattern of immunoreactivity was similar to that obtained with non-treated antibodies.

RESULTS

Characterization of anti-peptide antibodies

Polyclonal antibodies were raised against synthetic peptides derived from unique amino acid sequences of the N-terminal portion of GluRA (residues 253–267, code Ab-GluRA-N12[75]) and the C-terminal portion of GluRD (residues 869–881, code Ab-GluRD-CT13) subunits. To reduce nonspecific labelling, the antibodies were purified on an affinity column prepared with synthetic peptides.

The specificity of the antibodies was tested by immunoblotting of COS-7 cells transfected with cDNAs for one of the GluRA, GluRB, GluRC or GluRD glutamate receptor subunits. Antibodies Ab-GluRA-N12 and Ab-GluRD-CT13/8 reacted only with the GluRA and GluRD subunits, respectively (Fig. 1). To study the regional distribution of the GluRA and GluRD subunits in the CNS, membranes prepared from rat cerebral cortex, hippocampus and cerebellum were immunoblotted following sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Both antibodies stained a band of about 105,000 mol. wt in all brain regions. The value is slightly higher than those predicted from the deduced amino acid sequence which are 99,769 mol. wt for the GluRA and 101,034 mol. wt for the GluRD subunit.[47,57] The difference may be due to post-translational glycosylation at the possible *N*-glycosylation sites of these subunits. Differences were seen in the intensity of immunostaining obtained from different brain areas; antibody Ab-GluRA-N12 gave stronger reaction in membranes from hippocampus and cerebral cortex than antibody Ab-GluRD-CT13/8 which gave the strongest reaction in the cerebellum (Fig. 1). This antibody also labelled more weakly a lower molecular weight band that does not seem to be related to the GluRD subunit, because it could also be detected in COS cells that were not transfected with cDNA for GluRD. A similar low molecular weight band is also detected[59a] by another antibody to the GluRD subunit.[120] Following preincubation of the antibody with fixative-treated rat liver sections, the labelling of this band was greatly reduced even under conditions which greatly increased the labelling of the band corresponding to the GluRD subunit in cerebellar membranes (Fig. 1). Immunoreactivity with the antibodies was blocked by 10 μg/ml of the appropriate synthetic peptide (not shown).

Immunofluorescence analysis of the location of antibody binding sites

COS-7 cells were transfected with cDNAs for either the GluRA or GluRD subunits and 48 h later the coverslip cultures were fixed. They were incubated with or without 0.2% Triton X-100 detergent used to permeabilize the cells before antibody reaction (Fig. 2). Immunolabelling with Ab-GluRA-N12 raised against the putative extracellular region (residues 253–267) of the GluRA subunit resulted in surface labelling of GluRA transfected COS-7 cells without permeabilization (Fig. 2D). In contrast, almost no labelling was seen on GluRD transfected non-permeabilized COS-7 cells with Ab-GluRD-CT13/8 raised against the C-terminal end of the GluRD subunit (Fig. 2B). After permeabilization of cell membranes by detergent, both Ab-GluRA-N12 (Fig. 2C) and Ab-GluRD-CT13/8 (Fig. 2A) antibodies labelled the plasma membrane and intracellular organelles, presumably the endoplasmic reticulum, revealing a high level of expression in some of the cells. These results suggest that the carboxy terminus of the GluRD subunit is intracellular. Many presumably non-transfected cells remained immunonegative in the transfected cultures. Furthermore, no immunofluorescence signal was detected in permealized non-transfected control cultures, indicating that the lower molecular weight protein, reacting weakly with the antibodies and present in immunoblots of both transfected and non-transfected cultures, does not contribute to the immunofluorescence signal.

Immunoprecipitation of the GluRD subunit

The anti-peptide antibodies Ab-GluRD-CT13/7 and Ab-GluRD-CT13/8, attached to protein A-agarose, immunoprecipitated [^{3}H]AMPA binding sites from membranes of whole rat brain, cerebral cortex, hippocampus and cerebellum which had been solubilized under conditions shown to yield soluble binding sites for both AMPA and kainate.[75]

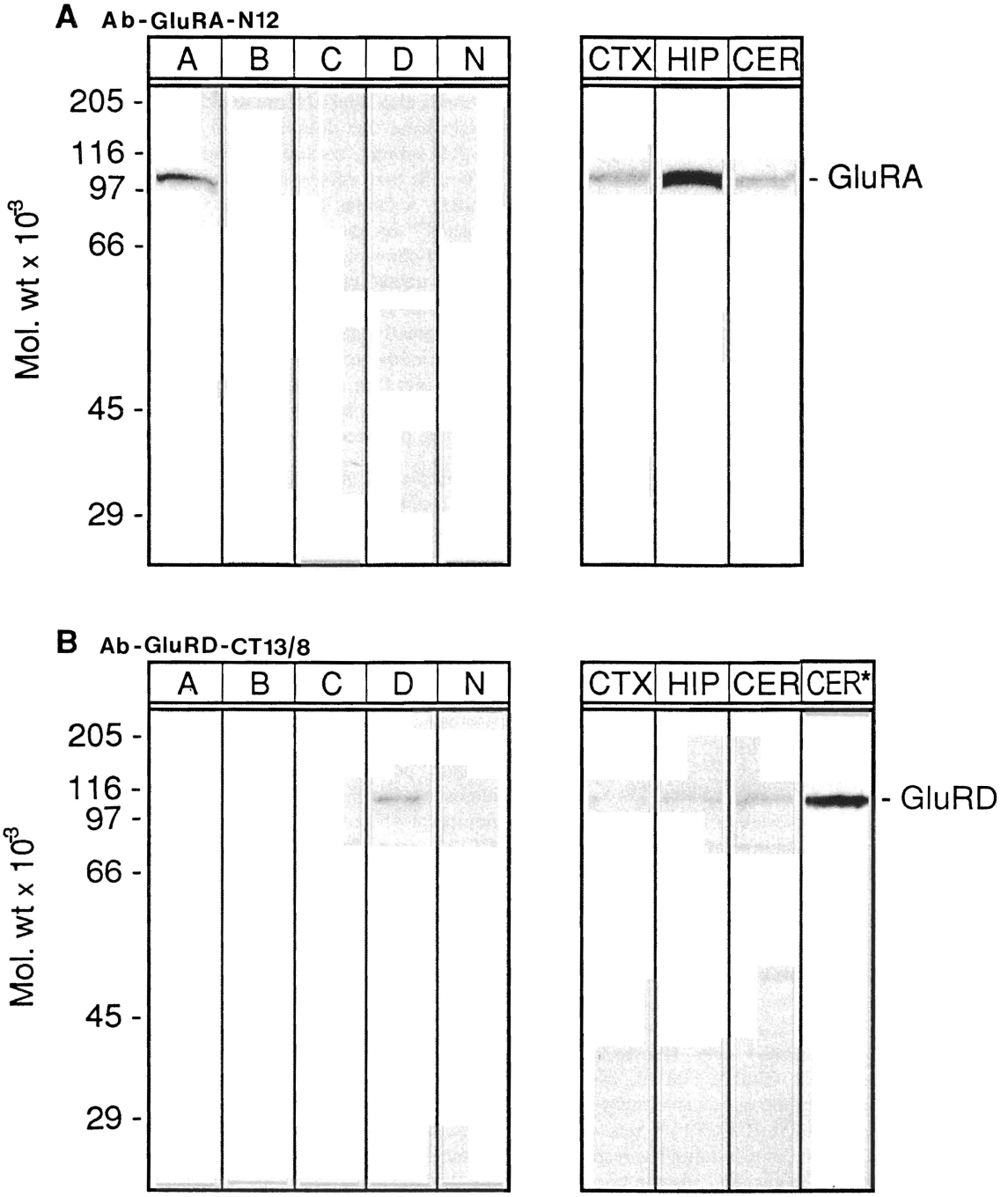

Fig. 1. Immunoblotting of proteins from membranes of COS-7 cells, transfected with cDNAs for GluRA (lane A), GluRB (lane B), GluRC (lane C), GluRD (lane D) subunits of glutamate receptor, and from membranes of rat brain. (A) Ab-GluRA-N12 (253–267) selectively labels a band that corresponds to the GluRA subunit of around 105,000 mol. wt. (B) Ab-GluRD-CT13/8 (869–881) selectively labels a similar band that corresponds to the GluRD subunit. This antibody also gives a variable reaction with a lower molecular weight band that is not related to GluRD. Lane N shows lack of labelling of non-transfected control cell membrane proteins. Crude membrane samples (50 μg protein/lane, except lane CER*) prepared from cerebral cortex (CTX), hippocampus (HIP) and cerebellum (CER) were used. For lane CER*, 100 μg cerebellar protein was applied and Ab-GluRD-CT13/8 (7 μg protein/ml) was incubated with fixative treated liver sections before applying it to the blot. The samples were subjected to electrophoresis on 9% sodium dodecyl sulphate-polyacrylamide gels and blotted on to polyvinylidene difluoride membrane. Affinity-purified primary antibodies were used at 2 μg protein/ml (except for lane CER*), and the bound antibodies were detected by reaction with alkaline phosphatase-conjugated anti-rabbit IgG. The blot in lane CER* was developed longer than the other lanes in the substrate solution. Positions of molecular mass standards (mol. wt $\times 10^{-3}$) are indicated on the left.

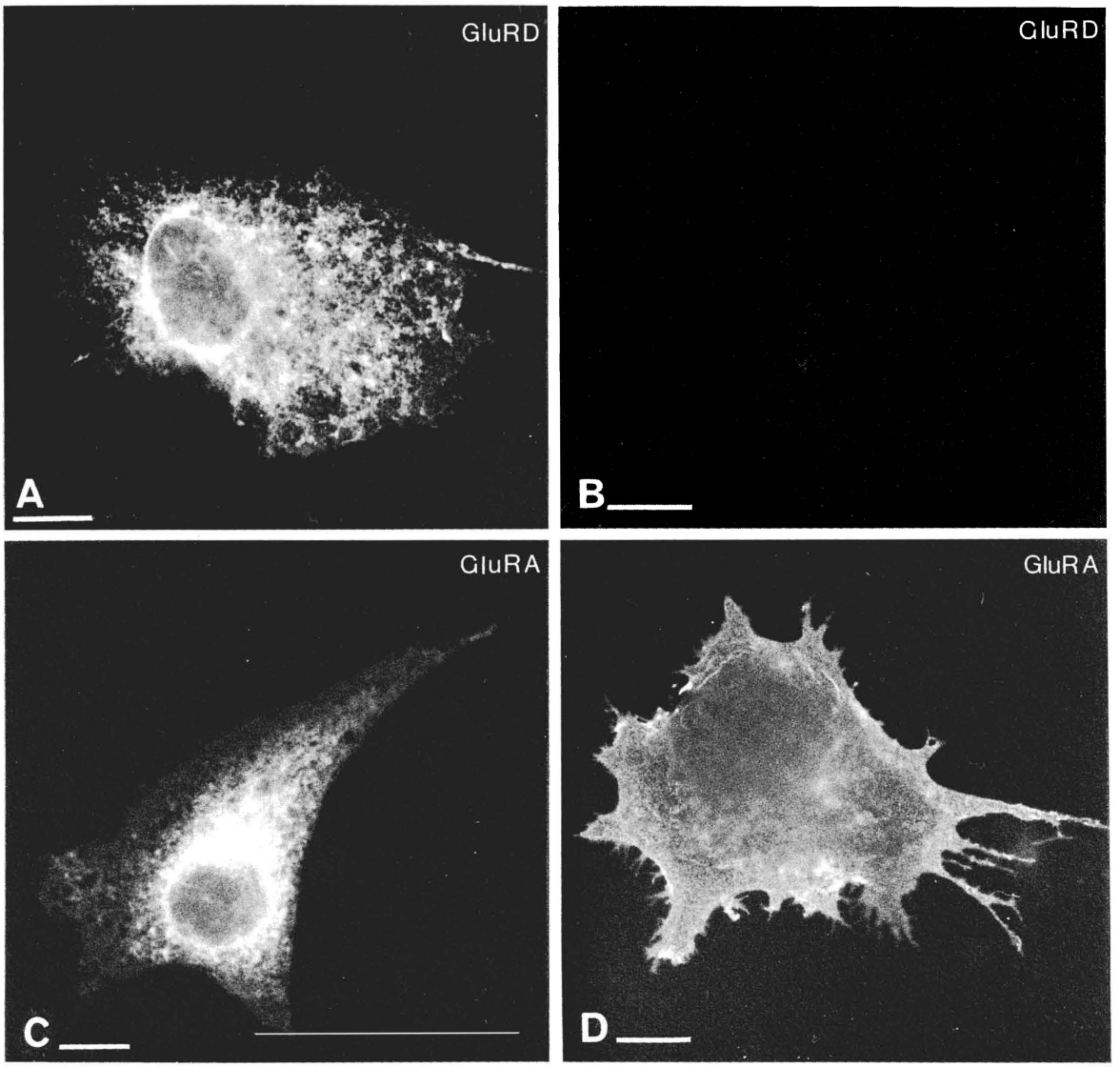

Fig. 2. Immunofluorescence labelling of COS-7 cells transfected with cDNAs for either GluRD (A, B) or GluRA (C, D) glutamate receptor subunits two days before reacting with antibodies Ab-GluRD-CT13/8 (A, B) or Ab-GluRA-N12 (C, D), respectively. Immunofluorescence labelling of the GluRA subunit in fixed cells with the amino-terminal domain antibody Ab-GluRA-N12 shows strong surface labelling (D), but no surface labelling is seen with the antibody raised against the C-terminal end of the GluRD subunit in cells expressing the GluRD subunit (B). After cells were permeabilized with detergent both antibodies labelled structures throughout the cytoplasm (A, C), revealing a high level of expression. Scale bars = 20 μm (A, B, D) and 10 μm (C).

