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BIOCHEMICAL AND IMMUNOCYTOCHEMICAL CHARACTERIZATION OF ANTIPEPTIDE ANTIBODIES TO A CLONED GluR1 GLUTAMATE RECEPTOR SUBUNIT: CELLULAR AND SUBCELLULAR DISTRIBUTION IN THE RAT FOREBRAIN

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Abstract—Antibodies were made to synthetic peptides corresponding to residues 253–367, 757–771 and 877–889 of the published amino acid sequence of the rat brain glutamate receptor GluR1 subunit [Hollmann *et al.* (1989) *Nature* **342,** 643–648]. The peptides were synthesized both as multiple copies on a branching lysyl matrix (multiple antigenic peptides) and conventional linear peptides using solid-phase synthesis. Rabbits were immunized with these peptides either without conjugation (multiple antigenic peptides) or following coupling to ovalbumin with glutaraldehyde (monomeric peptides). The antibodies from immune sera were then purified by affinity chromatography using reactigel coupled monomeric peptides. All the rabbits produced good antipeptide responses, and were characterized by immunoprecipitation of solubilized α-amino-3-hydroxy-5-methylisoxazole-4-propionate and kainate binding activity and by their staining patterns on immunoblots.

Antibody to peptide 253–267 specifically immunoprecipitated 12 ± 3, 50 ± 3 and 44 ± 4% of solubilized α-amino-3-hydroxy-5-methylisoxazole-4-propionate binding activity from cortex, hippocampus and cerebellum, respectively. Under identical conditions, antibody against the 877–889 peptide removed 23 ± 4, 9 ± 4 and 15 ± 9% of α-amino-3-hydroxy-5-methylisoxazole-4-propionate binding sites from these areas. On immunoblots of rat brain membrane samples separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, antibodies labelled a 105,000 mol. wt immunoreactive band. GluR1 was immunoaffinity-purified using subunit-specific antibodies against both N-terminal (253–267) and C-terminal (877–889) residues, covalently attached to protein A-agarose. Analysis of the purified product from each column showed a major immunoreactive band, recognized by both sera at 105,000 mol. wt and silver staining identified the same major protein. After exhaustive immunoprecipitation of solubilized membrane samples with antibody against the C-terminal of the subunit, a subpopulation of GluR1 was labelled with antibodies specific for the N-terminal part of the receptor. These observations suggest that the GluR1 subunit consists of at least two isoforms possessing a common N-terminal region but a distinct C-terminus.

Immunocytochemistry, using immunoperoxidase staining, was performed for the GluR1 subunit in rat forebrain with antisera raised against the N-terminal (253–267) and the C-terminal parts (877–889) of the molecule. Both antisera gave a similar distribution of immunoreactivity at the light-microscopic level. Immunoreactivity for the GluR1 subunit was selectively distributed throughout the rat forebrain. The hippocampus, septum, amygdala and olfactory bulb exhibited the strongest immunoreactivity. Immunoreactivity was found in cell bodies and processes of neurons and also in tanicytes, a specialized glial cell type of the hypothalamus.

The CAl region of the hippocampus was particularly rich in GluR1 subunit. Electron-microscopic examination of the CA1 area revealed that immunoreactivity for the GluR1 subunit was present within the endoplasmic reticulum of neurons, at a majority of type I synapses established on dendritic spines, and also at extrasynaptic sites along the dendritic membrane of the pyramidal cells. Immunoreactivity obtained with the antiserum to the N-terminal part (253–267) detected epitopes at the extracellular face of plasma membrane, whereas the antiserum raised against the C-terminal part of the GluR1 subunit (877–889) recognized epitopes at the intracellular face of postsynaptic membranes.

The results demonstrate that the antisera selectively react with GluR1 subunit(s) of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor. This subunit is distributed in an area and cell type specific manner in the forebrain and is present at synapses assumed to use excitatory amino acids as transmitters. Immunocytochemistry at the subcellular level suggests that the C-terminus of the subunit is located at the cytoplasmic face of the synaptic membrane contrary to the currently held model.

Abbreviations: ABC, avidin biotinylated complex; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; DHBT, 3,4-dihydro-4-oxobenzotriazine-3-oxy; DMF, *N,N*-dimenthylformamide; EDT, ethanedithiol; EGTA, 1,2-di(2-aminoethoxy)ethane-N,N′,N′,N′-tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; Fmoc, fluorenylethoxycarbonyl; g_{av}, average g; HPLC, high-performance liquid chromatography; MAP, multiple antigen peptide; NMDA, *N*-methyl-D-aspartate; OPfp, pentafluoreophenoxy; PB, phosphate-buffer; PBS, phosphate-buffered saline; PEG, polyethylene glycol 6000; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulphate; TBS, Tris-buffered saline; TFA, trifluoroacetic acid.

Glutamate receptors are generally thought to be the principal neurotransmitter receptors responsible for excitatory neurotransmission in the CNS. These receptors have been implicated in many CNS functions and neurological disorders.[9,11,15,37,40] Classically these receptors have been divided into different subtypes on the basis of their pharmacological properties. The ionotropic glutamate receptors were grouped into *N*-methyl-D-asparate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate receptors (for reviews, see Refs 1, 40) and metabotropic G-protein coupled glutamate receptors have also been described.[54]

The molecular structure underlying the pharmacological complexity of the glutamate receptors has recently begun to be defined with the cloning and sequencing of the genes coding for the ionotropic receptor subunits[7,17,24,30,44,58] and the metabotropic receptors.[27,35,66] From these studies considerable insights into the structure and protein composition of these receptors have been gained (for reviews, see Refs 1, 18, 19, 59).

The ionotropic AMPA receptor subunits have been subdivided into four main groups on the basis of their cloned gene sequences. GluR1-4 (also known as GluRA-D)[30] is a group of four protein subunits which form ion channels when expressed in cell lines or oocytes and show high-affinity binding for AMPA.[7,13,23,30,42,44,68] Sequence analysis of these proteins suggests that they have an extracellular N-terminus followed by three (possibly four) hydrophobic α-helices (TM1–3), a hydrophilic cytosolic domain, and a further hydrophobic α-helix (TM4) preceding the putative extracellular C-terminus.[25,26] Furthermore electrophysiological experiments in oocytes[58] and cross-linking studies using antisera to the receptor subunits[70] have indicated that these proteins form ion channels by association of subunits into heteromeric oligomers.

In addition to these AMPA receptor subunits, two other groups of proteins designated KA-1, KA-2 and GluR5, 6, 7 have been cloned and these genes may code for the high- and low-affinity kainate binding glutamate receptor subunits, respectively.[2,3,14,22,59,71] More recently the NMDA family of glutamate receptors has also been characterized.[31,43,64]

The mRNAs coding for the different excitatory amino acid receptor subunits have been located throughout the mammalian brain by *in situ* hybridization histochemistry. It is clear from these studies that the same cell type can express several different subunits of the AMPA receptor, but very little is known about their distribution on the surface of the cell, particularly in relation to specific synaptic inputs. For such studies immunocytochemistry with antisera raised against specific subunits is needed. To date at least three studies have utilized this approach to localize the GluR1 subunit. Two of them have used affinity-purified antisera to peptides derived from sequences at the C-terminus of the protein[6,48] and one has used antisera raised to large fusion proteins of this subunit.[52] In the reports by Rogers *et al.*[52] and Blackstone *et al.*[5] immunocytochemistry was performed only at the light microscopy level and the sera were characterized by western blotting of cells transfected with the gene for the subunit and rat brain membranes. The study of Petralia and Wenthold[48] involved both light- and electron-microscopic immunocytochemistry and the affinity-purified antibodies to the C-terminal peptides had been characterized both by western blotting and immunoprecipitation of AMPA binding activity.[70]

In this report we describe the production of antisera to synthetic peptides derived from sequences at both the N- and C-termini of the rat brain GluR1 subunit. Affinity-purified antibodies from these sera, raised to both linear and multiple antigenic peptides (MAPs) have been characterized by immunoblotting, immunoprecipitation of binding activity from different brain areas and immunoaffinity purification of solubilized glutamate receptor subunits. The affinity-purified antibodies have also been used for immunocytochemical staining of the GluR1 subunit at both light- and electron-microscopic levels.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]AMPA (60.0 Ci/mmol) and (vinilidene-^{3}H)-kainic acid (58.0 Ci/mmol) were purchased from Du Pont-New England Nuclear, Boston, MA, U.S.A.; AMPA was obtained from Tocris Neuramin, Bristol, U.K.; Triton X-100, protein A-sepharose CL 4B, kainic acid, high molecular weight markers for electrophoresis and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Company Ltd, Dorset, U.K. Lyophilized human γ-globulin was supplied by Calbiochem, La Jolla, CA, U.S.A.; polyethylene glycol 6000 and 6-amino-*n*-hexonoic acid were from BDH Chemicals Ltd, Poole, England. Reacti-gel (HW-65F) came from the Pierce Chemical Company, Rockford, IL, U.S.A.; peroxidase–conjugated swine immunoglobulins to rabbit immunoglobulins and alkaline phosphatase conjugated affinity-purified immunoglobulins to rabbit immunoglobulins from Dakopatts, Denmark. For solid phase peptide synthesis all reagents were purchased from Novabiochem, Nottingham, U.K.; MAP polylysyl-polydimethylacrylamide/kieselguhr resin was obtained from Peptide and Protein Research, University of Exeter, Exeter, U.K.

Solid-phase peptide synthesis

All peptides including MAPs (Table 1) were synthesized using fluorenylmethoxycarbonyl (Fmoc) chemistry and either pentafluorophenoxy- (OPfp) or 3,4-dihydro-4-oxo-benzotriazine-3-oxy- (DHBT) activated amino acids. In the case of arginine the freshly prepared symmetrical anhydride was used. Novasyn KA resin was used for the production of C-terminal peptides whereas internal sequences were prepared as peptide amides using Novasyn KR resin. MAPs were prepared using a polylysyl-polydimethylacrylamide resin substituted at a ratio of 0.13 mmol Fmoc/g resin.

Peptide synthesis was carried out on a 0.1 mmol scale and all resins were pre-swollen and de-fined in *N*,*N*-dimethylformamide (DMF) prior to loading into a synthesis column. This was attached to a Novasyn Gem semi-automatic peptide synthesis system fitted with counter ion monitoring (Novabiochem, Nottingham, U.K.). The synthesis was

Table 1. Synthetic peptides corresponding to different regions of the GluR1 receptor subunit[24]

Corresponding residues in GluR1 receptor subunit	Predicted location of area	Amino acid sequence of peptide antigen (one-letter code)	Overlapping regions in other subunits
253–267	EC	RTSDSRDHTRVDWKR	—
757–771	IC	KLKNKWWYDKGECGT	GluR2 (761–774) GluR3 (765–778) GluR4 (762–775)
877–889	EC	SHSSGMPLGATGL	—

started by de-protecting the resin with 20% piperidene in DMF and continued using a counter ion monitoring system[73] to determine the progress of the reactions.

