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EFFECTS OF CREATINE TREATMENT ON THE SURVIVAL OF DOPAMINERGIC NEURONS IN CULTURED FETAL VENTRAL MESENCEPHALIC TISSUE

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Abstract—Parkinson's disease is a disabling neurodegenerative disorder of unknown etiology characterized by a predominant and progressive loss of dopaminergic neurons in the substantia nigra. Recent findings suggest that impaired energy metabolism plays an important role in the pathogenesis of this disorder. The endogenously occurring guanidino compound creatine is a substrate for mitochondrial and cytosolic creatine kinases. Creatine supplementation improves the function of the creatine kinase/phosphocreatine system by increasing cellular creatine and phosphocreatine levels and the rate of ATP resynthesis. In addition, mitochondrial creatine kinase together with high cytoplasmic creatine levels inhibit mitochondrial permeability transition, a major step in early apoptosis. In the present study, we analyzed the effects of externally added creatine on the survival and morphology of dopaminergic neurons and also addressed its neuroprotective properties in primary cultures of E14 rat ventral mesencephalon. Chronic administration of creatine [5 mM] for 7 days significantly increased survival (by 1.32-fold) and soma size (by 1.12-fold) of dopaminergic neurons, while having no effect on other investigated morphological parameters. Most importantly, concurrent creatine exerted significant neuroprotection for dopaminergic neurons against neurotoxic insults induced by serum and glucose deprivation ($P<0.01$), 1-methyl-4-phenyl pyridinium ion (MPP^+) [15 μM] and 6-hydroxydopamine (6-OHDA) [90 μM] exposure ($P<0.01$). In addition, creatine treatment significantly protected dopaminergic cells facing MPP^+-induced deterioration of neuronal morphology including overall process length/neuron (by 60%), number of branching points/neuron (by 80%) and area of influence per individual neuron (by 60%). Less pronounced effects on overall process length/neuron and number of branching points/neuron were also found after 6-OHDA exposure ($P<0.05$) and serum/glucose deprivation ($P<0.05$). In conclusion, our findings identify creatine as a rather potent natural survival- and neuroprotective factor for developing nigral dopaminergic neurons, which is of relevance for therapeutic approaches in Parkinson's disease and for the improvement of cell replacement strategies.

Key words: creatine kinase, neuroprotection, Parkinson's disease, ventral mesencephalon, tyrosine hydroxylase, rat cell cultures.

Parkinson's disease (PD) is a neurodegenerative disorder mainly characterized by the progressive deterioration of dopaminergic neurons in the substantia nigra pars compacta, leading to clinical symptoms like bradykinesia, rigidity, tremor and postural instability (Lang and Lozano, 1998a; Lang and Lozano, 1998b). Despite recent advances, the etiologic factors of idiopathic PD still remain poorly defined. Strong evidence has accumulated that impairment of cellular energy metabolism, particularly a defective mitochondrial function, contributes to neuronal death in PD (Gu et al., 1998b; Mattson et al., 1999; Mizuno et al., 1995; Sheehan et al., 1997). Patients suffering from idiopathic PD demonstrated a reduced mitochondrial complex-I activity and consecutive production of cytotoxic reactive oxygen species (ROS) (Janetzky et al., 1994; Mann et al., 1992; Schapira et al., 1990). Maternally inherited forms of PD are associated with complex-I deficiency (Gu et al., 1998a; Sheehan et al., 1997; Simon et al., 2000). Improving the metabolic state of neuronal cells may therefore have therapeutic potential in PD.

Suboptimal metabolic conditions are regarded to be critically involved in poor survival of transplanted neuronal tissue in cell replacement strategies for PD. This approach is still experimental and its application obstructed not only by the limited availability of human fetal donor tissue and ethical issues, but predominantly by the abovementioned mediocre survival of the transplanted dopaminergic neurons and their suboptimal innervation of the host striatum (Borlongan et al., 1999; Freed et al., 2001, 2003). Strategies to improve cell replacement therapies therefore focus on establishing techniques for effective tissue storage, improvement of survival and integration of grafted dopaminergic neurons (Thajeb et al., 1997).

Aerobic glycolysis is the primary pathway of ATP synthesis in brain (Ames, 2000). With only small stores of glucose, glycogen and O_2, the rate of glycolysis in brain is closely related to cerebral blood flow and oxygen uptake. These rates in turn are coupled to the utilization of ATP

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Abbreviations: BB-CK, brain-specific cytosolic isoform of creatine kinase; CK, creatine kinase; DIV, day *in vitro*; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylenediamine tetraacetate; E14, embryonic day 14; HBSS, Hanks' balanced salt solution; HD, Huntington's disease; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; ir, immunoreactive; MPP^+, 1-methyl-4-phenyl pyridinium ion; PBS, phosphate-buffered saline; PCr, phosphocreatine; PD, Parkinson's disease; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; uMt-CK, ubiquitous mitochondrial isoform of creatine kinase; VM, ventral mesencephalon; 6-OHDA, 6-hydroxydopamine.

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(Holtzman et al., 1987). A rapid available alternative source for ATP synthesis in brain is the creatine kinase/phosphocreatine (CK/PCr) system, which can operate via substrate level phosphorylation to equilibrate adenine nucleotides with PCr and creatine. The CK/PCr system plays therefore an important role in cells with high and fluctuating energy demands like neurons (Hemmer and Wallimann, 1993; Wallimann et al., 1989, 1992). Creatine (1-methylguanidinoacetic acid) is synthesized in liver, kidney and pancreas (Wyss and Kaddurah-Daouk, 2000) and to some extent in brain (Braissant et al., 2001). At least 50% of the creatine content in the organism, however, is derived from dietary intake. The molecule is taken up into the cytoplasm against a concentration gradient via a specific sodium- and chloride-dependent transmembrane transporter (CRT) related to the family of GABA/betaine transporters (Guerrero-Ontiveros and Wallimann, 1998; Nash et al., 1994; Saltarelli et al., 1996; Wallimann and Hemmer, 1994). In brain cells, ATP levels are regulated by the brain-specific isoform of creatine kinase (BB-CK) along with the mitochondrial isoform (uMT-CK) and their substrates creatine and PCr (Hemmer and Wallimann, 1993). There is evidence for a direct functional coupling of CK with sodium potassium ATPase, neurotransmitter release, maintenance of membrane potentials and restoration of ion gradients after depolarization (Dunant et al., 1988; Hemmer and Wallimann, 1993; Guerrero et al., 1997). The high activity of CK in brain (Norwood et al., 1983) justifies the assumption that CK is a key enzyme in the energy metabolism of this tissue, which is supported by *in vivo* ^{31}P nuclear magnetic resonance transfer measurements, demonstrating that CK flux correlates with brain activity (Sauter and Rudin, 1993; Corbett and Laptook, 1994). It has been shown that creatine passes the blood–brain barrier (Dechent et al., 1999) and its systemic administration increases brain PCr concentrations and buffers against toxin-induced depletion (Matthews et al., 1998; Dechent et al., 1999).

Recent work has shown that creatine supplementation can protect neurons against neurotoxins *in vitro* (Brewer and Wallimann, 2000) and slow down the progression of a number of neurodegenerative disease states, as assessed in experimental animal models. Increased intracellular PCr levels, thereby buffering cellular ATP resources under metabolic or oxidative stress (Michaelis et al., 1999; Wilken et al., 1998, 2000; Woznicki and Walker, 1979, 1988) or brain ischemia (Zhu et al., 2004; Adcock et al., 2002), acting as a substrate for the CK/PCr energy shuttle (Chen et al., 1995; Hemmer et al., 1993; Hemmer and Wallimann, 1993) and inhibition of apoptosis by means of preventing the mitochondrial permeability transition (MPT) (Dolder et al., 2003; Brdiczka et al., 1998; O'Gorman et al., 1997) have been proposed to account for the mode of action of creatine in these studies.

