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Creatine Supplementation Improves Dopaminergic Cell Survival and Protects Against MPP+ Toxicity in an Organotypic Tissue Culture System

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Cell replacement therapy using mesencephalic precursor cells is an experimental approach for the treatment of Parkinson's disease (PD). A significant problem associated with this procedure is the poor survival of grafted neurons. Impaired energy metabolism is considered to contribute to neuronal cell death after transplantation. Creatine is a substrate for mitochondrial and cytosolic creatine kinases (CK) and buffers cellular ATP resources. Furthermore, elevated cellular creatine levels facilitate metabolic channeling and show anti-apoptotic properties. Exogenous creatine supplementation therefore might offer a tool for improvement of dopaminergic neuron survival. The present study aimed at investigating the effects of creatine on cell survival of rat embryonic day 14 (E14) ventral mesencephalic neurons grown as organotypic free-floating roller tube (FFRT) cultures. We found that the brain-specific isoform of CK (BB-CK) and the ubiquitous mitochondrial isoform (uMt-CK) are expressed at high levels in FFRT cultures and colocalize with tyrosine hydroxylase immunoreactive (TH-ir) cells. Exposure of these cultures to creatine induced an increase in the content of the BB-CK isotype. Creatine (5 mM) administration starting at day in vitro (DIV) 7 resulted in a significant increase (+35%) in TH-ir cell density at DIV21. In addition, we observed that creatine treatment provided neuroprotection against 1-methyl-4-phenyl pyridinium ion (MPP^+)-induced TH-ir cell loss in the FFRT culture system, resulting in a significantly higher density (+19%) of TH-ir neurons in creatine-treated cultures compared to corresponding controls. The decrease of TH-ir neurons in the MPP^+-treated group corresponded with an increase in immunoreactivity for active caspase-3, an effect that was not seen in the group receiving creatine supplementation. In conclusion, our data imply that creatine administration is beneficial for the survival of TH-ir neurons encountering harmful conditions.

Key words: Ventral mesencephalic cell cultures; Creatine; Tyrosine hydroxylase; Free-floating roller tubes; MPP^+; Parkinson's disease

INTRODUCTION

Transplantation of embryonal ventral mesencephalic cells is an experimental therapy for the treatment of medically intractable Parkinson's disease (PD) and has been shown to be effective in reinnervating the host striatum (31,32,54,69). However, limited availability of human embryonal tissue originating from legal abortions, ethical concerns, and the poor survival of the grafts impair a widespread clinical application. Collecting and pooling tissue from multiple donors, in vitro conditioning (18,59,89,91), and exposure to neurotrophins (48,91), antiapoptotic factors (42,65,80), or antioxidative agents (35,71) are promising strategies to reduce the amount of tissue required to achieve a sufficient number of surviving functional dopaminergic neurons in the grafts. Optimal metabolic conditions are required during the in vitro storage period to prevent cell loss due to metabolic and oxidative stress (88). Furthermore, release of toxic metabolites and neurotransmitters with excitotoxic potential, such as dopamine (10) and glutamate (33), from dying cells contributes to an unfavorable environment and potentiate cell death (79). So far, culture conditions are still suboptimal compared to the in vivo environment, resulting in an unsatisfactory survival of dopaminergic neurons.

The phosphocreatine/creatine kinase system has been described to play an important role in cellular energy homeostasis in cells with high and fluctuating energy demands, like neurons, by acting as a temporal and spatial ATP buffer (93,94). Creatine (1-methyl-guanidinoacetic acid) supplementation has been shown to in-

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crease the intracellular phosphocreatine level and buffers cellular ATP resources under metabolic and/or oxidative stress (61,95,96,97), to act as a substrate for a cellular energy shuttle (20,43,44) and to inhibit apoptosis by means of stabilizing the mitochondrial permeability transition (14,44,68,73). Moreover, there is evidence that defects in creatine utilization are directly involved in the pathogenesis of several neurological diseases (3,4,11,82, 98). In line with these notions, recent studies demonstrated a neuroprotective effect of creatine supplementation in a variety of experimental models of neurological disorders including PD, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and ischemia (2,6, 7,23,57,58).

Knowledge on the functional effects of the creatine kinase/phosphocreatine system regarding the metabolism of embryonic neurons is limited. In rat embryonal brain tissue, the creatine kinase isoenzymes BB-CK (brain-specific cytosolic isotype) and uMt-CK (ubiquitous mitochondrial isotype) have been described to be present in moderate quantities at embryonic day 14 (E14), to decrease slightly during the further period in utero, and to increase rapidly up to adult levels in the early postnatal period (46). Enzyme activity assays have shown similar results for total CK activity in the developing rat brain (20,46), indicating an important role of CK and presumably also its substrate, creatine, for brain development and viability of brain cells. Indeed, in adult hippocampal neurons, creatine showed protection against glutamate excitotoxicity and β-amyloid precursor protein (15). Creatine supplementation may therefore also be effective in improving the metabolic state of embryonic neurons and neuronal precursors and thus constitute a means of improving cell viability and long-term survival of cells for subsequent transplantation.

Organotypic tissue cultures like the free-floating roller tubes (FFRT) culture system (89) offer advantages for transplantation by preserving the complex functional and structural interaction of the nigral neurons at least partially. In the present study, we investigated whether creatine supplementation has beneficial effects on the survival of embryonal mesencephalic dopaminergic neurons in FFRT cultures and addressed the possible protective properties against 1-methyl-4-phenylpyridinium ion (MPP^+)-induced cell death, which provides an effective model of PD in vitro.

MATERIALS AND METHODS

Preparation of Organotypic Cultures

After induction of deep pentobarbital anesthesia (Nembutal™, 100 mg/kg body weight; Abbott), E14 fetuses were removed by cesarean section from pregnant Sprague-Dawley rats (RCC Biotechnology, Füllinsdorf, Switzerland) and killed by decapitation. The fetal brains were carefully excised from the cranium (26), transferred to a petri dish with Dulbecco's modified Eagle medium (DMEM; Gibco), and the ventral mesencephali (VM) dissected according to standard procedures (89). The mesencephalic explants ($2 \times 1.5 \times 1$ mm in size) were cut into four equally large quadrants by a sagittal and a coronal incision and transferred into gas-permeable conical plastic tubes (Falcon), supplied with 1 ml of culture medium consisting of 55% DMEM, 32.5% Hank's balanced salt solution (HBSS; Gibco), 0.3% glucose, 10% fetal calf serum (FCS; Gibco), and 1% 0.01 M HEPES (Merck). The tubes were placed in a slowly (1 rev/min) rotating roller-drum (36) placed in an incubator at 37°C in a 5% CO_2 atmosphere as described in detail previously (89). The culture tubes were randomly assigned to either the creatine-treated or to the control group. Maintenance medium was changed every fourth day with antibiotics/antimycotics (No. 061-05240 D; Gibco) present during the first 4 days in culture. All animal procedures were approved by the Animal Care and Use Committee at the University of Berne.

