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Research Report

Creatine promotes the GABAergic phenotype in human fetal spinal cord cultures

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ABSTRACT

In the present study, we investigated the expression pattern of cytosolic brain specific-BB-CK and ubiquitous mitochondrial-creatine kinases (uMt-CK) in developing human spinal cord. Consequently, we studied the effects of creatine treatment on cultured fetal human spinal cord tissue. We found that both CK isoforms were expressed in fetal spinal cord at all time points investigated (5 to 11.5 weeks post conception) and correspondingly specific CK activity was detected. Chronic creatine exposure resulted in significantly higher densities of GABA-immunoreactive neurons in the cultures, while total neuronal cell density was not altered, suggesting a differentiation inducing mechanism of creatine supplementation. Taken together, our observations favour the view that the creatine phosphocreatine system plays an important role in the developing CNS.

1. Introduction

Creatine is a substrate for the different isoforms of creatine kinases (CK's). In the adult central nervous system of mammalian species, cytosolic brain specific creatine kinase (BB-CK) and ubiquitous mitochondrial creatine kinase (uMt-CK) are predominantly abundant. The phosphocreatine (PCr)/CK's system plays a key role in modulating ATP metabolism by increasing PCr in the cell and enhancing the function of a cellular energy shuttle, coupling sites of ATP production and ATP consumption (for review see Hemmer and Wallimann, 1993; Wallimann and Hemmer, 1994; Schlattner et al., 2006). We have previously shown that creatine treatment provides neuroprotection against various toxic insults in fetal rat ventral mesencephalic and striatal cultures (Andres et al., 2005a,b,c). In addition, we observed that creatine promoted the GABAergic phenotype of cultured striatal neuronal precursors (Andres et al., 2005b). Creatine supplementation has also been reported to have protective effect after spinal cord injury (Hausmann et al., 2002) and in transgenic mouse-model of amyotrophic lateral sclerosis (Dupuis et al., 2004; Klivenyi et al.,

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Abbreviations: ATP, adenosine triphosphate; BB-CK, dimeric cytosolic brain-type CK present in brain and neuronal, as well as other non-muscle tissues; ChAT, choline acetyltransferase; CK's, creatine kinases; CNS, central nervous system; DAB, metal-enhanced 3,3′-diaminobenzidine; DIV, days in vitro; DMEM, Dulbecco's modified Eagle medium; GABA, γ-aminobutyric acid; ir, immunoreactive; MAP2, microtubule-associated protein 2; PBS, phosphate-buffered saline; p.c., post conception; PCr, phosphocreatine; SDS, sodium dodecylsulfate; S.E.M., standard error of the mean; TBS, Tris-buffered saline; uMt-CK, ubiquitous MtCK present in brain and neuronal, as well as other-non-muscle tissues

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2004a; Zhang et al., 2003). Improving cellular energy metabolism by creatine administration may therefore offer a possible way to reduce neuronal deterioration during spinal cord injury and to influence neuronal differentiation and survival in the context of cell replacement strategies. In embryonic rat brain tissue, BB-CK contents have been described to decrease slightly during embryonal development in utero and to reach adult levels quickly during the early postnatal period (Chen et al., 1995). These data are in line with findings of an earlier report on functional maturation of CK's in rat brain (Holtzman et al., 1993). Little is known, however, about the expression, function and distribution of CK's during development in human brain and spinal cord.

The present study was designed to investigate the expression levels and isoenzyme composition of CK's in the human spinal cord during development with special emphasis on the phenotypical characterization of those spinal cord cells that are expressing CK's. Moreover, we examined the effects of creatine exposure on survival and differentiation of cultured human spinal cord precursor cells.

2. Results

2.1. *Expression of both non-muscle creatine kinase isoenzymes in fetal human spinal cord*

Immunohistological detection for cytosolic BB-CK and mitochondrial uMt-CK in tissue sections of human spinal cord showed widespread distributed immunoreactivity for BB-CK and uMt-CK predominantly in the gray matter, as demonstrated by Nissl staining and immunohistochemistry for microtubule-associated protein 2 (MAP2) a neuronal cytoskeleton marker (Fig. 1).

