Dev Neurosci 1993;15:261–270

Functional Maturation of Creatine Kinase in Rat Brain

D. Holtzman [a]
M. Tsuji [b]
T. Wallimann [c]
W. Hemmer [d]

Departments of
[a] Neurology, and
[b] Pediatrics (Neonatology), Children's Hospital and Harvard Medical School, Boston, Mass., USA;
[c] Department of Cell Biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland;
[d] Department of Chemistry, University of California-San Diego, La Jolla, Calif., USA

Key Words
ATP
Bergmann glia
Brain development
Cytosolic creatine kinase
Hypoxia
Mitochondrial creatine kinase
Nuclear magnetic resonance
Phosphocreatine
Purkinje neurons

Abstract
The physiological role of the phosphocreatine (PCr)/creatine kinase (CK) system has been studied in rat brain by comparing maturational changes in in vivo CK-catalyzed reaction rate and activities of CK isoenzymes. The CK-catalyzed reaction rates, measured by ^{31}P-nuclear magnetic resonance spectroscopy, increased 4-fold between 12 and 17 days of age. The mitochondrial CK (Mi-CK) isoenzyme, as a percentage of total CK, increased to the same extent over this relatively narrow age period. Cytosolic CK (B-CK) was active earlier and, with the total CK activity, increased steadily over a longer time course. An immunohistochemical study of cerebellum showed Mi-CK predominantly in gray matter, while the cytosolic CK was present in rather large concentrations in both gray and white matter. In the molecular layer, B-CK was most prominent in the Bergmann glial cells, while Mi-CK was more prominent in Purkinje neurons. During development a redistribution of Mi-CK from the Purkinje cell bodies to their processes was observed. These results point to regional differences in CK content and in isoenzyme-specific localizations. The increase in CK activity is temporally coincident with the maturational appearance of closely coupled decreases in brain PCr and ATP during hypoxia. These maturational changes suggest that the activity of the PCr/CK system, particularly the Mi-CK isoenzyme, is central in regulation of brain ATP.

Introduction

Brain energy metabolism is characterized by high and rapidly fluctuating rates of ATP synthesis and utilization [1]. In vivo ^{31}P nuclear magnetic resonance (NMR) spectroscopic studies have demonstrated that the ATP concentration is constant under conditions of energy deficit (moderate hypoxia) and high energy demand (seizures) [2, 3]. Like other cell systems characterized by fluctuating energy requirements, the brain contains both cytosolic creatine kinase (B-CK) and mitochondrial CK (Mi-CK) isoenzymes [4, 5]. In analogy with the heart, it has been suggested that the CK catalyzed reaction rate in brain, measured in vivo by NMR spectroscopy, may re-

This paper was presented at the Satellite meeting entitled 'Functional Aspects of Energy Metabolism in Neural Tissue' which was sponsored by the International Society for Neurochemistry and held from August 28th to September 1st, 1993, in Carcassonne, France. This paper has undergone the Journal's usual peer review.

David Holtzman MD, PhD
Department of Neurology
Children's Hospital
300 Longwood Avenue
Boston, MA 02115 (USA)

flect energy demand and ATP turnover more directly than do the concentrations of ATP or PCr [6, 7]. Two primary physiological functions have been suggested for the PCr/CK system in these tissues [5, 8]. The first is a temporal buffer role in which PCr contributes phosphoryl groups for the resynthesis of ATP. The second is a transport function (spatial buffer) in which PCr synthesized at the mitochondrion resynthesizes ATP at the functional sites of high ATPase activity. A third, more recent proposal is that the CK system, including Mi-CK complexed with the ATP-ADP translocase, functions to closely regulate ATP and ADP concentrations by closely coupling ATP demand and synthesis [5]. The first and, possibly, the second of these models would be characterized by a sequential loss of PCr and ATP during a condition of energy deficit. The third mechanism could be characterized by coincident losses of both reactants.

In vivo maturational studies may provide further insights into the physiology of the brain PCr/CK system. The CK catalyzed reaction rate increases 4-fold in the narrow age period of 12–15 days in the lightly anesthetized mouse brain [9]. Over this same age period, dramatic changes occur in the coupling of decreases in PCr and ATP during hypoxia [10, Tsuji, et al., in preparation]. With very frequent NMR spectral acquisitions, almost complete loss of brain PCr is associated with only a small loss of ATP during hypoxia in rats younger than 12 days of age. This sequential loss of PCr and ATP, which is similar to the pattern seen in muscle, suggests a physiological temporal energy buffering role for the PCr/CK system. In more mature rats prolonged hypoxia produces an initial 50% decrease in PCr with a 20% decrease in ATP, followed by simultaneous decreases in PCr and ATP. Coincident with this brief developmental age period, rat cerebral cortical slices show marked increases in stimulated rates of aerobic glycolysis and the efficiency of oxidative phosphorylation increases significantly in brain mitochondria [11–13].

The studies described in the present collaborative report compare directly the maturational time course of the increases in CK isoenzymes with the CK catalyzed fluxes measured in vivo in rat cerebral hemispheres. Initial observations on the localization of CK isoenzymes in rat cerebellum will be described. These results show regional variation of the isoenzyme localization which complicate comparisons of isoenzyme activities with an in vivo averaging technique such as NMR.

Methods

Animals. Long-Evans hooded rats were used for all studies. Dated litters or postweanling male rats were obtained from Charles River Breeding Laboratories (Wilmington, Mass., USA). The animals were maintained with 5 weanling rats or one litter per cage at 23 °C and constant light-dark periods. For both the NMR and isoenzyme studies, animals were studied in age groups 4–6, 11–13, and 18–20 days. For the NMR studies an oldest group of 30 days wa used as the 'adult' group. For the enzyme analyses, the oldest group was 40 days.

^{31}P-NMR. Brain spectra were acquired in the Fourier transform mode at 145,587 MHz using a custom-built spectrometer and an Oxford 8.9 cm vertical bore superconducting magnet (8.45 T), as described previously [9]. The rat was anesthetized with chloral hydrate (100 mg/kg, intraperitoneal) and wrapped with adhesive tape. The bound animal was positioned in the NMR probe with the single turn copper surface coil (0.8 cm outer diameter) centered over the skull behind the supraorbital ridges. The surface coil antenna was matched and tuned to the ^{31}P frequency with the animal in place. The magnetic field was optimized in the usual manner and the 90° pulse was determined [9]. An initial spectrum was acquired with a 12-second recycle delay using the previously described spectral parameters.