Creatine Treatment and Tissue Processing

FFRT cultures were kept in standard culture medium. In the treatment groups, creatine monohydrate (Creapure™; SKW Trostberg) was added to the culture medium at a final concentration of 5 mM from day in vitro 7 (DIV7) to DIV21 at each medium change, while no creatine was added to the control groups. The concentration of 5 mM was chosen due to the maximal effects found on survival of dopaminergic neurons in mesencephalic cultures in a pilot study (Andres et al., unpublished data). Furthermore, Brustovetsky and coworkers reported creatine concentrations of 3 and 10 mM to exert neuroprotection against an energetic insult in hippocampal and striatal cultures, while lower doses turned out to be ineffective (19). Analyses proved that the concentration of the added creatine in the culture medium remained stable over the interval between the medium changes (48 h). Levels of endogenous creatine derived from the FCS content in the maintenance culture medium were determined to be much lower (45 μmol/L) as determined to be effective in influencing survival of neurons (i.e., >1 mM; Andres and Widmer, unpublished data). Creatine contents in the culture medium were assessed using standardized clinical enzymatic assays (Chemical Central Laboratories, University Hospital, Berne).

Cultures for immunohistochemistry were washed twice with ice cold HBSS before fixation in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 45 min at room temperature. Control cultures were fixed at DIV7 ($n = 12$, two independent experiments), DIV14 ($n = 24$, two independent experiments), and DIV21 ($n = 48$, three independent experiments), while

creatine-treated cultures were stopped and fixed at DIV14 ($n = 12$, two independent experiments) and DIV21 ($n = 48$, three independent experiments). The four cultures corresponding to one VM were pooled. Tissue was then rinsed three times in 0.1 M PBS, equilibrated in 15% sucrose in 0.1 M phosphate buffer solution containing 0.01% NaN_3, and cut at 20 µm on a freezing microtome (Frigocut 2800 N; Reichert-Jung). Slices were mounted on gelatine chrome-alum precoated glass slides.

To assess the protective potential of creatine against dopaminergic cell loss induced by MPP^+ (Sigma), cultures ($n = 72$, two independent experiments) were grown in vitro for 7 days. MPP^+ was added to the culture medium at a final concentration of 15 µM at DIV3 and DIV5 without ($n = 24$) or with concurrent creatine supplementation (5 mM) ($n = 24$), added 2 h prior to MPP^+ (74). Cultures receiving no MPP^+ in the absence of creatine served as controls ($n = 24$). For analysis of active caspase-3-immunoreactive cells in the cultures, experiments were performed with an identical setup ($n = 24$, two independent experiments). Cultures were stopped and fixed at DIV7 as described above.

For time course analysis of BB-CK expression cultures were grown for 7, 14, or 21 days in vitro ($n = 4$, for each group, two independent experiments) and processed for immunoblot analysis as described below.

Tyrosine Hydroxylase Immunohistochemistry

Every sixth section was selected for tyrosine hydroxylase (TH) immunohistochemistry. After three rinses in PBS, tissue sections were preincubated in 0.3% Triton X-100 in PBS plus 10% horse serum (HS) for 60 min, washed, and incubated overnight with the primary antibody (mouse monoclonal anti-TH, 1:500; Boehringer Mannheim) in 0.1% Triton X-100 in PBS plus 2.5% HS at 4°C. Following three washes, sections were incubated with a biotinylated secondary antibody (horse anti-mouse 1:200; Vector Labs) in 0.1% Triton X-100 in PBS plus 2.5% HS for 90 min. Endogenous peroxidase was blocked by 3% H_2O_2 and 10% methanol in PBS for 10 min. Following incubation with an avidin-peroxidase complex (1:150; Vector Labs) for 45 min, specifically bound antibody was visualized with a metal-enhanced 3,3′-diaminobenzidine (DAB) substrate kit (No. 34065; Pierce) for 3–5 min. Sections were dehydrated in alcohol, cleared in xylene, and mounted in Eukitt.

Detection of Active Caspase-3

Every sixth section was selected for active caspase-3 immunohistochemistry. After four rinses in PBS, tissue sections were preincubated in 0.1% Triton X-100 in PBS plus 10% HS for 120 min, washed, and incubated overnight with the primary antibody (anti active-caspase-3, 1:1000; Promega) in 0.1% Triton X-100 in PBS plus 2.5% HS at 4°C. Following four washes, sections were incubated with a biotinylated secondary antibody (horse anti-rabbit 1:200; Vector Labs) in 0.05% Triton X-100 in PBS plus 2.5% HS for 90 min. Endogenous peroxidase was blocked by 3% H_2O_2 and 10% methanol in PBS for 10 min. Following incubation with an avidin-peroxidase complex (1:150; Vector Labs) for 45 min, specifically bound antibody was visualized with a metal-enhanced DAB substrate kit (No. 34065; Pierce) for 3–5 min. Sections were dehydrated in alcohol, cleared in xylene, and mounted in Eukitt.

TH Colocalization of TH With BB-CK or uMt-CK

To perform colocalization studies, tissue sections were preincubated after three rinses in PBS in a buffer solution containing 0.5 M NaCl, 50 mM Tris-HCl, 5% bovine serum albumin (BSA; Sigma), and 2% milk powder in aqua destillatum (20) for 60 min, washed, and incubated overnight with the primary antibodies [rabbit anti-BB-CK or rabbit anti-uMt-CK, respectively, 1:1000 (81), and mouse anti-TH, 1:500; Boehringer Mannheim] in the buffer solution at 4°C. Following three washes, sections were incubated with the secondary antibodies (FITC-labeled anti-mouse 1:50 and TRITC-labeled anti-rabbit 1:100; DAKO, Denmark) in the buffer solution for 2 h. Slides were coverslipped in PBS containing 25% glycerol and immunofluorescence was performed at excitation wavelengths of 490 and 557 nm on an epifluorescence microscope (DMRB; Leica). Specifity of BB-CK and uMt-CK immunostaining was determined by incubation with preimmune serum.

