Expression of creatine kinase isoenzyme genes during postnatal development of rat brain cerebellum: evidence for transcriptional regulation

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Transcription and accumulation of brain-type creatine kinase (CKB) mRNA and its protein was examined during postnatal development of rat brain cerebellum, the brain region containing highest CKB mRNA in the adult. CKB protein was extremely low at day 1, increased about 10-fold until week 4 and remained constant until week 10. This time course was paralleled by cerebellar CKB mRNA, which was also extremely low at day 1 and increased 5-fold during the first 3 weeks and then remained constant. High levels of CKB protein were also detected in cultured primary cerebellar granular neurons. Nuclear run-on assays directly showed that CKB mRNA accumulation during postnatal cerebellar development was due to increased transcription. When compared with cerebrum and whole brain, cerebellar CKB mRNA accumulation during postnatal development was temporally delayed. Analysis of myocyte enhancer factor (MEF)-2 and Sp1, factors known to initiate or sustain CKB transcription in tissues other than brain, revealed that MEF-2 in cerebellum was low at week 1 but increased 3.5-fold by week 7, while Sp1 remained unchanged. The increase in CKB protein during cerebellar postnatal development was coincident with that of the ubiquitous mitochondrial CK protein and mRNA, indicating that a functional phosphocreatine energy shuttle probably exists for efficient ATP regeneration in the cerebellum. This should be beneficial for the many energy-demanding requirements during cerebellar development, as indicated by the observed temporal co-expression of CKB with myelin basic protein, which is involved in axon myelination by oligodendrocytes.

Key words: brain creatine kinase, energy metabolism, glial cell, myelin basic protein, transcription.

INTRODUCTION

The creatine kinase (CK) isoenzymes catalyse the synthesis of phosphocreatine (PCr) and its subsequent use in the regeneration of ATP in cell types where the consumption of ATP is rapid and/or sudden. Previous reports have suggested that in the brain the different CK isoforms constitute an energy shuttle wherein ATP produced in the mitochondria is used by a mitochondrial CK [e.g. ubiquitous mitochondrial creatine kinase (uMi-CK) in brain] to generate PCr, which is then transported and used by a cytoplasmic CK [e.g. brain creatine kinase (CKB) in brain] to regenerate ATP at discrete cellular sites of high ATP turnover [1,2]. CKB is expressed in a number of tissues and is most abundant in adult brain, approx. 5-fold lower in the stomach, 10-fold lower in the heart and barely detectable in liver [3,4]. In brain, whereas most CKB has been shown to be cytosolic [5], several of the reactions requiring CKB are membrane-associated. CKB appears to have a role in regenerating ATP needed for the transport of ions and neurotransmitters since CKB has been localized to brain synaptic plasma membranes, possibly coupled to Na^+/K^+-ATPase [6] and acetylcholine receptor-rich membranes [7]. In primary cultures of rat cerebral astrocytes, a fraction of CKB was found at the plasma membrane in a complex with the cytoplasmic domain of the thrombin receptor (PAR1) [8]. CKB activity was found to be essential in regenerating the ATP needed during the morphological change of stellate astrocytes that is triggered by thrombin activation of PAR1 [8]. In agreement, our studies of primary cell cultures from rat brain have shown that CKB mRNA levels in neonatal cerebral astrocytes and oligodendrocytes were much higher (15–17-fold) than in embryonic neurons [9]. This is probably related to the large energy requirements for the transport of ions [10] and glutamate by astrocytes [11–14] and for myelin formation in oligodendrocytes involving myelin basic protein (MBP) [12,15]. In adult rat brain, significant amounts of CKB protein are also expressed in some neurons, principally localized in the neuronal processes and enriched in the synaptic layers of the cerebellum and hippocampus and in the process of cortical pyramidal cells [1,16]. This suggests that as some neurons mature, expression of CKB increases.

We have recently shown in adult rats that the cerebellum contains the highest level of CKB mRNA compared with the 16 other brain regions analysed [17]. However, during postnatal development, little is presently known of (i) the timing of CKB mRNA and protein expression in different brain regions or (ii) the mechanisms which regulate CKB transcription during development and differentiation of glial and neuronal cells (e.g. the CKB promoter elements and their cognate factors). In rat, human and chicken, the CKB proximal promoter contains two CCAAT boxes (at -80 bp and -50 bp), a TA-rich element (TATAAATA; at -60 bp) and a non-consensus TATA box (TTAA; at -28 bp) [4]. Both CCAATs and the (-60) TA-rich element bind *trans*-acting (protein) factors that activate CKB transcription which is initiated at the downstream start site ($+1$), under direction from the (-28) TTAA [18,19]. The CKB (-60) TA-rich element binds the TA-rich DNA-binding protein (TARP) [18], which is possibly similar to the MADS box proteins myocyte enhancer factor (MEF)-2A and -2D that are expressed

Abbreviations used: CGN, cerebellar granular neuron; CK, creatine kinase; CKB, brain creatine kinase; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde phosphate dehydrogenase; mAb, monoclonal antibody; MBP, myelin basic protein; MEF, myocyte enhancer factor; PCr, phosphocreatine; RPA, RNase protection assay; SCG 10, superior cervical ganglion gene 10; uMi-CK, ubiquitous mitochondrial creatine kinase; TARP, TA-rich DNA-binding protein.

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in the brain [20,21]. Another factor, Sp1, was shown to bind two 5′ upstream Sp1 elements located between −568 bp and −523 bp in CKB [22]. While it is not established if these transcription factors regulate the CKB promoter in the brain, such knowledge would be of basic and practical importance. Elevating the production of PCr by CK, via creatine administration, has recently been shown to act as a neuroprotective agent for neuronal cells by preventing ATP depletion [23] and to greatly diminish neuronal degeneration in animal models of amyotrophic lateral sclerosis [24], Huntington's disease [25] and Parkinson's disease [26].

In this report, we investigated mRNA and protein levels of both CK isoenzymes expressed in brain, CKB and uMi-CK, during cerebellar postnatal development, as well as some of the transcription factors which may be involved. Both isoenzymes showed low abundance of mRNA and protein during the early postnatal period, which dramatically increased thereafter and reached a stable plateau value by postnatal week 4. The increase in cerebellar CKB appears to be principally regulated by transcription and was coincident with expression of MBP.

EXPERIMENTAL

Isolation of brain tissue and cytosolic fractions

Adult male and female Long–Evans breeder rats were obtained from Harlan Sprague Dawley and maintained *ad libitum* on pellet rat food and tap water as described in [17]. Rat pups of both sexes were killed by decapitation. For most series of experiments, cerebella and cerebra were removed from postnatal day 1 to postnatal week 10. For another series, whole brain (including the brain stem), cerebellum and cerebrum from littermates at weeks 1 and 8 were used. Tissue was rinsed in isotonic PBS (137 mM NaCl, 2.7 mM KCl, 9.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and 1 mM $MgCl_2$) and the meninges were carefully removed [27]. Cerebella and cerebra were weighed separately and homogenized in a Dounce homogenizer (10 strokes with a loose pestle followed by 15 strokes with a tight pestle) in 4 vol. of lysis buffer [100 mM Tris/HCl (pH 7.5), 0.25 M sucrose, 0.5 mM dithiothreitol and 0.2 mM PMSF]. Usually one-third of each cerebellar and cerebral whole cell lysate was used to isolate total cellular RNA, as described below. The remaining lysate was either (i) frozen in liquid N_2 and stored at −80 °C for preparation of whole cell lysates analysed in immunoblots for CKB and uMi-CK protein or (ii) used immediately to prepare a postmicrosomal (cytosolic) protein fraction by centrifugation at 116000 ***g*** as described in [5]. The cytosol as well as whole cell lysates were used to quantify CKB immunoreactive protein by immunoblots as indicated in the Figure legends.

Culturing primary astrocytes, oligodendrocytes and cerebellar granular neurons (CGNs)

Single-cell cultures of mixed glial cells prepared from postnatal day 2 cerebral hemispheres [9] were cultured for 5 days in Dulbecco's modified essential medium containing 10 % fetal calf serum plus biotin (10 ng/ml), insulin (50 μg/ml), tri-iodo-L-thyronine (15 nM) and SeO_2 (30 nM). Oligodendrocytes were separated from astrocytes by the shake-off procedure of Cole and de Vellis [28] and cells were harvested for preparation of total RNA and protein. Microscopy and cell staining indicated that > 90 % of the released cells were oligodendrocytes and > 90 % of the attached cells were astrocytes, determined as described previously [9]. Primary single-cell cultures of CGNs were prepared from postnatal day 8 cerebella as described by Levi et al. [29] and cultured in poly-L-lysine-coated (5 μg/ml) dishes (10 cm) in basal Eagle's medium with Earle's salts, free of glutamine (Gibco) and supplemented with gentamicin (100 μg/ml), KCl (25 mM), L-glutamine (2 mM) and 10 % fetal calf serum at 37 °C in 10 % CO_2/90 % air. After 24 h, cytosine arabinoside (10 μM) was added to the medium which was renewed every 2 days. When harvested after 5 days in culture, more than 95 % of the cells were CGNs [30].