The CK-catalyzed reaction rate was measured in vivo using the saturation transfer (ST) experiment, as previously described [9]. In order to measure the phosphoryl transfer rate from PCr to ATP, selective saturation of the γ-phosphorus signal from ATP (γ-ATP) was achieved using a 5-second low power pulse centered on the γ-ATP resonance frequency. Following this saturating pulse, the usual 90° high power, nonselective acquisition pulse was applied and the free induction decay was acquired to record the net z-magnetization. The total interpulse interval was less than 6 s.

Phosphorus flux in the complex CK-catalyzed reaction

$$PCr + ADP + H^+ \leftrightarrow Cr + ATP$$

was analyzed using the following idealized first-order exchange model,

$$PCr \underset{k2}{\overset{k1}{\leftrightarrows}} ATP$$

In this model the unidirectional pseudo-rate constant, k, is equal to the ratio of the chemical flux, J, to the substrate concentration [PCr]. Use of the pseudo-rate constant does not imply that the reaction is first order. The ratio of chemical flux to substrate concentration is measured directly in the ST experiment. Thus, the rate constant was obtained from the ST experiment as

$$k_f = \frac{J}{[PC]} = \frac{1}{(T_1)_{PC}}\left[\frac{M_o}{M_s} - 1\right]$$

where T_1 is the longitudinal relaxation time of PCr in the absence of chemical exchange, M_s is the PCr signal in the presence of the γ-ATP saturating pulse, and M_o is the PCr signal with the saturating pulse centered an equal distance on the opposite side of the PCr peak. The T_1 value of 3 s for brain PCr was taken from modified progressive saturation experiments in mouse pups over this age period [9].

Tissue Preparation for Enzyme Analyses. Whole frozen rat brains (1 volume) were thawed in 2.6 volumes of ice-cold hypotonic buffer A (0.5 m*M* EGTA, 1 m*M* PMSF, 2 m*M* beta-mercaptoethanol) and homogenized with a glass-teflon homogenizer. The homogenate was kept for 30 min on ice to allow mitochondrial swelling. Buffer B (same as buffer A, but with additional 1 *M* NaH_2PO_4 pH 8.4) was added to give a final concentration of 0.1 *M* phosphate and a tissue to volume ratio of 1:4. At the same time, Triton X100 was added to a final concentration of 0.05% (w/w). The high phosphate concentration together with the Triton was optimal for the complete solubilization of the CK isoenzymes, which is especially critical for Mi-CK. Homogenates were frozen on liquid nitrogen and thawed on ice upon usage. These homogenates were analyzed either directly or centrifuged at 178,000 *g* in a Beckmann-Air-Fuge for 15 min. The supernatants are referred to as extracts.

CK Activities by the pH-stat Method. Total CK activities (IU/mg protein) of the different brain homogenates were measured by the pH stat method [14]. One IU corresponds to 1 µmol PCr transphosphorylated per minute at 25 °C.

CK Isoenzyme Concentrations by Immunoblot Analysis. Homogenates were analyzed on 10% polyacrylamide SDS gels [15]. Blotting was performed with semi-dry blotters (JKA-Biotech, Denmark) with the continuous buffer system. Transfer efficiency was controlled by staining protein on the nitrocellulose membrane with Ponceau S prior to blocking nonspecific binding sites of the nitrocellulose with blocking buffer (1% BSA in PBS). Specific labelling of antichicken B-CK and Mi-CK on the blots was achieved with antibody solutions of 1:800 and 1:250, respectively, in the same buffer, followed by peroxidase-conjugated goat antirabbit IgG (Nordic) diluted at 1:3,000 using 4-chloro-1-naphthol and H_2O_2 as substrates.

Analysis of Developmental Changes in Specific CK Isoenzyme Activities. Electrophoresis and CK activity staining were performed as previously described [16] except that 0.3 m*M* (instead of 0.01 m*M*) P^1,P^5-di(adenosine-5')-pentaphosphate was used for blocking adenylate kinase activity. The Mi-CK, which can exist in octameric and dimeric states, was dimerized by adding 1 vol buffer C (16 m*M* ADP, 20 m*M* $MgCl_2$, 80 m*M* creatine, 200 m*M* KNO_3) prior to electrophoresis to get only a single Mi-CK band corresponding to the dimer [5]. The staining intensity was quantified with a Molecular Dynamics Computing Densitometer (Model 300A) using a volumetric mode, taking care that the colorimetric assays were in a linear range. The CK bands were further identified by omitting PCr in the overlayed staining gel [not shown]. Samples were loaded either containing the same protein concentration (4.6 mg/ml) or the same total enzyme activity (10.3 IU/ml). As a standard marker a mitochondria-enriched fraction of rat brain, containing B-CK and dimerized Mi-CK, was applied.

Localization of CK Isoenzymes by Indirect Immunofluorescence. Whole rat brains were obtained after perfusion fixation with 4% paraformaldehyde in 0.1 *M* phosphate buffer and paraffin embedding. Indirect immunofluorescence on the sectioned rat cerebella was performed as described earlier for chicken retina [17] and chicken brain [18]. Rabbit antichicken B-CK antibody (diluted 1:150) or rabbit antichicken Mi-CK antibody (diluted 1:300) were used as primary antibodies, and FITC-conjugated goat antirabbit IgG (diluted 1:100) served as a second antibody [19]. Stained sections were examined with a Zeiss-Standard-18 epifluorescence microscope and photographed on Ilford HP-5 film.