Antibodies raised in rabbits 7 and 8, immunoprecipitated $26 \pm 9\%$ and $25 \pm 9\%$ of the total AMPA binding activity of solubilized whole rat brain membranes respectively (Table 1). Ab-GluRD-CT13/8 removed similar proportions of the AMPA binding activity from samples of hippocampus ($25 \pm 5\%$) and cerebellum ($26 \pm 9\%$), but very little if any, from the cerebral cortex. The [^{3}H]kainate binding activity of brain samples was not changed by either antibody (Table 1).

Terminology and identification of structures in brain sections

Many of the conclusions drawn in this study depend on the correct identification of neuronal elements in different hippocampal layers, and so the criteria and limitations in the predictability of the origin of postsynaptic elements will be summarized briefly. The pyramidal, granule and hilar mossy cells of the hippocampus, collectively called *principal cells*,

Table 1. Selective immunoprecipitation of [^{3}H]AMPA binding activity with GluRD subunit-specific antibodies (Ab-GluRD-CT13)

			[^{3}H]AMPA binding			
Epitope of Ab (residues)	Immunized rabbit	Source of solubilized receptor	Control (pmol/mg)	Not bound (pmol/mg)	Yield (%)	%[^{3}H]AMPA binding immunoprecipitated
GluRD (869-881)	7	Whole brain	1.26 ± 0.10	0.93 ± 0.10	74 ± 9	26 ± 9
	8	Whole brain	1.17 ± 0.06	0.88 ± 0.11	75 ± 9	25 ± 9
	8	Cortex	1.93 ± 0.13	1.80 ± 0.09	93 ± 6	7 ± 6
	8	Hippocampus	2.16 ± 0.08	1.63 ± 0.09	75 ± 5	25 ± 5
	8	Cerebellum	0.89 ± 0.10	0.66 ± 0.05	74 ± 9	26 ± 9
			[^{3}H]kainate binding			
Epitope of Ab (residues)	Immunized rabbit	Source of solubilized receptor	Control (pmol/mg)	Not bound (pmol/mg)	Yield (%)	%[^{3}H]AMPA binding immunoprecipitated
GluRD (869-881)	7	Whole brain	133 ± 3.5	128 ± 3.1	96 ± 3	4 ± 3
	8	Whole brain	104 ± 6.5	101 ± 8.8	97 ± 7	3 ± 7

Immunoprecipitation of ligand binding activity was measured with [^{3}H]AMPA (20 nM) in the presence of 100 mM potassium isothiocyanate, or with [^{3}H]kainate (10 nM). Receptors were solubilized from membranes with 1% Triton X-100 and 0.2% digitonin. The solubilized samples were incubated overnight with protein-A bound antibody beads and the percentage of ligand binding activity remaining in the supernatant (Yield %) was determined. The proportion of ligand binding specifically immunoprecipitated was calculated by subtracting the yield value from the total binding activity (100%). The immunoprecipitation removed a significant proportion of the total [^{3}H]AMPA binding activity, but there was no significant change in the [^{3}H]kainate binding activity of the same samples. All determinations of binding were performed in triplicate and the values are the mean ± S.D. from three independent determinations.

are all thought to use glutamate as neurotransmitter. Throughout this paper the term *non-principal cell* will be used to include all other cell types some of which may have hippocampofugal axons, although they are often called *interneurons*. The non-principal cells can be divided into many subclasses according to their synaptic connections, neurochemistry and the position of their cell bodies. In the present report no attempt was made to differentiate between the cell types. The dentate *hilus*, in addition to mossy cells, contains many non-principal cell bodies, and dendrites of different origins. The cell bodies of mossy cells are not easily distinguishable from pyramidal cells at the hilar end of the CA3c area. However, the two dendritic layers of the CA3c area, surrounding the cell bodies of pyramidal cells, can be distinguished from the hilus in immunostained material. It appears that the dendrites of mossy and CA3c pyramidal cells remain segregated and differ in their $GABA_A$[85] as well as glutamate[75] receptor immunoreactivity.

Light microscopic distribution of immunoperoxidase labelling for subunits of the AMPA receptor

The distribution of immunolabelling from this series of animals is in agreement with previous data describing immunoreactivity for the GluRA subunit using the same antibodies.[75] Immunoreactivity for the GluRA subunit was more prominent in strata oriens and radiatum of the CA1 area, in stratum lacunosum moleculare of CA1 and CA3 areas and in the molecular layer of the dentate gyrus, than in other layers of the CA3 area, with the stratum lucidum showing the weakest labelling.[75,98] Immunolabelling for the GluRB/C subunits resembled the earlier results reported with the same antibody[90] and using different antibodies,[68] but some differences were apparent. In our preparations, immunoreactivity for the GluRB/C subunits was more uniformly distributed in the neuropil throughout the hippocampus, with the exception of the hilus, than reported previously.[68,90] The difference between CA1 and CA3 regions seen for the GluRA subunit was not present for the GluRB/C subunits. The neuropil of the hilus was weakly labelled. Clear difference was seen in the immunoreactivity of the different principal cell bodies and proximal dendrites, the labelling decreasing in the order as follows, CA3 pyramid = mossy cell > CA1 pyramid > granule cell. Hilar mossy cells were identified on the basis of their angular shape, density and location.[1] Immunoreactive non-principal cells were

Fig. 3. Light micrographs of immunoreactivity for the GluRD subunit of the AMPA type glutamate receptor in the hippocampus. (A, B) Immunoreactivity is present in all layers, being most intense in stratum oriens (so) and radiatum (sr) of the CA1 area and the molecular layer (ml) of the dentate gyrus. The hilus (h), the stratum lucidum (sl) and the stratum lacunosum moleculare (slm) are less reactive. The cell bodies of pyramidal and granule cells are moderately reactive. Some non-principal cells (arrows in B) and their dendrites (double arrow) in stratum pyramidale (sp) and in the alveus (alv) react very strongly for this subunit. (C) At higher magnification of the stratum radiatum (CA1) the weakly reacting apical dendrites (arrows) are surrounded by punctate labelling in the neuropil. (D) In the stratum lucidum (sl) of the CA3 area, the weakly reacting dendrites and pyramidal cells (p) are surrounded by strongly reacting large rosettes (e.g., arrowheads), whereas the stratum radiatum (sr) shows small punctate immunostaining. (E) A large hilar neuron is surrounded by stained rosettes (arrowheads) at the origin of its main dendrites. c, capillaries; gcl, granule cell layer. Scale bars = 200 μm (A), 50 μm (B), 10 μm (C–E).

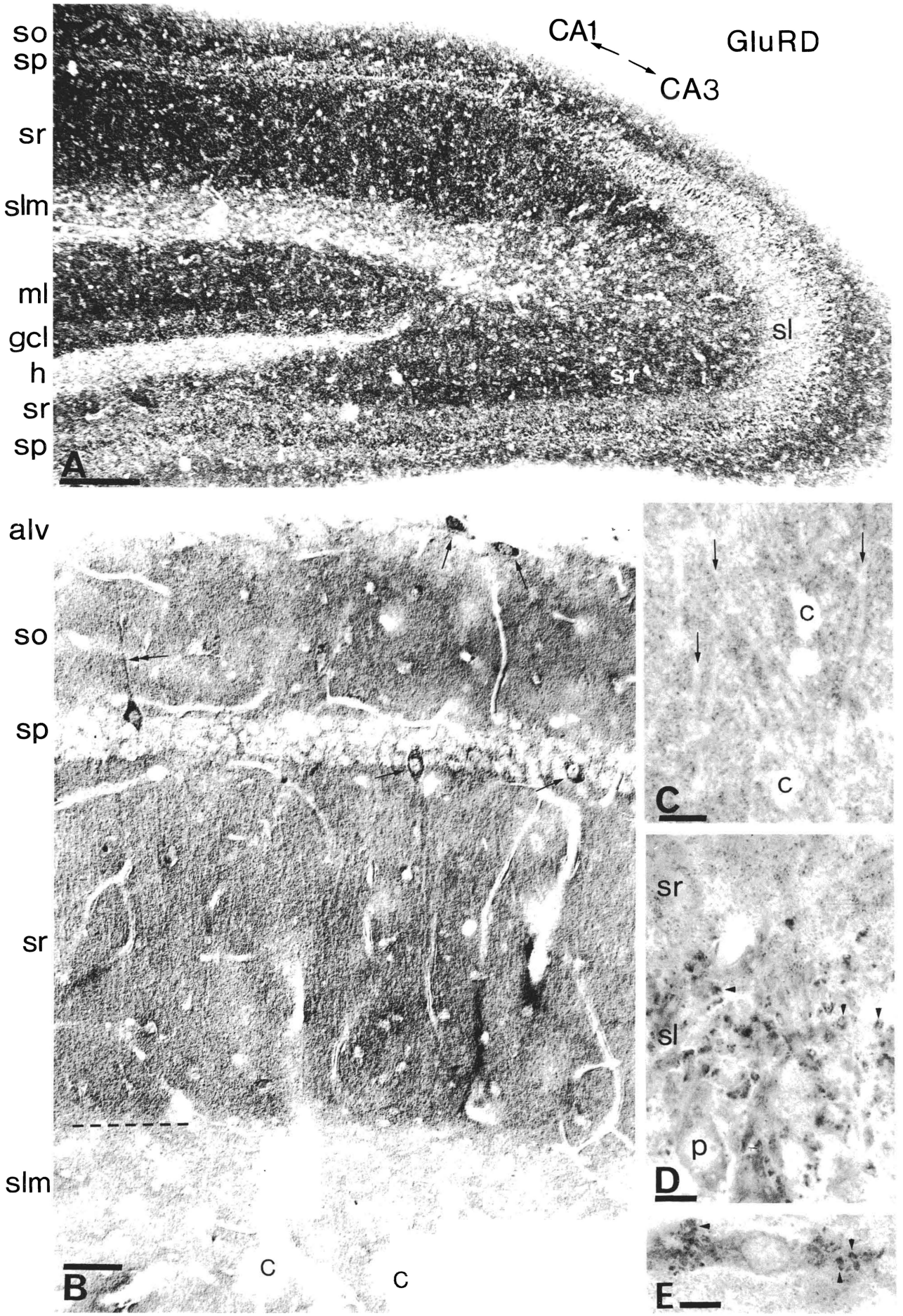
so
sp
sr
slm
ml
gcl
h
sr
sp
CA1
CA3
GluRD
sl
sr
A
alv
so
sp
sr
slm
B
c
c
c
c
C
sr
sl
p
D
E

Fig. 3.

recognizable in all layers of the CA1 and CA3 areas, but they were less strongly labelled relative to principal cells than for the GluRA subunit.

The distribution of immunolabelling for the GluRD subunit with our antibodies differed substantially from that reported earlier,[68,90] and so it is described in more detail (Fig. 3). The difference in the pattern obtained with Ab-GluRD-CT13/8 is unlikely to be due to crossreactivity with a lower molecular weight protein, responsible for the labelling of an additional weak band in immunoblots, since the immunoreactivity pattern in the hippocampus did not change following the pretreatment of the antibodies that lead to the virtual elimination of this band (Fig. 1B). Immunoreactivity was present in all layers, being most intense in stratum oriens and radiatum of the CA1 area and in the molecular layer of the dentate gyrus (Fig. 3A, B). At higher magnification, the weakly reacting apical dendrites were surrounded by punctate labelling in the neuropil of the stratum radiatum of the hippocampus (Fig. 3C). The hilus, the stratum lucidum of the CA3 area and the stratum lacunosum moleculare of both the CA1 and CA3 areas were less strongly labelled. The cell bodies of pyramidal and granule cells were only moderately reactive; the hilar mossy cells were more strongly labelled. Mossy cells were also surrounded by strongly stained rosettes probably corresponding to complex dendritic spines or excrescences (Fig. 3E). Some non-principal cells and their dendrites in the pyramidal cell layer and in the alveus reacted very strongly for this subunit (Fig. 3B). In stratum lucidum of the CA3 area, the weakly reacting dendrites and pyramidal cells were surrounded by strongly reacting large rosettes corresponding to complex dendritic spines, whereas in the stratum radiatum small punctate immunostaining was observed (Fig. 3D).

