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'Hot Spots' of Creatine Kinase Localization in Brain: Cerebellum, Hippocampus and Choroid Plexus

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Abstract
Creatine kinase (CK) isoenzymes, with emphasis on the mitochondrial CK isoenzymes, were characterized and localized in chicken cerebellum. Chicken cerebellar extracts analyzed by two-dimensional gels, using anti-peptide antibodies specific for sarcomeric muscle-type mitochondrial CK (Mi_b-CK) and revealed the presence of a Mi_b-CK variant in avian cerebellum. This CK isoform was localized by immunofluorescence staining exclusively in the Purkinje neurons. The co-expression of this Mi_b-CK together with cytosolic muscle-type MM-CK, as observed in the same Purkinje neurons, may reflect the specific energy requirements associated with highly fluctuating Ca^{2+} levels (Ca^{2+} spiking) in these specialized neurons. Ubiquitous brain-type mitochondrial Mi_a-CK was found together with cytosolic BB-CK mainly in the glomeruli structures of the cerebellar granular layer. BB-CK, but much less so Mi_a-CK, however, was also very prominent in Bergmann glial cells of the molecular layer. Thus, an isoenzyme-specific differential localization of the two mitochondrial Mi-CK isoenzymes in the chicken cerebellum is demonstrated. Other hot spots of CK localization were the granule and pyramidal cells of the hippocampus in rat. There, a developmental stage-dependent immunofluorescence staining, especially with antibodies against Mi_a-CK, was noted. Epithelial cells of the choroid plexus were also highly enriched in CK. The possible implications of a CK/PCr circuit at these various cellular locations of the brain are discussed with respect to normal brain physiology and pathology.

Introduction

Brain energy metabolism is characterized by high and fluctuating rates of ATP synthesis and utilization. High levels of creatine kinase (CK, EC 2.7.3.2), catalyzing the reversible transfer of the N-phosphoryl group of phosphorylcreatine (PCr) to ADP in order to regenerate ATP, were found in brain [1]. Besides in brain, CK isoenzymes are also expressed in other tissues with high and fluctuating energy demands, e.g. in skeletal and cardiac muscle, retina photoreceptor cells, *Torpedo* electrocytes and in spermatozoa of sea urchin and fowl, where cytosolic as

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well as mitochondrial CK isoenzymes are usually coexpressed [2]. In vertebrates, three cytosolic CK isoenzymes form dimeric molecules (MM-CK, MB-CK and BB-CK), each composed of two types of subunits (M standing for the 'muscle' isoform; B standing for the 'brain' isoform). These CK isoforms are either cytosolic or associated with subcellular structures, e.g. with the myofibrillar M-band, the sarcoplasmic reticulum or the plasma membrane, where they are functionally coupled to the actomyosin ATPase [3, 4], the Ca^{2+}-ATPase [5, 6] or the Na^+/K^+-ATPase [7], respectively. In contrast, the mitochondrial CK (Mi-CK) isoenzymes are associated with the outer side of the inner mitochondrial membrane [8] and form both dimeric and octameric molecules that are readily interconvertible [9]. Two different Mi-CK isoenzymes were found in all vertebrates examined, a sarcomeric (Mi_b-CK) and a ubiquitous isoform (Mi_a-CK), which are expressed predominantly in striated muscle and brain, respectively [10– 13]. Studies on isolated mitochondria from muscle and brain have shown that mitochondrial oxidative phosphorylation and the Mi-CK reaction are functionally coupled, that is, Mi-CK preferentially utilizes the ATP synthesized through oxidative phosphorylation for PCr synthesis [2, 14].

The two major CK isoforms in brain, cytosolic B-CK [15] and ubiquitous Mi_a-CK [11] have been purified and characterized from chicken brain. The observed microheterogeneity of B-CK was shown to be derived from phosphorylation and alternative initiation [16], as well as from autophosphorylation [17], indicating a connection of CK with signalling pathways of the brain. In addition, a marked upregulation of B-CK transcription involving cyclic AMP-dependent protein kinase A was reported in glioblastoma cells [18].

In rat, the cerebellum, striatum and pyramidal tracts showed significantly higher total CK activity than whole brain, and higher specific CK activities, higher levels of B-CK mRNA, as well as higher PCr/ATP ratios were measured in cultured oligodendrocytes and astrocytes compared to neurons [19–22]. This is in agreement with dissection experiments measuring by immunoassays 26, 18, 17, 13 and 12 μg of B-CK per mg soluble protein of cerebellar white matter, cerebellar cortex, brain stem, cerebral cortex and cerebral white matter, respectively [23], as well as with localized ^{31}P-NMR saturation transfer measurements, showing, in contrast to previous measurements [24], a higher PCr/NTP ratio in white compared to gray matter [25], and a higher CK-catalyzed reaction flux in white versus gray matter of the cerebral hemispheres [26].

With respect to the cellular and subcellular localization of CK isoenzymes in brain, rat and chicken cerebellum has been studied most thoroughly so far. In the cerebellar molecular layer, immunohistochemical localization studies showed, for example, strong staining with the antibody against B-CK of Bergmann glial cells but also of other cells of the cerebellar cortex, e.g. astrocytes and glomerular structures, as well as of cells of the deeper nuclei, whereas mitochondrial CK was primarily localized to the cerebellar glomerular structures in the granule cell layer and in Purkinje neurons of chicken and rat (see [27–29 and refs therein]). In the granular layer of the cerebellum, strong anti-B-CK immunostaining of astrocytes and Golgi 2 cells was seen in chicken and rat [27–30], whereas the myelinated white matter fiber tracts of rat cerebellum were also clearly positive for B-CK [27–29].

Curiously, small amounts of muscle-type MM-CK have also been detected in rat [31], human [32], as well as in chicken brain [28]. Using a set of isoenzyme-specific antibodies against chicken CK isoenzymes, the very small fraction of muscle-type cytosolic M-CK found in chicken, has been specifically localized to the Purkinje neurons of the cerebellum in chicken [28]. The specific mitochondrial Mi-CK isoenzyme in these cells is unknown.

In this study, we characterized and localized the CK isoenzymes in chicken cerebellum with emphasis on the mitochondrial CK isoenzymes, using specific antipeptide antibodies discriminating between the two Mi-CK isoenzymes of chicken, sarcomeric Mi_b-CK and ubiquitous Mi_a-CK [33, 41]. In addition, we report on the cellular localization of CK isoenzymes in rat hippocampus and choroid plexus.