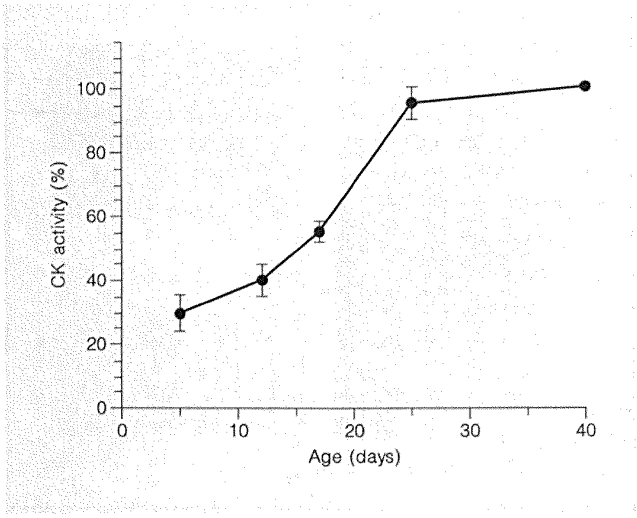

Fig. 1. Increase of CK activity in total brain homogenates during postnatal development of the rat. The CK activity of rat brain extracts was measured by the pH-stat method at different developmental time points (days 5, 12, 17, 25 and 40). Extractions of CK isoenzymes, including Mi-CK, were optimized to yield maximal values of total CK activity present in this tissue (see Methods). Note the marked increase of total CK activity by a factor of 3–4 during postnatal development of the rat brain between days 5 and 25.

Results

The total CK activity, measured enzymatically by the pH-stat method in rat brain homogenates, increased 3- to 4-fold between 5 and 25 days of age (fig. 1). Adult CK levels varied from 4 to about 10 IU/mg protein depending on whether the activity measurements are referred to total untreated homogenates or to supernatants after the 178,000 *g* centrifugation, respectively. The specific CK activity was ≈360 ± 40 IU/g wet weight for the homogenates from adult rat brains (P 40).

Quantities of total CK and of the isoenzymes, B- and Mi-CK, were measured in brain homogenates during postnatal development. Total homogenates were analyzed by SDS-PAGE (80 µg/lane) and stained with Commassie blue (fig. 2a) or blotted onto nitrocellulose and stained by affinity-purified antichicken B-CK antibody (fig. 2b) or antichicken Mi-CK antibody (fig. 2c). Anti-B-CK staining (fig. 2b) revealed a single peroxidase-stained band at the expected apparent molecular weight (≈43 kD, labelled by an asterisk). Anti-Mi-CK staining (fig. 2c) led to the staining of a protein with slightly higher electrophoretic mobility in SDS gels compared to B-CK, which is a common feature for Mi-CK [5]. With

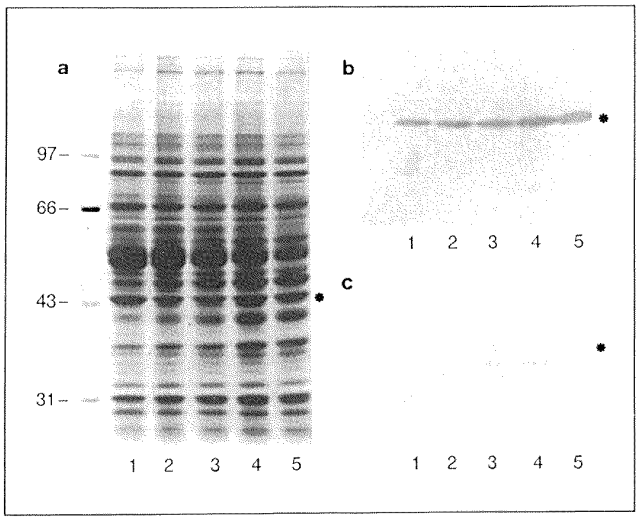

Fig. 2. Expression of creatine kinase isoenzyme subunits visualized by immunoblot analysis during postnatal development of the rat brain. **a** Total rat brain homogenates (80 µg protein loaded/lane) at different time points of postnatal development (lane 1 is day 5; lane 2, day 12; lane 3, day 17; lane 4, day 25 and lane 5, day 40) are shown after separation of proteins by denaturing electrophoresis in 10% polyacrylamide gels in the presence of SDS (SDS-PAGE) and staining for protein with Coomassie blue. The electrophoretic mobilities of molecular weight standards are indicated in kDa on the left. **b** Relevant sections of a parallel gel as shown in **a**, after electrophoretic transfer of proteins to nitrocellulose membranes and subsequent immunostaining with affinity purified rabbit antichicken brain-type (B-CK) antibody at 1:800 dilution, followed by peroxidase-coupled second antibody at 1:3,000 dilution and staining for peroxidase activity. The B-CK was present at an early age increasing slightly with time. **c** Relevant section of a parallel gel as shown in **a** and immunoblotted as in **b**, but stained with rabbit antimitochondrial-type (Mi-CK) antibody at 1:250 dilution. The postnatal appearance of Mi-CK is late relative to B-CK in rat brain, where significant amounts of Mi-CK can only be detected after day 12 of postnatal development. An asterisk represents the position of B-CK. Note the slight cross-reactivity on these Western blots of anti-Mi-CK antibody with B-CK subunits after SDS-PAGE, with the latter CK subunit showing a slightly higher apparent M_r compared to Mi-CK.