Following completion of the synthesis the resins were washed with 100 ml DMF, glacial acetic acid and diethylether and stored at $-20°C$ after drying in vaccuo. Peptides were cleaved from the resin (0.1 g) by treatment with 10 ml 95% trifluoroacetic acid (TFA): 5% distilled water or 20 ml in the case of a MAP for 2–4 h. The following scavengers were used for the indicated peptides: RTSDSRDHTRVDWKR 95% TFA: 2.5% anisole: 2.5% ethanedithiol; SHSSGMPLGATGL 95% TRA: 5% ethanedithiol (EDT). Following cleavage TFA was removed by rotary evaporation, the peptide dissolved in 10% acetic acid and the organic scavengers and protecing groups removed by extraction into diethylether (3×20 ml).

The peptides were lyophilized and purification of the linear peptides were carried out by reverse phase high-performance liquid chromatography (HPLC) on a Pharmacia Pep RP 16/10 column using a gradient from 0% acetonitrile to 90% acetonitrile: 10% water containing 0.1% TFA over 45 min. The flow rate was 5 ml/min and the absorbance of the elutate was monitored at 230 nm. Routinely the major peak of absorbance was the peptide product which was collected by hand. Occasionally a second round of chromatography was needed to achieve the desired purity. The pooled fractions were lyophilized.

MAPs were re-dissolved in 1 ml of 10% acetic acid and desalted by size exclusion on Sephadex G-25 PD-10 columns. The absorbance of the eluate was monitored at 230 nm. Fractions (1 ml) were collected and routinely those eluting as the void fractions (fractions 3–5) were pooled to yield the product. These were lyophilized and used without further purification. The yield of both MAPs and linear peptides varied for each synthesis but the final yield of pure linear peptide was routinely between 5–8 mg/0.1 g resin and of pure MAP 4-10 mg.

The purity of the final products was assessed by mass spectrometry amino acid analysis, and if necessary by N-terminal sequencing.

Peptide–carrier conjugation

Peptide conjugates were prepared by coupling the peptides to ovalbumin with glutaraldehyde as described by Van Regenmortel *et al.*[67] Briefly ovalbumin (1 mg/ml) and 0.12 mg peptide were dissolved in phosphate-buffered saline (PBS) pH 7.2 and mixed with an equal volume of 2% glutaraldehyde. After 1 h the reaction was stopped by the addition of sodium borohydride ($NaBH_4$) to a final concentration of 10 mg/ml and mixed for 1 h. After dialysis against PBS the conjugate was stored in aliquots at $-70°C$.

Generation of antisera

New Zealand white rabbits were immunized with 0.3 mg ovalbumin conjugated peptide or 0.1 mg of multiple antigenic peptide dissolved in PBS (0.5 ml) and emulsified in two volumes of Freund's complete adjuvant. Injections were given subcutaneously at two different sites. Freund's incomplete adjuvant was used for the second and all subsequent injections which were started four weeks after the first injection and continued at two-weekly intervals. Bleeds were taken 10 days after the second and subsequent injections. The titre of antisera was determined by enzyme linked immunosorbent assay (ELISA) using both the MAP and the monomeric peptides bound to the ELISA plates.

Purification of antisera

Antibodies directed against the peptides were purified using the linear peptides immobilized on Reacti-gel (HW-65F) following the procedure recommended by the supplier (Pierce, Rockford, IL, U.S.A.). Antiserum (3–5 ml) was incubated with the immobilized peptide for 12–14 h at 2°C, then washed with 40 ml PBS and 10 ml 1 M NaCl. Following a second PBS wash (20 ml) the bound antibodies were eluted with 50 mM citrate buffer (pH 2.5) and immediately neutralized with 1 M Tris base. The elution of antibodies from, and the washing of, columns was monitored by following the light absorbance of fractions at 280 nm. Aliquots were then dialysed against PBS for 14–20 h and stored at $-70°C$.

Enzyme-linked immunosorbent assay

Peptides were dissolved in coating buffer (13 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) at 20 μg/ml and 50 μl/well used to coat microtitre plates (Falcon 3912 assay plates, Becton Dickinson and Co. Oxnard, CA, U.S.A.) at 4°C for 12 h. The plates were washed three times for 3 min each with 200 μl washing buffer containing 0.05% Tween 20 in PBS (pH 7.2). Blocking of non-specific protein binding was done with 100-μl aliquots of 1% bovine serum albumin in PBS for 1 h at room temperature.

The reaction with different antisera ($1:10^2$–$1:3 \times 10^6$ dilutions) was performed for 1 h at 23°C. The plates were then washed three times with 200 μl PBS containing 0.05% Tween 20 before incubation with 100 μl anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution in PBS) for 1 h. After washing as before the plates were developed by the addition of 100 μl *O*-phenylenediamine (1 mg/ml) and H_2O_2 (0.003%) substrate in 35 mM citric acid and 125 mM Na_2HPO_4 (pH 4.0). After 15 min the reaction was stopped with 50 μl 4 M H_2SO_4. The plates were then read at 492 nm on a Titertek Multiskan microtitre plate spectrophotometer (Flow Laboratories, Inc., McLean, VA, U.S.A.).

Membrane preparation

Whole brains or dissected cortical, hippocampal, cerebellar and spinal cord areas from female Wistar rats (Charles River U.K. Ltd; 200–250 g) were homogenized in 0.32 M sucrose (10 ml/g brain) with a glass–teflon homogenizer.[74] The homogenate was centrifuged at 1000 g_{av} for 10 min and the supernatant fraction removed and centrifuged at 20,000 g_{av} for 20 min. The membrane pellet was washed by resuspension in H_2O following centrifugation at 8000 g_{av} for 20 min. The supernatant and the upper layer of pellet were combined and then recentrifuged at 48,000 g_{av} for 20 min. The pellet was subsequently resuspended three times in 50 mM Tris–citrate (pH 7.4). After a final centrifugation at 54,000 g_{av} for 20 min, the resulting membrane pellet was stored in aliquots at $-70°C$ until use. All procedures for membrane preparation were carried out at 2°C.

Solubilization of membranes

Solubilization was performed according to Hunter *et al.*[28] and Hampson *et al.*[21] 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. PMSF and EGTA were added to a final concentration of 0.1 and 1.0 mM, respectively. The membranes were solubilized by the addition of Triton X-100 to a final concentration of 1% (v/v). The mixture was incubated with constant agitation at 2°C for 30 min and centrifuged at 54,000 g_{av} for 20 min. The resulting supernatant was diaysed against a 100-fold excess of 50 mM Tris–citrate (pH 7.0), containing 0.1% Triton X-100, 20% glycerol and 0.1 mM PMSF, with three changes over a 20-h period. The dialysate was centrifuged at 100,000 g_{av} for 1 h and stored at −70°C until use. Under these conditions 70–74% of the proteins became soluble.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Samples taken for electrophoresis were diluted with equal volume of sample buffer containing 2% sodium dodecylsulphate (SDS), 50 mM Tris–HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol and 0.1% Bromophenol Blue. After incubation for 5 min at 100°C, aliquots containing 50–100 μg protein were applied for gel electrophoresis. Electrophoresis was performed essentially according to Laemmli[32] using 10% polyacrylamide gels. Myosin (205,000 mol. wt), β-galactosidase (116,000 mol. wt), phosphorylase b (94,000 mol. wt), bovine serum albumin (69,000 mol. wt), ovalbumin (43,000 mol. wt) and carbonic anhydrase 29,000 mol. wt) were used as molecular weight markers. Proteins were stained with silver as described by Molnár *et al.*[39]

Electrophoretic transfer and immunoblot analysis

After SDS–polyacrylamide gel electrophoresis the proteins were electroblotted onto poly(vinylidene difluoride) microporus transfer membrane (Immobilon; Millipore Co. Bedford, MA, U.S.A.) using the Multiphor II NovaBlot Electrophoretic Transfer Unit with a discontinuous buffer system and transfer conditions as recommended by the manufacturer (LKB Produkter AB, Bromma, Sweden). Molecular weight markers were stained with Coomassie Blue as described by Matsudaira.[36]

Following transfer, the nitrocellulose sheets were immersed in a solution of 5% (w/v) non-fat dry milk and 1:50 dilution of normal swine serum in PBS (blocking solution) for 12–20 h at 2°C. The sheets were then incubated for 12–20 h with either a 1:200 dilution of the various antisera, or 5 μg/ml of affinity-purified antibody in blocking solution at 2°C. After washing twice for 5 min with PBS containing 0.05% Tween 20, and once with blocking solution the membrane filters were incubated for 2 h at room temperature with alkaline phosphatase conjugated anti-rabbit IgG diluted 1:1000 in blocking solution. The reaction was terminated by washing the sheets three times for 5 min each with PBS containing 0.05% Tween 20. The bound, conjugated IgG was visualized by reaction with a solution containing 20 ml 0.1 M Tris (pH 9.5), 0.1 M NaCl, 0.05 M $MgCl_2$, 50 μl of 150 mg/ml nitroblue tetrazolium in 70% DMF and 35 μl 50 mg/ml 5-brom-4-chlor-3-indolyl-phosphate toluidine salt (Boehringer Mannheim Gmbh, Germany) in DMF for 30 min at room temperature. When the bands became visible the immobilon membranes were rinsed with distilled water and dried for subsequent analysis.

Immunoprecipitation

Immunoprecipitation of GluR1 receptor subunit was carried out using antibody bound to protein A-Sepharose.[69] Briefly, 200 μl of antiserum was incubated with 200 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 PMSF, the antibody–protein A–sepharose complex was incubated overnight with 1.4 ml of Triton X-100 solubilized membrane preparations from different regions of the rat brain. Beads were removed by centrifugation (1000 g_{av} for 2 min), and the supernatants used for [^{3}H]AMPA and [^{3}H]kainate binding assay.

Radioligand binding

Radioligand binding studies were carried out by a centrifugation assay, using polyethylene glycol 6000 (PEG) precipitation. The reaction medium was incubated in a final volume of 0.2 ml in 50 mM Tris–citrate (pH 7.4) for 30 min at 2°C, using either 20 nM [^{3}H]AMPA in the presence of 100 mM KSCN or 10 nM [^{3}H]kainate. Nonspecific binding was determined using 100 μM AMPA or kainate, respectively. Following incubation, 50 μl of 8 mg/ml IgG and 0.2 ml of 30% PEG were added and the samples centrifuged at 10,000 g_{av} in an Eppendorf microfuge for 2 min. The supernatant was aspirated and the pellet rinsed twice superficially using 0.4 ml ice-cold 15% PEG. The pellets were suspended in 1% SDS and then transferred to scintillation vials and counted after the addition of 4 ml Liquiscint (National Diagnostics, Manville, NJ, U.S.A.).