High-resolution immunogold labelling

Membrane topology of immunolabelling. For the N-terminal antibody to the GluRA subunit, immunoparticles were located along the external face of the extrasynaptic plasma membrane when detected with the pre-embedding method (Figs 5A, 8A). Only very rarely and only in tissue that was disrupted by the freeze–thaw procedure could particles be found in the synaptic cleft (Fig. 8A). In contrast, immunoparticles were located along the cytoplasmic side of the synaptic plasma membrane when antibodies to the C-terminal part of the GluRD subunit were used (Figs 4A–C, 8B, C). These results demonstrate the extracellular location of the N-terminal and the intracellular location of the C-terminal parts of subunits of the AMPA type receptor subunits.[75,90]

Synapse types. Synaptic junctions in the CNS were classified on the basis of the electron-microscopic appearance and extent of the postsynaptic density. Type 1 (often called asymmetrical) synapses have extensive postsynaptic densities, and type 2 (often called symmetrical) have small postsynaptic densities.[35] Electron-microscopic studies demonstrated that in the hippocampus, entorhinal, pyramidal and granule cell axon terminals, which produce monosynaptic fast excitatory postsynaptic potentials in postsynaptic cells, make type 1 synapses.[3,16,80] Most, but not all, synaptic boutons which make type 1 synapses are rich in glutamate[19,62] which is considered to be the transmitter at these synapses. In the present study synaptic labelling was only seen at type 1 synapses.

Synapses were considered immunopositive if the junctional membrane specialization was contacted by more than one immunoparticle. Along with synapses labelled by multiple particles, many additional synapses were labelled only by one particle in material that showed negligible background labelling, thus the above criterion leads to an underestimate of immunolabelled synapses. Nevertheless, restricting the immunolabelled population for synapses labelled by two or more particles does not change the pattern of immunolabelling described here. Interestingly, in the same area that contained strongly immunopositive synaptic junctions (Fig. 5C–D) most synapses were unlabelled and some showed labelling only by one particle. In the pre-embedding condition one could argue that the antibodies did not penetrate to the immunonegative synaptic junctions (Fig. 5A), but this explanation cannot apply to the post-embedding condition. The most parsimonious explanation of the great range of labelling intensity of type 1 synapses for AMPA type glutamate receptors is that the synapses express widely differing levels of the receptor.

Synapses on dendritic spines in the CA1 and CA3 areas. Most glutamatergic terminals make synaptic junctions with dendritic spines, and most of the spines originate from principal cells, however, non-principal cells may also have sparsely or densely spiny dendrites.[37,39,41,112] Although spines of non-principal cells contribute only a small fraction of the total spine population and these spines are more irregular in shape than principal cell spines, they cannot be differentiated from pyramidal cell spines without identification of the parent dendrite. Therefore, the origin of any single spine in electron micrographs is uncertain and the conclusions described below about principal cell spine labelling are derived from observations of the whole population.

Using the *pre-embedding* method, immunolabelling was generally associated with spines receiving type 1 synapses in strata radiatum, oriens and lacunosum moleculare (Figs 4A–C, 5A). The subcellular distribution of immunoparticles under pre-embedding condition was different for the GluRD subunit from that for the GluRA subunit and GluRB/C subunits. For the GluRD subunit, particles were present both at the cytoplasmic side of postsynaptic densities (Fig. 4A–C) and less frequently along the extrasynaptic spine membrane. Based on the criterion of more than one particle per synapse, up to 5% of spines had

labelled postsynaptic densities. For the GluRA (Fig. 5A) and GluRB/C (not shown), subunits particles were only present at the extrasynaptic membrane, often located just at the edge of the postsynaptic densities (Fig. 5A). Immunoreactivity could also be seen at the extrasynaptic membrane of

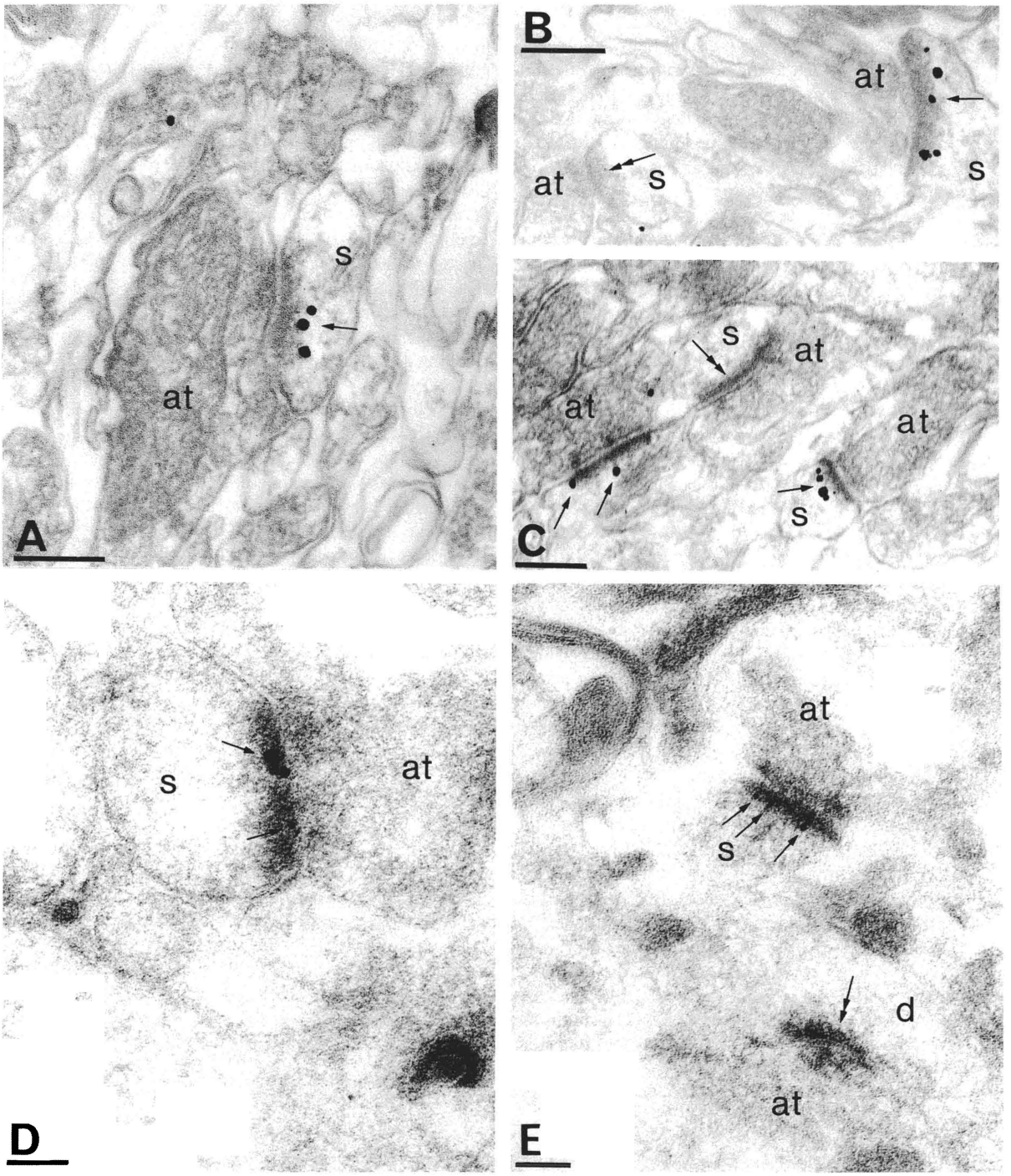

Fig. 4. Electron micrographs of immunoreactivity for the GluRD subunit of the AMPA receptor. (A–C) Pre-embedding silver intensified immunogold reaction in stratum radiatum of the CA1 area. Immunoparticles are located at the cytoplasmic side of postsynaptic densities of some type 1 synapses (arrows) made by axon terminals (at) with dendritic spines (s). Other synapses are immunonegative (double arrows). (D, E) Postembedding silver intensified immunogold reaction in stratum radiatum of the CA1 area (D) and stratum oriens of the CA3 area (E). Immunoparticles (arrows) are present on the postsynaptic densities of type 1 synapses. A type 1 synapse on a dendritic shaft (d) in E is immunonegative (double arrow). Scale bars = 0.2 μm (A–C), 0.1 μm (D, E).

dendritic spines and shafts, but in no obvious relationship to synapses (Fig. 5A). Some presynaptic labelling, associated with the cytoplasmic face of nerve terminals making type 1 synapses, was also present for the GluRD (not illustrated), but not for the other subunits.

Using the *postembedding* method, immunolabelling was mainly localized to the postsynaptic densities for the GluRB/C and D subunits (Figs 4D, E, 5B–E, 7B, 8C), and to the postsynaptic density as well as the synaptic cleft for the GluRA subunit (Fig. 6A, B). Immunoparticles do not seem to have a preferential distribution at the postsynaptic membrane specialization, since they could be found at any position along the junction. However, they usually do not occupy the whole length of the membrane specialization, even at high labelling density (Figs 4D, 5C, D, E). Based on the criterion of more than one particle per synapse, less than 1% of spines were immunopositive in the best reacting material, but synapses on dendritic shafts were more frequently labelled. Antibodies to the GluRB/C subunits were the most effective at labelling synapses. Immunoparticles were rarely seen at extrasynaptic membranes and not at all within the spine away from the plasma membrane.

Synapses on dendritic shafts. Pyramidal cells receive type 2 synapses on their dendritic shafts and these were immunonegative for all subunits. Many types of non-principal cell receive a high density of both types 1 and 2 synapses on their dendritic shafts; therefore, in electron micrographs dendrites with many type 1 synaptic junctions should be considered as originating from non-principal cells. The type of non-principal cell (e.g., axo-axonic cell, bistratified cell, etc.) cannot be determined from electron micrographs at present, as only the visualization of their axonal arbors allows subtype identification.[20]

The *postembedding* method revealed many strongly immunopositive axo-dendritic synapses for the GluRB/C (Fig. 5B, 7) subunits and less strongly labelled ones for the GluRA (Fig. 6B) and GluRD (not shown) subunits. The most frequent synaptic labelling was obtained with antibodies to the GluRB/C subunits. However, immunonegative axodendritic type 1 synapses were also observed on the same dendrites (Fig. 7A). Some axo-dendritic synapses were consistently more strongly labelled than the axo-spinous synapses (see, e.g., Fig. 7) in the same area of the section, probably due to a higher density of AMPA receptor in synapses of interneurons.

Mossy fibre synapses. These synapses are concentrated on the proximal dendrites of CA3 pyramidal cells, where the presynaptic terminals can be recognised on the basis of their size and the high density of synaptic vesicles (Fig. 8). Here, in contrast to other parts of the dendritic tree, a homogeneous population of synapses can be analysed for the different receptor subunits. Light-microscopic analysis of immunoperoxidase labelling for the GluRD subunit revealed large rosettes in the termination zone of the mossy fibres (Fig. 3D, E). Electron microscopy of the same peroxidase immunoreacted material showed that the labelling was mainly postsynaptic and largely restricted to the complex dendritic spines of pyramidal cells (not shown). The postsynaptic labelling was also confirmed by the pre-embedding immunogold method (Fig. 8A–C), which also showed non-synaptic labelling along the dendritic shafts. Interneurons also receive synapses from mossy fibres and some of these were heavily labelled for the GluRD subunit along the cytoplasmic side of the postsynaptic density (Fig. 8C). The GluRA subunit could only be demonstrated by the pre-embedding method (Fig. 8A), rarely in the synaptic junctions and more frequently at the extrasynaptic spine and dendritic membranes. A possible reason for this is the lower amount of GluRA subunit at mossy fibre synapses, which could not be detected by the postembedding method due to its lower sensitivity. Immunoparticles were distributed along the external face (Fig. 8A) of the plasma membrane. The GluRB/C subunits could only be localized in the mossy fibre synapses with the postembedding method and immunoparticles showed a concentration in the synaptic membrane specialization, with a sharp decrease in labelling at the edge of the junctions (Fig. 8D, E).

DISCUSSION

The immunocytochemical results demonstrate that the AMPA type glutamate receptor is concentrated in synaptic junctions that have extensive postsynaptic membrane specialization and have been classified anatomically as type 1 or asymmetrical synapses.[35] This conclusion was reached on the basis of the enrichment of immunoparticles on the synaptic

Fig. 5. Electron micrographs of AMPA receptor immunoreactivity in the CA1 area. (A) Immunoreactivity for the GluRA subunit, pre-embedding silver intensified immunogold reaction. Two spines (s), one of them connected to a pyramidal cell dendritic shaft (d), receive type 1 synapses from axon terminals (at). Immunoparticles are located at the periphery of postsynaptic densities (arrows) and at extrasynaptic sites on the dendritic shaft and dendritic spines (open arrows). The location of immunoparticles mainly at the external face of membranes indicates the extracellular position of the epitope(s). (B–E) Immunoreactivity for the GluRB/C subunits, postembedding silver intensified immunogold reactions. Immunoparticles are present on the postsynaptic densities (arrows) of type 1 synapses established by axon terminals (e.g., at). The postsynaptic elements are a dendritic shaft (d in B) in stratum oriens, and spines (s) in stratum radiatum. Note that the immunolabelled synapses on spines are amongst the largest ones and nearby smaller synapses (double arrows) are immunonegative. Scale bars = 0.2 μm.