Immunoblot analysis

Protein samples (10–30 μg as indicated in the Figure legends) were denatured and separated by SDS/PAGE (4.5 % polyacrylamide stacking/10 % polyacrylamide resolving), as described by Wilson et al. [31]. Proteins were electrophoretically transferred to a nitrocellulose membrane (Amersham Biosciences), which was incubated at 4 °C in blocking solution [PBS containing 5 % (w/v) non-fat milk and 0.3 % (v/v) Tween 20] for 8–12 h. CKB-specific monoclonal antibody (mAb) CK-BYK/21E10 (diluted 1:5000 in blocking buffer), which recognizes residues 4–20 at the N-terminus of CKB [32], was incubated with the membrane overnight at 4 °C. In separate experiments, a rabbit polyclonal CKB-specific antibody (diluted 1:5000 in blocking buffer), raised against amino acid residues 258–270 of CKB [1], was used. After washing, secondary peroxidase-conjugated anti-mouse IgG (or anti-rabbit IgG for the polyclonal antibody; Sigma) was incubated with the membrane for 1 h at 22 °C and the membrane was washed three times with PBS plus 0.3 % Tween 20 and twice with PBS. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) method according to manufacturer's instructions (Amersham Biosciences) and the intensity of fluorescence was directly quantified with a ChemiImager TM 4000 (Alpha Innotech Corporation). To control for equal sample loading, 5 μg of cerebellar cytosolic protein at each postnatal time point was electrophoresed as above, stained with silver [33] and quantified by densitometric scanning of all protein bands. Alternatively, membranes were probed with a rabbit polyclonal antibody to actin (Sigma A2066) as described in Figure legends.

In immunoblot analyses of uMi-CK, 15 μg of total cerebellar lysate was used since uMi-CK is present in the mitochondrial fraction; membranes were probed with a rabbit polyclonal antibody specific for uMi-CK as shown by Schlattner et al. [34]. In analyses of MEF-2 proteins, positive controls for recombinant MEF-2A and MEF-2C were generated using a coupled transcription/translation reticulocyte lysate system (Promega) with plasmids pCGNC4 or pCMVMEF-2C, respectively, as described previously [35]. HeLa cell nuclear extracts served as a source for MEF-2D [35]. In immunoblots of MBP, 30 μg of total cerebellar lysate was used since most of the MBP is membrane-bound [15]. A primary rabbit polyclonal anti-MBP antibody (diluted 1:1000 in blocking buffer) was used as above followed with a secondary peroxidase-conjugated anti-rabbit IgG and visualized by enhanced chemiluminescence.

Isolation and quantification of total cellular RNA

Total cellular RNA was isolated from brain region lysates using the acid guanidinium thiocyanate/phenol/chloroform procedure [33] and stored in sterile water at −80 °C. RNA was quantified by absorbance at 260 nm and its integrity checked by electrophoresis in a 1 % agarose gel after RNA denaturation in formaldehyde/formamide [31]. Before measuring levels of CKB mRNA by the RNase protection assay (RPA), the levels of 18 S rRNA were determined by RPA to confirm RNA concentrations.

Riboprobe DNA templates

The riboprobe plasmid containing the genomic rat CKB exon 7, intron 7 and exon 8 DNA has been described elsewhere [36]. After digestion at the *Pml*I site in exon 7, T7 RNA polymerase generated a 1140 nt full-length probe of which 377 nt were protected by CKB exon 8 RNA and 122 nt were protected by exon 7 RNA [36]. In some assays, the same plasmid was digested at the *Mvn*I site in intron 7 to generate a CKB antisense riboprobe which protected the 377 nt in CKB exon 8 RNA. The riboprobe template for 18 S rRNA was purchased from Ambion; T7 RNA polymerase generated a 116 nt full-length probe of which 80 nt were protected by 18 S rRNA. Plasmid containing rat MBP exon 1 cDNA [37] was obtained from Dr F. Art McMorris; after digestion with *Eco*RI, SP6 RNA polymerase generated a 201 nt full-length probe of which 160 nt were protected by MBP exon 1 RNA.

Synthesis of antisense riboprobes and RPAs

Synthesis of antisense [^{32}P]riboprobes for CKB, uMi-CK and MBP and the RPAs were performed as described in [9,36]. For the 18 S rRNA riboprobe, the unlabelled GTP was increased to 80 μM to decrease probe-specific activity and help ensure probe saturation. Unless stated otherwise in Figure legends, hybridizations contained 4 μg of total cerebellar RNA and 4×10^5 c.p.m. of antisense probe for either CKB or MBP mRNA in 30 μl of 1 × hybridization buffer [80 % formamide, 0.4 M NaCl, 40 mM Pipes (pH 6.4) and 1 mM EDTA]. Separate reactions contained 5 ng of total cellular RNA and 4×10^5 c.p.m. of antisense probe for 18 S rRNA or 10 μg of total RNA and 4×10^5 c.p.m. of probe for uMi-CK mRNA. The level of RNase-resistant [^{32}P]probe was analysed on a 6 % polyacrylamide/8 M urea gel, autoradiographed and bands were quantified by scintillation counting or with a PhosphorImager-SI (Molecular Dynamics).

To be certain that RPAs were conducted in probe excess, titration experiments were performed. For CKB mRNA, 1–16 μg of the rat cerebellar RNA were hybridized with 4×10^5 c.p.m. of CKB exons 7,8 probe, resulting in a linear increase in protected CKB probe with increasing RNA input ($R^2 > 0.99$). For 18 S rRNA, 0.5–200 ng of cerebellar RNA were hybridized with 4×10^5 c.p.m. of 18 S probe and the amount of protected 18 S probe increased linearly with the input RNA ($R^2 > 0.99$).

Nuclear run-on assay

Rat cerebellar nuclei were prepared as described in [4], resuspended in 200 μl of storage buffer [50 mM Tris/HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM dithiothreitol and 40 % (v/v) glycerol] and stored in liquid N_2. The number of nuclei was determined both by counting in a haemocytometer and determining, in an aliquot of nuclei, the amount of DNA present using the fluorometric assay of Barres et al. [38] with Hoechst dye 33258 and dividing by 6.6 pg of DNA/rat cell [39]. To isolate 1×10^8 nuclei from cerebella at day 1 required 50 brains (5 litters) and at week 6 required 2–3 brains. Nuclear run-on assays were performed with 1×10^8 nuclei as described by Mukherjee and Molloy [40]. Typically, $(20–30) \times 10^6$ c.p.m. of RNA were obtained from nuclei at day 1 and week 6 and the total yield of [^{32}P]RNA from day 1 and week 6 was used for hybridization to a nylon membrane (Amersham Biosciences) containing 10 μg of cDNA to either CKB, glyceraldehyde phosphate dehydrogenase (GAPDH), actin, MBP or superior cervical ganglion gene 10 (SCG 10) and an 80 bp fragment of 18 S rRNA as described in [40]. The relative intensity of the hybridized bands was quantified by the PhosphorImager-SI. The level of [^{32}P]CKB transcripts at day 1 was compared directly to that at week 6 without normalizing to transcripts from the other cDNAs, in the event that the latter might vary during postnatal development.

Preparation of nuclear extracts and electrophoretic mobility-shift assays (EMSAs)

Nuclei from cerebella and cerebra were isolated [4] from which nuclear extracts were prepared according to Zhao et al. [41]. EMSAs were carried out by preincubating 15 μg of nuclear extract with 1.0 μg of poly(dI-dC) in binding buffer [8.5 mM Hepes (pH 7.9), 30 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dithiothreitol, 0.3 mM PMSF and 4 % Ficoll 400] and, where indicated, a 100-fold molar excess of unlabelled Sp1 probe for 10 min at 4 °C. ^{32}P-Labelled Sp1 probe (5×10^4 c.p.m. and 2.5 ng; 5′-TCGATCGGGGCGGGGCGAG-3′; Santa Cruz Biotechnology catalogue no. sc 2502) was added and incubation continued for 30 min at 22 °C in a final volume of 20 μl. The reaction was loaded on to a 5 % polyacrylamide gel (40:1, acrylamide/bis-acrylamide) in 0.5 × Tris/borate/EDTA buffer [45 mM Tris/borate (pH 8.0) and 1 mM EDTA], electrophoresed at 200 V for 4 h, dried and exposed to a PhosphorImager screen.

Data analysis

Each postnatal experiment was repeated at least three times, each time with cerebella and cerebra isolated at postnatal day 1 to week 10 from rats of the same litter. All results are expressed as means ± S.E.M. Data were analysed using STATMOST software initially for ANOVA and, where appropriate, followed by Student–Newman–Keul analysis. Differences were set to be significant only for $P < 0.05$.