Immunoaffinity purification

Affinity-purified antibody (0.5 mg) was covalently attached to 0.6 ml of protein A-agarose by cross-linking with dimethyl pimelimidate dihydrochloride.[53] Ten millilitres of detergent-solubilized rat membranes (2 mg protein/ml) were incubated with the affinity support for 12 h at 2°C. The resin was washed with 30 ml of 50 mM Tris–citrate (pH 7.4) containing 0.1% Triton X-100, 20% glycerol and 0.1 mM PMSF and bound GluR1 was eluted with 10 ml of 1 M KSCN dissolved in the same medium. The eluted material was dialysed against 3 × 1000 ml 50 mM Tris–citrate (pH 7.4), 0.1% Triton X-100, 20% glycerol and 0.1 mM PMSF before the measurement of [^{3}H]AMPA and [^{3}H]kainate binding activity. For SDS–polyacrylamide gel electrophoresis analysis samples were dialysed extensively against 0.01% SDS in water and then lyophilized.[69,70]

Determination of protein concentration

The protein concentration of membrane samples was measured according to Lowry *et al.*[34] Protein concentration of immunoaffinity purified proteins was estimated by colloidal gold protein assay.[63]

Immunocytochmistry

Thirteen adult female Wistar rats (Charles River U.K. Ltd; 100–200 g) were deeply anaesthetized with sodium pentobarbital (150 mg/kg, i.p.). They were perfused through the aorta with NaCl solution (0.9%, 1 min) followed by 100–200 ml of ice-cold fixative. The fixative contained 4% paraformaldehyde, 0.025–0.1% glutaraldehyde and picric acid (approximately 0.2%) made up in 0.1 M phosphate buffer (PB, pH 7.2).[61] The duration of perfusion varied from 9 to 30 min. Whilst all fixation methods gave some immunoreaction the best results were achieved by using low glutaraldehyde concentrations and a short perfusion time. After perfusion the brain was removed, cut into 3-mm blocks, extensively washed in PB for several hours and then immersed in PB containing 30% sucrose at 4°C for cryoprotection. The blocks were frozen in liquid nitrogen and immediately thawed in PB. This procedure was adopted to improve the penetration of immunoreagents. Sagittal and frontal sections were obtained either with a Vibratome (70–100 μm thick) or with a cryostat (20–40 μm thick), and collected in PB.

Immunocytochemistry was performed using the avidin biotinylated horseradish peroxidase complex (ABC) method. Sections were incubated in 20% normal goat serum (Vector Laboratories) diluted in 50 mM Tris–HCl (pH 7.4), containing 0.9% NaCl (TBS) for 1 h at room temperature or overnight at 4°C. Several dilutions of the purified antibodies were tested, but the best results were obtained with affinity-purified antibodies at a final protein concentration

of 1 μg protein/ml for the antiserum raised against the N-terimal part of the subunit (253–267), and of 4 μg protein/ml for the antiserum raised against the C-terminal part (877–889). After several washes in TBS, sections were incubated in biotinylated goat anti-rabbit IgG 1:50 (Vector Laboratories), then in ABC, both diluted in TBS, for 2 h. Triton X-100 (0.3%) was present only in the buffers used for the light-microscopic studies. Peroxidase reaction was carried out with 3,3′-diaminobenzidine tetrahydrochloride (0.05% in 50 mM Tris–HCl buffer, pH 7.6) as chromogen and 0.01% of hydrogen peroxide as oxidant. Sections for light-microscopy were mounted on gelatine-coated slides, allowed to dry overnight, dehydrated in graded ethanol and xylene, and finally covered in DePeX under coverslips. Usually the peroxidase reaction end-product was slightly enhanced by treatment with OsO_4 solution (approx. 0.04% in PB, 10 min) before dehydration. Occasionally, sections were treated with a stronger OsO_4 solution (1% in PB, 40 min) before dehydration.

Sections for electron-miscroscopic studies were successively immersed in OsO_4 solution (1% in PB, 1 h), in distilled water for washing, in uranyl acetate solution (1% in distilled water, 1 h), in graded ethanol and propylene oxide for dehydration, and finally impregnated overnight in Epoxy resin (DURCUPAN ACM, Fluka Chemicals Ltd, Buchs, Switzerland). The sections were flat-embedded between slide and coverslip and the resin was allowed to polymerize at 56°C for 48 h. After removing the coverslip, areas of the CA1 region of the hippocampus were re-embedded for ultrathin sectioning. No lead staining was used.

Control experiments for immunocytochemistry were performed with the following outcome. The omission of primary antiserum during the immunocytochemical procedure resulted in the removal of all staining. There was no reaction with the pre-immune sera (1:1000 dilution) that resembled the reaction obtained with the purified antibodies. Pre-absorption of the primary antisera with the corresponding linear synthetic peptide (20 μg peptide/ml, 5 h incubation at room temperature) before application to the sections resulted in the disappearance of specific labelling. All of these experiments confirmed the specificity of the immunocytochemical method.

RESULTS

Antibody production

To produce antibodies selective for the GluR1 subunit, synthetic peptides corresponding to specific sequences were used as antigens. Amino acid sequences of peptides used for antibody production are listed in Table 1. Residues 253–267 are located on the predicted large extracellular amino terminal part of the protein, whilst residues 877–889 are near to the carboxyl terminus. Neither of these sequences have homologies with the other GluR2-4 subunits. Residues 757–771 are present on the putative intracellular loop between transmembrane domains III and IV. This sequence is common to the other glutamate receptor subunits, i.e. GluR2 (761–774), GluR3 (765–778) and GluR4 (762–775). Since all of these regions of the molecule are hydrophilic and two of them are proposed to be exposed to the surface of the cell[24,30] it was expected that antisera to these sequences would recognize the native receptor subunit and detect the GluR1 subunit by immunocytochemistry.

The efficacy of immunization with MAPs was compared to the conventional approach using monomeric peptides coupled to ovalbumin. MAPs consist of multiple copies of peptides that are synthesized as single units on a branching lysyl matrix using solid-phase peptide synthesis.[38,65] The molecular weight of the MAP is large (typically 13,000–15,000 mol. wt from a 15-amino acid sequence) and can elicit an immune response without coupling to carrier protein.[38] Peptides GluR1 253–267, 757–771 and 877–889 were also synthesized as conventional monomeric peptides and shown to be >95% pure by HPLC analysis. Each was coupled to ovalbumin and the conjugate employed for the production of anti-peptide antibodies in rabbits. Two rabbits were immunized with each peptide and the overall immune response in each animal was similar. The immune sera were initially screened by ELISA using both the immobilized MAP and the monomeric peptide as the target antigen. The immune response induced by MAPs was generally faster and gave a higher titre serum than that against ovalbumin-conjugated peptides (not shown). An example of the immune response for a MAP peptide (GluR1 253–267) is illustrated in Fig. 1. Antisera raised against MAPs also showed stronger immunoreactivity to the GluR1 subunit on western blots and gave

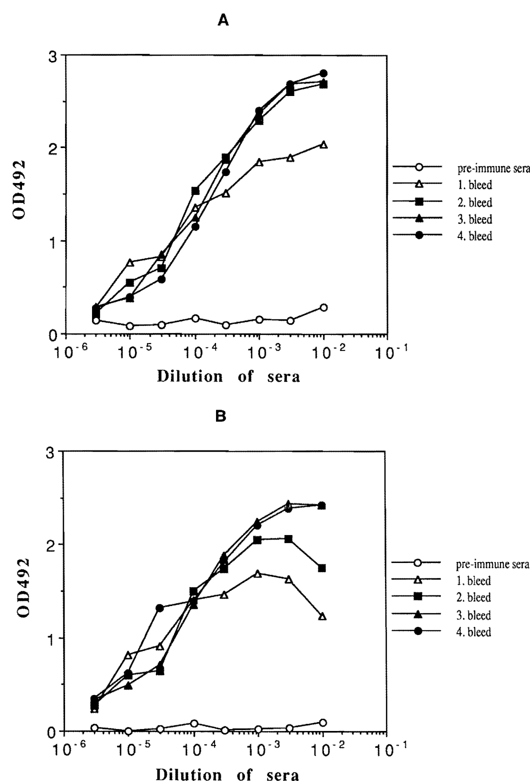

Fig. 1. Development of the immune response following immunization of rabbits with MAP peptide GluR1 253–267. Rabbits were injected as described in Experimental Procedures at 14-day interval and bled 10 days following the second and subsequent immunizations. The immune response was measured by ELISA with either MAP (A) or conventional monomeric peptide (B) as antigen and all determinations were performed in triplicate.

a more specific immunocytochemical detection of the protein in brain sections than those directed against the linear peptides. Therefore MAP antisera were used for all further experimentation. Antibodies raised in all immunized rabbits were affinity-purified from different bleeds and were tested and gave similar results. In this report we used the bleed-out sera of rabbits 012 (4. bleed on Fig. 1) and 017 immunized with MAPs 253–267 and 877–889, respectively.

*Antibodies to GluR*1 *identify a* 105,000 *mol. wt protein in the rat CNS*

Affinity-purified antibodies raised against residues 253–267 and 877–889 recognized a major band (105,000 mol. wt) in immunoblots which corresponds to the predicted molecular weight of the GluR1 subunit (Fig. 2). The labelling was completely prevented when the antibody was reacted in the presence of the appropriate peptide (10 μg/ml; not shown). Although all the rabbits produced good antipeptide responses in ELISA, sera raised against residues 757–771 did not recognize the GluR1 subunit in rat brain membranes, nor did they react in any other tests and therefore these sera were not examined further.

