The Journal of Neuroscience, May 1995, 15(5): 3840–3851

Differential Spatiotemporal Expression of K^+ Channel Polypeptides in Rat Hippocampal Neurons Developing *in situ* and *in vitro*

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Hippocampal neurons are highly plastic in their excitable properties, both during development and in the adult brain. As voltage-sensitive K^+ channels are major determinants of membrane excitability, one mechanism for generating plasticity is through regulation of K^+ channel activity. To gain insights into the regulation of K^+ channels in the hippocampus, we have analyzed the spatiotemporal expression patterns of five K^+ channel polypeptides in rat hippocampal neurons developing *in situ* and *in vitro*. Delayed rectifier-type channels (Kv1.5, Kv2.1, and Kv2.2) are expressed on all neuronal somata and proximal dendrites, while A-type channels (Kv1.4 and Kv4.2) are present distally on distinct subpopulations of neurons. The development of these patterns *in situ* is monotonic; that is, while the time and spatial development varies among the channels, each K^+ channel subtype initially appears in its adult pattern, suggesting that the mechanisms underlying spatial patterning operate through development. Immunoblots confirm the differential temporal expression of K^+ channels in the developing hippocampus, and demonstrate developmentally regulated changes in the microheterogeneity of some K^+ channel polypeptide species. Temporal expression patterns of all five K^+ channels observed *in situ* are retained *in vitro*, while certain aspects of cellular and subcellular localization are altered for some of the K^+ channel polypeptides studied. Similarities in K^+ channel polypeptide expression *in situ* and *in vitro* indicate that the same regulatory mechanisms are controlling spatiotemporal patterning in both situations. However, differences between levels of expression for all subtypes studied except Kv2.1 indicate additional mechanisms operating *in situ* but absent *in vitro* that are important in determining polypeptide abundance.

[Key words: ion channels, gene expression, molecular weight, primary neuronal culture, development]

Received Aug. 22, 1994; revised Dec. 5, 1994; accepted Dec. 21, 1994.

We thank Sean Trowbridge for technical assistance with immunostaining *in situ*, and Kimberley Lopez and Erik Runko for technical assistance with the cultures. We thank Drs. Morgan Sheng and Lily Jan for the generous gifts of the Kv1.4 and Kv4.2 antibodies, and for critical reading of the manuscript, and Drs. Paul Hwang and Solomon Snyder for the Kv2.2 antibodies. We also thank Dr. Gary Banker and Dr. Michael Caplan for advice during the initiation of this project, and Drs. Paul Adams and Joel Levine for critical reading of the manuscript. This research was supported in part by grants to J.S.T. from the National Institute of Health (NS29876) and from the Council for Tobacco Research. This work was done during the tenure of an Established Investigatorship from the American Heart Association (J.S.T.).

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Voltage-sensitive K^+ channels are integral plasma membrane proteins of major physiological and pathological importance (Hille, 1992). During development, K^+ currents are the first voltage-gated currents to appear in neurons (Spitzer, 1991), and more than a dozen physiologically distinct K^+ currents are observed during development of the rat brain, with many neurons exhibiting more than one physiologically defined K^+ channel type (Halliwell, 1990). Expression of K^+ currents varies substantially among specific classes of neurons isolated from either different regions of the brain, or from a given region at different stages of development (Ribera and Spitzer, 1992). The time of expression, cellular abundance and subcellular distribution of specific K^+ channel polypeptides are important factors that can influence the physiological properties of neuronal K^+ currents.

Hippocampal neurons are highly plastic in their excitable properties, both during development and in the adult brain. One mechanism for generating this plasticity is the regulation of the activity of voltage-sensitive K^+ channels (Ribera and Spitzer, 1992). Here we detail and contrast the spatiotemporal developmental pattern of five K^+ channel polypeptides in rat hippocampal neurons differentiating *in situ* and *in vitro*, in order to gain insights into extrinsic factors important in the regulation of K^+ channels during development. The *in vitro* system has several advantages over *in situ* studies including the presence of only a limited number of neurons, the wide spacing between the individual cells, the absence of afferent axons from remote sites, and the absence of the wide variety of hormonal and humoral influences which are present *in situ*. The inability to control such factors *in situ*, either at baseline or after experimental interventions, often confounds the interpretation of results. Primary neuronal cultures also have obvious advantages over other *in vitro* systems (e.g., pheochromocytoma cells, transfected fibroblasts, etc) in expressing the precise complement of transmitters, growth factors, receptors and other neural-specific proteins essential to the establishment and maintenance of the authentic neuronal phenotype. We have focused these studies on a well-characterized primary culture system for hippocampal neurons (Goslin and Banker, 1991) which provides low density, long-lasting, and almost pure neuronal culture. Stages of differentiation (Bartlett and Banker, 1984a,b), development of polarity (Dotti et al., 1988), morphology (Benson et al., 1994), neurotransmitter content (Mattson, 1988), and electrophysiological properties (Segal and Barker, 1984) of neurons in this system have been well characterized, and correspondence to the same parameters *in situ* have been impressive (Fletcher and Banker, 1989; Kleiman et al., 1990; Pietrini et al., 1992).

Here we report the distinctive features of K^+ channel poly-

peptide spatiotemporal expression patterns in hippocampal neurons developing both *in situ*, and in these *in vitro* cultures. Distinct patterns of temporal expression, and spatial distribution at both the cellular and subcellular levels are observed for each of the K^+ channel subtypes. Generally, temporal expression patterns observed *in situ* are retained *in vitro*, while certain aspects of cellular and subcellular localization are altered. This indicates that complex regulatory mechanisms present *in situ* but not *in vitro* govern the cellular expression and subcellular targeting of K^+ channel polypeptides.

Materials and Methods

Materials

Timed pregnant rats were from Taconic Farms (Germantown, NY). Horse serum was obtained from JRH Bioscience (Lenexa, KS). Fluorescein, rhodamine, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit second antibodies were from Organon Teknika (West Chester, PA), and biotinylated goat second antibody and avidin and biotin conjugated peroxidase from Vector Labs (Burlingame, CA). Enhanced chemiluminescence (ECL) reagent was from Amersham (Arlington Heights, IL). Prestained molecular weight standards are from Sigma (St. Louis, MO). All other reagents were from Sigma or Boehringer Mannheim (Indianapolis, IN).

Animals

Fetal (E19) and newborn Sprague–Dawley albino rats were perfused/sacrificed under anesthesia (Equithesin, 1.0 ml/100 gm body weight, for adults) or deep hypothermia (young animals). Brains were removed, and processed for biochemical or morphological analyses.

Preparation of hippocampal sections

Fetal brains were immersed in 2% paraformaldehyde in 0.1 M PBS (pH 7.4) for 4 hr. Postnatal animals were perfused through the aorta with the same solution for ages postnatal day (P) 1 to P12 and 4% paraformaldehyde in the same buffer for adult, delivered at 140 torr pressure for 5 min (Lenn and Beebe, 1977). Thereafter, brains were removed from the skull and immersed in the same fixative solution used for perfusion. Fixation was continued for 4 hr at 4°C. Brains were then transferred to 10% sucrose in 0.1M PBS at 4°C until they sank, followed by 20% and 30% sucrose until they sank. They were dissected into coronal blocks, which were quickly frozen on powdered dry ice and mounted on chilled chucks covered with OCT. They were stored frozen in the cryostat, sectioned at 30 μm, and stored in PBS at 4°C until used within a week.

We have analyzed the spatiotemporal pattern of K^+ channel polypeptide expression in anterior, middle, and posterior hippocampal sections within the same brain for all five K^+ channel subtypes. No differences were observed between the staining pattern among these regions for the same antibody.

Primary cell cultures

Preparation of primary cultures was done according to the method of Banker and Cowan (1977).

Astrocyte cultures. Cerebral hemispheres from neonatal rat pups (P1) were dissected in HEPES buffered, calcium and magnesium-free balanced salt solution (HBSS), pH 7.35. Tissue was incubated in HBSS with 0.25% trypsin and 1% DNase, 15 min at 37°C. Cell suspension was diluted 1:1 with glial plating medium (minimal essential medium (MEM) with 10% horse serum, 0.6% glucose, 100 U/ml penicillin and 100 μg/ml streptomycin). Cells were then collected by centrifugation, resuspended in a glial plating medium and plated at 10^6 per 10 cm tissue culture dish, coated with 0.1 mg/mL poly-smd-lysine. Cultures were fed twice a week. When confluent, some dishes were used for neuronal cultures within 2 weeks, and some were replated at the original density, for use 15 d later. Subculture was done only once. One to several days before using the glial cultures, their medium was changed to 16 ml of neuronal maintenance medium (below).

