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Creatine Treatment Promotes Differentiation of GABA-ergic Neuronal Precursors in Cultured Fetal Rat Spinal Cord

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Creatine is a substrate of cytosolic and mitochondrial creatine kinases. Its supplementation augments cellular levels of creatine and phosphocreatine, the rate of ATP resynthesis, and improves the function of the creatine kinase energy shuttle. High cytoplasmatic total creatine levels have been reported to be neuroprotective by inhibiting apoptosis. In addition, creatine has direct antioxidant effects, which may be of importance in amyotrophic lateral sclerosis. In the present study, we investigated the effects of creatine [5 mM] on survival and differentiation of cultured GABA-immunoreactive (-ir) and choline acetyltransferase (ChAT)-ir rat spinal cord neurons. Furthermore, we addressed the neuroprotective potential of creatine supplementation against 3-nitropropionic acid (3-NP) induced toxicity. General cell survival and total neuronal cell density were not altered by chronic creatine treatment. We found, however, after chronic creatine and short-term creatine exposure a significantly higher density of GABA-ir neurons hinting to a differentiation-inducing mechanism of creatine. This notion is further supported by a significant higher content of GAD after creatine exposure. Creatine supplementation also exerted a partial, but significant neuroprotection for GABA-ir neurons against 3-NP induced toxicity. Interestingly, chronic creatine treatment did not alter cell density of ChAT-ir neurons but promoted their morphologic differentiation. Cell soma size and number of primary neurites per neuron were increased significantly after creatine supplementation. Taken together, creatine supplementation promoted the differentiation or the survival of GABAergic neurons and resulted in partial neuroprotection against 3-NP induced toxicity. The data suggest that creatine may play a critical role during development of spinal cord neurons.

Key words: creatine supplementation; neuroprotection; morphology; rat; GABA

Large amounts of energy are required to maintain the functional activities of the brain and nervous tissues, e.g., for maintenance of membrane potential, calcium homeostasis, neurotransmitter processing, intracellular signalling, and axonal as well as dendritic transport (Ames, 2000). Impaired energy homeostasis is suggested to contribute to neuronal deterioration in various neurological disease states (Tarnopolsky and Beal, 2001). Creatine is a substrate for cytosolic brain specific creatine kinase (BB-CK) and ubiquitous mitochondrial creatine kinase (uMt-CK) and thereby modulating ATP metabolism in the cell by increasing the phosphocreatine (PCr) pool (Woznicki and Walker, 1979; Wallimann and Hemmer, 1994) and enhancing the function of a cellular energy shuttle, coupling sites of ATP production and ATP consumption (Bessman and Geiger, 1981; Wallimann et al., 1989, 1992, 1999). Neurons are known to have a low capacity to synthesize creatine (Braissant et al., 2005), hence they rely on creatine supply from glia cells and from the blood stream (Tachikawa et al., 2004).

Uptake of creatine into the cells is accomplished by specific high-affinity, sodium and chloride-dependent creatine transporters (Nash et al., 1994). Exogenous creatine supplementation for 3 days has been reported to increase intracellular creatine and PCr levels (Holtzman et al., 1998) and to provide neuroprotection in several models of acute and chronic disease states (Klivenyi et al., 1999; Matthews et al., 1999; Brewer and Wallimann, 2000; Ferrante et al., 2000). A new window of creatine mediated effects has been opened by a very recent report that showed that creatine is not only synthesized and

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taken up by neurons of the central nervous system but also released in an action-potential dependent exocytotic manner (Almeida et al., 2006). This observation suggests that creatine can also play a role as a neuromodulator. Most recently, creatine was shown to protect cultured cells from hyperosmotic shock, by a significant increase of creatine uptake into cells, indicating that creatine can work as a compensatory osmolyte (Alfieri et al., 2006)

We have shown previously neuroprotective properties of creatine against toxic insults induced by 1-methyl-4-phenyl pyridinium (MPP+) (Andres et al., 2005c) or 6-hydroxydopamine (6-OHDA) treatment (Andres et al., 2005a) in rat ventral mesencephalic cultures. Creatine supplementation has also been shown to have protective effect after spinal cord injury (Hausmann et al., 2002; Rabchevsky et al., 2003) and in transgenic mouse-model of amyotrophic lateral sclerosis (ALS) (Klivenyi et al., 1999; 2004; Zhang et al., 2003; Dupuis et al., 2004), where the direct antioxidant properties of creatine may be implicated (Lawler et al., 2002). Mitochondrial defects and glucose use impairments are implicated in the pathogenesis of motor neuron degeneration in ALS but their precise contribution to the disease mechanism remains unclear. Nevertheless, a marked reduction in cerebral cortex ATP levels was detected in ALS mouse model well before symptom onset (Browne et al., 2006).

We showed recently that creatine promoted the GABA-ergic phenotype of cultured rat striatal neuronal precursors (Andres et al., 2005b). Improving cellular energy metabolism by creatine supplementation may furthermore offer a feasible approach not only to reduce neuronal deterioration during spinal cord injury, but also to influence neuronal differentiation and survival in the context of cell replacement strategies. Hence we examined in the present study the effects of creatine on survival and differentiation of neuronal precursor cells in tissue cultures of rat spinal cord.

MATERIALS AND METHODS

Preparation of Rat Cell Cultures

Rat fetuses staged at embryonic day (E) 14 (E0 = day of vaginal plug) were removed from deeply anaesthetized (ketamine 100 mg/kg b.w. and xylazine 10 mg/kg b.w.) pregnant Wistar rats (Janvier Breeding Center, France). Using microscopic guidance, the spinal cords were dissected in cold DMEM (Gibco, Carlsbad, CA) according to procedures described previously (Kleitman et al., 1998) with minor modifications, i.e., the dorsal root ganglia were removed carefully. Spinal cord tissue was rinsed with cold DMEM and cell suspension prepared by means of mechanical dissociation in DMEM (Andres et al., 2005b). For immunocytochemistry and viability assays, aliquots were pipetted (seeding density of 600 viable cells/mm^2) in 24-well plates (Multiwell 3047, 14-mm diameter per well; Falcon, Becton Dickinson, Franklin Lakes, NJ) containing glass coverslips of 12-mm diameter (Assistent, Germany). For immunoblot analysis, aliquots were pipetted (seeding density of 600 viable cells/mm^2) in 6-well plates (Multiwell 3046, 34 mm diameter per well; Falcon). Culture wells were coated with a solution of poly-L-lysine (0.1 mg/ml; Sigma, St. Louis, MO) in 0.15 M sodium borate buffer, pH 8.3 over night and washed three times with sterile PBS before adding culture medium. All animal procedures were approved by the Animal Research Ethics Committee of the Canton Berne, Switzerland and the University of Berne Animal Care and Use Committee, Switzerland.

Treatment Regimen

The cells were grown 7 days in vitro (DIV) in culture medium consisting of 55% DMEM (Gibco), 32.5% Hank's balanced salt solution (Gibco), 1.5% glucose, 10% FCS (Gibco), and 1% 0.01 M HEPES. Medium was changed at DIV2 and DIV5. Antibiotics/antimycotics (1:100 diluted cocktail of stock containing streptomycin [10,000 μg/ml], penicillin [10,000 U/ml], and amphotericin B [25 μg/ml]; Gibco 15240-062) were present for the first 2 days in culture (Spenger et al., 1995). Cells were incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere.

The culture wells were assigned randomly to each of the experimental groups: control, [5 mM] creatine chronic for 7 days, [5 mM] creatine for 2 days (DIV5–7). Untreated cultures served as controls. Neurotoxicity was brought about by adding 3-NP [5 mM] at DIV5. At DIV7, the cultures were either fixed for histologic assessments or further processed for viability assays and Western blotting.