To study the regional distribution of the GluR1 subunit in the CNS, membranes prepared from rat cerebral cortex, hippocampus, cerebellum and spinal cord were immunoblotted following SDS–polyacrylamide gel electrophoresis. Both antibodies stained a band at 105,000 mol. wt in cerebral cortex, hippocampus and cerebellum (Fig. 2), but did not detect any protein in the spinal cord. However, despite loading equal quantities of membrane protein in each track, differences were seen in the signals derived from different brain areas. Both

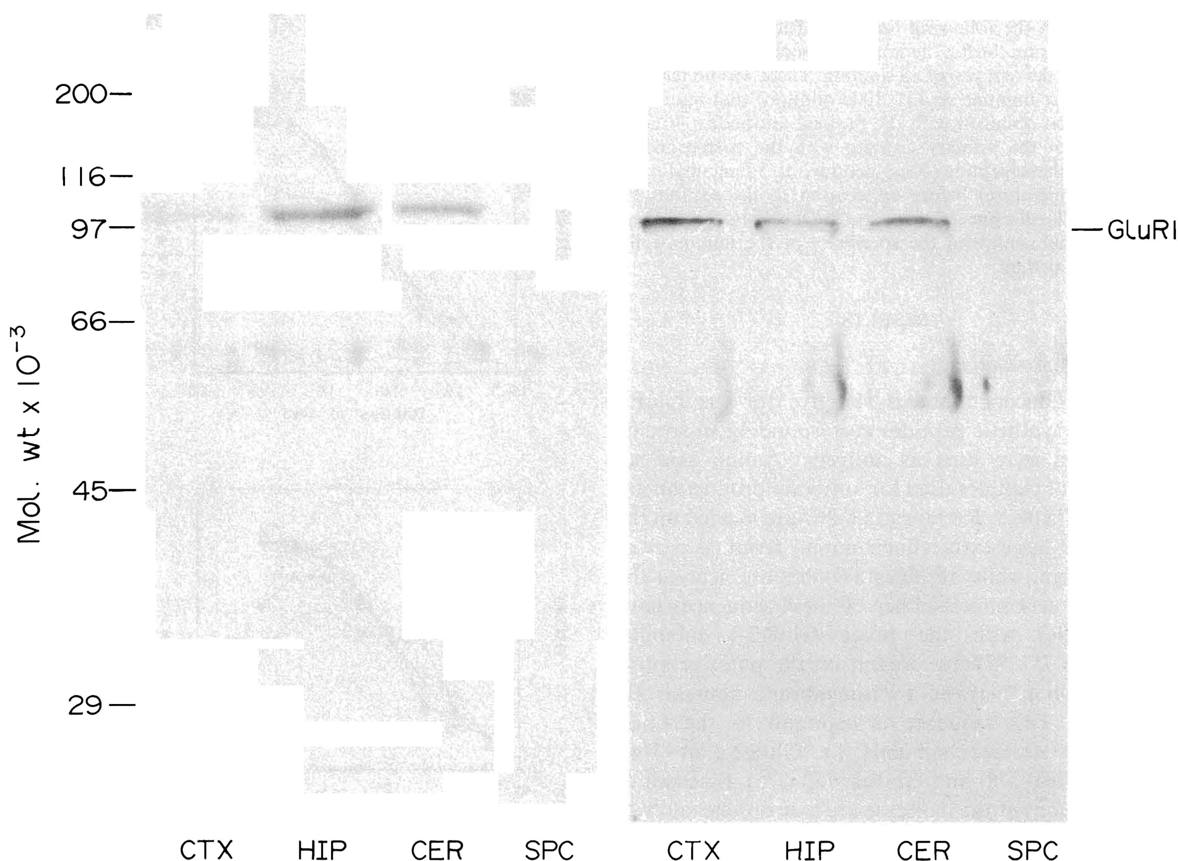

Fig. 2. Immunolabelling of GluR1 subunit from different regions of rat brain by anti-peptide antibodies raised against residues 253–267 and 877–889. Crude membrane samples prepared from cerebral cortex (CTX), hippocampus (HIP), cerebellum (CER) and spinal cord (SPC) were immunolabelled with purified anti-GluR1 253–267 and 877–889 antibodies. One hundred microgrammes of membrane protein was applied to each lane. For immunoblot analysis 5 μg/ml of affinity-purified antibody was used. The positions of the molecular weight standards are indicated on the left.

antibodies gave strong reactions in membranes from hippocampus and cerebellum. The antibody raised against residues 877–889 gave stronger reactions in membranes from cerebral cortex than the antibody against residues 253–267.

Immunoprecipitation of GluR1 subunit

Anti-peptide antibodies attached to protein A-agarose were used to immunoprecipitate [^{3}H]AMPA binding sites from whole rat brain, cerebral cortex, hippocampus and cerebellar membranes which had been solubilized under conditions shown to yield soluble binding sites for both AMPA and kainate.[21,28] The use of this solubilization protocol allowed the determination of the effect of immunoprecipitation on the binding of both ligands in the same antibody-treated sample. Solubilized membrane preparations were incubated with the antibodies coupled to protein A-Sepharose[69,70] and antibody binding was assessed by determining the remaining [^{3}H]AMPA or [^{3}H]kainate binding in the supernatant relative to controls prepared with pre-immune sera. Antibodies to both GluR1 253–267 and GluR1 877–889 were able to recognize the solubilized receptor subunit in a native conformation, as demonstrated by immunoprecipitation of AMPA binding activity from whole rat brain (Table 2).

The anti-GluR1 253–267 antibody immunoprecipitated $37 \pm 6\%$ of the total AMPA binding activity of whole rat brain whereas anti-GluR1 877–889 antibody removed only $10 \pm 4\%$ of all AMPA binding sites. In the membranes dissected from different brain areas anti-GluR1 253–267 removed AMPA binding as follows: $12 \pm 3\%$ in cortex, $50 \pm 3\%$ in hippocampus and $44 \pm 4\%$ in cerebellum (Table 2). In contrast the anti-GluR1 877–889 removed different proportions of the AMPA binding activity from the same areas, namely $23 \pm 4\%$ in cortex, $9 \pm 4\%$ in hippocampus and $15 \pm 9\%$ in cerebellum (Table 2). In addition the [^{3}H]kainate binding activity of these immunoprecipitated samples was tested and it was found that there was no significant change in this activity with either antiserum (data not shown).

Purification of the GluR1 subunit by immunoaffinity chromatography

The detergent-solubilized membrane preparation was incubated with the antibodies which were covalently attached to protein A–sepharose. Both columns retained [^{3}H]AMPA binding sites albeit to different degrees (Table 3). The bound protein was eluted with 1 M KSCN and the released [^{3}H]AMPA binding activity measured (Table 3). Approximately 28 and 40% of the retained binding sites were eluted from the anti-GluR1 253–267 and 877–889 immunoaffinity columns, respectively.

Eluates from these affinity columns were subjected to 10% SDS–polyacrylamide gel electrophoresis and western blot analysis with purified GluR1 specific anti-253–267 and anti-877–889 antibodies (5 μg/ml). As demonstrated in Fig. 3 a specific band at 105,000 mol. wt corresponding to the GluR1 subunit, was observed in samples immunopurified on both affinity resins and each column eluate yielded an immunoreactive band at 105,000 mol. wt following western blotting with both anti-peptide antibodies (253–267 and 877–889). Occasionally aggregated proteins at the top of the gel and a band present in both silver-stained and immunostained receptor eluates were observed at 60,000 mol. wt. Since the alkaline phosphatase-conjugated second antibody reacts weakly with this 60,000 mol. wt band in the absence of a specific antibody, it probably represents IgG heavy chain derived from antibody which leached from the columns. Similar antibody leaching has also been reported in previous studies.[52,70]

Table 2. Immunoprecipitation of GluR1 subunit

Epitope of antibody (residues)	Source of solubilized receptor	[^{3}H]AMPA binding activity: Control (pmol/mg)	Not bound to antibody (pmol/mg)	Yield (%)	% [^{3}H]AMPA binding immuno-precipitated
GluR1 (253–267)	Whole-brain	0.95 ± 0.12	0.60 ± 0.04	63 ± 6	37 ± 6
	Cortex	1.87 ± 0.03	1.65 ± 0.12	88 ± 3	12 ± 3
	Hippocampus	3.80 ± 0.13	1.89 ± 0.14	50 ± 3	50 ± 3
	Cerebellum	1.00 ± 0.09	0.56 ± 0.03	56 ± 4	44 ± 4
GluR1 (877–889)	Whole-brain	1.06 ± 0.02	0.95 ± 0.06	90 ± 4	10 ± 4
	Cortex	1.60 ± 0.05	1.23 ± 0.09	77 ± 4	23 ± 4
	Hippocampus	2.04 ± 0.06	1.87 ± 0.10	91 ± 4	9 ± 4
	Cerebellum	0.68 ± 0.08	0.58 ± 0.07	85 ± 9	15 ± 9

Immunoprecipitation of GluR1 subunit, measured with [^{3}H]AMPA (20 nM). Receptors were solubilized from rate whole-brain, cortical, hippocampal and cerebellum 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 AMPA binding remaining (Yield) was determined as described in Experimental Procedures. The percentage of AMPA binding specifically immunoprecipitated was calculated by subtraction of this value from the total binding activity (100%). There was no significant change in the [^{3}H]kainate (10 nM) binding activity of the same samples (not shown). All determinations of binding were performed in triplicate and the values are the mean $\pm$ S.D. from three independent determinations.

Table 3. Purification of the GluR1 subunit by immunoaffinity chromatography

Epitope of antibody (residues)	Receptor	Protein (mg)	[^{3}H]AMPA binding activity		
			pmol/mg	Total (pmol)	Yield (%)
GluR1	Solubilized	20	1.11 ± 0.05	22.3 ± 0.9	100
(253–267)	Not bound to column	20	0.91 ± 0.02	18.2 ± 0.4	82 ± 2
	Bound to column	ND	ND	4.1 ± 0.6	18 ± 2
	Column eluate	0.017 ± 0.012	174 ± 160	1.0 ± 0.6	5 ± 3
GluR1	Solubilized	20	0.96 ± 0.10	19.3 ± 2.0	100
(877–889)	Not bound to column	20	0.87 ± 0.13	17.4 ± 2.6	90 ± 4
	Bound to column	ND	ND	3.7 ± 1.3	10 ± 4
	Column eluate	0.018 ± 0.012	82 ± 48	0.9 ± 0.1	4 ± 1

Glutamate receptor subunits were solubilized from rat brain using 1% Triton X-100, 0.5 M potassium phosphate (pH 7.0), 20% glycerol, 0.1 mM PMSF 1.0 mM EGTA, and GluR1 subunit purified on anti-253–267 or anti-877–889 antibody affinity columns as described in Experimental Procedures. Specific [^{3}H]AMPA and [^{3}H]kainate-binding activity was determined by centrifugation assay using PEG precipitation. There was no significant removal of [^{3}H]kainate binding activity from the solubilized samples during immunoaffinity purification (not shown). ND, not determined.