The application of the neurotoxins 1-methyl-4-phenyl pyridinium ion (MPP^+) and 6-hydroxydopamine (6-OHDA) provide effective models for assessment of neuroprotection on dopaminergic cells. Both MPP^+ and 6-OHDA compete with dopamine for uptake via the plasma membrane transporter (Decker et al., 1993). MPP^+ utilizes an energy-dependent mechanism for concentration within mitochondria, where it inhibits complex I of the respiratory chain and alpha-ketoglutarate dehydrogenase (KGDH) of the tricarboxylic acid (TCA) cycle. Consequently, ATP depletion, elevation of ROS, and increased glutamate levels precede neuronal death (McNaught et al., 1998). After MPP^+ administration, a rapid concentration-dependent reduction in intracellular PCr levels is observed, followed by a slower decline in ATP levels in combination with a decrease in cell viability. These findings provide evidence for an important role of PCr in stabilizing cellular ATP levels (Marini and Nowak, 2000). In contrast to MPP^+, 6-OHDA has been assumed to cause neuronal death by a free radical mediated mechanism. There is some evidence that 6-OHDA induces rather apoptosis *in vitro*, while MPP^+ is thought to provoke both apoptotic and necrotic cell death in dopaminergic neurons (Lotharius et al., 1999; Bilsland et al., 2002).

In the developing rat brain, both BB-CK and uMt-CK are expressed as early as embryonic day 14 (E14) and a considerable increase of total CK activity has been detected during embryonic brain development (Chen et al., 1995; Holtzman et al., 1993). Hence, embryonic CNS tissue does possess a functional CK/PCr system and may benefit from amelioration of the energetic state. Given that rational, in the present work we aimed at investigating the possible neuroprotective and survival-enhancing properties of creatine administration in cultured embryonic ventral mesencephalic neurons.

EXPERIMENTAL PROCEDURES

Preparation of primary neuronal cultures

After induction of deep pentobarbital anesthesia (Nembutal™, Abbott, North Chicago, IL, USA, 100 mg/kg body weight), E14 fetuses were removed by cesarean section from pregnant Sprague–Dawley rats (RCC Biotechnology, Füllinsdorf, Switzerland) and killed by decapitation. The fetal brains were excised in toto (Dunnett and Björklund, 1992), transferred to a Petri dish with Dulbecco's modified Eagle medium (DMEM; Gibco, Basel, Switzerland) and the ventral mesencephali (VM) were dissected according to standard procedures (Spenger et al., 1994). The mesencephalic explants were rinsed twice in DMEM and mechanically dissociated in 2 ml of ice-cold DMEM by gently triturating using a 1 ml sterile pipette tip. Immediately after dissociation, 10 ml of medium was added to the suspension and the undispersed tissue pieces were allowed to settle down for 1 min. The supernatant containing the individual cells was transferred to a second tube, centrifuged at 1200 r.p.m. for 4 min at 16 °C and resuspended in 1 ml of culture medium (Widmer et al., 2000).

Aliquots corresponding to 0.8 VM were pipetted per well (seeding density of 100′000 viable cells per mm^2) in 24-well plates (Multiwell 3047, 14 mm diameter per well; Falcon, Lincoln Park, NJ, USA) with 0.5 ml growth medium/well. Culture wells contained glass coverslips with a diameter of 12 mm (Assistent, Sondheim, Germany) previously coated with a solution of poly-L-lysine (0.1 mg/ml; Sigma, St. Louis, MO, USA) in 0.15 M sodium borate buffer, pH 8.3. The wells were washed three times with sterile phosphate-buffered saline (PBS, pH 7.4; Life Technologies, Grand Island, NY, USA) before adding medium. Cell viability as assessed using the Trypan-Blue dye exclusion method was higher than 75% for cultures derived from E14 embryos.

The neurons were grown in culture medium consisting of 55% DMEM, 32.5% Hanks' balanced salt solution (HBSS; Gibco, Basel, Switzerland), 0.3% glucose, 10% fetal calf serum (Gibco, Basel, Switzerland) and 1% 0.01 M HEPES (Merck, Darmstadt, Germany). Antibiotics/antimycotics (No. 061-05240 D; Gibco, Basel, Switzerland) 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 was changed 24 h after plating and, subsequently, every second day. Creatine and creatinine contents in the culture medium were determined using standardized clinical assays (Chemical Central Laboratory, University Hospital, Berne).

All animal procedures were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and aproved by the local Animal Care and Use Committee at the University of Berne. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Treatment regimens

Wells were randomly assigned to either the treatment group or to the control group. Cultures were exposed to creatine-monohydrate (Creapure™, SKW Trostberg, Germany) at a final concentration of 5 mM from day *in vitro* (DIV) 0–7 (n=16, four independent experiments), from DIV0-DIV3 (n=6, two independent experiments) or from DIV5-DIV7 (n=8, two independent experiments), respectively. Untreated cultures served as controls. For analysis of TH-ir cell densities, cells were fixed at DIV7 and processed as described below.

For assessment of creatine-mediated neuroprotective effects against a metabolic insult, cultures were kept in standard culture medium up to DIV5 when the medium was changed to serum- and glucose-free conditions. Cells were consequently grown for additional 2DIV in absence or presence of creatine [5 mM] (n=16, two independent experiments). Cultures were fixed at DIV7 and compared with untreated controls.

The effect of creatine exposure against MPP^+ and 6-OHDA exposure was investigated using two different settings. First, MPP^+ (Sigma, St. Louis, MO, USA) was added to the culture medium at a final concentration of 15 μM from DIV5-7 with or without concurrent creatine addition [5 mM] (n=16, two independent experiments). In the second series of experiments cultures were exposed to 6-OHDA (Sigma, St. Louis, MO, USA) at a final concentration of 90 μM from DIV6-7 with or without concurrent creatine addition [5 mM] (n=9, three independent experiments). Cells were grown in standard conditioning medium. TH-ir cell densities were assessed at DIV7 and compared with untreated controls.

Immunocytochemistry

Maintenance medium was aspirated and the cultures were briefly rinsed in 0.1 M PBS and fixed in 0.1 M PBS containing 4% paraformaldehyde for 20 min at room temperature. After three further rinses in PBS, the cultured tissue was preincubated in 0.1% Triton X-100 in PBS plus 10% horse serum for 30 min, washed and incubated overnight with the primary antibodies (mouse monoclonal anti-TH, 1:500, or mouse monoclonal anti-glial fibrillary acidic protein (GFAP), 1:800; Boehringer Mannheim, Germany) in PBS containing 2.5% HS at 4 °C. Following three washes in PBS, wells were incubated with a biotinylated secondary antibody (horse anti-mouse, 1:200; Vector Laboratories, Burlingame, CA, USA) in PBS containing 2.5% HS for 60 min. After incubation with an avidin–peroxidase-complex (1:150; Vector Laboratories, Burlingame, CA, USA) for 45 min, bound antibodies were visualized using a metal-enhanced 3,3′-diaminobenzidine substrate kit (No. 34065; Pierce, Rockford, IL, USA). Cultures were rinsed in PBS and mounted on slides using Aquamount (BDH Chemicals, Poole, UK).

