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Effects of creatine treatment on survival and differentiation of GABA-ergic neurons in cultured striatal tissue

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Abstract

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder, characterized by a prominent loss of GABA-ergic medium-sized spiny neurons in the caudate putamen. There is evidence that impaired energy metabolism contributes to neuronal death in HD. Creatine is an endogenous substrate for creatine kinases and thereby supports cellular ATP levels. This study investigated the effects of creatine supplementation (5 mM) on cell survival and neuronal differentiation in striatal cultures. Chronic creatine treatment resulted in significant increased densities of GABA-immunoreactive (-ir) neurons, although total neuronal cell number and general viability were not affected. Similar effects were seen after short-term treatment, suggesting that creatine acted as a differentiation factor. Inhibitors of transcription or translation did not abolish the creatine-mediated effects, nor did omission of extracellular calcium, whereas inhibition of mitogen-activated protein kinase and phosphatidylinositol-3-kinase significantly attenuated the creatine induced increase in GABA-ir cell densities. Creatine exhibited significant neuroprotection against toxicity instigated either by glucose- and serum deprivation or addition of 3-nitropropionic acid. In sum, the neuroprotective properties in combination with promotion of neuronal differentiation suggest that creatine has potential as a therapeutic drug in the treatment of neurodegenerative diseases, like HD.

Keywords: creatine, creatine kinase, differentiation, GABA, ganglionic eminence, neuroprotection.

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Huntington's disease (HD) is an inherited, autosomal dominant neurodegenerative disorder characterized by involuntary, uncontrollable choreiform movements of the body coupled with progressive emotional and cognitive disturbances (Sanberg and Coyle 1984; Hefter *et al*. 1987). Recent studies on the neuronal pathology of HD have uncovered an intricate mosaic of related and interdependent neurochemical and histopathological changes in the striatum and related structures. The disease phenotype in HD is caused by expansion of a polyglutamine tract in the protein huntingtin (The Huntington's Disease Collaborative Research Group 1993), leading to the accumulation and aggregation of cleaved N-terminal fragments in the cytoplasm and nucleus (Mangiarini *et al*. 1996). Widespread degeneration of the GABA-ergic medium-sized spiny projection neurons in the caudate putamen is the characteristic pathological hallmark of HD. Recently, evidence has grown that impaired energy metabolism due to reduced mitochondrial complex-II and complex-III activity contributes to neuronal death during the course of the disorder (Gu *et al*. 1996; Calabresi *et al*. 2001). An impaired cerebral metabolism has been shown by magnetic resonance spectroscopy in the basal ganglia and the occipital cortex of HD patients (Jenkins *et al*. 1993), who

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Abbreviations used: BB-CK, brain-type cytosolic CK; CK, creatine kinase; DARPP-32, dopamine- and cAMP-regulated phosphoprotein-32; DIV, day *in vitro*; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GE, ganglionic eminence; HBSS, Hank's balanced salt solution; HD, Huntington's disease; ir, immunoreactive; MAPK, mitogen-activated protein kinase; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NeuN, neuronal nuclei; 3-NP, 3-nitropropionic acid; PBS, phosphate-buffered saline; PtdIns3K, phosphatidylinositol 3-kinase; uMt-CK, ubiquitous mitochondrial CK.

also show a reduced phosphocreatine/P_i ratio in skeletal muscle (Koroshetz *et al*. 1997). Further evidence for the involvement of mitochondrial dysfunction in the pathogenesis of HD has been obtained using transgenic mouse models of HD (Tabrizi *et al*. 2000). Yet, despite considerable research efforts, a wide gap remains between knowledge about the neuronal substrates of HD and the ability to prevent or alleviate the progression of the disorder.

Transplantation of embryonic striatal tissue has been shown to restore deficits in experimental animal models of HD (Deckel *et al*. 1983; Isacson *et al*. 1986; Dunnett *et al*. 1988) as well as in first trials in patients (Bachoud *et al*. 2000a; Freeman *et al*. 2000). A preliminary study has shown that striatal transplantation can promote functional recovery in HD patients (Bachoud *et al*. 2000b). However, the limited availability of tissue suitable for transplantation, ethical issues and poor survival and integration in the host brain of the transplanted neurons still prevent widespread clinical application. Recent research has, therefore, focused on establishing alternatives to fetal tissue, particularly stem cells and neuronal precursors (Snyder and Macklis 1995; Martinez and Bjorklund 1997; McKay 1997). Drugs that influence the differentiation of stem cells and neuronal precursors into a specific neurochemical phenotype therefore have potential for improving cell-replacement techniques.