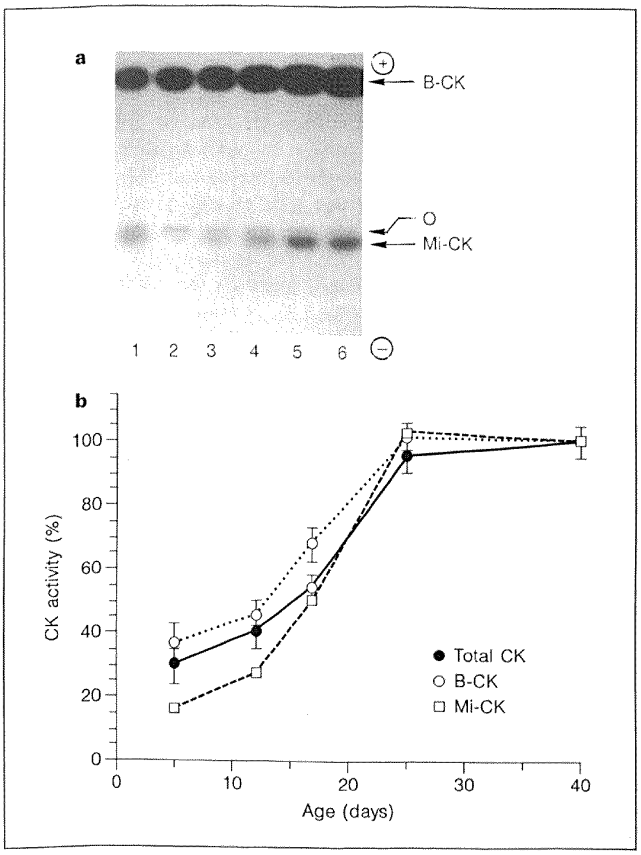

Fig. 3. Zymograms of the expression of CK isoenzyme activities during postnatal rat brain development. Separation of CK isoenzymes, brain-type cytosolic CK (B-CK) and mitochondrial CK (Mi-CK) of total rat brain extracts (4.6 mg protein/ml) (lane 1 is a fraction of enriched mitochondria from adult rat brain containing Mi-CK and B-CK as standards; lane 2, day 5; lane 3, day 12; lane 4, day 17; lane 5, day 25; and lane 6, day 40 of postnatal brain development), after electrophoretic separation on cellulose polyacetate strips under native conditions and subsequent staining for CK activity (**a**); followed by densitometic scanning and quantitation of total CK activity; B-CK activity, and Mi-CK activity, all given in%, as function of time during postnatal development (**b**). Adenylate kinase activity was suppressed by addition of 300 µm of di-adenosine-penta-phosphate (Ap5A). (+) and (–) indicate the anode and cathode, respectively; (o) indicates the sample application point. Since prior to the electrophoresis the samples were incubated with transition-state inducing substrates (MgADP, creatine and nitrate), Mi-CK is completely converted into the dimeric form (see Methods) [5]. Note that all CK-related parameters increase markedly between days 5 and 25, with Mi-CK showing the steepest maturational increase (by a factor of 6, compared to a factor of 3–4 for B-CK). The increase in Mi-CK is greatest between days 12 and 25. Peak levels of both isoenzymes are reached at day 25 postnatally (see also fig. 1).

maturation the staining intensity and band width increased for both B- and Mi-CK. However, in contrast to B-CK, which was already prominent at early stages of postnatal development, Mi-CK only started to appear between day 12 and 17 postnatally (compare fig. 2b and c). On a semi-quantitative basis, the relative increase of Mi-CK was greater than the relative increase of B-CK during the same period of postnatal development. In figure 2c a slight cross-reactivity of the anti-Mi-CK antibody with B-CK (upper band, labelled by an asterisk)

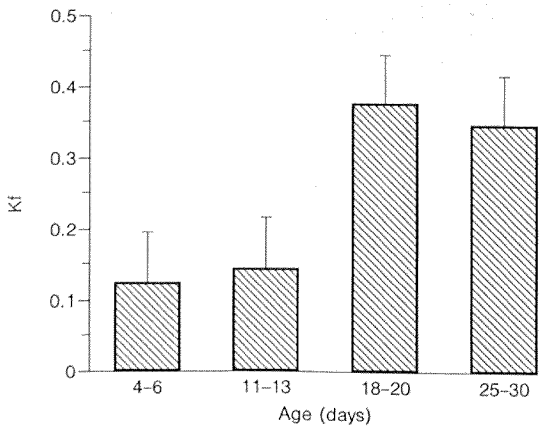

Fig. 4. The pseudo first-order rate constant (Kf) for the forward CK-catalyzed reaction rates in the developing rat brain are shown. Each value is the mean ± standard error of the mean for 5–6 animals at each of the ages indicated. Compared to the oldest group, the rate constants for the two youngest are significantly smaller ($p < 0.001$) using Student's t test corrected for multiple comparisons.

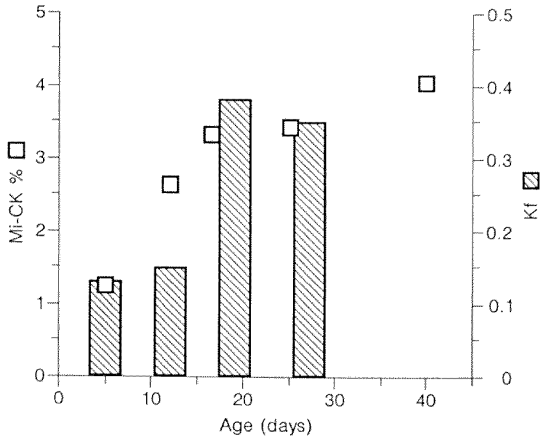

Fig. 5. The pseudo first-order rate constants (Kf) for the CK-catalyzed reaction rates in the developing rat brain (taken from fig. 4) are compared with the fraction of brain CK activity represented by the Mi-CK isoform. The Mi-CK is expressed as a percentage of the total CK activity (Mi-CK/(Mi-CK + B-CK) × 100 as determined by densitometry of zymograms similar to that shown in figure 3.

was detected, due to possible differential immunoreactivities.

In order to obtain quantitative information on the maturation of CK isoenzyme activities, brain extracts and homogenates were subjected to cellulose poly-acetate electrophoresis followed by CK activity staining. The staining was analyzed by computer densitometry. By this method it was possible to separate the CK isoenzymes (fig. 3a) and to quantify the relative staining intensity of the B-CK and Mi-CK bands. Homogenates and extracts revealed very similar results, but the staining patterns of extracts (e.g., fig. 3a) were much clearer than those of the homogenates [not shown]. The quantitative maturational increases of B-CK and Mi-CK activities also were compared to the increase in total activity measured by the pH-stat method (fig. 3b). Within experimental error, B-CK activity increased parallel to total CK activity which is in accordance with the observation that B-CK activity accounts for most of the total CK activity. Consistent with the immunoblot experiments (fig. 2b), the Mi-CK activity increased about 6 times between days 12 and 25 compared to an increase of about 3 times for the B-CK activity.

