***In situ* Hybridization Histochemistry of Ca²⁺/Calmodulin-Dependent Protein Kinase in Developing Rat Brain**

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Oligonucleotide DNA probes were used to determine the distribution of mRNAs encoding the α - and β -subunits of Ca²⁺/calmodulin-dependent protein kinase type II (CaM-KII) in developing rat brain. The regional and temporal distribution of these mRNAs closely paralleled the distribution and developmental appearance previously reported for their respective protein subunits. α -Subunit mRNA was barely detectable in sagittal sections at 4 d postnatal but increased as much as 10-fold in frontal cortex by day 16. β -Subunit mRNA, on the other hand, was readily detected at 4 d postnatal and changed only slightly during development. Telencephalic structures exhibited the highest levels of CaM-KII mRNA and the brain stem displayed the least. α -Subunit mRNA was not observed in cerebellar granule cells and was barely detectable in Purkinje cells, while the β -mRNA was easily detected in both neuronal types.

mRNAs for both α - and β -subunits were present in many neuronal cell bodies; however, only the α -subunit mRNA was localized to molecular layers of the hippocampus and lamina I of the frontal cortex. These layers of neuropil are relatively cell sparse and contain extensive dendritic arborizations and synaptic contacts. Since polyribosomes have been observed near hippocampal dendritic spines, the localization of α -subunit mRNA to dendrites of pyramidal and dentate granule cells suggests that this subunit is synthesized *in situ* at postsynaptic sites. The co-localization of translational machinery and high concentrations of CaM-KII in postsynaptic elements suggests an important relationship between α -subunit synthesis and the maintenance and plasticity of postsynaptic structures.

Multifunctional Ca²⁺/calmodulin-dependent protein kinases have been found in a wide variety of organisms and tissues, each expressing similar yet distinct tissue-specific subunits (Shenolikar et al., 1986; Fukunaga et al., 1988). In mammalian brain, this enzyme is referred to as Ca²⁺/calmodulin-dependent protein kinase II (CaM-KII) and appears to be a multimeric holoenzyme composed of nonstoichiometric amounts of 2 principle subunits, α (50 kDa) and β (58/60 kDa). The doublet at

58/60 kDa is believed to be due to alternative splicing of the β -subunit mRNA (Bennett and Kennedy, 1987). The α - and β -subunits are greater than 85% homologous at the amino acid level (Bulleit et al., 1988), share nearly identical calmodulin-binding and catalytic domains (Hanley et al., 1987; Bulleit et al., 1988), and undergo autophosphorylation following activation by Ca²⁺/calmodulin (Bennett et al., 1983). Despite these similarities, recently sequenced cDNA clones have established that the α - and β -subunits are clearly products of distinct genes (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987). Moreover, studies have shown that the subunits are differentially expressed in cerebellum and forebrain. The holoenzyme isolated from adult forebrain appears to consist of 9 α - and 3 β -subunits, while that purified from cerebellum expresses a composition of about 2 α - and 8 β -subunits (McGuinness et al., 1985; Miller and Kennedy, 1985). Immunochemical analyses suggest that CaM-KII is much more highly expressed in neurons than glia (Ouimet et al., 1984; Scholz et al., 1988), while subcellular fractionation studies have shown that in forebrain, CaM-KII is enriched in synaptic junction (SJ) fractions (Kelly et al., 1985; Suzuki and Tanaka, 1986) and is the single most abundant protein in isolated postsynaptic densities (Kelly et al., 1984). This enrichment in postsynaptic structures has implicated CaM-KII in signal-transduction mechanisms (Miller and Kennedy, 1986) and memory storage (Lisman and Goldring, 1988).

Developmental studies have shown that levels of the β -subunit increase slowly during forebrain postnatal development, doubling in SJs between postnatal days 5 and 25. During this same period, however, α -subunit levels increase about 10-fold in SJs, with the most rapid increase occurring during the second and third postnatal weeks (Kelly and Vernon, 1985). Coincident with increases in α -subunit is a change in the subunit composition of the holoenzyme from an α - to β -subunit ratio of about 1:7 at postnatal day 5 to the adult ratio of 3:1 (Kelly et al., 1987). During this period of rapid increase in α -subunit levels, CaM-KII appears to shift from a predominantly cytosolic enzyme to one more tightly associated with neuronal membranes and SJs (Kelly and Vernon, 1985).

Immunohistochemical studies have demonstrated that CaM-KII is nonuniformly distributed in rat brain (Ouimet et al., 1984; Erundu and Kennedy, 1985). In light of regional differences in subunit ratios for the holoenzyme, the present study was undertaken to define the regional and cellular distribution of each subunit's mRNA and to examine their co-localization within discrete neuronal populations. Furthermore, since CaM-KII is highly concentrated in postsynaptic structures, we were inter-

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ested in determining whether its mRNAs were present in dendritic fields or restricted to neuronal cell bodies.

In this paper, we show that levels of mRNA for both CaM-KII subunits change during development in parallel with previously reported protein levels. We demonstrate that, in addition to finding mRNA in cell bodies, the mRNA for the α - but not β -subunit is also localized in dendrites. This selective dendritic localization suggests that α -subunit mRNA is targeted for dendrites where the subunit may be synthesized *in situ* at post-synaptic structures.

Materials and Methods

Sprague-Dawley rats at various postnatal ages were decapitated under ether anesthesia and whole brains were rapidly removed and frozen on dry ice. Brains were stored at -80°C until sectioned. Then, 20 μm sagittal cryostat sections were thaw-mounted onto twice-gelatinized slides (1% gelatin/0.05% chrom alum), air-dried for 5–10 min, and fixed for 15 min in 3% paraformaldehyde in PBS (pH 7.2). Sections were rinsed twice in PBS and delipidated through graded ethanol (50, 70, 95%) for 5 min each before drying and storing at -80°C .