Neuronal cultures. E19 hippocampi dissected in HBSS were digested with 0.25% trypsin, 15 min at 37°C and dispersed by trituration with a constricted Pasteur pipette 15–20 times to produce a homogeneous suspension. Cells were plated at 400,000 per 10 cm tissue culture dish, containing 12 1 mg/ml poly-L-lysine coated coverslips in MEM with 10% horse serum. After 4 hr for the cells to adhere to the substrate, coverslips were transferred into the dishes containing the confluent layer of astrocytes. Neurons did not contact the glia, due to the presence of paraffin wax pedestals. Cultures were maintained in serum-free MEM with N2 supplements, 0.1% ovalbumin and 0.1 mM sodium pyruvate (Goslin and Banker, 1991). After 3 d 5 μM cytosine arabinoside was added to inhibit the proliferation of non-neuronal cells. Cultures were kept at 36°C in a humidified atmosphere of 95% air, 5% CO_2. One-third of the culture medium was changed once a week.

Brain membrane preparations

A crude membrane fraction from freshly dissected adult whole brain and hippocampi of rats of different ages [E19, P1, P5, P7, P9, P12, and P92 (adult)] was prepared essentially as described (Trimmer, 1993). Samples were homogenized in 0.3 M sucrose in 10 mM sodium phosphate, pH 7.4, containing 1 mM EDTA and a protease inhibitor cocktail (1 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml antipain, 10 μg/ml benzamidine, and 0.5 mM PMSF). The homogenate was centrifuged at 3000 × *g* for 10 min to remove nuclei. The pellet was then resuspended in homogenization buffer and respun. Combined supernatants were centrifuged at 45,000 × *g* for 90 min. The pellet, which represents crude membrane fraction, was resuspended in homogenization buffer and stored at −80°C. Protein content was determined using the bicinchoninic acid method (BCA; Pierce, Rockford, IL) with bovine serum albumin as a standard.

Preparation of antibodies

In this study we have used affinity-purified rabbit polyclonal antibodies against Kv1.4, Kv1.5, Kv2.1, Kv2.2, and Kv4.2, a mouse monoclonal antibody against Kv1.5 and site-specific polyclonal antisera raised against two different portions of Kv2.1 (Trimmer, 1991). Antibodies against Kv1.4 and Kv4.2 were provided from Drs. M. Sheng and L. Jan, University of California, San Francisco School of Medicine (Sheng et al., 1992). Polyclonal anti-Kv.1.4 antibody recognizes N-terminal portion of Kv1.4, while two polyclonal anti-Kv4.2 antibodies, Kv4.2N and Kv4.2C, recognize N- and C-terminal portions of Kv4.2, respectively. Polyclonal antibodies against C-terminal and N-terminal portions of Kv2.2 were obtained from Drs. P. Hwang and S. Snyder, Johns Hopkins School of Medicine (Hwang et al., 1992). All other antibodies and antisera were prepared in our laboratory: polyclonal anti-Kv2.1 antibodies KC and pGEX-drk1 (Trimmer, 1991), polyclonal anti-Kv1.5 antibody pGEX-K41C (Takimoto et al., 1993), and monoclonal anti-Kv1.5 antibody K4/209 (J. S. Trimmer, unpublished data). Each batch of antiserum and antibody, whether produced here or elsewhere, was tested for specificity and cross-reactivity by immunostaining of K^+ channel-transfected COS-1 cells and immunoblot analysis of transfected COS-1 cell lysates and brain membrane preparations (Shi et al., 1994; Maletic-Savatic et al., unpublished observations). Control experiments in which antibodies were preincubated with appropriate peptides or fusion proteins were also performed to demonstrate the specificity of antibody staining.

SDS/polyacrylamide gels and immunoblot analysis

For immunoblots, 30–100 μg of hippocampal crude membrane sample was added to reducing SDS sample buffer (Maizel, 1971), boiled for 5 min and size-fractionated on 7.5% polyacrylamide-SDS gels. Each lane of the individual blots had the same amount of membrane protein, although the amount differed for each K^+ channel polypeptide (Kv2.1, 50 μg; Kv2.2, 100 μg; Kv1.5, 100 μg; Kv1.4, 30 μg). Our SDS gel system utilizes an SDS mixture from Sigma (#L5750) that has been previously shown to promote electrophoretic separation of highly related polypeptides (e.g., α and β tubulin, Best et al., 1981), or different phosphorylation states of the same protein (e.g., particulate guanylate cyclase, Ward et al., 1985), that are not possible with pure SDS. Proteins are electrophoretically transferred to nitrocellulose membranes as described (Trimmer, 1993). Nonspecific protein-binding sites were blocked by incubation in Blotto [5% (w/v) nonfat dry milk, 0.15 M NaCl, 20 mM Tris-HCl pH 7.5 with 0.01% Antifoam A (Johnson et al., 1984) for 30 min at room temperature. Membranes were incubated with antisera (against Kv2.1) or affinity-purified antibodies (against Kv1.4, Kv1.5, Kv2.2, and Kv4.2) diluted in Blotto, for 2 hr at room temperature. Appropriate dilution for each antibody/antiserum was determined by titration. Dilutions have been anti-Kv1.4, 1:1000; rabbit anti-Kv1.5,

1:25; anti-Kv2.1, 1:1000; and anti-Kv2.2, 1:10. After several washes in Blotto, membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:2000 in Blotto) for 1 hr at room temperature, followed by several washes in PBS. The blots were then incubated 1 min in the substrate for enhanced chemiluminescence (ECL) and autoradiographed on Fuji RX x-ray film. Determination of the apparent M_r was based on relative mobilities of prestained molecular weight standards (Sigma).

Immunostaining

Immunostaining of hippocampal sections. Sections were placed in mesh-bottom baskets, approximately 10 sections/basket, and incubated 2 hr at room temperature with the primary antibody, diluted in PBS containing 1% normal goat serum and 0.3% Triton X-100 (PBSGT). The optimal concentration of each antibody/antisera was determined by titration. Dilutions were as follows: polyclonal anti-Kv1.4 and anti-Kv4.2C affinity-purified antibodies, 1:1000; mouse monoclonal anti-Kv1.5 antibody tissue culture supernatant, 1:2; polyclonal anti-pGEXdrk1 and anti-KC antisera, 1:500; polyclonal anti-Kv2.1 and anti-Kv2.2 affinity-purified antibodies, 1:75. After washing three times in PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Labs) in PBSGT for 1 hr at room temperature, followed by washes in PBS. Tissue was then incubated 1 h at room temperature with avidin and biotin conjugated peroxidase (ABC; Vectastain, Vector Labs.), 9 μl of each reagent diluted in 1 ml of PBS. After washes in PBS, sections were preincubated in 0.07% diaminobenzidine (DAB) in Tris-HCl buffer pH 7.5, containing 0.015% H_2O_2 for approximately 5 min. Sections were then rinsed in PBS, mounted onto subbed slides, dried overnight, dehydrated, cleared, and coverslipped. An adult section was processed along with the sections from younger animals within each basket, for comparison of the staining intensity. A negative control for the primary antibody (preimmune serum from the same rabbit and/or PBSGT without primary antibody) was run with each batch, although these were consistently negative.

Immunostaining of cultured hippocampal neurons. Coverslips were removed from culture dishes with forceps and processed in 35 mm plastic petri dishes. Coverslips were washed in PBS, fixed in 3% paraformaldehyde in PBS for 20 min, washed in PBS again, permeabilized with 0.3% Triton X-100 in PBS for 5 min, rinsed again, and stored at 4°C in PBS with 0.05% sodium azide. Nonspecific protein-binding sites were blocked by incubation in Blotto (see above) containing 0.1% Triton X-100 (Blotto-T) for 30 min at room temperature. Incubation with primary antibody appropriately diluted (as above) in Blotto-T was for 2 hr at room temperature. After several washes in Blotto-T, coverslips were incubated with the appropriate secondary, rhodamine-conjugated antibody (1:500 dilution in Blotto-T) for 1 hr and washed again in PBS containing 0.1% Triton X-100. The samples were mounted in 10% (vol/vol) glycerol in PBS with 0.1% *p*-phenylenediamine to retard photobleaching. Primary antibody was omitted for controls. Cultured neurons immunostained with anti-Kv4.2 antibody were counted in the following way: 20–25 neurons at four corner fields of six coverslips, each bearing different cultures, were counted under the Zeiss epifluorescence microscope at 40X magnification. They were scored for immunofluorescent staining and the percentage of Kv4.2-positive cells was calculated.