Co-localization of TH with BB-CK or uMt-CK

Cultures were fixed as described above and then preincubated in a buffer solution containing 0.5 M NaCl, 50 mM Tris–HCl, 5% bovine serum albumin (Sigma, St. Louis, MO, USA) and 2% milk powder (Chen et al., 1995) for 60 min, washed and incubated overnight with the primary antibodies (rabbit polyclonal anti-BB-CK or rabbit polyclonal anti-uMt-CK, respectively, 1:1000 (Schlattner et al., 2002), in combination with mouse monoclonal anti-TH, 1:500; Boehringer Mannheim, Germany) in the buffer solution at 4 °C. Following three washes, cultures were incubated with the secondary antibodies (fluorescein isothiocyanate-labeled anti-mouse 1:50 and tetrarhodamine isothiocyanate-labeled anti-rabbit 1:100; DAKO, Glostrup, Denmark) in the buffer solution for 2 h. Cultures were mounted on slides in PBS containing 25% glycerol and immunofluorescence was performed at excitation wavelengths of 490 and 557 nm on an epifluorescence microscope (DMRB; Leica, Heidelberg, Germany).

Western blot analysis

Ventral mesencephalic tissue from E14 and E18 embryos was dissected as described above. Immunoblot analyses were carried out according to the method of Laemmli (1970) with some modifications. The tissue was rinsed twice with 7 ml sterile ice-cold HBSS and transferred into an Eppendorf tube with 150 μl of lysis buffer consisting of leupeptin (1 μl/ml), phenylmethylsulfonyl fluoride (1 mM), beta-mercaptoethanol (1 mM), EDTA (0.2 mM) and Triton X-100 (1 μl/ml) in HBSS. Cells were sonificated during 3 min and the lysate centrifuged for 2 min at 14,000 r.p.m. Protein content in the samples was determined according to the method of Bradford using the Bio-Rad™ protein assay (Bradford, 1976). Sample buffer (7.5 μl; 0.2 M Tris–HCl, pH 7.0, 30% glycerol, 6% sodium dodecyl sulfate (SDS), 15% beta-mercaptoethanol and 0.0075% Bromphenol Blue) was then added to 15 μl of the supernatant and incubated at 95 °C for 5 min. SDS-polyacrylamide gel electrophoresis was performed using a 12% SDS-polyacrylamide separating gel with 1–10 μg of sample protein per lane. Proteins were blotted using the semi-dry technique on a 0.45 μm nitrocellulose membrane (BA85; Schleicher & Schüll, Dassel, Germany) 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. Transfer efficiency and equal sample loading were checked by Coomassie Blue staining of the gels and reversible Ponceau S staining of blotting membranes, respectively. After decolorization by rinsing twice in Tris-buffered saline (TBS) buffer (20 mM Tris, pH 7.5, 150 mM NaCl) for 5 min, the blot was blocked for 30 min with 4% fat-free milk powder in TBS and incubated with the primary antibodies (mouse monoclonal anti-BB-CK, 1:1000 (Sistermans et al., 1995) and rabbit polyclonal anti-uMt-CK, 1:1000 (Schlattner et al., 2002)) for 60 min at 22 °C in the same solution. The blots were washed twice in TBS containing 0.2% Tween-20 (Merck, Darmstadt, Germany) and subsequently incubated with an affinity purified horseradish peroxidase-coupled secondary antibody (donkey anti-rabbit, 1:20,000; Jackson Immunoresearch, West Grove, PA, USA) for 45 min at 22 °C in TBS containing 4% milk powder. Blots were washed three times in 0.2% Tween-20 containing TBS and incubated for 1 min in chemiluminescent substrate (Western Lightning, Perkin-Elmer Life Science, Hünenberg, Switzerland). Amplified chemiluminescence was detected and quantified using a digital imaging system (ImageStation; Kodak, Rochester, NY, USA).

Cell counts and morphological analyses

Assessment of TH-ir cell densities was carried out using a bright field microscope (Axioplan; Zeiss, Oberkochen, Germany) connected to a digital image processing system (Lucia 4.50; Nikon,

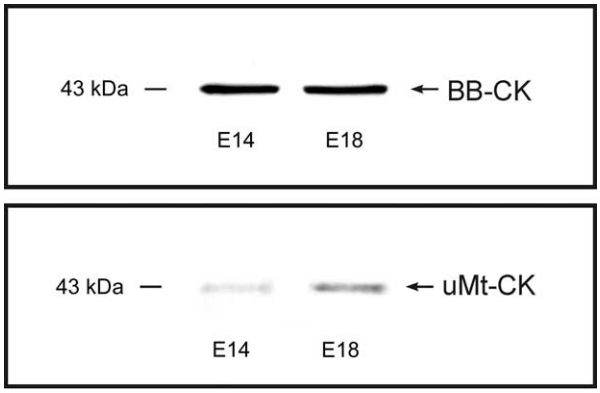

Fig. 1. Western blot analysis of the brain-specific isoforms of CK in the cytosol (BB-CK) and the mitochondria (uMt-CK) in rat ventral mesencephalic tissue from E14 and 18.

Tokyo, Japan). Only cells with distinct immunoreactivity, clear neuronal shape and a nucleus were counted as TH-positive neurons. To obtain cell counts, 5–10% of the glass slide surface in the culture dish were analyzed in a randomized fashion using a ×10 objective (Widmer et al., 2000).

For quantitative morphometry, the Eutectic NTS three dimensional neuron tracing system (Eutectic Electronics, Raleigh, NC, USA) was used as previously described (Studer et al., 1995; Widmer and Hefti, 1994). For chronic treatment, 96 neurons randomly selected from treated and untreated cultures, were analyzed (48 per group, two independent experiments). In the MPP^+ experiments, a total number of 144 cells was analyzed (48 from controls, MPP^+-treated and combined MPP^+- and creatine-treated cultures; two independent experiments). In the 6-OHDA and in the metabolic insult experiments, a total number of each 72 cells was analyzed (24 from controls, 6-OHDA-treated and combined 6-OHDA- and creatine-treated cultures and 24 from controls, metabolic insult-exposed and combined metabolic insult- and creatine-exposed cultures, respectively; from each two independent experiments). Parameters assessed for individual TH-ir neurons included cell soma area (μm^2), total length of processes (μm), number of primary neurites emerging from the cell body (n), total number of branching points (n) and size of neurite field per neuron (μm^2).

Statistical evaluation

For statistical analysis, a commercially available software package was used (Statistica 5.0; StatSoft, Tulsa, OK, USA). The experimental groups were compared by multivariate analysis of variance, followed by nonparametric post hoc Mann-Whitney test. The statistical significance level was set at $P<0.05$. Values are presented as mean±S.E.M.

RESULTS

Creatine and creatinine content in culture medium

The concentrations of added creatine were found to be stable over the interval between the medium changes (1.045±0.026 mmol/l and 1.052±0.003 mmol/l for a 1 mM solution and 10.166±0.063 mmol/l and 9.960±0.142 mmol/l for a 10 mM solution added at medium change and 48 h thereafter, respectively; mean±S.E.M.; $n=3$). A slight but not significant increase was observed in the concentration of creatinine, the major catabolite of creatine originating from spontaneous non-enzymatic cyclization over the same time period (data not shown). Determination of creatine content in the maintenance culture medium revealed a concentration of 46.33±0.67 μmol/l (mean±S.E.M.; $n=3$) hence levels much lower as determined to be effective in influencing survival of neurons, i.e. >1 mM.

