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Stable Maintenance of Glutamate Receptors and Other Synaptic Components in Long-Term Hippocampal Slices

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ABSTRACT: Cultured hippocampal slices retain many in vivo features with regard to circuitry, synaptic plasticity, and pathological responsiveness, while remaining accessible to a variety of experimental manipulations. The present study used ligand binding, immunostaining, and in situ hybridization assays to determine the stability of AMPA- and NMDA-type glutamate receptors and other synaptic proteins in slice cultures obtained from 11 day postnatal rats and maintained in culture for at least 4 weeks. Binding of the glutamate receptor ligands [^{3}H]AMPA and [^{3}H]MK-801 exhibited a small and transient decrease immediately after slice preparation, but the binding levels recovered by culture day (CD) 5–10 and remained stable for at least 30 days in culture. Autoradiographic analyses with both ligands revealed labeling of dendritic fields similar to adult tissue. In addition, slices at CD 10–20 expressed a low to high affinity [^{3}H]AMPA binding ratio that was comparable with that in the adult hippocampus (10:1). AMPA receptor subunits GluR1 and GluR2/3 and an NMDA receptor subunit (NMDAR1) exhibited similar postcutting decreases as that exhibited by the ligand binding levels, followed by stable recovery. The GluR4 AMPA receptor subunit was not evident during the first 10 CDs but slowly reached detectable levels thereafter in some slices. Immunocytochemistry and in situ hybridization techniques revealed adult-like labeling of subunit proteins in dendritic processes and their mRNAs in neuronal cell body layers. Long-term maintenance was evident for other synapse-related proteins, including synaptophysin, neural cell adhesion molecule isoforms (NCAMs), and an AMPA receptor related antigen (*GR53*), as well as for certain structural and cytoskeletal components (e.g., myelin basic protein, spectrin, microtubule-associated proteins). In summary, following an initial and brief depression, many synaptic components were expressed at steady-state levels in long-term hippocampal slices, thus allowing the use of such a culture system for investigations into mechanisms of brain synapses.

KEY WORDS: organotypic culture, AMPA receptor, NMDA receptor, *GR53*, NCAM, synaptophysin

INTRODUCTION

The use of organotypic cultures to study the cellular and synaptic properties of the brain has a number of advantages over other types of experimental systems. Accessibility and strict control over surrounding fluid composition, as well as the retention of native neural circuitry, make organotypic brain slice cultures preferable over in vivo conditions and dissociated neuronal cultures. Hippocampal slice cultures, in particular, have been shown to develop normally with regard to neuronal organization (Beach et al., 1982; Gähwiler, 1984a,b; Frotscher and Gähwiler, 1988; Caesar and Aertsen, 1991; Stoppini et al., 1991; Bahr et al., 1994a; Kunkel et al., 1994) and the formation of synaptic connections (Gähwiler, 1984b; Buchs et al., 1993; Muller et al., 1993; Robain et al., 1994; Debanne et al., 1995; Frotscher et al., 1995). It has further been demonstrated that cultures prepared by the interface method (see Stoppini et al., 1991; Bahr, 1995) develop the ability to express normal synaptic waveforms as well as long-term potentiation in a manner seemingly identical to acute slice or in vivo preparations (Stoppini et al., 1991; Vanderklish et al., 1992, 1995; Muller et al., 1993; Bahr et al., 1994a). Thus, cultured slices of hippocampus retain many in vivo features and can easily be submitted to experimentation.

Since organotypic slices typically survive for many weeks, they are ideal for studying long-lasting (i.e., days to weeks) physiological changes that would otherwise be difficult or impossible to measure with short-lived acute slices. For example, development of axonal projections between brain regions (see Gähwiler, 1988; Li et al., 1993), effects of translational suppression of neurotransmitter receptor subunits (Vanderklish et al., 1992),

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regulation of growth factor gene expression (Rivera et al., 1994), and stable expression of foreign genes via viral vectors (see Bergold et al., 1993; Ozaki et al., 1993; Bahr et al., 1994b) have been described with slice cultures and co-cultures, most of which were prepared from hippocampus. The introduction of the long-term hippocampal slice technique has also advanced the understanding of pathological processes such as those involved in (1) ischemia (Newell et al., 1990, 1995; Hsu et al., 1994; Strasser and Fischer, 1995), (2) acidosis (Shen et al., 1995), (3) excitotoxicity (Rimvall et al., 1987; Vornov et al., 1991; Casaccia-Bonnefil et al., 1993; del Cerro et al., 1994; Bahr et al., 1995b; Takahashi et al., 1995), (4) epileptic activity (McBain et al., 1989; Müller et al., 1993b), (5) damage from neurotoxins and venoms (Bahr et al., 1995b), (6) lysosomal dysfunction (Bahr et al., 1994a), (7) free radical damage (Müller et al., 1993a), and (8) lesion-induced sprouting (Stoppini et al., 1993; Muller et al., 1994).

Recent studies using long-term slices have focused on the molecular aspects of synaptic mechanisms underlying plasticity (Vanderklish et al., 1992, 1995; Fukunaga et al., 1993) and pathophysiology (Bednarski et al., 1995) in hippocampus. Essential for such endeavors is an experimental system that possesses a steady population of healthy synapses, that is, those with stable concentrations of molecular components. Certain synapse-specific proteins have been shown to exhibit sufficient stability in long-term hippocampal slices, thus allowing for experimental manipulation. However, while the presynaptic component synaptophysin has been shown to be quite stable for up to 60 days in long-term slices (Bahr et al., 1994a,b), the development and maintenance of excitatory transmitter receptors have not been well characterized in such cultures. Accordingly, the present study examined the development, maintenance, and localization of two types of excitatory amino acid receptors, that is, AMPA-* and NMDA-type glutamate receptors. Also, other components of synapses as well as selected structural proteins important for synaptic functioning were assessed for their stability in the cultures. Several of these characterizations were conducted at both the protein and mRNA levels.

MATERIALS AND METHODS

Materials

Plasmids containing the cDNAs of different glutamate receptor subunits and synaptophysin were generously provided by Drs. S. Heinemann and J. Boulter (Salk Institute, La Jolla, CA; GluR2-4), Dr. R. Neve (McLean Hospital, Harvard Medical School, Belmont, MA; NMDAR1), Dr. T. Südhof (University of Texas, Dallas; synaptophysin), and Dr. K. Sumikawa (University of California, Irvine; GluR1). Antibodies developed in rabbit against mouse neural cell adhesion molecules (anti-NCAM) were provided by Dr. B. Murray (University of California, Irvine). Rabbit antibodies to AMPA receptor and NMDA receptor subunits (e.g., anti-GluR1) were prepared by injecting rabbits with the respective carboxy-terminal sequences and then subjecting the antisera to affinity purification as described (Wenthold et al., 1992; Bahr et al., 1995a). The peptide SHSSGMPLGATGL was used for GluR1, EGYNVYGIESVKI for GluR2/3, RQSSGLAVIASDLP for GluR4, and AIEREEGQLQLCSRHRES for NMDAR1. A subset of anti-GluR4 antibodies (anti-GluR4a) eluted from affinity columns with acidic buffer was used to detect both GluR4 and the AMPA receptor related antigen *GR53* (Bahr et al., 1995a). A second preparation of affinity purified IgG to NMDAR1 (against KRRAIEREEGQLQLCSRHRES) was obtained from Upstate Biotechnology Incorporation (Lake Placid, NY).

Protease inhibitors and monoclonal antibodies to synaptophysin, the growth-associated protein GAP-43, glial fibrillary acidic protein (GFAP), microtubule-associated protein 2 (MAP2), myelin basic protein (MBP), neurofilament-200, and tau were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). Other protease inhibitors were from Chemicon International, Incorporated (Temecula, CA) and Sigma Chemical Company (St. Louis, MO). *DL*-α-amino-3-hydroxy-5-[methyl-^{3}H]isoxazole-4-propionic acid ([^{3}H]AMPA; 50–70 Ci/mmol) and (+)-[3-^{3}H]MK-801 ([^{3}H]MK-801; 15–30 Ci/mmol) were purchased from NEN/Du Pont Company (Boston, MA). Millicell-CM culture inserts with Biopore membranes were from Millipore Corporation (Bedford, MA). Other cell culture supplies were obtained from Sigma Chemical Company (St. Louis, MO). Nitrocellulose paper and alkaline phosphatase-conjugated antibodies against rabbit and mouse IgGs were from Bio-Rad Laboratories (Richmond, CA). Phosphatase and peroxidase substrate kits were purchased from Vector Laboratories (Burlingame, CA).

Organotypic Cultures of Hippocampal Slices

Slice cultures can be prepared from brain tissue at the second or third postnatal week of development and thus incorporate a number of mature features. In this study, rat pups of 11–12 days postnatal were quickly decapitated and the brains were rapidly cooled. The hippocampi were removed and briefly incubated in ice-cold slice buffer containing (in mM) 124 NaCl, 3 KCl, 2 $CaCl_2$, 4 $MgSO_4$, 1.25 KH_2PO_4, 26 $NaHCO_3$, 10 *D*-glucose, and 2 ascorbic acid (pH 7.2) before being sliced transversely from the septal to the temporal end at a thickness of 400 μm. Groups of 6–8 slices were distributed randomly onto porous (0.4 μm), transparent, low protein-binding Biopore insert membranes that were in contact with culture medium composed of 50% Basal Medium Eagle, 25% Earl's balanced salt solution, 25% regular horse serum, and supplemented to the following final concentrations: 136 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$, 4 mM $NaHCO_3$, 3 mM glutamine, 40 mM glucose, 0.4 mM ascorbic

*Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GAP-43, growth-associated protein 43 kDa; GFAP, glial fibrillary acidic protein; GluR, AMPA-type glutamate receptor subunit; *GR53*, glutamate receptor related antigen of 53 kDa; HRP, horseradish peroxidase; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; NCAM, neural cell adhesion molecule isoform; NMDA, N-methyl-D-aspartate; NMDAR1, NMDA receptor subunit 1. Single letter notations for amino acids are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

acid, 20 mM HEPES buffer (pH 7.3 at 23°C), 1 mg/l insulin (Sigma Chemical Co., 24 I.U. per mg), 5 units/ml penicillin, and 5 mg/l streptomycin. The culture medium was changed every 2–3 days, and the surface of the slices was exposed to humidified air plus 5% CO_2 at 37°C. The insert membrane was used as a support matrix to maintain the hippocampal tissue at the air–medium interface. In this way the slices were never submerged in culture medium nor allowed to dry. The surface of the slice cultures were continuously covered by a thin layer of medium, which diffuses through the insert membrane and ensures tissue survival. Note, no cytosine arabinoside was included in the preparation of the long-term slices.