Results

In situ expression of K+ channel subtypes in the developing hippocampus: immunoblot analysis

Equal amounts of membranes prepared from whole brain and hippocampi of rats of six ages [embryonic day 19 (E19), postnatal day 1 (P1), P5, P7, P9, and P92 (adult)] were analyzed for the expression levels of distinct K+ channel polypeptides by immunoblot analysis using the appropriate rabbit polyclonal antibodies (Fig. 1).

Delayed rectifier-type K+ channel polypeptides

Kv2.1. Adult whole brain membranes show a microheterogeneous population of Kv2.1 species, with two principal bands of M_r = 105–115 kDa and 120–130 kDa, as previously reported (Trimmer, 1991, 1993). In the hippocampus, three bands of M_r = 105, 120, and 125–130 kDa are present at P1 and subsequent ages. All detected species of Kv2.1 have larger M_r than predicted for the core polypeptide (95.3 kDa; Frech et al., 1989). The developmental expression of these Kv2.1 isoforms in the hippocampus, as in whole brain (Trimmer, 1993), is complex, indicating expression of distinct molecular isoforms at various times during development. Differential phosphorylation of the Kv2.1 polypeptide appears to be the basis of at least some of the observed heterogeneity (G. Shi, S. A. Keilbaugh, and J. S. Trimmer, unpublished data).

Kv2.2. Two prominent bands of M_r = 125–130 and 130–135 kDa are present in adult whole brain membranes. In hippocampus, the same bands appear simultaneously at P5 and persist thereafter. On some gels additional faint bands at M_r = 95 and 110 kDa are present. The predicted molecular weight of the core Kv2.2 polypeptide is 90.7 kDa (Hwang et al., 1992), which indicates that posttranslational modifications contribute to the mature Kv2.2 polypeptide found in brain.

Kv1.5. A single, homogeneous band of M_r = 63 kDa is obtained on immunoblots of adult whole brain membranes. In the hippocampus, Kv1.5 is not detectable at E19, P1, or P5, appears faintly at P7 and P9, and at adulthood is equal to the signal from adult whole brain. These data indicate a regulatory mechanism acting later in development than for the other K+ channel polypeptides studied. The expression of Kv1.5 in the hippocampus parallels observed developmental increases in Kv1.5 mRNA in whole brain (Swanson et al., 1990). The size of Kv1.5 in brain (M_r = 63 kDa) is similar to the predicted molecular weight deduced from the Kv1.5 cDNA (66.5 kDa; Swanson et al., 1990).

A-Type K+ channel polypeptides

Kv1.4. For adult whole brain membranes, a single immunoreactive band is present at M_r = 95 kDa, larger than the predicted value for the core polypeptide (73.4 kDa; Stühmer et al., 1989), but in agreement with that previously reported for rat brain Kv1.4 (Sheng et al., 1992). In hippocampal membranes from E19 and P1, no immunoreactivity is detected. At P5, a sharp band of M_r = 95 kDa appears. The intensity of this band increases with further development such that the band observed in adult hippocampal membranes is as intense as with adult whole brain membranes.

Kv4.2. Membranes from whole brain and hippocampus at all ages are negative in immunoblots using anti-Kv4.2N and anti-Kv4.2C polyclonal antibodies at a range of applied concentrations, as previously reported (Sheng et al.,1992). However, the specificity of these antibodies has been established both previously (Sheng et al., 1992), and in assays on Kv4.2 transfected COS-1 cells (Maletic-Savatic, unpublished observation).

In situ expression of K+ channel subtypes in the developing hippocampus: immunohistochemical analysis

Figures 2 and 3 illustrate the different spatiotemporal expression patterns of K+ channel polypeptides at several ages. Increases in staining intensity with age from P1 through P12 to adult agree well with the immunoblot data. The following description therefore gives mainly spatial distribution features, with some comparisons to adult findings where helpful. Table 1 summarizes these data. More detailed analysis of the expression patterns of these K+ channel polypeptides in the adult rat hippocampus is presented in another paper (Maletic-Savatic, Trimmer, and Lenn, unpublished observations). Although we have no direct ultra-

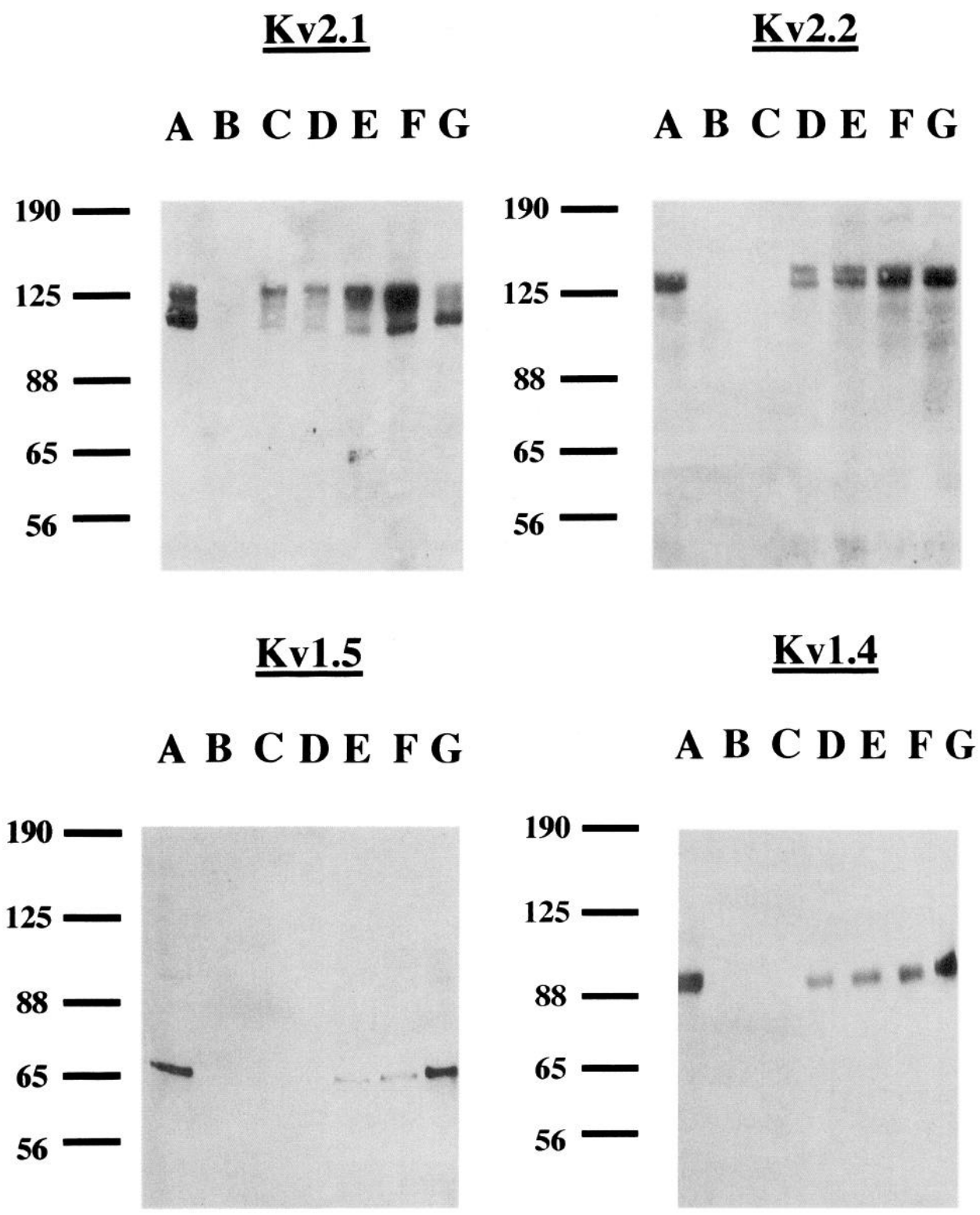

Figure 1. Immunoblot analysis of K^+ channel polypeptides in the developing hippocampus. Membrane protein prepared from adult whole brain (*lane A*) or hippocampi isolated from different aged rats [*lanes B*, E19; *C*, P1; *D*, P5; *E*, P7; *F*, P9; *G*, P92 (adult)] were fractionated on 7.5% SDS gels, and analyzed by immunoblots using the indicated subtype-specific antibodies (anti-Kv2.1, anti-Kv2.2, anti-Kv1.5, and anti-Kv1.4) and autoradiography with ECL. *Numbers on left of each panel* refer to mobility of prestained molecular weight standards. While each polypeptide increases during development, they vary in temporal pattern as well as molecular weight and degree of heterogeneity.