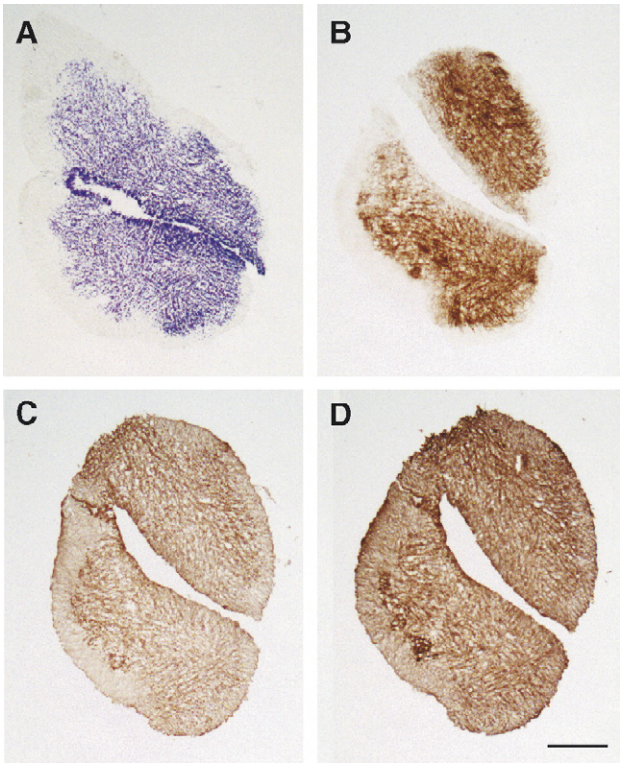

Fig. 1 – Representative photomicrographs of sections from fetal human spinal cord (8.5 weeks p.c.) immunocytochemically stained for BB-CK (C) and uMt-CK (D). Sections were Nissl stained (A) and immunohistochemically for MAP2 (B) to demonstrate the general cellular structure of the tissue. Scale bar: 100 μm.

2.2. *Expression and activity of creatine kinases in developing human spinal cord tissue*

Western blot analysis revealed that both BB-CK and uMt-CK were expressed at all stages of embryonic development studied (Fig. 2). Analysis of protein level revealed that BB-CK showed a slight tendency ($r^2=0.092$; $p=0.12$) to decrease with age (Fig. 3A), while uMt-CK (Fig. 3B) showed no significant variation of expression during this developmental period ($r^2=0.003$; $p=0.86$). Similarly, we detected specific CK activity in all samples studied with rather high levels at early stages and a significant decrease thereafter ($r^2=0.457$; $p<0.05$) (Fig. 3C). CK specific activity showed a non-significant tendency ($r^2=0.274$; $p=0.15$) to correspond with BB-CK levels (Fig. 3). Both isoforms of creatine kinase and corresponding specific CK activity were also detected in other regions investigated of the developing human CNS, i.e., rhombencephalon, ventral mesencephalon, ganglionic eminence and cerebral cortex (data not shown).

2.3. *Effects of chronic creatine exposure on density of GABA-ir neurons and on total neuronal cell in human spinal cord cultures*

First, by means of double-immunolabeling, it was demonstrated that GABA-immunoreactive (-ir) neurons expressed BB-CK and that all BB-CK-ir cells also coexpressed uMt-CK (Fig. 4A). Moreover, it was found that choline acethyl-transferase (ChAT)-ir neurons colocalized with both creatine kinases (Fig. 4B). Whether these ChAT-ir cells are motoneurons is not possible to finally state. We assume that different subsets of cholinergic neurons were present in our cultures with a predominance of motoneurons.