Western Blot Analysis

Immunoblot analyses were carried out according to the method of Laemmli (53) with some modifications. Four cultures of the same group were pooled; the culture medium was dipped off and the tissue was rinsed twice with 7 ml sterile ice-cold HBSS and transferred into an Eppendorf tube with 150 µl of lysis buffer containing leupeptin (1 µl/ml), phenylmethylsulfonyl fluoride (PMSF, 5 µl/ml), β-mercaptoethanol (1 mM), EDTA (0.2 mM), and Triton X-100 (1 µl/ml) in HBSS. Cells were sonicated for 3 min and the lysate centrifuged for 2 min at 14,000 rpm; 7.5 µl of sample buffer [0.2 M Tris-HCl, pH 7.0, 30% glycerol, 6% sodium dodecyl sulfate (SDS), 15% β-mercaptoethanol, and 0.0075% bromphenol blue] was added to 15 µl of the supernatant and incubated at 95°C for 5 min. SDS-PAGE was performed using a 12% SDS-polyacrylamide separating gel with 1–10 µg of sample protein per lane. Proteins were blotted using the semidry technique on a 0.45 µm nitrocellulose membrane (BA85; Schleicher & Schüll) previously equilibrated in blotting buffer. Electrophoretic transfer was performed at a constant current of 120 mA during

45 min in blotting buffer consisting of 39 mM glycine, 48 mM Tris, 0.375% SDS, and 20% methanol. Sufficient transfer efficiency was checked by Coomassie blue staining of the gels for residual protein bands. After rinsing twice in TBS buffer (200 mM Tris, pH 7.5, 1.5 M 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 (87); mouse monoclonal anti-tyrosine hydroxylase, 1:500, Pharmingen; rabbit polyclonal anti-neuron-specific enolase, 1:500, Chemicon; mouse monoclonal anti-β-III-tubulin, 1:2000, Promega; mouse monoclonal anti-α-tubulin, 1:20000, Sigma] for 60 min at 22°C in the same solution. The blots were washed twice in TBS and subsequently incubated with antibody–peroxidase conjugates (goat anti-mouse or goat anti-rabbit, 1:20000; Boehringer Mannheim) for 45 min at 22°C in PBS containing 4% milk powder. Blots were washed three times in 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, USA). Band intensities were normalized to α-tubulin.

Analysis of Histological Sections and Statistics

Cell counts and measurements of culture volumes were carried out in a blinded experimental design under bright field illumination using a microscope (Axioplan; Zeiss) connected to a digital image processing system (Lucia 4.50; Nikon). Only cells with a distinct immunoreactivity, clear neuronal shape, and a nucleus were counted as TH-positive or active caspase-3-positive neurons, respectively. Cell numbers were corrected for double counting according to Abercrombie's formula (1), using the mean diameter of the nuclei. Estimations of cell numbers were carried out as described previously (45,60). In brief, the density of cultured TH-ir neurons was assessed by counting the numbers on every sixth culture section throughout the explant. The number of active caspase-3-ir neurons was estimated by counting three 20-μm sections within a 240-μm range, representing the central portion of the culture. Culture volumes were assessed by computer-assisted image analysis. As FFRT cultures, the VM tissue pieces assume a spherical shape within a few days and therefore culture volumes can be approximated by measuring the area of the most central section through the culture and calculating the volume of the corresponding sphere (89).

For statistical analysis, a commercially available software package was used (Statistica 5.0; StatSoft). Cell counts and measurements were expressed as the mean ± SEM. The experimental groups were compared by multivariate analysis of variance (MANOVA), followed by nonparametric post hoc Mann-Whitney test. The statistical significance level was set at $p < 0.05$.

RESULTS

Quantification of BB-CK and TH

The brain-specific cytosolic isoenzyme of creatine kinase (BB-CK) was present in detectable amounts at all time points assessed. Compared with untreated controls, chronic creatine treatment induced significantly higher levels of BB-CK in the cultures at DIV7 (+45%), while having no significant effects at later time points (Fig. 1A). Analysis of TH expression in untreated control cultures revealed a significant increase from DIV7 to DIV14 and from DIV14 to DIV21. Chronic creatine exposure did not result in altered TH levels (Fig. 1B).

Colocalization of TH With Creatine Kinases

All TH-ir neurons showed distinct colocalization with BB-CK and uMt-CK (Fig. 2). Beside cells with neuronal morphology, glial cells were also found to be immunoreactive for both creatine kinase isoenzymes (data not shown). No effects of creatine treatment on the distribution of CK expression between neuronal and nonneuronal cells was seen. Low signals were obtained for both BB-CK and uMt-CK as well as for TH in the central area of the cultures. This finding is consistent with the fact that centrally located cells in FFRT cultures are subject to necrosis.

Effects of Creatine on Culture Volumes, TH-ir Cell Numbers, and TH-ir Cell Density

In cultures kept in vitro up to DIV14, the mean density of TH-ir neurons was 64.4 ± 2.9 cells per mm² in the group receiving standard maintenance medium and 62.4 ± 5.3 cells per mm² in the group supplemented with creatine. Culture volumes (0.46 ± 0.03 vs. 0.53 ± 0.01 mm³) and number of TH-ir cells per culture (1361 ± 83 vs. 1187 ± 51 cells) did not differ between the treated and the untreated group. Therefore, no significant effects of the creatine treatment could be observed at this time point.