Quantification and localization of CK isoenzymes

Cytosolic BB-CK and uMt-CK were found to be expressed both in the E14 and the E18 VM, as assessed at day of dissection (Fig. 1). For uMt-CK, a stronger signal was obtained in E18 than in E14 ventral mesencephalic tissue, likely indicating an upregulation of uMt-CK expression during this later developmental stage (Fig. 1),

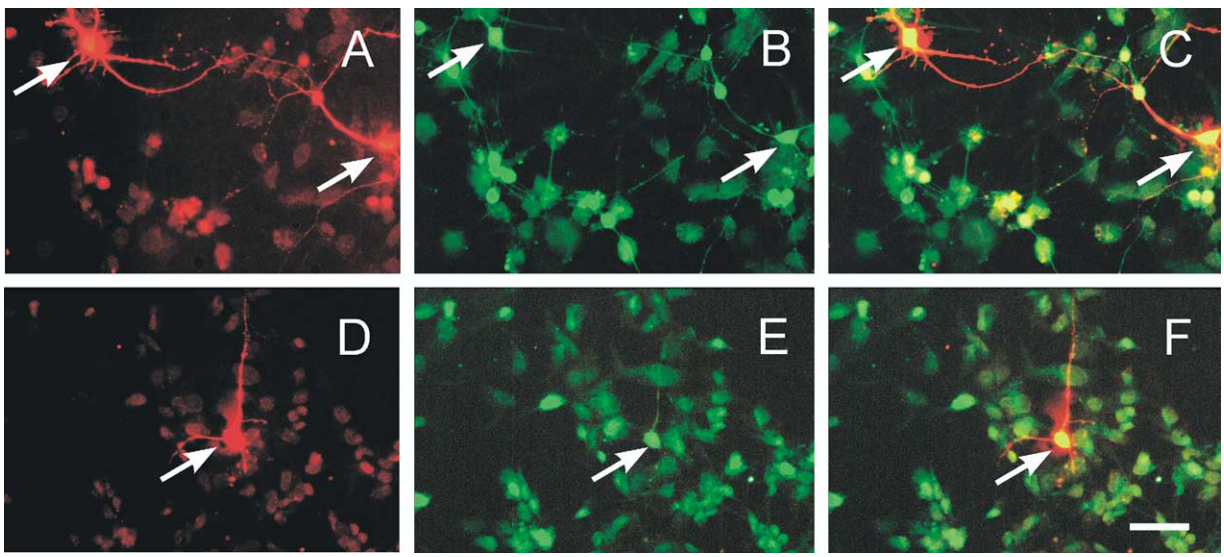

Fig. 2. Digitalized photomicrographs of double immunofluorescence for TH (A, D) and brain-type CK (B), or ubiquitous mitochondrial CK (E) in untreated ventral mesencephalic cultures grown for 1 week. Note: TH-ir neurons were found to express both CK isoenzymes. Scale bar=50 μm.

Table 1. Quantification of TH-ir cell densities in primary dissociated mesencephalic cultures exposed to creatine for different time periods

Treatment period	Control	Creatine
DIV0-7	11.7±0.2	15.5±0.2*
DIV0-3	11.7±0.3	15.0±0.5*
DIV5-7	12.5±0.4	12.9±0.7

Creatine [5 mM] was administered for DIV0-7, DIV0-3 or DIV5-7. Values are given as mean±SEM (two independent experiments) and are expressed as number of TH-ir neurons per mm^2. * Indicates significance at $P<0.05$ vs. corresponding control.

with relatively higher levels of BB-CK throughout development.

Colocalization studies using the double immunolabeling technique revealed that tyrosine hydroxylase immunoreactive (TH-ir) neurons in ventral mesencephalic cultures also expressed BB-CK and uMt-CK (Fig. 2). No differences were observed in the distribution pattern of CKs between TH-ir and TH-negative neurons using semiquantitative analyses.

Effects of creatine treatment on the survival of TH-ir neurons

In cultured mesencephalic tissue treated with creatine for 1 week, the number of TH-ir neurons was significantly higher as compared with controls (Table 1; Fig. 3). This increase was already seen when adding creatine only during the first 3 days in culture (DIV0-3), but not when adding creatine only at the end of the observation period (DIV5-7) (Table 1).

Effects of creatine treatment on morphological parameters of TH-ir neurons

The effect of chronic creatine exposure on morphological differentiation of TH-ir neurons was quantitatively analyzed in the ventral mesencephalic cultures at DIV7. TH-ir neurons from creatine-treated cultures demonstrated a moderate but significant increase in cell soma area, while neuritic length per neuron, number of branching points per neuron, number of primary neurites emerging from an individual soma and the size of neurite field of an individual neuron did not differ significantly from untreated controls (Table 2).

Effects of creatine treatment on mesencephalic TH-ir neurons exposed to MPP^+

Exposure of the cultures to the neurotoxin MPP^+ [15 μM] for 2 days (DIV5-7) resulted in significantly lower numbers of TH-ir neurons in the cultures (by 25%, Fig. 4a) and alterations in their morphology (Fig. 5, Fig. 6), as assessed at DIV7 and compared with untreated controls. Concurrent creatine administration lent to a significant neuroprotection of TH-ir neurons against MPP^+-exposure in absence of creatine (Fig. 4a), and also to a more preserved neuronal morphology (see below).

Effects of creatine treatment on TH-ir neurons exposed to 6-OHDA

Significantly lower numbers of TH-ir neurons (by 40%) as compared with untreated controls were observed in the ventral mesencephalic cultures after exposure to the neurotoxin 6-OHDA [90 μM] from DIV6-7. Similarly to the effects seen for MPP^+-induced neurotoxicity (vide supra), creatine administration engendered a distinct protection against the neurotoxic insult with significant higher numbers of TH-ir cells detected in the cultures than observed after 6-OHDA-exposure in absence of creatine (Fig. 4b), and also to a more preserved neuronal morphology (Fig. 7).

Effects of creatine treatment on TH-ir neurons undergoing a metabolic insult

Serum- and glucose deprivation from DIV5-7 resulted in a marked decrease in overall number of TH-ir cells (by 30%) and in an altered neuronal morphology as compared with untreated control cultures (Fig. 4c, Fig. 8). Creatine treatment protected TH-ir cells in the ventral mesencephalic cultures from this metabolic insult. Significantly higher numbers of TH-ir neurons were detected as compared with those found in the serum and glucose deprived cultures

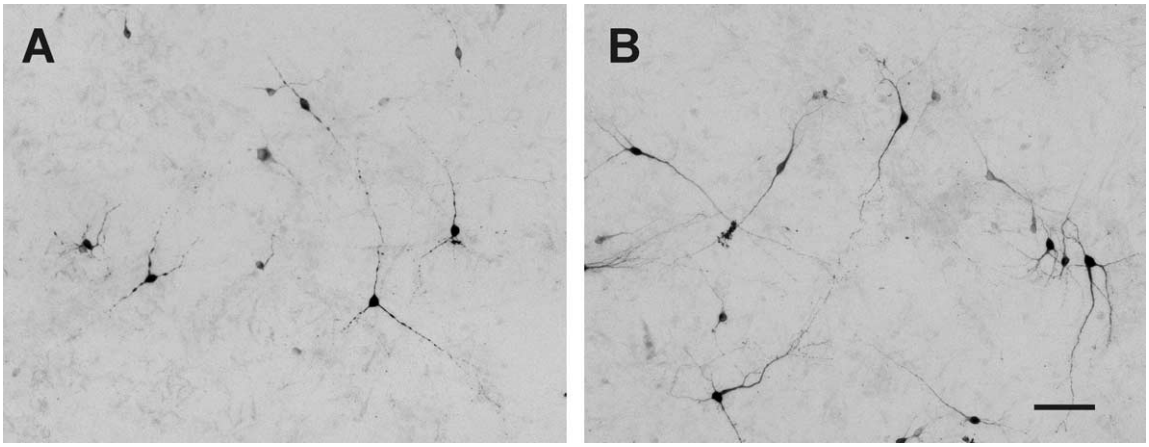

Fig. 3. Representative photomicrographs of TH-ir neurons in ventral mesencephalic cultures grown for 1 week without (A) and with addition of creatine (B). Note: Creatine treatment [5 mM] resulted in higher cell TH-ir cell densities. Scale bar=50 μm.