Preparation of radiolabeled DNA. Synthetic oligonucleotide probes specific to the α - and β -subunits of CaM-KII and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are delineated in Figure 1. The α -subunit sense strand oligonucleotide is identical to nucleotides 1439–1470 of the α -subunit mRNA and has the sequence 5'-GGACCAGGC-CAGGGTCCCTGCGCTCTCGTCGC-3' (Lin et al., 1987). The anti-sense oligonucleotide is complementary to this sequence. The β -subunit and GAPDH anti-sense oligonucleotides are complementary to nucleotides 1090–1128 and 1128–1159 of their respective mRNAs (Fort et al., 1985; Bennett and Kennedy, 1987). The β -subunit probe has the sequence 5'-GGCGGCAGGAGGGAGGGATCCTTTGGGGCTGG-TGATGGC-3', while the GAPDH probe has the sequence 5'-GGGCT-CCCCAGGCCCTCCTGTGTATGGGG-3'. Oligonucleotides are numbered based on the AUG start codon of each mRNA being nucleotides 1–3.

Oligonucleotides were 3' end-labeled with ^{32}S -dATP (New England Nuclear) by terminal deoxynucleotidyltransferase for 20 min at 37°C in tailing buffer [50 mM potassium cacodylate (pH 7.2), 2 mM CoCl_2 , 0.2 mM DTT]. Radiolabeled probes were separated from unincorporated nucleotides by ethanol precipitation. Specific activity of the probes was routinely between 10^8 – 10^9 CPM/ μg DNA.

Random primed ^{32}P -labeled α -subunit cDNA probes were prepared with a labeling kit (Boehringer Mannheim Biochem.) according to the manufacturer's instructions and used for slot-blot hybridization (see below). The template DNA was a 3.8 kb fragment corresponding to nucleotides 225–4010 of the α -subunit mRNA. Unincorporated nucleotides were separated from labeled DNA by chromatography through Sephadex G-50. The specific activity of the probe was routinely 10^9 CPM/ μg DNA. Probes were boiled for 5 min and rapidly placed in an ice bath immediately prior to use.

In situ hybridization. Hybridizations were performed essentially as described by Young et al. (1986). Briefly, sections were thawed, hydrated through graded ethanol (95:70:50:20%) and incubated for 1 hr at 37°C in $2\times$ SSC with or without 100 $\mu\text{g}/\text{ml}$ RNase A. Sections were rinsed twice in $2\times$ SSC and acetylated for 10 min at room temperature in 0.25% acetic anhydride, 0.1 M triethanolamine (pH 8.0). After rinsing twice in $2\times$ SSC, sections were dehydrated in 70% ethanol and air-dried. Hybridization buffer contained 10 mM HEPES (pH 7.5), 600 mM NaCl, 100 mM DTT, 1 mM EDTA, 50% formamide, 10% dextran sulfate, $1\times$ Denhardt's, 100 $\mu\text{g}/\text{ml}$ each of sonicated salmon sperm DNA and yeast tRNA. Each slide was incubated with 50 μl of hybridization solution containing approximately 500,000 CPMs of probe (~ 5 ng, 10 nM). Hybridizations were performed for 12–16 hr at 37°C in a humidified chamber. Following hybridization, sections were washed twice for 10 min at room temperature in $2\times$ SSC, twice for 45–60 min at 45°C in $0.2\times$ SSC, and twice for 10 min at room temperature in $2\times$ SSC. Sections were rinsed briefly in 70% ethanol and air-dried prior to placing under Kodak XAR-5 or Agfa Curix RP-1 X-ray film. After obtaining appropriate X-ray film exposures, sections were dipped in NTB-2 photographic emulsion diluted 1:1 with distilled H_2O . Sections were exposed for 2–6 weeks at -80°C , developed for 2 min in Dektol developer and fixed in Kodak fixer.

RNA purification. Rat brains at various ages were harvested and spe-

cific brain regions were rapidly dissected on a chilled glass plate as previously described (Johnston et al., 1987). Total RNA was purified from the brain tissue using the acid-guanidinium-phenol-chloroform method of Chomczynski and Sacchi (1987). Briefly, the tissue was homogenized in a glass-Teflon homogenizer in denaturing solution containing 4 M guanidinium thiocyanate, 25 mM Na-citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. The homogenate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). Nucleic acid remaining in the aqueous phase was twice precipitated with isopropanol and washed twice with ice-cold 70% ethanol. The pellet was dried in a speed-vac and resuspended in diethylpyrocarbonate-treated water. RNA was quantitated by absorbance at 260 nm.

Slot-blot hybridization. RNA from dissected brain regions was diluted to 2 $\mu\text{g}/400$ μl in denaturing solution D (6.8% formaldehyde $5\times$ SSC) and heated at 50°C for 1 hr. The denatured RNA was cooled to room temperature and 4 serial dilutions (1:1) were made in solution D. After loading RNA onto nitrocellulose, membranes were baked at 80°C in a vacuum oven for 2 hr. Blots were pretreated for 2–4 hr at 45°C in hybridization buffer containing $5\times$ SSC, $2.5\times$ Denhardt's, 50 mM Na_2PO_4 (pH 6.5), 50% formamide, 0.1% SDS, and 200 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA. Radiolabeled CaM-KII α -subunit cDNA probe was added to the hybridization solution and hybridized overnight at 45°C . Unbound probe was removed by washing twice in $2\times$ SSC for 10 min at room temperature, followed by 2 washes in $0.2\times$ SSC/0.1% SDS for 45–60 min at 50°C .