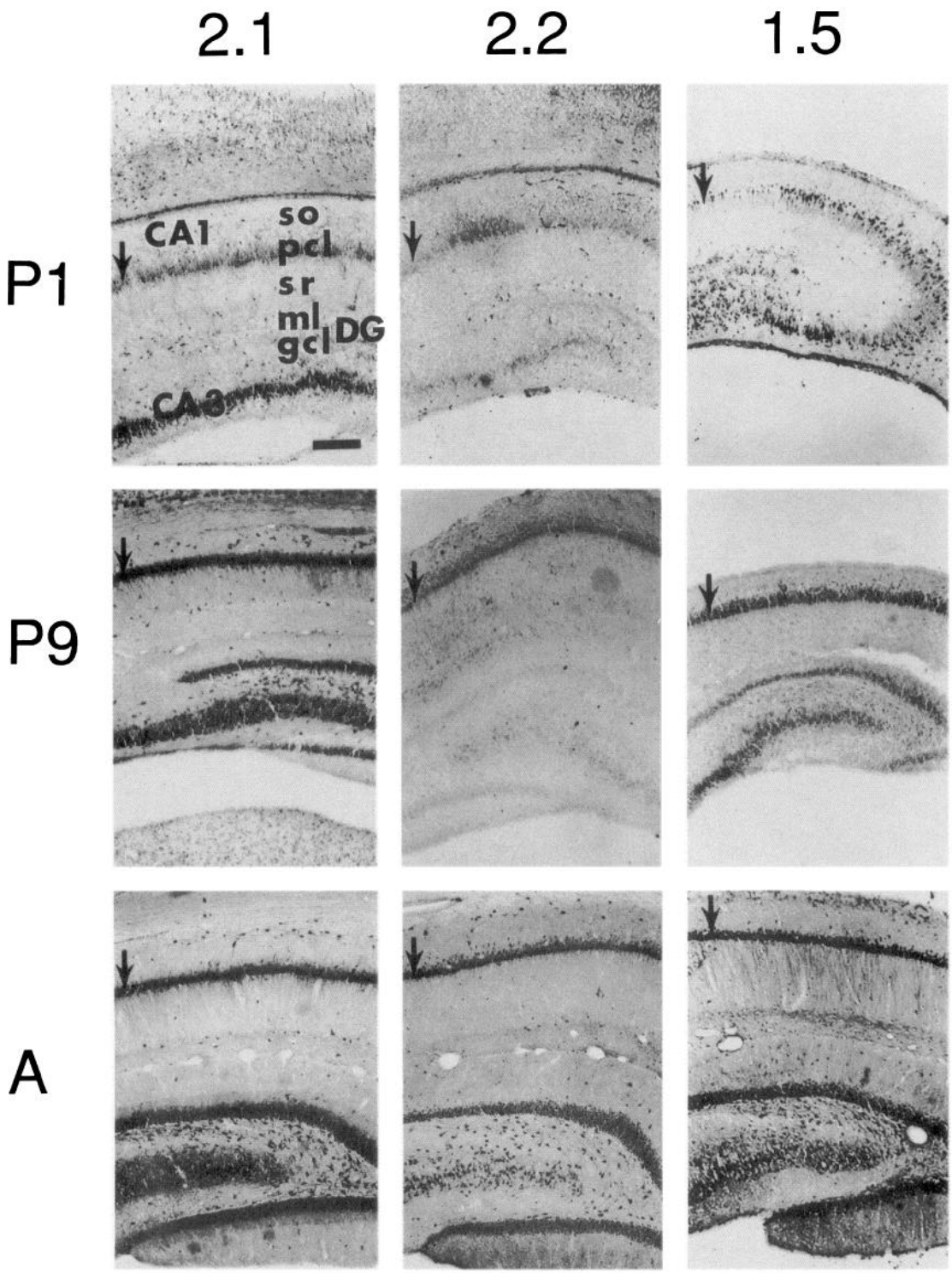

Figure 2. Immunohistochemical analyses of K^+ channel polypeptides in the developing hippocampus. Low power photomicrographs of normal rat hippocampus immunostained for the three delayed-rectifier K^+ channel polypeptides, visualized with peroxidase reaction. The pyramidal cell layer of CA1 is marked (*large solid arrow* at upper left corner) on each photograph, for orientation. Kv2.1 is expressed first at P1, as light staining of cell bodies and proximal dendrites. At P9, plasma membrane patches are present and the immunostaining is like in adult, although less in intensity. Kv2.2 is absent at P1. It is first observed at P9 as homogeneous stain in the soma and proximal dendrites. Interneurons are stained more strongly than other cells only in CA3. At adulthood, Kv2.2 is expressed in all neurons, but more in interneurons of all hippocampal layers. Kv1.5 appears at P1 in all pyramidal cell bodies in CA1 and CA3, but not in DG. At P9 and adult the intensity of Kv1.5 immunostaining increases and is more uniform among all hippocampal cells. *so*, stratum oriens (contains neuropil); *pcl*, pyramidal cell layer (contains cell bodies of pyramidal cells and some interneurons); *sr*, stratum radiatum (contains proximal dendrites of pyramidal cells and interneurons); *ml*, molecular layer of dentate gyrus (contains granule cell dendrites and projections from entorhinal cortex and ipsi- and contralateral dentate gyrus); *gcl*, granule cell layer (contains cell bodies of granule cells). CA1, CA3, and dentate gyrus (DG) correspond to the appropriate fields described by Lorente de No (1934). Note that the micrographs representing the expression of K^+ channel polypeptides at P1 are overdeveloped in order to make the stain visible after reproduction. Scale bar, 100 μm.