RESULTS

Expression of CKB protein increases in rat cerebellum during postnatal development

Cerebella were isolated at postnatal day 1 and at weekly intervals thereafter during the first 10 weeks of development. The cytosolic protein fraction was analysed by immunoblot analysis (Figure 1A) with a mAb shown to specifically recognize the N-terminus of CKB [32]. Quantification of the CKB protein (Figure 1B) showed extremely low levels at day 1 that increased during weeks 1–3 and attained a maximal value at week 4, which remained constant until week 10 (ANOVA; $P < 0.05$). Further analyses showed that CKB protein levels at week 4 (and thereafter) were 10-fold higher than at day 1 (Student–Newman–Keul's test; $P < 0.01$). CKB levels were expressed per μg of protein analysed. To ensure equal loading, the protein profile was obtained by silver staining the gel (Figure 1C); densitometric scanning showed the summation of absorbance of all protein bands did not vary by more than 10 % between samples. A similar pattern of CKB protein expression during cerebellar development was observed in immunoblots using a polyclonal antibody raised against amino acid residues 258–270 in CKB [1] (results not shown). This essentially eliminated the possibility that CKB protein modifications diminished reactivity of the mAb.

CKB mRNA accumulates in rat cerebellum during postnatal development

From the same cerebella analysed by immunoblots, total cellular RNA was isolated and CKB mRNA was analysed by RPA using the rat CKB exon 7,8 probe (Figure 2A). This probe protects all

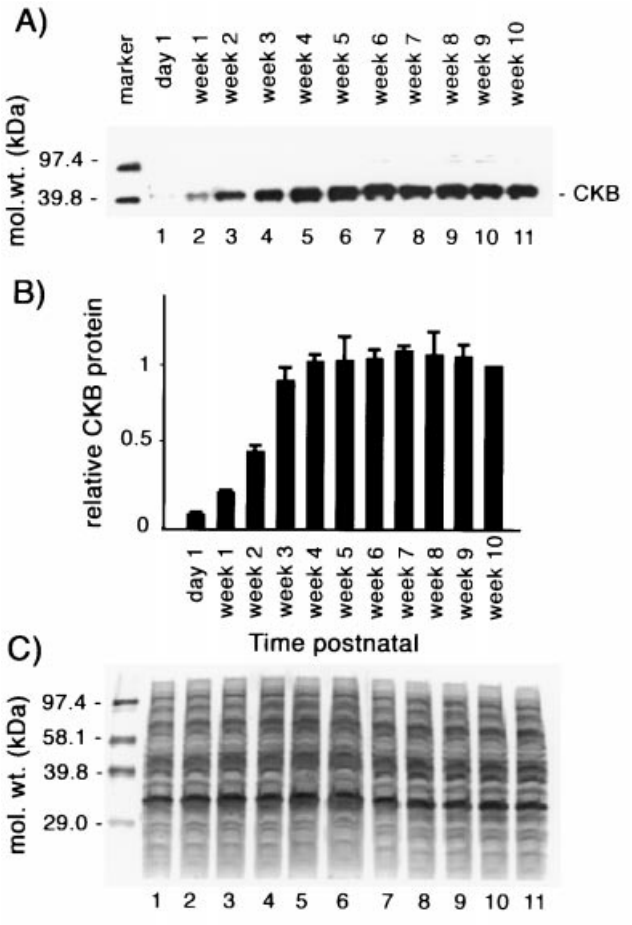


Figure 1 CKB immunoreactive protein levels in rat cerebella during postnatal development

(**A**) Immunoblot analysis for CKB protein during postnatal cerebellar development. Cytosolic protein (10 μg) were electrophoresed in each lane and analysed for CKB protein with an anti-CKB mouse mAb recognizing amino acid residues 4–20 in CKB. Lanes 1–11 are samples from day 1 to week 10. The position of the 47 kDa CKB band is indicated. (**B**) Histogram showing CKB protein levels during cerebellar postnatal development; all values were normalized to that at week 10 (which was set at 1) and are expressed as the mean $\pm$ S.E.M. ($n = 3$). (**C**) Cytosolic protein (5 μg) was electrophoresed and stained with silver. Lanes correspond to lanes 1–11 in (**A**).

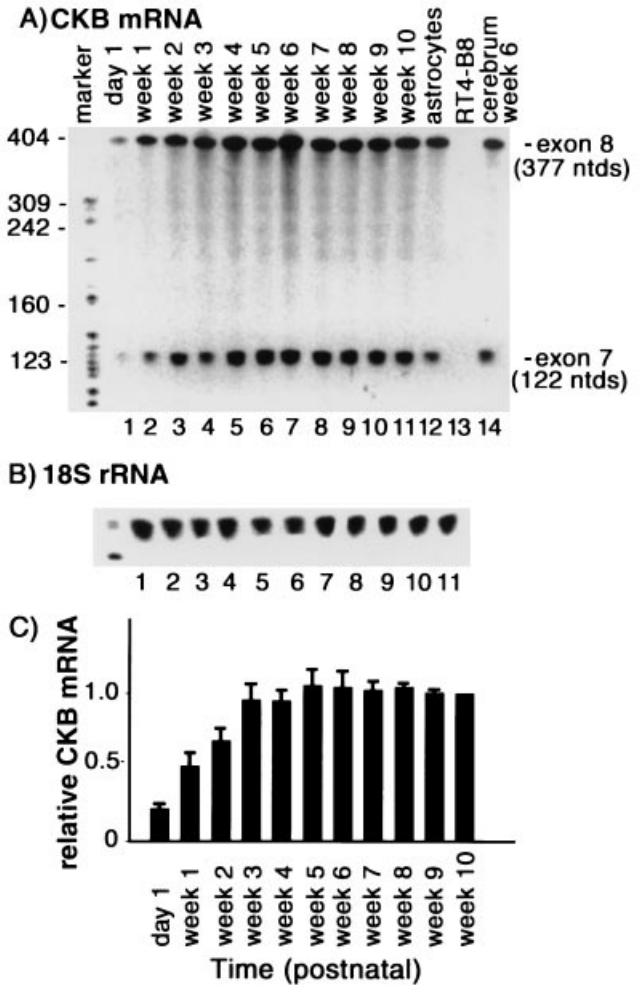


Figure 2 CKB mRNA levels in rat cerebellum during postnatal development

(**A**) Total cerebellar RNA (4 μg) was hybridized with the [^{32}P]CKB exon 7,8 antisense riboprobe in the RPA. Lanes 1–11 are samples from day 1 to week 10. Lanes 12 and 14 are positive controls from primary astrocytes and week 6 cerebrum, respectively. Lane 13 is a negative control RNA sample from RT4-B8 rat neuroblastoma. The marker lane is the molecular-mass marker pBR322/MspI. The positions of the RNase-protected CKB exon 8 (377 nt) and the exon 7 (122 nt) are indicated. (**B**) The level of 18 S rRNA was measured by RPA in a 5 ng aliquot of the cerebellar RNAs; lanes 1–11 correspond to lanes 1–11 in (**A**). (**C**) The level of CKB mRNA in each sample was adjusted for the amount of 18 S rRNA present, then normalized to the CKB mRNA level at week 10 (which was set at 1) and was plotted as the mean $\pm$ S.E.M. as determined from three separate litters.

377 nt of exon 8 and 122 nt of exon 7 in the completely spliced CKB mRNA [36]. The level of CKB mRNA at each time was divided by the amount of 18 S rRNA, which was quantified in the same sample (Figure 2B), since recent reports indicate that rRNA varies less than other internal RNA controls tested (e.g. GAPDH, actin) [42]. Cerebellar CKB mRNA was extremely low at day 1, increased from week 1 to week 3 and reached a plateau level which remained constant from week 3 to week 10 (Figure 2C; ANOVA; $P < 0.01$). Further analyses showed CKB mRNA levels at week 3 (and thereafter) were 5-fold higher than at day 1 (Student–Neuman–Keul's test; $P < 0.01$). The maximal levels of CKB mRNA in the postnatal cerebellum (i.e. at week 3 and thereafter) were twice as high as in cerebrum at week 6 (Figure 2A, lane 14). This agrees with our previous quantification of CKB mRNA in 17 different brain regions of the adult rat [17]. CKB mRNA in cultured primary cerebral astrocytes (Figure 2A, lane 12) had similar levels as cerebrum at week 6. As a negative control, CKB mRNA was essentially undetectable in cultured rat RT4-B8 neuronal cells (Figure 2A, lane 13), as we have previously shown [31].