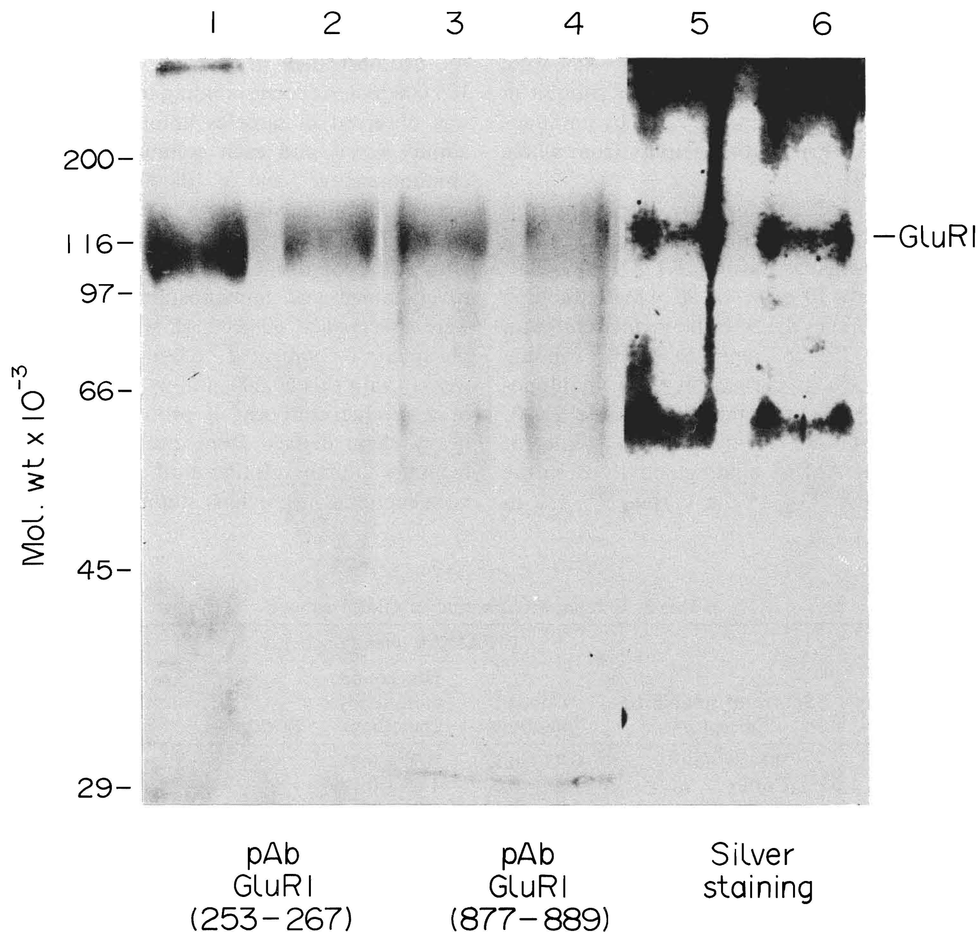

Fig. 3. Immunoaffinity purification of GluR1 subunit by antibody affinity chromatography. Solubilized membranes of rat brains were applied to an anti-GluR1 253–267 (lanes 1, 3, 5) or 877–889 (lanes 2, 4, 6) antibody affinity column. The columns were washed to remove nonspecifically bound proteins, and the receptor subunit eluted by 1 M KSCN as described in Experimental Procedures. The purified receptor subunits were immunoblotted with affinity-purified anti-GluR1 253–267 (lanes 1, 2) or 877–889 (lanes 3, 4) antibody at a final concentration of 5 μg/ml. Lanes 5 and 6 are silver-stained affinity-purified GluR1 subunits. The left-hand side shows the positions of the molecular weight standards.

Exhaustive immunoproecipitation of solubilized membrane samples with anti-GluR1 antibodies

Solubilized rat brain membranes (500 μg) were incubated for 12 h at 2°C with either an anti-GluR1 253–267 or an anti-GluR1 877–889 antibody affinity support. The solubilized membranes were then subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis using both affinity-purified GluR1 antibodies (Fig. 4). The antibody raised against residues 253–267 immunoprecipitated all the GluR1 subunits since neither antibody detected any protein in the solubilized membranes immunoadsorbed with this antibody (Fig. 4; lanes 3, 6).

However, immunoadsorbtion of the membranes by the C-terminal antibody 877–889, whilst removing all immunoreactivity to itself, failed to remove all the protein immunoreactive with the N-terminal antibody (Fig. 4; tracts 2, 5). These results could suggest the existence of at least two isoforms of GluR1 possessing a common N-terminal region but a distinct C-terminus.

Immunocytochemical localization of the GluR1 subunit in the rat forebrain

The distribution of the GluR1 subunit was examined throughout the rat forebrain using both antisera to the N- and the C-terminal part of the GluR1 subunit. The distribution of immunoreactivity obtained with these two affinity-purified antibodies was similar. The immunolabelling for the GluR1 subunit was selectively distributed, and the hippocampus exhibited the strongest immunoreactivity (Fig. 5A).

Immunoreactive cells and processes could be observed in all layers of the cortex except in layer I

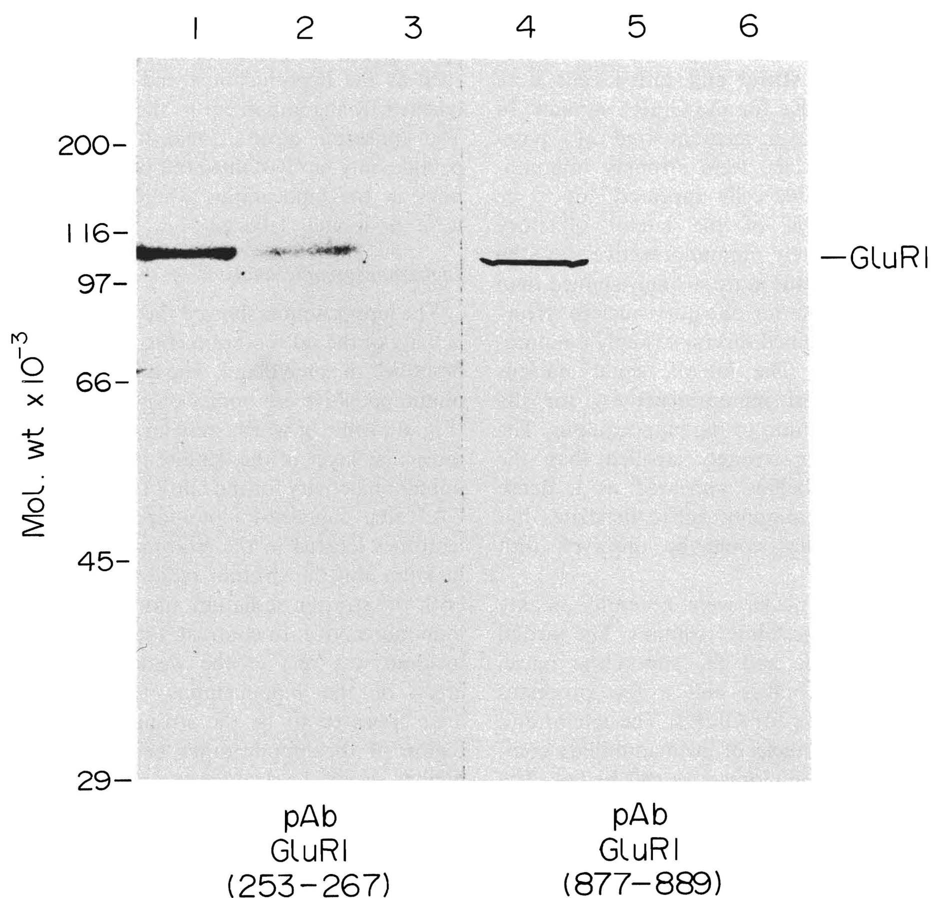

Fig. 4. Exhaustive immunoprecipitation of solubilized membrane samples with antibodies raised against residues 253–267 and 877–889 of the GluR1 subunit. Affinity-purified antibodies (0.5 mg) were covalently attached to 0.6 ml of protein A-agarose by cross-linking with dimethyl pimelimidate dihydrochloride.[53] A small amount of detergent solubilized rat brain membrane (250 μl, 2 mg protein/ml) was incubated with the affinity support for 12 h at 2°C. Following incubation, samples were centrifuged at 2,000 g_{av} for 2 min, and the resulting supernatant was subjected to SDS–polyacrylamide gel electrophoresis. To each lane 50 μg protein was applied. For immunoblot analysis of the solubilized membrane samples 5 μg/ml of affinity purified GluR1 antibody was used. Lanes 1 and 4, control solubilized membrane samples; lanes 2 and 5, solubilized samples after exhaustive immunoprecipitation with anti-GluR1 877–889 antibody; lanes 3 and 6, solubilized samples after exhaustive immuno-precipitation with anti-GluR1 253–267 antibody. Lanes 1–3 are immunostained with anti-GluR1 253–267 whilst lanes 4–6 are labelled with anti-GluR1 877–889 antibody. The positions of the molecular weight standards are indicated on the left.

(Fig. 5C). The neuropil of layers II, III, V and VI were most intensively labelled than that of layer IV, or layer I which was the weakest cortical layer (Fig. 5C). Of all cortical areas the piriform and entorhinal cortex exhibited the strongest immunoreactivity. Numerous non-pyramidal cell bodies and their dendrites were more strongly labelled (Fig. 5B) than the pyramidal cells.

All the nuclei of the amygdaloid complex were immunoreactive for the GluR1 subunit but showed varying intensities of labelling. Immunoreactivity was present in both cell bodies and neuropil. The basolateral, basomedial, intercalatus and cortical nuclei showed the strongest staining, whereas the central and anterior nuclei were only moderately immunoreactive. The lateral and medial nuclei were weakly labelled.

The olfactory system related structures were rich in GluR1 subunit. In the olfactory bulbs, the glomeruli and the external plexiform layer were strongly labelled whilst the internal granular layer was weakly stained. Mitral and tufted cells were strongly immunoreactive for the GluR1 subunit. In the internal granular layer, medium-sized cells, possibly the short-axon cells, were strongly immunoreactive but the granule cells appeared not to be labelled. The neuropil of the lateral olfactory nucleus was moderately immunoreactive for the GluR1 subunit, but it was more strongly stained than the neuropil of the anterior olfactory nucleus. However, both nuclei contained several strongly immunoreactive cell bodies. The lateral septal nucleus exhibited the strongest immunoreactivity for the GluR1 subunit in addition to the hippocampus. The dorsal part was more strongly labelled than the ventral part. The labelling appeared as a dense network of strongly immunoreactive dendrites but no positive cell bodies could be observed (not shown).

The hypothalamic nuclei were generally weakly immunoreactive for the GluR1 subunit. The medial magnocellular preoptic and the supraoptic nuclei contained large cell bodies and a few processes weakly immunoreactive for GluR1. The lateral and the tuberomammilary nuclei of the mammillary complex also exhibited immunoreactive cell bodies. The arcuate nucleus was immunonegative apart from a very strong reaction in the tanicytes.

The habenular complex was the only structure of the thalamus containing detectable immunoreactivity for the GluR1 subunit. Here the staining was more intense in the medial habenular nucleus than in the lateral habenular nucleus and both cell bodies and the neuropil were labelled.

The neuropil of the caudate–putamen and the accumbens nuclei were moderately immunoreactive and contained several small and medium-sized immunoreactive cell bodies. In contrast, the globus pallidus remained immunonegative. The subthalamic nucleus showed significant and diffuse immunoreactivity in its neuropil.

Generally the two antisera gave the same pattern of immunoreactivity in the rat brain, however the intensity of staining with antiserum to the C-terminal peptide was weaker than that obtained with antiserum to the N-terminal peptide. This meant that brain areas weakly labelled with the latter antiserum, such as the hypothalamus and habenula, were not labelled by the antiserum to the C-terminal peptide. The antiserum directed against the the C-terminal peptide only rarely stained cell bodies, apart from the hilus in the hipocampus where non-principal cells were moderately labelled.