Electron Microscopy

Cultured slices were incubated with fixative containing 1.5% glutaraldehyde, 1% paraformaldehyde, and 0.1 M phosphate buffer (pH 7.4; PB) for 2 h at 4°C. Slices were then rinsed in PB and removed from the culture insert by cutting the Biopore membrane. The slices were postfixed in PB containing 2% OsO_4 for 1 h at 20°C, rinsed for 10 min in PB, and dehydrated through a graded series of ethanol and propylene oxide concentrations. The slices were then incubated in increasing concentrations (30–100%) of PolyBed 812 (Polysciences; Warrington, PA) for 1 h each and left overnight in fresh 100% PolyBed 812 in a desiccator. Polymerization was done at 60°C for 3 days and 1-μm sections were stained with toluidine blue to localize CA1 pyramidal cells. Ultra-thin (65 nm) sections were prepared with a Sorvall MT6000 ultramicrotome, picked up on formvar/carbon-coated copper grids, and stained in 2% uranyl acetate in water containing Reynolds lead citrate. The sections were examined with a Zeiss 10CR electron microscope.

Horseradish Peroxidase (HRP) Labeling

A semi-dry slurry was prepared with 20 mg HRP (Type VI-A; Sigma Chemical Co.) in slice buffer containing 2 M sucrose. A small amount of the slurry at the end of a fine-tip electrode was gently placed in stratum pyramidale in region CA1 of a cultured hippocampal slice. After 2 h at 37°C, treated slices were extensively washed in PB, fixed in PB with 4% paraformaldehyde for 1.5 h, and mounted on glass slides. The slices were then stained for 5–10 min with a peroxidase substrate kit containing diaminobenzidine and washed in water. Some slices were then counterstained with Nissl, and all were rapidly dehydrated through ascending concentrations of ethanol, cleared in AmeriClear (Baxter; McGaw Park, IL), and coverslipped with Permount.

Immunoblot Analyses

Cultured slices were harvested by flooding the culture insert with ice-cold homogenization buffer (HB), which contained 0.32 M sucrose, 5 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3, 10 mg/l antipain, and 2 mg/l each of leupeptin, aprotinin, and pepstatin. The slices (6–8 per sample) were gently removed with a soft brush and rinsed in HB by centrifugation. The slice samples were then subjected to one freeze-thaw cycle and homogenized by mild tip sonication in 30–50 μl of hyposmotic buffer containing 8 mM HEPES (pH 7.4), 1 mM EDTA, 0.3 mM EGTA, and the protease inhibitors mentioned earlier. Membranes were isolated and washed by repeated centrifugation-resuspension cycling; protein content was determined using the Bio-Rad Protein Assay with a BSA standard. Membrane samples (40 μg protein) were treated with 2.5% (wt/vol) sodium dodecyl sulphate (SDS) in the presence of 3% (vol/vol) 2-mercaptoethanol and were incubated at 100°C for 5 min. The denatured samples were then subjected to electrophoresis on polyacrylamide linear gradient gels (3–17% wt/vol) and transferred to nitrocellulose (0.2 μm pore size) for 1–2 h or 24 h with transfer blot systems from Bio-Rad Laboratories. Immunodetection of transferred proteins utilized the following antibodies diluted to the indicated concentration in 1.5% (wt/vol) nonfat dry milk: anti-GAP-43 (0.5–1 μg/ml), anti-GluRs (2–6 μg/ml), anti-MAP2 (1 μg/ml), anti-myelin basic protein (anti-MBP; 4 μg/ml), anti-NCAM (5 μg/ml), anti-NMDAR1 (2–4 μg/ml), anti-spectrin (1:200 dilution of serum), and anti-synaptophysin (50 ng/ml). Incubation of the nitrocellulose with diluted antibody was carried out at 4°C with agitation for 12–16 h. Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates, and color development used either the 5-bromo-chloro-3-indolyl phosphate and nitro blue tetrazolium substrate system or the Vector Substrate Kit II (Vector Laboratories). Color development of immunoreactive bands was terminated well before maximal intensity was reached in order to avoid saturation and to allow comparative studies within each blot. The optical densities and image areas of the labeled antigens were quantitatively compared within single immunoblots using a computerized image analysis system; the specific immunoreactivity [(optical density − background) × image area)] for each blot lane was determined from these values. The immunostaining of each antigen was initially tested for the respective optical density range, which exhibited a linear relationship with an increasing amount of brain tissue applied to the gel. Subsequent experiments used the linearity information to determine the amount of hippocampal slice protein needed for sufficient labeling intensity. Immunoreactivity vs. culture day studies usually required two to three immunoblots; thus the data were normalized to identical samples of CD 20–25, which were present on each blot at equal protein content. Calibration of immunoblots using prestained protein molecular weight standards allowed the determination of the M_r for pertinent species.

Receptor Binding Assays

Binding assays were carried out with a membrane fraction prepared from cultured slices by conventional methods involving homogenization, differential centrifugation, and hyposmotic lysis (see Hall et al., 1992). [^{3}H]AMPA binding was determined by the centrifugation method. Aliquots of 20–30 μg slice membrane protein in a volume of 100 μl tris-acetate buffer (100 mM, pH 7.2; TAB) were equilibrated with [^{3}H]AMPA for 40–60 min at 0°C in the presence of 50 mM potassium thiocyanate (KSCN). To determine binding constants, AMPA concentrations were chosen between 2 nM and 1500 nM. Concentrations between 2 and 50 nM were measured by increasing the concentration of radio-

labeled AMPA, while those above 50 nM were reached by adding unlabeled AMPA. Nonspecific binding was defined as that which occurred in the presence of 2.5 mM unlabeled L-glutamate. Samples were centrifuged at 48,000 *g* (4°C) for 20 min, then placed in the ice water bath. The supernatants were aspirated within 10–20 min, and the pellets were superficially rinsed with 0.4 ml of ice-cold assay buffer and then dissolved in 10 μl Beckman tissue solubilizer. Radiolabel content was assayed by liquid scintillation spectroscopy with a counting efficiency of 0.40 in 1 ml of aqueous counting scintillant. Nonspecific binding increased from approximately 15% to 75% of the total binding as AMPA concentration increased from 2 nM to 1500 nM. One- and two-site analysis of binding data was performed by nonlinear regression using the Inplot program by GraphPAD Software, Inc.; comparisons for one- vs. two-site fits were made using the F-squared test. For [^{3}H]MK-801 binding, the filtration method was utilized. Twenty micrograms of sample was incubated at 25°C for 4 h with 2 nM of radiolabeled MK-801 in the presence of 10 μM glycine and 10 μM glutamate. Nonspecific binding was determined with the addition of 10 μM unlabeled MK-801.

Ligand-Binding Autoradiography

Autoradiography was performed on cryostat sections (20 μm) of cultured hippocampal slices. The slices had been frozen and stored overnight in the presence of desiccant until sectioning. The sections were placed on gelatin-coated slides and preincubated in TAB for 90 min at 0°C to wash endogenous glutamate out of the tissue. For AMPA receptor autoradiography, sections were then covered with 20–30 μl of an incubation solution containing 50 nM [^{3}H]AMPA and 50 mM KSCN in TAB, and left on ice for 60 min. Nonspecific binding was determined by adding 5 mM L-glutamate to the incubation solution for some sections. At the end of the incubation, the sections were washed for a total duration of 30 s by passing the slides through two baths of TAB plus 25 mM KSCN and by a final 2-s rinse in distilled water; all wash solutions were near 0°C. For NMDA receptor autoradiography, slices were incubated in TAB containing 10 nM [^{3}H]MK-801, 100 μM glycine, and 100 μM L-glutamate for 50 min at 25°C; nonspecific binding was determined by inclusion of 10 μM MK-801. After the washes, the slides were immediately dried with hot air and exposed to tritium-sensitive film (Hyperfilm-^{3}H; Amersham Corporation, Arlington Heights, IL) for 6–12 days.

Immunocytochemistry

Slices on culture inserts were rinsed with PB and then were fixed for 2 h in cold PB plus 3% paraformaldehyde. Slices were subsequently incubated in PB with 20% sucrose for 8–12 h, and then were carefully removed from the insert for microtome sectioning (15–20 μm thickness) on a flat stage of frozen 20% sucrose. Sections mounted on gelatin-coated slides were immunostained with the following antibodies: anti-GFAP (0.4–1 μg/ml), anti-GluRs (5–10 μg/ml), anti-NCAM (6–10 μg/ml), anti-neurofilament-200 (1 μg/ml), and anti-tau (3–4 μg/ml). The avidin-biotin-peroxidase technique was used with the enzymes, substrate, and method recommended by Vector Laboratories, Inc. Lastly, the slices were rapidly dehydrated through ascending concentrations of ethanol, cleared in AmeriClear (Baxter; McGaw Park, IL), and coverslipped with Permount.