Quantification of autoradiographic images. X-ray film images were quantitated for optical density (O. D.) with an Eikonix digitizing system at a resolution of approximately 50 μm . Average O. D. values were calculated for specific brain regions to quantify region-specific changes in mRNA levels during development. Each value was expressed as a percentage of the appropriate adult value and was calculated independently for each experiment before averaging. Each experiment contained brain sections of several ages, including adult, all of which were hybridized with the same probe at the same concentration and processed under identical conditions. Slot-blot autoradiograms using purified RNA were analyzed in a similar manner, except that O. D. values were expressed as units of α -subunit mRNA/ μg total RNA. One unit is defined as the signal from 1 μg of total cellular RNA purified from adult rat forebrain using CsCl gradients (Maniatis et al., 1982). This RNA standard was included in each blot.

Results

Synthetic oligonucleotide probes

Because the entire coding region of the α -subunit shares homology with most of the β -subunit's coding region, the α -subunit probe corresponds to a unique 3' noncoding sequence. The β -subunit probe corresponds to a segment of coding region which is unique to the β -subunit (Fig. 1A). This region is absent in the putative 58 kDa β -sequence (Bennett et al., 1987), as well as in 2 novel Ca^{2+} /calmodulin-dependent protein kinases which have recently been described in brain, namely, CaM-KII " γ -subunit" (Tobimatsu et al., 1988) and "CK-Gr" (Ohmsted et al., 1989). Oligonucleotides were also selected for high G-C content to increase the strength of hybridization.

As a positive control, a 32-mer anti-sense oligonucleotide was synthesized corresponding to the 3' noncoding region of GAPDH mRNA. The mRNA for this housekeeping enzyme was anticipated to be a good marker for cell density. As negative controls, adjacent sections were pretreated with RNase A (100 $\mu\text{g}/\text{ml}$) for 1 hr prior to hybridization. To control against nonspecific hybridization due to high G-C content, a sense strand oligonucleotide was prepared corresponding to the same region of the α -subunit from which the anti-sense probe was made. Figure 1B shows the Northern hybridization pattern for each of the probes using rat forebrain poly A⁺ RNA. The sense strand control exhibited no hybridization, while each of the other probes hybridized to a single distinct band. Using ^{32}P - λ DNA standards, the α and β probes hybridized to RNA species of approximately

Figure 1. Specificity of oligonucleotide probes. **A**, Graphic representation of mRNAs depicting the regions of identity for each oligonucleotide (see Materials and Methods for nucleotide sequences). Stippled boxes represent coding regions, and the black boxes represent β -subunit specific sequences. The α -subunit mRNA probes correspond to nucleotides 1439–1470, just 3' to the stop codon. The β -subunit anti-sense oligonucleotide corresponds to nucleotides 1090–1128. The GAPDH probe is complementary to nucleotides 1128–1159. **B**, Northern hybridization analysis of forebrain poly-A⁺ RNA. Poly-A⁺ RNA from adult forebrain (2.5 μ g/lane) was separated by glyoxal/DMSO agarose gel electrophoresis and transferred to a Nytran Nylon membrane (Schleicher and Schuell). The membrane was hybridized as described in Materials and Methods to each of the oligonucleotide probes: lane 1, α -subunit anti-sense probe; lane 2, β -subunit anti-sense probe; lane 3, GAPDH probe; lane 4, α -subunit sense strand probe; S, Bst EII digested and ³²P end-labeled lambda DNA standards. Exposure times were 22–35 hr.

5.3 and 4.3 kb, respectively. The GAPDH probe hybridized to a message of 1.5 kb.

In situ hybridization analysis in adult brain

Previous immunohistochemical studies have shown that CaM-KII is not uniformly distributed in rat brain. In the present study, *in situ* hybridization histochemistry was used to ascertain whether mRNA levels correlated with the observed distribution and relative expression of CaM-KII protein. Figure 2 shows typical sagittal sections of adult rat brain hybridized with each of the oligonucleotide probes. The strongest hybridization for both CaM-KII probes was seen in telencephalic structures, with very light hybridization in the brain stem. In contrast to the α -subunit probe, the β probe hybridized strongly in the granule

and Purkinje cell layers of the cerebellum. The hybridization of the GAPDH probe reflected overall cell density throughout the brain.

Pretreatment with RNase A eliminated virtually all hybridization of CaM-KII probes, while GAPDH hybridization decreased about 80% (data not shown). It is not known whether the GAPDH message is partially resistant to RNase A treatment, if it is sequestered in an inaccessible compartment such as mitochondria, or if the probe displays a small amount of nonspecific hybridization which is unaffected by RNase treatment.

As expected from the Northern analysis, the α -subunit sense-strand probe did not hybridize to any forebrain structures. However, there was prominent punctate hybridization in the floor of the fourth ventricle and in the trapezoid bodies. This hy-

Figure 2. *In situ* hybridization to adult rat brain. Digitized images of sagittal sections of adult rat brains (1–2 mm from midline) were prepared as described and hybridized with the indicated oligonucleotide probe: *Alpha*, α -subunit anti-sense probe; *Beta*, β -subunit anti-sense probe; *GADPH*, *GADPH* anti-sense probe; *Ctl*, α -subunit sense strand probe. Autoradiographic exposures were 3–6 d (white represents hybridization). The lower-left panel diagrams major anatomical landmarks: FC, frontal cortex; HC, hippocampus; DG, dentate gyrus; C-P, caudate-putamen; CB, cerebellum; cc, corpus callosum; OC, olfactory cortex; IC, inferior colliculus. The area of the hippocampus enclosed by the box is analyzed in Figure 3.

bridization pattern was developmentally regulated, first appearing at about 12–16 d postnatal and was completely abolished by RNase pretreatment (data not shown). It is not known whether hybridization in these structures is to an unrelated mRNA or possibly to a distinct mRNA derived from the opposite strand of the α -subunit gene.