MTT Assay

The water-soluble, yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of active mitochondria to purple formazan crystals that are insoluble in aqueous solution, and hence, are often used to quantify metabolic activity and cell survival in cultures (Rubinstein et al., 1990). In brief, the MTT (Sigma) solution (5 mg/ml in sterile PBS), prepared just before use, was added to the spinal cord cultures (final concentration = 0.5 mg/ml) for 4 hr at 37°C in the incubator. After aspiration of the medium, 500 μl DMSO was added to resolve the resulting MTT-formazan product (Widmer and Hefti, 1994). The culture plates were gently agitated on a shaker at room temperature for 10 min. For quantification, 500 μl of DMSO was added and after a good mix, the absorbance of the resulting supernatant was measured at 540 nm and read against DMSO in the linear range of the absorption curve using a microplate reader (Benchmark Plus; Bio-Rad, Hercules. CA).

Western Blotting

Expression of proteins was determined by immunoblot analyses carried out according to the method of Laemmli (Laemmli, 1970) with some modifications. Cultured spinal cord cells were rinsed twice with sterile ice-cold HBSS and lysis buffer containing leupeptin (1 μl/ml), phenylmethylsulphonyl fluoride (PMSF, 5 μl/ml) in HBSS was added to culture wells. The cells for each sample were scraped from a single 6-well culture dish and the cell suspension transferred in an Eppendorf tube and stored at −80°C. Lysates were sonicated for 1 min and then centrifuged for 5 min at 10,000 rpm

on a cooled Eppendorf centrifuge. The resulting supernatants were used for protein content determination according to the method of Bradford using the Bio-Rad protein assay (Bradford, 1976). The supernatants were diluted in 25% sample buffer containing 0.2 M Tris-HCl, pH 7.0, 30% glycerol, 6% sodium dodecyl sulfate (SDS), 15% β-mercaptoethanol, and 0.0075% bromophenol blue. This solution was incubated at 95°C for 5 min. SDS-PAGE was carried out using a 10 or 12% SDS-polyacrylamide separating gel with 10 μg of sample protein per lane. Proteins were blotted using the dry technique on a 0.2 μm Sequi-Blot PVDF membrane (Bio-Rad) equilibrated previously in methanol and then in blotting buffer. Electrophoretic transfer was carried out at a constant current of 120 mA during 1 hr in blotting buffer consisting of 39 mM glycine, 48 mM Tris, 0.375% SDS, and 20% methanol. Equal sample loading and transfer efficiency and were checked by Coomassie Blue staining of the gels and by reversible Ponceau S staining of the blotting membranes, respectively. After decolorization by rinsing twice in TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl) for 5 min, the blots were blocked for 1 hr with 5% fat-free milk powder, 0.2% Tween-20 (Merck, Darmstadt, Germany) in TBS, and incubated with the primary antibodies: mouse monoclonal anti-BB-CK, 1:1,000 (kindly provided by Dr. B. Wieringa, University of Nijmegen) (Sistermans et al., 1995); rabbit polyclonal anti-uMt-CK, 1:1,000 (Schlattner et al., 2002); mouse monoclonal anti-MAP-2, 1:500 (Sigma); rabbit polyclonal anti-glutamate decarboxylase 65/67 ($GAD_{65/67}$) (Chemicon, Temecula, CA); mouse monoclonal anti-β-III-tubulin, 1:2000 (Promega, Madison, WI); mouse monoclonal anti-NeuN, 1:700 (Chemicon); and mouse monoclonal anti-α-tubulin, 1:20,000 (Boehringer-Mannheim, Mannheim, Germany) overnight at 4°C in the same solution. The blots were washed twice in TBS containing 0.2% Tween-20 (Merck) and incubated subsequently with an affinity purified HRPO-coupled secondary antibody (donkey anti-mouse, 1:4,000 or 1:20,000; donkey anti-rabbit, 1:4,000 or 1:10,000; Jackson ImmunoResearch, West Grove, PA) in TBS containing 5% milk powder for 2 hr at room temperature. Blots were washed three times in 0.2% Tween-20 containing TBS and incubated for 1 min in chemiluminescent substrate (SuperSignal West Pico Trial Kit; Pierce, Rockford, IL). Amplified chemiluminescence was detected and films were scanned. Band densities were quantified using Image J software (NIH, Bethesda, MD). For normalization of data, total BB-CK and total uMt-CK data were normalized to α-tubulin data before expressing as mean ± SEM. All measurements were carried out in triplicates.

Immunohistochemistry

At the end of the culture period, cultures were washed twice in 0.1 M PBS (pH; 7.4) and fixed in 4% paraformaldehyde in 0.1 M PBS for 20 min at room temperature. After 4 × 10 min washes in PBS, cultures were preincubated for 60 min in PBS containing 0.1% Triton X-100 and 10% horse serum. After a brief wash in PBS, cultures were incubated overnight at 4°C with primary antibodies: rabbit polyclonal anti-GABA, 1:5,000 (Sigma); mouse monoclonal anti-BB-CK, 1:500; rabbit polyclonal anti-uMt-CK (Andres et al., 2005b), 1:500; polyclonal goat anti-ChAT, 1:500 (Chemicon); mouse monoclonal anti-β-III-tubulin, 1:1000 (Promega); mouse monoclonal anti-GFAP, 1:800 (Boehringer-Mannheim); mouse monoclonal 1:2500 anti-MAP-2 (Sigma); mouse monoclonal anti-NeuN, 1:500 (Chemicon) diluted in PBS containing 0.05% Triton X-100 and 2.5% horse serum. Subsequent to 3 ×15 min washes in PBS, cultures were incubated for 2 hr with biotinylated secondary antibodies (anti-mouse, anti-rabbit, or anti-goat antibodies; Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS containing 0.05% Triton-X-100 and 2.5% horse serum. Cultures were then washed in PBS (3 × 10 min) and incubated with an avidin-biotin conjugate of peroxidase (Vector Laboratories) for 60 min. Visualization of tissue bound antibodies was carried out using a metal-enhanced 3,3′-diaminobenzidine substrate kit (Pierce). After a wash in PBS cultures were rinsed in aqua destillata, the glass slides removed from the culture wells and embedded in Aquamount (BDH, Poole, UK) on regular glass slides for light microscopy. For double immunofluorescence analyses cultures were incubated with mixtures of primary antibodies as described above and exposed subsequently for 2 hr at room temperature to a proper mixture of secondary antibodies (Alexa Fluor anti-rabbit 594 nm, Alexa Fluor anti-mouse 488 nm, 1:250; Molecular Probes, Eugene, OR). Cultures were then mounted in PBS containing 50% glycerol and examined under a Leitz epifluorescence microscope.

Quantification of Cells

Quantification of GABA-ir neurons in the cultures was carried out using bright field microscopy (Leica, Heerbrugg, Switzerland). Cell counts were carried out in six areas selected randomly per well (×10 objective) (Andres et al., 2005b). To be quantified, cells had to fulfill the following criteria: 1) show a dark staining pattern well above that determined in negative control cultures; 2) have at least one visible process; and 3) a well-defined pericaryon. Cell counts were done in a blinded experimental design (Schaller et al., 2005). The variation in cell densities between different cultures based on SD was 13%. Mean densities in control cultures for GABA-ir and ChAT-ir neurons were 76.8 ± 6.7 cells/mm^2 and 0.16 ± 0.02 cells/mm^2 (mean ± SEM), respectively (n = 11–18 cultures from three to five independent experiments). For assessment of total neuronal cell number, randomly chosen visual fields (six per culture) in each of the cultures from control cultures were examined using phase contrast microscopy (×40 objective) (Andres et al., 2005b). Because we detected 722.4 ± 21.3 cells/mm^2 total neuronal cells (mean ± SEM), GABA-ir cells represented about 10% and ChAT-ir cells about 0.2‰ of total neurons in our cultures, respectively.