In Situ Hybridization

Riboprobes to the mRNAs of AMPA receptor GluR subunits, the NMDA receptor subunit NMDAR1, and synaptophysin were prepared as follows. For GluR1, two plasmids have been described previously that generated antisense cRNA probes of 720 and 1442 nucleotides complementary to the 5′ end of the GluR1 message (Gall et al., 1990). For GluR2 antisense probes, cRNAs were transcribed from the GluR2$_{flop}$ clone pRB14 (Boulter et al., 1990) digested with either *Sph*I or *Bam*HI, generating riboprobes of 400 and 900 nucleotides in length, respectively; these probes are complementary to the 3′ end of the GluR2 mRNA, including the noncoding region. GluR3 cRNAs were transcribed from either *Bgl*II, *Sal*I, or *Sac*I digests of the GluR3$_{flop}$ clone pRB12 (Boulter et al., 1990), generating respective probes complementary to 250, 720, and 1860 nucleotides of the 3′ end. For GluR4, a 774-nucleotide cRNA was transcribed from an *Eco*RI digest of the GluR4$_{flip}$ clone pK46 (obtained from Dr. Heinemann); the riboprobe is complimentary to both the flip and flop versions of the GluR4 message. For NMDAR1 (Moriyoshi et al., 1991), the cRNA was transcribed from a *Hin*dIII digest of a pGEM7 vector that contains in its *Sma*I-*Xho*I site the rat NMDAR1 clone extending from the starting methionine to the *Xho*I site at bp ~1170. For synaptophysin, a full length (1200 nucleotides) cRNA was transcribed from a *Kpn*I and *Bam*HI digest of the synaptophysin expression vector obtained from Dr. T. Südhof.

All riboprobes were transcribed with T3 or T7 RNA polymerase in the presence of [^{35}S]UTP. Cultured slices were sectioned at a thickness of 20 μm, mounted on glass slides coated with Vectabond (Vector Laboratories), and processed as previously described (Gall et al., 1994; Guthrie et al., 1993) at 60°C for 20–24 h with individual radiolabeled cRNAs at a density of 10^7 cpm/ml. Following hybridization, the tissue was treated with ribonuclease A and washed through descending concentrations of saline sodium citrate buffer (SSC) to a final rinse in 0.1 × SSC for 1 h at 60°C. The tissue was then processed for film autoradiography (Amersham β-max film) as described by Rivera et al. (1994). GluR1 and GluR2 sense probes were shown not to produce any hybridization above background levels in adjacent sections to those exhibiting antisense labeling. In addition, Pellegrini-Giampietro et al. (1991) and Gold et al. (1994) showed that unlabeled sense RNAs for GluR1-3 anneal only to their respective antisense counterpart to block specific in situ hybridization.

RESULTS

Cellular Organization of Hippocampal Slice Cultures

The morphological characteristics of slice cultures prepared from rats 11–12 days postnatal and maintained in culture for 3–8 weeks resembled those of adult tissue. Figure 1A shows the Nissl staining of a horizontal section from a typical slice at culture day

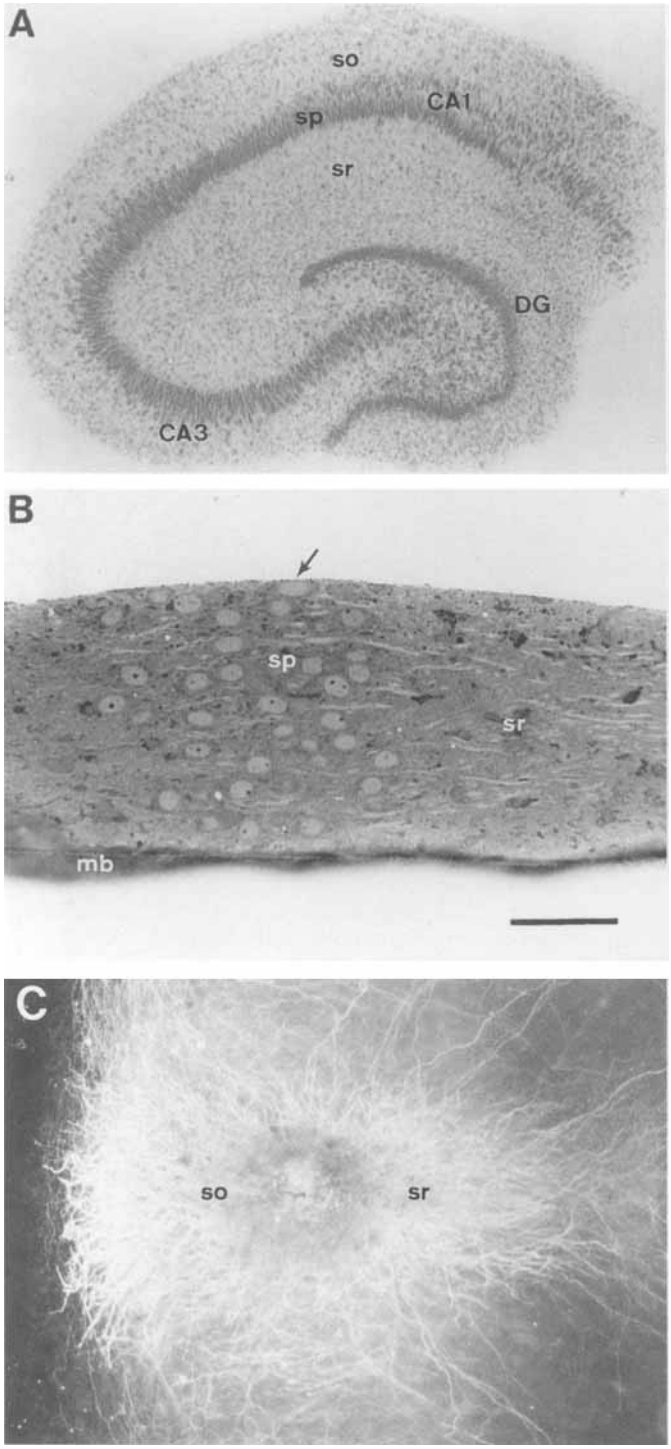

FIGURE 1. Morphological characteristics of long-term hippocampal slice cultures. A: A cultured slice prepared from a postnatal day 12 hippocampus was maintained in culture for 3 weeks, then fixed, sectioned, and stained with cresyl violet. B: A transverse semi-thin section of a similar slice culture was cut perpendicular to the Millicell insert membrane (mb), sectioning across the CA1 cell layer. Staining shows the cell layer to include 10–14 pyramidal neurons across the depth of the slice (~170 μm thick). C: An elaborated dendritic arborization was revealed in a 3-week cultured slice by extracellular application of HRP to a small group of CA1 neurons (dark-field microscopy). Scale bar: A, 600 μm; B, 65 μm; C, 175 μm. DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum.

(CD) 21. The major subfields of the hippocampus are present, including CA1 and CA3 zones containing the pyramidal cell layers and the dentate gyrus with an organized granule cell layer; such cellular organization was also retained in 8-week cultures. Counterstaining with an antibody against the astroglial marker glial fibrillary acidic protein (GFAP) revealed the native organization of astroglia with elaborated processes in and around the pyramidal cell body layers and throughout the neuropil (not shown; see Bahr, 1995). Stable maintenance of the GFAP concentration after CD 7 has been previously shown (Bahr et al., 1994a).

A transverse semi-thin section through the CA1 field of a cultured slice (i.e., cut perpendicular to the Biopore insert membrane) revealed that after 3 weeks in culture, the stratum pyramidal is still organized in a vertical layer 10–14 neurons high and 5–7 neurons wide (Fig. 1B). As indicated by the arrow in Fig. 1B, the neuronal cell bodies extend from the insert membrane to the upper surface of the slice and contribute to a dense network of dendritic processes. Dense dendritic fields stemming from pyramidal and granule neurons were previously described with the use of MAP2 staining (Bahr et al., 1994a). For the pyramidal cells, the densities of apical and basal dendrites were better appreciated when labeled with HRP deposited in the CA1 stratum pyramidale (Fig. 1C). Dendrites and axonal arborizations from the pyramidal neurons, as well as the mossy fiber axons of the granule cells, were also stained with antibodies to neurofilament-200 and tau (Bahr, 1995). Higher magnification of the HRP-labeled dendrites revealed an intricate array of spines (not shown) with a density matching that of axon terminals outlining dendritic processes in cultured slices (Bahr et al., 1994a; Bahr, 1995). In electron micrographs taken from stratum radiatum of a CD 25 slice, spine synapses were abundant, with well-developed spine heads frequently associated with spine apparati and occasionally possessing perforated postsynaptic densities (Fig. 2).

To examine the homogeneity of axon terminal density across different depths of the cultured slices, CD 15–25 explants (~150 μm final thickness) were sectioned by cryostat in order to assess the distribution of two presynaptic markers. Sections were combined into three groups representing the top third (material from the slice surface to ~50 μm below the surface), the middle third (~50 to ~100 μm depth), and the bottom third (~100 μm deep to the material in contact with the insert membrane) of the slices. The three groups were assessed for synaptophysin (Floor and Feist, 1989) and GAP-43 (a growth-associated nerve terminal protein; see Skene et al., 1986). Immunoblot analyses showed that both proteins are more concentrated in sections near the slice surface (Fig. 3). The differences between the section groups were significant for both antigens according to one-way ANOVAs ($P < 0.03$, $F = 4.7$ and $P < 0.02$, $F = 5.1$, respectively).