Localization in the hippocampal formation. The hippocampus displayed high levels of hybridization for all anti-sense probes (Fig. 3, *A, B, D*). Both *GADPH* and β -subunit probes hybridized almost exclusively to the pyramidal and granule cell layers. The *GADPH* probe also hybridized to occasional cell bodies in the molecular layers (Fig. 3*D*). The punctate pattern of hybridization for this probe indicated that *GADPH* mRNA is localized to cell somas. The α -subunit probe hybridized strongly to the cell layers and exhibited robust hybridization throughout the molecular layers of the hippocampus and dentate gyrus (Fig. 3*A*). The molecular layers contain few neuronal cell bodies and are composed almost exclusively of neuronal processes. The diffuse yet strong hybridization of the α -subunit probe, in contrast to the punctate pattern of the other antisense probes, indicates that α mRNA is not restricted to cell bodies. There appeared to be little α -subunit mRNA in hippocampal axons, since the hilar region adjacent to dentate granule cells and the

alveolar region of the hippocampus were relatively free of hybridization (Figs. 3*A, 4A*). These regions contain the exiting axons of granule and pyramidal cells and very few dendrites. Further comparisons of dark-field images of α -subunit hybridization in hippocampal molecular layers (Fig. 4*A*) with bright-field localization of cell bodies revealed by toluidene blue staining (Fig. 4*B*) demonstrated that an overwhelming majority of this mRNA was present in neuropil. In fact, there appeared to be a rather striking negative correlation between cell body staining and *in situ* hybridization in the strata radiatum and lacunosum (Fig. 4, *A, B*, arrows). Together, these results indicate that the localization of α -subunit mRNA in hippocampal molecular layers is dendritic.

Localization in forebrain. Both β -subunit and *GADPH* probes hybridized evenly throughout the cellular laminae of the cerebral cortex and showed little, if any, hybridization to subcortical white matter (Fig. 2, see cc). The α -subunit probe hybridized throughout the cortex but in a distinct laminar distribution (Fig. 5*A*). It hybridized about twice as intensely in laminae II and III than in laminae IV–VI. Lamina I, which is cytoarchitecturally similar to the hippocampal molecular layers, and displayed strong hybridization with the α -subunit probe but not with either the β -subunit or *GADPH* probe. Both CaM-KII probes hybridized

Figure 3. Dark-field photomicrographs of *in situ* hybridization. Sections were hybridized as described in Materials and Methods and exposed with NTB2 emulsion. Areas CA1 and CA4 of the hippocampus with associated regions of the dentate gyrus (see boxed region, Fig. 2, lower-left panel) were photographed under dark-field optics at 50 $\times$ magnification. *A*, α -Subunit antisense probe; *B*, β antisense probe; *C*, α -subunit sense strand; and *D*, GAPDH antisense probe; *g* (arrowheads), granule cells in dentate gyrus; *p* (short arrows), pyramidal neurons; long arrows, dendritic fields of the molecular layers of CA1 and the dentate gyrus; *alv* (brackets), alveolar region of the hippocampus.

strongly to the pyramidal cell layer of the olfactory cortex (Fig. 2). The caudate-putamen, amygdala, and thalamus showed moderate hybridization with both CaM-KII probes, while the inferior colliculus hybridized almost exclusively to the β -subunit probe (Fig. 2).

Localization in cerebellum and brain stem. Levels of α -subunit mRNA in the cerebellum were extremely low. However, long exposures of emulsion-coated slides displayed modest hybridization in the Purkinje cell layer (Fig. 5*C*). In contrast, the β -subunit mRNA was readily detected in both the granule and Purkinje cell layers (Figs. 2, 5*D*). Neither of the CaM-KII probes exhibited hybridization above background to the cerebellar molecular layer. The hybridization pattern of the GAPDH probe in the cerebellum was similar to the CaM-KII β probe with the exception of additional hybridization in the molecular layer

(data not shown). None of the probes hybridized to white matter. In the brain stem, both CaM-KII mRNAs were detected in several discrete nuclei, including the paraventricular nucleus, pars compacta of substantia nigra, and the spinal nucleus of nerve V (data not shown).

Developmental changes in CaM-KII and GAPDH mRNAs

Levels of α -subunit mRNA (Hanley et al., 1987) and protein (Kelly and Vernon, 1985; Kelly et al., 1987) have been shown to increase dramatically in rat forebrain during the second and third postnatal weeks. Using *in situ* hybridization, we sought to determine which brain regions displayed these developmental changes. Figure 6 shows computer-digitized images of autoradiograms from different aged animals hybridized with either α - or β -subunit probes. The developmental changes in mRNA levels

Figure 4. Hybridization of α -subunit probe in hippocampus. Panel *A* of Figure 3 is shown at higher magnification under dark-field (*A*) and bright-field (*B*) illumination (scale bar in *B*, 100 μ m). Robust hybridization of the α -subunit probe can be seen in the pyramidal cell layer (Pyr) and in neuropil associated with pyramidal cell dendrites: basal dendrites in stratum oriens (*or*) and apical dendrites in strata radiatum and lacunosum (*rad/lac*). There was no hybridization to several large cells in the molecular layers (*arrowheads*) nor to endothelial cells surrounding a blood vessel coursing through the section (*large arrow*). The alvius (*alv*), containing the axons of pyramidal cells, was also relatively devoid of hybridization.

for the 2 CaM-KII subunits were quite distinct. The level of α -subunit mRNA was barely detectable at 4 d but increased dramatically in forebrain regions during the second postnatal week and by day 16 approached adult values (Fig. 6, compare days 8 and 16). β -Subunit mRNA, on the other hand, was readily detected at 4 d, thereafter decreasing slightly to adult levels.

To quantify the developmental changes seen with *in situ* hybridization, images were digitized on an Eikonix digitization system and average signal intensity was calculated for several discrete regions. In each experiment, values were normalized against either adult (90–110 d of age) or 32 d postnatal brain sections included in the experiment; values represent a minimum of 6 sections at each age and were obtained from 3 (α) or 4 (β) independent experiments.