The differential and isoenzyme specific localization of CK in brain, with BB-CK and mitochondrial Mi_a-CK in the grey matter and predominantly BB-CK in white matter, as well as the functional maturation of the CK system during brain development [29, 34] point to an important role of the CK system in the energetics of synaptic transmission, ion homeostasis, K^+ resorption, metabolite and neurotransmitter trafficking, calcium spiking, as well as myelination [22].

Materials and Methods

Cerebellum Extract

Postmortem chicken cerebella were extracted as described previously [28] with minor modifications. Cerebella were homogenized with 2.6 vol (per gram cerebellum) ice-cold hypotonic buffer A

(0.5 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 m*M* 2-mercaptoethanol) using a manual glass-Teflon homogenizer. After the extract was kept for 20 min on ice, buffer B (buffer A with 1 *M* NaH_2PO_4, pH 8.4 at 4 °C) was added to a final concentration of 0.1 *M* phosphate and a tissue to volume ratio of 1:4. The CK isoenzymes were extracted for 2 h on ice in the presence of 0.05% Triton X-100 (final concentration). The crude extract was aliquoted and stored at −20 °C. This procedure guarantees almost quantitative extraction of B-CK and mitochondrial membrane-bound Mi-CK, which is critical for Mi-CK, first by hypoosmotic swelling of mitochondria in buffer A, followed by high-pH phosphate extraction of Mi-CK (buffer B) facilitated by low concentrations of Triton.

Antibodies

The following primary antibodies against purified CK isoenzymes, generated and characterized previously in this laboratory [11, 12, 33–37], were used for immunolocalization and staining of Western blots: rabbit anti-B-CK serum [35], rabbit anti-Mi_a-CK serum [11] which was crossabsorbed against Mi_b-CK for use in this study [33, 41], rabbit anti-Mi_b-CK serum [36], monoclonal mouse anti-Mi_b-CK 30a supernatant [12], and rabbit anti-M-CK serum [37]. In control experiments, preimmune sera were used at the same dilution as the corresponding primary antibodies.

Since there were no specific antibodies against Mi_a-CK available, the specificity of the polyclonal antibody raised against Mi_a-CK was increased significantly by crossabsorption against Mi_b-CK [33]. For this purpose, Mi_b-CK was bound to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) and the serum was incubated with this affinity matrix. The supernatant of the incubation mixture contained the crossabsorbed antibody. In addition, we raised specific antibodies against the Mi-CK isoenzymes by choosing an antipeptide antibody approach. Since the N-terminal peptides of the Mi-CKs are highly diverse, also in comparison to the cytosolic CK isoenzymes [11], they were selected as antigens for the generation of antibodies. Synthetic peptides made according to standard solid phase procedures, followed by HPLC purification (a kind gift of Thomas Vorherr, Hoffmann-LaRoche AG, Basel), corresponding to the Mi_b-CK amino acids sequence 1-15 and the Mi_a-CK sequence 1-15 and 175-183, were coupled separately to keyhole limpet hemocyanin at room temperature as follows [38]: hemocyanin (Pierce) was dissolved in 50% glycerol (10 mg protein in 80 µl solution) and mixed with 420 µl of buffer C (140 m*M* NaCl, 1.6 m*M* KCl, 1.1 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, pH 7.4). Subsequently, 20 µl of freshly prepared 100 m*M* disuccinimidyl suberate in dimethylsulfoxide was added, and the mixture was stirred for 10 min. Then, 6.3 mg of solid peptide was added and stirring continued for 90 min. Finally, the mixture was diluted with 500 µl of buffer C. After stirring for another 30 min, 160-µl aliquots were frozen and stored at −20 °C.

Rabbits were injected intracutaneously with a mixture of 160 µl of coupled peptide, 340 µl of buffer C and 500 µl of either Freund's complete adjuvant (day 0) or incomplete adjuvant (days 14, 28, 42, 67 and 91). Rabbits were bled by ear puncture on days 35, 42, 55, 67, 84, 91, 98 and 105. Sera were analyzed for cross-reaction with other CK isoenzymes by ELISA (data not shown) and Western blots and were stored in aliquots at −20 °C. As far as terminology is concerned, the term 'polyclonal antibody against a particular CK isoenzyme' stands for antibodies raised against the purified entire CK molecule, whereas polyclonal antibodies raised against N-terminal peptides of the Mi-CK isoenzymes, or against the 175-183 peptide of Mi_a-CK, are referred to as 'antipeptide antibodies' [33].

Electrophoretic Techniques and Immunoblotting

For two-dimensional gel electrophoresis cerebellar extracts were dissolved in 9 *M* urea. 2-D gel electrophoresis was performed according to O'Farrell et al. [39] with nonequilibrium pH-gradient electrophoresis (NEPHGE; pH range 3–10, Pharmalyte, Pharmacia) for a limited running time of 6 h at 400 V in the first dimension (using a Bio-Rad Model 175 Tube Cell and 160 mm tubes with 2.4 mm inner diameter) and standard SDS-PAGE [40] in the second dimension. Proteins separated by the 2-D gel electrophoresis technique were either silver stained or semi-dry-blotted at a constant current of 1 mA/cm^2 for 90 min onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore AG) followed by 'staining' with specific antibodies against the different CK isoenzymes (referred to as 2-D Western blots).

Immunofluorescence Microscopy

Paraffin sections of chicken cerebella were prepared as described previously [28] with minor modifications. Cerebella were fixed by immersion in a mixture of methanol/acetic acid (9/1) or in Carnoy's solution (chloroform/acetic acid/ethanol, 3/1/6) for 60 min at 4 °C. The tissue was then immersed in chloroform and embedded in paraffin. 5-µm-thick sections were deparaffinized with xylene, rehydrated by a reverse graded series of ethanol and washed with Tris-buffered saline (TBS; 150 m*M* NaCl, 50 m*M* Tris, pH 7.4). To lower the background, sections were washed for 10 min with TBS containing 0.05% sodium borohydride and for 10 min with TBS containing 50 m*M* ammonium chloride, prior to blocking of the nonspecific binding sites with TBG (TBS with 1% bovine serum albumin and 0.2% gelatine) for 30 min. Samples were incubated with primary antibodies (in the dilutions indicated in the individual experiments) for 2 h in a moist chamber, and then washed with three changes of TBG for 10 min each. Rhodamine-conjugated goat antirabbit IgG (Pierce, 1:500 diluted) or fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (Nordic, 1:100 diluted) served as secondary antibodies (1 h incubation) and were diluted in TBG. After three washes with TBG, the specimens were mounted either in buffered polyvinyl alcohol medium (Lenette) in the presence of *p*-phenylene diamine (1 mg/ml) as antifading agent or in n-propyl gallate medium. For rat brain, whole rat brains were exised after perfusion fixation with 4% paraformaldehyde in 100 m*M* phosphate buffer and embedded in paraffin. Indirect immunofluorescence on the sectioned rat brains was performed as described above.