In a second series of experiments, effects of creatine treatment were assessed over a prolonged time period of 21 days in vitro. An untreated control group was stopped at DIV14 to ensure comparability with the previous experiments. In untreated controls stopped at different time points, the culture volumes significantly decreased from 0.95 ± 0.03 at DIV7 to 0.53 ± 0.01 at DIV14 and subsequently to 0.26 ± 0.02 mm³ at DIV21 (Fig. 3A), with a corresponding decrease in the number of TH-ir neurons (2722 ± 491, 1361 ± 83, and 458 ± 53, respectively; Fig. 3B) and in the density of TH-ir cells (73.1 ± 5.2, 64.4 ± 2.9, and 44.8 ± 4.1 cells per mm², respectively; Fig. 3C). Chronic creatine exposure starting at DIV7 resulted in a

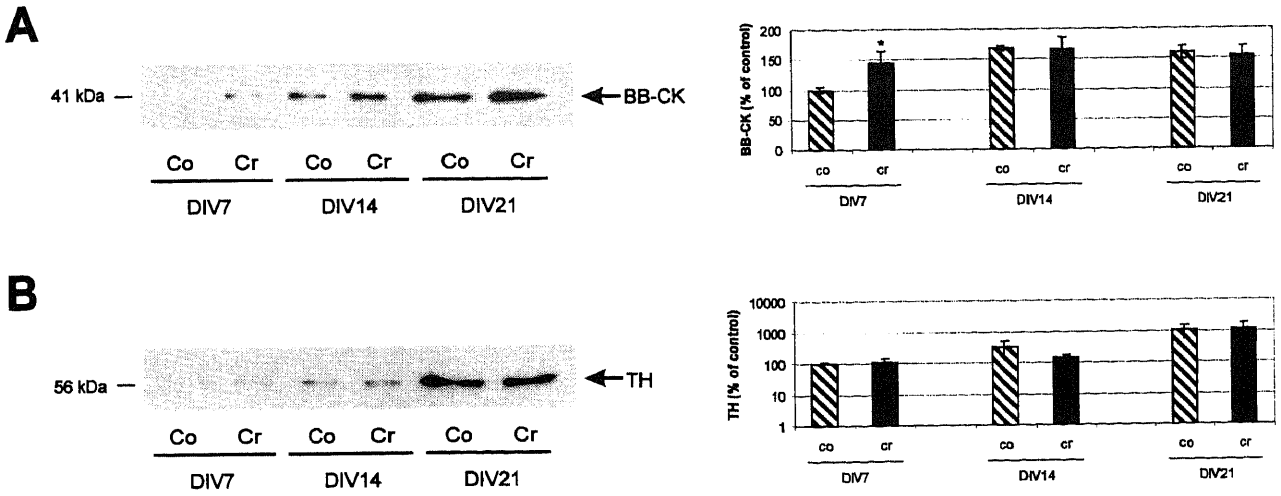

Figure 1. Western blot analysis of the effects of creatine on levels of brain-specific isoform of creatine kinase (BB-CK) (A) and tyrosine hydroxylase (TH) (B) in rat ventral mesencephalic FFRT cultures, as assessed at day in vitro (DIV) 7, 14, and 21. Corresponding bar graphs of digital quantification of the Western blots are presented to the right (two independent experiments). Chronic creatine treatment (Cr, filled bars) resulted in significantly higher levels of BB-CK, as assessed at DIV7 and compared to controls (co, hatched bars). No significant effects of creatine treatment were observed at later time points. Creatine exposure did not affect TH expression levels in the cultures. Data in the bar graphs are given as a percentage of control values at DIV7 and are presented as mean ± SEM. $*p < 0.05$ versus corresponding control.

significant increase in the number of TH-ir cells per culture, as assessed at DIV21 and compared against untreated controls (620 ± 67 vs. 458 ± 53 cells, 35% increase; Fig. 3E). The VM culture volume at DIV21 was not significantly affected by the creatine treatment, compared against controls (0.28 ± 0.02 vs. 0.26 ± 0.02 mm^3, 7.7% increase; Fig. 3D). TH-ir cell density was calculated and a significant increase was found in the treated group compared to controls (57.6 ± 2.4 vs. 44.7 ± 4.1 cells per mm^2, 29% increase, Fig. 3F, Fig. 4).

Effects of Creatine After MPP⁺ Exposure

Exposure of the cultures to the neurotoxin MPP⁺ (15 μM) resulted in a significantly lower density of TH-ir neurons, as assessed at DIV7 and compared to untreated controls (53.3 ± 2.9 vs. 65.6 ± 5.5 cells per mm^2, 19% decrease). Creatine administration resulted in a protection against the insult with significant higher densities of TH-ir cells compared to cultures exposed to MPP⁺ but not receiving creatine (70.2 ± 4.6 vs. 53.3 ± 2.9 cells per mm^2) (Fig. 5, Fig. 6A). In cultures exposed to MPP⁺, a higher number of active caspase-3-ir cells was found compared to untreated controls (165.7 ± 11.1 vs. 136.6 ± 9.2 cells per mm^2, 21% increase), as assessed at DIV7. However, this effect was not statistically significant. Creatine treatment showed a markedly attenuated increase in the number of cells immunoreactive for active caspase-3 after exposure to MPP⁺ than cultures not creatine supplemented (Fig. 6B).

A significant decrease in the tissue content of TH protein after MPP⁺ exposure and partial protection by concurrent creatine treatment was also confirmed by Western blotting analysis of FFRT cultures at DIV7 (Fig. 7). No effect of MPP⁺ and creatine was found on expression levels of the neuronal markers neuron-specific enolase (NSE) and β-III-tubulin (Fig. 7).

DISCUSSION

During the last decade, strong evidence has grown that impaired cellular energy metabolism, particularly defective mitochondrial function, contributes to neuronal death in PD (13,40,63,76) as well as in other neurodegenerative disorders, such as Huntington's disease (HD) (9,17,38), progressive supranuclear palsy (PSP) (5,12,56), Alzheimer's disease (AD) (3,8,47,72), and amyotrophic lateral sclerosis (ALS) (13,16,22,30).

Reduced mitochondrial complex-I activity and consecutive production of cytotoxic reactive oxygen species (ROS) has been found in patients suffering from idiopathic PD (49,55,77). Maternally inherited forms of PD associated with complex-I deficiency furthermore support the hypothesis concerning a mitochondrial involvement in the pathogenesis of the disorder (39,84,85). Administration of the neurotoxin MPP⁺, which acts as a potent complex-I inhibitor and exerts its neurotoxic potential through disruption of the mitochondrial respiratory chain (86), induces lesions similar to those found in PD in the brains of exposed animals and humans.