Table 2. Effects of chronic creatine treatment on morphological parameters of TH-ir neurons in primary dissociated ventral mesencephalic cultures

	Soma size (μm^2)	Process length (μm)	Mean tree origins (n)	Branching points (n)	Area of influence (μm^2)
Control	101.7±3.3	684.6±32.5	4.4±0.2	26.6±1.6	17791±1351
Creatine	114.3±4.8*	672.0±33.2	4.1±0.2	25.1±1.7	16511±1288

Cultures were grown for 1 week in absence (control) or presence of creatine [5 mM]. Parameters per individual TH-ir neurons were assessed as described in Experimental Procedures. Values are given as mean±SEM (two independent experiments). * Indicates significance at $P<0.05$ vs. corresponding control.

(Fig. 4c). Moreover, in the presence of creatine the morphology of TH-ir neurons was less affected (see below).

Effects of creatine treatment on morphology of TH-ir neurons exposed to MPP^+

To investigate the morphological changes of TH-ir neurons provoked by MPP^+ and their alleviation by creatine treatment in more detail, we conducted a quantitative analysis. MPP^+-exposed cultures demonstrated markedly lower values for morphological parameters, i.e. overall process length (by 60%), number of branching points per neuron (by 70%) and the mean size of neurite field of an individual neuron (by 70%) as compared with untreated controls (Fig. 5; Fig. 6). The presence of creatine in the culture medium significantly protected TH-ir neurons against MPP^+-induced morphological deterioration (Fig. 5; Fig. 6). Other parameters including the number of primary neurites emerging from an individual soma (4.2±0.2, 3.7±0.2 and 4.3±0.2 for untreated controls, MPP^+-treated cultures and cultures exposed to both MPP^+ and creatine, respectively; mean±S.E.M.) and the cell soma area (134.4±4.3, 109.0±3.9, 117.1±3.8 μm^2 for untreated controls, MPP^+-treated cultures and cultures exposed to both MPP^+ and creatine, respectively; mean±S.E.M.) were not significantly affected either by MPP^+ exposure or by the combined MPP^+/creatine treatment.

Effects of creatine treatment on morphology of TH-ir neurons exposed to 6-OHDA

Quantitative analyses of 6-OHDA-exposed cultures demonstrated significantly lower values for morphological parameters, i.e. overall process length (by 30%), number of branching points per neuron (by 40%) and the mean size of neurite field of an individual neuron (by 40%) as compared with untreated controls (Fig. 7). The presence of creatine in the culture medium significantly protected TH-ir neurons against 6-OHDA-induced morphological deterioration of overall process length and number of branching points per neuron, and showed a tendency for preservation of mean size of neurite field of an individual neuron ($P<0.05$) (Fig. 7). Other parameters including the number of primary neurites emerging from an individual soma (4.2±0.2, 4.0±0.2 and 4.3±0.2 for untreated controls, 6-OHDA-treated cultures and cultures exposed to both 6-OHDA and creatine, respectively; mean±S.E.M.) and the cell soma area (116.6±7.6, 115.5±9.3, 125.6±10.9 μm^2 for untreated controls, 6-OHDA-treated cultures and cultures exposed to both 6-OHDA and creatine, respectively; mean±S.E.M.) were not significantly affected either by 6-OHDA exposure or by the combined 6-OHDA/creatine treatment.

Effects of creatine treatment on morphology of TH-ir neurons undergoing a metabolic insult

Cultures undergoing a metabolic insult, induced by serum- and glucose deprivation, demonstrated significantly lower values for morphological parameters of TH-ir neurons, i.e. overall process length (by 30%), number of branching points per neuron (by 50%) and the mean size of neurite field of an individual neuron (by 30%) as compared with untreated controls (Fig. 8). The presence of creatine in the culture medium significantly protected TH-ir neurons

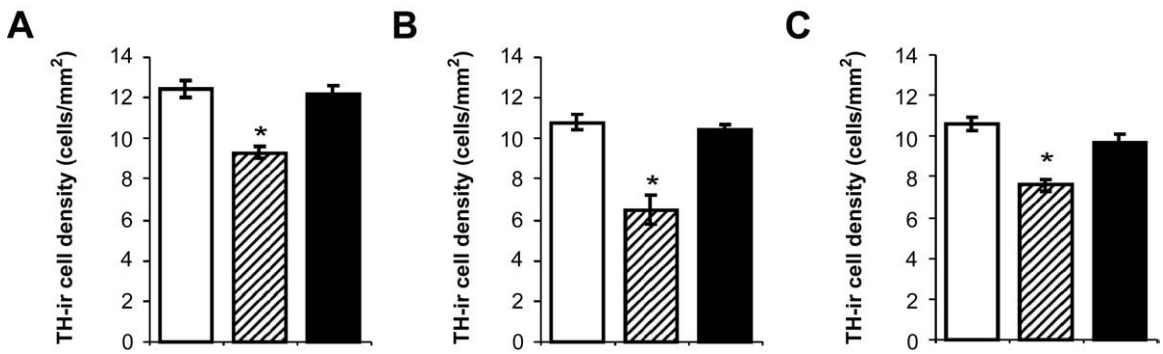

Fig. 4. Effects of creatine treatment on TH-ir cell densities in ventral mesencephalic cultures facing a MPP^+ (A), or 6-OHDA (B) -induced neurotoxicity and a metabolic insult (C). Cultures were grown for 7DIV. MPP^+ [15 μM] exposure (A) or serum and glucose deprivation (C) was performed from DIV5-7 and 6-OHDA [90 μM] treatment (B) from DIV6-7 in absence (hatched bars) or presence of creatine [5 mM] (filled bars). Untreated cultures served as controls (open bars). Notably creatine administration showed a significant protection against the induced TH-ir cell loss in all three paradigms. Values are given as mean±S.E.M. and are expressed as percentage of the corresponding untreated controls. * Indicates significant difference ($P<0.05$) vs. corresponding control and creatine-treated cultures.

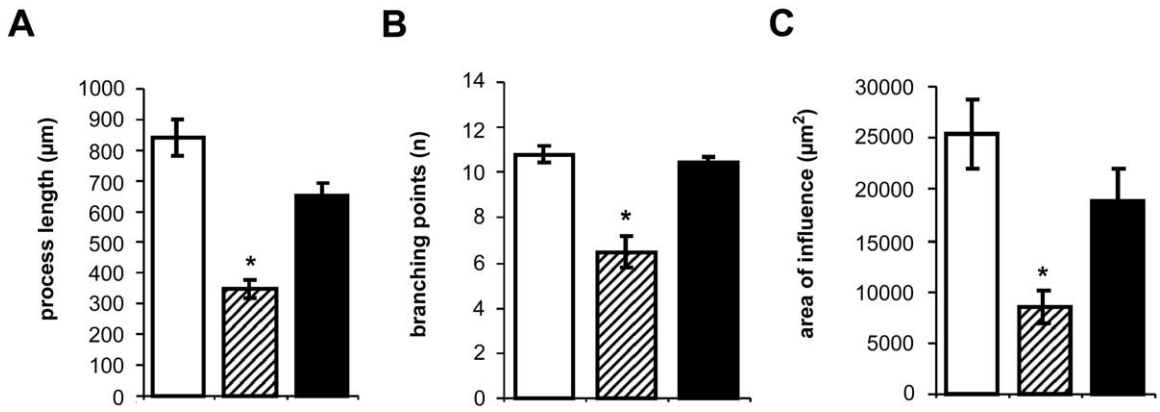

Fig. 5. Effects of creatine treatment on the morphological parameters of TH-ir neurons in ventral mesencephalic cultures exposed to MPP^+. Cultures were grown for 7DIV. MPP^+ [15 µM] exposure was performed from DIV5-7 in absence (hatched bars) or presence of creatine [5 mM] (filled bars). Untreated cultures served as controls (open bars). Note: MPP^+ administration resulted in decreased process length/neuron (A), number of branching points/neuron (B) and a smaller area of influence/neuron (C). Creatine improved all investigated parameters. Data are given as mean±S.E.M. and are expressed as percentage of the corresponding untreated controls. * Indicates significant difference ($P<0.05$) vs. corresponding control and creatine treatment.