Results

Production of Specific Antibodies against Mi-CK Isoenzymes

For the identification and localization of the CK isoenzymes in chicken cerebellum, specific antibodies against all chicken CK isoenzymes were needed. Several polyclonal antibodies against these chicken CK isoenzymes and monoclonal antibodies against Mi_b-CK [12] were available in our laboratory. The polyclonal antibody against Mi_a-CK, the predominant Mi-CK isoform in brain, cross-reacted also with purified Mi_b-CK on Western blots [41] and with several CK isoenzymes in

ELISA [33]. To improve the specificity of this latter antibody, it was crossabsorbed against Mi_b-CK (see 'Materials and Methods'). In ELISA experiments, this crossabsorbed antibody did no longer crossreact with Mi_b-CK and other CK isoenzymes [33]. In an attempt to get highly specific antibodies against Mi-CK isoenzymes, additional polyclonal antibodies were raised in rabbits also against N-terminal or internal peptides of Mi_b-CK and Mi_a-CK, respectively. As an antigen for the antipeptide antibodies, synthetic peptides corresponding to Mi_b-CK amino acids 1–15 [41], to Mi_a-CK amino acids 1–15 [41], and 175–183, respectively, were used. Control experiments revealed that the antipeptide antibody against Mi_b-CK 1–15 was highly specific for Mi_b-CK and did not crossreact at all with other CK isoenzymes in ELISA tests under native conditions, as well as in immunoblotting experiments under denaturing conditions [33, 41]. The antipeptide antibody Mi_a-CK 1–15 [33] and antipeptide antibody against Mi_a-CK 175–183 crossreacted slightly with Mi_b-CK in ELISAs and were therefore also crossabsorbed against Mi_b-CK.

Identification of CK Isoenzymes in Chicken Cerebellar Extracts by Two-Dimensional Gel Electrophoresis

In a previous study [28], the CK isoenzymes of chicken cerebellum were investigated by using the zymogram technique, i.e. electrophoresis under native conditions on cellulose polyacetate strips, followed by enzyme activity staining. No additional mitochondrial CK isoenzyme, except Mi_a-CK, could be detected in this way, whereas the small amount of M-CK was just barely detectable. However, by 2-D isoelectric focusing in the pH range 5–8, the presence of M-CK in chicken cerebellum could be unambiguously demonstrated [28]. However, using this pH range, molecules with basic pI like the mitochondrial CK isoenzymes, cannot be detected since they do not enter the isoelectric focusing gels. Therefore, our approach was to apply cerebellar extracts to nonequilibrium pH gradient electrophoresis (NEPHGE) in the pH range 3–10, enabling us to resolve the mitochondrial Mi-CK isoenzymes.

Three identical samples of chicken cerebellum extract were applied to NEPHGE. One of the resulting gels was silver stained (fig. 1A), whereas the other two were semidry blotted onto PVDF membranes and subsequently stained with specific antibodies against the CK isoenzymes. The antipeptide antibody against Mi_b-CK 1–15 stained one spot in the center of the blot (fig. 1B, asterisk). This staining was much enhanced by using

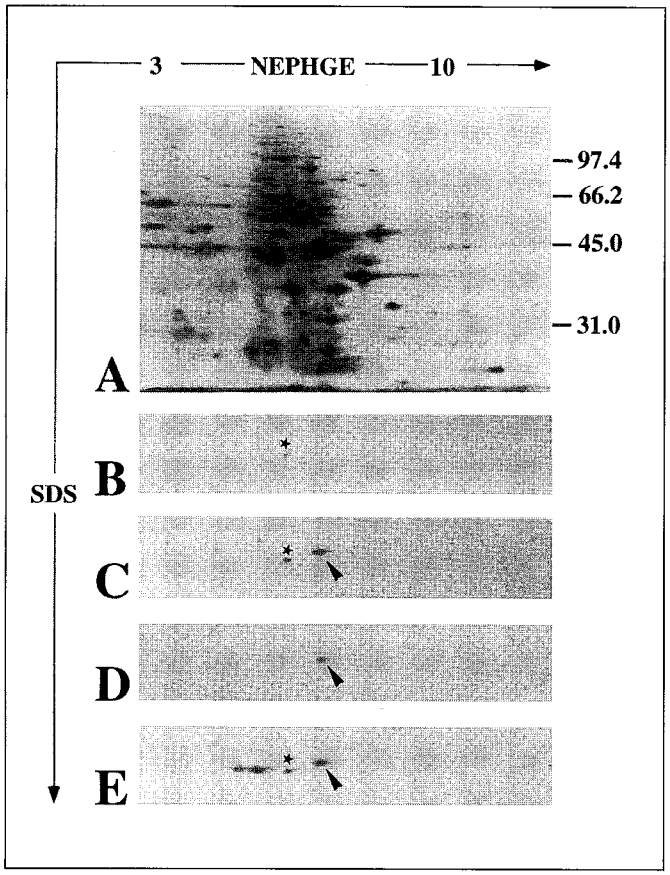

Fig. 1. 2-D gel electrophoresis and Western blots of chicken cerebellum extracts. Chicken cerebellum proteins were separated by nonequilibrium pH gradient electrophoresis in the first dimension, followed by conventional SDS-PAGE in the second dimension. One gel was silver stained (**A**), other parallel gels were blotted onto PVDF membranes followed by visualization of the CK isoenzymes by staining with the antipeptide antibody against Mi_b-CK 1–15 (**B**, 1:200 dilution), with the cross-absorbed antipeptide antibody against Mi_a-CK 175–183 (**C**, 1:500 dilution), with cross-absorbed polyclonal antibody against Mi_a-CK (**D**, 1:1,000 dilution), and with polyclonal antibody against B-CK (**E**, 1:1,000 dilution). Note that the blot shown in **B** was subsequently stained with antipeptide antibodies against Mi_a-CK 175–183 (**C**) and with the polyclonal antibody against B-CK (**E**). Molecular weight markers (Bio-Rad) comprising phosphorylase B (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD) and bovine carbonic anhydrase (31 kD) are indicated on the right side of **A**. Asterisks and arrowheads point to the minor sarcomeric Mi_b-CK variant and to major ubiquitous Mi_a-CK, respectively, found in avian cerebellum.