Creatine is a substrate for cytosolic and mitochondrial creatine kinases (CK) and thereby modulating ATP metabolism in the cell by increasing the phosphocreatine pool (Woznicki and Walker 1979) 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, 1999). In line with this notion using hippocampal cell cultures, creatine supplementation has been shown to increase the phosphocreatine to ATP ratio, therefore providing an effective energy shuttle (Brewer and Wallimann 2000).

In addition, there is evidence for a direct anti-apoptotic effect of elevated cellular creatine concentrations by inhibition of the mitochondrial permeability transition pore opening (O'Gorman *et al*. 1997; Dolder *et al*. 2003). Because neurons have only a very low capacity to synthesize creatine, they rely on a creatine supply from glia cells or from the blood stream (Tachikawa *et al*. 2004). Administration of exogenous creatine has previously been shown to result in higher cerebral phosphocreatine levels (Matthews *et al*. 1998), which are reduced in HD patients and correlate with the clinical severity of the disorder (Aronin *et al*. 1995). Protective effects of creatine treatment have been reported in the R6/2 transgenic mouse model of HD (Ferrante *et al*. 2000; Dedeoglu *et al*. 2003).

Improving cellular energy metabolism by creatine supplementation may, therefore, offer a feasible approach not only to reduce neuronal deterioration during the course of HD, but also to influence neuronal differentiation and survival in the context of cell-replacement strategies. We therefore examined the effects of creatine on survival and differentiation of striatal precursor cells in tissue culture.

Materials and methods

Preparation of dissociated cultures

After induction of deep pentobarbital anaesthesia (100 mg/kg body weight i.p.; Nembutal™, Abbott, North Chicago, IL, USA), embryonic day 14 (E14) fetuses (E0 = day of vaginal plug) were removed by cesarean section from pregnant Wistar rats (Janvier, Le Genest Saint Isle, France). In brief, the whole ganglionic eminence (GE) was dissected bilaterally in cold Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) according to standard procedures (Dunnett and Björklund 1992). Cell suspensions were prepared according to previously published procedures, with slight modifications (Widmer and Hefti 1994). Tissue pieces were washed twice in DMEM and then mechanically dissociated in 2 mL culture medium by gently pipetting through a 1 mL sterile pipette. Immediately after this trituration, $\approx$ 10 mL of medium was added and the undispersed tissue pieces were allowed to settle. Most of the supernatant, containing the individual cells, was then transferred to a second tube. About 1.5 mL medium were left in the tube for a second trituration. The cells were spun down (Hereaus centrifuge, 130 *g*, 4 min) and resuspended in 2 mL of DMEM. Cell viability, as assessed using the Trypan Blue dye exclusion method in a Neubauer chamber, was always above 65%. For immunocytochemistry and GABA-uptake assays, aliquots corresponding to one GE were pipetted per well (seeding density of 600 viable cells per mm^2) in 24-well plates (Multiwell 3047, 14 mm diameter per well; Falcon, Lincoln Park, NJ, USA) containing glass coverslips of 12 mm diameter (Assistent, Sandheim, Germany). For immunoblot analysis, aliquots corresponding to six GE were pipetted per well (seeding density of 600 viable cells per mm^2) in six-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, USA) in 0.15 M sodium borate buffer, pH 8.3 over night and washed three times with sterile phosphate-buffered saline (PBS) before adding culture medium.

The neurons were grown in 0.5 and 2.5 mL, for 24- and 6-well plates, respectively, of standard culture medium consisting of 55% DMEM, 32.5% Hank's balanced salt solution (HBSS; Gibco, Basel, Switzerland), 1.5% glucose, 10% fetal calf serum (FCS; Gibco) and 1% 0.01 M HEPES (Merck, Darmstadt, Germany). The plates were placed in an incubator at 37°C in a 5% CO_2 atmosphere. Medium was changed 24 h after plating and, subsequently, every other day. Antibiotics/antimycotics (No. 061-05240D; Gibco) were present during the first three days in culture.