Maintenance of AMPA Receptors in Hippocampal Slice Cultures

Development and maintenance of AMPA-type glutamate receptors in long-term hippocampal slices were measured over 30 days in culture. Slices were prepared from eight rats 11 days postnatal and placed randomly onto Millicell culture plate inserts (6–8

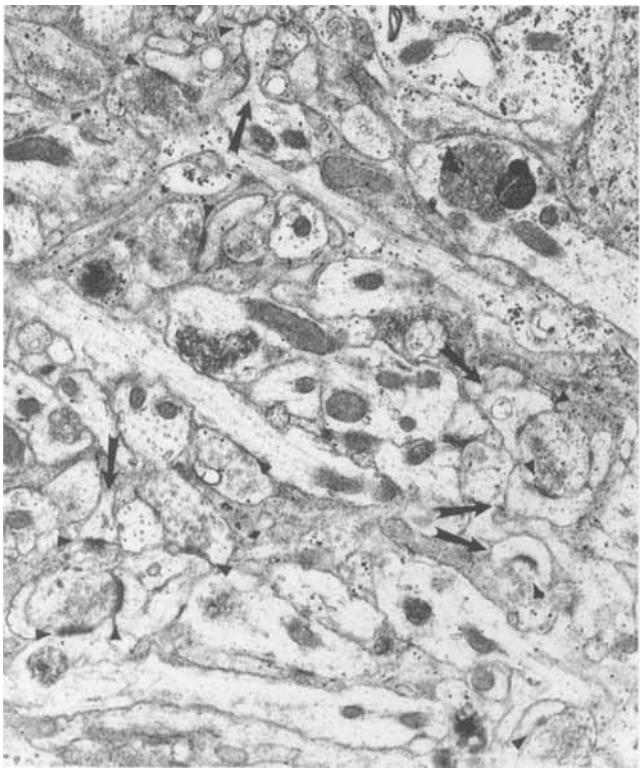

FIGURE 2. Synaptic density in the stratum radiatum of a cultured slice. Numerous synaptic contacts (arrowheads) are illustrated in the electron micrograph of a long-term hippocampal slice maintained in culture for 25 days. Well-developed spine heads and synapses are indicated by long arrows, some with spine apparati and perforated postsynaptic densities (see middle-right arrow). The length of each arrow = 0.6 μm.

slices/insert); some slices were frozen in buffer immediately after preparation in order to determine control values (CD 0; Table 1). Over time, slices were either harvested into three groups of eight slices each for the preparation of membranes or were fixed in paraformaldehyde for subsequent immunocytochemistry and in situ hybridization analyses. Aliquots of the membrane samples were used to measure specific [^{3}H]AMPA binding.

Figure 4A shows that AMPA receptor binding activity exhibited a modest reduction 1–2 days after slice cutting, but then recovered by CD 5 to a level similar to that found in adult hippocampal membranes (see Hall et al., 1992) and remained relatively stable through at least CD 30. There was a slow but significant decrease in binding between CD 10–30 as determined by one-way ANOVA ($P = 0.02$, $F = 4.77$), and the binding level at CD 25 was significantly lower than that at CD 1 ($P < 0.02$; unpaired t-test, two-tailed). However, the level of bound [^{3}H]AMPA at CD 30 establishes that the AMPA receptors are well maintained in cultured slices as compared with sister slices stored frozen from time of cutting (80% maintenance) and with adult hippocampal membranes (64%; see Table 1). Scatchard analyses using pooled slice material of CD 10 and CD 20 both determined that long-term slices express more low affinity (K_D = 580 ± 220 nM, B_{MAX} = 6.6 ± 0.9 pmol/mg protein; means ± SEM) than high affinity (K_D = 13 ± 6 nM, B_{MAX} = 0.7 ± 0.2 pmol/mg) [^{3}H]AMPA binding sites (see insert in Fig. 4A). The ratio between the low and high affinity sites (approximately 10:1) is closer to that found in membranes from adult rats than to that measured in neonates (see Hall and Bahr, 1994).

A second set of membrane aliquots were subjected to electrophoretic separation and immunoblotting with antibodies to different AMPA receptor subunits (anti-GluR1, anti-GluR2/3, and anti-GluR4a; see Bahr et al., 1995a). Densitometric analyses with a digital imaging system (at 10–15× magnification of the blot lanes) were used to measure the level of immunoreactivity for the individual antigens. Immunoreactivity for GluR1 (Fig. 4B) and the GluR2/3 doublet (Table 2; see blot insert in Fig. 4C) went through a transient decrease between 0.4 and 2 CDs, as did the ligand binding. The profiles also exhibited a similar recovery, which began after CD 2 and reached a plateau at CD 5–10. Immunoreactivity values for the upper and lower bands of the GluR2/3 doublet were plotted separately in Figure 4C in order to visualize changes that may provide information about individual subunits or about subunits with different levels of glycosylation or phosphorylation (Note: Boulter et al. [1990] reported that the GluR3 protein is slightly larger than GluR2 [i.e., 98 vs. 96 kDa]).

The separate profiles followed the same general pattern as that of the GluR2/3 doublet measured as a single antigen (Table 2). However, Table 3 shows that the relative amount of the top band

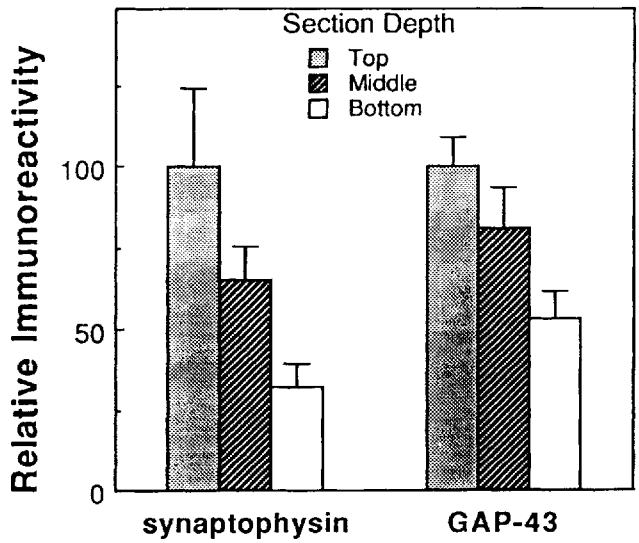

FIGURE 3. Synaptic markers are located toward the upper surface of organotypic hippocampal slice cultures. Approximately 650 slices of culture day 11–16 were divided into 6–8 separate groups, frozen in 2-methylbutane at −30°C for 1 min, and stored at −80°C. Each group was then cryostat sectioned starting from the bottom (membrane adherent) surface of the slice, and sections were grouped to yield samples from the top (upper ~50 μm), middle (~50 to ~100 μm depth), and bottom (~100 to ~150 μm depth) sectors, which were prepared for immunoblot analyses using monoclonal antibodies to synaptophysin and GAP-43. The mean immunoreactivity values (± SEM) are plotted with the data for the "middle" and "bottom" subgroups normalized to the values from the "top" samples.

TABLE 1.

*Maintenance of Synaptic Markers in Long-Term Hippocampal Slices**

Marker	Percent maintenance	
	vs. uncultured slices	vs. adult hippocampus
[^{3}H]AMPA binding	80 ± 14 (3)	64 ± 14 (3)
GluR1	50 ± 6 (6)	n.d.
GluR2/3 (doublet)	40 ± 11 (3)	n.d.
GR53	96 ± 55 (3)	n.d.
MBP	U.M.	86 ± 15 (6)
[^{3}H]MK-801 binding	73 (2)	67 (2)
$NCAM_{180}$	85 ± 39 (3)	89 ± 11 (6)
$NCAM_{140}$	132 ± 16 (4)	71 ± 7 (4)
$NCAM_{120}$	U.M.	80 (2)
NMDAR1	43 ± 11 (3)	n.d.
Synaptophysin	43 ± 6 (6)	n.d.

*Synaptic and structural markers were assessed in hippocampal slices harvested after 30–45 days in culture (CD 30–45) as described in the Methods section; the number of groups of six to eight slices examined are shown in parentheses. The markers were compared with those in either uncultured sister slices (CD 0) or adult hippocampal membrane samples; within-blot comparisons were used for immunolabeled antigens.