Morphometric Analyses

GABA-ir neurons (n = 96 cells per group) and ChAT-ir neurons (n = 47 cells per group), selected randomly in control and creatine treated [5 mM] cultures (8–10 cultures per group; 12 cells per culture for GABA-ir neurons and 8–11 cultures per group; 13–17 cells per cultures for ChAT-ir neurons), were analyzed (two to three independent experiments). In brief, for quantitative morphometry only clearly visible pri-

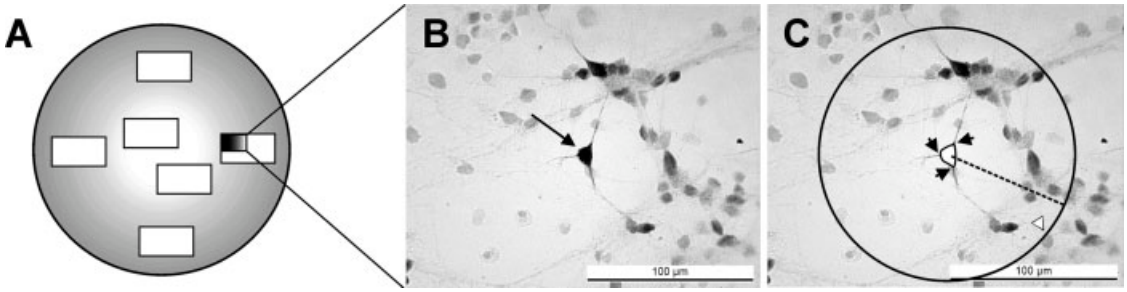

Fig. 1. Schematic drawing indicating the six randomly chosen areas of 0.9 mm^2 (boxes) for morphologic analysis of neurons (**A**). The photomicrographs show an individual GABA-immunoreactive neuron (arrow, **B**) and visualize how the different morphologic parameters were analyzed (**C**). The total number of primary neurites emerging from the cell body (arrows) were counted, the cell soma area outlined (white), and the longest process of the individual GABA-ir neuron identified (white arrowhead) and the length measured as depicted by the dotted line (C).

mary neurites were included for measurements when they could be undoubtedly assigned to single neurons. The Lucia Neuron Tracing System (Nikon) was used in combination with a Leitz microscope (×20 objective). GABA-ir neurons selected randomly in control and creatine-treated cultures were analyzed. The following parameters were investigated for all neurons: cell soma area [μm^2], number of primary neurites emerging from the cell body [n] and the neurite length delineated by the longest process of the individual neuron [µm] (indicated by the white arrowhead in Fig. 1C) was identified by means of a radius measure (Ducray et al., 2006). In case that process length exceeded the visual frame the visual field was adjusted (Fig. 1).

GABA-ir neurons from control cultures (192 cells analyzed from 14 cultures, three independent experiments) showed a mean cell soma area of 93.3 ± 1.8 μm^2, 3.2 ± 0.1 primary neurites emerging from the cell body, and a mean neurite length/neuron of 86.0 ± 2.1 µm (mean ± SEM). The variability between different cultures was as follows: SD in percentage of mean for soma size, longest process and number of primary neurites per neurons was 15.6%, 19.2%, and 13.8%, respectively, in control cultures. ChAT-ir neurons from control cultures (47 cells analyzed from 11 cultures, three independent experiments) showed a mean cell soma area of 163.3 ± 8.4 μm^2, 3.6 ± 0.2 primary neurites emerging from the cell body and a mean neurite length/neuron of 118.4 ± 4.5 µm (mean ± SEM).

Statistical Evaluation

Statistical analysis of cell densities and morphometric analyses were carried out by using a software package (Statistica 5.0; StatSoft, Tulsa, OK) available commercially. The experimental groups were compared using analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test. Morphologic parameters were analyzed using the two-sided non-parametric Mann-Whitney *U*-test. Differences were considered statistically significant at $P < 0.05$. Values are presented as mean ± SEM.

RESULTS

Expression of BB-CK and uMt-CK Isoenzymes in Rat Spinal Cord Tissue and In Vitro

Both isoforms of CK, i.e., cytosolic brain-specific (BB-CK) and the mitochondrial (uMt-CK) isoforms, were found to be expressed in rat spinal cord tissue at the day of dissection. Notably, only a faint staining was observed in white matter (Fig. 2E,F). Both CKs were also found to be expressed in cultured spinal cord neurons as assessed by colocalization analyses for BB-CK and uMt-CK with the neuronal markers microtubule-associated protein 2 (MAP-2) and neuronal nuclei (NeuN) (Fig. 3A–D). In accordance, Western blot analysis illustrated signals for both CK isoforms (Fig. 4A). As compared to controls, creatine treatment resulted in significantly higher levels of uMt-CK in spinal cord cultures (Fig. 4B). In contrast, expression levels of BB-CK did not differ significantly between groups (Fig. 4B).

Effects of Creatine Supplementation on Expression of MAP-2, NeuN, and β-III-Tubulin in Spinal Cord Cultures

Chronic and 2-day creatine exposure resulted in an increased expression levels of MAP-2, a general marker for neurons that is reported to represent a dendritic and somal marker (Matus et al., 1986) (Fig. 5A). Notably, the rate of increase differed markedly between the experiments, which resulted in a rather high variation with an outcome of 210 ± 82% (mean ± SEM). In line with this observation, MAP-2-positive neurons were stained more heavily in creatine-treated cultures as compared to controls (Fig. 5B). This finding likely accounted for the higher densities of MAP-2 positive neurons detected after chronic creatine treatment with 100.0 ± 8.2% and 137.3 ± 9.2% for control and creatine treatment, respectively (mean ± SEM; n = 7 from two independent experiments). The creatine treatment also resulted in an increase of expression levels of neuronal nuclei (NeuN) (Fig. 5A). Appearance of the marker protein NeuN is coincident with neuronal differentiation and the cells exit from the