chemiluminescence detection (data not shown) and therefore corroborating the expression of Mi_b-CK in cerebellum shown also by immunofluorescence (fig. 3A). The antipeptide antibody against Mi_a-CK 175–183, which had been crossabsorbed against Mi_b-CK, stained a

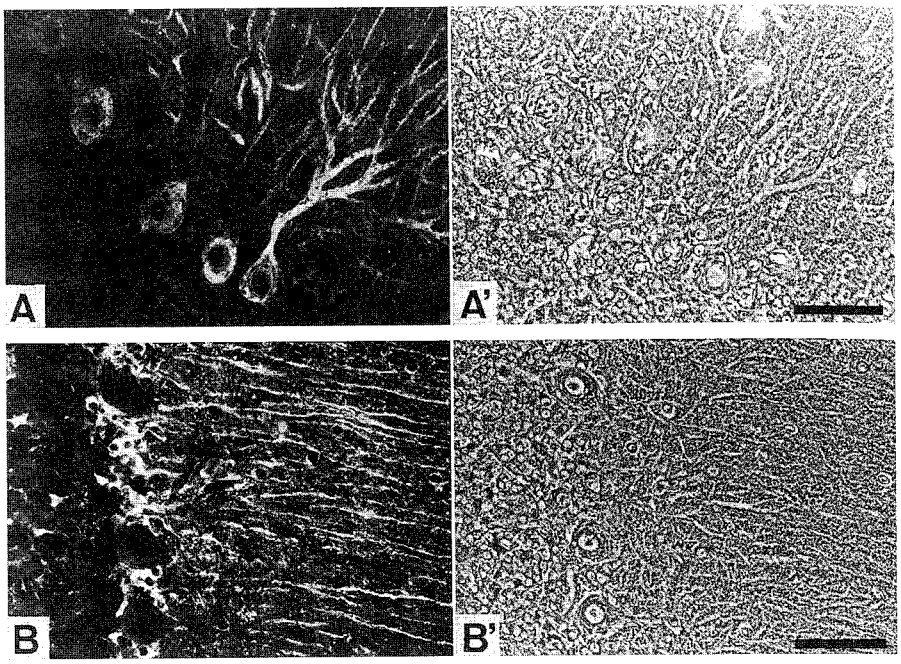

Fig. 2. Immunofluorescence localization of cytosolic M- and B-CK isoenzymes in chicken cerebellum. Immunofluorescence microscopy of paraffin sections of chicken cerebellum, fixed either with Carnoy's solution (chloroform/ethanol/acetic acid, 3/1/6; A and A') or with methanol/acetic acid (9/1; B and B') before embedding. Labelling with polyclonal antibody against M-CK (1:100 dilution) (**A**), and with polyclonal antibody against B-CK (1:100 dilution) (**B**). The corresponding phase-contrast images are shown for comparison in panels **A'**, and **B'**. Note that the antibody against M-CK stains exclusively and specifically only Purkinje neurons, whereas the antibody against B-CK labelled predominantly the Bergmann glial cells. Control staining with preimmune serum or antigen-adsorbed antibodies was negligible (not shown). Bars = 50 μm.

spot on the basic side of the blot (fig. 1C, arrowhead). This very same spot was stained on a parallel blot by the polyclonal antibody against Mi_a-CK, which had also been crossabsorbed against Mi_b-CK, thereby confirming that this spot is genuine Mi_a-CK (fig. 1D, arrowhead). Absolutely no crossreaction with sarcomeric muscle-type Mi_b-CK could be detected with both of the two antibodies against Mi_a-CK, even after using chemilumenescent enhancer. Interestingly, the spot stained by the antipeptide antibody against sarcomeric Mi_b-CK 1–15 was at a more acidic position compared to ubiquitous Mi_a-CK. This result differs from previous findings mainly obtained with isolated, purified Mi-CKs from chicken brain and cardiac muscle [14], where Mi_b-CK migrated more basic than Mi_a-CK [9]. This was the reason for coining the original names, Mi_a-CK and Mi_b-CK (a, for acidic, b, for basic). In analogy with recent results obtained with chicken spermatozoa [41], it can be postulated that the muscle-type Mi_b-CK in cerebellum represents a variant of genuine sarcomeric muscle Mi_b-CK.

The polyclonal antibody against B-CK recognized two spots on the acidic side of the blot (fig. 1E, note that this blot was already used in fig. 1B and C). These two spots correspond to the well-known B_a- and B_b-CK subunit species of cytosolic brain-type BB-CK, which have been identified, isolated and characterized from chicken brain and gizzard [15]. These minor subspecies of B-CK are attributed to phosphorylation and autophosphorylation of this CK isoenzyme [16, 17].

Staining of 2-D Western blots with polyclonal antibodies against cytosolic muscle-type M-CK and native zymograms confirmed the presence of small amounts of M-CK in chicken brain and cerebellar extracts [28; fig. 7, not shown here]. Thus, in conclusion, besides the two major CK isoenzymes, B- and Mi_a-CK, chicken cerebellum contains small amounts of cytosolic muscle-type M-CK and sarcomeric Mi_b-CK.

Localization of the Cytosolic CK Isoenzymes in Chicken Cerebellum

In agreement with earlier results [28], the polyclonal antibody against cytosolic muscle-type M-CK, representing a very minor CK species in brain which is barely detectable in brain extracts [see fig. 7 and 8 in 28], stained exclusively and specifically the Purkinje neurons (fig. 2A). Staining was seen in primary dendrites, dendritic arborizations and perikarya. The cell bodies of Purkinje neurons were less intensively labelled by anti-M-CK antibodies compared to staining with antibodies against Mi_b-CK (cf. fig. 3A).