Percent maintenance data are expressed as the mean ± SEM.

n.d., not determined; U.M., unable to measure the low antigen level in uncultured slices.

as compared with the bottom band (within-sample comparisons) decreased sharply during the first few days in culture before recovering to a similar level as that found in uncultured slices (CD 0); the change in the top:bottom band ratio is apparently the result of the post-cutting transition period. While the top band of

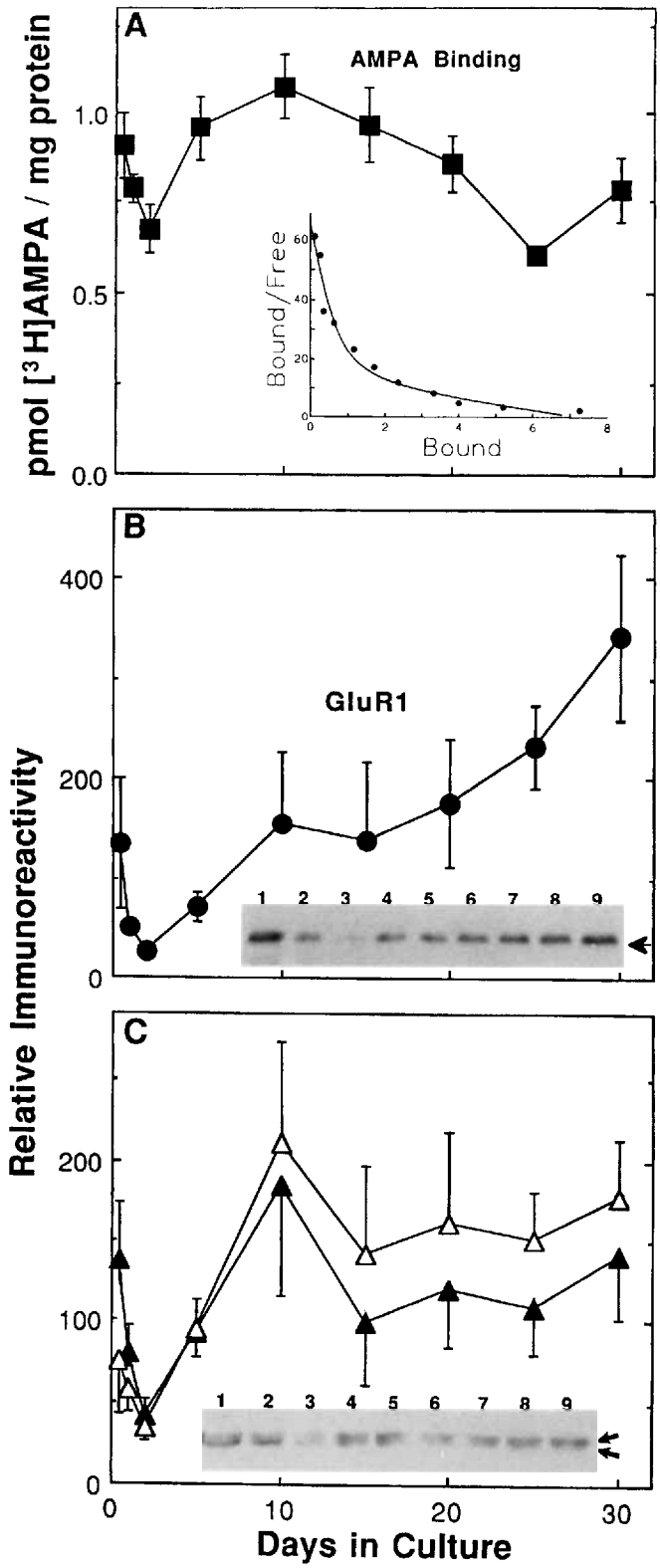

FIGURE 4. Development and maintenance of AMPA receptor binding activity and subunits in long-term hippocampal slices. Slices were harvested over time in culture and groups of eight slices were homogenized and lysed membranes prepared (n = 3 groups). A: Equal protein aliquots from the membrane samples were analyzed by a centrifugation binding assay using 50 nM [^{3}H]AMPA. Nonspecific binding in the presence of 2 mM glutamate was also determined and subtracted from the total binding at each time point. Specific binding activity values are plotted ± SEM. Insert: Scatchard plots of [^{3}H]AMPA binding to pooled membrane samples from culture days 10 and 20 were combined and shown. Each point represents the mean of three independent determinations, each performed in triplicate (SEM <10% of mean value). The units for the x and y axes are pmol/mg and pmol/mg/μM, respectively. B,C: Equal amounts of protein (22 μg/lane) from membrane samples were analyzed by immunoblot using antibodies to the AMPA receptor subunits GluR1 and GluR2/3. Typical immunoblot results obtained with anti-GluR1 (insert in B) and anti-GluR2/3 (insert in C) are shown. Lanes 1–9, culture day 0.4, 1, 2, 5, 10, 15, 20, 25, and 30, respectively. Anti-GluR2/3 labeled a doublet pattern (see insert arrows in panel C). The plotted profiles for the top (open triangles) and bottom (closed triangles) bands of the doublet may represent separate subunits or different glycosylation or phosphorylation states of the subunits. The two profiles followed a similar pattern as that of the GluR2/3 doublet measured as a single antigen (see Table 2). Each value plotted represents the mean specific immunoreactivity of the antigen determined by image analysis [(density − background) × area)] ± SEM.

TABLE 2.

*GluR2/3 Immunoreactivity in Hippocampal Slices Across Days in Culture**

Culture day	GluR2/3 doublet optical density × area
0.4	203 ± 58
1	127 ± 21
2	71 ± 14
5	180 ± 22
10	381 ± 130
15	224 ± 83
20	280 ± 103
25	255 ± 56
30	322 ± 86

*Long-term slices analyzed in Figure 4 (three groups of 8 slices each) were assessed for their GluR2/3 content. The optical density and image area of the immunolabeled doublet antigen (see insert in Fig. 4C) was measured by computerized image analysis and was expressed as [(density − background) × area)] ± SEM.

the doublet was less pronounced than the lower band in harvested slices of <5 CDs (−25 ± 12% when normalized to the lower band; mean ± SEM, n = 9), it was more abundant than the lower band at >10 CDs (+ 37 ± 6%; n = 12), a difference that was found to be significant when analyzed by the nonparametric Mann-Whitney U-test ($P < 0.001$; U-statistic = 7.50). Whether this switch reflects a change in the R2:R3 ratio or in the post-translational state of the subunits remains to be determined. The GluR1 subunit had a tendency to increase gradually in concentration between CD 10 and 30, but this increase was not found to be significant by one-way ANOVA. Notwithstanding, however, the slow decrease in [^{3}H]AMPA binding after CD 10 (see Fig. 4A and earlier) may be related to a gradual change in the subunit stoichiometry. The maintenance of GluR1 and GluR2/3 epitopes in CD 30 slices compared with uncultured slices is lower than that of [^{3}H]AMPA binding (Table 1); this may be explained by the difference in threshold of detection between immunoblot and binding assays (Hall and Bahr, 1994).

Anti-GluR4a antibodies did not detect the 105 kDa subunit in the first 10 CDs, and only one to two slice groups developed detectable levels in CD 15–30 samples (Fig. 5). This result was expected since, in contrast to GluR1 and GluR2/3, GluR4 has been shown to be present at low levels in rat hippocampus (Petralia and Wenthold 1992; Bahr et al., 1995a). Interestingly, anti-GluR4a also recognized a glutamate receptor related antigen of 53 kDa (*GR53*) that exhibited a maintenance profile in cul-

TABLE 3.

*Change in the Relative Concentrations of the Top and Bottom Bands of the GluR2/3 Doublet Across Days in Culture**

Culture day	Top vs. bottom, % difference
0	27 ± 4
0.4	−48 ± 11
1	−21 ± 23
2	−5 ± 27
5	5 ± 23
10	33 ± 25
15	42 ± 2
20	28 ± 15
25	42 ± 8
30	36 ± 21

*The three groups of eight slices from Figure 4C were analyzed for the difference between the top and bottom bands of the GluR2/3 doublet using [(density − background) × area)] values.

The paired data are shown as the mean difference as a percentage of the bottom band ± SEM.

One-way ANOVA: $P = 0.026$, $F = 2.81$.

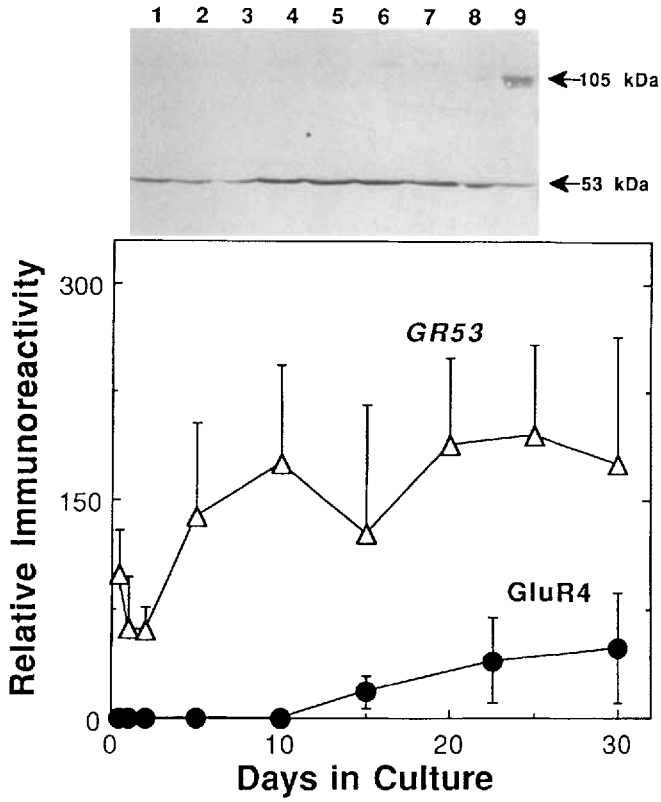

FIGURE 5. GluR4 and *GR53* develop differently in cultured hippocampal slices. Slices harvested at different culture days were prepared as in Figure 4 (n = 3 groups of 8 slices). Equal protein aliquots from membrane samples (22 μg/lane) were subjected to SDS-PAGE and immunoblotted with anti-GluR4a antibodies. A typical immunoblot is shown (top), and the 105 kDa GluR4 subunit and the 53 kDa *GR53* antigen are marked with arrows. Lanes 1–9, culture days 0.4, 1, 2, 5, 10, 15, 20, 25, and 30, respectively. Each value represents the mean specific immunoreactivity of the antigen determined by image analysis [(density − background) × area)] ± SEM.

tured slices similar to that of GluR1 and GluR2/3 (Fig. 5). The *GR53* antigen has been previously characterized as also being recognized by antibodies raised against an amino-terminal domain of GluR1 (Bahr et al., 1992), and as a brain-specific protein concentrated in synaptosomal membranes and postsynaptic densities throughout the rat brain (Bahr et al., 1995a). It is of note that *GR53* was highly preserved in culture when compared with control uncultured tissue (Table 1).