In frontal cortex (Fig. 7*A*), the level of β -subunit mRNA decreased by half from day 4 to adult. Most of this decrease occurred between days 6 and 8. In contrast, α -subunit mRNA increased 10-fold between day 4 and adult, primarily between days 8 and 16. This is in general agreement with previous studies with purified RNA (Hanley et al., 1987). GAPDH mRNA, like the β -subunit, decreased by half in frontal cortex between day 4 and adult.

Changes in α -subunit mRNA in hippocampus were qualitatively similar to the cortex, increasing about 5-fold during postnatal development (Fig. 7*B*). The level of α mRNA in the molecular layer closely paralleled the changes for the whole hippocampus. β -Subunit mRNA levels did not change significantly between postnatal day 4 and adult. The apparent levels of GAPDH mRNA in the hippocampus decreased about 10-fold between postnatal day 4 and adult (Fig. 7*B*). The average size of the hippocampus in sagittal sections increased about 3.5-fold during postnatal development, mostly in the cell-sparse molecular layers. This may account for some of the decrease in relative levels of GAPDH mRNA in the hippocampus.

Both CaM-KII mRNAs increased about 50% in the cerebellum between day 4 and adult (Fig. 7*C*). This increase coincides with neurogenesis and synaptogenesis within the cerebellum. While the percentage increases for α - and β -subunit mRNA were approximately equal, it should be noted that the absolute increases for the 2 mRNAs were quite different (Fig. 6). Furthermore, virtually all the developmental increases of the β -subunit mRNA in the cerebellum were in the granule and Purkinje cell layers. These layers comprised only about 1/3 of the entire cerebellar areas which were quantified. Therefore, after correcting for the contribution of hybridization in the molecular layer, white matter, and deep cerebellar nuclei, levels of β -subunit mRNA in the granule and Purkinje cell layers increased approximately 200% during development. GAPDH mRNA increased with neurogenesis in the cerebellum and then decreased after day 12 to adult levels.

To further analyze developmental changes in α -subunit mRNA, total RNA was isolated from microdissected brain regions at various ages and examined by slot-blot hybridization. Figure 8 shows the results of this analysis for 5 separate regions. At all ages studied, the highest levels of α mRNA were detected in frontal cortex and hippocampus. These regions increased in α -subunit hybridization during development by 7- and 10-fold, respectively. The septal region increased approximately 5-fold during development but expressed a much lower level of α -subunit mRNA than the other 2 forebrain regions. Neither brain-stem nor cerebellar RNA displayed significant hybridization to the α -subunit probe and therefore showed very little developmental change.

Discussion

Distribution of CaM-KII mRNA

Previous studies have revealed substantial heterogeneity in the distribution and subunit ratios of CaM-KII in discrete regions

Figure 5. Dark-field photomicrographs of *in situ* hybridization. Sections were hybridized and analyzed as described in Figure 3. *A* and *B*, Paracentral frontal cortex at 60 $\times$ (scale bar, 250 μ m). *C* and *D*, Cerebellum at 260 $\times$ (scale bar, 50 μ m). *A* and *C* were hybridized with α -subunit anti-sense probe; *B* and *D* with β -subunit anti-sense probe. Layers I–VI of the frontal cortex are designated in *A* and *B*; molecular (*mol*), Purkinje (*Pur*), and granule cell (*Gr*) layers and white matter (*wm*) are designated in *C* and *D*. The diagonal dotted line in *C* and *D* delineates the Purkinje and molecular layers; the short dotted line in *D* delineates the granule cell layer and the underlying white matter.

of adult brain (Ouimet et al., 1984; Erond and Kennedy, 1985). The highest levels of immunoreactivity were found in forebrain structures, while hindbrain regions expressed the lowest levels. Subcellular localization showed strong CaM-KII immunoreactivity in postsynaptic densities (PSDs) (Ouimet et al., 1984), and biochemical studies have estimated that CaM-KII comprises as much as 40% of the protein in isolated PSDs (Kelly and Montgomery, 1982; Kelly et al., 1984; Miller and Kennedy, 1985). Using slot-blot analysis and *in situ* hybridization histochemistry, we have shown that the distribution of CaM-KII mRNA levels in adult brain closely approximates the distribution of the protein seen in previous studies. Studies at the protein level utilized monoclonal antibodies that were specific to the α -subunit (Erond et al., 1985) or cross-reacted with both the α - and β -subunits (Ouimet et al., 1984). We have employed synthetic oligonucleotides which are specific for each subunit and which clearly demonstrate their distinct distribution and developmental expression.

The hippocampus contains the highest levels of CaM-KII in brain, where it may comprise as much as 2% of total protein (Erond and Kennedy, 1985). In agreement with this observation, we found that the hippocampus expresses the highest levels of CaM-KII mRNA. While probes specific to either subunit hybridized to the pyramidal and granule cell layers, only the α -subunit probe hybridized to the molecular layers. The localization of the α -subunit mRNA to molecular layers was not described in a previous report by Lin et al. (1987). Hippocampal molecular layers contain few cell bodies, as demonstrated by the sparsity of GAPDH hybridization but contain extensive dendritic arborizations of the pyramidal and granule cells. The most prominent morphological feature of these dendrites is the presence of numerous spiny protrusions which contain asymmetric postsynaptic densities. The dendrites of hippocampal neurons have been shown to contain polyribosomes which appear to be closely associated with dendritic spines (Steward and Levy, 1982; Steward and Falk, 1985). In addition,

ALPHA SUBUNIT

BETA SUBUNIT

Figure 6. *In situ* hybridization in developing rat brain. Sagittal sections of rat brains at indicated ages were prepared as in Figure 2 and hybridized with α -subunit anti-sense probe (left column) or β -subunit anti-sense probe (right column). Exposure times were 12 d (left) and 16 days (right). Autoradiographic images were digitized and photographed.