Quantification with the RPA is reliable

Because the cerebellum contains the highest level of CKB mRNA of any region in the adult rat brain [17], we verified that all samples were measured within the linear range and below the saturation level of the RPA. Figures 3(A) and 3(B) show that 4×10^5 c.p.m. of CKB exon 7,8 probe were sufficient to give a linear response for quantification of CKB mRNA with up to 16 μg of total cellular RNA (from week 6 cerebellum), while we routinely assay only 4 μg of RNA. Similarly, 4×10^5 c.p.m. of antisense 18 S rRNA probe (adjusted to a 3-fold lower specific activity) were sufficient to give a linear response for quantification

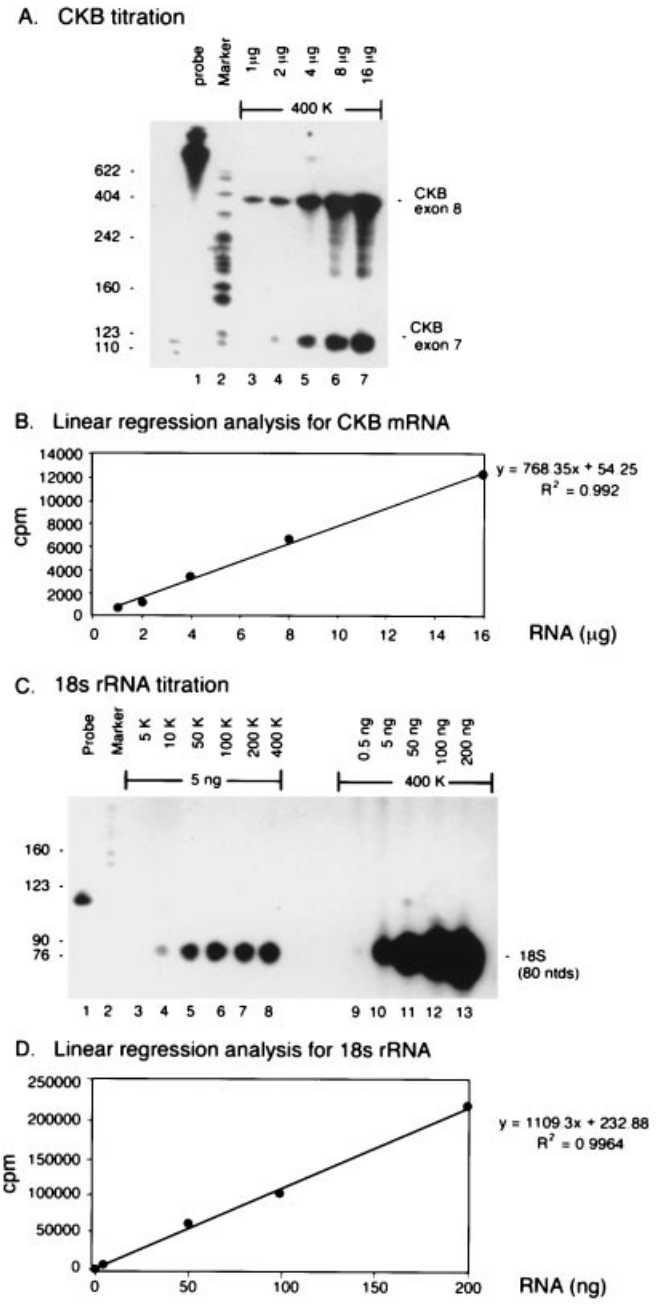


Figure 3 Antisense probe saturation for CKB mRNA and 18 S rRNA

(**A**) RPA of CKB mRNA. Lanes 1 and 2 are the full-length CKB probe and the pBR322/MspI marker, respectively. Lanes 3–7 show the hybridization of 1, 2, 4, 8 and 16 μg, respectively, of week 5 cerebellar total cellular RNA with 4×10^5 c.p.m. of [^{32}P]CKB exon 7,8 antisense riboprobe. (**B**) The RNase-protected CKB exon 8 bands (377 nt) were excised and counted by scintillation and analysed by linear regression using the Microsoft Excel program. (**C**) RPA of 18 S rRNA. Lanes 1 and 2 are the full-length probe for 18 S and the pBR322/MspI marker, respectively. In lanes 3–8, RNA (5 ng) from week 5 cerebellum was hybridized with 5×10^3, 1×10^4, 5×10^4, 1×10^5, 2×10^5 and 4×10^5 c.p.m. of probe, respectively. In lanes 9–13, 4×10^5 c.p.m. of 18 S probe were hybridized with 0.5, 5, 50, 100 and 200 ng of RNA, respectively. (**D**) The protected 18 S rRNA bands (80 nt) were excised and counted by scintillation and analysed by linear regression with Microsoft Excel.

of 18 S rRNA present in as much as 200 ng of total cellular RNA (Figure 3C, lanes 9–13, and Figure 3D), while we routinely assayed only 5 ng of RNA.

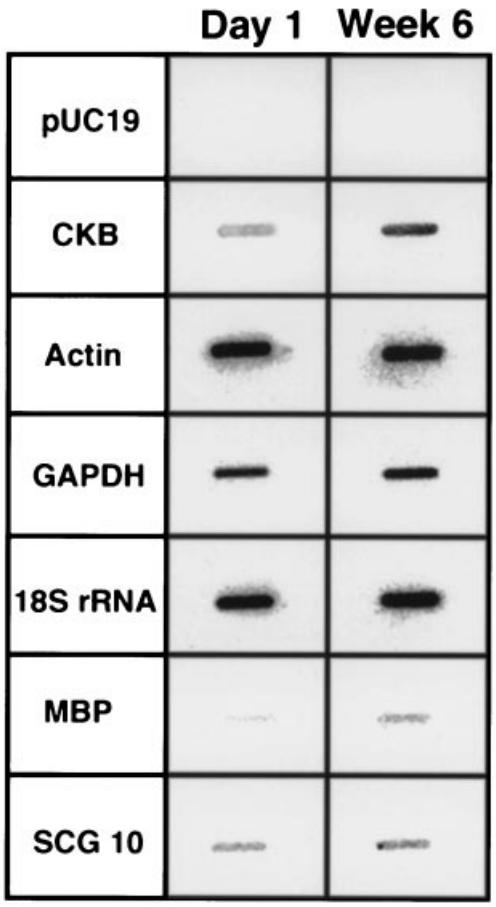


Figure 4 Nuclear run-on assay of CKB transcription from cerebellar nuclei isolated at either postnatal day 1 or week 6

A total of 1×10^8 cerebellar nuclei isolated at day 1 or week 6 were used. Column 1: the positions of the six different cDNAs and vector pUC 19 bound to the filters are indicated. Typically, (20–30) $\times 10^6$ c.p.m. of total nascent [^{32}P]RNA was generated by the day 1 and week 6 nuclei, all of which was hybridized to the filter. Column 2: nascent ^{32}P-labelled specific RNAs transcribed in nuclei isolated from day 1 cerebella. Column 3: nascent [^{32}P]RNAs transcribed in nuclei isolated from week 6 cerebella.

CKB mRNA accumulation in rat cerebellar nuclei is due to transcriptional regulation

To determine whether the increase in CKB mRNA during postnatal cerebellar development is due to elevated transcription, a nuclear run-on assay was used to directly measure the transcription of nascent CKB mRNA. An equal number (1×10^8) of nuclei were isolated from rat cerebella at day 1 and week 6 and the incorporation of [^{32}P-α]GTP into nascent CKB mRNA transcripts was subsequently determined by filter hybridization. Transcription of nascent CKB mRNA in day 1 cerebella (Figure 4, column 2) was much lower than in week 6 cerebella (Figure 4, column 3). As internal controls, nascent transcripts from the 'housekeeping' genes of actin and GAPDH as well as 18 S rRNA were present at essentially equal levels in day 1 and week 6 cerebella. It should be noted that while the signal of hybridized 18 S rRNA might appear lower than expected, this was because the cDNA probe contained only 80 bp of 18 S rDNA. The empty cloning vector pUC19 served as a negative control and showed that non-specific hybridization to the filter was extremely low. Furthermore, we measured the nascent transcripts of the

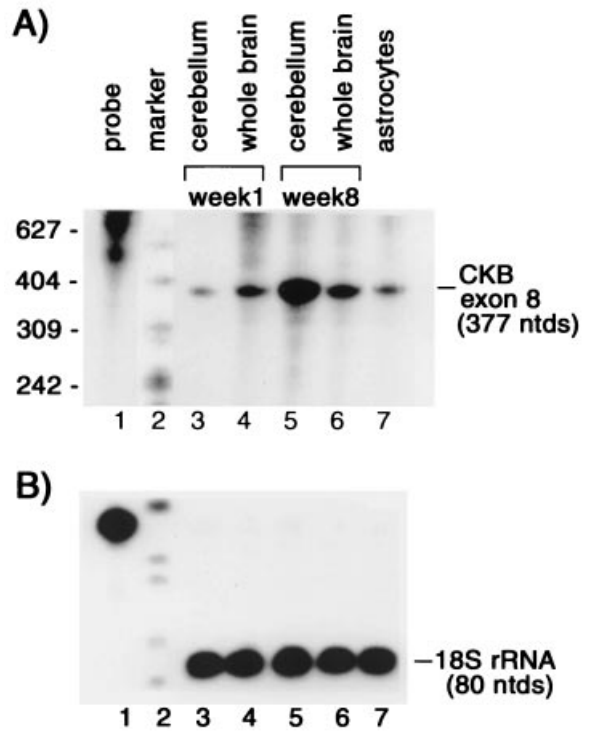


Figure 5 Comparison of CKB mRNA levels in the cerebellum relative to CKB in whole brain at postnatal week 1 and week 8

(**A**) Lanes 1 and 2 are the full-length CKB exon 8 probe and the pBR322/MspI marker, respectively. All samples contained 3 μg of total cellular RNA hybridized to 4×10^5 c.p.m. of CKB exon 8 probe. Lanes 3 and 4 contain RNA from cerebellum or whole brain, respectively, isolated at week 1. Lanes 5 and 6 contain RNA from cerebellum or whole brain, respectively, isolated at week 8. Lane 7 contains RNA from cultured primary astrocytes. The position of the protected CKB exon 8 band is indicated. (**B**) The level of 18 S rRNA was measured by RPA in a 5 ng aliquot of these RNAs; lanes 3–7 correspond to the same lanes in (**A**).