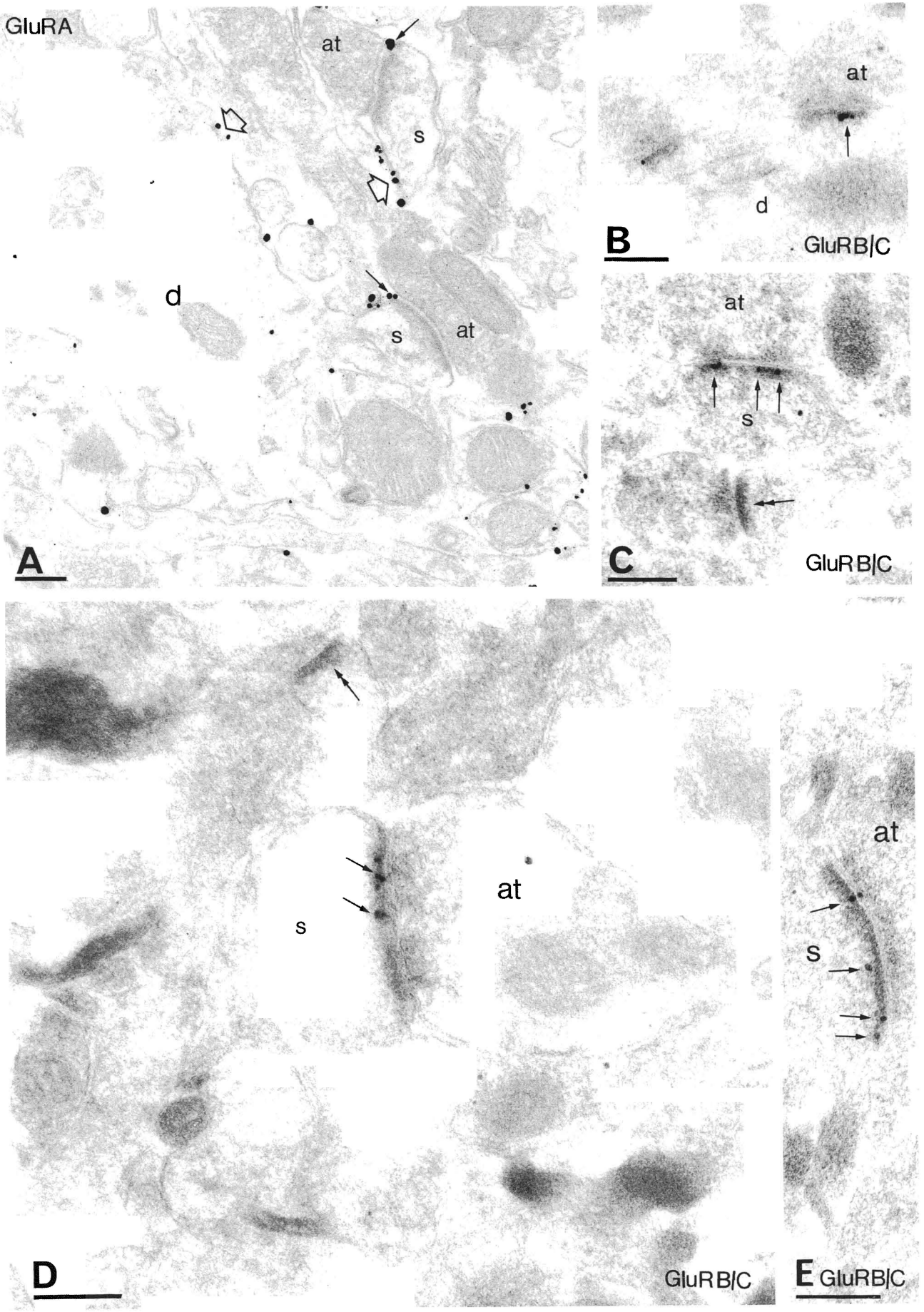
GluRA
at
s
d
s
at
A
at
d
B
GluRB/C
at
s
C
GluRB/C
at
s
D
GluRB/C
at
s
E GluRB/C

Fig. 5.

membrane specialization in the brain, as demonstrated by *the postembedding immunogold method*, which has been found to be *the only reliable method to demonstrate synaptic receptors unequivocally* with most antibodies. Since this is the first study to apply the postembedding method to the hippocampus it is important to compare the merits of the different techniques when making comparisons with other studies.

Pre-embedding versus postembedding methods for the localization of synaptic receptors

Previous electron-microscopic immunoperoxidase localization of AMPA receptors, using antibodies to the C-terminal part of the polypeptides, showed that postsynaptic densities were covered by peroxidase reaction endproduct and this has been generally interpreted as demonstrating synaptic receptor immunoreactivity.[6,31,68,75,76,90,107,116] The illustrations in these studies also show an identical precipitate on intracellular organelles such as mitochondria and on non-synaptic plasma membrane. The latter has been shown to contain non-synaptic receptors by the present immunogold method as well as by recording glutamate receptor-mediated membrane currents by patch recording.[27,54,114] We could reproduce previously published immunoperoxidase labelling of postsynaptic densities in the hippocampus,[68,90] either with antibodies used by those authors[31,90] or with other antibodies.[6,75,76] However, we found it impossible to establish whether the peroxidase reaction precipitate on the postsynaptic specialization was formed there, or diffused to the postsynaptic density from non-synaptic sites. Evidence for the latter event was obtained from the immunolocalization of a metabotropic glutamate receptor, which is absent from the main body of postsynaptic specialization when revealed by particulate immunomarkers.[8,84] Nevertheless, when shown by the peroxidase technique, the postsynaptic densities were covered by precipitate in a pattern[69] indistinguishable from that obtained for the AMPA receptor subunits. Furthermore, in our immunoperoxidase localization of glutamate receptors, using antibodies to intracellular epitopes, the reaction endproduct was also found on synaptic specializations that were formed by presumed GABAergic terminals on dendritic shafts and spines as long as the postsynaptic element was immunopositive. It is noteworthy that no immunonegative synapse has been demonstrated on immunopositive postsynaptic structures in previous peroxidase labelling studies. Unless one assumes that glutamate receptors are equally concentrated in all synaptic junctions, the most parsimonious explanation of the labelling of all synapses is that the reaction product can cover any synaptic junction within a postsynaptic element. It can be concluded that the postsynaptic specialization can concentrate the peroxidase reaction product wherever it is formed. Therefore, a high density of peroxidase reaction product on the postsynaptic density is consistent with, but does not necessarily represent, the visualization of synaptic receptors.

We have tried previously an alternative strategy for the synaptic localization of glutamate receptors using antibodies to predicted extracellular epitopes, hoping that the narrow extracellular space will restrict the peroxidase reaction product to sites of high densities of receptor. The results for both glutamate[6,75,76] and GABA receptors[36,108,111,113] showed a relatively uniform deposition of reaction product in the synaptic cleft as well as along the extrasynaptic plasma membrane. Although the reaction signal in the synaptic cleft was taken as evidence for the presence of the receptor in the junction, in retrospect, the possibility that all the reaction product was formed extrasynaptically and that some of it then diffused into the synaptic cleft cannot be excluded. Indeed when a non-diffusible immunosignal, namely silver intensified immunogold, is used for the localization of extracellular receptor epitopes, the same antibodies that give an immunoperoxidase signal in the synaptic cleft, do not show an immunoparticle signal in synaptic junctions.[85,86] Thus either the primary antibody, or the 1.4-nm gold particle-coupled secondary antibody are unable to enter the synaptic cleft. The effect of detergent treatment is as yet unclear.

Immunofluorescence detection of glutamate receptors produced spectacular results on cultured neurons[12,28,29] showing that under such conditions there is clustering of the immunosignal on dendritic spines for AMPA type receptors. Both dendrites and somata showed clustered distribution of NMDA receptors detected with a fluorescent derivative of conantokin-G toxin.[12] Clustering of receptors, however, can occur in the absence of synaptic input[23] and the resolution of the fluorescent signal does not allow the differentiation of pre- and postsynaptic membranes or for us to decide how much of the signal originates from outside the synaptic membrane specialization. Nevertheless immunofluorescent methods appear to provide a more discrete signal than immunoperoxidase labelling.

Fig. 6. Electron micrographs of AMPA receptor immunoreactivity for the GluRA subunit in the CA3 area, as detected with the postembedding method. (A) Two groups of immunoparticles (arrows) label the postsynaptic membrane at a type 1 synapse established between a spine (s) and an axon terminal (at) in stratum oriens. (B) Immunoparticles (arrows) are located in the synaptic cleft at the external face of the postsynaptic membrane at a type 1 synapse established between a dendrite (d) and an axon terminal in stratum lucidum. The pale line next to the particles is the lipid bilayer of the postsynaptic membrane. Scale bars = 0.1 μm.

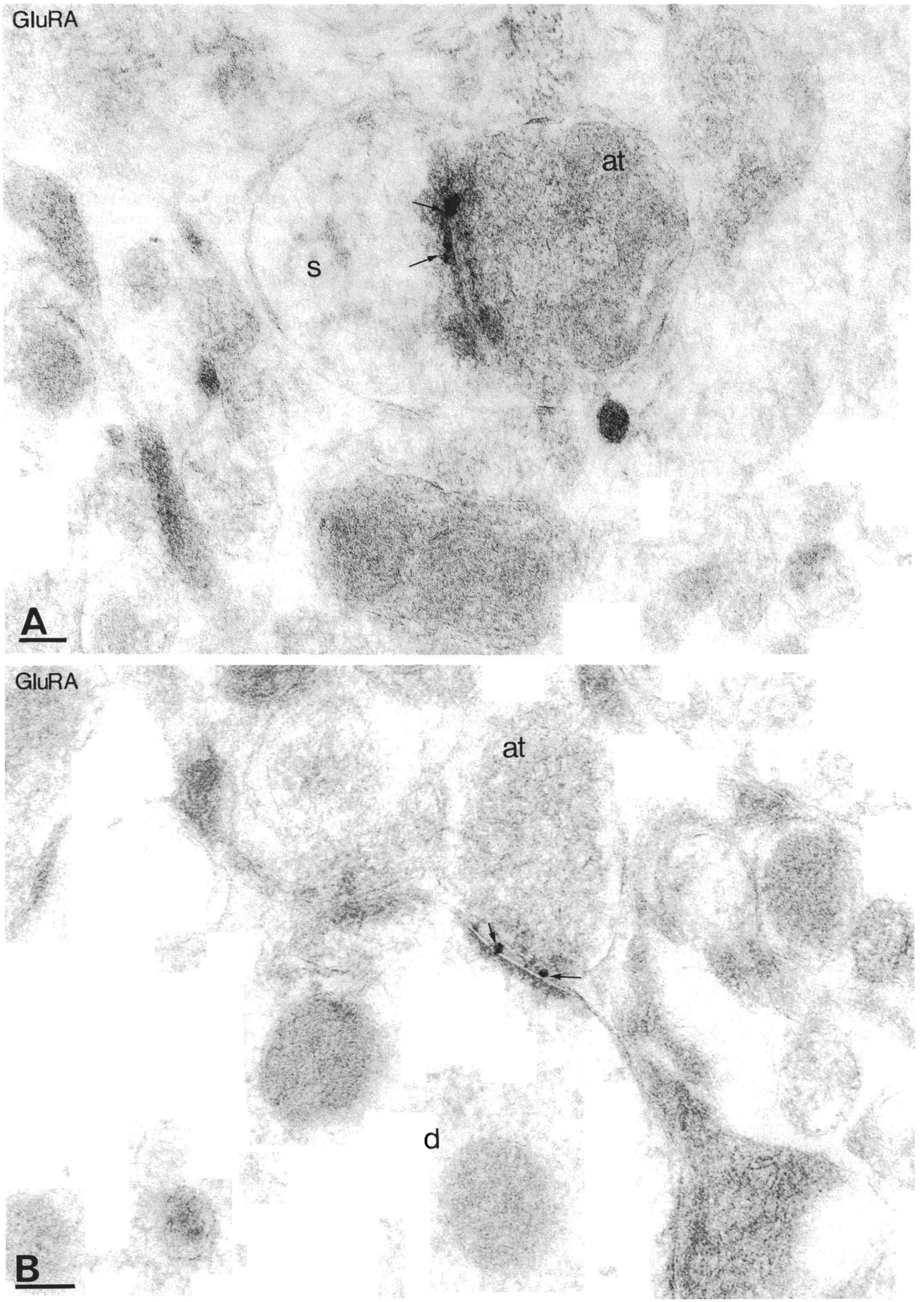

Fig. 6.