against morphological deterioration induced by serum and glucose deprivation (Fig. 8). No effects were observed on number of primary neurites emerging from an individual soma (3.7±0.2, 3.3±0.2 and 3.4±0.2 for untreated controls, serum- and glucose-deprived cultures and cultures exposed to both the metabolic insult and creatine, respectively; mean±S.E.M.). Interestingly, the cell soma area was detected to be significantly smaller in cultures deprived of serum and glucose (by 20%), and the creatine treatment failed to provide neuroprotection (157.0±13.2,

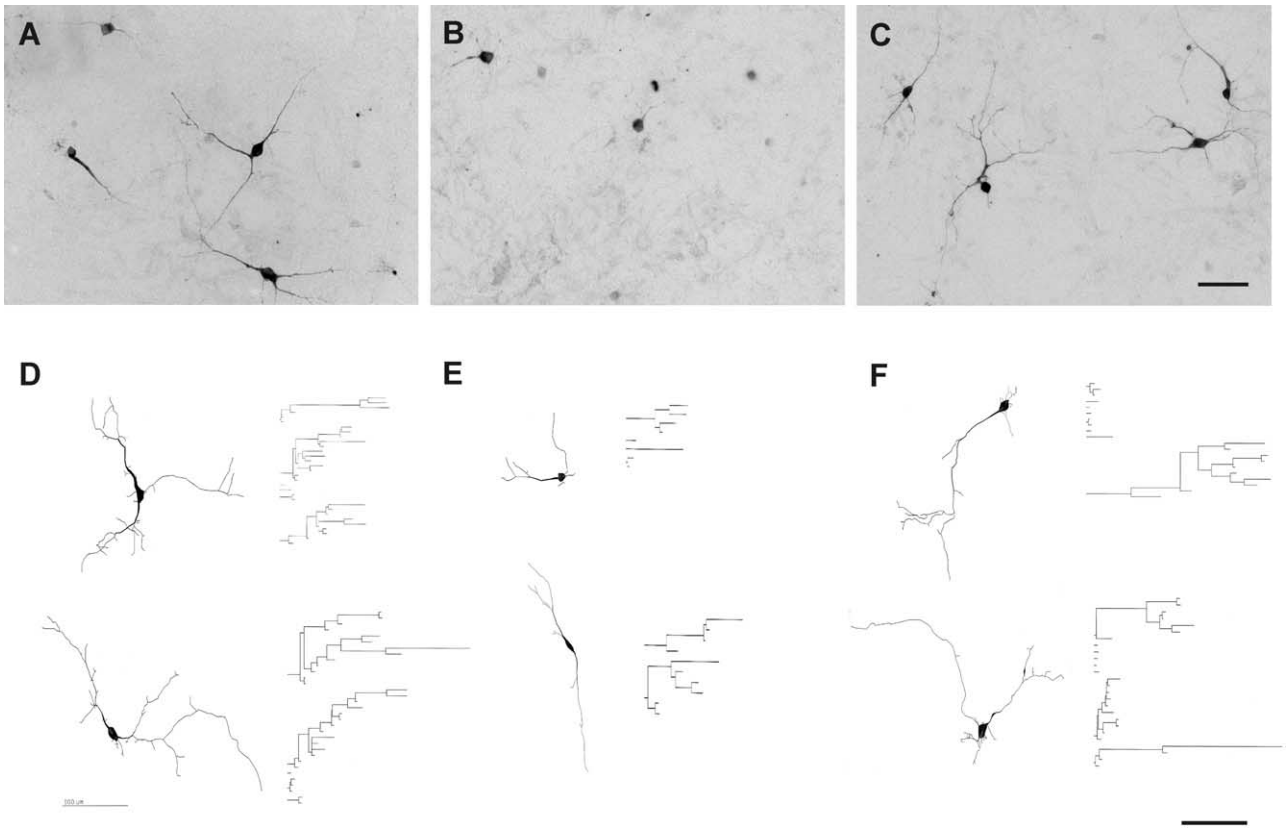

Fig. 6. Representative photomicrographs (upper panel) and computer-assisted reconstructions of two representative TH-ir neurons (lower panel) and their corresponding dendrograms (the *y* axis is chosen arbitrarily) from a control (A, D), a MPP^+-treated (B, E) and a combined MPP^+- and creatine-exposed culture (C, F). Dissociated ventral mesencephalic cultures were treated as described in Fig. 5. Note: Creatine treatment during the insults provided distinct neuroprotection for the TH-ir neurons. Scale bars=50 µm.

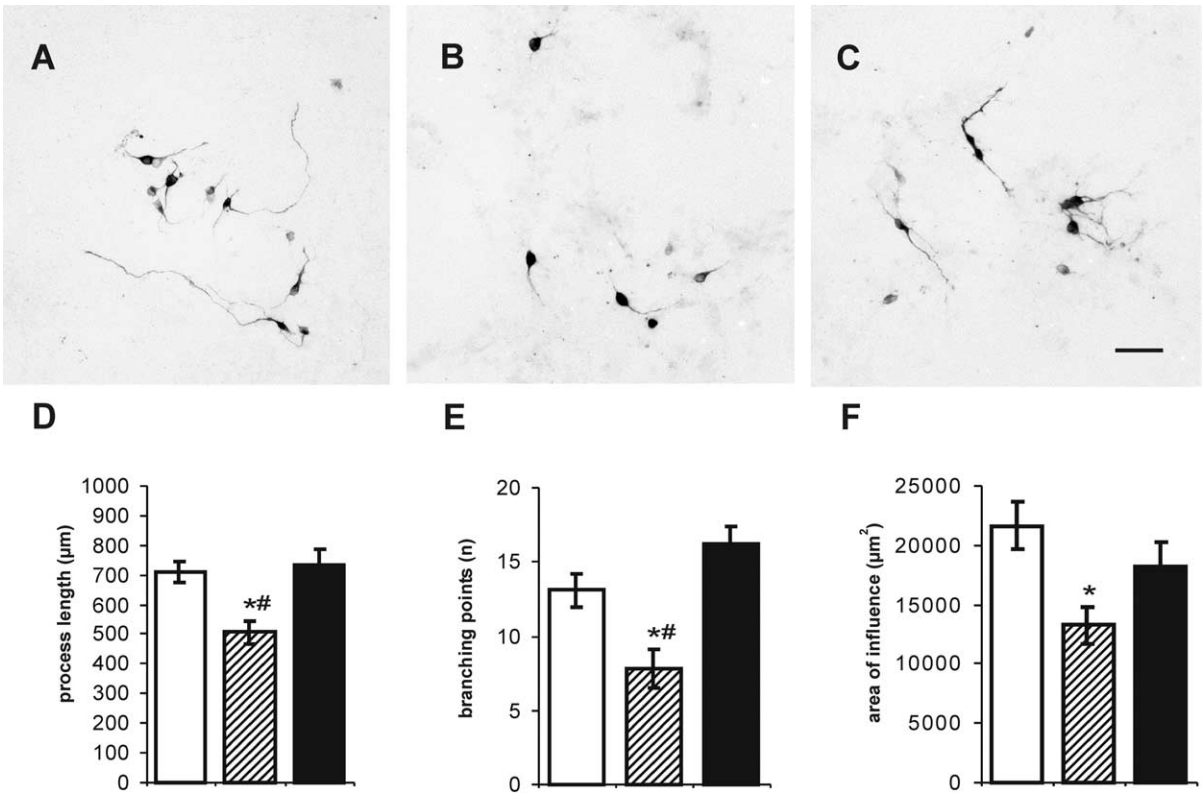

Fig. 7. Effects of creatine treatment on the morphological parameters of TH-ir neurons in ventral mesencephalic cultures exposed to 6-OHDA. The representative photomicrographs (upper panel) show TH-ir neurons from control (A), 6-OHDA- (B) and 6-OHDA- and creatine-exposed (C) cultures. Scale bar=50 μm. Cultures were grown for 7DIV. 6-OHDA [90 μM] exposure was performed from DIV6-7 in absence or presence of creatine [5 mM]. Untreated cultures served as controls (open bars). Note: 6-OHDA administration (hatched bars) resulted in decreased process length/neuron (D), number of branching points/neuron (E) and a smaller area of influence/neuron (F). Creatine (filled bars) improved process length/neuron and number of branching points/neuron, and showed a tendency for a larger area of influence/neuron. Data are given as mean±S.E.M. and are expressed as percentage of the corresponding untreated controls. * Indicates significant difference ($P<0.05$) vs. corresponding control and # Indicates significant difference ($P<0.05$) vs. corresponding creatine treatment.