Localization of AMPA Receptor Subunits and Their mRNAs

To examine the AMPA receptor localization in long-term hippocampal slices, [^{3}H]AMPA binding autoradiography and GluR immunohistochemistry were conducted on thin sections. In Figure 6A it can be seen that the ligand binding sites are concentrated in dendritic fields of CA1 and CA3, as well as in the molecular layer of the dentate gyrus (see arrow). This distribution is characteristic of adult hippocampus (Blackstone et al., 1992; Magnusson and Cotman, 1993). Nonspecific binding measured by inclusion of 2 mM glutamate in the incubation mixture was barely detectable (<10% total binding). Fixed tissue sections processed with antibodies to GluR1 and GluR2/3 similarly revealed that AMPA receptors are densely distributed throughout the dendritic fields of long-term slices, whereas lower levels of immunoreactivity are present in pyramidal and granule cell body layers (Fig. 6B,C). Both antibodies also labeled hilar interneurons and their processes (see Fig. 6C), while no staining was evident throughout slices incubated with pre-immune serum or secondary antibodies alone.

In contrast to the dendritic localization of GluR subunits, in situ hybridization experiments with fixed slice sections determined that GluR mRNAs are confined to neuronal cell bodies (Fig. 7). Individual [^{35}S]cRNA antisense probes specific for GluR1 (panel A) and GluR2 (panel B) transcripts produced robust and nearly equal labeling among pyramidal and granule cell layers of CD 15–20 slices, while the GluR3 [^{35}S]cRNA produced less hybridization in CA1 and little labeling in other neuronal fields (panel C). Slices at CD 1–2 expressed consistently lower levels of GluR1-3 mRNAs, consistent with the ligand binding and immunoblot maintenance profiles (see Fig. 4). No detectable hybridization in CD 12–25 slices was observed with a GluR4 riboprobe (data not shown), which would explain the low and rather sporadic expression of GluR4 protein (see Fig. 5). The GluR4 [^{35}S]cRNA used did yield positive hybridization in cerebellum and the reticular thalamic nucleus, areas known to be enriched in the GluR4 subunit (Keinanen et al., 1990). Overall, the relative distribution of AMPA receptor subunit mRNAs in long-term slices nearly matches that found in the adult hippocampus (see Keinanen et al., 1990).

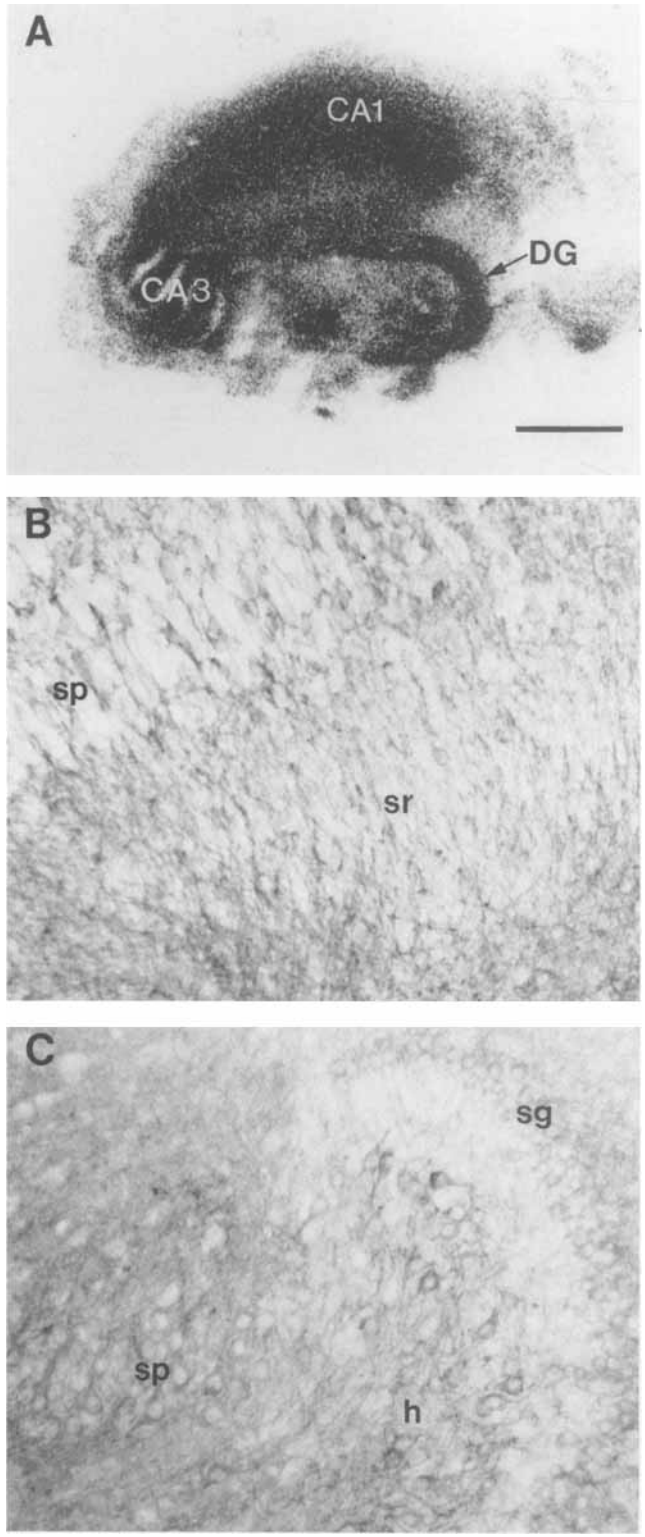

FIGURE 6. [^{3}H]AMPA autoradiography and GluR immunohistochemistry in hippocampal slice cultures. A: Cryostat sections from cultured slices of CD 10–20 were incubated with 50 nM [^{3}H]AMPA, washed, and exposed to film. B: Fixed sections were treated with antibodies that recognize the GluR1 subunit of the AMPA-type glutamate receptor; dense staining is evident in the CA1 dendritic field. C: Immunostaining with anti-GluR2/3 antibodies is visible in the neuropil surrounding CA3 pyramidal neurons and cell bodies of hilar interneurons. Scale bar: A, 840 μm; B and C, 90 μm. DG, molecular layer of the dentate gyrus; h, hilus of the dentate gyrus; sg, stratum granulosum; sp, stratum pyramidale; sr, stratum radiatum.

Long-Term Maintenance of NMDA Receptors

Aliquots of slice membrane samples were used to measure [^{3}H]MK-801 binding (a noncompetitive ligand specific for NMDA receptors) and NMDAR1 immunoreactivity with two antibodies against the subunit's carboxy terminus. As in the case of [^{3}H]AMPA binding, the [^{3}H]MK-801 binding activity exhibited a small decrease after slice cutting followed by recovery and stability (Fig. 8C). It appears from the maintenance profiles that the two classes of glutamate receptor have comparable degrees of preservation (see Figs. 4A and 8C). Bound [^{3}H]MK-801 at CD 25 also confirms that the NMDA receptors are well maintained in cultured slices (see Table 1) as compared with binding values from uncultured sister slices and adult hippocampal membranes (values obtained from Wong et al., 1988; Tamaru et al., 1991). Similarly, immunoblotting of slice membranes revealed that the 118 ± 4 kDa (mean ± SD, n = 16) NMDAR1 subunit experiences a transient post-cutting decrease, quick recovery, and good stability through CD 30 (see Table 1).

NMDA receptor localization using [^{3}H]MK-801 autoradiography found concentrated binding sites in stratum oriens, stratum radiatum, and the molecular layer of the dentate gyrus (Fig. 8B). The binding level was low in the pyramidal cell body layer (see arrow in Fig. 8B) and the dendritic labeling was blocked by pretreating the cryostat sections with unlabeled MK-801 (10 μM; not shown). In situ hybridization with a [^{35}S]cRNA probe specific for NMDAR1 mRNA showed that the transcript is uniformly distributed throughout neuronal cell body layers (CD 10–20; Fig. 8A). The distributions of the binding sites and mRNA are characteristic of those for NMDA receptors in adult hippocampus (Moriyoshi et al., 1991; Magnusson and Cotman, 1993).

Maintenance of Other Proteins in Long-Term Slices

Several proteins important for the functioning of brain synapses were examined, including some that are involved in synaptic connections, neurotransmitter storage, myelination, and structural integrity of dendrites and axons. Figure 9 shows that maintenance profiles for the 180 and 140 kDa NCAM isoforms ($NCAM_{180}$ and $NCAM_{140}$, respectively) are similar to those for GluR subunits 1–3 (i.e., a transient decrease followed by stable recovery). Some of the $NCAM_{180}$ measures had a large variance (Fig. 9A) due to smeared immunoreactivity, which was variably present and is presumably due to glycosylation. The densitometric analyses attempted to exclude the smeared glycosylation, since the latter went through a dramatic maturation process across culture days, comparable with that seen during development in vivo (Hoffman et al., 1995).