The postembedding immunogold method is reliable to localize receptors at synaptic sites with high resolution and to reveal relative quantitative differences in receptor density along the plasma membrane.[84,86] This method has demonstrated the enrichment of AMPA receptors in anatomically defined type 1 synaptic junctions. The preservation of tissue and immunoreactivity can be uneven within the slam-frozen blocks used for postembedding labelling, resulting in variable proportion of immunopositive asymmetrical synapses. In addition to a genuine absence of receptors, the lack of immunoreactivity in some asymmetrical synapses could be due to either the inaccessibility of receptors in tangentially cut synapses which do not reach the surface of the section, or to receptor levels below the detectability of our method. Therefore, only the positive immunolabelling results could be conclusively interpreted. In the postembedding method, the sectioned membrane is uniformly exposed to the reagents in contrast to the pre-embedding methods which often produce a decreasing gradient of reactivity as a function of depth in the thick section. In addition, the rarity of immunogold labelling of the glutamatergic synaptic junctions, using the pre-embedding method and antibodies recognizing extracellular epitopes, strongly suggests that antibodies may not have access to the synaptic cleft due to its composition.

Both the pre-embedding immunoperoxidase and immunogold methods are reliable for the visualization of extrasynaptic receptors, which are less frequently detected with the postembedding method, due to its lower sensitivity. Thus, the three methods provide complementary information and are best used in combination in order to define the precise distribution of receptors.

Immunocytochemical localization of receptors in comparison with other methods

The presence of the AMPA type receptors in hippocampal principal and non-principal cells was expected from the expression of mRNA for these subunits by these cells.[18,57,100,109] The regional distribution of [^{3}H]AMPA binding[77,121] also showed dense labelling of the dendritic layers in the hippocampus. This appears similar to our light microscopic immunohistochemical results[75,76] and is in agreement with some previous studies which revealed strong homogeneous immunostaining of the dendritic layers.[14,68,98] However, other studies[48,90] showed the preferential staining of pyramidal cell bodies and main dendrites, which contrasts with the expected concentration of receptor at synaptic sites in the dendritic layers.

Membrane topology of AMPA type receptor subunits

Since the cloning of the first glutamate receptor subunit,[47] several models have been suggested for the membrane organization of the subunit protein.[13,46,97,115,117,119] Using antibodies to residues 253–267 or 877–889 of the GluRA subunit in an immunoperoxidase procedure, we first reported that the reaction deposit was on the extracellular (residues 253–267)[6,75,76] face of the plasma membrane, and confirmed the result of Petralia and Wenthold[90] that an antibody to residues 877–889 produced intracellular labelling along the plasma membrane.[6,75,76] We suggested the revision of the model based on hydropathy plots which predicted both the C- and N-terminal ends of the polypeptide to be extracellular. On the basis of the different location of immunocytochemical signals and recent biochemical studies,[13,97] it is generally accepted that the C-terminal end is indeed cytoplasmic, although the overall membrane conformation is open to several interpretations. Concerning the labelling in the hippocampus, using the pre-embedding immunogold method which is the most suitable immunocytochemical method to predict the membrane topology of epitopes, we have now confirmed the immunoperoxidase results and have demonstrated that the epitope(s) representing residues 253–267 in the GluRA subunit are indeed extracellular, whereas epitopes in the C-terminal 850–862 (GluRB/C) and 869–881 (GluRD) residues are at the cytoplasmic side of the plasma membrane. Interestingly, the GluRD subunit could be revealed at the cytoplasmic side of the postsynaptic density by the pre-embedding method, whereas the GluRB/C subunits could not. The GluRD subunit has a longer C-terminal intracellular segment after the last transmembrane domain than the GluRB and GluRC subunits and our results suggest that it is in the cytoplasm further from the membrane than the C-terminal parts of the other two subunits.

AMPA type receptor subunits are concentrated at type 1 *synapses in the hippocampus*

The presence of high concentrations of AMPA type glutamate receptors in the main body of anatomically defined type 1 postsynaptic membrane

Fig. 7. Electron micrographs showing immunoreactivity for the GluRB/C subunits of the AMPA receptor in stratum lucidum (A, B) and stratum oriens (C) of the CA3 area. (A) Two dendrites (d) with characteristics of interneurons receive several immunoreactive type 1 synapses (thick arrows) from large boutons. (B) Immunoparticles are distributed all along the synaptic membrane (thin arrows) on the postsynaptic density. Note the presence of Taxi's bodies (e.g., arrowhead) at most of the synapses of both dendrites. (C) Synapses (arrows) are consistently labelled on this interneuron dendrite (d). The synapse at the right upper corner is cut tangentionally. Since particles are on only one surface of the section, some of them falsely appear over the presynaptic terminal. At cross-section of the synapses they are centred close to the postsynaptic membrane. Scale bars = 0.2 μm.

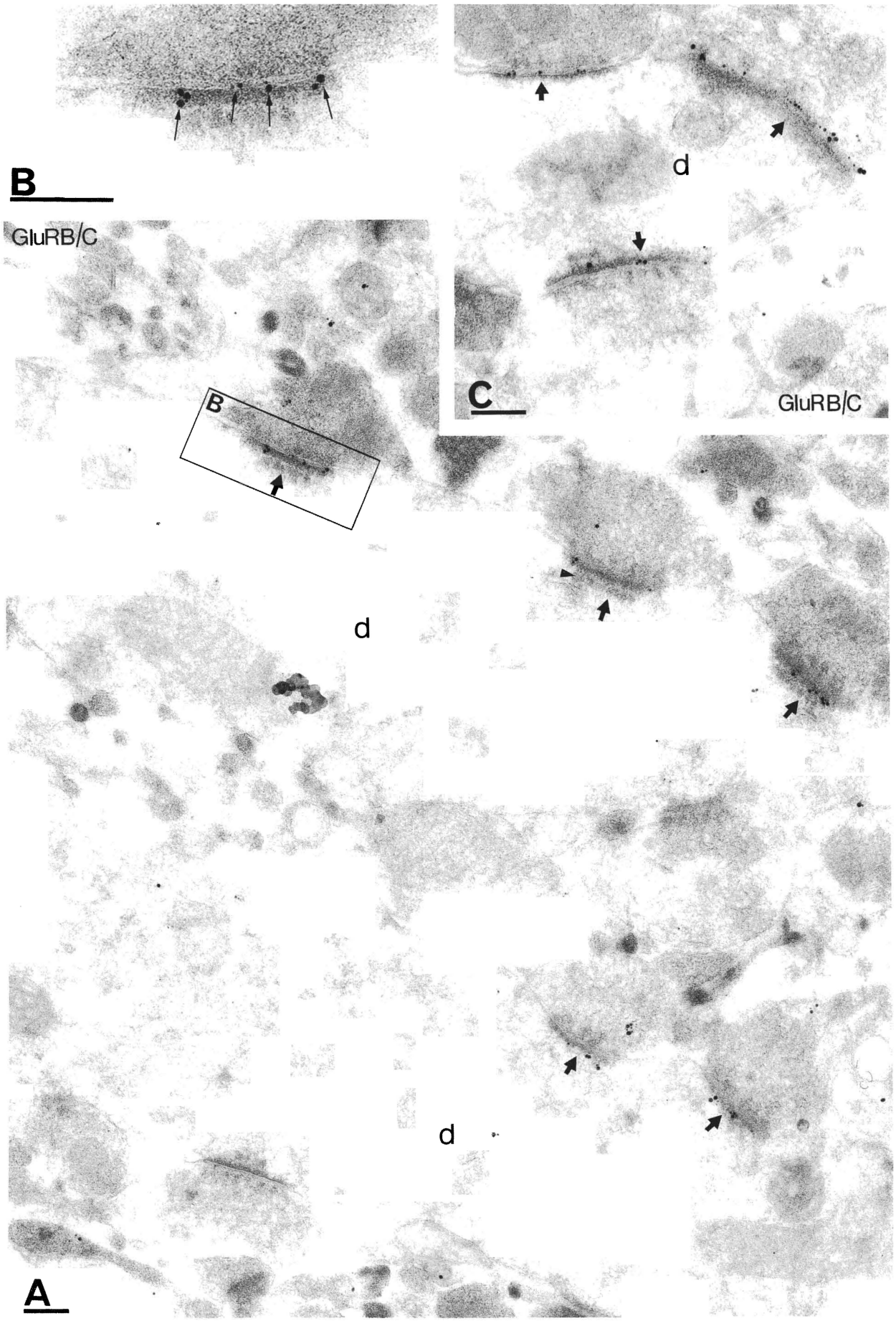

Fig. 7.

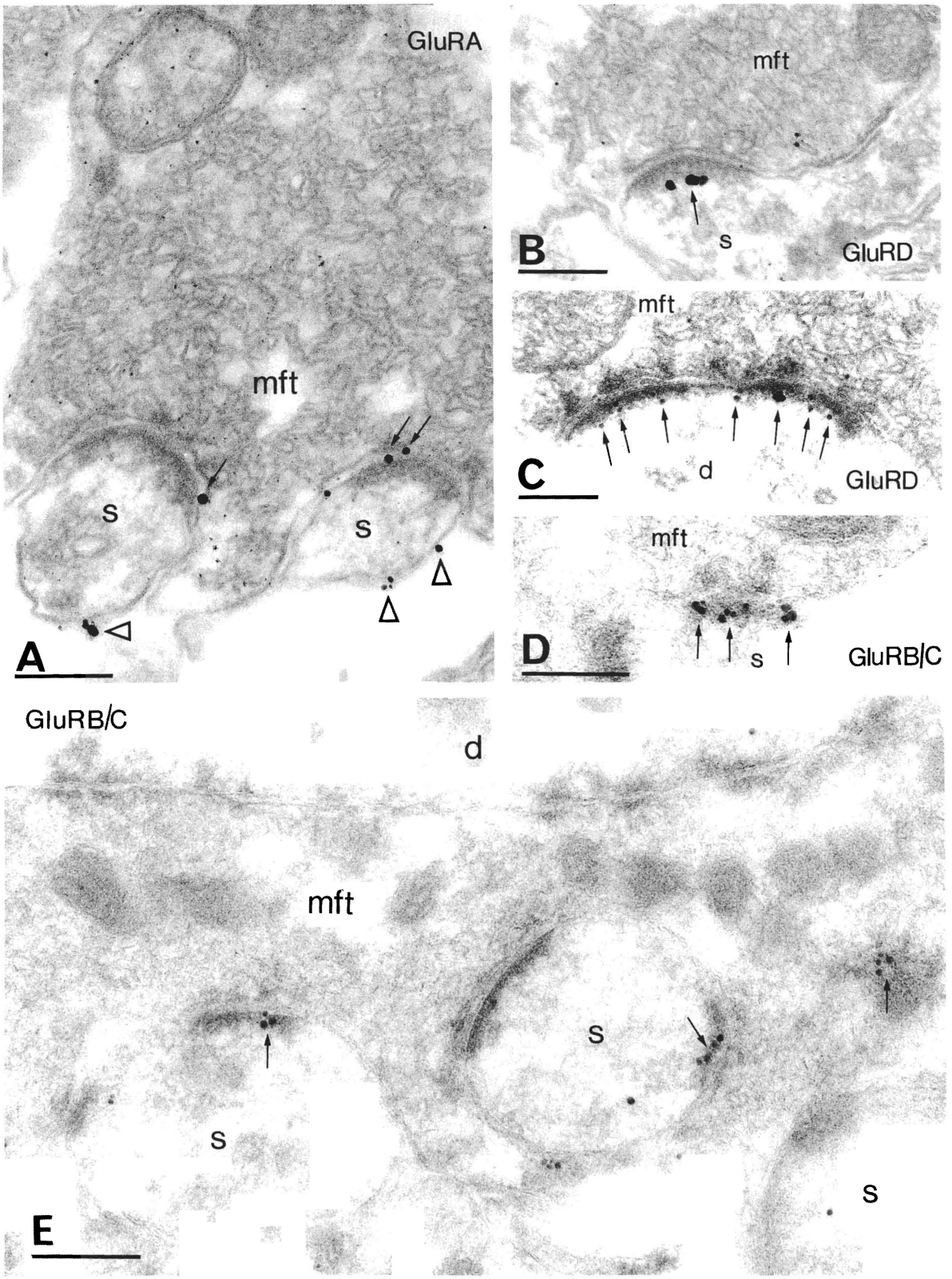

Fig. 8. Electron micrographs of AMPA receptor immunoreactivity at synapses made by mossy fibre terminals (mft) in stratum lucidum of the CA3 area. (A–C) Pre-embedding, (D, E) postembedding silver intensified immunogold reactions. (A) Immunoreactivity for the GluRA subunit, showing two spines (s) receiving type 1 synapses. Immunoparticles are present along the extracellular face of the spine membrane at synaptic (arrows) and extrasynaptic sites (open triangles). (B) Immunoreactivity for the GluRD subunit, showing a spine (s) and immunolabelling of the synaptic specialization (arrows). (C) Immunolabelling for the GluRD subunit along the synaptic specialization of a dendritic shaft (arrows) probably originating from an interneuron. (D) Heavily immunolabelled mossy fibre synapse with a spine (s). (E) Immunoreactivity for the GluRB/C subunits, showing spines establishing immunoreactive type 1 synapses with a mossy fibre terminal lying along the apical dendrite (d) of a pyramidal cell. Immunoparticles (arrows) are distributed along the postsynaptic densities at the internal face of the synaptic membrane. The immunolabelled synapse to the right is cut tangentially. Scale bars = 0.2 μm.

specializations facing the transmitter release site is in line with the fast onset and rise time of non-NMDA receptor generated synaptic currents.[44,52,67] Furthermore, AMPA type glutamate receptors have relatively low affinity for L-glutamate on hippocampal cells,[88] suggesting that they are close to the synaptic junction where glutamate reaches millimolar concentration.[24] Clustering of AMPA receptors has been demonstrated in the anatomically defined synaptic junctions in the cerebellar cortex[84] and spinal cord,[92] for glycine receptors in the spinal cord[104] and for $GABA_A$ receptors in the cerebellum[86] and hippocampus.[85] Although in the hippocampus extrasynaptic AMPA receptors are clearly present on both dendritic shafts and spines, their concentration seems to be much lower since they are rarely seen with the postembedding method. The latter technique reveals a sharp decrease of receptor density at the edge of the membrane specialization which demonstrates that at a given level of glutamate only a well defined number of receptors can be activated. Even if glutamate diffuses out of the cleft, a much lower density of receptor will be reached, probably contributing little to the synaptic current.