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 regimens

Wells were randomly assigned to either the treatment group or the control group. Cultures were exposed to creatine-monohydrate (Creapure™, SKW Trostberg, Trostberg, Germany) at a final concentration of 5 mM from day *in vitro* (DIV) 0 to 7 ($n = 16$, five independent experiments), from DIV6 to 7 ($n = 18$, five

independent experiments) or from DIV5 to 7 ($n = 7$, two independent experiments), respectively. Untreated cultures served as controls. For analysis of GABA-, or dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32)-ir cell densities, cultures were fixed at DIV7 and processed as described below. In addition, GABA-uptake measurements ($n = 12$, three independent experiments) and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays were carried out at DIV7 ($n = 6$, two independent experiments). In the experiments investigating effects of a transient creatine treatment cultures were exposed at DIV6 to creatine (5 mM) for 24 h. Thereafter cells were maintained for three further days in regular maintenance medium and processed for histology and the MTT reduction assay at DIV10 ($n = 6$–12, three independent experiments).

For dose–response analysis, cultures were exposed to creatine at final concentrations of 0.25, 1, 5 and 20 mM from DIV0 to 7, fixed at DIV7 and processed for GABA immunocytochemistry ($n = 8$, two independent experiments).

To assess the effects of creatine administration under serum- and glucose-free conditions, cultures were grown in maintenance culture medium up to DIV5 when the medium was changed for the rest of the culturing period, i.e. up to DIV7, to Locke's solution consisting of 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$ and 5 mM HEPES, pH 7.2 (Cheng and Mattson 1994). Controls were kept in regular maintenance medium or in the same buffer solution but with 20 mM glucose added. Cells were consequently grown for additional two days *in vitro* in the absence or presence of creatine (5 mM). Cultures were fixed at DIV7 ($n = 22$, seven independent experiments).

To investigate the effect of creatine treatment against neurotoxicity induced by 3-nitropropionic acid (3-NP; Sigma) exposure, cultures were kept in maintenance medium up to DIV5, when 3-NP was added at a final concentration of 10 mM with or without concurrent creatine addition (5 mM) ($n = 13$, four independent experiments). Controls were grown in standard conditioning medium. At DIV7 cultures were fixed and processed for immunochemical analyses (see below).

To investigate whether transcription or translation might interfere with creatine-mediated effects on GABA-ergic cell density, actinomycin D (3 μM) ($n = 8$, two independent experiments) or cycloheximide (10 μM) ($n = 7$, two independent experiments) were added to the culture medium at DIV6 with or without concurrent creatine addition (5 mM). Cells were grown for another 24 h in standard conditioning medium and the cultures fixed and processed for immunochemical analyses (see below) at DIV7.

In order to examine the role of extracellular calcium, the maintenance medium was changed to Ca^{2+}-free Locke's medium (Cheng and Mattson 1994) containing 20 mM glucose from DIV6 to 7 with or without concurrent creatine treatment (5 mM) ($n = 7$–8, two independent experiments). GABA-ir cell densities were assessed at DIV7. Cultures that were not calcium deprived served as controls (Cheng and Mattson 1994).

To analyse the involvement of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PtdIns3K) pathways in creatine-mediated effects, cultures grown in standard conditioning medium were supplemented with creatine at a concentration of 5 mM from DIV6 to 7 and concurrently exposed to selective inhibitors of PtdIns3K (LY294002, 50 μM; Biomol, Plymouth Meeting, PA, USA), MAPK (PD98059, 50 μM; Calbiochem, San Diego, CA, USA) or a combination thereof, fixed at DIV7 and processed for immunocytochemistry ($n = 16$–24, four independent experiments).

Immunocytochemistry for cell-density assessments

Cultures were rinsed three times in 0.1 M PBS and fixed in 0.1 M PBS containing 4% paraformaldehyde for 20 min at room temperature. After three rinses in PBS, cultures were pre-incubated in 0.1% Triton X-100 in PBS plus 10% horse serum for 30 min, washed in PBS and incubated overnight with primary antibodies [rabbit polyclonal anti-GABA, 1 : 5000, Sigma Immunochemicals (St Louis, MO, USA); rabbit polyclonal anti-DARPP—32, Chemicon (Temecula, CA, USA); and mouse monoclonal anti-(neuronal nuclei) (NeuN), 1 : 500, Chemicon] in 0.1 M PBS containing 0.1% Triton X-100 and 2.5% horse serum overnight at 4°C. Following three washes, wells were incubated with a biotinylated secondary antibody (goat anti-rabbit or horse anti-mouse, 1 : 200; Vector Laboratories, Burlingame, CA, USA) in PBS containing 2.5% horse serum for 30 min. Following incubation with an avidin–peroxidase complex (1 : 150; Vector Laboratories) for 45 min, specifically bound antibody was visualized with a metal-enhanced 3,3′-diaminobenzidine substrate kit (No. 34065; Pierce, Rockford, IL, USA) for 3–5 min. Cultures were rinsed in PBS and mounted on slides using Aquamount (BDH, Chemicals, Poole, UK).