Figure 7. Changes in mRNA levels during brain development. Brain sections at indicated ages were prepared and hybridized as in Figure 2. Images were digitized and quantitated as described in Materials and Methods. Specific regions were outlined on the digitized image, and average signal intensity was calculated. Frontal cortex included the area between the dorsal surface and the corpus callosum, from anterior to posterior poles. The hippocampal formation included the hippocampal pyramidal cell layer, the dentate gyrus, and associated molecular layers. The cerebellum included the cerebellar cortex and the deep nuclei: A, frontal cortex; B, hippocampal formation (note log scale); C, cerebellum. Values are expressed as percentages of adult brain (error bars represent the SEM).

cultured hippocampal pyramidal neurons have been reported to transport ^3H -UTP into dendrites in an energy-dependent manner (Davis et al., 1987). Our results indicate that CaM-KII α - but not β -subunit mRNA is transported into dendrites, there-

Figure 8. Developmental changes in α -subunit mRNA in brain regions. RNA was prepared from the indicated brain regions as described in Materials and Methods and analyzed by slot-blot hybridization. Each point represents the average of at least 3 separate samples analyzed at 5 dilutions. Error bars represent the SEM. Values are arbitrary units based on hybridization to the same positive RNA standard included in each experiment (see Materials and Methods).

by allowing its translation in close proximity to spine PSDs where CaM-KII is concentrated.

Garner et al. (1988) and Tucker et al. (1989) have recently reported the presence of MAP2 mRNA in the molecular layers of the hippocampus and dentate gyrus. Like CaM-KII, MAP2 protein is concentrated in the dendrites of these neurons. Furthermore, MAP2 is highly phosphorylated by CaM-KII *in vitro* (Schulman, 1984). Thus, mRNA for a prominent synaptic enzyme and an apparent endogenous substrate protein appear to be co-localized in dendrites of forebrain neurons. It should be noted that MAP2 mRNA appears to be restricted to proximal portions of dentate granule cell dendrites, while it is distributed more distally in dendrites of hippocampal pyramidal neurons. Together, these observations suggest that the extent of dendritic translocation of specific mRNA species varies among different neuronal types.

In agreement with immunohistochemical studies (Ouimet et al., 1984; Erondy and Kennedy, 1985), both CaM-KII mRNA probes hybridized strongly within cerebral cortex in a distinct laminar pattern. Ouimet et al. (1984) reported strong immunoreactivity throughout the cortex, with the strongest staining in layer I. Our results with α -subunit mRNA probes support these observations, except that layers II and III showed the strongest hybridization. Ouimet found that the large pyramidal neurons of layer V were the most immunoreactive cell bodies in the cortex. These neurons extend numerous processes to the cortical surface, where they make synaptic contacts within layer I. Layer I is analogous to the molecular layers of the hippocampal formation; it contains few cell bodies but is rich in processes and synapses. Like hippocampal molecular layers, layer I also hybridized α -subunit mRNA probe, suggesting dendritic localization of α -subunit mRNA in cortical neurons. Consequently, the strong hybridization signal in laminae II and III may result from additional hybridization to mRNA in dendrites of layer V neurons, rather than a higher expression of mRNA in cells of layers II and III.

Previous studies (McGuinness et al., 1985; Miller and Kennedy, 1985) have shown that the α - to β -subunit ratio in fore-

brain is about 3:1, while in the cerebellum it is about 1:4. Much of the difference is due to greatly decremented levels of the α -subunit in cerebellum. Using an α -subunit specific antibody, Erondy and Kennedy (1985) reported that the only immunoreactivity in the cerebellum was due to apparent nonspecific staining in the Purkinje layer. Recently, Walaas et al. (1988) reported that α -subunit immunoreactivity was restricted to Purkinje cells. Using mutant mice deficient in either Purkinje or granule cells, they observed a decrease in α -subunit levels only in Purkinje cell-deficient mutants. Our results support these observations. Hybridization of the α -subunit mRNA probe was detected only in the Purkinje cell layer and only with very long autoradiographic exposures (Fig. 5C). Furthermore, we found little hybridization of α -subunit mRNA probe in the molecular layer, suggesting that Purkinje cells, unlike forebrain pyramidal neurons, do not transport α -subunit mRNA into dendrites. We cannot discount the possibility that α -subunit mRNA undergoes dendritic transport in these neurons but at levels which escape detection with ^{35}S -labeled probes. However, as evidenced with MAP2 (Garner et al., 1988; Tucker et al., 1989), the same mRNA may be transported differentially among different neuronal populations. We have consistently observed higher background hybridization with ^{32}P -labeled probes and therefore used ^{35}S -labeled probes throughout our *in situ* studies. Lin et al. (1987) observed low levels of hybridization of a ^{32}P -labeled α -subunit riboprobe in cerebellar granule and molecular layers; however, the possible contribution of nonspecific hybridization was not reported.

Using a monoclonal antibody directed against both the α - and β -subunits, Ouimet et al. (1984) described weak immunoreactivity in both Purkinje and granule cells of the cerebellum. Our results with the β -subunit mRNA probe agree with those of Ouimet et al.; however, we found the level of hybridization to be quite high in these neurons (Fig. 2). The difference in signal intensity revealed by antibody staining versus oligonucleotide hybridization may be due to limited accessibility of the antibody to the epitope or possibly to inefficient translation of β -subunit mRNA by granule cells.