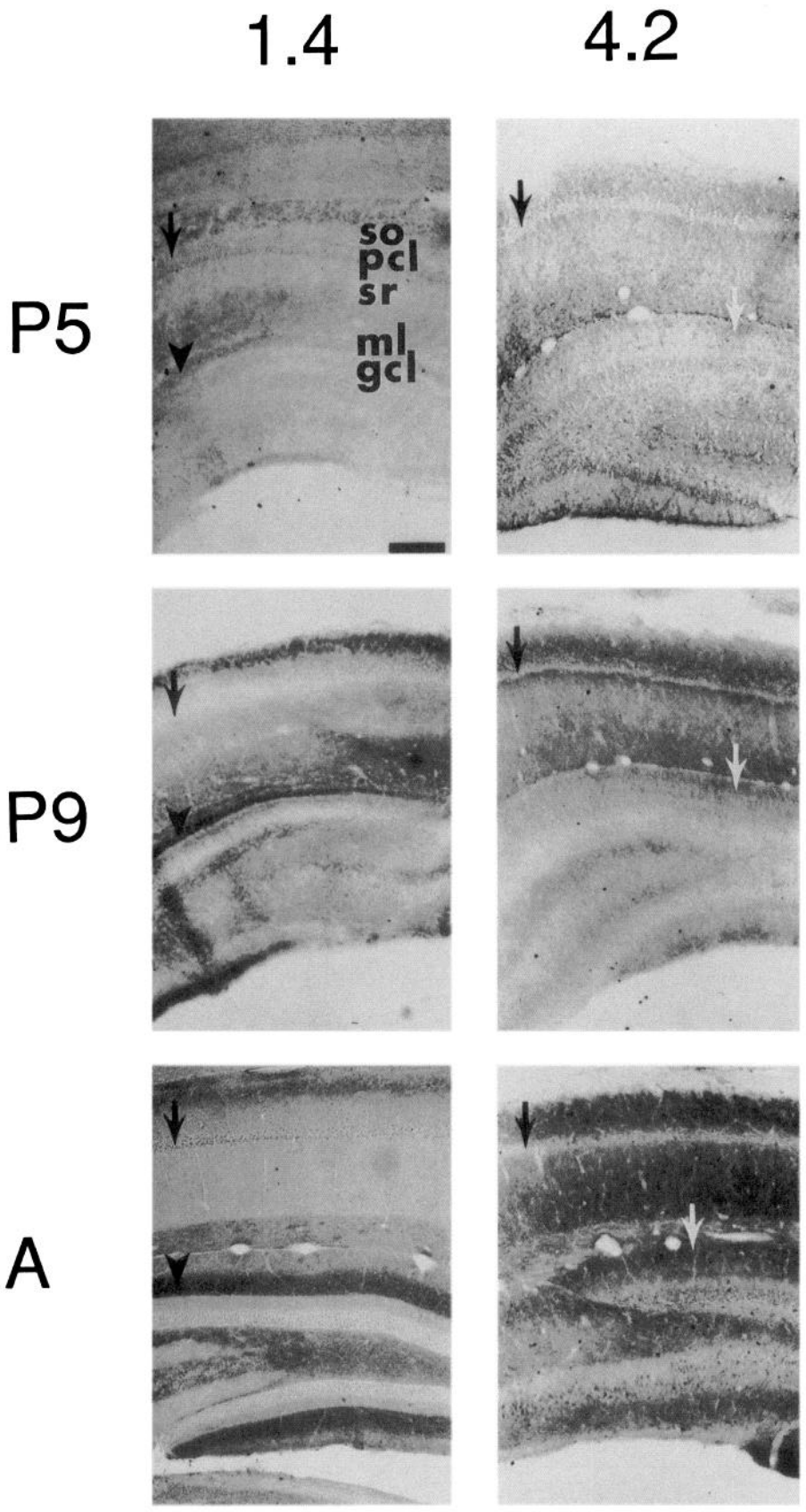

Figure 3. Immunohistochemical analyses of K^+ channel polypeptides in the developing hippocampus. Low power photomicrographs of normal rat hippocampus immunostained for the two A-type K^+ channel polypeptides, visualized with peroxidase reaction. The pyramidal cell layer of CA1 is marked (*large solid arrow at upper left corner*) on each photograph, for orientation. Kv1.4 is first expressed at P5 as light staining of CA1 neuropil, the mossy fibers in CA3 and DG, and the mid-molecular layer of DG. At P9, although less intense, the expression of Kv1.4 is the same as in the adult hippocampus. Kv4.2 is first detected at P5 as moderate staining of a few cells, with no definite neuropil staining in any area. At P9 and adult, the intensity of staining increased in both plasma membranes and neuropil (SO, SR and the outer molecular layer of DG, *arrow at the center*), as well as in interneurons (*arrow at lower left*, compare to Table 1). *so*, stratum oriens (contains neuropil); *pcl*, pyramidal cell layer (contains cell bodies of pyramidal cells and some interneurons); *sr*, stratum radiatum (contains proximal dendrites of pyramidal cells and interneurons); *ml*, molecular layer of dentate gyrus (contains granule cell dendrites and projections from entorhinal cortex and ipsi- and contralateral dentate gyrus); *gcl*, granule cell layer (contains cell bodies of granule cells). CA1, CA3 and dentate gyrus (DG) correspond to the appropriate fields described by Lorente de No (1934). Scale bar, 100 μm.

Table 1. Immunostaining of K+ channel polypeptides in adult rat hippocampus *in situ*

K+ channel polypeptide	Kv 2.1	Kv 2.2	Kv 1.5	Kv 1.4	Kv 4.2
Neuronal somas and proximal dendrites CA1, CA2, CA3	+	+	+	−	−
DG	+	+	+	−	−
Interneurons accentuated CA1, CA2, CA3	−	++	−	−	−
DG	−	++	−	−	−
Only interneuron somas and proximal dendrites CA1, CA2, CA3	−	−	−	−	+
DG	−	−	−	−	+
Axonal processes in DG and CA3	−	−	−	+	−
neuropil accentuated	−	−	−	+	−
Distal dendritic processes accentuated in neuropil CA1, CA2, CA3	−	−	−	−	+
DG	−	−	−	−	+

CA1, CA2, CA3, DG = divisions of the hippocampus; see text for further details.

structural or biochemical data to support specific assignments of subcellular localization, for brevity we will hereafter describe immunostaining present at or near the periphery of neuronal cell bodies and processes, as opposed to that which appears to be intracellular or deep within the soma, as "associated with the plasma membrane."

Delayed rectifier-type K+ channel polypeptides

Kv2.1. Kv2.1 in adult brain is expressed in all neurons, in a characteristic pattern of plasma membrane patches. Developmentally, Kv2.1 is absent at E19. At P1, there is light staining of cell bodies and proximal dendrites in CA1, CA3 and dentate gyrus (DG). At P5, staining is more intense, with plasma membrane accentuations on a minority of cell bodies, especially in CA3 (not shown). The neuropil of the inner half of SO stains homogeneously. In DG all granule cells stain moderately and molecular layer (ML) lightly. At P9 Kv2.1 has increased in intensity, with membrane accentuations on all cell bodies and proximal dendrites, which in CA1 extend across stratum radiatum (SR), but are smaller and sparse compared to the adult. Neuropil staining has become moderate, but still less than the adult.

Kv2.2. Like Kv2.1, Kv2.2 is expressed in all neurons in the adult hippocampus. Unlike Kv2.1, Kv2.2 is expressed more in interneurons than in pyramidal cells. It is first observed at P9 as a faint, homogeneous stain in the soma and proximal dendrites of CA1, CA3, and DG. Interneurons stain more strongly than other cells only in CA3 at this age. At P12 (not shown), staining has increased further, and now interneurons are stained more strongly than pyramidal cells in CA1 as well as CA3. Kv2.2 expression at P12 differs from adult only in lower intensity and fewer plasma membrane accentuations.

Kv1.5. There is homogeneous Kv1.5 expression in all neuronal cell bodies and proximal dendrites throughout the adult hippocampus, accentuated on the plasma membranes in a uniform distribution. We first detect Kv1.5 at P1 as light, occasionally moderate, staining of all pyramidal cell bodies in CA1 and CA3. This suggests a slightly greater sensitivity of immunostaining than present in the immunoblot analysis (Fig. 1), which shows no detectable staining at this age, probably due to the expression of the polypeptide in a limited number of small neurons. Kv1.5 is not observed in DG at this age. At P9, the intensity of Kv1.5 immunostaining on the cell bodies has slightly increased and is more uniform among cells, still with no neuropil or dendritic staining. At P12 (not shown), the adult pattern is present, except the staining of dendrites in CA1–3 is less extensive.

A-Type K+ channel polypeptides

Kv1.4. The distribution of Kv1.4 in the adult hippocampus is distinctive. It is detected in the ML of DG, with a strong staining in the middle third, and in mossy fibers in CA3. All neuronal cell bodies and proximal dendrites are unstained. Kv1.4 is first observed developmentally at P5 as very light staining of SO and stratum lacunosum moleculare (SLM) in CA1, the mossy fibers in CA3 and DG, and the medial entorhinal projection in the middle third of the ML. For comparison, staining in substantia nigra and globus pallidus (data not shown) is already quite strong. At P9, although less intense, the expression of Kv1.4 has the same distribution as in the adult hippocampus. At this age, outer half of SO is more homogeneous with all neuronal cell bodies and proximal dendrites unstained, the neuropil of SO is lightly stained while the mossy fibers and endings in CA3 are strongly stained, and there is a distinct pattern in DG with strong staining of medial entorhinal cortex terminals.

Kv4.2. Kv4.2 in adult hippocampus is expressed on distal dendrites and neuropil of CA1–3. It is first detected at P5, as moderate staining of a few cells in CA1–3, with no definite neuropil staining in any area. At P9, the plasma membrane staining of positive cells is increased, and now neuropil in the SO, inner SR, SLM of CA1 only, and ML are lightly and homogeneously stained. At P12 (not shown), light staining of the hilar neuropil is added. Between P12 and adult, there is a general increase in the intensity of staining, with a relative increase in the outer half of SR.