Light-microscopic analysis of the hippocampus

The hippocampus showed the strongest immunoreactivity of the rat forebrain (Fig. 5A) and is therefore described in some detail. The hilus contained several immunopositive cell bodies of non-principal neurons (Fig. 6), some of which were large and fusiform. The molecular layer of the dentate gyrus exhibited dense immunoreactivity for the GluR1 subunit (Fig. 6). The CA3 area contained immunopositive non-principal neurones located in the stratum oriens, the stratum lucidum and the stratum radiatum. The neuropil of both the stratum radiatum and oriens were strongly immunoreactive. In contrast, the neuropil of stratum lucidum was one of the weakest immunoreactive areas of the hippocampus (Fig. 6). The CA1 area appeared to be the strongest immunoreactive region of the hippocampus (Fig. 6). All the CA1 regions except the stratum pyramidale, were intensely

Fig. 5. Micrographs showing the distribution of immunoreactivity for the GluR1 subunit detected in the rat forebrain (frontal sections) by the antiserum to the N-terminal part of the subunit. (A) The hippocampus (H) exhibits the strongest immunoreactivity. The nuclei of the posterior region of the amygdala show differing degrees of immunoreactivity; BL, basolateral nucleus; BM, basomedial nucleus; C, central nucleus; Co, cortical nucleus; L, lateral nucleus; M, medial nucleus. Arrows indicate digits made by the intercalatus nucleus. The caudate–putamen (CP) and the subthalamic nucleus (S) are moderately immunoreactive for the GluR1 subunit, but the globus pallidus (G) does not show labelling. No positive labelling can be observed in the thalamus (T) apart from the habenulae (Hb). (B) Higher magnificantion of the piriform cortex (PCx), seen also in A shows numerous strongly immunoreactive non-pyramidal neurones (black arrows). The basolateral nucleus of the amygdala (BL) exhibits very strongly immunoreactive neuropil where occasional strongly labelled cell bodies can be seen (white arrows). (C) In the temporal area of the neocortex several cell bodies (arrows) are strongly immunoreactive for the GluR1 subunit. Peroxidase reaction end-product was enhanced with 1% osmium tetroxide solution. Scale bars = 1 mm (A); 200 μm (B); 100 μm (C).

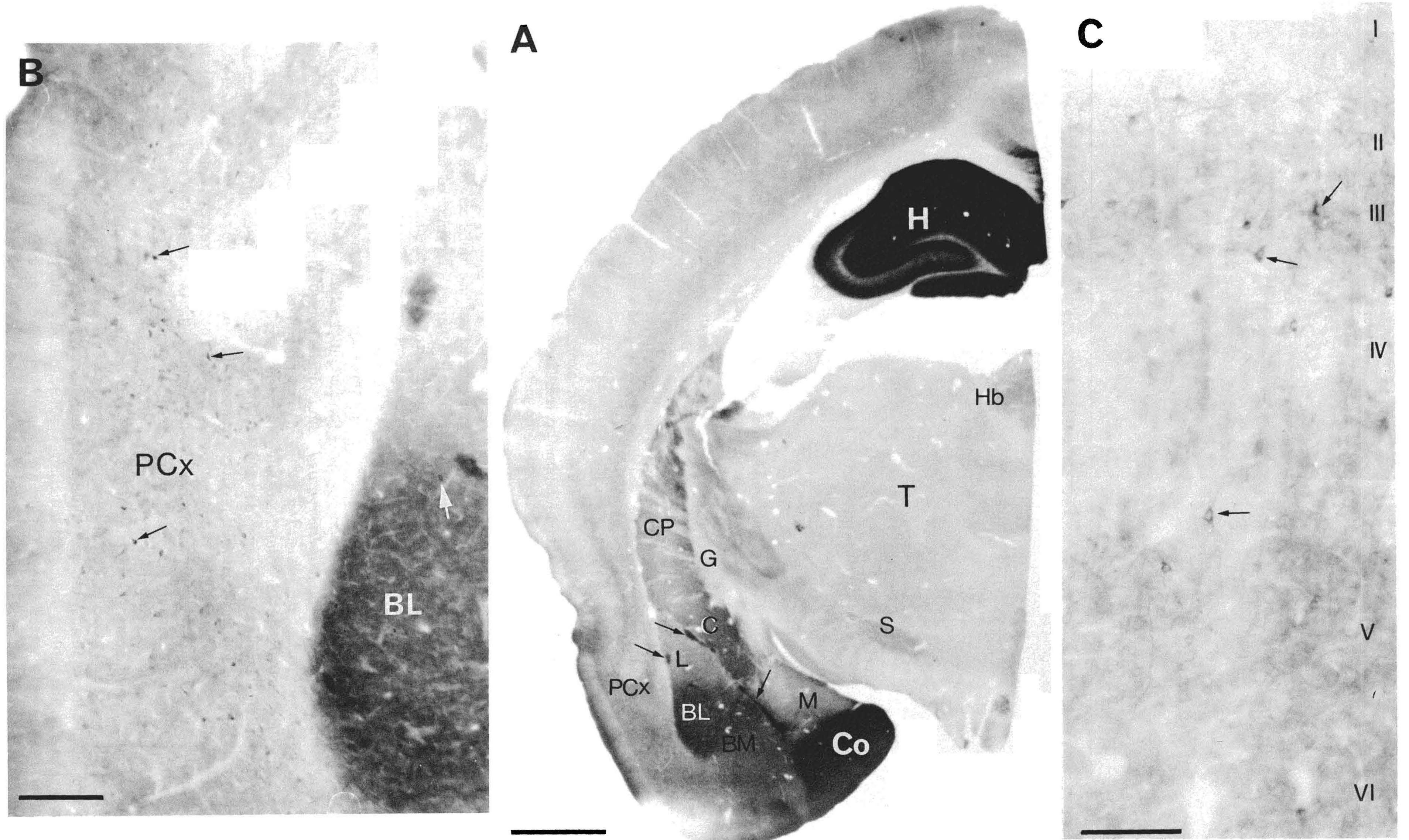

Fig. 5

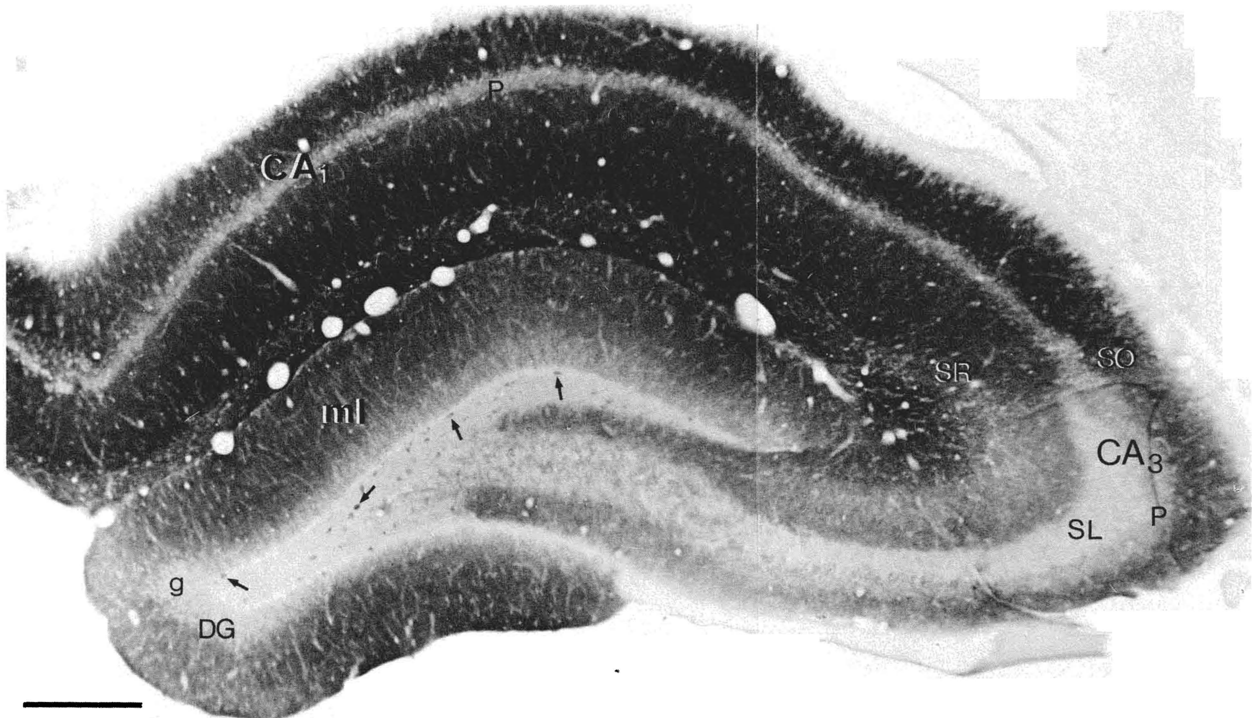

Fig. 6. Immunoreactivity detected by the antiserum to the N-terminal part of the GluR1 subunit in a frontal section of the dorsal hippocampus. The molecular layer (ml) of the dentate gyrus (DG) and the dendritic field of the pyramidal cells [stratum oriens (SO)] stratum radiatum (SR) of the CA1 area are strongly immunoreactive, but the layer of the granule cells (g) and the stratum pyramidale (P) exhibit weak immunoreactivity. The CA3 area is labelled more weakly and in this region, the termination area of the mossy fibres (stratum lucidum, SL) is very weakly immunoreactive for the GluR1 subunit. Strongly immunopositive non-principal neurons are observed in all parts of the hippocampus but they are more conspicuous in the hilus (arrows) whose neuropil shows very weak immunoreactivity. Scale bar = 300 μm.

immunoreactive. In order of decreasing intensity of staining, the stratum oriens, the stratum radiatum and the stratum lacunosum-moleculare could be delineated. In both CA1 and CA3 areas, the stratum pyramidale was weakly labelled and peroxidase reaction end-product could be seen around the pyramidal cell bodies. The neuropil of the subiculum was very strongly labelled and contained numerous immunoreactive cell bodies and processes.

Subcellular localization of immunoreactivity in the CA1 region of the hippocampus

Immunoreactivity detected with antiserum raised against the N-*terminal part of the subunit* (*residues*: 253–267). Immunoreactivity for the GluR1 subunit could be observed in the soma of non-pyramidal cells in all laminae (Fig. 7A). The peroxidase end-product was found inside the cisternae of the endoplasmic reticulum (Fig. 7B) and within the nuclear membrane (Fig. 7A, B). Occasionally immunoperoxidase reaction could be observed at the extracellular face of the somatic membranes of these non-pyramidal neurons, but this staining was never associated with a synaptic contact or found apposed to an axon terminal. In the alveus and in both the stratum oriens and radiatum most type I synapses[20] between spines and presynaptic axon terminals were immunoreactive. Here peroxidase end-product was located inside the synaptic cleft (Fig. 8A) but sometimes it could also be observed at the extracellular surface of non-synaptic membranes of the spine. The peroxidase reaction end-product was also found between directly apposed proximal dendrites of pyramidal cells (Fig. 8B) or between dendrites and soma of pyramidal cells. At these sites there was no evidence for the presence of synapses or even for the presence of axon terminals and this suggested the presence of immunoreactivity for the GluR1 subunit at non-synaptic locations. No immunoreaction could be observed within glial cells, axon terminals or axons in the CA1 region of the hippocampus.