Within the postnatal time period from day 12 to 17, the pseudo-rate constant of the CK-catalyzed reaction rate measured by the ST experiment increased 3–4 times (fig. 4). This time course and quantitative increase were the same as seen in the mouse [9]. Because the PCr concentration increases over this age period, the increase in reaction rate is even greater. During this same time period, Mi-CK activity also increased by a factor of three (fig. 3c), while B-CK activity barely doubled (fig. 3b). This temporal relationship suggests that the increase of the CK reaction rate reflects the increase in B-CK and, perhaps more directly, the increase in Mi-CK activity. As shown in figure 5, the fraction of total CK activity represented by the Mi-CK activity shows a developmental increase which is very close to that of the in vivo CK-catalyzed reaction rate.

Initial results using immunohistochemical localization of CK isoenzymes in cerebellum suggest that caution is necessary in interpretating the cellular and regional bases of in vivo NMR studies. At the earliest stages of postnatal development, B-CK immunoreactivity was generally very weak (fig. 6d) while Mi-CK was undetectable (fig. 6a). At day 12 a clear B-CK signal was visible particularly in the molecular layer (fig. 6e). The Mi-CK appeared to be concentrated in the Purkinje cell bodies (fig. 6b). By day 25, Mi-CK immunoreactivity was strongest in the granular layer, perhaps in the cellular

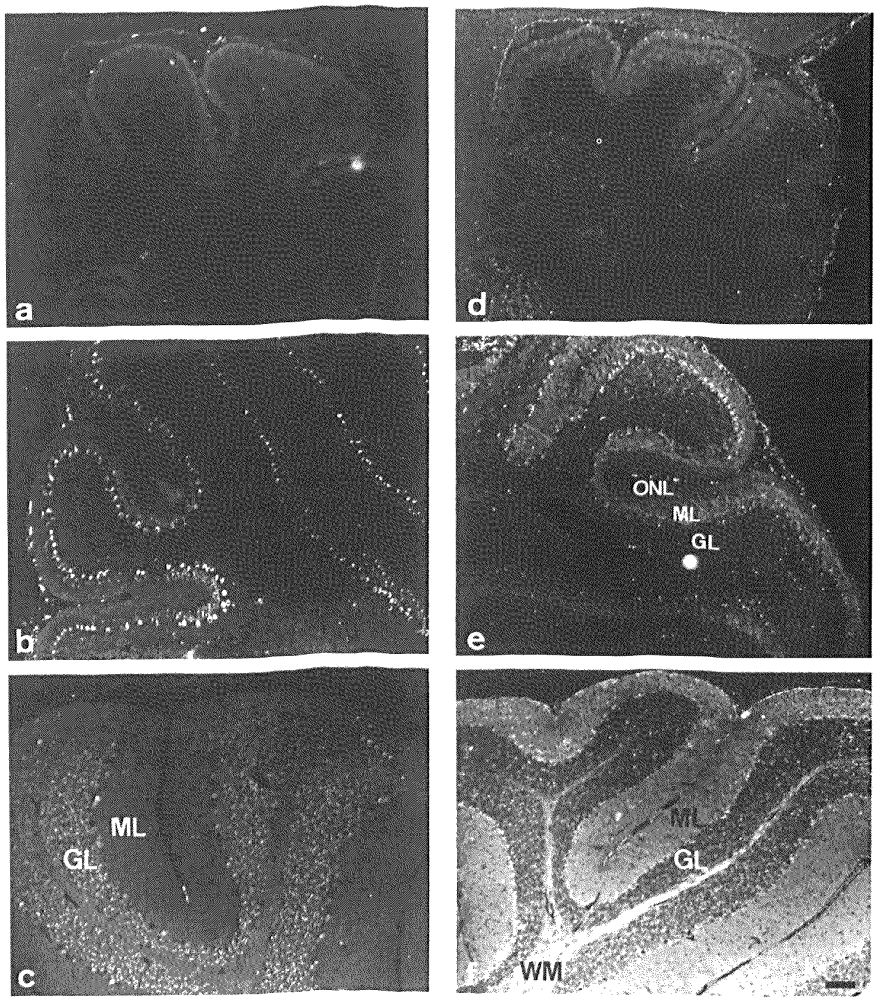

Fig. 6. Immunolocalization of CK isoenzymes in postnatally developing rat cerebellum. Immunolocalization of Mi-CK, using rabbit antichicken Mi-CK antiserum at 1:300 dilution, followed by 1:100 diluted FITC-conjugated goat-antirabbit IgG as the second antibody (**a, b, c**), and of B-CK, using a rabbit antichicken B-CK IgG at 1:150 dilution, followed by the same second antibody (**d, e, f**) in rat cerebellum at different ages (**a, d** 5 days; **b, e** 12 days; **c, f** 25 days). Note the very weak staining for Mi-CK and the rather weak staining for B-CK in rat cerebellum at day 5 postnatally and the marked increase in fluorescence intensity obtained both with anti-Mi-CK and anti-B-CK at day 12. The Mi-CK was concentrated mainly in the cell bodies of the Purkinje cells and B-CK appeared in the molecular layer (ML), indicating inhomogeneous localization of CK isoenzymes in different regions of the cerebellum as well as subcellular compartmentation of CK isoenzymes. Note also the relocation of Mi-CK from Purkinje cell bodies (**b** at day 12) into the processes of the same cells, as well as into the granular layer (GL), presumably also into the glomeruli regions (**c** at day 25). The B-CK appeared most prominently in the ML as well as in distinct areas of the GL and in the glomeruli. The B-CK showed strong staining in white matter (WM), which may be due in part to nonspecific staining by the second antibody in this region (see text). Bar = 100 μm.

processes of maturing Purkinje cells. The Mi-CK also was seen in a more spotted staining of the glomeruli in the granular layer which are known to be rich in mitochondria [20]. The B-CK staining was most prominent in the molecular layer with strong staining of the entire Bergmann glial cells, including the cell bodies, linear processes, and the membrana limitans gliae. The B-CK staining was strong also in white matter where little or no Mi-CK was seen (fig. 6f). Control staining with pre-immune sera was weak, except for some nonspecific staining of white matter often observed with the second FITC-conjugated antibody alone [not shown]. Thus, some of the anti-B-CK staining observed in white matter probably was nonspecific. At higher magnification, the specific cell layers and the prominent individual cell types of the cerebellum were confirmed (fig. 7). These results further confirm the distinct localization of CK isoenzymes in specific brain cells with strong differences between white and gray matter. The details of the immunolocalization of the CK isoenzymes in cerebellum were very similar to those found in the chicken cerebellum [18].