Developmental changes in CaM-KII mRNA

Previous studies have measured developmental changes of CaM-KII levels in forebrain by several criteria (Kelly and Vernon, 1985; Kelly et al., 1987). They have shown that α -subunit levels are very low at birth but increase dramatically during the second and third postnatal weeks, coinciding with the most active period of synaptogenesis in forebrain. The β -subunit, on the other hand, is readily detected at birth and increases only slightly thereafter. During the period of greatest α -subunit accumulation, CaM-KII shifts from being predominantly cytosolic to being concentrated in SJ-enriched particulate fractions. In hippocampus the burst in α -subunit mRNA expression parallels the most active period of synapse formation (Crain et al., 1973). Thus, cell-cell recognition may contribute to the regulation of this gene, or vice versa. We have used *in situ* and slot-blot hybridization analyses to demonstrate that mRNA levels for CaM-KII subunits change during development in synchrony with previously reported protein levels. These coordinated changes in mRNA and protein levels strongly suggest that transcription is the primary mechanism for regulating levels of CaM-KII during brain development.

Related isoforms of CaM-KII have been described in a variety of tissues, but these proteins appear to be distinct by several

criteria (Shenolikar et al., 1986; Fukunaga et al., 1988). Moreover, among all tissues examined, the mammalian brain is by far the most abundant source of CaM-KII. In brain, CaM-KII is most highly, if not exclusively, expressed in neurons compared with non-neuronal cells (Scholz et al., 1988). Thus, it appears that CaM-KII may be regulated by a brain-specific transcriptional element. The distinct expression of the mRNAs encoding the 2 subunits suggests that their respective genes are under additional controls that are activated at different times during development within a single neuron (e.g., hippocampal pyramidal cells). In addition, subunit-specific genes display very different transcriptional activities among neuronal types in the adult brain (e.g., pyramidal vs Purkinje cells). These results suggest that extrinsic factors such as cell-cell recognition and/or synaptic connectivity may contribute to the neuron-specific expression of CaM-KII subunits.

Functional considerations of dendritic localization of α -subunit mRNA

The concentration of α -subunit in forebrain homogenates does not change significantly after the third postnatal week; however, there is a redistribution of the enzyme among subcellular fractions (Kelly and Vernon, 1985; Rostas et al., 1986). CaM-KII continues to accumulate in SJs well into adulthood, while soluble levels decrease during the same period. It has been speculated that SJ-associated CaM-KII is derived from a cytosolic holoenzyme pool during development (Kelly and Vernon, 1985) and that the α -subunit may play a role in anchoring CaM-KII to PSDs (Miller and Kennedy, 1985). Interestingly, the α : β ratio of SJ-associated CaM-KII (7:1) is about twice that of the cytosolic enzyme (3:1; Kelly et al., 1987). Therefore, additional α -subunits derived from dendritic polyribosomes may be involved in the translocation of the cytosolic holoenzyme to PSDs. In another setting, namely, reactive synaptogenesis in the dentate gyrus, dendritic polyribosomes become more closely associated with spines and are postulated to facilitate the synthesis of important postsynaptic proteins (Steward, 1983). One such protein could be the α -subunit of CaM-KII, which may be involved in targeting the soluble enzyme to regenerating PSDs.

CaM-KII exhibits a unique mechanism of autoregulation that has been implicated as a molecular "switch" in memory storage (Miller and Kennedy, 1986). Moreover, recent results by Malenka et al. (1989) and Malinow et al. (1989) have implicated CaM-KII in the induction of long-term potentiation, a cellular analog of learning and memory, in CA1 neurons of the hippocampus. Lisman and Goldring (1988) have presented a theoretical treatise describing how CaM-KII's properties might allow for a graded and stable memory storage system resistant to protein turnover. A central tenet of their theory is that CaM-KII subunits in PSDs are replaced individually, not as holoenzymes. Consequently, there would need to be a source of individual subunits for the PSD-associated enzyme to draw from. The presence of α -subunit mRNA in dendrites supports the hypothesis of localized turnover of this subunit. Thus, α -subunits synthesized in cell bodies might be assembled into cytosolic holoenzymes while dendritically synthesized α -subunits may be targeted for the turnover of individual subunits at PSDs. The selective localization of α -subunit mRNA in pyramidal and hippocampal granule, but not Purkinje, cell dendrites suggests that the local synthesis of this protein may be important in the formation, function, and plasticity of postsynaptic elements in some, but not all, neuronal populations.