Importantly, it was observed that chronic creatine treatment resulted in significantly higher densities of GABA-ir neurons as compared to controls (by 40%; $n=29$ cultures, 8 independent experiments) (Fig. 5). The creatine induced effect was not dependent on developmental stage of spinal cord tissue as significant differences in density of GABA-ir neurons were seen in cultures from tissue taken before 6 weeks p.c. and more than 9 weeks p.c. Interestingly, creatine treatment did not affect the total number of neuronal cells in the cultures ($n=6$ cultures; three independent experiments) (Fig. 5D).

3. Discussion

This report represents (to our knowledge) the first measurements of specific CK activity, as well as a comparison of BB-CK and uMt-CK expression levels in the developing human spinal cord. While uMt-CK levels remained unchanged, we observed that BB-CK levels and the CK activity showed a slight tendency to decrease over time. These findings suggest that the CK isoforms may play a distinct role during the process of spinal cord development. Coincidentally, we found both isoforms of

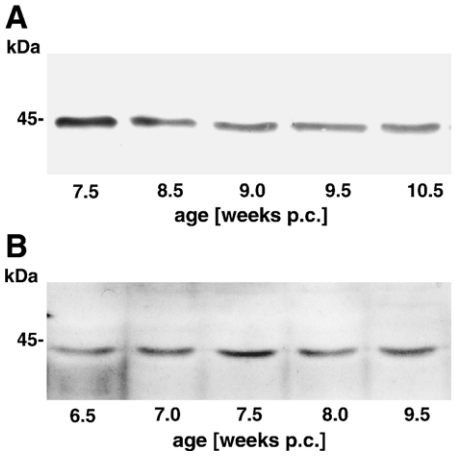

Fig. 2 – Representative Western blots for cytosolic brain-specific (BB-CK) (A) and ubiquitous mitochondrial (uMt-CK) (B) creatine kinases in human fetal spinal cord.

CK's to be expressed in the human spinal cord cell cultures. These findings let us to assume that the PCr/CK system is operational in developing spinal cord and that exogenous creatine administration should be effective in improving their metabolic state.

Developmental changes in CK activity and CK expression have been studied in detail in the rat brain (Holtzman et al., 1993). The authors reported that CK-catalyzed reaction rate and uMt-CK expression levels increased 4-fold between postnatal days 12 and 17, whereas BB-CK was found to be active earlier and increased steadily over a longer time course. The level of expression of CK's may reflect the endogenous need of cells for a reservoir of high-energy phosphate and, therefore, characterize neurons with high and fluctuating energy requirements (Wallimann et al., 1992). In another study, a decrease in uMt-CK expression has been described during the early postnatal period in cortex and hippocampus of mice with persisting high levels of expression of uMt-CK in restricted subsets of neurons that continue development postnatally suggesting that uMt-CK plays an important role during the developmental maturation of neuronal cells and that changes in energy demands induce uMt-CK expression (Boero et al., 2003).

We observed that creatine treatment resulted in a significant increase in the density of GABA-ir neurons in human spinal cord cultures, while total neuronal cell number was not affected. These findings strongly point to an induction of a differentiation process towards the GABAergic phenotype and are in agreement with the outcome seen for creatine treatment in fetal rat striatal (Andres et al., 2005b) and spinal cord cultures (Ducray et al., submitted). Moreover, expression of BB-CK was reported as highly dynamic, with the enzyme frequently being expressed in specific cells for a short period only, indicating cell-type specific function in zebrafish embryo (Dickmeis et al., 2001). Based on these findings, the authors suggested that BB-CK may be involved in creatine phosphate metabolism related to differentiation of neurons in the spinal cord (Dickmeis et al., 2001). The fact that transgenic CK double knock-out mice that do no longer express BB-CK and uMt-CK show a rather severe brain- and central nervous system-related phenotype (Streijger et al., 2005), indicates that CK is important for normal brain development and function. This is corroborated by the phenotype of recently discovered patients with a creatine deficiency syndrome, who present with severe neurological symptoms (Kleefstra et al., 2005).