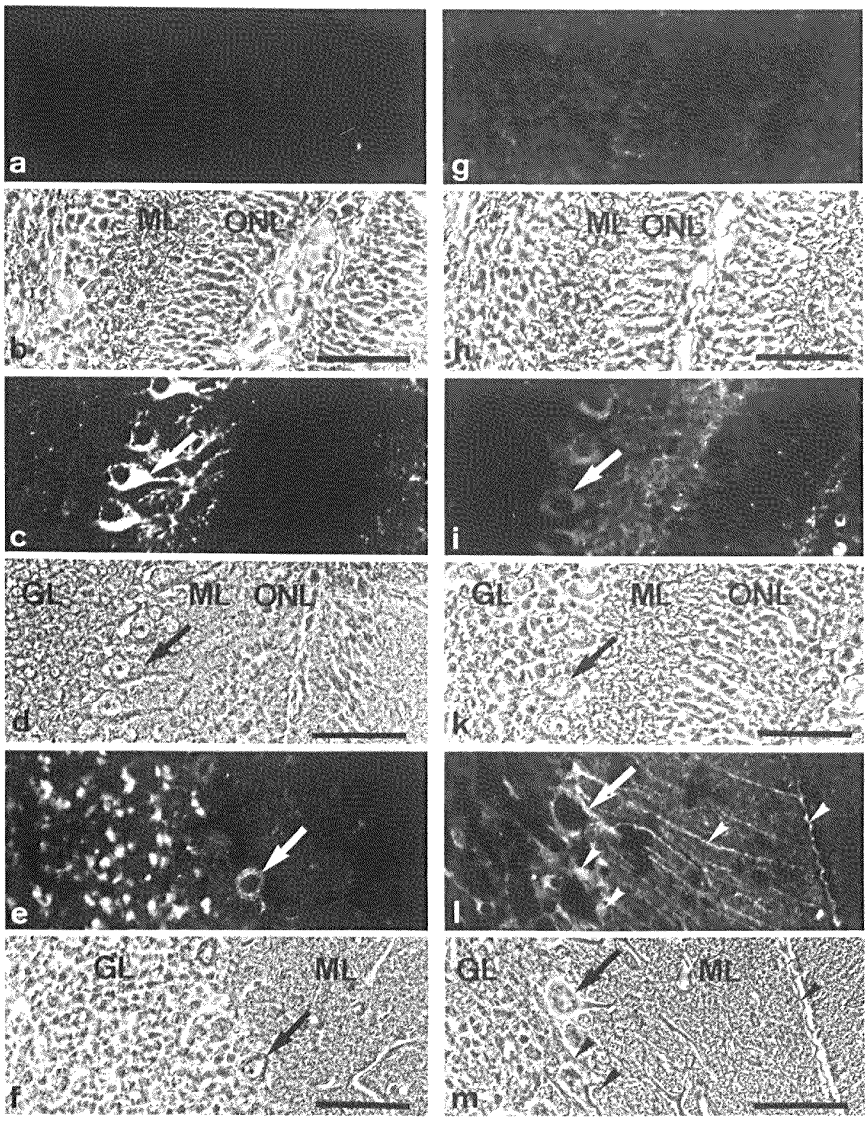

Fig. 7. Detailed immunolocalization of CK isoenzymes in postnatally developing rat cerebellum. Immunolocalization of Mi-CK, using rabbit antichicken Mi-CK antiserum at 1:300 dilution, followed by 1:100 diluted FITC-conjugated goat antirabbit IgG as the second antibody (left-hand panels **a, b, c, d, e, f**); and of B-CK, using a rabbit antichicken B-CK IgG at 1:150 dilution, followed by the same second antibody (right-hand panels **g, h, i, k, l, m**) in rat cerebellum at different postnatal ages (**a, b, g, h** = 5 days; **c, d, i, k** = 12 days; **e, f, l, m** = 25 days postnatally, with fluorescence pictures each above the corresponding phase contrast pictures.
Note the very weak staining of cerebellar sections at 5 days postnatal with both anti-Mi-CK and anti-B-CK, strong staining by anti-Mi-CK of Purkinje cell bodies (**c, d**), relatively weak staining of the same Purkinje cell bodies plus cells in the outer nuclear layer (ONL) by anti-B-CK antibody at 12 days postnatal (**i, k**) and relocation of Mi-CK from the Purkinje cell bodies into the Purkinje cell processes as well as into the glomeruli regions at 25 days of postnatal development (**e, f**). Note also the prominent anti-B-CK staining of the Bergmann glia cell bodies (**l** Small arrowheads, left) and their linear processes (**l** Small arrowhead, middle) as well as distinct staining of the membrana limitans gliae (**l** Small arrowhead, right) at the same postnatal age (25 days). ML = Molecular layer; GL = granular layer; large arrows pointing to individual Purkinje cells; small arrowheads pointing Bergmann glia cell bodies and their processes as well as to the membrana limitans gliae (**l, m**). Bar = 50 μm.

Discussion

These results represent the first developmental comparison of the predominant brain CK isoenzymes, the cytosolic B-CK and the mitochondrial Mi-CK, with an in vivo measure of the brain CK catalyzed reaction rate in the same rat strain and at the same ages. The increase in CK-catalyzed flux from PCr to ATP and the time course of this increase in the rat are the same as previously described in the mouse [9]. The postnatal increase in total CK activity measured in brain homogenates also is in accordance with previous results. The adult rat brain total CK activity is the same as reported by Norwood et al. [21] and about 50% greater than reported by Booth and Clark [22]. The 3–4 times increase in total CK activity is similar to reports from other laboratories [22, 23]. These similar results between studies have been found in spite of differences in methods used for extraction and measurement of the CK activities.