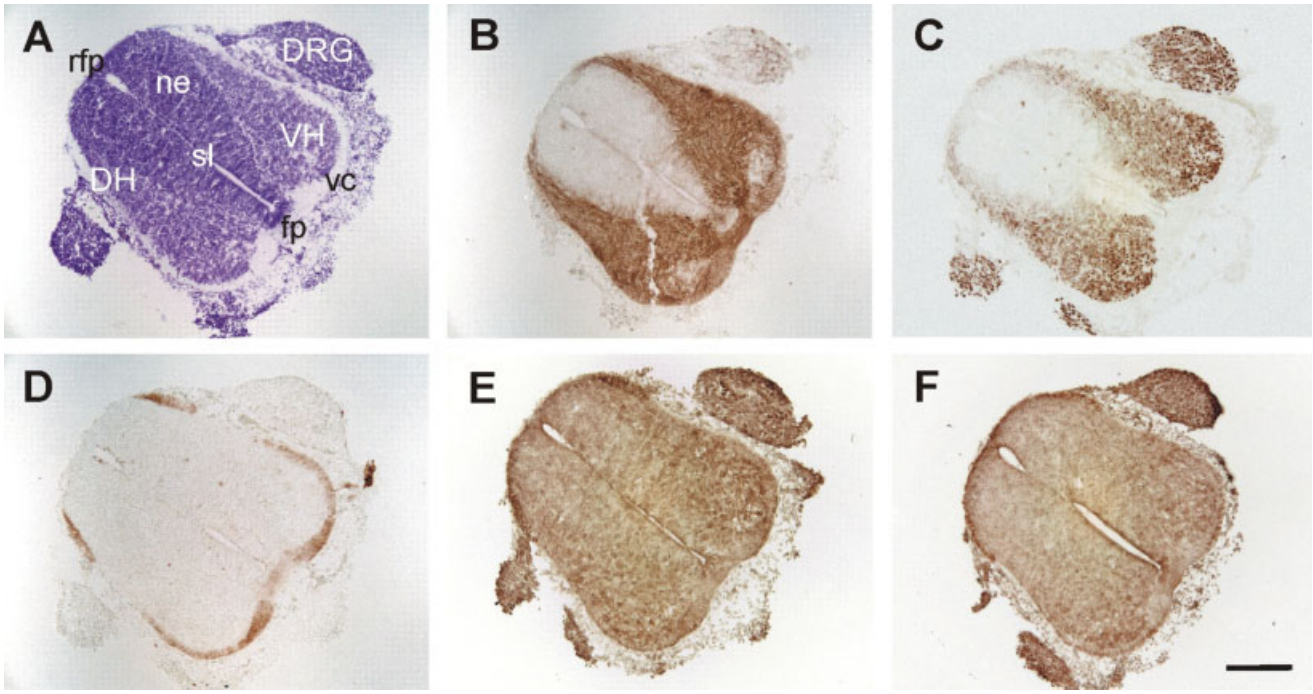

Fig. 2. Digitalized photomicrographs of sections from E14 rat spinal cord stained for Nissl (**A**), microtubule-associated protein 2 (**B**), neuronal nuclei (**C**), neurofilament 200 kD (**D**), ubiquitous mitochondrial creatine kinase (**E**), and cytosolic brain specific creatine kinase (**F**). Scale bar = 200 μm. DH, dorsal horn; DRG, dorsal root ganglion; fp, floor plate of s.c. neuroepithelium; ne, neuroepithelium; rfp, roof plate of s.c. neuroepithelium; sl, sulcus limitans; vc, ventral commisure of s.c.; VH, ventral horn.

cell cycle (Mullen et al., 1992). In contrast, no differences were detected for β-III-tubulin protein levels between groups (Fig. 5A). It has been reported that neuronal precursor cells (NPC) plated for differentiation undergo a sequential pattern of protein expression recapitulating the developmental differentiation or regeneration of neurons. The sequential expression of nestin, and then β-III-tubulin show the first steps of differentiation of these NPC into more mature neurons (Gingras et al., 2007). Similarly to the effects seen for β-III-tubulin, unchanged levels for the astroglial marker glial fibrillary acidic protein (GFAP) were observed (data not shown).

Effects of Creatine Supplementation on General Survival in Spinal Cord Cultures

Creatine treatment did not affect the total number of neuronal cells in the cultures with 100.0 ± 6.6% and 100.7 ± 4.9% for control and creatine treatment, respectively (mean ± SEM; n = 20 from two to three independent experiments). Similarly, creatine did not alter measures of the MTT assay with 100.0 ± 0.4% and 98.5 ± 1.8% for control and creatine treatment, respectively (mean ± SEM; n = 12 from two independent experiments). Semi-quantitative measures showed no difference in cell density of β-III-tubulin and GFAP positive cells for control and creatine-treated cultures (Fig. 6). Total protein levels were found to be increased significantly in the creatine-treated cultures (by 1.3-fold) (data not shown).

Effects of Creatine Supplementation on Cell Densities and Morphology of GABA-ir Neurons in Spinal Cord Cultures

Western blot analysis showed an upregulation (1.2-fold) in expression of glutamate decarboxylase 65 (GAD_{65}), after chronic creatine treatment, whereas no effect was observed for GAD_{65} after creatine exposure from DIV5–7 (Fig. 5A). Signal intensity for GAD_{67} did not differ between all groups studied (Fig. 5A). Chronic creatine treatment resulted in significantly higher densities of GABA-ir neurons as compared to control (Fig. 7). Interestingly, and in contrast to what was observed in the Western blots for GAD levels, a significant increase was also seen when creatine was present only for 2 days, i.e., from DIV5–7 (Fig. 7C).

The analyses of morphologic parameters of GABA-ir neurons showed no significant differences between the chronically creatine-treated group and untreated controls. Soma size and neurite length were unaltered, whereas a moderate 8.9% lower number of primary neurites/neuron was detected (Table I).

Effects of Creatine Supplementation Against a Neurotoxic Insult Induced by 3-Nitropropionic Acid

We then aimed at investigating the neuroprotective potential of creatine against 3-NP induced toxicity in spinal cord cultures. Exposure of spinal cord cultures to the

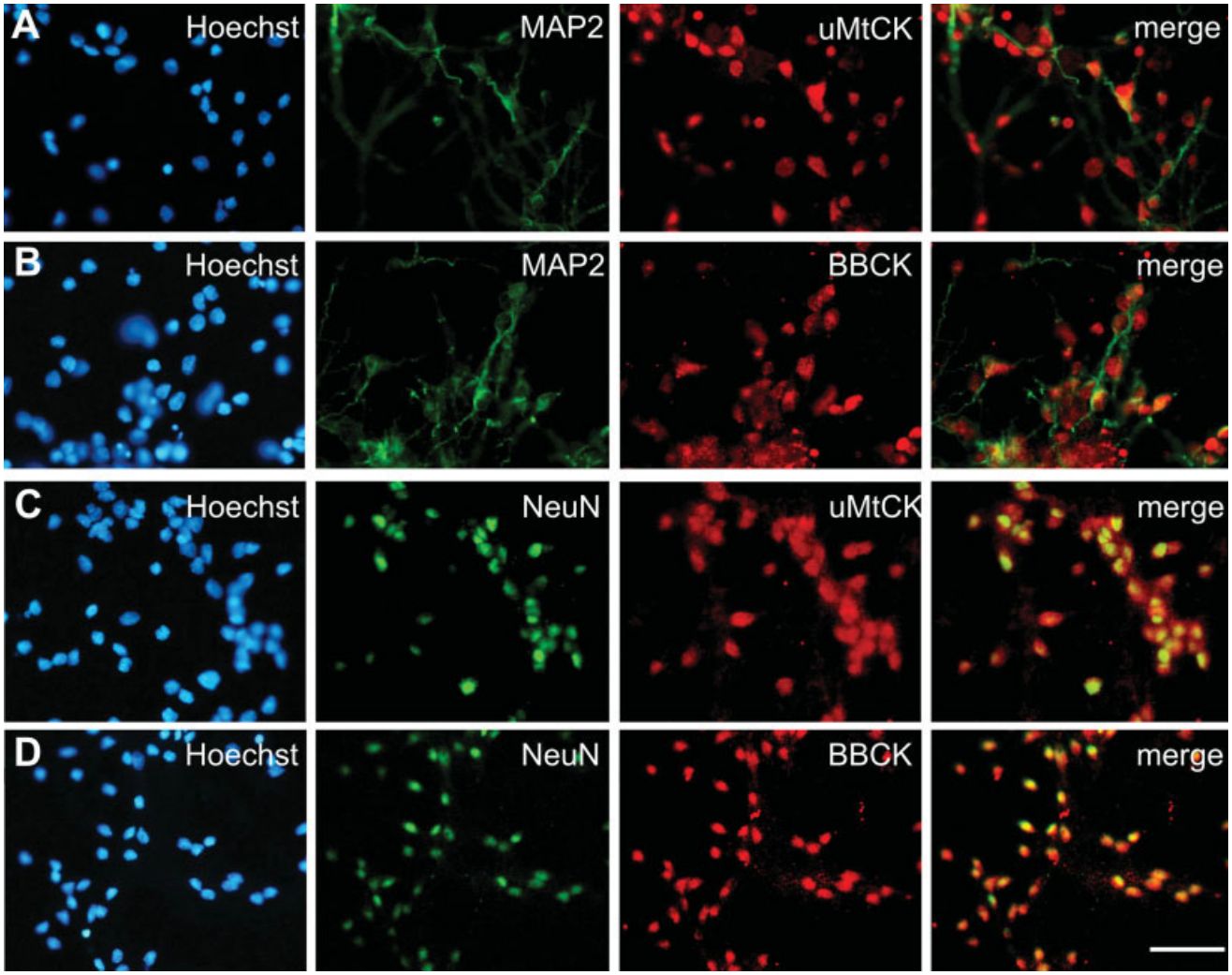

Fig. 3. Colocalization analyses for cytosolic brain specific creatine kinase (BBCK) and ubiquitous mitochondrial creatine kinase (uMtCK) with the neuronal markers microtubule-associated protein 2 (MAP2) and neuronal nuclei (NeuN) in spinal cord cultures grown for 7 days. The digitalized photomicrographs of double immunofluorescence for MAP2 and uMtCK (**A**) or BBCK (**B**) and NeuN and uMtCK (**C**) or BBCK (**D**) and corresponding merges show that all neurons expressed both isoforms of CK. Hoechst stain was used to indicate number of cells in the cultures. Scale bar = 50 μm.