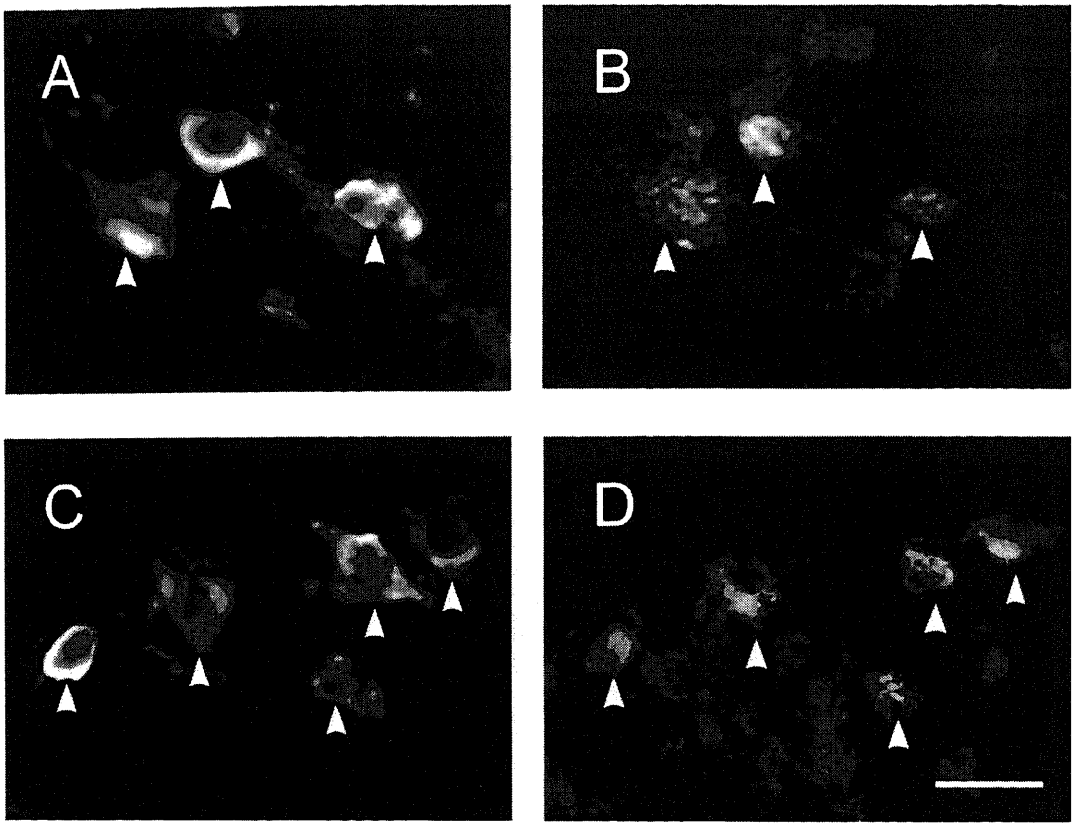

Figure 2. Digitalized photomicrographs (original magnification ×400) of double immunofluorescence for tyrosine hydroxylase (A, C), the brain-specific isoform of creatine kinase (B), and ubiquitous mitochondrial creatine kinase (D) in untreated ventral mesencephalic cultures grown for 14 days. Tyrosine hydroxylase-expressing neurons were found to colocalize with both creatine kinase isoenzymes (arrows). Scale bars: 15 μm.

Improving the metabolic state of neuronal cells may therefore offer an additional therapeutic strategy for the treatment of PD.

Several studies have reported that creatine supplementation has neuroprotective effects in various disorders associated with premature neuronal death. Protective effects have been found in normal hippocampal cells against neurotoxins (15) as well as in different animal models of neurodegeneration (6,29,51,57,83), ischemia (96,100), and trauma (78,90). Creatine-induced neuroprotection has also been demonstrated in several in vitro models of neurotoxicity and metabolic insults (15,19,61,92). Therefore, creatine is suggested to have potential as a therapeutic molecule for the treatment of PD (28,50). Regarding these findings, it should be rational to assume a creatine-mediated benefit on embryonic neuronal precursor cells. In the present study, we investigated the possible effects on cultured embryonal mesencephalic tissue. Long-term survival of TH-ir neurons in culture is a key factor for the efficiency of neurotransplantation approaches in PD.

The presence of sufficient amounts of functional CK in the treated tissue is a mandatory condition for cellular benefit of creatine supplementation. Our analyses showed that the cytosolic BB isotype of CK was present in FFRT-cultured E14 rat ventral mesencephalon in respectable amounts (about 2% of total tissue protein content) and increased considerably during the in vitro period up to DIV21. Corresponding results have previously been shown for the in vivo BB-CK content in the developing rat brain, where it appears to decrease slightly during embryonal development in utero and rapidly reaches adult levels by a quadruple increase in the early postnatal period (20,46,67). Regarding the fact that the early postnatal period corresponds to the days following DIV7 in tissue derived from E14 embryos, the pattern of BB-CK expression seems to show only minor quantitative changes in an organotypic ex vivo environment. However, creatine supplementation resulted in a significant upregulation of BB-CK expression in the treated cultures grown for 1 week. Because the BB-CK content in the cultured tissue was substantial and colocalization studies confirmed its abundant presence in TH-ir neurons at DIV14, we postulate that the missing effects of

chronic creatine treatment on dopaminergic cell density up to DIV14 are not due to insufficient presence of creatine kinases.

The observation that creatine exposure failed to increase survival of dopaminergic neurons in cultures up to DIV14 but was effective between DIV14 and DIV21 may be based on the fact that we used an organotypic culture system. In monolayer cultures of embryonic rat VM origin a continuous loss of TH-ir cells, most prominent during the first days in culture, has been demonstrated (27,99). In contrast, our study comprised tissue cultured using the FFRT method, which allows storage of embryonic VM prior to transplantation without a major loss of dopaminergic neurons for a time period of at least 1 week (45). This is likely due to the preservation of cell to cell interactions and of the natural cytoarchitecture in the organotypic culture system. Hence, the applicability of this particular model for the investigation of cell survival of dopaminergic neurons by creatine may be not appropriate for relatively short culture periods. However, we were able to demonstrate protective effects of creatine on dopaminergic cell survival in a period when significant cell loss occurs in the FFRT cultures (i.e., after 12 days in vitro) (45). Furthermore, it has to be considered that the cellular energy metabolism reserves of dopaminergic cells may be sufficient under standard culture conditions in the early in vitro period, but creatine addition might boost ATP resources and facilitate metabolic channeling at a later time point, when axonal and dendritic outgrowth and synaptogenesis occur and much higher energy demands result due to augmented protein synthesis, impaired spatial configuration of the metabolic pathways, and beginning electrophysiological activity. Based on this notion, it is suggested that