At present it seems that there are various mechanisms by which neurons respond to increased creatine levels (Brewer and Wallimann, 2000; Brustovetsky et al., 2001; Friedman and Roberts, 1994; Wallimann et al., 1999). There is evidence for a direct anti-apoptotic effect of elevated cellular creatine levels by preventing or delaying mitochondrial permeability transition pore opening (Dolder et al., 2003). Evidence that creatine mediated neuroprotection can occur independent of changes

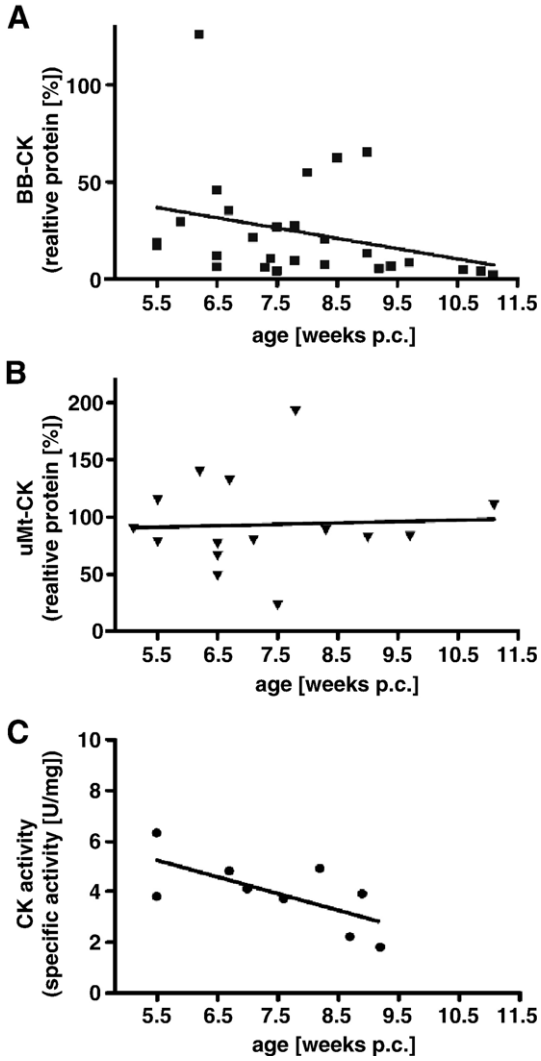

Fig. 3 – Developmental expression of BB-CK (A, filled squares), uMt-CK (B, filled triangles) and levels of CK activity (C, filled circles) in human fetal spinal cord. BB-CK and uMt-CK expression levels, as assessed using digital quantification of immunoblots, are presented as relative intensity of the particular bands as compared to total protein and CK activity as U/mg/time.