122.3±13.9, 126.8±17.0 μm^2 for untreated controls, serum- and glucose-deprived cultures and cultures exposed to both the metabolic insult and creatine, respectively; mean±S.E.M.).

DISCUSSION

A number of reports have highlighted the protective potential of creatine supplementation in various disorders associated with premature neuronal death, including animal models of neurodegeneration, ischemia and trauma, as described above. In the present study, we investigated the possible effects of creatine administration on cultured embryonal mesencephalic tissue.

The presence of functional CK in the targeted tissue is necessary that cells can benefit from creatine treatment. Our analyses showed that both the brain-specific cytosolic (BB-CK) as well as the ubiquitous mitochondrial (uMt-CK) isotype of CK were present in cultured E14 rat mesencephalon in respectable amounts (about 2% of total tissue protein content). This is consistent with previously published results about the *in vivo* BB-CK content in rat embryonic brain tissue. CK appears to decrease slightly during embryonal development in utero and reaches adult levels thereafter quickly by a quadruple increase in the early postnatal period (Chen et al., 1995; Manos et al., 1991; Holtzman et al., 1993; Norwood et al., 1983). Enzyme activity assays have shown a corresponding increase for total CK activity in the same period (Chen et al., 1995; Holtzman et al., 1993). Using double-labeling immunofluorescence, we were also able to demonstrate that both BB-CK and uMt-CK are expressed in TH-ir neurons, hence the dopaminergic cells in our cultures are able to be potentially targeted by exogenous creatine supplementation.

In a first series of experiments, we investigated the effects of chronic creatine treatment on survival and morphology of TH-ir neurons. Chronic creatine treatment resulted in significantly higher numbers of surviving TH-ir neurons in our cultures. Except for an increase in neuronal soma size, probably due to osmotic reasons, morphological parameters of TH-ir neurons were not altered by creatine exposure. This may be indicative that creatine enhanced survival, but did not interfere with TH-ir cell differentiation, at least on a morphological basis. We did, however, not study other markers of differentiation like TH activity or dopamine production in the present experimental paradigm. This finding is in contrast to our observations

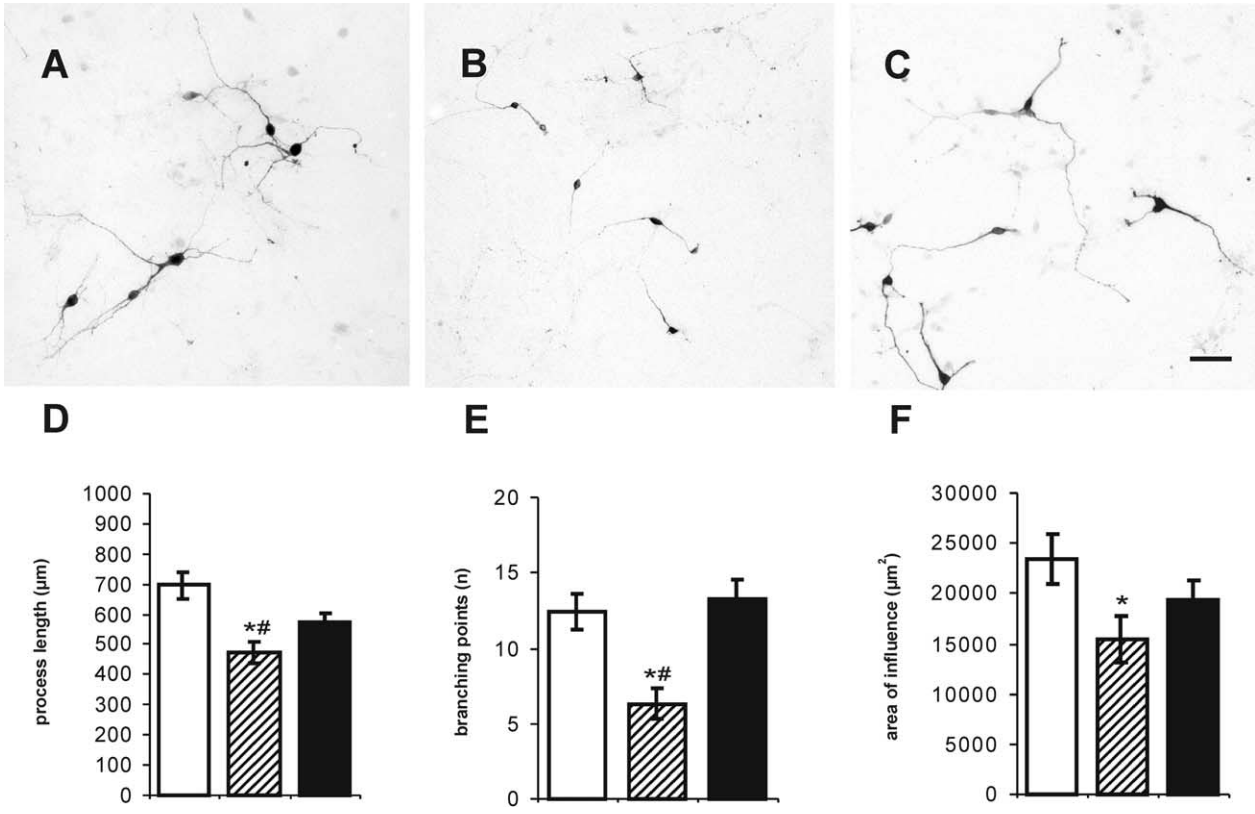

Fig. 8. Effects of creatine treatment on the morphological parameters of TH-ir neurons in ventral mesencephalic cultures exposed to a metabolic insult. The representative photomicrographs (upper panel) show TH-ir neurons from control (A), metabolic insult-exposed (B) and combined metabolic insult- and creatine-exposed cultures (C) cultures. Scale bar=50 μm. Cultures were grown for 7DIV. Serum and glucose-deprivation was performed from DIV5-7 in absence or presence of creatine [5 mM]. Untreated cultures served as controls (open bars). Note: The metabolic insult (hatched bars) resulted in decreased process length/neuron (D), number of branching points/neuron (E) and a smaller area of influence/neuron (F). Creatine (filled bars) improved process length/neuron and number of branching points/neuron, but only showed a tendency for a larger area of influence/neuron. Data are given as mean±S.E.M. and are expressed as percentage of the corresponding untreated controls. * Indicates significant difference ($P<0.05$) vs. corresponding control and # indicates significant difference ($P<0.05$) vs. corresponding creatine treatment.

made using glial cell line-derived neurotrophic factor, which promoted cell survival as well as morphological differentiation of dopaminergic neurons (Widmer et al., 2000). It has been previously reported that death of TH-ir neurons in dissociated VM cultures predominantly occurs during the first days after explantation, suggesting a distinct vulnerability in the early period of adaptation to adverse environmental factors or lack of appropriate trophic support (Fawcett et al., 1995). In accordance with these findings, we observed a continuous loss of TH-ir cell densities over the culture period, which was most prominent for the first 3 days. Thereafter, little changes in TH-ir cell densities were found in the cultures. The outcome with significantly higher TH-ir cell densities seen at the end of the culture period after short-term creatine exposure from DIV0-3, with no significant effects on cells treated from DIV5-7, further supports the idea of a role as a survival factor for creatine.