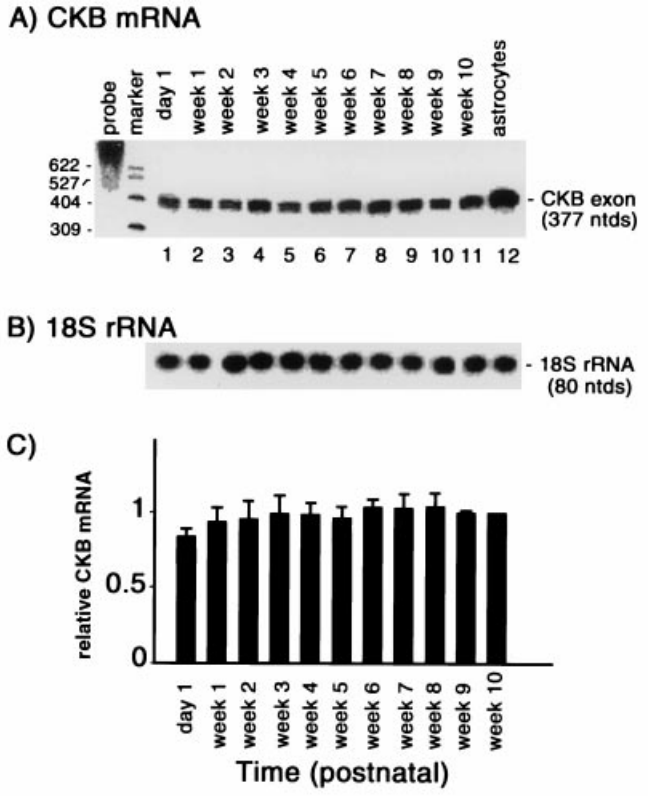


Figure 6 CKB mRNA levels in rat cerebra during postnatal development

(**A**) Total cerebral RNA (3 μg) was assayed for CKB mRNA using the rat CKB exon 8 antisense probe in the RPA at the indicated postnatal times (lanes 1–11). Lane 12 contains RNA (3 μg) from cultured primary astrocytes as a positive control. The full-length probe and the pBR322/MspI marker are indicated to the left. (**B**) The level of 18 S rRNA was measured by RPA in a 5 ng aliquot of these RNAs; lanes 1–12 correspond to lanes 1–12 in (**A**). (**C**) Histogram showing CKB mRNA levels during postnatal development. The level of CKB mRNA in each sample was adjusted for the amount of 18 S rRNA present, then normalized to the CKB mRNA level at week 10 (which was set at 1) and plotted as the mean $\pm$ S.E.M. as determined from three separate litters. There was no significant difference in CKB mRNA levels during postnatal cerebral development (ANOVA; $P = 0.48$).

oligodendrocyte-specific gene MBP and the neuron-specific gene SCG 10 during cerebellar development. Both were increased at week 6 relative to day 1 (rows 6 and 7) as expected.

CKB mRNA accumulation in cerebellum is delayed relative to the cerebrum and the whole brain

The increase of CKB mRNA in the cerebella from the low levels seen at postnatal day 1 (Figure 2) was unexpected, since in whole rat brain samples the CKB mRNA levels at day 1 and week 1 were previously reported to be very similar and only slightly lower than in the adult at week 8 [43]. Together these findings suggested that CKB mRNA production in cerebellum is delayed as compared with other brain regions. Therefore, we directly compared CKB mRNA levels in total cellular RNA from cerebellum and whole brain (Figure 5A). All samples were also analysed for 18 S rRNA to ascertain that they contained the same amount of RNA (Figure 5B). The amount of CKB mRNA in the cerebellum as compared with whole brain was much lower at week 1 (Figure 5A, lanes 3 and 4), while it was an average of 3.5-fold higher at week 8 (Figure 5A, lanes 5 and 6). In other words, during the first 8 weeks of postnatal development the amount of CKB mRNA increased 5-fold in cerebellum but only by 40 % in whole brain. Since the cerebrum constitutes the largest brain region, expression of CKB mRNA in the cerebrum may significantly determine the mRNA pattern seen in whole brain (Figure 5A). To test this, total cellular RNA was isolated from the cerebra of the same rats analysed in Figures 1 and 2 and the level of CKB mRNA was measured using the exon 8 probe (Figure 6A). RNA from cultured primary astrocytes served as a positive control (Figure 6A, lane 12). Figure 6(B) shows that an equal amount of RNA taken from these different samples contained similar levels of 18 S rRNA. When expressed per μg of total cellular RNA assayed or normalized to the amount of 18 S rRNA present, cerebral CKB mRNA levels at the early postnatal period (day 1, week 1 and week 2) were not statistically different from week 3 to week 10 when analysed by ANOVA ($P = 0.48$; Figure 6C). Taken together, the accumulation (and probably the transcription) of CKB mRNA during postnatal development is delayed in the cerebellum relative to the cerebrum and the whole brain.

CKB and uMi-CK accumulate coincidently during cerebellar development

Total cerebellar lysates, rather than cytosolic fractions, were prepared during postnatal development to allow immunodetection of both CK isoenzymes expressed in brain, CKB and