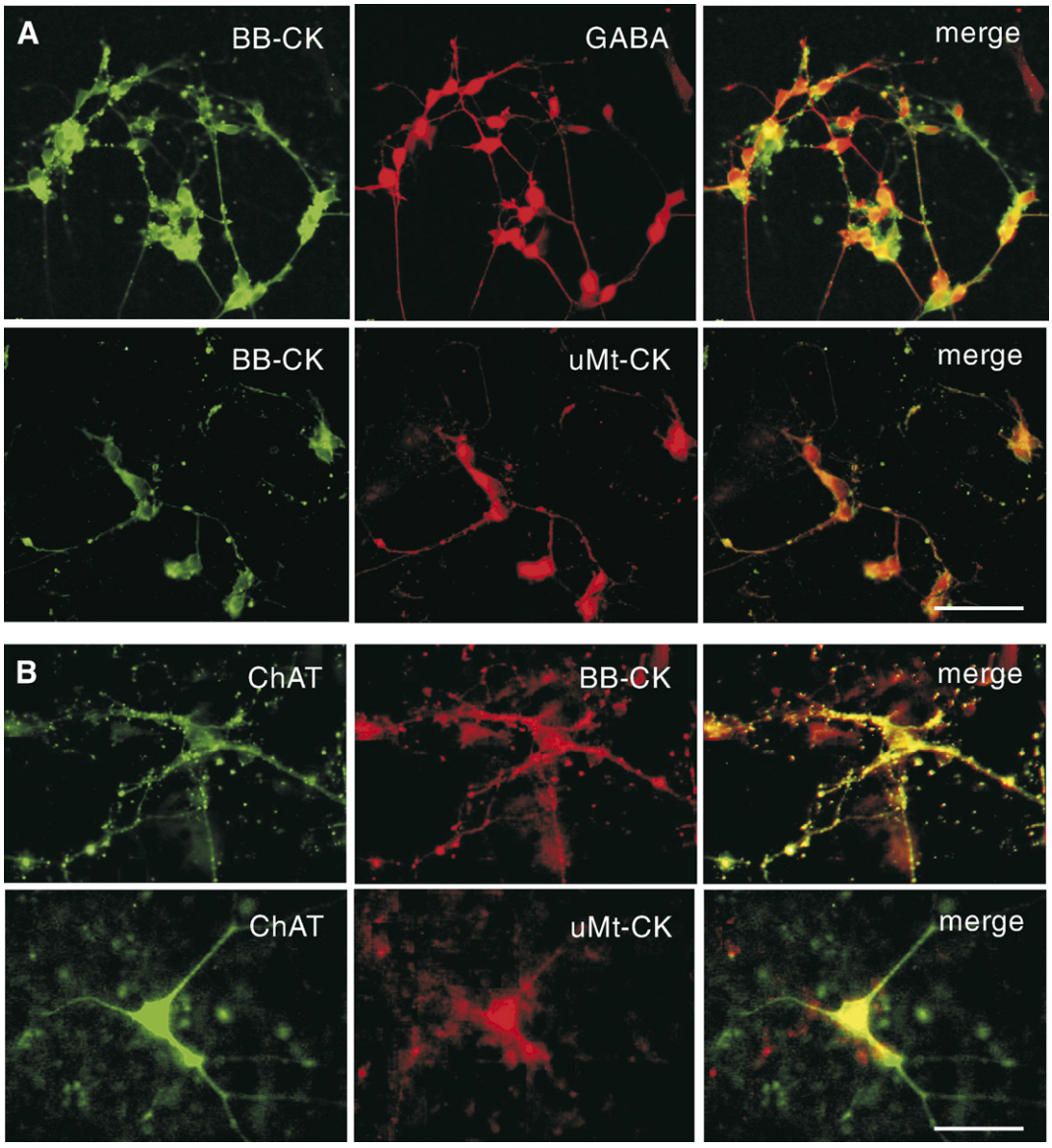

Fig. 4 – Digitalized photomicrographs of double immunofluorescence for BB-CK and GABA or uMt-CK (A) and ChAT and BB-CK or uMt-CK (B) in untreated control cultures from human fetal spinal cord (7 weeks p.c.). Scale bars: 50 μm.

in the bioenergetic status of brain tissue was recently reported (Prass et al., 2006). Nevertheless, Klivenyi and co-workers demonstrated neuroprotective mechanism of creatine in mice deficient in uMt-CK (Klivenyi et al., 2004b). More recently, it was shown that creatine is also released in an action-potential dependent exocytotic manner suggesting a role as a neuromodulator (Sakellaris et al., 2006), and that creatine provided remarkable prevention of complications related to traumatic brain injury in children and adolescents (Almeida et al., 2006).

Finally, there is great interest in cell replacement strategies to repair the damaged central nervous system. Embryonic spinal cord tissue transplants are a rich source of neural-restricted and glial-restricted progenitors and it has been shown that grafted cells can survive, migrate, and differentiate into neurons and oligodendrocytes (Nakamura et al., 2005). In certain motoneuron diseases, lower motoneurons are destroyed and muscle function cannot be restored except by reinnervation from alternate motoneuron sources. Interestingly, reduced CK activity has been reported in transgenic amyotrophic lateral sclerosis mice (Wendt et al., 2002). Our observation that BB-CK was expressed in choline acetyltransferase-ir neurons in cultured tissue suggests that concurrent creatine treatment may offer a way for enhanced functional capacity of transplanted neurons.