The polyclonal antibody against B-CK stained the molecular layer strongly. At higher magnification one can see that Bergmann glial cells (also known as Golgi epithelial cells [42]) are stained most intensively

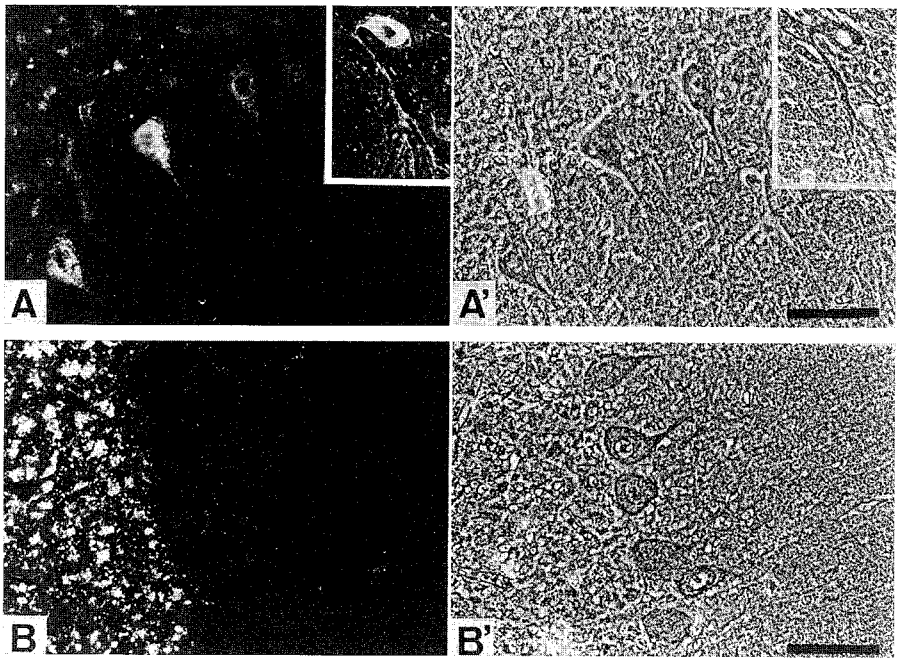

Fig. 3. Immunofluorescence localization of the mitochondrial CK isoenzymes in chicken cerebellum. Paraffin sections of chicken cerebellum, previously fixed in methanol/acetic acid (9/1) for 7 h at room temperature (see 'Material and Methods'), were labelled either with the antipeptide antibody against Mi_b-CK 1-15 (**A**, 1:500 dilution) or with the polyclonal antibody against Mi_a-CK which had been cross-absorbed against Mi_b-CK (**B**, 1:50 dilution). **A'** and **B'** show the corresponding phase-contrast pictures. The inset in **A** and **A'** displays a Purkinje neuron in plane showing, besides the heavy staining of the cell body, also some minor staining of the dendrites. Note that the antibody against Mi_b-CK stained mainly Purkinje neurons whereas the antibody against Mi_a-CK stained mitochondria present in large quantities in the glomerular structures (neuropils) of the granular layer. Bars = 50 µm.

(fig. 2B). To a somewhat lesser extent stellate and basket cells surrounding the Purkinje neurons were also stained by anti-B-CK antibodies, whereas Purkinje neurons themselves remained unstained. The antibody against B-CK also stained astrocytes, as well as the glomerular structures in the granular layer, where the antibody against Mi_a-CK also stained intensively (fig. 3B) [28]. However, some of this staining seen in the granular layer with the antibody against B-CK may be nonspecific due to minor crossreactivity with the relatively abundant Mi_a-CK present in this stratum of the chicken cerebellum. These observations corroborate earlier immunofluorescence localization work done with chicken cerebellum [28].

Localization of the Mitochondrial CK Isoenzymes in Chicken Cerebellum

Expression of sarcomeric Mi_b-CK was thought to be restricted entirely to heart and skeletal muscle in chicken [10]. However, Mi_b-CK was shown recently to be the major CK isoenzyme in chicken spermatozoa [41]. Therefore, we readdressed the question of whether Mi_b-CK may also be expressed in chicken cerebellum, since Purkinje cells were shown to contain cytosolic muscle-type M-CK (fig. 2A) [28]. Indeed, like the antibody against cytosolic muscle-type M-CK, the specific antipeptide antibody against sarcomeric Mi_b-CK 1–15 stained selectively and specifically the cerebellar Purkinje neurons (fig. 3A). The cell bodies of the Purkinje neurons, known to contain large numbers of mitochondria, were stained heavily, whereas the dendrites of the Purkinje neurons were only stained occasionally and with lower intensity. A very faint, but nevertheless specific staining of the Purkinje neurons was also observed using the monoclonal anti-chicken Mi_b-CK [12], antibody (not shown). Thus, the sarcomeric Mi_b-CK variant identified by 2-D Western blots (fig. 1), plus cytosolic M-CK, constituting the muscle CK isoforms for a phosphocreatine shuttle [2], are coexpressed within the Purkinje neurons. Minor staining with the antibody against Mi_b-CK was also observed in the granular layer, probably originating from remaining slight crossreactivity with denatured Mi_a-CK (fig. 3A). Similar results were obtained with polyclonal antibodies against Mi_b-CK, raised against the isolated intact molecule, but these immunofluorescence stainings were accompanied by significantly stronger crossreactivity with Mi_a-CK (data not shown). If both polyclonal rabbit antichicken M- and Mi_b-CK antibodies were applied together, followed by a second fluorescent antirabbit IgG, this colabelling resulted in a strong, complementary staining of the entire Purkinje neurons, that is, of cell bodies as well as processes (not shown), corroborating the colocalization of M- and Mi_b-CK within the same cerebellar Purkinje neuron.