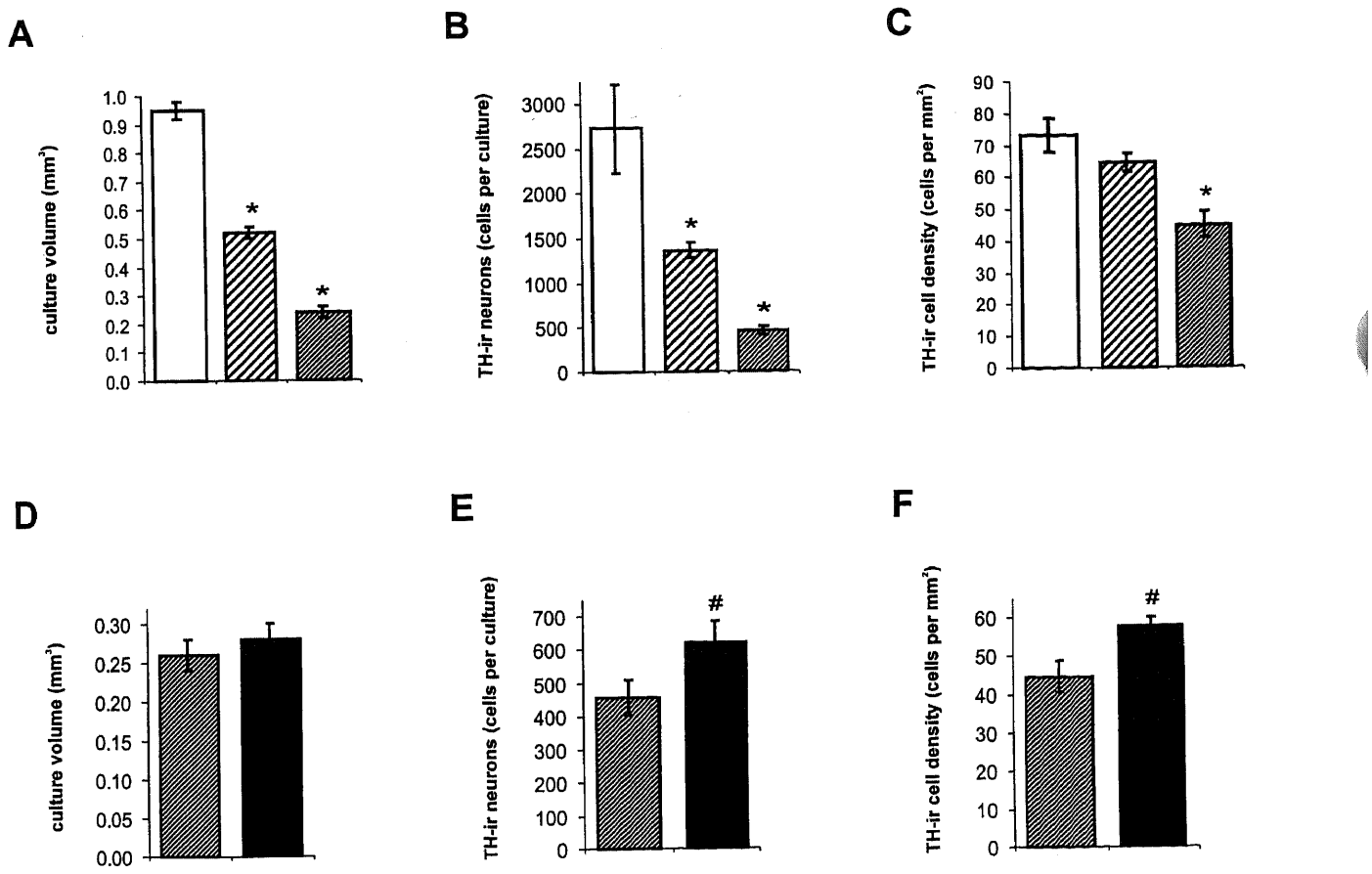

Figure 3. Time course analysis of culture volume, number of TH-immunoreactive (-ir) neurons per culture, and TH-ir cell density as assessed at DIV7 (open bars), DIV14 (hatched bars), and DIV21 (densely hatched bars), and effects of creatine on these parameters as analyzed at DIV21. Culture volume (A) and the number of TH-ir neurons per culture (B) decreased significantly over the time period analyzed, while TH-ir cell density (C) was only significantly decreased at DIV21 compared to values determined at DIV7. Creatine treatment (filled bars) had no effect on culture volume (D), but significantly increased the number (E) and the cell density (F) of TH-ir neurons in the cultures grown for 21 days compared to controls (densely hatched bars). Values are presented as mean ± SEM. $*p < 0.05$ versus corresponding cultures at DIV7. $\#p < 0.05$ versus corresponding untreated controls.

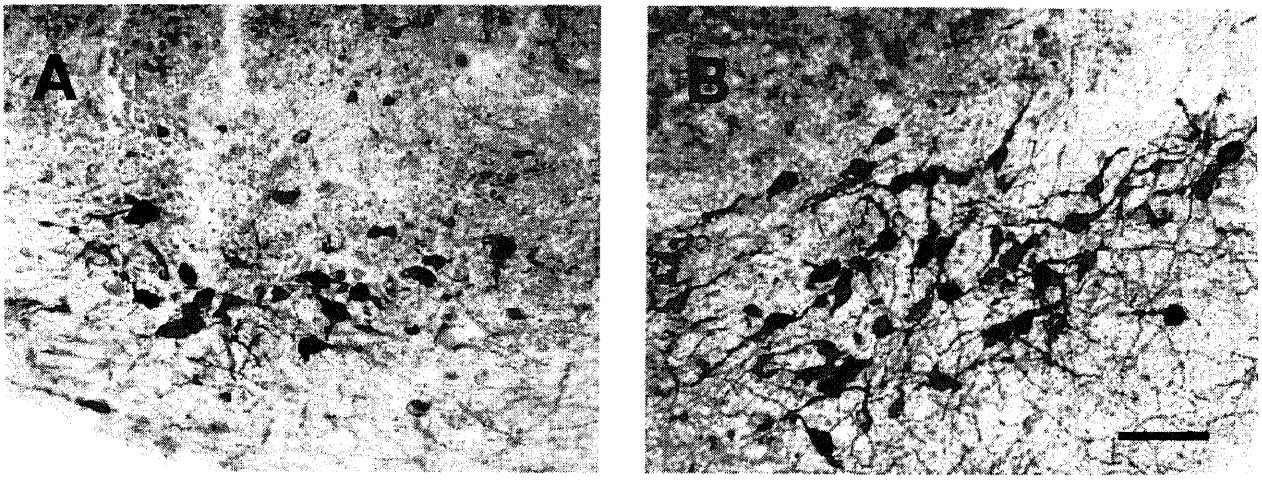

Figure 4. Representative photomicrographs (original magnification ×200) from TH-immunoreactive neurons in ventral mesencephalic FFRT cultures grown in absence (A) or presence (B) of creatine (5 mM) at DIV21. Scale bar: 40 μm.