The high maintenance of $NCAM_{180}$ in CD30–45 slices as compared with uncultured slices was similar to that for AMPA receptor markers (Table 1). Moreover, the content of $NCAM_{180}$ in CD 45 slices was ~90% of that found in fresh adult hippocampal samples (Table 1) and CD 60 slices contained 78 ± 13% (mean ± SEM; n = 4) of the adult concentration. $NCAM_{140}$ also exhibited stable maintenance (see Table 1), while the 120 kDa $NCAM_{120}$ isoform gradually increased in concentration over initial measures that were just above detectability (see Fig. 9B). These observations on NCAM isoforms across culture days closely match those found during telencephalic development (Hall and Bahr, 1994). It is also noteworthy that immunohisto-

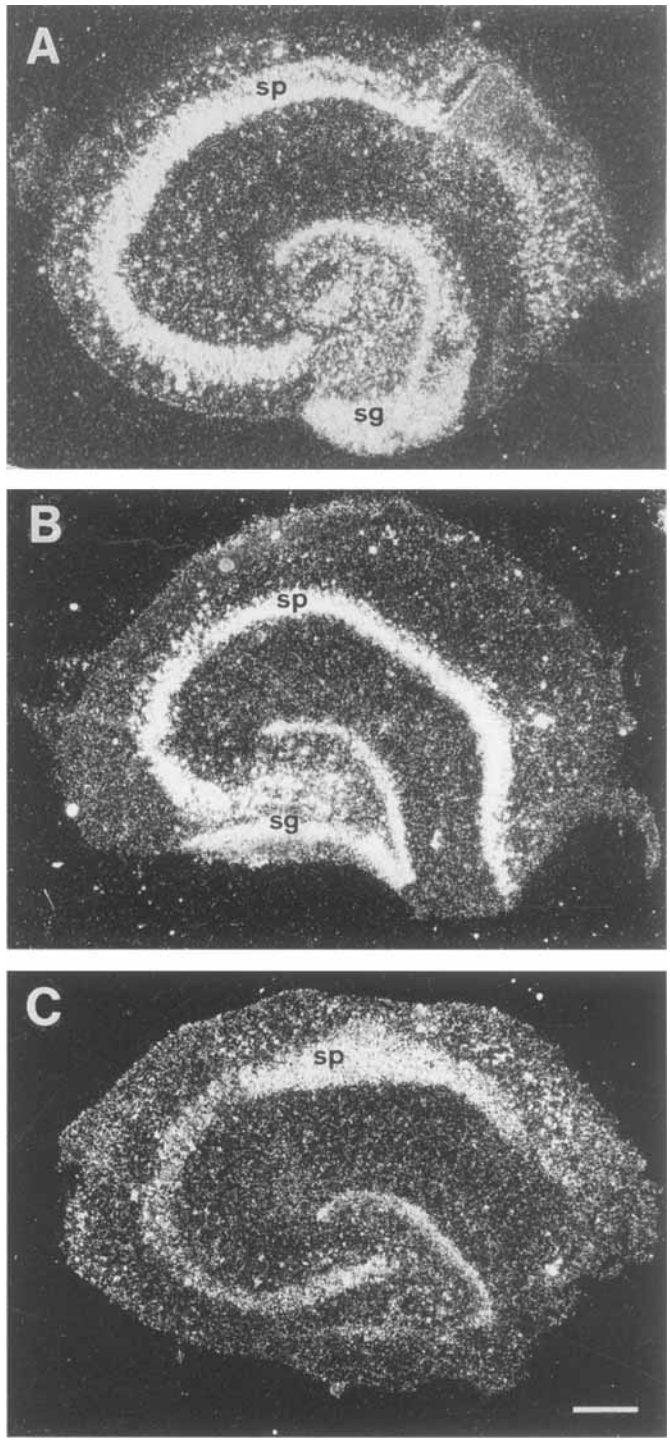

FIGURE 7. Localization of hybridization of GluR cRNAs in long-term hippocampal slices. Slices maintained for ~15 days in culture were processed for in situ hybridization with [^{35}S]cRNA probes that bind the GluR1 (A), GluR2 (B), and GluR3 (C) mRNAs. Dark-field photomicrographs show the highest densities of hybridization (evident in white) in the stratum granulosum (sg) and stratum pyramidale (sp). Scale bar = 370 μm.

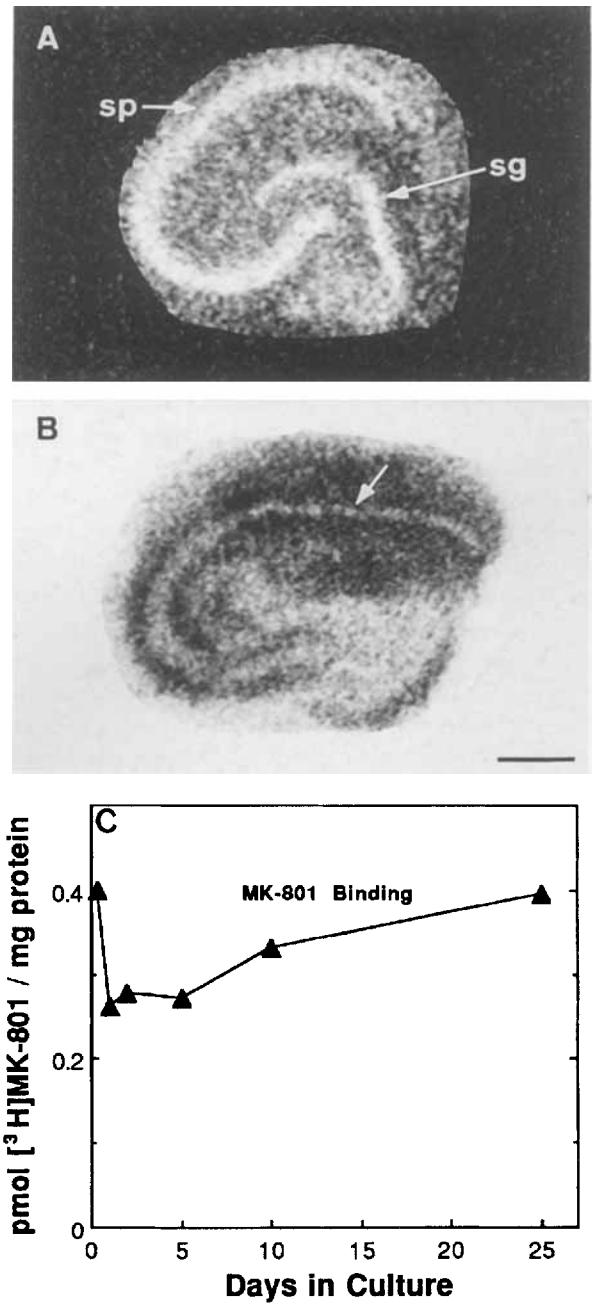

FIGURE 8. Development and maintenance of NMDA receptor markers in cultured hippocampal slices. A,B: Slices maintained in culture for 10–20 days were processed for *in situ* hybridization with [^{35}S]cRNA complementary to NMDAR1 mRNA, and by [^{3}H]MK-801 binding autoradiography with 50 nM ligand. The dark-field photomicrograph in panel A shows the highest densities of NMDAR1 mRNA (evident in white) in the stratum granulosum (sg) and stratum pyramidale (sp). In contrast, [^{3}H]MK-801 densely labeled dendritic fields and molecular layers without labeling neuronal cell body layers (see arrow in panel B). Scale bar = 560 μm. C: Slices were harvested over time in culture, and groups of eight slices were homogenized and lysed membranes prepared (n = 2–3 groups). Equal protein samples (20 μg) were analyzed by a filtration binding assay using 2 nM [^{3}H]MK-801. Nonspecific binding in the presence of 500 μM nonradiolabeled MK-801 was also determined and subtracted from the total binding at each time point. The average specific binding activity values are plotted.

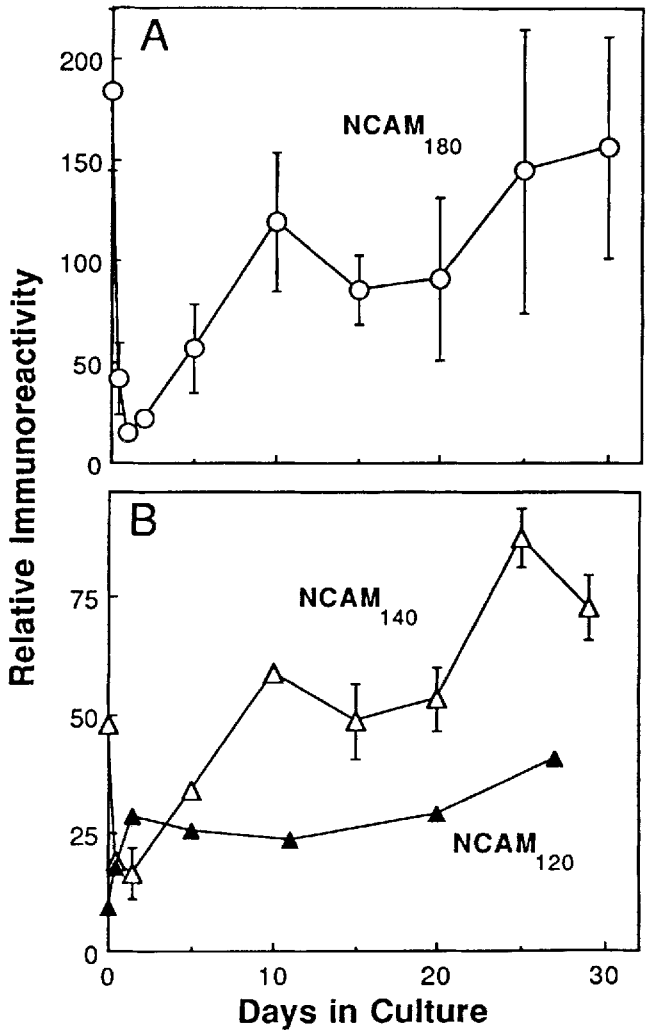

FIGURE 9. Neural cell adhesion molecule isoforms (NCAMs) develop differently in cultured hippocampal slices. Slices harvested at different culture days were prepared as in Figure 4 (n = 3 groups of 8 slices). Equal protein aliquots were subjected to SDS-PAGE and immunoblotted with anti-NCAM antibodies in order to measure the content of the 180 kDa isoform excluding the characteristic smeared antigenicity (A), as well as the 140 and 120 kDa isoforms (B). Each value plotted represents the mean specific immunoreactivity of the antigen determined by image analysis [(density − background) × area)] ± SEM.

logical techniques revealed native distribution of NCAM epitopes in and around pyramidal neurons of the slices (not shown). Patterned immunoreactivity was evident within the stratum radiatum, where immunostaining was especially heavy at the base of apical dendrites. In summary, stable maintenance of mature NCAM species indicates that cultured hippocampal slices are suitable for the analysis of NCAM biochemistry.