Immunoreactivity detected with antiserum raised against the C-*terminal part of the subunit* (*residues*: 877–889). The immunoreactivity detected with the antiserum to the C-terminal part of the subunit was located intracellularly at the inner face of postsynaptic membranes. Most of the type I synapses established between spines and axon terminals were immunoreactive (Fig. 8C). The peroxidase reaction end-product covered the postsynaptic densities of the spines and also diffused into the cytoplasm of the spine with decreasing intensity (Fig. 8C). No positive

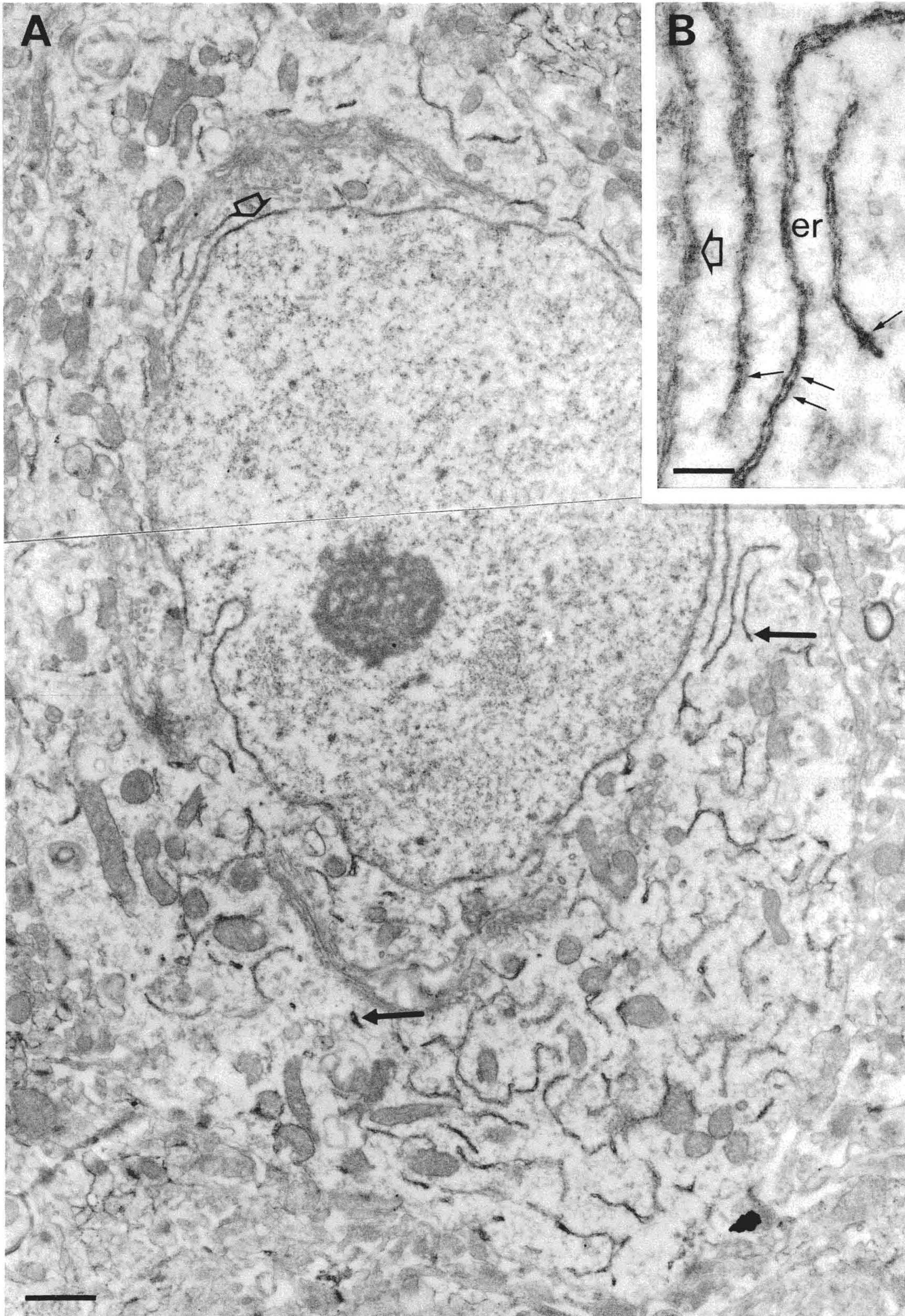

Fig. 7. Intracellular distribution of the immunoreactivity for the GluR1 subunit using the antiserum raised to the N-terminal part of the GluR1 subunit. (A, B) Electron micrographs of a non-principal neuron in the CA1 area of the hippocampus showing immunoreactivity for the GluR1 subunit in the endoplasmic reticulum (er) (arrows) or in the nuclear membrane (open arrows). At higher magnification (B) it can be seen that the reaction end-product is within the cisternae demonstrating epitopes within the cisternal face of the endoplasmic reticulum membrane. Scale bars = 1 μm (A); 0.25 μm (B).

labelling could be observed in large dendrites or soma.

DISCUSSION

Characterization of antibodies

In this report the production and characterization of two different antisera to synthetic peptides containing sequences specific for the GluR1 subunit have been described. These sera were raised using both the linear peptide[46,72] and the MAP system described by Tam and Zavala.[65] The use of the latter system yielded higher-titre sera faster than the linear peptide immunizations as has been reported previously.[38] In addition the MAP antisera gave better reactivity towards the native and denatured proteins. One potential problem in the use of the MAP peptides for immunization is the difficulty of purifying them and eliminating any deletion sequences which may have occurred during synthesis. The strategy adopted here of immunizing with a partially purified MAP peptide and then affinity-purifying the serum with an immobilized linear peptide obviates that problem since the linear peptide can be purified to homogeneity. We believe that the novel strategy described here will be generally useful for the production of antibodies to neurotransmitter receptors.

The peptides used for immunization were chosen for their hydrophilicity from hydropathy plots based on the cloned sequence.[24] Two of these peptides were to internal sequences of the protein and one was to the C-terminal 13 amino acids (Table 1). Two of the three, peptides 253–267 and 877–889, yielded sera that reacted with both the denatured and native protein as determined by western blotting and immunoprecipitation of the solubilized receptor. These sera would be expected to be GluR1 specific, since both of these sequences are unique to this subunit when compared to presently known proteins. The mol. wt of 105,000 observed following western blotting is close to the predicted molecular weight for this receptor subunit,[24] and in this finding our antibodies were similar to the others produced to GluR1 specific sequence of C-terminal peptides[5,6,70] and fusion proteins.[52]

Both of the antisera described here specifically immunoprecipitated [^{3}H]AMPA binding from solubilized rat brain membranes, and neither of them removed [^{3}H]kainate binding, which is in agreement with the ligand binding characteristics of the cloned GluR1 receptors.[30] Similar results were obtained in the study of Wenthold *et al.*[70] in which antisera to sequences of all four of the GluR1-4 subunits were produced successfully to C-terminal peptides. Since both the antiserum to the C-terminal and to the N-terminal sequences (253–267) can immunoprecipitate the solubilized receptor, this suggests that this portion of the molecule is exposed in the solubilized receptor preparations. However, in the study of Blackstone *et al.*,[6] an antiserum produced to the C-terminal sequence of GluR1 did not react with the native protein in solubilized form. This discrepancy may be explained by the different membrane solubilization protocol used by these authors. As in the study of Wenthold *et al.*,[70] both of our antisera were suitable for immunoaffinity purification of the receptor, albeit with a low yield of binding activity. The purified protein from both affinity columns had a molecular weight of 105,000 mol. wt and was recognized by both antibodies which indicates that the antibodies are indeed reacting with the same molecule.

The extent to which the antiserum to the C-terminus of the subunit was able to immunoprecipitate AMPA binding activity from solubilized whole-brain membranes ($10 \pm 4\%$) was comparable to that reported by Wenthold *et al.*[69,70] Similar results were obtained with the serum to the N-terminal sequence (253–267) only here more of the AMPA binding activity ($37 \pm 6\%$) was removed by immunoprecipitation. Immunoprecipitation studies on dissected brain areas showed that the N-terminal antiserum preferentially removed AMPA binding from the hippocampus and cerebellum, whilst the C-terminal antiserum removed binding optimally from the cortex (Table 2). Neither antibody removed kainate binding from any brain area. These results together with the affinity purification data above, suggest that the two sera, whilst reacting with the same molecule, could be seeing different isoforms of the protein and this was tested by exhaustive immunoprecipitation, with each serum, of solubilized whole brain membranes. The results clearly show that the N-terminal serum to peptide 253–267 removed all of the GluR1 subunit

Fig. 8. Synaptic and extrasynaptic distribution of immunoreactivity for the GluR1 subunit using antiserum to the N-terminal part of the subunit. Electron micrographs of the stratum radiatum in the CA1 region of the hippocampus. (A) Strongly immunoreactive type I synapse between a dendritic spine (s) and an axon terminal (at). The peroxidase reaction end-product fills the synaptic cleft (sc), between the pre- and postsynaptic membranes, but does not label the thick postsynaptic density (pd), demonstrating that the epitopes are at the extracellular face of the plasma membrane. (B) Immunoreactivity is present at extrasynaptic membrane (arrows) between two directly apposed apical dendrities (D1 and D2) where there is no evidence for a synaptic contact. (C) Immunoreactivity detected with the antiserum to the C-terminal part of the GluR1 subunit. Two type I synapses between dendritic spines (s1, s2) and axon terminals (at1, at2), are immunoreactive for the GluR1 subunit. The peroxidase reaction end-product labels the postsynaptic density (arrows) demonstrating the intracellular location of the epitopes. Scale bars = 0.1 μm (A); 0.5 μm (B); 0.1 μm (C).