References

- Bennett MK, Kennedy MB (1987) Deduced primary structure of the β -subunit of brain type II Ca^{2+} /calmodulin-dependent protein kinase determined by molecular cloning. *Proc Natl Acad Sci USA* 84:1794–1798.
- Bennett MK, Erondy NE, Kennedy MB (1983) Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J Biol Chem* 258:12735–12744.
- Bulleit RF, Bennett MK, Molloy SS, Hurley J, Kennedy MB (1988) Conserved and variable regions in the subunits of brain type II Ca^{2+} /calmodulin-dependent protein kinase. *Neuron* 1:63–72.
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Crain B, Cotman C, Taylor D, Lynch G (1973) A quantitative electron microscopic study of synaptogenesis in the dentate gyrus of the rat. *Brain Res* 63:195–204.
- Davis L, Banker G, Steward O (1987) Selective dendritic transport of RNA in hippocampal neurons in culture. *Nature* 330:477–479.
- Erondy NE, Kennedy MB (1985) Regional distribution of type II Ca^{2+} /calmodulin-dependent protein kinase in rat brain. *J Neurosci* 5:3270–3277.
- Fort P, Marty L, Piechaczyk M, El Sabrouty S, Dani C, Jeanteur P, Blanchard JM (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res* 13:1431–1442.
- Fukunaga K, Goto S, Miyamoto E (1988) Immunohistochemical localization of Ca^{2+} /calmodulin-dependent protein kinase II in rat brain and various tissues. *J Neurochem* 51:1070–1078.
- Garner CC, Tucker RP, Matus A (1988) Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. *Nature* 336:674–677.
- Hanley RM, Means AR, Ono T, Kemp B, Burgin KE, Waxham MN, Kelly PT (1987) Functional analysis of a complementary DNA for the 50-kilodalton subunit of calmodulin kinase II. *Science* 237:293–297.
- Johnston MV, Rutkowski JL, Wainer B, Long J, Mobley WC (1987) NGF effects on developing forebrain cholinergic neurons are regionally specific. *Neurochem Res* 12:985–994.
- Kelly PT, Montgomery P (1982) Subcellular localization of the 52,000 molecular weight major postsynaptic density protein. *Brain Res* 233:265–286.
- Kelly PT, Vernon P (1985) Changes in the subcellular distribution of calmodulin-kinase II during brain development. *Dev Brain Res* 18:211–224.
- Kelly PT, McGuinness TL, Greengard P (1984) Evidence that the major postsynaptic density protein is a component of a Ca^{2+} /calmodulin-dependent protein kinase. *Proc Natl Acad Sci USA* 81:945–949.
- Kelly PT, Yip RK, Shields S, Hay M (1985) Calmodulin-dependent protein phosphorylation in synaptic junctions. *J Neurochem* 45:1620–1634.
- Kelly P, Shields S, Conway K, Yip R, Burgin K (1987) Developmental changes in calmodulin-kinase II activity at brain synaptic junctions: alterations in holoenzyme composition. *J Neurochem* 49:1927–1940.
- Lin C, Kapiloff M, Durgerian S, Tatemoto K, Russo A, Hanson P, Schulman H, Rosenfeld MG (1987) Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. *Proc Natl Acad Sci USA* 84:5962–5966.
- Lisman JE, Goldring MA (1988) Feasibility of long-term storage of graded information by the Ca^{2+} /calmodulin-dependent protein kinase molecules of the postsynaptic density. *Proc Natl Acad Sci USA* 85:5320–5324.
- Malenka RC, Kauer JA, Perke D, Mauk M, Kelly PT, Nicoll RA, Waxham MN (1989) Long-term potentiation: an essential role for postsynaptic calmodulin and protein kinase activity. *Nature* 340:554–557.
- Malinow R, Schulman H, Tsien RW (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* 245:862–866.
- Maniatus T, Fritsch E, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- McGuinness TL, Lai Y, Greengard P (1985) Ca^{2+} /calmodulin-dependent protein kinase II: isozymic forms from rat forebrain and cerebellum. *J Biol Chem* 260:1696–1704.
- Miller SG, Kennedy MB (1985) Distinct forebrain and cerebellar isoforms of type II Ca^{2+} /calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *J Biol Chem* 260:9039–9046.
- Miller SG, Kennedy MB (1986) Regulation of brain type II Ca^{2+} /calmodulin-dependent protein kinase by autophosphorylation: a Ca^{2+} -triggered molecular switch. *Cell* 44:861–870.
- Ohmsted C, Jensen KF, Sahyoun N (1989) Ca^{2+} /calmodulin-dependent protein kinase enriched in cerebellar granule cells. *J Biol Chem* 264:5866–5875.
- Ouimet CC, McGuinness TL, Greengard P (1984) Immunocytochemical localization of calcium/calmodulin-dependent protein kinase II in rat brain. *Proc Natl Acad Sci USA* 81:5604–5608.
- Rostas J, Weinberger R, Dunkley P (1986) Multiple pools and multiple forms of calmodulin-stimulated protein kinase during development: relationship to postsynaptic densities. *Prog Brain Res* 69:355–371.
- Scholz WK, Baitinger C, Schulman H, Kelly P (1988) Developmental changes in Ca^{2+} /calmodulin-dependent protein kinase II in cultures of hippocampal pyramidal neurons and astrocytes. *J Neurosci* 8:1039–1051.
- Schulman H (1984) Phosphorylation of microtubule-associated proteins by a Ca^{2+} /calmodulin-dependent protein kinase. *J Cell Biol* 99:11–19.
- Shenolikar S, Lickteig R, Hardie DG, Soderling TR, Hanley RM, Kelly P (1986) Calmodulin-dependent multifunctional protein kinase. *Eur J Biochem* 161:739–747.
- Steward O (1983) Alterations in polyribosomes associated with dendritic spines during the reinnervation of the dentate gyrus of the adult rat. *J Neurosci* 3:177–188.
- Steward O, Falk PM (1985) Polyribosomes under developing spine synapses: growth specializations of dendrites at sites of synaptogenesis. *J Neurosci Res* 13:75–88.
- Steward O, Levy WB (1982) Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. *J Neurosci* 2:284–291.
- Suzuki T, Tanaka R (1986) Characterization of Ca^{2+} /calmodulin-dependent protein kinase associated with rat cerebral synaptic junction: substrate specificity and effect of autophosphorylation. *J Neurochem* 47:642–651.
- Tobimatsu T, Kameshita I, Fujisawa H (1988) Molecular cloning of the cDNA encoding the third polypeptide of brain calmodulin-dependent protein kinase II. *J Biol Chem* 263:16082–16086.
- Tucker RP, Garner CC, Matus A (1989) *In situ* localization of microtubule-associated protein mRNA in the developing and adult rat brain. *Neuron* 2:1245–1256.
- Walaas SI, Lai Y, Gorelick FS, DeCamilli P, Moretti M, Greengard P (1988) Cell-specific localization of the α -subunit of calcium/calmodulin-dependent protein kinase II in Purkinje cells in rodent cerebellum. *Mol Brain Res* 4:233–242.
- Young WS, Mezey E, Siegel R (1986) Quantitative *in situ* hybridization histochemistry reveals increased levels of corticotropin-releasing factor mRNA after adrenalectomy in rats. *Neurosci Lett* 70:198–203.