In vitro expression of K+ channel subtypes in the developing hippocampus: immunohistochemical analysis

To determine the correspondence between our results obtained with hippocampal tissue *in situ* and hippocampal neurons differentiated *in vitro*, we immunostained fixed and permeabilized cultured cells with K+ channel polypeptide-specific antibodies. All cultures utilized neurons from the hippocampi of E19 rats, analyzed at 2, 4, 6, 8, 10, 14, 18, 20, 22, and 26 d *in vitro* (DIV). No staining was seen with any K+ channel antibody to cultured astrocytes in similar experiments. Figures 4 and 5 illustrate the patterns of K+ channel polypeptide expression at 10 and 22 DIV, and Table 2 summarizes the data for seven time points.

Delayed rectifier-type K+ channel polypeptides

Kv2.1. Kv2.1 is first observed at 8 DIV as small bright spots within the cell bodies of all neurons (not shown). At 10 DIV, plasma membranes of somas and very proximal processes show accentuated staining. At 14 DIV, Kv2.1 is expressed more, especially on plasma membranes which show distinct patches (not shown). The staining also outlines the thick, proximal part of

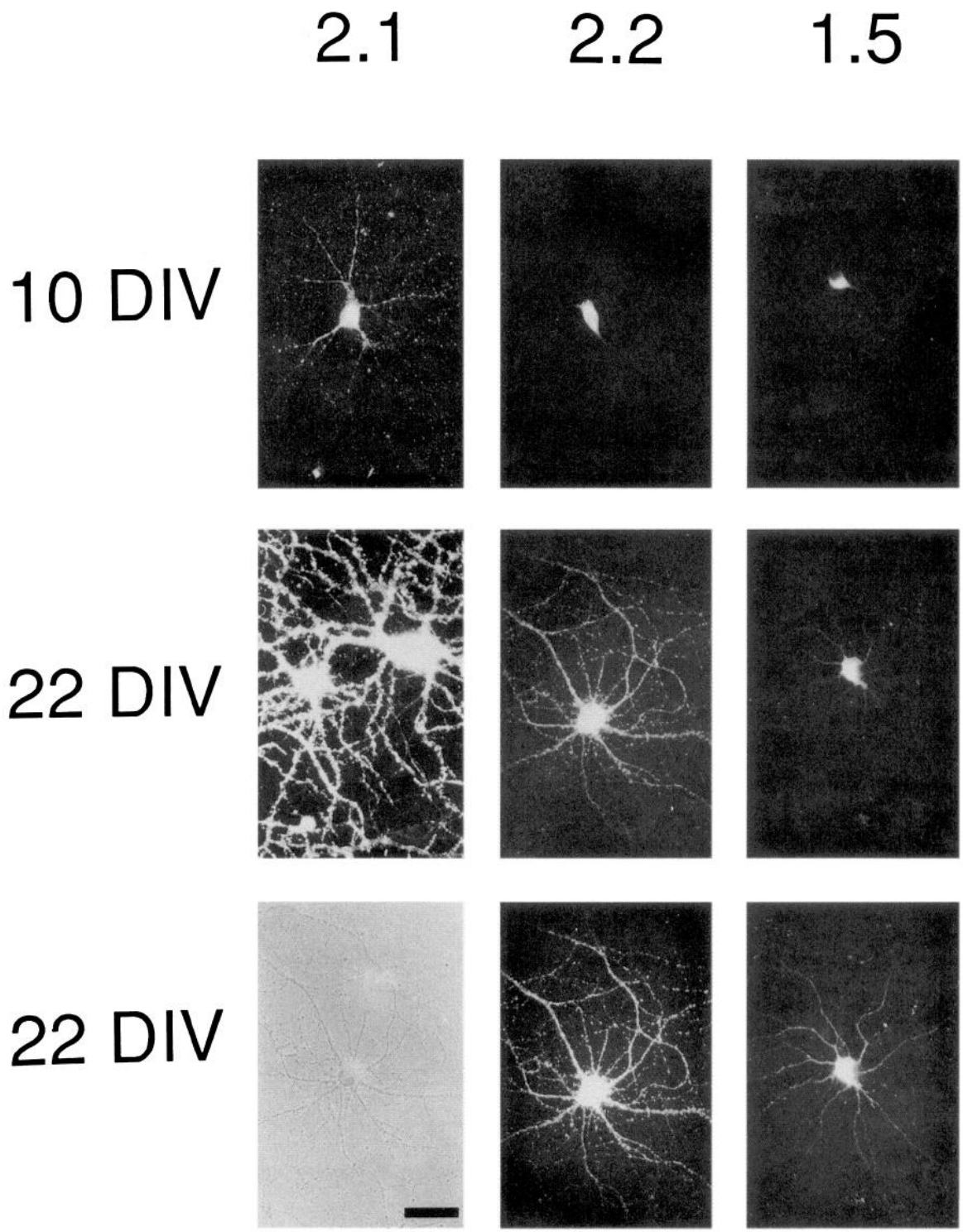

Figure 4. Spatiotemporal expression of three delayed rectifier K^+ channel polypeptides in cultured hippocampal neurons at 10 and 22 DIV. Neurons on coverslips were visualized with indirect immunofluorescence. *First row*, 10 DIV, optimal exposure times (Kodak, 400 ISO). *Second row*, 22 DIV; immunofluorescence recorded with the same exposure time for each polypeptide (8 sec), the optimal exposure time for Kv2.1. *Third row*, 22 DIV; phase contrast micrograph showing morphology of a pyramidal neuron immunostained for the expression of Kv2.2 polypeptide and photomicrographs of the neurons stained with anti-Kv2.2 and anti-Kv1.5 antibodies. Immunofluorescence recorded with the optimal exposure times (15 sec). Note intense immunoreactivity of Kv2.1 compared to the lower immunoreactivity of the other K^+ channel polypeptides. Detail in text and Table 2. Scale bar, 30 μm.