Usually not the whole cut length of the postsynaptic membrane specialization of dendritic spines was occupied by immunoparticles even when they were clustered at high density on a part of the synapse. This may indicate that patches of different densities of glutamate-gated AMPA type channels exist within a synaptic specialization, or that AMPA type channels may be totally absent from some parts of the membrane specialization. These regions may be occupied by other types of glutamate receptors. The preferential location of the AMPA type receptor at postsynaptic densities provides a site where Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) and/or other protein kinases can modulate glutamate receptor activity.[15,58,71,96] Postsynaptic densities contain a high concentration of CaMKII[123] and all subunits of the AMPA type receptor contain consensus phosphorylation sites for CaMKII and protein kinase C.[57] The phosphorylation state of receptors at extrasynaptic sites where the necessary enzymes may be absent could therefore be different from synaptic receptors.

How the AMPA type receptors are targeted to specific synapses in the plasma membrane is not known. It is generally thought that plasma membrane receptors are carried to the surface by transport vesicles from the Golgi apparatus, but whether these vesicles target synaptic or extrasynaptic sites is not known. The possibility has been raised that packets of receptor may be inserted into the synaptic membrane upon potentiation of glutamatergic synapses.[59,61,61a,125] We did not observe any significant number of intracellular immunoparticles for AMPA receptors inside dendritic spines; therefore, if present, any intracellular pool of receptors near synapses is much lower then the synaptic pool. However, the presence of extrasynaptic receptors outside the synaptic membrane specialization provides a readily recruitable pool of receptors. The lateral mobility of AMPA type glutamate receptors has not been estimated, but for NMDA receptors it was calculated, on the basis of their observed mobility, that a highly mobile pool could diffuse across a spine head in about 2 s.[12]

The hypothesis that AMPA type receptors may be inserted at synapses which had no such receptors prior to long term potentiation predicts that some synapses lack AMPA receptors.[59,61,61a,125] The absence of a protein cannot be proven by immunocytochemistry, but the results with the postembedding immunogold method, demonstrating synapses labelled by multiple particles amongst synapses having no label at all, predict that there must be great variation in AMPA type glutamate receptor density on different dendritic spines. Interestingly, often the largest, perforated synapses were the most strongly immunolabelled and these may be particularly effective in synaptic transmission.

Subunits of the AMPA type receptor at distinct synapses of pyramidal cells

Many synapses on dendritic spines were immunoreactive for the GluRA, GluRB/C and GluRD subunits. It is reasonable to assume that most of these dendritic spines belong to pyramidal cells. The results demonstrate that these subunits of the AMPA type glutamate receptor are present in many of the glutamatergic synapses formed by the entorhinal, CA3 pyramidal and mossy fibre terminals.

The spines in strata radiatum and oriens receive synapses mainly from axon terminals arising from ipsi- and contralateral pyramidal cells of the CA3 area.[2,60,64,74,95] Quantitative immunogold localization revealed that these terminals are enriched in glutamate[19] which is considered to be a neurotransmitter at these synapses. The presence of immunoreactivity for subunits of the AMPA receptor at synapses on dendritic spines in the CA1 area is in agreement with the results of physiological studies demonstrating that Schaffer collateral, commissural and intra-areal, associational synaptic transmission is mediated partially through non-NMDA receptors.[25,26,30,44,94,118] Furthermore, currents evoked by brief application of glutamate to pyramidal cells had fast rise time and rapid desensitisation[27] and were blocked by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), an antagonist of non-NMDA receptors. When dendritic membrane patches were tested, kainate-preferring receptors did not contribute to glutamate evoked responses which could be mimicked by responses to AMPA.[114] Therefore, AMPA receptors are likely to be the main non-NMDA receptors mediating responses to glutamate.

Our results demonstrate that the GluRA, GluRB/C and GluRD subunits are present at

synapses of pyramidal cells in the mature hippocampus. However, even the postembedding immunogold method that has a resolution of about 20 nm is inadequate to test whether the different subunits are in the same channel. Co-expression of AMPA receptor subunits *in vitro*[21,45,57,82] and immunoprecipitation of native receptors[120] strongly suggest that native receptors are hetero-oligomeres. Moreover, clusters immunoreactive for the GluRA subunit have been shown to co-localize with clusters immunoreactive for the GluRB/C subunits on cultured rat hippocampal neurons.[28]

One antibody used by us recognizes both the GluRB and the GluRC subunits on immunoblots;[120] therefore, immunoreactivity observed here on postsynaptic densities could represent either or both subunits. The absence of the edited version of the GluRB subunit[109] from expressed receptors results in highly Ca^{2+} permeable AMPA-gated channels and a non-linear *I–V* relationship.[21,45] Activation of AMPA type non-synaptic receptors by glutamate in hippocampal principal cells is characterized by a linear *I–V* relationship and low Ca^{2+} permeability,[27,54,63,70,114] thus the edited version of the GluRB subunit probably contributes to native receptors. Although the properties of synaptic receptors may be different, it is likely that a major part of the immunolabelling of type 1 synapses of pyramidal cells with the antiserum to the GluRB/C subunits involved the B subunit.

Responses of pyramidal cells to mossy fibre activation are mainly mediated by non-NMDA glutamate receptors[52] and the presence of immunoreactivity for the GluRA, B/C and D subunits in the synaptic specializations established by mossy fibres with spines in stratum lucidum supports pharmacological evidence that AMPA receptors are involved in synaptic transmission. Previous immunoperoxidase studies also suggested the presence of AMPA receptors in the complex spines of stratum lucidum.[75,90] The fast component of the synaptic response has the characteristics of edited GluRB-containing AMPA-gated channels. This again correlates well with our findings, strongly suggesting that the GluRB subunit is concentrated at the postsynaptic densities opposite to glutamatergic mossy fibre terminals.[19,62] Spiny interneurons in the CA3 area also receive synaptic contacts from mossy fibres;[37,112] therefore, some immunoreactive synapses observed in CA3 stratum lucidum may belong to interneurons. However, the spines of interneurons in stratum lucidum are thin and mostly lack distinct enlarged heads in contrast to the spines of pyramidal cells that we illustrated and described above.

AMPA type receptors are concentrated at synapses of hippocampal interneurons

Our results demonstrate that type 1 synapses of some hippocampal interneurons contain the GluRA, B/C and D subunits of the AMPA type glutamate receptor similarly to pyramidal cells, but that not all these subunits may be expressed by all cells. Excitatory synaptic responses of many interneurons exhibit both NMDA and non-NMDA components in the hippocampus[63,70,99] and the visual cortex.[43] However, not all interneurons may have the same excitatory amino acid receptor composition at their excitatory synapses and exclusively non-NMDA responses have been reported in some cells.[99] Interneurons are also heterogeneous in their non-NMDA glutamate receptor-mediated responses. In the neonatal CA3 region, some stratum radiatum interneurons exhibited mainly linear *I–V* relationship in response to kainate (type I), others (type II) showed strong inward rectification both to applied kainate and of their miniature excitatory postsynaptic currents (mEPSCs).[70] Interneurons (type II) with inwardly rectifying *I–V* relationship, high Ca^{2+} permeability of their non-NMDA receptors and expressing only the GluRA and D subunits of the AMPA type glutamate receptor have also been found in hippocampal cultures.[17,51] The interneurons with high GluRB/C subunit immunoreactivity in synapses demonstrated here are unlikely to be the type II variety, and clearly a more complete definition of interneurons expressing particular AMPA receptor subunit combinations is required according to their synaptic connections or neurochemical markers. In the neocortex, some GABAergic interneurons also have low levels of mRNA for the GluRB subunit accompanied by highly Ca^{2+}-permeable AMPA receptors, but little inward rectification.[53]

Synaptic responses of pyramidal and non-pyramidal cells differ in the rise-time, decay-rate and desensitization of the AMPA receptor-mediated component,[43,63,70,72] but similarities have also been suggested.[99] The kinetics of excitatory postsynaptic currents (EPSCs) are faster in interneurons than in principal cells in the visual cortex,[43] the hilus[63,63a] and the CA3 area.[70] Single-channel conductance is higher in aspiny interneurons (27 pS) than in pyramidal cells (9 pS) in the visual cortex.[43] A similar conclusion was reached for aspiny vs the spiny mossy cells in the dentate hilus.[63] A higher concentration of the GluRB/C subunit(s) of the AMPA type receptor in synapses of interneurons, demonstrated by the immunogold method, suggests that a higher number of AMPA-gated channels are present at synapses of some interneurons in the CA1 and CA3 areas. This, in combination with a higher channel conductance, would increase the efficacy of the feed-forward activation of interneurons prior to pyramidal cells by the same afferent input.[22] Indeed, interneurons can be brought to firing threshold by an action potential from a single pyramidal cell,[72] although each pyramidal cell makes few, often only one, synaptic junctions with an interneuron,[20,38] and therefore may require a large synaptic current at these excitatory synapses.

Relationship of AMPA-type receptors to other glutamate receptors

Hippocampal neurons express mRNAs for at least three subunits of the NMDA receptor[78,79] in addition to AMPA type receptors. Synaptically released glutamate activates NMDA, AMPA type and metabotropic glutamate receptors and interactions amongst their effects are likely to occur.[4,11] The two ionotropic receptor subtypes have not been demonstrated in the same postsynaptic membrane specialization, but it is generally assumed that they occur together, although there are indications that they may also be segregated to some extent.[56,59] It is not known whether in the adult hippocampus all three classes of receptor are present at all synapses receiving glutamatergic innervation and even if they do occur together, whether their ratio is similar across the synapses of a single neuron. In cultures of cortical cells, where neuronal processes are at low density and there is little if any glial coverage, NMDA- and non-NMDA-receptor-mediated responses to glutamate were largest near to synaptic vesicle containing varicosities,[55] suggesting that both receptors occurred at the same synapses. More direct evidence for the synaptic co-localization of the two ionotropic receptor classes in cultured cells comes from the presence of both NMDA and non-NMDA components in mEPSCs,[10,70] although mEPSCs having only one component were also recorded. Previous immunoperoxidase localization of NMDA receptors reported labelling of postsynaptic densities together with homogeneous labelling of the cytoplasm,[50,89,91] but the extrasynaptic origin of the labelling of postsynaptic densities was not excluded. Thus, it remains to be determined at high resolution whether NMDA receptors are also concentrated in the anatomically defined synaptic specialization and whether they are intermingled with AMPA type glutamate receptors.

Subunits of glutamate receptors binding the agonist kainate with high affinity, GluR6, KA-1 and KA-2 are also highly expressed in the CA1 and CA3 areas.[122] The presence of kainate-preferring glutamate receptors with characteristics of expressed receptors,[42,102] remains to be fully demonstrated at synapses in the CNS. However, it remains possible that subunits of the kainate and AMPA-preferring receptors are present at the same synapses in the hippocampal formation.