Experimental *in vitro* models are important tools to better understand the pathogenesis of PD and to test therapeutic approaches. Different pathological mechanisms that converge upon damage of dopaminergic neurons are discussed in distinct models. We assessed the protective effects of creatine supplementation against neuronal cell death induced by exposure to the neurotoxins MPP^+ and 6-OHDA, which represent established *in vitro* models of PD (see above), and during periods of metabolic stress induced by glucose and serum deprivation. Interestingly, we found that creatine administration resulted in distinct neuroprotection against TH-ir cell loss and morphological deterioration induced by MPP^+, 6-OHDA and the metabolic insult. The three insult paradigms used, however, showed different effects on dopaminergic neurons. While TH-ir cell densities were observed to be decreased by 25%, 40% and 30% for MPP^+, 6-OHDA and metabolic insult, respectively, the attenuation of process length per neuron was found to be 60% for MPP^+ treatment as compared with 30% in the latter two groups. The observation that MPP^+-treated neurons showed a marked decrease in neuritic complexity of individual TH-ir neurons, as compared with untreated controls, is in line with previous reports (Michel and Hefti, 1990). MPP^+ is actively accumulated into dopaminergic neurons via the dopamine re-uptake system (vide supra). As a consequence, a decreased dopamine uptake of TH-ir neurons is noticed, followed by a decrease in process length and a decrease

in TH activity (Michel et al., 1990). Hence, MPP^+ treatment offers a good model to study potential effects of creatine on morphology of dopaminergic neurons. The protection against the deterioration of neuronal morphology in addition to the effects observed on cell survival may therefore indicate that creatine exerts a similar mode of action on both features of TH-ir neurons and also hints to the idea that energetic processes are required for maintaining complex neuronal structures. It is to note, however, that the experimental design used in the present study cannot exclude that the preservation of morphology by creatine could also be of transient nature. The finding that creatine provided significant neuroprotection against MPP^+ toxicity may also point to the involvement of a specific anti-apoptotic mechanism described before (Dolder et al., 2003). Nevertheless, it has to be taken in mind that apoptotic processes are also prevented by protection of the cellular energy metabolism. We propose that creatine treatment mediated its neuroprotective effects primarily by increasing the intracellular PCr pool, which is compromised after MPP^+ administration (see above). Notably, in hippocampal cell cultures creatine administration was reported to alter the PCr to ATP ratio rather than enhancing ATP levels, therefore providing an effective energy shuttle (Brewer and Wallimann, 2000). The same authors suggest that a rise of the PCr to ATP ratio may serve to power ion pumps, whose activities are required for a number of crucial cellular functions (Brewer and Wallimann, 2000; Wallimann and Hemmer, 1994).

In contrast to MPP^+, 6-OHDA has been assumed to cause neuronal death by a free radical-mediated mechanism as well as inhibiting mitochondrial complexes I and IV (Glinka et al., 1997). There is some evidence that 6-OHDA induces rather apoptosis *in vitro*, while MPP^+ is thought to provoke both apoptotic and necrotic cell death in dopaminergic neurons (Mazzio and Soliman, 2003; Choi et al., 1999; Lotharius et al., 1999; Bilsland et al., 2002). As compared with MPP^+-treated cultures, the protective effect of creatine in this paradigm seems to be less pronounced for TH-ir cell morphology. While 6-OHDA-induced deterioration of overall process length and number of branching points per neuron was found to be counteracted by creatine treatment, only a tendency toward a protection was observed on the area of influence of an individual neuron. Assuming that indeed apoptosis played a substantial role after 6-OHDA exposure in our cultures, it might be hypothesized that the antiapoptotic effects of creatine are less important than its effects on cellular energy metabolism. Nevertheless, the report that creatine protected permeability transition pore opening in mitochondria (O'Gorman et al., 1997), may indicate that some of the effects observed for creatine in our cultures are also due to stabilization and protection of the permeability transition pore and mitochondrial integrity (Brdiczka et al., 1998; Brewer and Wallimann, 2000).

A metabolic insult induced by serum and glucose deprivation leads to a decrease in cellular ATP levels and consecutively to neuronal death, which can take place both by necrosis and apoptosis (Lemasters et al., 1999). Furthermore, it has been reported that glucose deprivation resulted in increased intracellular calcium levels (Cheng and Mattson, 1994). Serum and glucose deprivation resulted in a significant lower number and an altered morphology of TH-ir neurons in our cultures. Concurrent creatine addition to the culture medium during the period of the insult significantly protected against loss of TH-ir cells and also attenuated the morphological deterioration of these neurons in our experiments. However, the effects of creatine on morphological parameters were less prominent than in cultures facing MPP^+ toxicity. Neuroprotective actions of creatine have been previously illustrated against glutamate excitotoxicity *in vivo* (Andreassen et al., 2001b) as well as *in vitro* (Brewer and Wallimann, 2000). In line with the reports that excitotoxicity contributes to TH-ir cell loss in mesencephalic tissue (Schierle and Brundin, 1999; Zhang et al., 1998), it is tempting to speculate that creatine interacted with such events occurring during serum and glucose deprivation.

Protective effects of creatine treatment on neuronal cells have previously been reported in *in vitro* and in *in vivo* studies as well as in first clinical trials. Our findings are in line with neuroprotective effects that were found in experimental models of PD (Matthews et al., 1999), Huntington's disease (HD) (Ferrante et al., 2000; Shear et al., 2000; Andreassen et al., 2001a), amyotrophic lateral sclerosis (Klivenyi et al., 1999), spinocerebellar ataxia (Kaemmerer et al., 2001), as well as in tissue hypoxia (Wilken et al., 1998; Jensen et al., 1993; Whittingham and Lipton, 1981), ischemia (Toner and Stamford, 1999; Plaschke et al., 2000; Zhu et al., 2004) and traumatic brain injury (Sullivan et al., 2000). A recent clinical trial reported that after 8–10 weeks of creatine diet glutamate levels were significantly reduced in HD patients, indicating that creatine has a impact on HD brain metabolism (Bender et al., 2005). Furthermore, the present findings may also have significant implications in cell replacement strategies for the treatment of PD (Winkler et al., 2005). We hypothesize that combining transplantation of tissue and creatine supplementation may provide an appropriate way to enhance graft survival and function and also to slow down the progressing cell loss in PD.

The precise mechanisms underlying the effects of creatine on neuronal survival still remain unclear (Brustovetsky et al., 2001). In particular, it remains to be elucidated whether neuroprotective effects are primarily induced by support of the cellular energy metabolism alone or if there is significant involvement of direct antiapoptotic effects as reported in other studies (O'Gorman et al., 1997; Wallimann et al., 1998). Nevertheless, creatine administration at a concentration of 5 mM was found to be beneficial for the survival of TH-ir neurons in our cultures and to protect them against neurotoxic and metabolic insults. Hence, our data indicate that the CK/PCr system plays a key role in the energy metabolism of dopaminergic neurons.

Taken together, our findings identify creatine as a potent endogenous survival and neuroprotective factor for dopaminergic neurons, which may have important implications for the therapy of PD and other neurodegenerative

disorders as well as for improving the cell transplantation approaches.

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