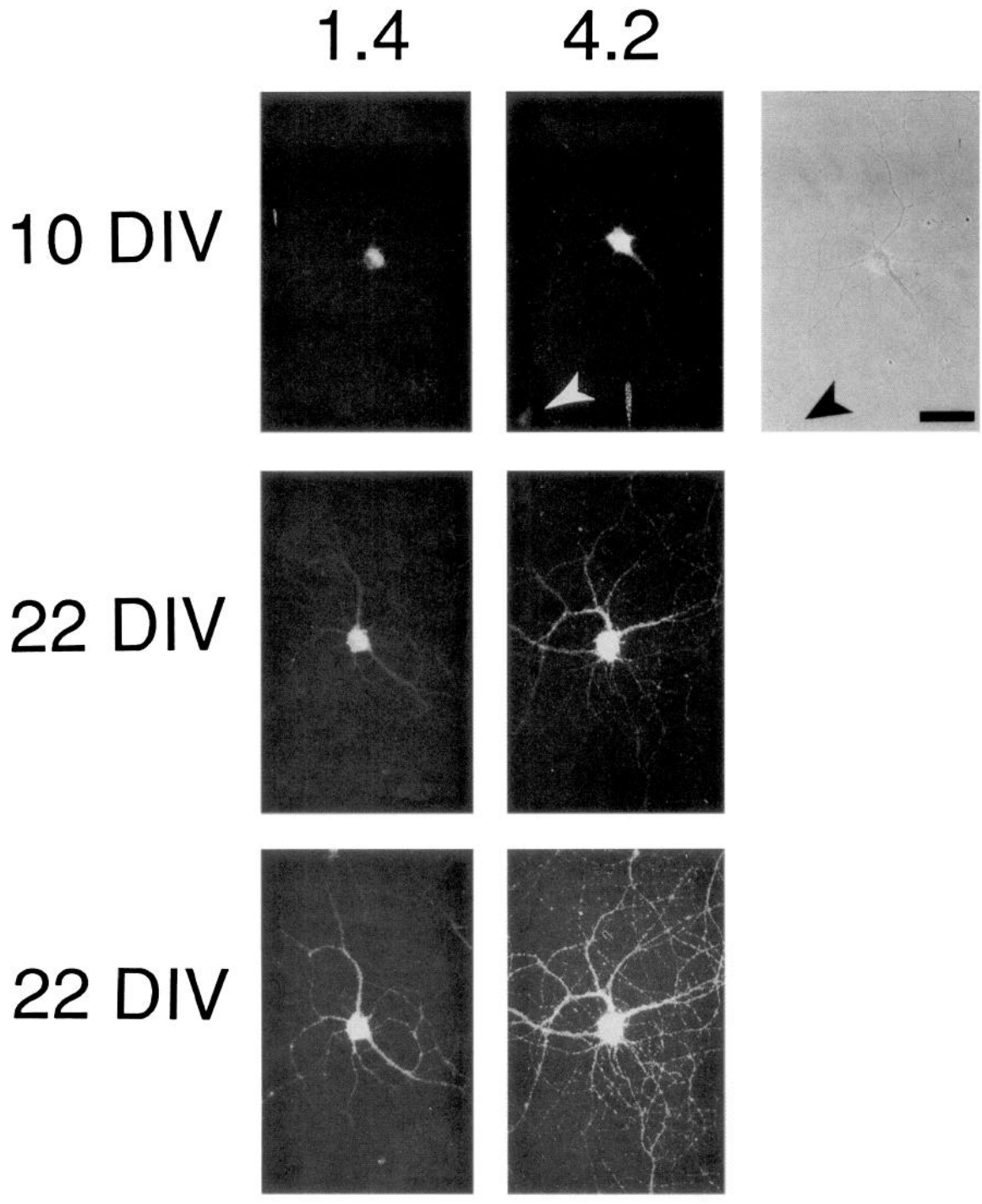

Figure 5. Spatiotemporal expression of two A-type K^+ channel polypeptides in cultured hippocampal neurons at 10 and 22 DIV. *First row*, 10 DIV, phase contrast micrograph showing morphology of a 10 DIV old pyramidal neuron and a neuron unstained with anti-Kv4.2 (*arrow*; Kodak, 400ISO). *Second row*, immunofluorescence recorded with the same exposure time for both polypeptides (8 sec), the optimal exposure time for Kv2.1. *Third row*, 22 DIV; immunofluorescence recorded with the optimal exposure time for each polypeptides (15 sec). Note intense immunoreactivity of Kv2.1 (Fig. 4) compared to the lower immunoreactivity of the other K^+ channel polypeptides. Detail in text and Table 2. Scale bar, 30 μm.

processes. Most processes at 14 DIV are dendrites based on their morphology and confirmed by double immunostaining for Kv2.1 and MAP2, a dendritic marker (not shown). At 18 DIV, very bright membrane patches of Kv2.1 outline the full length of almost all dendrites (not shown). The number, size and intensity of the patches increase further at 20, 22, and 26 DIV. These features are very similar to those *in situ*.

Kv2.2. Kv2.2 is first observed at 8 DIV as faint intracellular staining (not shown) which increases at 10 DIV. A marked change in the pattern of Kv2.2 expression from 10 DIV is seen at 14 DIV, when all cell bodies and processes are strongly stained, but some more stained than the rest (not shown). The latter are large, with a small number of smoothly curved dendrites, and are darker in phase microscopy, corresponding to the

Table 2. Summary of normal expression of K^+ channel polypeptides in rat hippocampal neurons *in vitro*

	Kv2.1	Kv2.2	Kv1.5	Kv1.4	Kv4.2
6 DIV	—	—	—	—	C
8 DIV	C	C	C	—	C
10 DIV	CS	C	C	C	CS
14 DIV	CSP	CSP	C	C	CSP
18 DIV	CSP	CSP	CSP	CS	CSP
22 DIV	CSP	CSP	CSP	CSP	CSP
26 DIV	CSP	CSP	CSP	CSP	CSP

—, No detectable strain; C, intracellular compartments; S, soma plasma membrane; P, processes.

established morphology of GABA interneurons in these cultures (Benson et al., 1994). Plasma membrane immunostaining is uniform, with a few bright accentuations on processes. This pattern and intensity of staining persist at 18, 20, and 22 DIV. Both pyramidal neurons and interneurons express Kv2.2 (Fig. 4, pyramidal cell at 22 DIV). The pattern, with all neuron cell bodies and proximal processes stained, is the same as found *in situ*, although *in situ* the difference in intensity between interneurons and pyramidal cells is greater.

Kv1.5. Kv1.5 exhibits the weakest staining of the K^+ channel subtypes studied *in vitro*, although clearly above controls and with specific changes in localization with time. First detected in somas at 8 DIV (not shown), it is unchanged at 10 and 14 DIV. At 18 and 22 DIV, Kv1.5 is present throughout the somatodendritic compartment, with some processes brighter than others. At each time point, the observed immunostaining is diffuse and intracellular. Plasma membrane staining, if present, is unpatterned. The gradual increase in staining in all neurons is seen *in vitro* and *in situ*, but the intensity is much less at all DIV.

A-Type K^+ channel polypeptides

Kv1 4. Kv1.4 is first detected at 10 DIV as faint spots arranged in a crescent inside the cell bodies. At 14 DIV, Kv1.4 is uniformly expressed throughout the soma and its staining intensity is increased (not shown). The intensity of Kv1.4 immunostaining increases further at 18, 20, and 22 DIV, but it does not reach the intensity observed *in situ*. At 18 DIV, immunostaining is accentuated on soma plasma membranes (not shown) and at 22 DIV outlines some processes, although lightly compared to Kv2.1 immunostaining. This is consistent with its limited expression *in situ* in the classes of neurons which are present in the cultures.

Kv4.2. The special characteristic of Kv4.2 expression pattern is the presence of both stained and unstained neurons at all time points. At 6 DIV, occasional Kv4.2 immunoreactive spots appear intracellularly in some neurons (not shown). At 8 DIV, diffuse immunoreactivity is present inside the cells (not shown). The intensity of intracellular Kv4.2 immunostaining increases at 10 DIV, and in addition staining is accentuated on the plasma membranes of the positive somas. At 14 DIV, Kv4.2 is expressed more, particularly on plasma membranes (not shown). The pattern and intensity are unchanged at 18, 20, and 22 DIV. There is a similarity between these observations and the *in situ* pattern in that only some neurons express Kv4.2, although the proportion of positive cells appears higher *in vitro* (88.7% ± 4.4% (mean ± SD; n = 6 samples of 20–25 cells/sample).

Discussion

The principal findings of this study are (1) the striking differences among the spatiotemporal expression patterns of five K^+ channel subtypes in the developing hippocampus, (2) the similarities in the temporal expression of each K^+ channel polypeptide *in situ* compared to *in vitro*, and (3) the differences in the level of expression and subcellular distribution between some, but not all, K^+ channel polypeptides *in vitro* versus *in situ*. Our data demonstrate differential regional, cellular, and subcellular distribution and differential temporal expression of K^+ channel subtypes in the developing hippocampus, indicating their different functions and different mechanisms of targeting within neurons. Furthermore, we show the distinct spatiotemporal expression patterns of K^+ channel subtypes in hippocampal neurons differentiating *in vitro*, and identify aspects of expression that may be regulated by extrinsic factors *in situ* but not *in vitro*.

Spatial distribution of K^+ channel polypeptides in situ. The spatial distribution of examined K^+ channel subtypes reveals distinct differences in hippocampal cells, including differential regional, cellular, and subcellular distribution between channels associated with delayed rectifier and A-type currents. The development of these patterns is monotonic; that is, while the time and spatial development varies among the different channels, each K^+ channel subtype appears in place, followed only by developmental increases in abundance. These findings indicate that the mechanisms controlling cellular distribution and subcellular targeting of channels operate through development, as the same patterns are seen at all ages at which the channels are expressed.

The three delayed rectifier-type K^+ channel polypeptides (Kv2.1, Kv2.2, Kv1.5) are present in virtually all neurons, with the greatest concentration on the somatodendritic plasma membrane. However, the relative intensity of expression among neurons of the hippocampus at various ages are distinctive. A-type K^+ channel polypeptides (Kv1.4, Kv4.2) differ in that they are expressed in distinct subpopulations of neurons, Kv1.4 on the distal axons of the entorhinal and granule cell projections and Kv4.2 on the distal dendritic processes of pyramidal cells and somatodendritic region of a small population of interneurons, as resolved by confocal microscopy on adult brain sections (Maletic-Savatic et al., unpublished observations). These patterns hold true throughout development, suggesting that the different mechanisms of targeting within the neurons operate continuously.