neurotoxin 3-NP [5 mM] for 2 days (DIV5–7) resulted in significantly lower GABA-ir cell densities (Fig. 8A–D) and overall viable cell numbers as assessed using the MTT assay, as compared to untreated controls and assessed at DIV7 (Fig. 8E). Concomitant creatine treatment exerted a partial but significant neuroprotection with significantly higher densities of GABA-ir neurons, as compared to cultures not exposed to creatine (Fig. 8D). In contrast, creatine treatment failed to exert a protective profile on overall viable cell numbers (Fig. 8E). 3-NP treatment also affected morphology of GABA-ir cells. We observed that soma size and neurite length of GABA-ir neurons were reduced significantly after exposure to 3-NP, whereas numbers of primary neurites per GABA-ir neuron were unaffected (Table II). Concomitant creatine supplementation exerted a partial but significant neuroprotective effect on neurite length of GABA-ir neurons (1.2-fold) but did not counteract the reduction of soma size caused by the 3-NP treatment (Table II).

Effects of Creatine Supplementation on Cell Densities and Morphology of ChAT-ir Neurons in Spinal Cord Cultures

In cultured rat spinal cord tissue chronic creatine supplementation [5 mM] had no significant effect on densities of ChAT-ir neurons as compared to controls (Fig. 9). Interestingly, a slight tendency for an increase in cell density of ChAT-ir neurons was observed when creatine was present from DIV5–7 (1.2-fold) (Fig. 9).

In contrast, morphology of ChAT-ir neurons was affected significantly after chronic creatine supplementa-

tion when a significant increase (1.3-fold) of the soma size of ChAT-ir cells and a higher number of primary neurites per ChAT-ir neuron was detected (Fig. 10, Table III). Two days of creatine exposure (DIV5-7), however, failed to exert an effect on investigated morphologic parameters of ChAT-ir neurons (Table III).

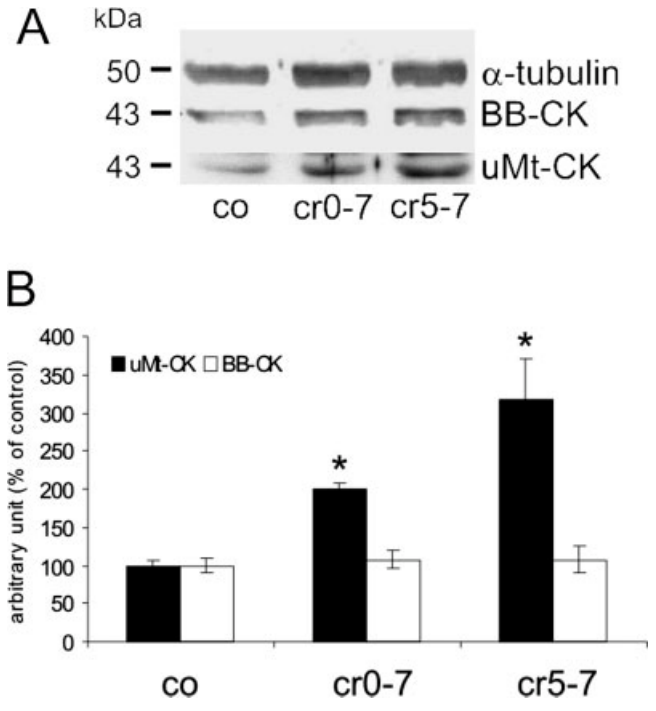

Fig. 4. Western blot analyses for levels of both cytosolic brain-specific (BB-CK) and ubiquitous mitochondrial (uMt-CK) creatine kinases (**A**), in spinal cord cultures grown for 1 week in absence (control, co) or presence of creatine [5 mM] for 1 week (cr0–7) or from DIV5–7 (cr5–7). Note the upregulation in the expression of uMt-CK (filled bars) for both creatine treatment regimen whereas no significant changes were observed for BB-CK (**B**). Membranes were reprobed with α-tubulin as loading control.

DISCUSSION

We have shown previously that creatine exposure protected cultured rat neurons against neurotoxic insults (Andres et al., 2005a–c) and that differentiation of cultured striatal precursor cells was promoted by creatine supplementation (Andres et al., 2005b). These results accommodated functional evidence for the presence of CKs in cultured brain tissue of rat origin. In line with this notion, we found that both isoforms of CK, i.e., BB-CK as well as uMt-CK, are expressed in the spinal cord cultures. Although we detected that all neurons colocalized with BB-CK and uMt-CK in our spinal cord cultures, previous reports have shown that CKs are also present in oligodendrocytes and astrocytes (Molloy et al., 1992; Kaldis et al., 1996; Andres et al., 2005b).

In the present study, we show that chronic and DIV5-7 creatine treatment resulted in a moderate but significant increase in the density of GABA-ir neurons in spinal cord cultures, whereas the total neuronal cell number and general viability, as assessed by the MTT formazan assay, and levels of β-III-tubulin were not affected. The unchanged total neuronal cell numbers in combination with a significantly higher percentage of GABA-ir cells strongly points to a promotion of differentiation of the GABA-ergic phenotype due to creatine exposure. The moderately elevated GAD levels further support this assumption. Notably, we cannot rule out that creatine also regulated GABA-ergic neuron survival due to the rather modest magnitude of effects slight changes in cell death could affect GABA-ergic cell densities. It seems, however, that creatine supplementation does not affect morphologic differentiation of GABA-ir neurons, as no alterations were observed in comparison to control cultures. These findings are in agreement with the outcome

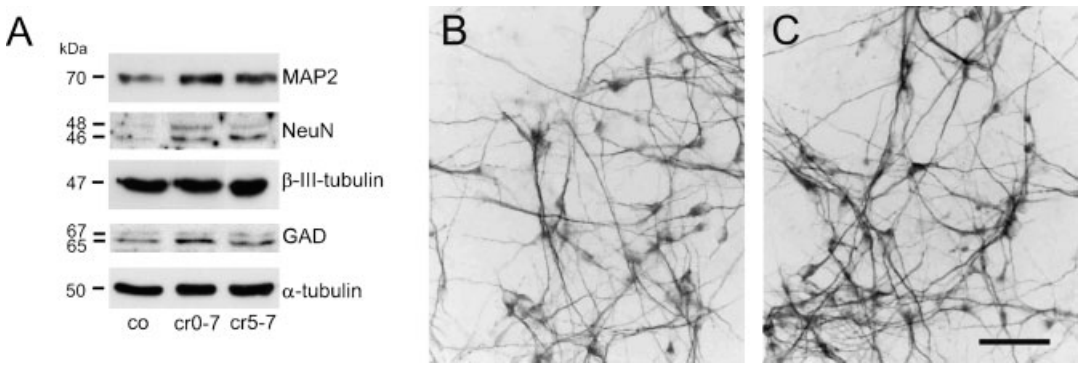

Fig. 5. Western blot analyses for levels microtubule-associated protein 2 (MAP2), Neuronal nuclei (NeuN), β-III-tubulin and glutamate decarboxylase (GAD), in spinal cord cultures grown for 1 week in absence (control, co) or presence of creatine [5 mM] for 1 week (cr0–7) or from DIV5–7 (cr5–7). Note the unchanged level of expression of β-III-tubulin, whereas an upregulation in the expression of MAP2 and NeuN for both creatine treatment regimen, and an increase in signal intensity for GAD_{65} (**A**) after chronic creatine treatment was observed. Membranes were reprobed with α-tubulin as loading control. The representative digitalized photomicrographs show MAP2 positive neurons in spinal cord cultures grown for 1 week in absence (**B**) or presence of creatine [5 mM] (**C**). Note the stronger staining intensity in the creatine exposed cultures. Scale bar = 50 μm.