the usage of cultures with a significantly smaller extent of cell–cell contact than the organotypic FFRT approach might result in a more pronounced effect of creatine treatment on dopaminergic neurons survival. However, this was not the primary goal of the recent study, which addressed ways for improvement of tissue quality prior to transplantation when tissue properties should not be too much altered.

The reasons for loss of dopaminergic cells in vitro are still debated. Pathophysiological mechanisms like excitotoxicity (41,75) or free radical generated toxicity (66) may contribute to cell death. Apoptosis has been reported to contribute to the loss of embryonic dopaminergic nigral cells in culture (21,62). Programmed cell death occurring as a natural phenomenon in a biphasic pattern with a first peak of apoptotic dopaminergic neurons around postnatal day 2 (70) has been shown in the developing rat substantia nigra. This time point would correspond to DIV9 for cultures initiated at E14, consistent with the period of pronounced cell loss between DIV8 and DIV12 we reported previously (45).

Despite intense research activities in this field during the last years, the precise mechanisms underlying the effects of creatine on neuronal survival still remain un-

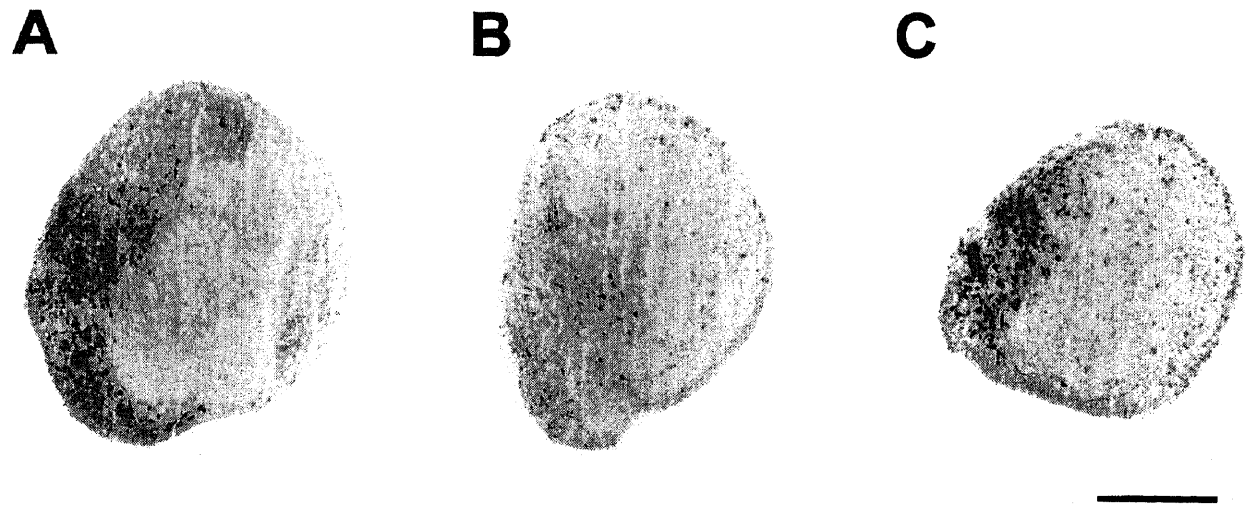

Figure 5. Representative photomicrographs (original magnification ×25) of TH-stained cryosections from ventral mesencephalic FFRT cultures grown for 7 days and exposed to MPP^+ (15 μM) from DIV3 to DIV7 in the absence (B) or presence of creatine (C). Untreated cultures served as controls (A). Creatine treatment protected TH-immunoreactive neurons against MPP^+-induced neurotoxicity. Scale bar: 250 μm.

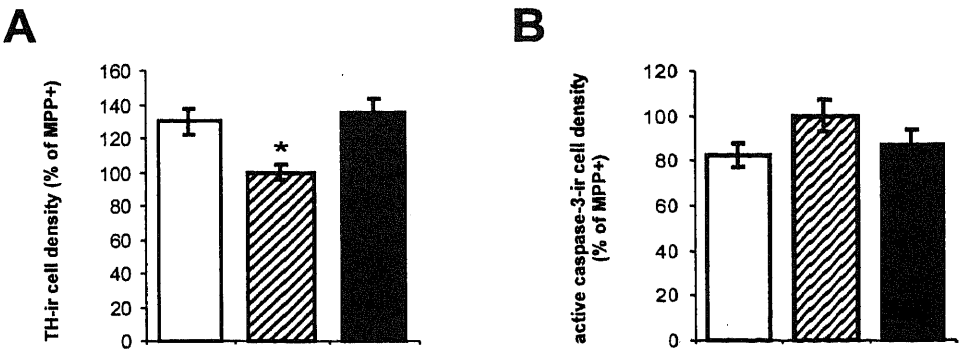

Figure 6. Effects of creatine treatment on TH-immunoreactive (TH-ir) cell density in FFRT cultures undergoing a neurotoxic insult. Cultures were grown for 7 days and exposed to MPP^+ (15 μM) from DIV3 to DIV7 in the absence (hatched bar) or presence of creatine (5 mM) (filled bar). Untreated cultures served as controls (open bars). Creatine treatment resulted in significant protection against MPP^+-induced cell loss (A). A nonsignificant increase in the number of cells immunoreactive for active caspase-3 was found in the cultures exposed to MPP^+, an effect that was less pronounced in cultures receiving concurrent creatine supplementation. Values are presented as mean ± SEM. $*p < 0.05$ vs. control and MPP^+ and creatine.