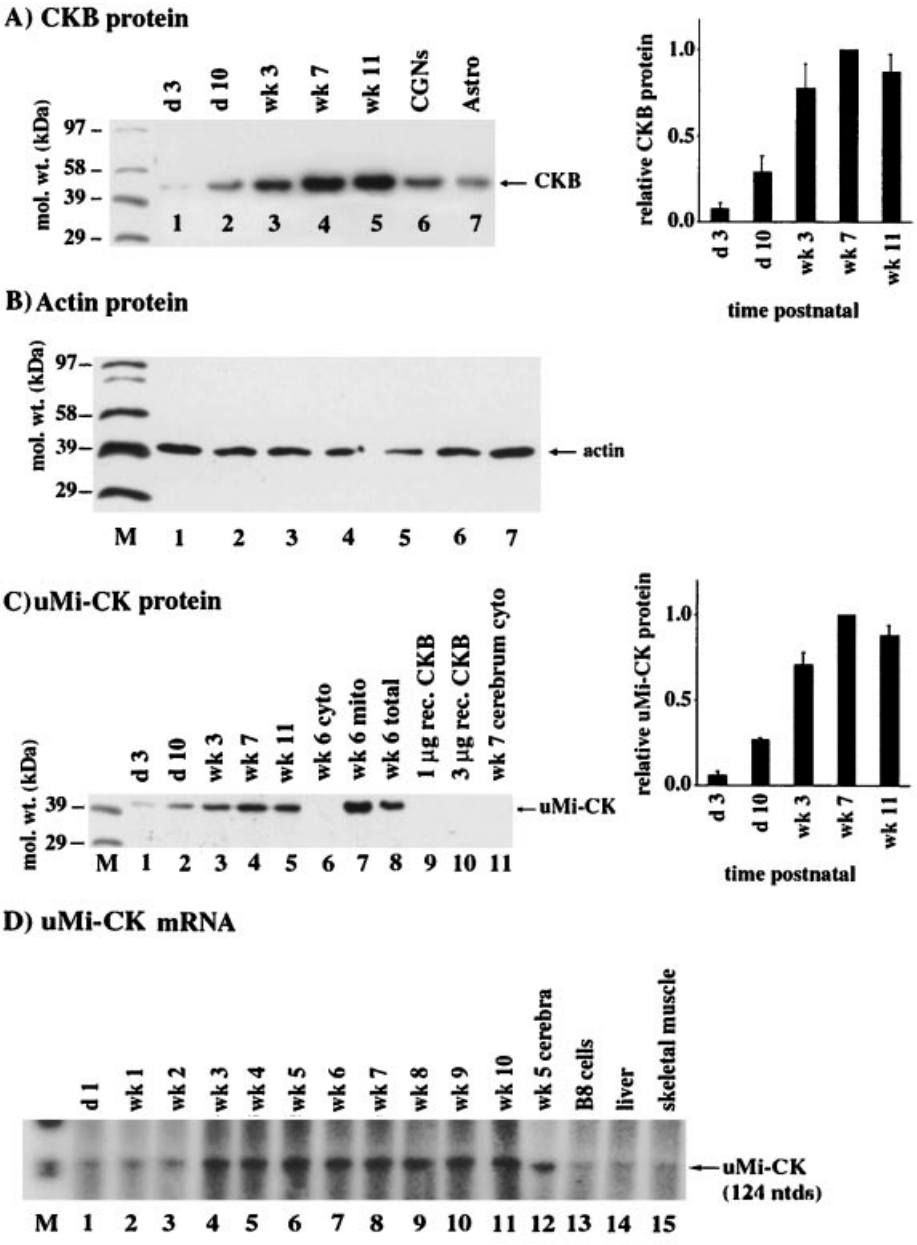
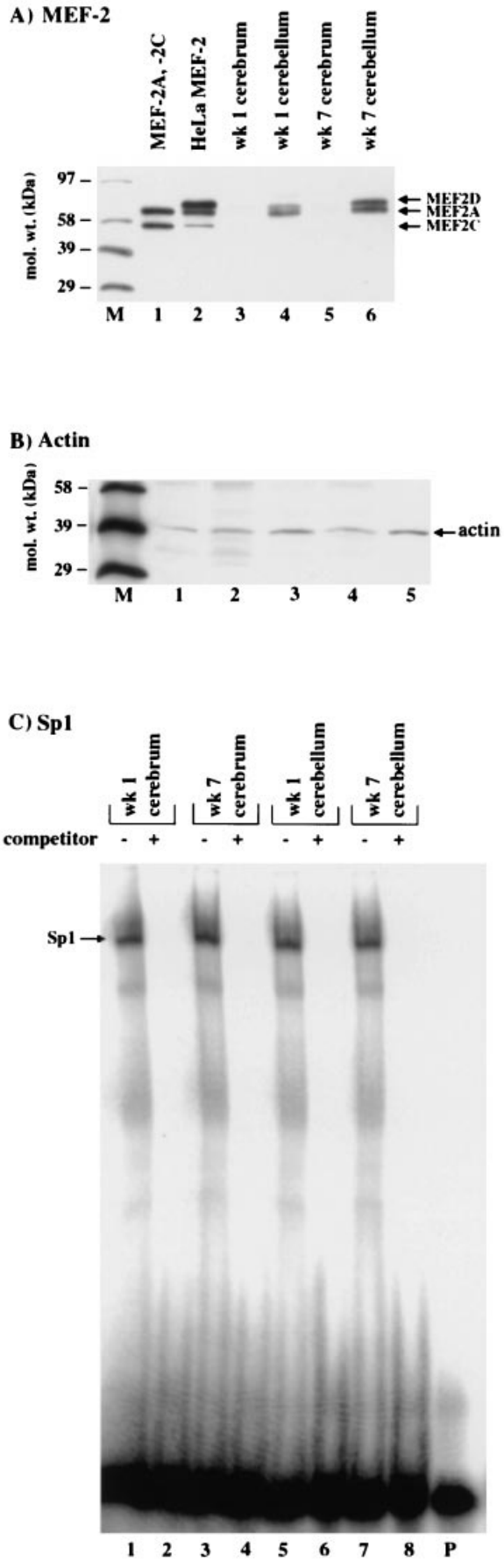


Figure 7 CKB and uMi-CK immunoreactive protein levels in total rat cerebellar lysates during postnatal development

(**A**) Immunoblot analysis for CKB protein in postnatal cerebellar samples and CGNs. Total protein (10 μg) was electrophoresed and analysed for CKB protein with the anti-CKB mAb. Lanes 1–5 contain days 3 and 10 and weeks 3, 7 and 11, respectively. All values were normalized to that at week 7 (which was set at 1) and are expressed as the mean $\pm$ S.E.M. ($n = 3$). Lanes 6 and 7 are from primary cultured CGNs and astrocytes, respectively. (**B**) Immunoblot analysis for actin protein in postnatal cerebellar samples. Protein (15 μg) was electrophoresed and analysed with a rabbit polyclonal anti-actin antibody; lanes 1–7 correspond to the same lanes in (**A**). (**C**) Immunoblot analysis for uMi-CK protein during postnatal cerebellar development. Total cerebellar lysate protein (20 μg) was electrophoresed and analysed with a rabbit polyclonal antibody that recognizes uMi-CK but not CKB. Lanes 1–5 contain days 3 and 10 and weeks 3, 7 and 11, respectively. All values were normalized to week 7 (set at 1) and are expressed as the mean $\pm$ S.E.M. ($n = 3$). Lane 6 contains 15 μg of week 6 cerebellum postmitochondrial, cytoplasmic protein as a negative control. Lane 7 has 15 μg of protein from a mitochondrial-enriched fraction of week 6 cerebellum. Lane 8 has 15 μg of total protein from week 6 cerebellum. Lanes 9 and 10 have 1 and 3 μg of recombinant CKB protein. Lane 11 has 15 μg of week 7 cerebral postmitochondrial, cytoplasmic protein as a negative control. Results shown in **A**–**C** are representative of three separate experiments. (**D**) Total cerebellar RNA (10 μg), from the same samples analysed in Figure 2, was hybridized with the [^{32}P]uMi-CK riboprobe in the RPA. Lanes 1–11 are samples from day 1 to week 10. Lane 12 is a positive control from week 5 cerebrum. Lanes 13–15 are negative controls from RT-4 B8 neuroblastoma, adult rat liver and adult rat skeletal muscle, respectively.

uMi-CK, in the same lysate. With the CKB mAb used in Figure 1, which does not react with uMi-CK [32], CKB was barely detectable at day 3 but increased during the first 3 postnatal weeks to reach a maximal level at week 7, which decreased slightly (20%) by week 11 (Figure 7A). CKB protein levels at week 7 were 12-fold higher than at day 3 (Figure 7A), consistent with Figure 1.

For immunoblot analyses of uMi-CK, we used a polyclonal antibody [34] shown to react with uMi-CK in total lysates and mitochondrial fractions (Figure 7C, lanes 7 and 8), but with



neither CKB from post-mitochondrial or cytoplasmic extracts (Figure 7C, lanes 6 and 11) nor pure recombinant CKB (Figure 7C, lanes 9 and 10). Similar control results were reported by Schlattner et al. [34]. In immunoblots, uMi-CK was barely detectable at day 3, increased at day 10 and reached a maximal level at week 7. The histogram in Figure 7(C) shows that uMi-CK protein levels at week 7 were 16.5-fold higher than at day 3. Actin protein levels in these samples were essentially constant, except for a reproducible small decrease at week 11 (Figure 7B).

Since uMi-CK protein increased dramatically from day 3 to week 7, we used the RPA to measure the levels of uMi-CK mRNA during postnatal development in the same RNA samples shown in Figure 2. uMi-CK mRNA was extremely low at day 1 and week 1, increased during weeks 2–4 and reached a plateau value at week 5 that remained constant until week 10 (Figure 7D). The plateau value was 2.6 ± 0.5-fold ($n = 3$) greater than that at day 1. The levels of 18 S rRNA in these samples are shown in Figure 2(B) and did not differ by more than 10 %.

Cultured primary CGNs contain high amounts of CKB

High amounts of CKB were detected in cultured CGNs (Figure 7A, lane 6), which exceeded those of cultured primary astrocytes (Figure 7A, lane 7). This is consistent with a role for CKB in CGNs. The level of CKB in CGNs may even be a low estimate, since the cell culture procedure for CGNs required preparation from postnatal day 8 cerebella, while expression of CKB may increase until week 3 or thereafter (see Figure 2).