The clustering of ion channel gating amino acid receptors in the postsynaptic membrane specialization has been revealed for the AMPA type glutamate receptors in synapses of the cerebellar cortex[84] and spinal cord[92] and for the $GABA_A$ receptors in the cerebellum[86] and hippocampus.[85] On the other hand, the G protein-coupled metabotropic glutamate receptors are concentrated in a perisynaptic annulus outside the postsynaptic density both in hippocampus[8,66] and cerebellum.[8,84] Consequently, it appears that the synaptic membrane is subdivided according to the type of glutamate receptor and this has been demonstrated directly in the cerebellum at the level of the single synapse.[84] In the hippocampus, some principal cells express the mGluR1β and/or c and all principal cells express the mGluR5 subtypes of metabotropic glutamate receptors which were found to be postsynaptic and highly concentrated in dendritic spines particularly in a perisynaptic annulus around the synaptic membrane specialization.[66] It has been suggested[8,84] that such a peripheral location of mGluRs contributes to their activation only at high-frequency presynaptic activity when more glutamate is released.[5,33,73] It will be interesting to see whether the same synapses shown here to contain high density of AMPA type receptors are the ones surrounded by a high density of mGluRs. Whether the subdivision of membrane territories applies also to the other postsynaptic ionotropic receptors that may be co-located in the same synapses remains to be determined by direct co-labelling experiments.[84] Nevertheless, the segregation of postsynaptic glutamate receptors may not be unexpected since a strict subdivision of the presynaptic terminal has been discovered by different classes of metabotropic glutamate receptors in the hippocampus.[105,106]

Acknowledgements—The authors thank Saul A. Richmond for the synthesis and purification of the GluRD (869-881) peptide and are grateful to Ms Diane Latawiec, Mrs Linda Lawes, Mrs Liz Norman and Mr J. David B. Roberts for excellent technical assistance. The authors also thank Mr Frank Kennedy and Mr Paul Jays for photographic assistance. Agnès Baude was supported by a fellowship of the Commission of European Communities, ERBCH-BICT920036. Zoltan Nusser was supported by a grant from Merck Sharp and Dohme Research Laboratories. The mammalian expression vectors containing the genes coding for the receptor GluRA–D were the generous gift of Dr Hannah Monyer at the Center for Molecular Biology, University of Heidelberg.

REFERENCES

1. Amaral D. G. (1978) A Golgi study of cell types in the hilar region of the hippocampus in the rat. *J. comp. Neurol.* **182,** 851–914.
2. Amaral D. G. and Witter M. (1989) The three-dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience* **31,** 571–591.
3. Andersen P., Blackstad T. W. and Lomo T. (1966) Location and identification of excitatory synapses on hippocampal pyramidal cells. *Expl Brain Res.* **1,** 236–248.
4. Aniksztejn L., Otani S. and Ben-Ari Y. (1992) Quisqualate metabotropic receptors modulate NMDA currents and facilitate induction of long-term potentiation through protein kinase C. *Eur. J. Neurosci.* **4,** 500–505.

5. Batchelor A. M., Madge D. J. and Garthwaite J. (1994) Synaptic activation of metabotropic glutamate receptors in the parallel fibre-Purkinje cell pathway in rat cerebellar slices. *Neuroscience* **63,** 911–915.
6. Baude A., Molnár E., Latawiec D., McIlhinney R. A. J. and Somogyi P. (1994) Synaptic and nonsynaptic localization of the GluR1 subunit of the AMPA-type excitatory amino acid receptor in the rat cerebellum. *J. Neurosci.* **14,** 2830–2843.
7. Baude A., Nusser Z., Roberts J. D. B., Molnár E., McIlhinney R. A. J., Mulvihill E., Jones R. S. G. and Somogyi P. (1993) Cellular and subcellular localization of ionotropic (GluR1) and metabotropic (mGluR1α) glutamate receptors in rat hippocampus. *Soc. Neurosci. Abstr.* **19,** 474.
8. 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 subpopulations as detected by immunogold reaction. *Neuron* **11,** 1–20.
9. Beesley J. E. (1989) Colloidal gold: A new perspective for cytochemical marking. In *Royal Microscopical Society Handbook*, pp. 8–9. Oxford University Press, Oxford.
10. Bekkers J. M. and Stevens C. F. (1989) NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus. *Nature* **341,** 230–233.
11. Ben-Ari Y., Aniksztejn L. and Bregestovski P. (1992) Protein kinase C modulation of NMDA currents: an important link for LTP induction. *Trends Neurosci.* **15,** 333–338.
12. Benke T. A., Jones O. T., Collingridge G. L. and Angelides K. J. (1993) *N*-Methyl-D-aspartate receptors are clustered and immobilized on dendrites of living cortical neurons. *Proc. natn. Acad. Sci. U.S.A.* **90,** 7819–7823.
13. Bennett J. A. and Dingledine R. (1995) Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. *Neuron* **14,** 373–384.
14. Blackstone C. D., Moss S. J., Martin L. J., Levey A. I., Price D. L. and Huganir R. L. (1992) Biochemical characterization and localization of a non-*N*-methyl-D-aspartate glutamate receptor in rat brain. *J. Neurochem.* **58,** 1118–1126.
15. Blackstone C., Murphy T. H., Moss S. J., Baraban J. M. and Huganir R. L. (1994) Cyclic AMP and synaptic activity-dependent phosphorylation of AMPA-preferring glutamate receptors. *J. Neurosci.* **14,** 7585–7593.
16. Blackstad T. W. and Kjaerheim A. (1961) Special axo-dendritic synapses in the hippocampal cortex: electron and light microscopic studies on the layer of mossy fibers. *J. comp. Neurol.* **117,** 133–159.
17. Bochet P., Audinat E., Lambolez B., Crepel F., Rossier J., Iino M., Tsuzuki K. and Ozawa S. (1994) Subunit composition at the single-cell level explains functional properties of a glutamate-gated channel. *Neuron* **12,** 383–388.
18. Boulter J., Hollman 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.
19. 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.
20. Buhl E. H., Halasy K. and Somogyi P. (1994) Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites. *Nature* **368,** 823–828.
21. Burnashev N., Monyer H., Seeburg P. H. and Sakmann B. (1992) Divalent ion permeability of AMPA receptor channel is dominated by the edited form of a single subunit. *Neuron* **8,** 189–198.
22. Buzsaki G. and Eidelberg E. (1982) Direct afferent excitation and long-term potentiation of hippocampal interneurons. *J. Neurophysiol.* **48,** 597–607.
23. Caruncho H. J., Puia G., Slobodyansky E., da Silva P. P. and Costa E. (1993) Freeze-fracture immunocytochemical study of the expression of native and recombinant $GABA_A$ receptors. *Brain Res.* **603,** 234–242.
24. Clements J. D., Lester R. A. J., Tong G., Jahr C. E. and Westbrook G. L. (1992) The time course of glutamate in the synaptic cleft. *Science* **258,** 1498–1501.
25. Colbert C. M. and Levy W. B. (1992) Electrophysiological and pharmacological characterization of perforant path synapses in CA1: mediation by glutamate receptor. *J. Neurophysiol.* **68,** 1–8.
26. Collingridge G. L., Herron C. E. and Lester R. A. J. (1988) Synaptic activation of *N*-methyl-D-aspartate receptors in the Schaffer collateral-commissural pathway of rat hippocampus. *J. Physiol.* **399,** 283–300.
27. Colquhoun D., Jonas P. and Sakmann B. (1992) Action of brief pulses of glutamate on AMPA/Kainate receptors in patches from different neurones of rat hippocampal slices. *J. Physiol.* **458,** 261–287.
28. 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.
29. Craig A. M., Blackstone C. D., Huganir R. L. and Banker G. (1994) Selective clustering of glutamate and γ-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. *Proc. natn. Acad. Sci. U.S.A.* **91,** 12,373–12,377.
30. Davies S. N. and Collingridge G. L. (1989) Role of excitatory amino acid receptors in synaptic transmission in area CA1 of rat hippocampus. *Proc. R. Soc. Lond. B* **236,** 373–384.
31. Eshhar N., Petralia R. S., Winters C. A., Niedzielski A. S. and Wenthold R. J. (1993) The segregation and expression of glutamate receptor subunits in cultured hippocampal neurons. *Neuroscience* **57,** 943–964.
32. Frens G. (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nature, Phys. Sci.* **241,** 20–22.
33. Gerber U., Luthi A. and Gahwiler B. H. (1993) Inhibition of a slow synaptic response by a metabotropic glutamate receptor antagonist in hippocampal CA3 pyramidal cells. *Proc. R. Soc. Lond. B* **254,** 169–172.
34. 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.
35. Gray E. G. (1959) Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.* **93,** 420–433.
36. Greferath U., Muller F., Wassle H., Shivers B. and Seeburg P. (1993) Localization of $GABA_A$ receptors in the rat retina. *Vis. Neurosci.* **10,** 551–561.
37. Gulyas A. I., Miettinen R., Jacobowitz D. M. and Freund T. F. (1992) Calretinin is present in non-pyramidal cells of the rat hippocampus—I. A new type of neuron specifically associated with the mossy fibre system. *Neuroscience* **48,** 1–27.

38. Gulyas A. I., Miles R., Sik A., Toth K., Tamamaki N. and Freund T. F. (1993) Hippocampal pyramidal cells excite inhibitory neurons through a single release site. *Nature* **366,** 683–687.
39. Halasy K. and Somogyi P. (1993) Subdivisions in the multiple GABAergic innervation of granule cells in the dentate gyrus of the rat hippocampus. *Eur. J. Neurosci.* **5,** 411–429.
40. Hampson D. R., Huie D. and Wenthold R. J. (1987) Solubilization of kainic acid binding sites from rat brain. *J. Neurochem.* **49,** 1209–1215.
41. Han Z.-S., Buhl E. H., Lorinczi Z. and Somogyi P. (1993) A high degree of spatial selectivity in the axonal and dendritic domains of physiologically identified local-circuit neurons in the dentate gyrus of the rat hippocampus. *Eur. J. Neurosci.* **5,** 395–410.
42. Herb A., Burnashev N., Werner P., Sakmann B., Wisden W. and Seeburg P. H. (1992) The KA-2 subunit of excitatory amino acid receptor shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron* **8,** 775–785.
43. Hestrin S. (1993) Different glutamate receptor channels mediate fast excitatory synaptic currents in inhibitory and excitatory cortical neurons. *Neuron* **11,** 1083–1091.
44. Hestrin S., Sah P. and Nicoll R. A. (1990) Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. *Neuron* **5,** 247–253.
45. Hollmann M., Hartley M. and Heinemann S. (1991) Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* **252,** 851–853.
46. Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. *A. Rev. Neurosci.* **17,** 31–108.
47. 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.
48. Hollmann M., Rogers S. W., O'Shea-Greenfield A., Deneris E. S., Hughes T. E., Gasic G. P. and Heinemann S. (1990) Glutamate receptor GluR-K1: structure, function, and expression in the brain. *Science* **249,** 1033–1037.
49. Hunter C., Wheaton K. D. and Wenthold R. J. (1990) Solubilization and partial purification of α-amino-3-hydroxy-methyl-4-isoxazole-propionic acid binding sites from rat brain. *J. Neurochem.* **54,** 118–125.
50. Huntley G. W., Vickers J. C., Janssen W., Brose N., Heinemann S. F. and Morrison J. H. (1994) Distribution and synaptic localization of immunocytochemically identified NMDA receptor subunit proteins in sensory-motor and visual cortices of monkey and human. *J. Neurosci.* **14,** 3603–3619.
51. Iino M., Ozawa S. and Tsuzuki K. (1990) Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones. *J. Physiol.* **424,** 151–165.
52. Jonas P., Major G. and Sakmann B. (1993) Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. *J. Physiol.* **472,** 615–663.
53. Jonas P., Racca C., Sakmann B., Seeburg P. H. and Monyer H. (1994) Differences in Ca^{2+} permeability of AMPA-type glutamate receptor channels in neocortical neurons caused by differential GluR-B subunit expression. *Neuron* **12,** 1281–1289.
54. Jonas P. and Sakmann B. (1992) Glutamate receptor channels in isolated patches from CA1 and CA3 pyramidal cells of rat hippocampal slices. *J. Physiol.* **455,** 143–171.
55. Jones K. A. and Baughman R. W. (1991) Both NMDA and non-NMDA subtypes of glutamate receptors are concentrated at synapses on cerebral cortical neurons in culture. *Neuron* **7,** 593–603.
56. Katz L. C and Dalva M. B. (1994) Scanning laser photostimulation: a new approach for analyzing brain circuits. *J. Neurosci. Meth.* **54,** 205–218.
57. Keinanen 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.
58. Keller B. U., Hollmann M., Heinemann S. and Konnerth A. (1992) Calcium influx through subunits GluR1/GluR3 of kainate/AMPA receptor channels is regulated by cAMP dependent protein kinase. *Eur. molec. Biol. Org. J.* **11,** 891–896.
59. Kullmann D. M. (1994) Amplitude fluctuations of dual-component EPSCs in hippocampal pyramidal cells: implications for long-term potentiation. *Neuron* **12,** 1111–1120.
59a. Kuriyama H., Jenkins O. and Altschuler R. A. (1994) Immunocytochemical localization of AMPA selective glutamate receptor subunits in the rat cochlea. *Hearing Res.* **80,** 233–240.
60. Li X. G., Somogyi P., Ylinen A. and Buzsaki G. (1994) The hippocampal CA3 network—an *in vivo* intracellular labeling study. *J. comp. Neurol.* **339,** 181–208.
61. Liao D., Jones A. and Malinow R. (1992) Direct measurement of quantal changes underlying long-term potentiation in CA1 hippocampus. *Neuron* **9,** 1089–1097.
61a. Liao D., Hessler N. A. and Malinow R. (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* **375,** 400–404.
62. Liu C.-j, Grandes P., Matute C., Cuénod M. and Streit P. (1989) Glutamate-like immunoreactivity revealed in rat olfactory bulb, hippocampus and cerebellum by monoclonal antibody and sensitive staining method. *Histochemistry* **90,** 427–445.
63. Livsey C. T., Costa E. and Vicini S. (1993) Glutamate-activated currents in outside-out patches from spiny versus aspiny hilar neurons of rat hippocampal slices. *J. Neurosci.* **13,** 5324–5333.
63a. Livsey C. T. and Vicini S. (1992) Slower spontaneous excitatory postsynaptic currents in spiny versus aspiny hilar neurons. *Neuron* **8,** 745–755.
64. Lopes da Silva F. H., Witter M. P., Boeijinga P. H. and Lohman A. H. M. (1990) Anatomic organization and physiology of the limbic system. *Physiol. Rev.* **70,** 453–511.
65. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement by the Folin phenolreagent. *J. biol. Chem.* **193,** 265–275.
66. Lujan R., Nusser Z., Roberts J. D. B., Shigemoto R. and Somogyi P. (1995) Perisynaptic location of the metabotropic glutamate receptor in the rat hippocampus. *Brain Res. Assoc. Abstr.* **12,** 114.
67. Malinow R. (1991) Transmission between pairs of hippocampal slice neurons: quantal levels, oscillations, and LTP. *Science* **252,** 722–724.
68. 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.