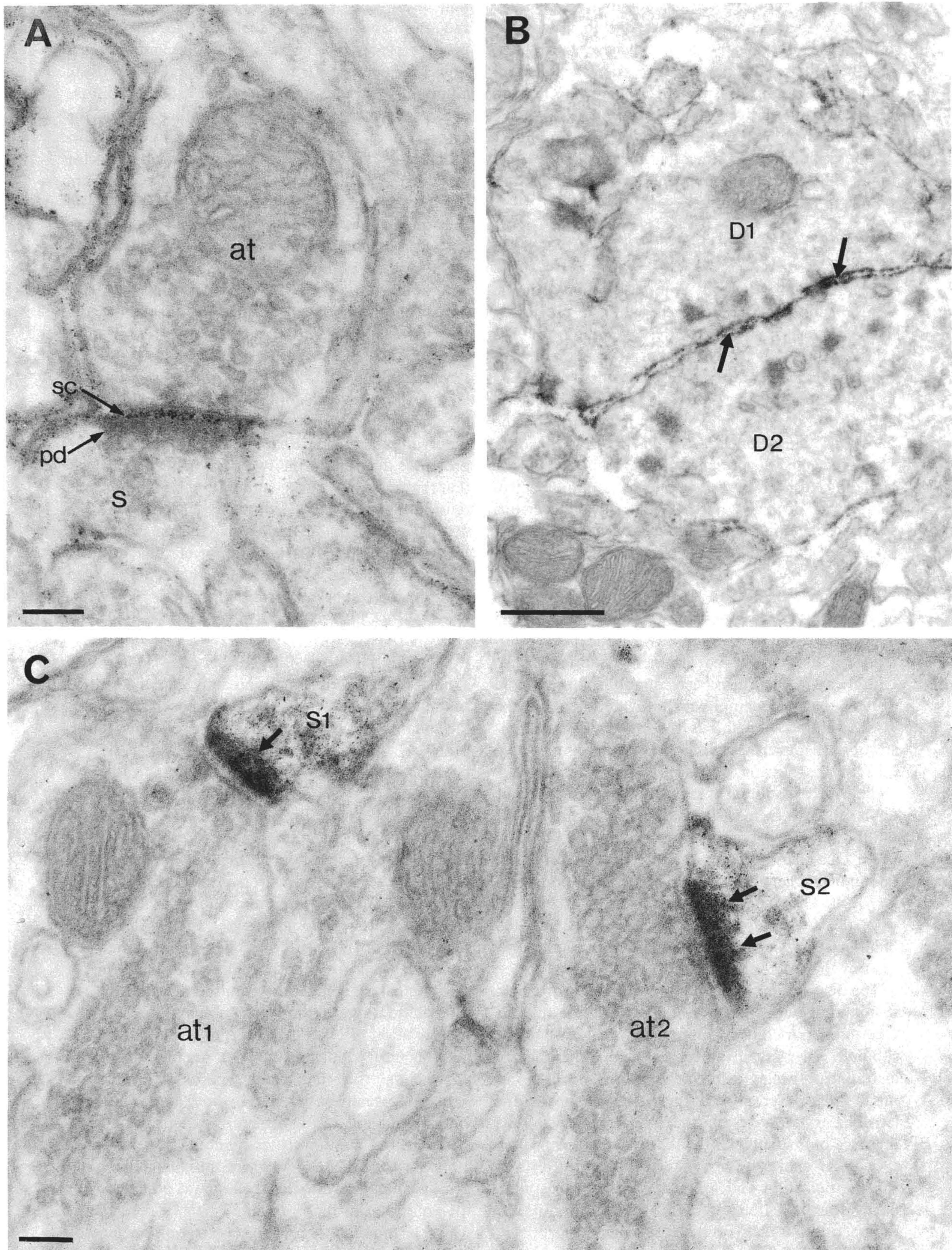

Fig. 8

but that the C-terminal peptide antiserum did not. Whilst we cannot exclude the possibility that these differences between the sera are due to C-terminal proteolysis of the subunit, we believe that taken together our results indicate the existence of more than one alternatively spliced form of GluR1. Such C-terminal alternatively spliced forms of GluR4 have been identified[17] and in the original report on GluR1 northern blot analysis suggested the existence of two minor mRNA bands which could give rise to alternative form of this subunit.[24]

Distribution of the GluR1 subunit in the forebrain of the rat

The regional pattern of immunoreactivity for the GluR1 subunit correlates well with the previously described distribution of mRNA coding for the GluR1 subunit.[7,16,30,47] The hippocampus, lateral septum, amygdala, striatum, cortex, medial habenular nucleus expressed the GluR1 mRNA at high levels and also showed intense immunoreactivity for the native protein. None of the *in situ* hybridization studies detected the presence of mRNA in the subthalamic nucleus, however a significant immunoreactive signal was observed both in this and a previous study,[48] suggesting a role for AMPA receptors in a nucleus of the basal ganglia receiving strong cortical input.

The expression of GluR1 mRNA in pyramidal cells of CA1 and CA3 regions and in granule cells of the dentate gyrus also correlates with the strong immunoreactivity observed in their dendritic fields. GluR1 mRNA has also been found in non-principal cells of the hippocampus in both the hilus and the CA3 region[47,58] and this is reflected in our demonstration of numerous immunopositive non-principal cells in these areas. However, whilst the mRNA coding for the subunit is uniformly expressed in the CA1 and CA3 regions, the immunoreactivity for the GluR1 subunit protein is stronger in CA1 than in CA3. This phenomenon has also been noted in other immunocytochemical studies.[6,52] The immunoreactivity for the GluR1 subunit nevertheless correlates well with the autoradiographic density of [^{3}H]AMPA binding sites which are greater in adult rat in the CA1 than in the CA3 region.[29,41,45,50]

The distribution of the immunoreactivity observed in our study with antisera raised against not only the C-terminal part but also the N-terminal part of the subunit, is largely in agreement with other neuroanatomical studies using antisera to the C-terminus.[6,48,52] However, none of these studies reported the labelling of tanicytes in the arcuate nucleus. The presence of a subunit of the AMPA receptor in such specialized glial cells is not surprising since both *in situ* hybridization and immunocytochemical studies have described the presence of GluR1 in the Bergmann glial cells of the cerebellar cortex.[6,30,47,48] The dentritic fields of hippocampal cells were very strongly immunoreactive along the plasma membrane. Indeed electron-microscopic analysis revealed that most of the dendritic spines in the statum radiatum and oriens of the CA1 region showed positive synapses. The results of our immunoreactions are in contrast with those of other studies using C-terminal specific antisera which showed diffuse peroxidase reaction end-product throughout the cytoplasm of the neurons covering all cellular organelles.[6,48,34a] There are two possible explanations for this difference. (1) Our C-terminal specific antiserum produces a weaker signal than that of others[6,34a,48] and thus only shows high concentrations of protein present at synapses. (2) The cytoplasmic reaction in other studies is due to nonspecific attachment to antibodies to the tissue and this reaction is superimposed on the specific staining at synapses. Only immunoreaction with particulate markers can resolve this question.

Whilst the pyramidal cells do not show intracellular labelling, several non-principal cells exhibit immunoreactivity in the endoplasmic reticulum. This observation may indicate that the turnover rate of the protein is different in the two cell types and suggests that the amount of GluR1 subunit in the endoplasmic reticulum of pyramidal neurons may be too low to be detected. Similar differences have been described for the $\alpha 1$ and $\beta 2/3$ subunits of the GABA/benzodiazepine receptor among the different cell types of the cerebellar cortex.[62]

Synaptic versus extrasynaptic receptors

Synaptic junctions formed between spines and axon terminals were immunoreactive for the GluR1 subunit of the AMPA receptor using both antisera to the N-terminal and C-terminal parts of the subunit. Most of the axon terminals establishing type I synapses on spines in CA1 stratum radiatum are thought to belong to the Schaffer collateral/commissural system (for review, see Ref. 33). Quantitative immunogold studies have shown that these boutons are enriched in glutamate[8] and that they probably use it as a neurotransmitter.[10,12,33,56] The presence of GluR1 subunit immunoreactivity at most of these synapses would suggest that at least one component of the excitatory innervation of the CA1 pyramidal neurons acts through AMPA receptors.[49] The Schaffer collateral/commissural pathway is thought to support long-term potentiation induced by activation of NMDA receptors of the CA1 pyramidal neuron,[10] but non-NMDA receptors also play an essential role in normal synaptic transmission.

Immunoreactivity detected by both antisera for the GluR1 subunit was generally associated with synapses, but immunoreactivity could also be found at non-synaptic sites along the plasma membrane. Most of the extrasynaptic labelling was observed with the antiserum to the N-terminal part of the subunit which is thought to be at the extracellular face of plasma membrane. Whilst the observed staining could be due to the diffusion of the peroxidase

reaction end-product from the synaptic sites the presence of immunoreactivity between directly apposed apical dendrites or on the somatic membrane of pyramidal neurons, which are known to receive only type II, GABAergic synapses,[51,60] suggests the presence of genuine extrasynaptic receptor. A similar distribution has also been described for the $\alpha 1$ and $\beta 2/3$ subunits of the $GABA_A$/benzodiazepine receptor,[57,62] but the $\alpha 6$ subunit shows a more restricted localization at synaptic junctions.[1a] It will be interesting to see if such differences also exist in the subcellular localization of the different AMPA receptor subunits.

Using the antiserum raised against the C-terminal part of the subunit, which recognized an intracellular epitope, extrasynaptic immunoreactivity was not obvious. This antiserum generally gave a weaker reaction with brain sections than the N-terminal antiserum, and the inability to detect non-synaptic receptors may simply reflect the different detection sensitivities of the two sera. An alternative explanation for the different patterns of immunoreaction found with the two antisera is that the antiserum to the C-terminal peptide detects fewer isoforms of the receptor and that the isoforms are differentially distributed on the cell. The biochemical data described earlier would be consistent with this hypothesis and clearly further work using different sera and immunocytochemical detection systems is needed to confirm it.

*The orientation of the GluR*1 *subunit polypeptide chain in the membrane as revealed by immunocytochemistry*

The current model for the transmembrane conformation of the ligand-gated ion-channel subunits, which includes the $GABA_A$/benzodiazepine, glycine, nicotinic and glutamate receptors, is based on their inclusion into a "receptor superfamily" as determined from their gene sequences.[1,4,19,55] According to this model the subunits of a ligand-gated ion channel would be made up of an extracellular N-terminal domain, four hydrophobic domains spanning the lipid bilayer and an extracellular C-terminal domain. However, this topology is largely conjectural and is based on both hydrophilicity analyses of the receptor amino acid sequences and on analogies with other receptor types.[24] In fact two possible models for the AMPA receptor containing five hydrophobic regions that may span the membrane have been proposed.[19,25] The immunoreactivity obtained in our study at the extracellular face of the plasma membrane with the antiserum raised against residues 253–267 would be in agreement with the location of this amino acid sequence in the N-terminal extracellular domain of the subunit as is predicted by the current model. This is further supported by the finding that this antiserum detects epitope(s) in the intracisternal face of the endoplasmic reticulum membrane. However, we have shown that the immunoreactivity obtained with the antiserum raised against the C-terminal residues 877–889 is located at the cytoplasmic face of the synaptic membrane as previously reported.[48] This is not in agreement with the predicted position of this region, which has been proposed to be extracellular, and suggests that the current model of these receptor subunits may require revision.

CONCLUSIONS

Using antibodies produced by the MAP approach to peptides at the N- and C-terminal regions of the GluR1 AMPA binding subunit, we have obtained subunit-specific antisera suitable for light- and electron-microscopic analysis of the distribution of this receptor subtype. The use of the two sera together in both the biochemical and immunocytochemical analyses of receptor distribution has enabled us to suggest that there may be different isoforms of the protein and that these may be differently distributed within neurons. The results also confirm and extend the earlier studies with anti-GluR1 antisera and show that the subunit is present in the membranes of cells in an area and cell type specific manner. Furthermore the organization of the polypeptide chain in the membrane at synapses may not be as currently postulated and the C-terminus of the molecule is at the cytoplasmic face of the plasma membrane.

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