Abundance of transcription factors MEF-2A and -2D increases coincidently with CKB in cerebellum, but not in cerebrum

Factor MEF-2 has been suggested to increase transcription of CKB mRNA [18,19]. We determined the amount of MEF-2 by immunoblot of nuclear extracts prepared from cerebella and cerebra at postnatal week 1 and week 7. The location on the blots of recombinant MEF-2C and -2A generated by *in vitro* transcription/translation is indicated in Figure 8(A), lane 1; MEF-2C migrated faster than MEF-2A (see Experimental and [35]). Nuclear extracts of HeLa cells contained a low level of MEF-2C but higher levels of MEF-2A and MEF-2D (Figure 8A, lane 2); as expected, MEF-2D had the slowest migration [35]. In cerebellum, only low levels of MEF-2A and -2D and no detectable MEF-2C were found at week 1. However, at week 7, MEF-2A and -2D increased 3.5 ± 0.5-fold ($n = 3$), while MEF-2C was still not detectable (Figure 8A, lane 6). Interestingly, in the cerebrum at week 1 and week 7, MEF-2 was too low to be detected (Figure 8A, lanes 3 and 5). This is consistent with the immuno-

Figure 8 MEF2 immunoreactive protein levels in nuclear extracts from rat cerebella and cerebra at postnatal weeks 1 and 7

(**A**) Immunoblot analysis for MEF2 protein in cerebellar and cerebral nuclear extracts. In lanes 2–6, 15 μg of nuclear extract protein were electrophoresed and analysed for MEF2 protein with a rabbit polyclonal anti-MEF2 antibody. Lane 1 contains positive control MEF-2A and MEF-2C protein samples generated using a coupled transcription/translation reticulocyte lysate system. Lane 2 is a HeLa nuclear extract known to contain MEF-2A, -2C and -2D. Lanes 3 and 4 are week 1 nuclear extracts from cerebrum and cerebellum, and lanes 5 and 6 are week 7 nuclear extracts from cerebrum and cerebellum, respectively. (**B**) Immunoblot analysis for actin protein in week 1 and 7 cerebellar and cerebral nuclear extract samples. Protein (15 μg) was electrophoresed in each lane and analysed with a rabbit polyclonal anti-actin antibody. Lanes 1–5 correspond to lanes 2–6 in (**A**). (**C**) EMSA of week 1 and 7 cerebral and cerebellar nuclear extracts incubated with a [^{32}P]oligonucleotide probe containing the consensus binding sequence for factor Sp1, in either the absence (odd-numbered lanes) or presence (even-numbered lanes) of 100-fold excess of the unlabelled Sp1 probe.

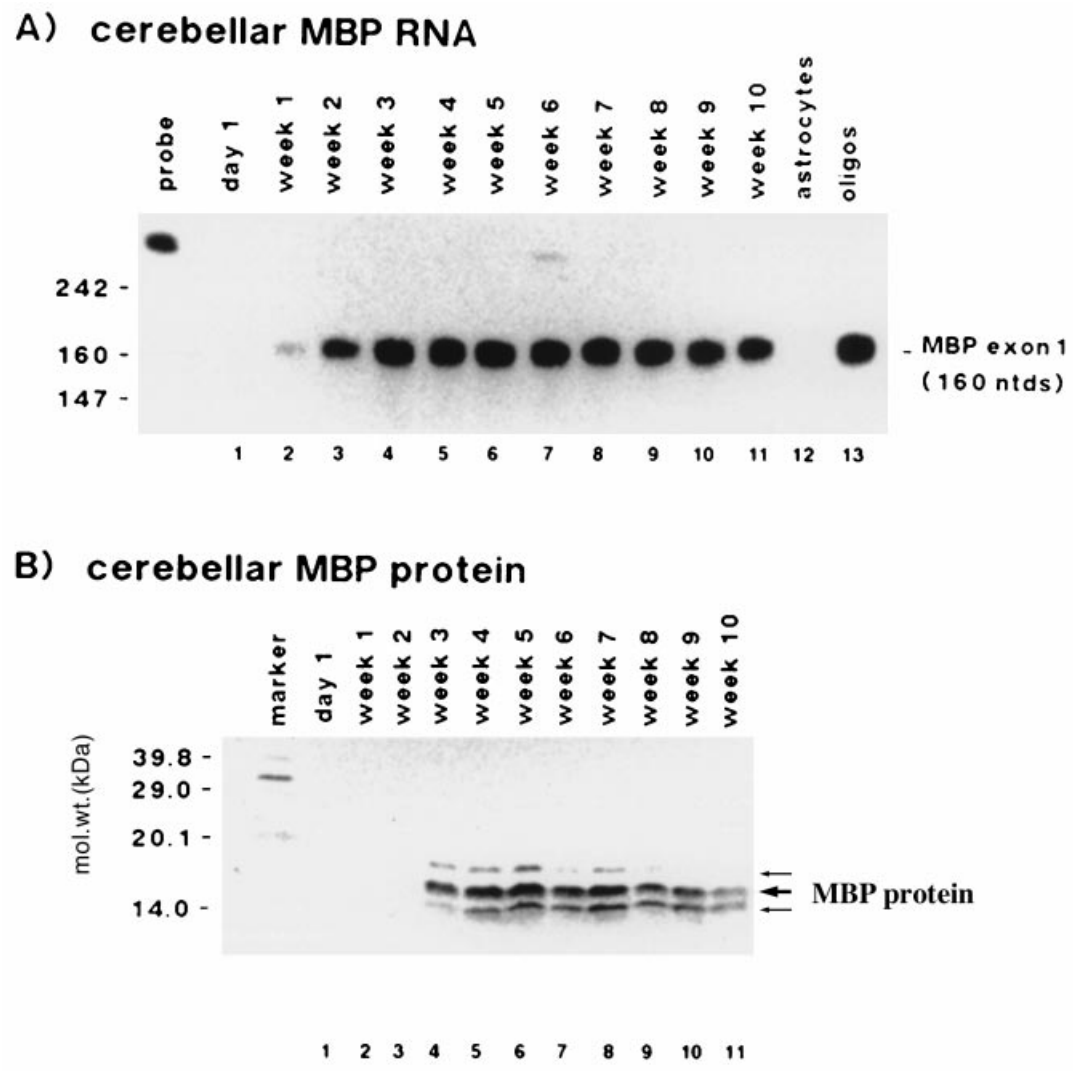


Figure 9 Expression of MBP mRNA and protein during rat cerebellar postnatal development

(**A**) RPA for MBP mRNA during cerebellar postnatal development. RNA (3 μg) from the same cerebellar samples used to measure CKB mRNA in Figure 2 were used to measure MBP mRNA. The location of the 160 nt RNase-protected probe for MBP exon 1 is indicated. Lanes 1–11 are samples from day 1 to week 10. Lane 12 is a negative control from primary astrocytes and lane 13 is a positive control from primary oligodendrocytes differentiated *in vitro* for 6 days as described in the Experimental section. The probe lane shows the full-length MBP exon 1 probe. (**B**) Immunoblot analysis for MBP protein during cerebellar postnatal development. Whole cerebellar lysates (20 μg) were electrophoresed in each lane and analysed for MBP with a rabbit anti-rat MBP polyclonal antibody. The four subtypes of MBP (21.5, 18.5, 17.0 and 14.0 kDa) are present; the two middle bands of 18.5 and 17.0 kDa migrated together (big arrow).

histochemistry results of Lin et al. [20] who showed MEF-2D and MEF-2A were abundant in postnatal cerebellum but much lower in cerebrum. Since Figure 6 showed that in the cerebrum CKB mRNA levels are unchanged at weeks 1 and 7 (and were approx. 50% of the value in week 7 cerebellum), factors other than MEF-2 may activate CKB transcription in the cerebrum. Immunoblot control experiments showed that all the nuclear extracts contained equivalent levels of actin protein (Figure 8B). The rat CKB 5′ upstream region has two elements located within -568 bp and -523 bp which bind another regulatory factor, Sp1 [22]. EMSAs showed that cerebellar and cerebral extracts at week 1 and week 7 contained equal amounts of Sp1 (Figure 8C, lanes 1, 3, 5 and 7); a 100-fold excess of unlabelled Sp1 probe completely eliminated all shifted bands (Figure 8C, lanes 2, 4, 6 and 8). Thus for CKB transcription in cerebellum and cerebrum, Sp1 by itself may not be a sufficient upstream factor but might contribute by co-operating with other factors.

MBP and CKB expression in rat cerebellum correlate during postnatal development

Manos et al. [5] have shown that CKB enzyme activity accumulates in oligodendrocytes during 3 weeks of *in vitro* culture and might function in the regeneration of ATP consumed during myelination, a very energy-demanding process [12,13]. Therefore, we compared the timing of expression of MBP mRNA and protein, relative to CKB, in the same postnatal cerebellar samples analysed in Figures 1 and 2. Figure 9(A) shows that MBP mRNA was essentially undetectable at day 1 and week 1, dramatically increased at week 2 and attained a maximal level at week 3, which decreased slightly by weeks 9 and 10. As expected, the negative control sample of astrocyte RNA (Figure 9A, lane 12) did not contain MBP mRNA and the positive control sample of RNA from oligodendrocytes differentiated *in vitro* contained a high level of MBP mRNA (Figure 9A, lane 13). Expression of

MBP followed behind MBP mRNA, although it was somewhat delayed, and reached a maximal level at week 4 (Figure 9B), which corresponds to the period of abundant myelination in rat brain [5]. Therefore, there is a coincidence in the timing of maximal expression of MBP and CKB protein in the cerebella which may be a further indication for a role of CKB in myelination. The delay in maximal expression of MBP relative to MBP mRNA is probably due to the transport of newly processed, cytoplasmic MBP mRNA to the peripheral, myelinating plasma membrane of the oligodendrocyte, which occurs prior to translation [44].