Comparisons of the maturational increases in CK isoenzyme activities with the increase in CK catalyzed fluxes in vivo provides tentative conclusions concerning the physiology of the complex PCr/CK system in brain. The maturational increase in total CK activity in brain

homogenates most closely parallels the increase in B-CK which makes up at least 95% of the total isoenzyme activity at any age. However, by antibody staining and quantitative activity measures, the increase in Mi-CK occurs later with a time course closer to that of the increase in CK activity in vivo. The closest association between the isoenzyme activities and the in vivo CK activity is with the calculated activity of Mi-CK as a percentage of the total CK isoenzyme activity (fig. 5). This temporal association can only be qualitative. A tentative conclusion from these observations is that the maturational appearance of the Mi-CK isoenzyme in functional association with some of the B-CK isoenzyme effects a large increase in the average flux catalyzed by the enzyme complex. Measuring the adult brain isoenzyme activities under conditions in which the in vivo CK catalyzed reaction rate is reduced to the rate in the immature brain will directly test this hypothesis on the roles of the CK isoenzymes in the total CK activity [7, 24].

The immunolocalization results suggest that comparison of in vivo measures of CK catalyzed reaction rates, a sum of activity over much of the cerebral hemispheres, with whole brain measures of CK isoenzyme activities must be approached with caution. Cerebellar white matter has little, if any, Mi-CK isoenzyme activity. In contrast, B-CK activity is high in both white and gray matter. The cerebellum was studied initially because of the well delineated structures. At present we must assume that the distribution of CK isoenzymes shows analogous gray-white differences in the cerebral hemispheres as in the cerebellum. If correct, the regional compartmentation suggests that the Mi-CK isoenzyme represents a much higher fraction of the total CK activity in cortical gray matter than indicated by our results in whole brain homogenates. This proposed interpretation is being tested directly using the NMR chemical shift imaging technique to compare the CK catalyzed reaction rates in the cerebral gray and white matter.

In the mature cerebellar gray matter, the two CK isoenzymes also are unevenly distributed suggesting a physiological heterogeneity of ATP regulation and coupling. In cerebellar gray matter, the B-CK is particularly prominent in Bergmann glial cells. The Mi-CK stains strongly in Purkinje cell processes in the granular layer as well as in glomeruli which are rich in mitochondria [20]. In these cells, the ratio of Mi-CK/B-CK may be particularly high, an interpretation applied to similar findings in chicken cerebellum [18]. The Mi-CK activity in the granular layer may be in either astrocytes or neurons.

The developmental immunolocalization results suggest that a cellular interpretation of in vivo CK studies in the developing brain must be complex. In cerebellum, Mi-CK redistributes from Purkinje cell bodies to processes coincident with the increase in cerebral CK catalyzed fluxes between days 12 and 25. The cellular factors effecting these changes in isoenzyme activities and their final distribution in the mature brain are unknown. Delineating these factors will be critical in understanding the roles of ATP regulation and, possibly, its physiological limits in determining the cellular physiology of energy requiring processes in developing mammalian brain.

The responsiveness of ATP synthesis to energy demand increases in rat cerebral cortical tissue over the same brief age period as the increase in CK catalyzed reaction rate (table 1). Between 12 and 17 days of age, large increases occur in the responses of cortical slice respiration to electrical stimulation, hyperthermia, or increased extracellular KCl [11, 12, 25]. Over this same age period, the fraction of respiration coupled to ATP synthesis and the efficiency of oxidative phosphorylation (ATP/O ratio) increase in cerebral cortical mitochondria [13, 26]. In a study of isolated cerebral hemisphere mitochondria in media of altered osmolarity, a subpopulation of mitochondria with contact sites between inner and outer membranes and resistance to separation of these membranes appears between 10 and 20 days of age [27, 28]. Approximately 50% of mature cerebral hemisphere mitochondria show these properties.

The results of this study and those summarized in table 1 are consistent with a compartmentalized model of energy metabolism in brain. This model assumes a physiologic role of Mi-CK in closely regulating mitochondrial ATP synthesis, a proposal based on the colocalization of Mi-CK with the ATP-ADP translocase [5]. The recently completed maturational study of hypoxia and brain ATP regulation has shown that PCr disappears while ATP shows a small but consistent decrease of 20% in the very immature brain [unpubl. results]. At ages in which the brain CK catalyzed reaction rate has increased, 50% of cerebral PCr is lost while ATP decreases 20%. The remaining PCr and ATP then disappear simultaneously, a pattern of energy loss not described in other tissues. If Mi-CK is necessary for the maturational appearance of the coincident loss of ATP and PCr during hypoxia, the cellular and regional distribution of Mi-CK may be a marker for this pattern of energy loss and for those regional, cellular and subcellular structures which possess the physiological capacity for close coupling of ATP synthesis and demand in the mature brain. Understand-

Table 1. Maturational changes in rat cerebral cortical mitochondrial and tissue properties which are temporally and, hypothetically, functionally related to increased in vivo creatine kinase activity (reference numbers refer to the references in this paper)

Metabolic changes	Preparation
Increased Mi-CK (this study)	Whole brain homogenates
Mitochondrial intermembrane contact sites [27, 28] Increased mitochondrial respiratory control [26] Increased mitochondrial ADP/O ratios [13]	Isolated cerebral hemisphere mitochondria
Increased stimulated aerobic glycolysis [11, 12] Increased stimulated lactate production [11] Increased Q_{10} for hyperthermia stimulated tissue respiration [25]	Cerebral cortical slices
Simultaneous loss of brain PCr and ATP with hyposia [Tsuji et al., unpubl. results]	In vivo brain NMR spectroscopy (not localized)

ing the complexity of ATP regulation in the developing and mature brain, by further study of this model, will be important for understanding the physiology of cellular energy metabolism and the regional and cellular pathophysiology of sequellae to common clinical conditions including hypoxia, hypoglycemia, and ischemia affecting children and adults.