Temporal expression patterns of K^+ channel polypeptides in situ. By immunoblots, Kv2.1 is expressed first, at P1, followed by Kv1.4 and Kv2.2 at P5, and Kv1.5 which is faintly present only at P7 and P9. Immunohistochemistry shows the same temporal pattern of expression, with Kv2.1 expressed earliest, at P1. Kv1.4, Kv1.5, and Kv4.2 are first detected at P5, with different patterns of increase after P5. Kv2.2 is detected last, at P9, even in the interneurons which are later strongly stained, with gradual increase in all neurons continuing past P12. However, by immunoblots, Kv2.2 appears at P5 as a moderately intense band (Fig. 1). This is probably due to the concentrated sample of brain membranes (100 μg per lane). Unlike spatial distribution, temporal expression patterns are not related to the A-type/delayed-rectifier type of current.

The microheterogeneity of Kv2.1 and Kv2.2 observed on immunoblots suggests extensive posttranslational modifications of these neuronal *Shab* family polypeptides. In addition, the exis-

tence of three separate bands of Kv2.1 in the hippocampus indicate that the heterogeneity of Kv2.1 species observed in whole brain preparations is present in these hippocampal samples. The remaining K^+ channel subtypes examined (Kv1.4 and Kv1.5, *Shaker* family) migrate as single bands on immunoblots, indicating that posttranslational modifications, if present, are relatively homogeneous compared to the *Shab* family channel polypeptides.

The sequential appearance of K^+ channel subtypes during hippocampal development indicates diverse regulatory mechanisms, either intrinsic or extrinsic, the nature of which are not yet known. However, epigenetic factors related to synaptogenesis and synaptic activity seem likely to have major effects on the expression of K^+ channel subtypes. The general increase in polypeptide quantity, observed for all K^+ channel polypeptides, but particularly for Kv1.5 after P9 in both immunoblots and immunohistochemical preparations, may be partly due to the postnatal increase in synaptogenesis. This increase in the amount of protein probably reflects both an increase in the number of molecules per unit of plasma membrane (i.e., increase of polypeptide density) and a relative increase in the plasma membrane because of more highly branched processes, both changes known to be influenced by synaptogenesis in some systems (Shatz, 1990).

Correlation of spatiotemporal expression of K^+ channel polypeptides in situ *and* in vitro. Spatiotemporal expression of all five K^+ channel polypeptides in cultured neurons correlates with their expression *in situ*. Of the five K^+ channel subtypes examined, Kv2.1 immunostaining is the strongest both *in situ* and *in vitro*. It is present on the cell bodies and dendrites of all neurons as distinct plasma membrane patches in both cases, giving a remarkable appearance compared to the other K^+ channel polypeptides tested. However, Kv2.1 immunoreactive patches are present throughout the whole length of dendrites in cultured neurons, indicating that factors that influence intracellular targeting may differ *in situ* and *in vitro*. Temporally, it is detected at 8 DIV in the cell body of all neurons. The number, size and intensity of Kv2.1 patches increase gradually thereafter, as they do *in situ*.

The expression of Kv2.2 *in vitro* correlates with the *in situ* pattern, with minor exceptions. Staining is present in all neurons and is found associated with the plasma membrane. However, *in vitro*, processes are more prominently stained than observed *in situ*. In addition, the notable difference between the expression levels in pyramidal cells and interneurons observed *in situ* is not evident *in vitro*. Relatively uniform staining of all neurons *in vitro* may result from a lesser expression in cultured interneurons compared to those *in situ*, possibly related to *in vitro* culture conditions, which lack the extrinsic inputs to the hippocampus.

Staining for Kv1.5 is observed *in vitro* at considerably lower levels then Kv2.1, even after 26 DIV, with the majority of staining associated with the cell body. This is in sharp contrast to Kv1.5 immunoreactivity *in situ*, where intense immunoreactivity comparable to Kv2.1 is observed, and processes are clearly stained. In addition, Kv1.5 is detected at 18 DIV, clearly later than *in situ* where it appears at P5. Its augmented expression *in situ* may be controlled by the extrinsic connections of the hippocampus or other factors not present *in vitro*, such as specific growth factors, neurotransmitters and neuropeptides. Identification of such factors through manipulation of these *in vitro* cultures will yield important insights into the mechanisms of K^+ channel expression.

We also observed differences between the *in situ* and *in vitro* expression of A-type K^+ channel polypeptides. In the cultures, Kv1.4 is present at low levels, and staining is not restricted to distal axons, as cell bodies are also stained. This finding is not surprising, since the cultures contain neither the neurons which give rise to the perforant path nor mossy fiber axons to which Kv1.4 is predominantly localized to distal axons *in situ* (Sheng et al., 1992, Maletic-Savatic et al., unpublished observations). Kv1.4 in the hippocampus *in situ* is also expressed in the neuropil of areas containing other afferents, but, in these areas it appears later and at low concentrations. This may correspond to its presence at low concentrations *in vitro* in the somata and some processes of all neurons, which are now more evident in the isolated cells *in vitro*. Kv1.4 is expressed relatively late (18 DIV) and does not increase noticeably up to 26 DIV.

Kv4.2 *in situ* is localized on the most distal dendrites of pyramidal cells in CA1–3 as observed by confocal microscopy (Maletic-Savatic et al., unpublished observations), and on the somata and dendrites of a small population of interneurons. *In vitro,* its presence on the somata and proximal dendrites of a subpopulation of neurons is retained (Fig. 5). Kv4.2 immunoreactive neurons are, however, relatively more numerous *in vitro* (88.7%) than *in situ*, and have the morphology of both pyramidal cells and GABAergic interneurons (Benson et al., 1994). In addition, there are no neurons *in vitro* that express Kv4.2 exclusively on small distal dendrites, which are the principal site for this K^+ channel polypeptide *in situ* (Maletic-Savatic et al., unpublished observations). During development these dendritic processes differentiate later, influenced by the formation of synapses. It is possible that these specific dendritic processes do not form in the cultures, perhaps due to the absence of particular afferent projections. It is interesting to note that Kv2.1 and Kv4.2 exhibit distinct spatial segregation on dendritic processes *in situ*, with Kv2.1 restricted to proximal portions of dendrites, while Kv4.2 is found only distal. This precise segregation indicates that distinct functional subdomains of the dendritic membrane are established by the differential sorting and/or retention of specific channel polypeptides. It should be noted that this spatial segregation does not arise for either channel *in vitro*.

Correlation of K^+ currents and K^+ channel polypeptides in hippocampus. Electrophysiological properties of K^+ currents in cultured hippocampal pyramidal cells have been studied in detail (Segal et al., 1984; Ficker and Heinemann, 1992). Storm (1990) lists seven main types of K^+ currents recorded from these neurons. Each of these currents has distinctive kinetics, voltage dependence and pharmacology. They are implicated in a number of electrical features of neurons, but in none of this work has a definite correlation of any K^+ current and a channel polypeptide been achieved.

During neuronal development delayed rectifier currents are the first outward currents to appear, followed by A-type currents (reviewed in Ribera and Spitzer, 1992). Our results show that Kv2.1, which yields a delayed rectifier current when expressed in *Xenopus* oocytes, is the first to appear both *in situ* and *in vitro*, followed by Kv4.2, which is an A-type channel. These may represent the major K^+ channels expressed in cultured hippocampal neurons, perhaps contributing to observed delayed rectifier and A-type currents in these cells. However, the presence of delayed rectifier K^+ current has been registered electrophysiologically earlier compared to our data (Bader et al, 1985;

Harris et al., 1988). It is possible that the level of expression of channel polypeptides required to give positive immunostaining is higher than the level required for electrophysiological recordings. In addition, formation of heterotetramers of α-subunits (Sheng et al., 1993; Wang et al., 1993) as well as the presence of β-subunits (Rettig et al., 1994) can significantly influence the characteristics of neuronal K^+ currents. Thus, a definitive correlation between the expression of a given channel polypeptide and a recorded current is difficult without more detailed information such as the precise subunit composition of neuronal K^+ channel complexes.

In conclusion, we have characterized the expression of K^+ channel subtypes during development of hippocampal neurons. Observed differences in the overall level of expression and subcellular distribution of some, but not all K^+ channels indicate the influence of factors present *in situ* but not *in vitro*. Identification of these factors, such as electrical activity, neurotransmitter and neuropeptide release, by manipulation of neuronal cultures will lead to new insights in the regulation of hippocampal activity during development and in the adult brain.

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