A stable maintenance profile similar to those for GluR subunits was exhibited by the synaptic vesicle marker synaptophysin (Table 1; also see Bahr et al., 1994a). Regarding the localization of the synaptophysin mRNA, in situ hybridization with a specific [^{35}S]cRNA probe showed that the transcript has a uniform distribution throughout neuronal cell layers, similar to that of the mRNAs for GluR1–2 and NMDAR1 (see Figs. 7 and 8).

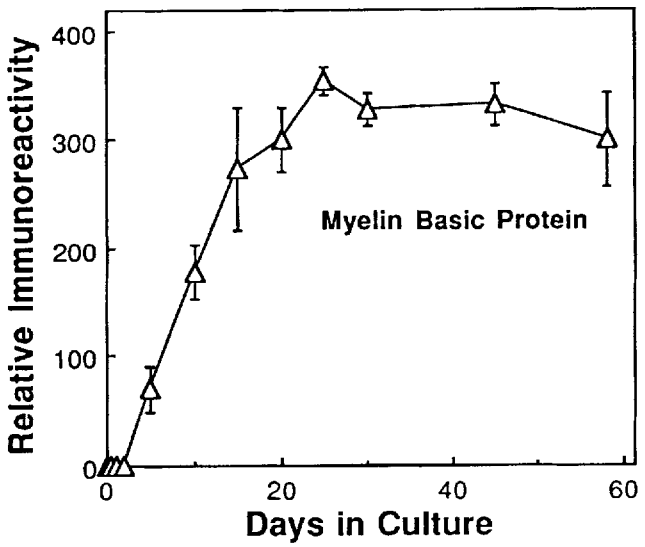

FIGURE 10. Development of a myelination marker in long-term slices. Hippocampal slices were prepared and maintained in culture for the indicated time periods and then were analyzed by immunoblot as described in Materials and Methods. Three groups of eight slices were harvested and homogenized, and equal aliquots of protein were subjected to electrophoresis in order to immunostain the oligodendrocyte marker myelin basic protein (MBP). The relative optical density and image area of the labeled antigen were quantitatively compared within single immunoblots by computerized image analysis [mean {(density − background) × area)} ± SEM].

The blots containing slice samples across culture days were subsequently stained for the structural proteins spectrin and MAP2; their profiles were also similar to those for GluRs (data not shown). The dense distribution of synaptophysin and MAP2 within the dendritic fields of long-term slices has been previously shown to closely resemble their native state in vivo (Bahr et al., 1994a). Spectrin epitopes, on the other hand, have been localized to neurons, blood vessels, and dendrites (Vanderklish et al., 1995).

Lastly, the development and maintenance of myelin basic protein (MBP) was measured in order to judge the myelination level in long-term slices. MBP serves as a marker for oligodendrocytes and has been shown to be localized to myelin membrane (Day and Potter, 1986; Goto et al., 1987). Figure 10 reveals that the ~19 kDa antigen labeled by anti-MBP monoclonal antibody exhibited low expression levels between CD 0 and 2, increased steadily from CD 5 to CD 15 and remained stable between CD 15 and 60. A similar profile was exhibited by a second labeled band of approximately 22 kDa. The MBP level in CD 30–60 slices was ~90% of the level expressed by fresh adult hippocampal samples (see Table 1).

DISCUSSION

The use of long-term hippocampal slices for studying synaptic mechanisms is predicated upon the assumption that the synapses formed in the cultured tissue are comparable with synaptic connections in the intact brain. There is a possibility that cultured neurons exist perpetually in an immature or otherwise "specialized" state, or that only a select population of cells survives the culturing process. In other words, organotypic cultures might be quite different from the in vivo systems for which they are intended to be a model. Evidence from many of the aforementioned studies indicates, however, that long-term slices possess many adult-like features, and the results presented in this paper add to this evidence with the observation that the cultured slices are similar to the in vivo hippocampus with regard to the density, distribution, and properties of glutamate receptors, the time-dependent changes in several developmentally regulated neuronal marker proteins, and the morphological organization of neurons, glia, and their processes. The cultures used here, in particular, do not exhibit the lateral spreading and thinning often seen with other explant culture methods. Moreover, these cultures possess the same high synaptic density that apparently allows brain networks not only to be able to store information but to also be vulnerable to pathological processes in the event of global depolarization. The maintenance of high levels of two types of glutamate receptors supports the latter. Consequently, the organotypic slice system described here provides an experimental in vitro system that is ideally suited to complement in vivo preparations and acute slices, and provides a means of studying both the physiological processes subserving synaptic plasticity, including the possibility of long-lasting changes in glutamate receptor function (see Vanderklish et al., 1992), and the effects of chronic excitotoxic insults that might contribute to subacute hippocampal neurodegeneration (see Bahr et al., 1994a).

Long-term slices are well suited to explore questions such as how synaptic properties are controlled by the composition of neurotransmitter receptors since they allow the manipulation of particular proteins by translational suppression (Vanderklish et al., 1992; Neve et al., 1993; Bednarski et al., 1995) and by the expression of foreign genes (Bergold et al., 1993; Bahr et al., 1994b). AMPA receptors are of particular interest in this regard since long-term potentiation (LTP) may result from a change in the properties of these receptors (Ambros-Ingerson et al., 1991, 1993) and since suppression of the AMPA receptor subunit GluR1 has been demonstrated to impair the ability of synapses in hippocampal field CA1 to express LTP (Vanderklish et al., 1992). It is not unlikely that the high ratio between GluR1 and GluR2/3 immunoreactivity levels, which is characteristic of the hippocampus in vivo (Bahr et al., 1995a), is also of consequence in this context. The density of the GluR4 subunit is conspicuously low in the in vivo hippocampus, yet long-term slices developed measurable levels of GluR4 after approximately 12 days in culture. Thus, another issue worthy of further investigations is whether this subunit confers novel properties to hippocampal AMPA receptors akin to those reported by Mosbacher et al. (1994).

Characterization of specific features of hippocampal synapses within the cultured slice model must also be attentive to the fact that presynaptic terminals are distributed toward the upper surface of the slice. An increase in the relative concentration of presynaptic proteins in superficial aspects of the slice may be a result of 1) clearing processes activated after slice preparation that remove damaged material from the surface more efficiently than

from lower areas, and/or 2) accumulation of glial cells and extracellular matrix components at the bottom of the slice that form a barrier between the nervous tissue and the culture medium (Buchs et al., 1993). While these events may not have physiological significance, their impact on the distribution of synapses is of particular importance for certain electrophysiological studies (e.g., Vanderklish et al., 1992).

It should be mentioned that AMPA receptors, NMDA receptors, and several other proteins exhibited a post-cutting decrease, but they generally recovered from this depression within a few culture days. The decrease is most likely attributable to the initial stress of slice preparation, which may have triggered a general depression in the rate of transcription and/or translation in association with the degradation of damaged cells and their elimination from the slice. Alternatively, spontaneous seizure activity provoked by the slicing procedure (via disruption of inhibitory inputs, etc.) may temporarily disrupt the transcription of some mRNAs, as has been shown to occur in vivo for the GluR1 subunit (Gall et al., 1990).

Overall, long-term slices possess the differentiated morphological characteristics of the hippocampus at the second postnatal week from which slices are prepared, and appear to undergo further maturation in culture to attain many adult-like qualities. This view is supported by a number of observations. First, myelin basic protein is increased over the course of 2 weeks from below detectability to a high level that was stably maintained for 2 months in culture. Thus, changes in this index of myelination matches the developmental time course reported by Cohen and Guarnieri (1976; see also Carson et al., 1983) for many regions of rat brain. Furthermore, Berger and Frotscher (1994) demonstrated that oligodendrocytes in hippocampal slice cultures have comparable distribution and morphological characteristics as in situ. Second, the astroglia marker GFAP increased steadily following the initial clearing period and reached a stable level by approximately CD 10 (Bahr et al., 1994a); this pattern of astrocyte proliferation closely matches that seen during brain development in vivo (Noetzel et al., 1985; Malloch et al., 1987). The culture method used here may have allowed glial development to follow a rather native course, since it does not include the use of cytosine arabinoside, an agent that is commonly used with other culture methods to quell astroglial growth (see Ronnett et al., 1991; Caeser and Aertsen, 1991). Third, the extent of glycosylation of the synaptic adhesion protein $NCAM_{180}$ greatly declined with increasing culture time; that is, the smeared immunoreactivity above the 180 kDa band on blots was reduced (Hoffman et al., 1995). This conversion is comparable to that seen during telencephalic development in vivo (Hall and Bahr, 1994) and may be a useful marker for the completion of synaptogenesis (Muller et al., 1994).

In conclusion, it is evident that long-term hippocampal slices attain many features of the mature hippocampus, including those that are notably important with regard to synaptic structure and function. The results suggest that the cultured slice is a valuable system for studying physiological mechanisms, especially those that operate in excitatory synapses. Long-term slices should also be of considerable utility as an intermediary link between in vivo experiments and those using dissociated cell cultures.

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