In sum, the presence of both CK isoforms and the demonstration of specific CK activity indicates that developing human spinal cord possesses an operational PCr/CK system. In line with this notion, our findings show that creatine exposure promotes the differentiation of cultured fetal human spinal cord GABAergic neurons. These data suggest that creatine may play an important role in cell fate decision during development of human spinal cord

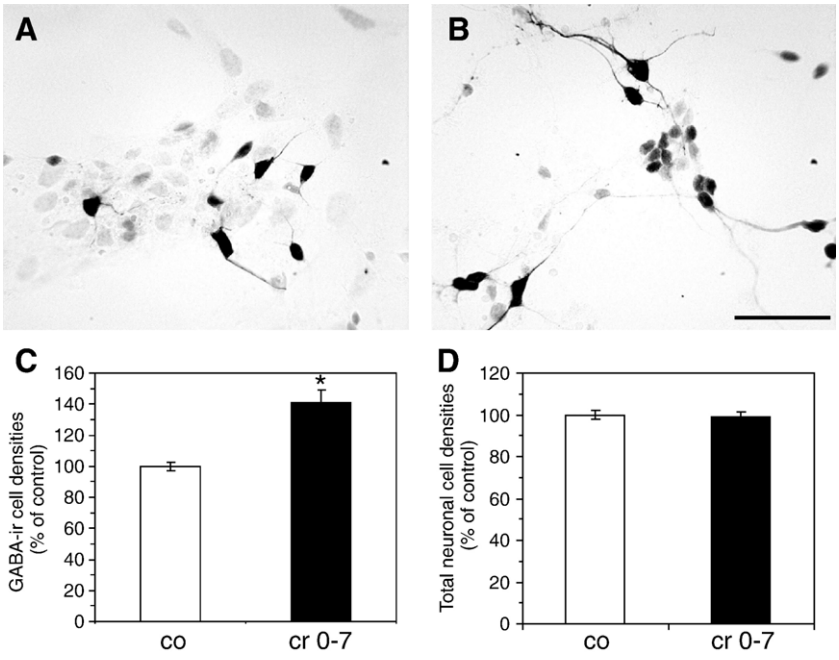

Fig. 5 – Effects of creatine supplementation on the density of GABA-ir neurons and on total neuronal cell density. Spinal cord cultures were grown for 1 week in absence (co, open bar) or presence of creatine [5 mM] (cr 0-7, filled bar). The representative photomicrographs show GABA-ir neurons in control (A) and creatine treated cultures (B). Scale bar: 100 μm. GABA-ir cells in creatine-free control cultures were found to represent around 15% of total neuronal cells. Quantitative analyses revealed that significantly higher GABA-ir cell densities were found after creatine treatment as compared to controls (C). Note that total neuronal cell densities were not affected by creatine treatment (D). Data are expressed as percentage of corresponding untreated controls and are given as mean ± S.E.M. * Indicates significant difference ($p < 0.05$) vs. corresponding control.

neurons. Moreover, the present observations may also have implications for cell replacement strategies after injury or in neurodegenerative disorders.

4. Experimental procedures

4.1. *Preparation of human fetal CNS tissue*

Human spinal cord tissue fragments were collected in a sterile collector assembly as described earlier (Spenger et al., 1994; Widenfalk et al., 1999). Written informed consent was given by the women seeking abortions and the experiments were approved by the Ethics Committee of the State Bern, Switzerland. The embryonic age of the specimens ranged from 5.0 to 11.5 weeks post conception (p.c.) determined as previously published (Evtouchenko et al., 1996; Widenfalk et al., 1999). Recovery of the nervous tissue required between 60 and 120 min. Identification and dissection of CNS tissue was performed under microscopic guidance.