69. 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.
70. McBain C. J. and Dingledine R. (1993) Heterogeneity of synaptic glutamate receptors on CA3 stratum-radiatum interneurones of rat hippocampus. *J. Physiol.* **462,** 373–392.
71. McGlade-McCulloh E., Yamamoto H., Tan S. E., Brickey D. A. and Soderling T. A. (1993) Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. *Nature* **362,** 640–642.
72. Miles R. (1990) Synaptic excitation of inhibitory cells by single CA3 hippocampal pyramidal cells of the guinea-pig *in vitro*. *J. Physiol.* **428,** 61–77.
73. Miles R. and Poncer J.-C. (1993) Metabotropic glutamate receptors mediate a post-tetanic excitation of guinea-pig hippocampal inhibitory neurones. *J. Physiol.* **463,** 461–473.
74. Miles R. and Wong R. K. S. (1986) Excitatory synaptic interactions between CA3 neurones in the guinea-pig hippocampus. *J. Physiol.* **373,** 397–418.
75. Molnár E., Baude A., Richmond S. A., Patel P. B., Somogyi P. and McIlhinney R. A. J. (1993) 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.
76. Molnár E., McIlhinney R. A. J., Baude A., Nusser Z. and Somogyi P. (1994) Membrane topology of the GluR1 glutamate receptor subunit: epitope mapping by site-directed antipeptide antibodies. *J. Neurochem.* **63,** 683–693.
77. 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.
78. Monyer H., Burnashev N., Laurie D. J., Sakmann B. and Seeburg P. H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12,** 529–540.
79. Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B. and Seeburg P. H. (1992) Heteromeric NMDA receptors—molecular and functional distinction of subtypes. *Science* **256,** 1217–1221.
80. Nafstad P. H. J. (1967) An electron microscope study on the termination of the perforant path fibres in the hippocampus and the fascia dentata. *Z. Zellforsch.* **76,** 532–542.
81. Naito S. and Ueda T. (1981) Affinity-purified anti-protein I antibody. *J. biol. Chem.* **256,** 10,657–10,663.
82. Nakanishi N., Schneider N. A. and Axel R. (1990) A family of glutamate receptor genes—evidence for the formation of heteromultimeric receptors with distinct channel proeprties. *Neuron* **5,** 569–581.
83. Nusser Z., Baude A., Lujan R., McIlhinney R. A. J., Molnár E., Roberts J. D. B., Shigemoto R. and Somogyi P. (1995) Subcellular and subsynaptic distribution of AMPA-type, NMDA-type and metabotropic glutamate receptors in rat hippocampus. *Eur. J. Neurosci., Suppl.* **8,** 108.
84. Nusser Z., Mulvihill E., Streit P. and Somogyi P. (1994) Subsynaptic segregation of metabotropic and ionotropic glutamate receptors as revealed by immunogold localization. *Neuroscience* **61,** 421–427.
85. Nusser Z., Roberts J. D. B., Baude A., Richards J. G., Sieghart W. and Somogyi P. (1995) Immunocytochemical localization of the $\alpha 1$ and $\beta 2/3$ subunits of the $GABA_A$ receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur. J. Neurosci.* **7,** 630–646.
86. Nusser Z., Roberts J. D. B., Baude A., Richards J. G. and Somogyi P. (1995) Relative densities of synaptic and extrasynaptic $GABA_A$ receptors on cerebellar granule cells as determined by a quantitative immunogold method. *J. Neurosci.* **15,** 2948–2960.
87. Ottersen O. P. and Storm-Mathisen J. (1989) Excitatory and inhibitory amino acids in the hippocampus. In *The Hippocampus—New Vistas* (eds Chan-Palay V. and Kohler C.), pp. 97–117. Alan R. Liss, New York.
88. Patneau D. K. and Mayer M. L. (1990) Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J. Neurosci.* **10,** 2385–2399.
89. Petralia R. S., Wang Y.-X. and Wenthold R. J. (1994) The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. *J. Neurosci.* **14,** 6102–6120.
90. 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.
91. Petralia R. S., Yokotani N. and Wenthold R. J. (1994) Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. *J. Neurosci.* **14,** 667–696.
92. Phend K. D., Rustioni A. and Weinberg R. J. (1995) An osmium-free method of epon embedment that preserves both ultrastructure and antigenicity for post-embedding immunocytochemistry. *J. Histochem. Cytochem.* **43,** 283–292.
93. Pow D. V. and Crook D. K. (1993) Extremely high titre polyclonal antisera against small neurotransmitter molecules: rapid production, characterization and use in light- and electron-microscopic immunocytochemistry. *J. Neurosci. Meth.* **48,** 51–63.
94. Radpour S. and Thomson A. M. (1991) Coactivation of local circuit NMDA receptor mediated EPSPs induces lasting enhancement of minimal Schaffer collateral EPSPs in slices of rat hippocampus. *Eur. J. Neurosci.* **3,** 602–613.
95. Ramon y Cajal S. (1893) Estructura del asta de ammon y fascia dentata. *Anal. Soc. espan. Historia natural.* **22,** 53–114.
96. Raymond L. A., Blackstone C. D. and Huganir R. L. (1993) Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity. *Trends Neurosci.* **16,** 147–153.
97. Roche K. W., Raymond L. A., Blackstone C. and Huganir R. L. (1994) Transmembrane topology of the glutamate receptor subunit GluR6. *J. biol. Chem.* **269,** 11,679–11,682.
98. Rogers S. W., Hughes T. E., Hollman M., Gasic G. P., Deneris E. S. and Heinemann S. (1991) The characterization and localization of the glutamate receptor subunit GluR1 in the rat brain. *J. Neurosci.* **11,** 2713–2724.
99. Sah P., Hestrin S. and Nicoll R. A. (1990) Properties of excitatory postsynaptic currents recorded in vitro from rat hippocampal interneurones. *J. Physiol.* **430,** 605–616.
100. Sato K., Kiyama H. and Tohyama M. (1993) The differential expression patterns of messenger RNAs encoding non-*N*-methyl-D-aspartate receptor subunits (GluR1–4) in the rat brain. *Neuroscience* **52,** 515–539.
101. Schwartzkroin P. A. and Mueller A. L. (1987) Electrophysiology of hippocampal neurons. In *Cerebral Cortex. Further Aspects of Cortical Function, Including Hippocampus* (eds Jones E. G. and Peters A.), pp. 295–343. Plenum, New York.
102. Seeburg P. H. (1993) The TINS/TIPS lecture—the molecular biology of mammalian glutamate receptor channels. *Trends Neurosci.* **16,** 359–365.

103. Seed B. and Aruffo A. (1987) Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. natn. Acad. Sci. U.S.A.* **84,** 3365–3369.
104. Seitanidou T., Nicola M.-A., Triller A. and Korn H. (1991) Immunohistochemical localization of glycine receptors and a linked polypeptide in the goldfish brain. *J. Rec. Res.* **11,** 359–370.
105. Shigemoto R., Roberts J. D. B., Ohishi H. and Somogyi P. (1995) High resolution immunolocalization of a presynaptic metabotropic glutamate receptor (mGluR) restricted to the site of glutamate release in the hippocampus. *Eur. J. Neurosci., Suppl.* **8,** 67.
106. Shigemoto R., Wada E., Ohishi H., Takada M., Mizuno N., Roberts J. D. B. and Somogyi P. (1995) Differential presynaptic localization of metabotropic glutamate receptor subtypes, mGluR2/3 and mGluR7, in the rat hippocampus. *Soc. Neurosci. Abstr.* (in press).
107. Siegel S. J., Janssen W. G., Tullai J. W., Rogers S. W., Moran T., Heinemann S. F. and Morrison J. H. (1995) Distribution of the excitatory amino acid receptor subunits GluR2(4) in monkey hippocampus and colocalization with subunits GluR5-7 and NMDAR1. *J. Neurosci.* **15,** 2707–2719.
108. Soltesz I., Roberts J. D. B., Takagi H., Richards J. G., Mohler H. and Somogyi P. (1990) Synaptic and nonsynaptic localization of benzodiazepine/$GABA_A$ receptor/Cl^- channel complex using monoclonal antibodies in the dorsal lateral geniculate nucleus of the cat. *Eur. J. Neurosci.* **2,** 414–429.
109. Sommer B., Keinanen 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.
110. 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.
111. Somogyi P., Takagi H., Richards J. G. and Mohler 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.
112. Soriano E. and Frotscher M. (1993) Spiny nonpyramidal neurons in the CA3 region of the rat hippocampus are glutamate-like immunoreactive and receive convergent mossy fiber input. *J. comp. Neurol.* **332,** 435–448.
113. Spreafico R., De Biasi S., Amadeo A. and De Blas A. L. (1993) $GABA_A$-receptor immunoreactivity in the rat dorsal thalamus: an ultrastructural investigation. *Neurosci. Lett.* **158,** 232–236.
114. Spruston N., Jonas P. and Sakmann B. (1995) Dendritic glutamate receptor channels in rat hippocampal CA3 and CA1 pyramidal neurons. *J. Physiol.* **482,** 325–352.
115. Stern-Bach Y., Bettler B., Hartley M., Sheppard P. O., O'Hara P. J. and Heinemann S. F. (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron* **13,** 1345–1357.
116. Tachibana M., Wenthold R. J., Morioka H. and Petralia R. S. (1994) Light and electron microscopic immunocytochemical localization of AMPA-selective glutamate receptors in the rat spinal cord. *J. comp. Neurol.* **344,** 431–454.
117. Taverna F. A., Wang L.-Y., MacDonald J. F. and Hampson D. R. (1994) A transmembrane model for an ionotropic glutamate receptor predicted on the basis of the location of asparagine-linked oligosaccharides. *J. biol. Chem.* **269,** 14,159–14,164.
118. Thomson A. M. and Radpour S. (1991) Excitatory connections between CA1 pyramidal cells revealed by spike triggered averaging in slices of rat hippocampus are partially NMDA receptor mediated. *Eur. J. Neurosci.* **3,** 587–601.
119. Tingley W. G., Roche K. W., Thompson A. K. and Huganir R. L. (1993) Regulation of NMDA receptor phosphorylation of alternative splicing of the C-terminal domain. *Nature* **364,** 70–73.
120. Wenthold R. J., Yokotani N., Doi K. and Wada K. (1992) Immunochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies. Evidence for a hetero-oligomeric structure in rat brain. *J. biol. Chem.* **267,** 501–507.
121. Westerberg E., Monaghan D. T., Kalimo H., Cotman C. W. and Wieloch T. W. (1989) Dynamic changes of excitatory amino acid receptors in the rat hippocampus following transient cerebral ischemia. *J. Neurosci.* **9,** 798–805.
122. Wisden W. and Seeburg P. H. (1993) A complex mosaic of high-affinity kainate receptors in rat brain. *J. Neurosci.* **13,** 3582–3598.
123. Wu K., Huang Y., Adler J. and Black I. B. (1992) On the identity of the major postsynaptic density protein. *Proc. natn. Acad. Sci. U.S.A.* **89,** 3015–3019.
124. Wyllie D. J. A. and Cull-Candy S. G. (1994) A comparison of non-NMDA receptor channels in type-2 astrocytes and granule cells from rat cerebellum. *J. Physiol.* **475,** 95–114.
125. Wyllie D. J. A., Manabe T. and Nicoll R. A. (1994) A rise in postsynaptic Ca^{2+} potentiates miniature excitatory postsynaptic currents and AMPA responses in hippocampal neurons. *Neuron* **12,** 127–138.
126. Wyllie D. J. A., Traynelis S. F. and Cull-Candy S. G. (1993) Evidence for more than one type of non-NMDA receptor in outside-out patches from cerebellar granule cells of the rat. *J. Physiol.* **463,** 193–226.
127. Zukin S. R., Young A. B. and Snyder S. H. (1974) Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system. *Proc. natn. Acad. Sci. U.S.A.* **71,** 4802–4807.

(*Accepted* 14 *August* 1995)