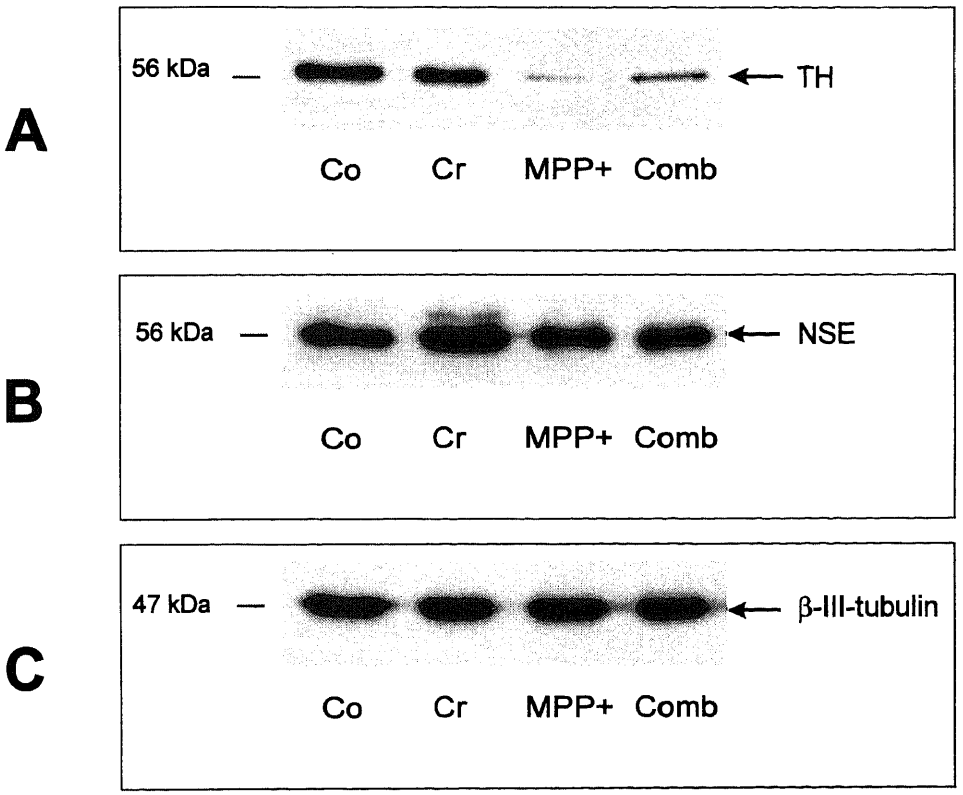

Figure 7. Western blot analysis of tyrosine hydroxylase (TH; A), neuron-specific enolase (NSE; B), and β-III-tubulin (C) in FFRT cultures grown for 7 days in vitro and exposed to MPP^+ (15 μM) at DIV3 and DIV5 in the absence (MPP^+) or presence of creatine (5 mM) (Comb). Cultures not exposed to MPP^+ grown without (Co) or with creatine (5 mM) (Cr) served as controls. Creatine treatment did not result in altered expression of TH, NSE, or β-III-tubulin. MPP^+ exposure resulted in significantly lower levels of TH protein expression in the cultures. Concurrent creatine treatment partially protected against this decrease.

clear (19). 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 (68,92). However, a recent publication failed to detect activity of this mechanism in isolated rat brain mitochondria pretreated with creatine (19). It should not be excluded, however, that attenuation of the mitochondrial permeability transition by creatine contributes to neuroprotection in intact neurons.

NMDA receptor-mediated excitotoxicity has been shown to contribute to TH-ir cell loss in vitro (79). There is evidence for a protective potential of creatine against NMDA excitotoxicity (19,57). However, addition of the noncompetitive NMDA antagonist dizocilpine hydrogen maleate (MK-801; RBI) at a concentration of 50 μM to the culture medium had no effect on the survival of dopaminergic neurons in untreated FFRT cultures nor did it influence the effects of creatine treatment in our setup (data not shown). It might be possible that the close glia–neuron interaction in organotypic cultures is responsible for the missing excitotoxic effects.

Apoptotic processes have recently been recognized to constitute a major mechanism of neuronal death in the context of neurodegenerative disorders (34). Studies of MPP^+ have shown that the neurotoxin induces apoptosis in dopaminergic neurons in vitro (24,64). TUNEL staining confirmed in a study by Dodel et al. (25) that TH-ir cells exposed to MPP^+ die via apoptosis. In line with these reports, treatment of our VM cultures with MPP^+ resulted in a significant decrease in the number of TH-ir neurons and in a corresponding trend towards higher active caspase-3 immunoreactivity. Creatine was able to significantly reduce the extent of MPP^+-induced cell loss in VM organotypic cultures as demonstrated by analysis of number of surviving TH-ir neurons. Accordingly, the number of cells immunoreactive for active caspase-3 was found to be decreased in MPP^+-treated cultures cotreated with creatine; however, this effect was not statistically significant. General neuronal viability, as assessed by analysis of NSE and β-III-tubulin expression, was not affected by MPP^+ or by creatine. Our results further confirm the neuroprotective property of creatine against MPP^+ toxicity reported in a previous study (57).

In conclusion, our data imply that creatine administration at a concentration of 5 mM is not beneficial for the survival of E14 rat ventral mesencephalic TH-ir neurons up to DIV14. However, a significantly better survival of TH-ir neurons was found in creatine-treated VM tissue kept in culture for longer than 14 days. This finding suggests that the time window for effective storage of dopaminergic tissue using the FFRT technique prior to grafting could be prolonged by creatine addition at a concentration of 5 mM to the culture medium. However, the optimal period for transplantation has been determined to be significantly earlier by previous studies. For rat ventral mesencephalic cultures grown longer than 1 week, poor graft survival and recovery is expected based on in vitro data (45), a fact that limits the potential of creatine treatment in this paradigm. On the other hand, creatine administration to the recipient of the grafts might be effective in improving survival and integration of dopaminergic cells after transplantation. We hypothesize that a combination of creatine treatment with transplantation of creatine-pretreated VM FFRT cultures may provide an appropriate method to further enhance graft survival. In addition, given the supposed protective effects of creatine on dopaminergic cells suffering compromised energy metabolism, the creatine diet may also slow down the progressing cell loss in PD. Notably, creatine supplementation shows low side effects even at high dosages (37,52). In combination with growth factors, effects of creatine may be found earlier and/or more pronounced due to the induction of differentiation and higher energetic demands.

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