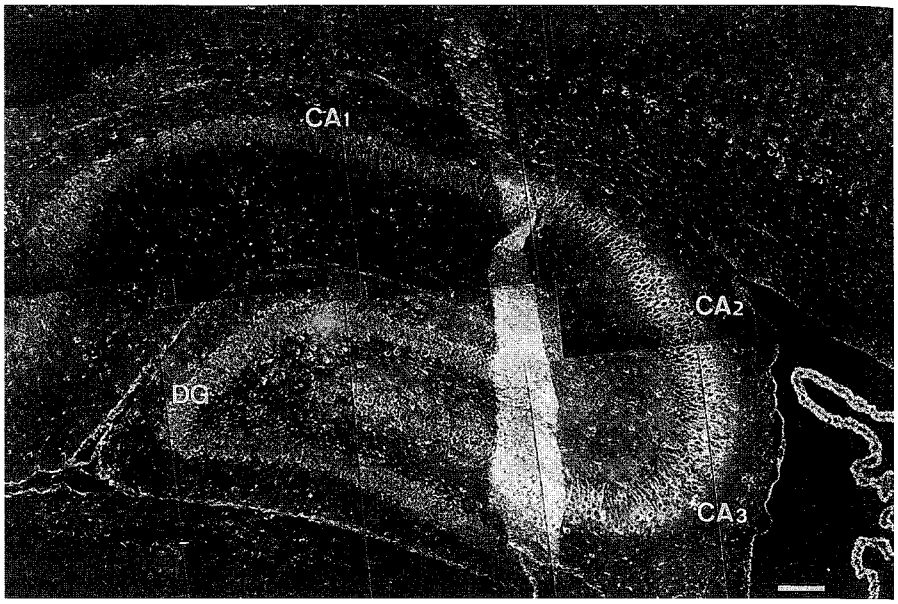

Fig. 4. Immunofluorescence localization of CK in rat hippocampus and choroid plexus. Perfusion-fixed, paraffin-embedded hippocampal region of rat brains at age 12 days postnatally were stained with the polyclonal antibody against chicken ubiquitous Mi_a-CK. Note the strong staining of granular cells in the dentate gyrus (DG) as well as of pyramidal cells in the CA_1-CA_4 regions of the hippocampus. Of further interest is the strong staining of epithelial cells along the choroid plexus (at lower right). Bar = 100 µm.

Previously, Mi_a-CK has been purified from chicken brain [11] and shown to be expressed in several other tissues besides brain [13]. On paraffin sctions of adult chicken cerebellum, as above, the crossabsorbed polyclonal antibody against ubiquitous Mi_a-CK stained specifically the granular layer (fig. 3B). The glomerular structures [43] were stained more intensively than the perinuclear areas of neurons or glial cells. Little staining was observed in the molecular layer and, in contrast to earlier findings with a nonabsorbed antibody against Mi_a-CK [28], no staining at all was found in Purkinje neurons with this crossabsorbed antibody. The clear differential immunofluorescence staining of Purkinje neurons, by our two new antibodies against Mi_a- and Mi_b-CK can thus be explained by the higher specificity of the crossabsorbed antibody against Mi_a-CK compared to the serum used before. Thus, we illustrate here for the first time the coexpression of cytosolic muscle-type M-CK together with sarcomeric mitochondrial Mi_b-CK in Purkinje neurons of chicken cerebellum, which can easily be recognized based on their location within the cerebellum and their cellular shape. In addition, an isoenzyme-specific differential localization of the two mitochondrial Mi-CK isoenzymes in the chicken cerebellum is demonstrated.

Presence of CK in Hippocampal Neurons and Epithelial Cells of the Choroid Plexus

During the course of a developmental study, investigating the sequential appearance of CK isoenzymes in postnatal rat brains [34], a strong immunofluorescence staining with the antibody against chicken ubiquitous Mi_a-CK of granular cells in the dentate gyrus (DG; fig. 4) and of the pyramidal cells in the CA_1-CA_4 region of the rat hippocampus became obvious (fig. 4). At postnatal age 12 days, staining for Mi-CK of the perinuclear regions of granular as well as pyramidal cells was most striking (fig. 4), whereas staining for B-CK was more uniform and generalized (not shown here). At later stages of rat brain development, anti-Mi_a-CK staining was generally weaker and more translocated towards the neuronal processes. This corresponds to the findings with rat cerebellar Purkinje neurons, where a redistribution of the immunofluorescence signal had been observed as a consequence of cell differentiation, e.g. when mitochondria are translocated from the cell body into the neuronal processes [34]. In these later stages of postnatal brain development in the rat, however, rather prominent staining of both granular and pyramidal cells could also be seen with our anti-chicken anti-B-CK antibody (not shown here) [23, 27].

Interestingly enough, very high immunofluorescence labelling with the antibody against Mi_a-CK, as well as

fair staining with the antibody against B-CK (not shown) was also observed in the epithelial cells along the choroid plexus (fig. 4, lower right), indicating a high energy requirement of this brain structure for ion homeostasis. Incubation of rat brain sections with control sera, applied at the same dilution as the antisera and followed by fluorescently conjugated second antibody, resulted in negligable background stainung (not shown).

Discussion

The CK isoenzymes of chicken cerebellum were characterized and localized, using 2-D gel electrophoresis, as well as immunofluorescence microscopy. The emphasis of the first part of this study was to localize and characterize the mitochondrial CK isoenzymes. The recently available antipeptide antibody against Mi_b-CK 1–15 was shown to be very specific in previous studies [33, 41], and on 2-D Western blots it stained specifically only one polypeptide spot (fig. 1B). The antipeptide antibody against Mi_a-CK 175–183, as well as the polyclonal antibody against Mi_a-CK, however, had to be crossabsorbed first against Mi_b-CK to exclude high levels of crossreactivity. The specificity of these antibodies can be judged from figure 1 and from the specific differential immunofluorescence staining of brain sections shown in figures 2 and 3.

BB-CK and Mi_a-CK, the Predominant CK Isoenzymes in Whole Brain and Cerebellum

In accordance with earlier results [28, 29], cytosolic B-CK was found in high amounts in whole brain and in the cerebellum of the chicken, where the enzyme was most prominent in the cerebellar molecular layer, particularly in Bergmann glial cell, but also in other cells of the cerebellar cortex, e.g. in astrocytes, glomerular structures and deeper nuclei of the granular layer, as well as in white matter tracts of rat cerebellum [34]. The possible physiological functions of B-CK with respect to ion homeostasis (K^+ resorption), metabolite and neurotransmitter trafficking and myelination have been discussed in detail [1, 29].