4.2. *Tissue processing and immunohistochemistry*

Tissue was fixed in 4% paraformaldehyde in 0.1 M PBS, washed in PBS and equilibrated in 15% sucrose/PBS. 20 μm sections were prepared on a freezing microtome (Leica CM1900), thawed to gelatine chrome-alum precoated glass slides and immunohistochemically stained as previously described (Mattaredona et al., 2003). In brief: Tissue was blocked for 60 min with PBS containing 0.1% Triton X-100 and 10% horse serum. After rinses in PBS, sections were incubated overnight at 4 °C with the primary antibodies (mouse monoclonal anti-human BB-CK, 1:1000 (kindly provided by Dr. B. Wieringa, University of Nijmegen; Sistermans et al., 1995), rabbit polyclonal anti-human uMt-CK, 1:1000 (Schlattner et al., 2002), monoclonal anti-MAP2, 1:2500 (Boehringer Mannheim) diluted in a solution consisting of 0.1% Triton X-100 in PBS plus 2.5% horse serum. Bound antibodies were visualized using the Vectastain method (Vector Labs, Burlingham, CA, USA) in combination with a metal-enhanced 3,3′-diaminobenzidine (DAB) substrate kit (Pierce, IL, USA). Sections were dehydrated in alcohol, cleared in xylene and mounted in Eukitt.

4.3. *Western blotting for CK isoforms*

Expression of CK proteins was determined by immunoblot analyses. Spinal cord tissue ($n = 27$) was frozen on dry ice and finely pulverized (1 min, 2000 rpm) using a Micro-Dismembrator (B. Braun Biotech International). The resulting powder was resuspended in ice-cold extraction buffer containing 50 mM Na-phosphate pH 9.0, 10 mM beta-mercaptoethanol, 0.05% Triton X-100 and a protease inhibitor mix (concentration according to manufacturer recommendations, Boehringer). The homogenate was centrifuged at 13,000 rpm for 5 min at 4 °C. The resulting supernatant was used for

Western blotting and protein content determination using the Bio-Rad™ protein assay (Andres et al., 2005b). The supernatant was diluted in 50% sample buffer containing 0.2 M Tris–HCl, pH 7.0, 30% glycerol, 6% sodium dodecyl sulfate (SDS), 15% beta-mercaptoethanol and 0.0075% bromphenol blue were added and this solution incubated at 95 °C for 5 min. SDS-PAGE was performed using a 12% SDS-polyacrylamide separating gel (10–15 μg protein per lane). Proteins were blotted using the semi-dry technique on a 0.45 μm nitrocellulose membrane (BA85; Schleicher and Schüll) previously equilibrated in blotting buffer. Electrophoretic transfer was performed at a constant current of 120 mA during 45 min in blotting buffer consisting of 39 mM glycine, 48 mM Tris, 0.375% SDS and 20% methanol (Andres et al., 2005b). After rinsing in TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl) for 5 min, the blots were blocked for 1 h with 5% fat-free milk powder in TBS and incubated with the primary antibodies (mouse monoclonal anti-BB-CK, 1:1000, rabbit polyclonal anti-uMt-CK, 1:1000; Andres et al., 2005b) for 60 min at 22 °C in the same solution. The blots were washed twice in TBS containing 0.2% Tween-20 (Merck) and subsequently incubated with affinity purified HRPO-coupled secondary antibodies (donkey anti-mouse or donkey anti-rabbit, 1:20,000; Jackson Immunoresearch) in TBS containing 5% milk powder for 45 min at 22 °C. Blots were washed three times in TBS containing 0.2% Tween-20 and incubated for 1–5 min in chemiluminescent substrate (Western Lightning, Perkin-Elmer Life Science, Switzerland). Amplified chemiluminescence was detected and quantified using a digital imaging system (ImageStation; Kodak, USA).