Acknowledgments

The NMR studies were supported by grants to DH from the Cerebral Palsy Foundation (R-4010) and from the National Institutes of Health (NS26371). M.T. was supported partially by a National Institutes of Health Training Grant (NS07264). These experiments were performed at the High Field NMR Center for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA, which is supported by a National Institutes of Health grant (RR00995) to Dr. Leo Neuringer. Graphics were supported partially by a Mental Retardation Research Center Grant (HD18655). We are grateful to Dr. Frances Jensen and Mr. Pieter Dikkes for perfusions of the rat brains. We are very grateful to Drs. Leo Neuringer (now deceased) and Eric McFarland for supportive, critical discussions during the course of this work and to Ms. Regena Bradeen for assistance in preparing this manuscript.

For the in vitro studies, we gratefully acknowledge the technical assistance of Else Zanolla. We also thank Prof. Hans M. Eppenberger, Institute for Cell Biology, ETH-Zürich, for continuous interest in and support for our work. This work was supported by SNF grants No. 3.376-0.86 and No. 31-26384.89 (to T.W.), by an ETH-graduate training grant (W.H.), and financial support by the Swiss Society for Muscle Diseases (to T.W.), as well as the H. Horten Foundation, Lugano, Switzerland.

References

1 Siesjo BK: Brain Energy Matabolism. Chichester, Wiley, 1978.
2 Prichard JW, Alger JR, Behar KL, Petroff OAC, Shulman RG: Cerebral metabolic studies in-vivo by ^{31}P NMR. Proc Natl Acd Sci USA 1983;80:2746-2751.
3 Petroff OAC, Prichard JW, Behar KL, Alger JR, Shulman RG: In vivo phosphorus nuclear magnetic resonance spectroscopy in status epilepticus. Ann Neurol 1984;16:169-177.
4 Jacobus WE, Lehninger AL: Creatine kinase of rat heart mitochondria. J Biol Chem 1973; 248:4803-4810.
5 Wyss M, Smeitink J, Wevers R, Wallimann T: Mitochondrial creatine kinase: A key enzyme of aerobic energy metabolism. Biochim Biophys Acta 1992;1102:119-166.
6 Sauter A, Rudin M: Determination of creatine kinase kinetic parameters in rat brain by NMR magnetization transfer. J Biol Chem 1993;268:13166-13171.
7 Holtzman D, Offutt M, Tsuji M, Neuringer LJ, Jacobs D: Creatine kinase reaction rates in the cyanide-poisoned mouse brain. J Cereb Blood Flow Metab 1993;13:153-161.
8 Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM: Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular homeostasis. Biochem J 1992;281:21-40.
9 Holtzman D, McFarland EW, Jacobs D, Offutt MC, Neuringer LJ: Maturational increase in mouse brain reaction rates shown by phosphorus magnetic resonance. Dev Brain Res 1991;58:181-188.
10 Jensen F, Tsuji M, Offutt M, Firkusny I, Holtzman D: Profound, reversible energy loss in the hypoxic rat brain. Dev Brain Res 1993;73:99-105.
11 Greengard P, McIlwain H: Metabolic response to electric pulses in mammalian cerebral tissues during development; in Waelsch H (ed): Biochemistry of the Developing Nervous System. New York, Academic Press, 1955, pp 251-260.

12 Holtzman D, Olson J, Zamvil S, Nguyen H: Maturation of potassium-stimulated respiration in rat cerebral cortical slices. J Neurochem 1982;39:274–276.
13 Holtzman D, Moore CL: Oxidative phosphorylation in immature rat brain mitochondria. Biol Neonate 1973;22:230–242.
14 Wallimann T, Schloesser T, Eppenberger HM: Function of M-line bound creatine kinase as an intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle. J Biol Chem 1984;259: 5238–5246.
15 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
16 Ishida Y, Wyss M, Hemmer W, Wallimann T: Identification of creatine kinase isoenzymes in the guinea-pig: presence of mitochondrial creatine kinase in smooth muscle. FEBS Lett 1991;283:37–43.
17 Wegmann G, Huber R, Zanolla E, Eppenberger HM, Wallimann T: Differential expression and localization of brain-type mitochondrial creatine kinase isoenzymes during development of the chicken retina: Mi-CK as a marker for differentiation of photoreceptor cells. Differentiation 1991;46:77–87.
18 Hemmer W, Zanolla E, Forter-Graves E, Eppenberger HM, Wallimann T: Creatine kinase isoenzymes in chicken cerebellum: Specific localization of brain-type CK in Bergmann glial cells and muscle-type CK in Purkinje cells. Eur J Neurosci, in press.
19 Hemmer W, Reisinger I, Wallimann T, Eppenberger HM, Quest AFG: Brain-type creatine kinase in photoreceptor cell outer segments: role of a phosphocreatine circuit in ROS energy metabolism and phototransduction. J Cell Sci, in press.
20 Palay SL, Chan-Palay V: Cerebellar Cortex: Cytology and Organization. New York, Springer, 1974.
21 Norwood WI, Ingwall JS, Norwood CR, Fossel ET: Developmental changes of creatine kinase in rat brain. Am J Physiol 1983;244: C205–C210.
22 Booth RFG, Clark JB: Studies on the mitochondrial bound form of rat brain creatine kinase. Biochem J 1978;170:145–151.
23 Manos P, Bryan GK, Edmond J: Creatine kinase activity in postnatal rat brain development and in cultured neurons, astrocytes, and oligodendrocytes. J Neurochem 1991;56: 2101–2107.
24 Holtzman D, McFarland E, Moerland T, Koutcher J, Kushmerick MJ, Neuringer LJ: Brain creatine phosphate and creatine kinase in mice fed an analogue of creatine. Brain Res 1989;483:68–77.
25 Holtzman D, Nguyen H, Zamvil S, Olson J: In-vitro cellular respiration at elevated temperatures in developing rat cerebral cortex. Dev Brain Res 1982;4:401–405.
26 Holtzman D, Magruder CS: Effects of oligomycin on respiration in developing rat brain mitochondria. Brain Res 1977,120: 373–378.
27 Holtzman D, Lewiston N, Herman MM, Desautel M, Brewer E, Robin E: Effects of osmolar changes in isolated brain versus liver mitochondria. J Neurochem 1978;30:1409–1419.
28 Holtzman D, Herman MM, Desautel M, Lewiston N: Effects of altered osmolarity on respiration and morphology of mitochondria from the developing brain. J Neurochem 1979;33:453–460.