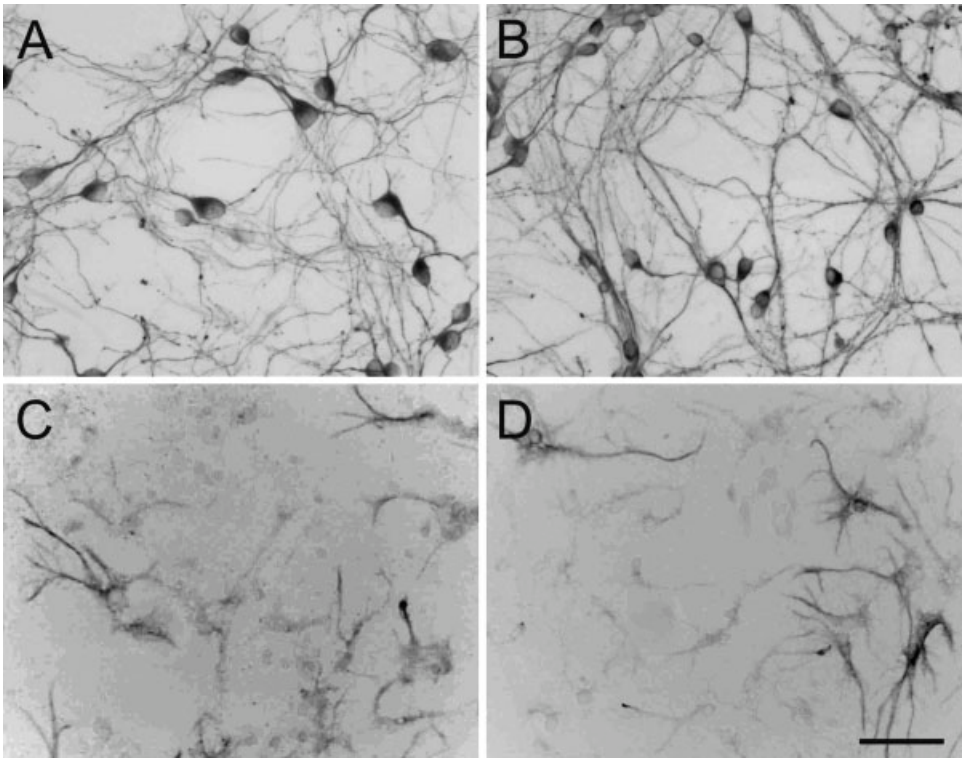

Fig. 6. The representative digitalized photomicrographs show β-III-tubulin-ir neurons (**A,B**) and GFAP-ir cells (**C,D**) in spinal cord cultures grown for 1 week in absence (A,C) or presence of creatine [5 mM] (B,D). Scale bar = 50 μm.

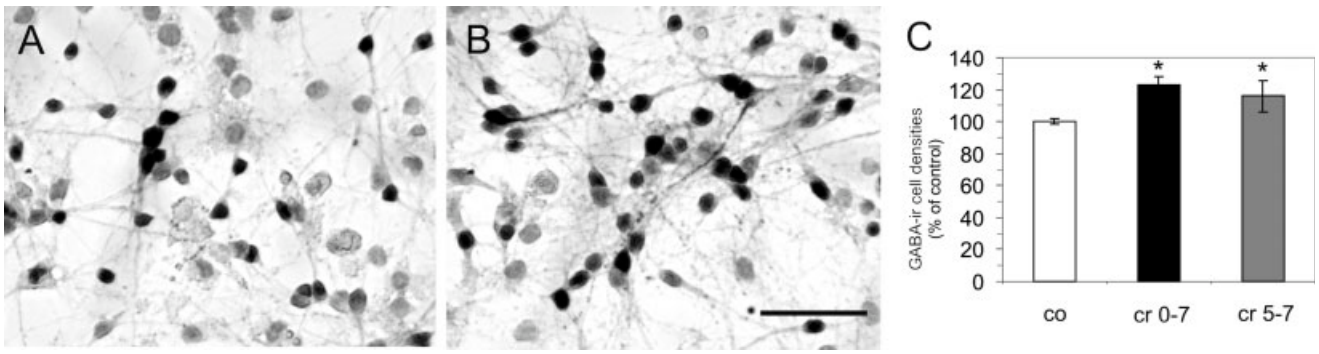

Fig. 7. Effects of creatine exposure on density of GABA-ir neurons in spinal cord cultures grown for 1 week. The representative photomicrographs show GABA-ir neurons in untreated controls (**A**) and after creatine [5 mM] treatment during the whole culture period (**B**). Scale bar = 50 μm. Quantitative analyses showed significantly higher GABA-ir cell densities in cultures exposed to creatine [5 mM] during the whole culture period (cr 0–7, filled bar) and for 2 days from DIV5–7 (cr 5–7, gray bar) as compared to untreated controls (co, open bar) (**C**). Data are expressed as percentage of untreated control values and are given as mean ± SEM (n = 12–34 from four to 10 independent experiments). *Indicates significance ($P < 0.05$) vs. control.

seen for creatine treatment in striatal cultures (Andres et al., 2005b). Nevertheless, the observations that levels of MAP-2 protein were higher in creatine-treated cultures as compared to controls together with no effects seen on morphology of GABA-ir neurons is somewhat puzzling as MAP-2 is reported to represent a dendritic and somal marker (Matus et al., 1986). We did not carry out colocalization studies for GABA and MAP-2 hence we can at present only suggest that in GABA-ir fibers and in the soma of GABA-ir neurons higher levels of MAP-2 were expressed even though morphology was not altered. Support for this notion is given by our semi-quantitative immunohistochemical analyses for MAP-2 that showed that in creatine-treated cultures staining intensity was stronger as compared to controls. Notably, it has been reported that BDNF exposure did not result in changes in distribution of MAP-2 but strengthened the intensity of labeling of developing cortical neurons (Fukumitsu et al., 1997). In addition, considering that GABA-ir cells contributed only to about 10% of total neuronal cell number in our cultures one could assume that the effects seen for creatine exposure on MAP-2 protein levels and correspondingly

TABLE I. Effects of Chronic Creatine Treatment on Morphologic Parameters of GABA-ir Neurons in Primary Dissociated Spinal Cord Cultures

	Soma size (n = 96) (μm^2)	Process length (n = 96) (μm)	Primary neurites (n = 120) (n)
Control	94.6 ± 2.6 [76.8–129.4]	88.3 ± 3.1 [71.4–132.8]	3.2 ± 0.1 [2.6–3.8]
Creatine	93.0 ± 2.8 [70.6–119.0]	93.1 ± 3.0 [78–109.9]	2.9 ± 0.1 [2.3–3.8]
Difference	1.7%	5.5%	8.9%

Cultures were grown for 1 week in absence (control) or presence of creatine [5 mM]. Parameters per individual GABA-ir neurons were assessed as described in Material and Methods. Values are given as mean ± SEM. Number in brackets show range of individual parameters per group.

on densities of MAP-2-positive neurons reflected an action on a larger number of neurons. Together with the observation of a higher total protein level and a stronger NeuN signal in creatine-treated cultures in absence of alteration in total neuronal cell numbers, we speculate that creatine promoted not only the maturation of GABA-ir cells but rather supported differentiation or maturation of a larger population of neuronal cells. This notion is furthermore supported by our observation that basically all MAP-2 and NeuN positive neurons showed colocalization with both isoforms of CKs. In addition, our results with unchanged levels of β-III-tubulin and increased expression levels of MAP-2 and NeuN may hint at the idea that the increase in GABA-ir cell density reflects an advanced maturation of already committed neuronal precursors.