The presence of large amounts of ubiquitous mitochondrial Mi_a-CK, together with B-CK, in the granular layer of the cerebellum, especially within the glomeruli structures, forming intimate synaptic as well as glial-neuron interactions, known to be rich in mitochondria [44], is shown here with a set of highly specific antibodies (fig. 2B, 3B). Thus, the localization of both B- and Mi_a-CK in these giant complexes of mossy fibres, Golgi cells and granule cell synapses, as well as glial cells suggests that a PCr circuit [1, 2] is needed for the energetics of restoration of K^+ gradients and for metabolite and neurotransmitter trafficking between neuronal and glial cells [44]. Our hypothesis of CK function in brain [1] is fully in line with the fact that supplementation of brain slices with creatine in the medium, especially under anoxic conditions, protects cerebral synaptic transmission and the maintenance of action potentials (via Na^+/K^+-ATPase) [45] by increasing baseline levels of PCr and thus reducing anoxia-induced decline of ATP, Ca^{2+} overload and cell death [46] which are observed otherwise.

The apparent discrepancies of relative CK and/or CK-mediated reaction flux in the gray compared to white matter of the brain [22–24, 26, 27, 30, 34] may depend on the methods of tissue fixation and on the antibodies used for inmmunohistochemistry, as well as on the developmental and physiological (stimulation by signalling pathways, see below) stage of the brain, on the sex and of the animal species, etc. Activation of glial cells by cAMP-dependent signalling pathways resulted in a 14-fold increase in B-CK expression [18]. Early data with different microdissected areas of the brain, show significant CK activity in white matter [23, 34] and more recent results verify that astroglial and oligodendrocytes in culture both show significantly higher levels of B-CK mRNA as well as B-CK enzyme activity than neurons [18, 21, 22, 47]. The same holds true for the phosphagen content, since PCr/ATP ratios were 1.0 and 0.33 in primary astrocytes and neuron cultures, respectively [19]. As far as immunofluorescence localization of the rather soluble B-CK in brain is concerned, we found significant variation of signal intensity due to washing-out of the enzyme. The latter could be prevented most efficiently by methanol/acetic acid or Carnoy's precipitation fixation, but not by paraformaldehyde alone [28].

These results together with recent findings that B-CK expression can be highly upregulated by cAMP-dependent processes [18], indicate that the CK system in brain shows developmental [34] and physiological state-dependent dynamics [18], and that B-CK plays an important function in astrocytes and oligodendrocytes [20, 22], and thus in the white matter of the brain [26]. Interesting in this respect is the finding that the amount of cytosolic muscle-type M-CK found in brain seems to be age-dependent [31].

Muscle-Type CK Isoenzyme Complement, M-CK and Mi_b-CK, in Cerebellar Purkinje Neurons

Surprisingly, besides the cytosolic muscle-type M-CK (fig. 2A), [28], also a sarcomeric muscle-type Mi_b-CK variant could be detected in chicken cerebellum extracts (fig. 1) and localized exclusively and specifically to the Purkinje neurons in the cerebellum (fig. 3). Since both antibodies against the entire Mi_b-CK protein, as well as an antibody against a synthetic N-terminal peptide of Mi_b-CK recognized one single polypeptide spot on 2-D Western blots, the protein is likely to be related to genuine Mi_b-CK. Because of its altered electrophoretic migration in NEPHGE gels (more acidic than purified Mi_b-CK from chicken heart), this Mi_b-CK is likely to represent a posttranslational modification, brought about, for example, by phosphorylation or autophosphorylation, as observed also for other CK isoenzymes [16, 17]. The expression of a full muscle-type complement of CK isoenzymes specifically in the Purkinje neurons may reflect important physiologic features of these highly specialized cells which are characterized by complex Ca^{2+} spiking and Ca^{2+}-induced long-term depressions (LTD) [28, 48]. Interestingly, chicken Purkinje neurons, representing the only nonmuscle cell type expressing bona fide calsequestrin [49–51], were shown to also preferentially express muscle-specific isoforms of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase [52, 53] and of the ryanodine receptor [54, 55].

The new findings that sarcomeric muscle-type CK isoenzymes are coexpressed in avian Purkinje neurons, together with a number of muscle-specific proteins involved in Ca^{2+} release, buffering and uptake is indicative for a crucial role of the CK/PCr circuit in the energetics of Ca^{2+} homeostasis in the highly specialized Purkinje neurons. The involvement of CK in the energetics of Ca^{2+} homeostasis is supported by a number of studies with muscle. For example, some fraction of M-CK is bound or functionally coupled to the Ca^{2+}-ATPase of the sarcoplasmic reticulum [5], to the plasmalemma Na^+/K^+-ATPase [7] and the myofibrillar actin-activated myosin ATPase [3, 4], and preferentially supplies the ATP-dependent ion pump with ATP [6, 56].

Summarizing, the CK/PCr circuit is keeping intracellular [ADP] low and by maintaining ATP/ADP ratios high at those subcellular sites where CK is functionally coupled to ATP-requiring processes [1, 2, 57], like ion pumps. These pumps are largely dependent on a high affinity of ATP hydrolysis, that is, they need a high local ATP/ADP ratio for efficient functioning. The improvement by the CK system of the thermodynamic efficiency of ATP hydrolysis is especially important for some ion pumps, and seems crucial for the sarcoplasmic (SR) Ca^{2+}-ATPase [2, 5, 6, 56].

Mammalian Cerebellar Purkinje Neurons

Even though, in mammalian Purkinje neurons, some of the proteins involved in Ca^{2+} homeostasis also have been shown to be of muscle type, e.g. the ryanodine receptor in mouse [54], no clear-cut evidence for the presence of muscle-type CK isoforms in these cells have been produced so far, most likely due to a lack of specific antibodies against all mammalian CK isoenzymes. Based on immunohistochemical results, one group has claimed the presence of M-CK in rat cerebellar Purkinje neurons [58], whereas most of the other researchers working in the field have shown high immunoreactivity of Purkinje neurons with polyclonal [23, 27, 34] or monoclonal antibodies against B-CK [30]. Our antibodies against chicken CK isoenzyme are not very isoenzyme-specific for rat CK isoenzymes showing some cross-reactivity. Therefore, the question of whether the cerebellar Purkinje neurons and some of the hippocampal cells in rat, e.g. the granular cells of the dentate gyrus and the pyramidal cells (CA1-CA4), which all give rather strong signals with antibodies against B-CK (not shown) [23, 27], may also contain MM-CK or MB-CK [31, 32], remains open. The message for the muscle M-CK isoform could not be detected in rat brain by regular Northern blotting, but only after amplification via PCR [32]. The amount of M-CK in brain therefore, may depend on the species, sex, and age of the animal [31].