4.4. *Analysis of CK-specific activity*

CK activity was determined with a photometer using a coupled enzyme assay (Schlattner and Wallimann, 2000). Briefly, generation of ATP by the CK reverse reaction was coupled by hexokinase (300 U/ml) and glucose-6-phosphate dehydrogenase (150 U/ml) to NADPH production, using 2 mM ADP, 5 mM $MgCl_2$, 20 mM PCr, 40 mM D-glucose and 1 mM NADP in 0.1 M triethanolamine buffer pH 7. The amount of enzyme activity required to reduce 1 μmol NADP per minute or to oxidize 1 μmol NADH per minute has been defined as 1U. The pH of the reactions were chosen in analogy to previous studies and according to established pH-optima. The Mg-salt concentrations were sufficient to maintain ADP and ATP entirely in their Mg^{2+}-complexed form. Changes in redox state of pyridine nucleotides were followed at 340 nm in a UV4 spectrophotometer (Unicam, Cambridge, UK) at 25 °C. Blank reaction activities were recorded before–and specific CK activities after–addition of PCr substrate.

4.5. *Spinal cord cultures and cell count analyses*

Using microscopic guidance, the spinal cords (from 9 embryos, aged 5 to 10 weeks p.c.) were dissected in cold DMEM (Gibco) (Kleitman et al., 1998), the dorsal root ganglia and meninges carefully removed and cell suspension prepared by means of mechanical dissociation in DMEM (Andres et al., 2005b; Hausmann et al., 2002). Aliquots of 2.4×10^5 cells were pipetted per 24-well containing poly-L-lysine coated cover-slips. The cells were grown for 7 days in vitro (DIV) in culture medium consisting of 55% DMEM, 32.5% Hank's balanced salt solution (HBSS; Gibco), 0.3% glucose, 10% fetal calf serum (Gibco) and 1% 0.01 M HEPES (Merck) in absence (controls) or presence of creatine [5 mM]. The concentration of 5 mM was chosen due to the maximal effects found on survival of GABA-ir neurons in striatal cultures (Andres et al., 2005b). Furthermore, Brustovetsky and co-workers reported creatine concentrations of 3 mM and 10 mM to exert neuroprotection against an energetic insult in hippocampal and striatal cultures, while lower doses turned out to be ineffective (Brustovetsky et al., 2001). Antibiotics/antimycotics (No. 061-05240 D; Gibco) were present during the first 2 days in culture. The plates were incubated at 37 °C in a 95% air/5% CO_2 humidified atmosphere. Medium change took place at DIV2 and thereafter every other day. At DIV7, the cultures were fixed in 4% paraformaldehyde in 0.1 M PBS and after a brief wash in PBS, incubated overnight at 4 °C with primary antibodies (rabbit polyclonal anti-GABA, 1:5000 (Sigma); mouse monoclonal anti-human BB-CK, 1:500; rabbit polyclonal anti-human uMt-CK, 1:500; polyclonal goat anti-ChAT, 1:500 (Chemicon) all diluted in PBS containing 0.05% Triton X-100 and 2.5% horse serum). Bound antibodies were visualized as described above. For double immunofluorescence analyses cultures were incubated for 2 h at room temperature with the proper mixture of secondary antibodies (Alexa Fluor anti-rabbit 594 nm, Alexa Fluor anti-mouse 488 nm, Alexa Fluor anti-goat 488 nm, 1:250 (Molecular Probes)). Cultures were then mounted in PBS containing 50% glycerol and examined under a Leitz epifluorescence microscope.

Quantification of neurons was performed in a blinded experimental design using bright field microscopy (Leica) as previously described (Andres et al., 2005b). Cell counts were performed in six randomly selected areas per well (Andres et al., 2005b). On average 300 GABA-ir neurons per single culture well were counted under control conditions. GABA-ir cells in creatine-free control cultures were found to represent around 15% of total neuronal cells in our set of experiments. For statistical analysis, a commercially available software package was used (Statistica 5.0; StatSoft). The experimental groups were compared using the two-sided non-parametric Mann–Whitney *U*-test. Pearson's coefficient of correlation (*R*) was calculated for CK isoforms levels and CK specific activity with developmental stage using a linear regression model for the quantification of correlations. Differences were considered statistically significant at $p<0.05$. Values are presented as mean ± S.E.M.

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