The fact that creatine could induce differentiation of neural precursors is supported by a report describing the

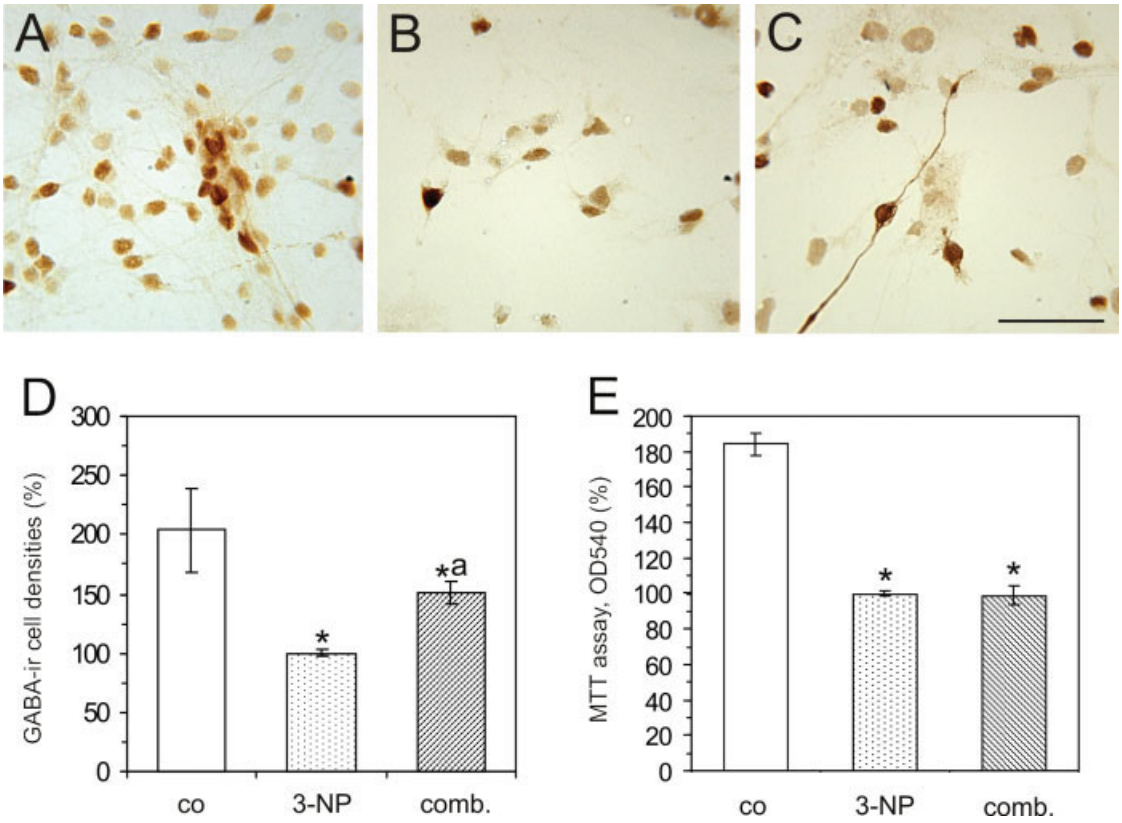

Fig. 8. Effects of creatine exposure on density of GABA-ir neurons and on general cell viability in cultures undergoing a neurotoxic insult. The representative photomicrographs show GABA-ir cells from control (**A**), 3-NP (**B**), and combination of creatine and 3-NP (**C**) treated cultures. Scale bar = 50 μm. Spinal cord cultures were grown for 1 week and exposed for 2 days from DIV 5–7 to 3-NP [5 mM] alone (3-NP, dotted bars) or a combination of creatine [5 mM] and 3-NP (comb, hatched bars). Untreated cultures served as controls (co, open bars). 3-NP exposure significantly lowered GABA-ir cell densities and overall viable cell numbers. (**D,E**). Concomitant creatine treatment resulted in a partial but significant protection on 3-NP induced GABA-ir cell loss (D) whereas no effects were observed for the MTT read out (E). Data are presented as a percentage of 3-NP values and are given as mean ± SEM (n = 27 from seven independent experiments for cell densities; n = 11 from three independent experiments for MTT assay). *Indicates significance ($P < 0.05$) vs. corresponding control, [a]Indicates significance ($P < 0.05$) vs. 3-NP group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II. Effects of Creatine Treatment on Morphologic Parameters of GABA-ir Neurons in Spinal Cord Cultures Exposed to 3-NP

	Soma size (n = 96) (μm2)	Process length (n = 96) (μm)	Primary neurites (n = 96) (n)
Control	92.1 ± 2.5 [43.9–153.6]	83.8 ± 2.9 [27.8–160.7]	3.1 ± 0.1 [2.0–8.0]
3-NP	69.9 ± 1.7[a] [36.6–136.2]	57.3 ± 2.9[a] [19.5–182.2]	3.0 ± 0.1[c] [2.0–6.0]
Comb	75.5 ± 1.9[a] [37.1–160.6]	68.0 ± 2.5[a,b] [32.5–160.9]	2.9 ± 0.1[c] [2.0–6.0]

Cultures were grown for 7 days in vitro. 3-NP [5 mM] exposure was carried out from DIV5–7 in absence (3-NP) or presence of creatine [5 mM] (comb). Untreated cultures served as controls. Parameters per individual GABA-ir neurons were assessed as described in Material and Methods. Values are given as mean ± SEM. Number in brackets show range of individual parameters per group.

[a]Indicates significance ($P < 0.05$) vs. corresponding control.

[b]Indicates significance ($P < 0.05$) vs. 3-NP group.

[c]Not significantly different.

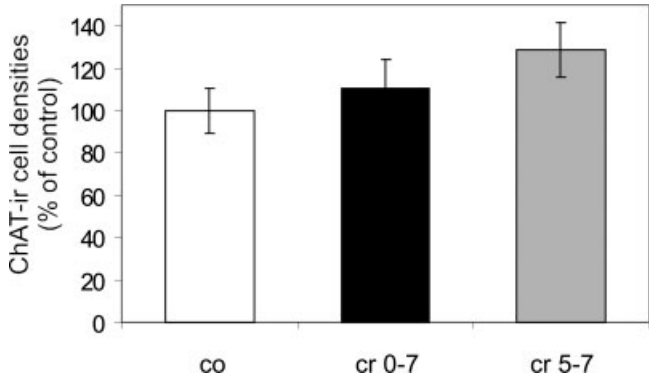

Fig. 9. Effects of creatine exposure [5 mM] on density of ChAT-ir neurons in spinal cord cultures grown for 1 week. Creatine exposure during the whole culture period (cr 0–7, filled bar) and for 2 days from DIV5–7 (cr 5–7, gray bar) did not significantly affect ChAT-ir cell densities as compared to untreated controls (co, open bar). Data are presented as a percentage of control values and are given as mean ± SEM (n = 7–8 from two independent experiments).

expression of BB-CK in developing zebrafish embryo. Expression of BB-CK was shown to be highly dynamic, often being expressed transiently in specific cells for a short period only, indicating a well-timed cell-type specific functions of CK in the zebrafish embryo (Dickmeis et al., 2001). Interestingly, BB-CK expression was absent in spinal cord of 2-day-old zebrafish embryos a stage when neuronal networks have to become functional. The authors suggested, based on their observations, that BB-CK may be involved in creatine phosphate metabolism related to differentiation of neurons in the spinal cord (Dickmeis et al., 2001).