Thus, in all likelihood, mammalian Purkinje neurons, in contrast to the avian counterpart, express high concentrations of brain-type B-CK, but, based on the present data, may or may not coexpress also some muscle-type M-CK. The final resolution of this problem awaits further detailed analysis. Although mammalian Purkinje neurons, clearly undergoing Ca^{2+} spiking, etc., are likely to have similar physiological functions in the cerebellum as avian ones, the avian counterpart with its clear muscle-type isoform characteristics may represent an evolutionarily and functionally specialized system adapted for avian motoric learning.

CK in Granule and Pyramidal Cells of the Hippocampus

In rat, remarkably high amounts of CK, especially of Mi_a-CK at stage 12 days postnatally, were found in granular as well as pyramidal cells of the rat hippocampus

(fig. 4). This developmental stage was chosen here since at later stages, when mitochondria migrate into the cell processes, the staining was no longer as brilliant as shown here in figure 4. It is still a matter of uncertainty whether granule and pyramidal cells of the rat hippocampus contain brain-type B-CK [27; this work] or possibly also muscle-type M-CK [32]. In rats, the establishment of neuronal networks, involving Ca^{2+} signalling, long-term depression (LTD) (most prominent during the first 10 days after birth) and long-term potentiation (LTP; preferentially found in rats that are more than 2 weeks old) happens during the first 3 weeks of postnatal development [59]. Incidentally, during rat brain development, CK activity, especially that of Mi-CK, as well as CK-catalysed reaction flux, increased concomitantly by a factor of 4, between days 12 and 17 postnatally [34]. The maturational changes of brain function coincide with the development of the PCr/CK system in brain, particularly with Mi-CK, suggesting that CK may be central in regulation of brain ATP.

CK in the Choroid Plexus

High levels of CK were also found in the epithelial cells along the choroid plexus where B-CK and Mi_a-CK were identified as the nonmuscle complement of CK isoenzymes. Whereas B-CK has been found earlier in choroid plexus of rat brain [23, 27], figure 4 represents the first demonstration of the presence of high concentrations of Mi_a-CK in the choroid plexus epithelium, where high levels of Na^+/K^+-ATPase are accumulated in the apical plasma membrane facing the lumen of the brain ventricles. This distribution of the sodium/potassium-pump results in a ATP-dependent net sodium secretion from the blood supply into the ventricles of the brain, and the secretion of cerebrospinal fluid [60]. Since CK has been shown to be localized at the plasma membrane of *Torpedo* electrocytes [61] and to be directly functionally coupled to the Na^+/K^+-ATPase [7], it is likely that a PCr-shuttle mediated by B-CK and Mi_a-CK [2, 57] is also at work in the choroid plexus of the brain.

CK and the Creatine Transporter in the Brain

In contrast to muscle, brain possesses the capacity to synthesize at least some creatine [62]. This may explain why PCr concentration is less affected by feeding with creatine analogues, like GPA [63]. On the other hand, it was shown that cultured astroglial cells, but not neuronal cells, depend on a creatine-uptake mechanism [64]. A Na^+/creatine cotransporter has recently been cloned, identified and partially characterized by heterologous expression [65–67]. Interestingly, the overall localization and the regional relative expression levels of the creatine transporter in brain, as visualized by in situ hybridization [67, 68], are strikingly similar to those of CK, obtained by immunohistochemistry [27, 28, 34], indicating that cells containing high levels of CK, accordingly also express high levels of creatine transporter. Besides strong in situ hybridization signals for creatine transporter in cerebellum and hippocampus [68], prominent signals were also obtained with several white matter structures [67]. The latter finding argues for the presence of relatively high concentrations of CK also in white matter.

CK Function in Brain Development, Health and Disease: Potential Benefits of Creatine Supplementation as Adjuvant Therapy for Neurodegenerative Diseases

Several lines of evidence point to a critical involvement of CK and PCr in the adult central and peripheral nervous system (see above), as well as in embryonic and postnatal brain development, during which a coordinated expression and compartmentalized localization of CK isoenzymes on a regional, cellular as well as subcellular level have been observed [34, 69], which takes place concomitantly with functional maturation of the brain [26, 34]. Already in embryonal rat brain, a differential compartmentation of B-CK and Mi_a-CK is evident, especially in large cells of the olfactory neuroepithelium [69]. Interestingly enough, in highly proliferating fetal neuronal cells, an immunostaining for B-CK of cell nuclei was also observed [69], indicating, in accordance with earlier studies on neuronal cell cultures [20], that nuclear CK may be critical to support nuclear biosynthesis in proliferating fetal brain cells. An essential role for B-CK during early development is supported by the fact that, unlike with the other CK isoenzymes, no viable transgenic B-CK knock-out mouse could be made so far [Wieringa, University of Nijmegen, pers. commun.].

Complete creatine deficiency in the brain of a newborn child is associated with a severe extrapyramidal disorder, presenting with hemiballism, seizures and muscle weakness [70]. These symptoms largely disappeared upon daily administration of creatine.

Most of the patients with mitochondrial cytopathies show also defective brain energy metabolism accompanied by varying degrees of brain symptoms [71]. At the same time, many of these patients show ragged red fibers and a compensatory overexpression of Mi-CK, leading to crystallization of the enzyme and formation of intra-

mitochondrial inclusions which are highly enriched in Mi-CK [72]. The fact that cells with a chronically lowered energy state show an overexpression of CK, an enzyme involved in energy metabolism, is a further argument for the importance of CK for cellular energetics. Many neurodegenerative disorders of the brain as well as age-related diseases, are accompanied by a deterioration of the cellular energy status [73]. Energy deficit could lead to membrane depolarizations, increase in the intracellular free calcium concentration, and finally to cell death by apoptosis [74]. Therefore, it is tempting to speculate that maintaining a high intracellular phosphorylation potential, e.g. with creatine supplementation, by increasing total creatine and PCr levels in the brain [46], may be a beneficial adjuvant therapy for a number of such neurodegenerative and age-related diseases, as was recently reported in a single case study with a MELAS patient [75]. The present knowledge about CK localization and function, and the new data concerning the creatine transporter in brain [67], are the foundations for such a rationale for creatine supplementation of patients in the future.

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