DISCUSSION

This study shows that the accumulation of CKB mRNA and protein during postnatal development of rat brain cerebellum is regulated principally, if not exclusively, at the level of CKB transcription. First, the steady-state level of CKB mRNA and CKB protein increased coincidentally during postnatal development. Second, nuclear run-on assays that directly measure the transcription of nascent CKB mRNA in nuclei from cerebella showed an increase of CKB transcription from day 1 to week 6 in cerebella. Nascent transcripts from the housekeeping genes of actin and GAPDH as well as 18 S rRNA were present at equal levels in day 1 and week 6 cerebella. Furthermore, we have shown that the timing of CKB mRNA production during postnatal development was delayed in the cerebellum compared with the cerebrum and with whole rat brain. In cerebrum and whole brain, CKB mRNA levels were already high at postnatal day 1 and, thereafter, did not increase significantly during development. The delayed expression of CKB in the cerebellum correlates with the late maturation of the cerebellum in rodents, (e.g. all interneurons of the cerebellar cortex, except in Golgi cells, are produced postnatally [45]) and that co-ordination of cerebellar-controlled movement occurs late after birth [46]. Indeed, the most dramatic increases in cerebellar size and complexity have been shown to occur after postnatal day 10 [47] with weeks 2–3 signalling the maturation of CGNs and Purkinje neurons [20,48], which have been shown by immunohistochemistry in adult rat brain to contain relatively large amounts of CKB protein [16]. By the end of week 3, the volume of the cerebellum has been shown to increase over 1000-fold [47].

Since we showed that the time course of mRNA and protein accumulation of both CK isoenzymes expressed in brain, CKB and uMi-CK, correlated during cerebellar postnatal development, it appears that a functional PCr/CK shuttle exists for energy buffering and transport in the cerebellum [2,16]. As discussed below, previous studies have described some energy-demanding events in neuronal and glial cells in the brain that begin during early postnatal development and which may either require or be accelerated by CKB. By immunohistochemistry CKB protein was detected in Golgi type I neurons in the cerebellum, red nucleus, pons and hippocampus in adult rat brain where it was concentrated in the neuronal processes in synaptic regions (e.g. the molecular layers of the cerebellar cortex and in the hippocampus) [1,16,49]. This location suggests a role for CKB in the regeneration of ATP to support events in synaptic transmission (e.g. protein phosphorylation, transport of ions and glutamate). The high level of CKB protein we have observed in cultured primary CGNs and the localization of some CKB in rat brain synaptic membranes probably coupled to Na^+/K^+ ATPase [6] are consistent with a role for CKB in synaptic transmission. Regarding synapses, recent reports indicate that astrocytes are required for synapse formation and maintenance and synaptic efficiency [14,50]. Astrocyte processes ensheathe synapses and take up ions and glutamate released into the synaptic cleft as a result of neuronal activity [11,14,50]. The energy requirements of this uptake may also explain the abundant levels of CKB mRNA and protein in astrocytes seen in this study (Figures 2 and 5–7) and previously [5,9]. Glutamate released into the synapse is taken up by astrocytes along with Na^+ ions; to re-establish the Na^+ gradient, astrocytes activate Na^+/K^+ ATPase which consumes ATP [11]. In addition, astrocytes take up extracellular K^+ discharged by depolarizing neurons by steps involving Na^+/K^+ ATPase [10]. Therefore, by coupling CKB protein to Na^+/K^+ ATPase, astrocytes can optimize the regeneration of ATP in the transport of ions and glutamate. Interestingly, glutamate uptake may also contribute to ATP production since glutamate has been shown to increase glucose uptake and glycolysis in cultured astrocytes [11]. Any additional ATP needed for ion transport could be generated by the increased glycogenolysis shown to be activated in cultured astrocytes by extracellular K^+ and a number of neurotransmitters [51].

A separate study of the ontogeny of CKB expression in rodent brain by Holtzman et al. [52], using non-invasive ^{31}P-NMR spectroscopy, showed that postnatal weeks 2 and 3 display the largest increase in CK-catalysed reaction rates which also coincided with an increase in glycolysis and tissue respiration in the brain. During this period, there was an increase in the concentration of PCr and the flux of phosphate from PCr into ATP. Holtzman et al. [52] suggested that the coincident increase in CKB activity and brain energy metabolism represents a mechanism for coupling energy production (ATP) and energy storage (PCr) to sudden changes in cellular energy requirements. Indeed, Walliman et al. [2] have proposed that the CK energy shuttle facilitates both (i) increased ATP synthesis by removing ATP from the mitochondria via uMi-CK, thus shifting the equilibrium towards further synthesis and (ii) an increase in efficiency of ATP utilization due to CKB-mediated regeneration of ATP at discrete cytoplasmic sites of high energy consumption. Our results in Figure 7 showing a coincident increase during cerebellar postnatal development in the CKB and uMi-CK proteins support the concept of a CK shuttle in the cerebellum. Future *in situ* immunohistochemistry experiments will be required to establish that CKB and uMi-CK proteins are co-expressed in the same cells.

Some of the increase in CKB during cerebellar postnatal development may also reflect CKB expressed in oligodendrocytes. MBP protein was essentially undetectable until week 3 but increased thereafter to a maximal value at weeks 4 and 5. This corresponded to the period of active myelination in rat brain, a process known to be energy demanding [12,13], as well as the period of maximal CKB protein expression and thus may suggest a role for CKB in myelination of axons by oligondendrocytes [5]. This is supported by our separate study, which has shown that expression of CKB mRNA and protein increased significantly when cultured cerebral $A2B5^+/O4^-$ oligodendrocyte progenitor cells differentiated *in vitro* into MBP^+ oligodendrocytes (W. Shen, D. Willis and G. R. Molloy, unpublished work). However, thus far we have not examined this in the cerebellum. These results are consistent with the studies of Manos et al. [5] who observed that primary oligodendrocytes prepared from postnatal day 2 cerebra and cultured for 3 weeks contained levels of CKB enzyme activity that were 4-fold greater than in co-cultured astrocytes and in embryonic cerebral neurons; however, the levels of CKB immunoreactive protein and mRNA were not measured.

Little is known of the CKB promoter elements and their cognate factors which regulate CKB transcription during brain

development. The CKB proximal promoter of human, chicken and rat contains two CCAAT boxes and a TA-rich element that are binding sites for *trans*-acting transcription factors. The TA-rich element binds the TARP protein [18], which is probably similar to the MADS box proteins MEF-2A and -2D expressed in the brain [20,21], while two other 5′ upstream elements bind factor Sp1 [22]. We have previously shown that the TA-rich MEF-2 element can bind a MEF-2-like factor present in whole rat brain and HeLa cells [18] and was an important *cis* element activating transcription of the CKB gene transfected into HeLa cells [19]. The present report shows that the levels of transcription factors MEF-2A and -2D increased coincidently with transcription of CKB mRNA during postnatal development in cerebellum, but not in cerebrum. Therefore, in the cerebellum, MEF-2A/-2D may be important for increasing transcription initiation of CKB. By contrast, the constant level of factor Sp1 suggests that its function for CKB transcription might depend on its co-operation with other factors (e.g. MEF-2A/-2D bound at −60 bp) whose concentrations increase during cerebellar development. Further analysis of the CKB promotor (e.g. in transgenic experiments) will identify *cis* elements and transcription factors involved in CKB expression in different brain regions and cell types. Recently, additional novel CKB regulatory elements have been reported in exon 1 [53,54] and the 5′ upstream promoter [55]. The mechanisms by which CKB transcription is activated in different brain regions or cell types are of great interest and practical importance in the future, since creatine administration has been shown to elevate production of PCr by uMi-CK and to prevent ATP depletion [23] and to greatly diminish neuronal degeneration in models studying amyotrophic lateral sclerosis [24], Huntington's disease [25] and Parkinson's disease [26]. The cerebellum during postnatal development provides a useful physiological system for future investigations since CKB transcription is initially low and increases significantly during development.

This research was initiated with a grant from the American Heart Association (grant 9951419U to G.R.M.) and the Swiss National Science Foundation (grant 31-62024.00 to T.W. and U.S.). We thank Dr F. Art McMorris of the Wistar Institute (Philadelphia, PA, U.S.A.) for advice in preparing primary astrocyte and oligodendrocyte cultures, for providing plasmid containing the MBP exon 1 cDNA, antibody to MBP and helpful discussions and encouragement. We thank Dr Be Wieringa (University of Nijmegen, Nijmegen, The Netherlands) for monoclonal antibody CK-BYK/21E10 to CKB and Dr Mark Payne (Wake Forest University, Winston-Salem, NC, U.S.A.) for polyclonal anti-CKB antibody. We are grateful to Dr Franca Cambi (Jefferson University Medical School, Philadelphia, PA, U.S.A.) for helpful discussions and Margie Barret for help in preparing the figures.

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Received 6 May 2002/3 June 2002; accepted 1 July 2002
Published as BJ Immediate Publication 1 July 2002, DOI 10.1042/BJ20020709