Mitochondrial synthesis of phosphocreatine is restricted to those neurons that express uMtCK. Elevated levels of creatine in neurons (after creatine supplementation) together with the activity of uMt-CK can protect these cells during periods of increased energy demands (Schlattner et al., 2006). Such a cell protection by creatine is achieved through uMt-CK-dependent fast and efficient cycling of mitochondrial ADP (Meyer et al., 2006). In a recent study, an overall decrease or unchanged levels in uMt-CK expression has been described during the early postnatal period in mice, but with persisting high levels of expression in brain regions that continue development postnatally indicating that uMt-CK plays an important role during the developmental maturation of neuronal cells (Boero et al., 2003). The high expression of uMt-CK in these brain regions seems related to neuronal activity, e.g., seizures can induce elevated uMt-CK expression in these brain regions (Boero et al. 2003). Whether the availability of extracellular creatine would also affect this pattern in vivo remains unknown, but in our neuronal cell cultures at least, we observed a marked upregulation of uMt-CK in vitro after creatine administration, indicating that this CK substrate can also affect, by as of yet unknown mechanisms, the regulation of uMt-CK enzyme expression in neurons. As mentioned above on the outcome seen for creatine on MAP-2 expression this latter finding hints to an effect of creatine not only on GABA-ir cells but on a larger number of subpopulations of neurons.

Treatment with the irreversible succinate dehydrogenase inhibitor 3-NP was reported to result in death of GABA-ergic neurons with depletion of cellular energy reserves as an initiating step (Beal et al., 1993; Greene et al., 1998). The finding that creatine treatment provided partial neuroprotection against 3-NP toxicity in our spinal cord cultures supports the idea of an improved cellular energy metabolism of GABA-ir neurons. The cellular and molecular mechanisms by which neurons respond to increased creatine levels remain, however, equivocal (Wallimann et al., 1992; 1999; Friedman and Roberts, 1994; Wallimann and Hemmer, 1994; Brewer and Wallimann, 2000; Brustovetsky et al., 2001). Reduced CK activity, due to oxidative damage of CK has been reported in transgenic ALS mice (Wendt et al., 2002). There is also evidence for a direct anti-apoptotic effect of creatine in conjunction with uMt-CK by preventing or delaying mitochondrial permeability transition pore opening, an early trigger event for apoptosis (O'Gorman et al., 1997; Dolder et al., 2003). Notably, we did not detect a protective effect by creatine on general cell viability in our spinal cord cultures. Overall number of cells in the cultures include neuronal, glial, and the pool of stem/precursor cells. MTT is also metabolized by glial cells and little is known about metabolization by stem/precursor cells. Hence we assume that 3-NP toxicity was exerted mainly in more mature neurons such that the partial neuroprotective effect seen on GABA-ir neurons with creatine supplementation was masked.

The neuroprotective effect of creatine in our culture paradigm was rather modest. It may well be that longer culture periods or tissue from older fetuses would have

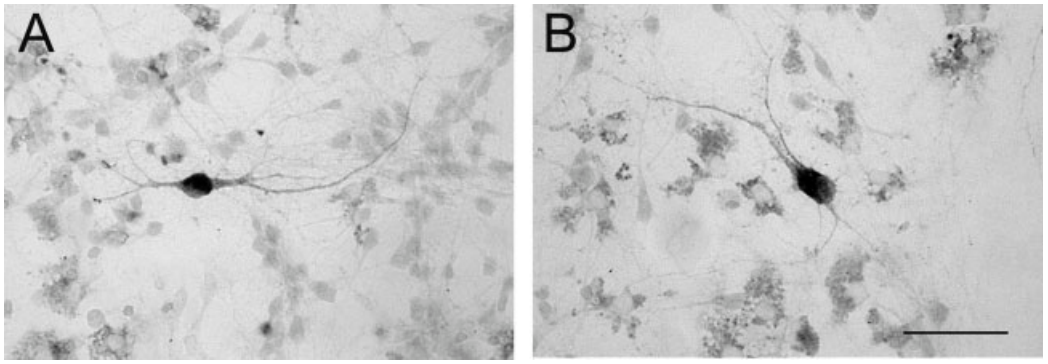

Fig. 10. Effects of creatine exposure on morphology of ChAT-ir neurons in spinal cord cultures grown for 1 week. The representative digitalized photomicrographs show ChAT-ir neurons grown in the absence (**A**) or presence of creatine [5 mM] from DIV0–7 (**B**). Scale bar = 50 μm.

TABLE III. Effects of Creatine Exposure on Morphologic Parameters of ChAT-ir Neurons in Primary Dissociated Spinal Cord Cultures

	Soma size (n = 31–47) (μm^2)	Process length (n = 15–38) (μm)	Primary neurites (n = 31–47) (n)
Control	163.3 ± 8.4 [60.0–300.0]	118.4 ± 4.5 [84.0–168.0]	3.6 ± 0.2 [2.0–7.0]
Cr 0–7	204.1 ± 8.6[a] [125.0–320.0]	130.3 ± 5.8[b] [96.2–182.0]	4.7 ± 0.3[a] [2.0–7.0]
Cr 5–7	176.3 ± 10.9[b] [60.6–363.3]	120.0 ± 5.1[b] [70.7–193.5]	3.6 ± 0.2[b] [2.0–6.0]

Spinal cord cultures were grown for 1 week in absence (control) or presence of creatine [5 mM] from DIV0–7 (Cr 0–7) or DIV5–7 (Cr 5–7). Parameters per individual ChAT-ir neurons were assessed as described in Materials and Methods. Values are given as mean ± SEM. Number in brackets show range of individual parameters per group.
[a]Indicates significance ($P < 0.05$) vs. corresponding control.
[b]Not significantly different.

shown a more distinct effect exerted by creatine. Moreover, a report by Hanson et al. (1998) observed that a large variety of peptides was able to significantly promote survival of although only by about 20–35% whereas combinations thereof resulted in marked increases. These findings together with the effect observed for creatine treatment on our rat spinal cord cultures suggest that creatine should be combined with other neuroprotective factors to achieve a substantial neuroprotection. Such combinations may include neurotrophic factors like neurotrophins or members of the glial cell line-derived neurotrophic factor family of growth factors (Zurn et al., 1994, 1996).

There is great interest in using 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. To evaluate the potential of spinal cord progenitor cells, Nakamura et al. (2005) used in vitro-expanded neurospheres and showed that these cells grafted into lesioned neonatal rat spinal cord can survive, migrate, and differentiate into neurons and oligodendrocytes, but rarely into astrocytes. Furthermore, transplantation of these cells promoted functional recovery of locomotion and reflex responses (Nakamura et al., 2005). In certain motoneuron diseases, such as ALS and spinal muscular atrophies, lower motoneurons are destroyed and muscle function cannot be restored except by reinnervation from alternate motoneuron sources. In this context, Erb et al. (1993) reported that transplanted motoneurons survived up to 18 weeks and reinnervated the denervated target muscle. We did not observe a significant effect of creatine supplementation on ChAT-ir cell densities in our cultures, however, creatine treatment increased soma size significantly and number of primary neurites per ChAT-ir neuron. This favors the view that creatine promoted neuronal differentiation rather than survival. Notably, the differentiation inducing actions exerted by creatine seem to differ depending on the targeted neuronal cell type, i.e., induction of GABA-ergic phenotype but not of morphologic complexity whereas morphology was altered but not density of ChAT-ir neurons. Based on the outcome of our study, it can be speculated that concurrent creatine supplementation may offer a way for enhanced reinnervation capacity of transplanted spinal motoneurons.

Our data show that creatine supplementation promoted the differentiation of, and had a neuroprotective effect on, cultured rat spinal cord GABAergic neurons. The data suggest that creatine may play an important role during development of spinal cord neurons. The present findings may also have implications for cell replacement strategies and for the treatment of spinal cord injuries.

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