Brain-type cytosolic CK and uMT-CK co-localization with GABA

Cultures were treated as described above and then incubated overnight at 4°C with primary antibodies (chicken egg yolk IgY anti-BB-CK, 1 : 500; Schlattner *et al*. 2002a, and rabbit polyclonal anti-GABA, 1 : 5000; Sigma Immunochemicals, or rabbit polyclonal anti-uMt-CK, 1 : 500; Schlattner *et al*. 2002b, and mouse monoclonal anti-GABA, 1 : 100; Sigma Immunochemicals, respectively) in 0.1 M PBS containing 0.1% Triton X-100 and 2.5% HS. Following three washes in PBS, the cells were incubated with PBS containing 2.5% rabbit serum for 30 min and subsequently with the secondary antibodies (FITC-labelled rabbit anti-IgY, Alexa Red™-labelled donkey anti-mouse or anti-rabbit, respectively, each 1 : 100; Jackson Immunoresearch, West Grove, PA, USA) for 2 h at 22°C. After rinsing three times in PBS, 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).

GABA uptake assay

The GABA uptake assay was performed as described before (Widmer and Hefti 1994). In brief, after aspiration of the culture medium, cells were rinsed twice with 5 mM glucose and 0.5 mM ascorbic acid in sterile PBS and pre-incubated for 5 min at 37°C with 250 μL of incubation solution (5 mM glucose, 10 μM aminooxyacetic acid and 1 mM β-alanine) in PBS. [^{3}H]-GABA (TRK527, 94 Ci/mmol; Amersham, Little Chalfont, UK) was added to 50 nM and the cultures incubated for another 4 min. Blanks were obtained by incubating cells at 0°C. Uptake was stopped by removal of the incubation mixture, followed by five washes with ice-cold PBS. The cultures were set on ice, mechanically lysed by trituration in 0.5 M NaOH and ^{3}H was measured by liquid scintillation (Ultima Gold; Packard Instrument Co., Meriden, CT, USA) counting.

MTT assay

The water-soluble, yellow tetrazolium salt MTT is reduced to purple formazan crystals insoluble in aqueous solution by the succinate dehydrogenase system of active mitochondria, and hence, specifically used to assay for viable cells, such as measurement of cell activation, proliferation, cytotoxicity, and viable cell number (Mosmann 1983; Rubinstein *et al*. 1990). Cultures were incubated with 250 μg MTT (Sigma) in 500 μL sterile PBS (final concentration of 0.5 mg/mL, solution prepared just before use) at 37°C for 4 h. After aspiration of the medium, 500 μL dimethylsulfoxide (DMSO) was added to resolve the resulting MTT-formazan product. This solution was then transferred into standard photometric cuvettes and diluted with 500 μL DMSO. The absorbance at 540 nm was read against DMSO in the linear range of the absorption curve using a photospectrometer (810; Kontron Instruments, Everett, MA, USA).

Western blot analysis

Immunoblot analyses were carried out according to the method of Laemmli (1970) with some modifications. After rinsing three times with 4 mL sterile ice-cold HBSS, cultures were supplied with 160 μL of lysis buffer containing leupeptin (2 μL/mL), phenylmethylsulfonyl fluoride (10 μL/mL), beta-mercaptoethanol (1 mM), EDTA (0.2 mM) and Triton X-100 (1 μL/mL) in ice-cold HBSS and the tissue detached from the plate using a cell scraper. The suspension was transferred into an Eppendorf tube and immediately set on ice. Cells were sonicated during 3 min, the lysate centrifuged for 2 min at 18 000 *g*, 7.5 μL 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) added to 15 μL of the supernatant and incubated at 95°C for 5 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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, 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. Sufficient transfer efficiency was checked by Coomassie Blue staining of the gels for residual protein bands while total protein on the blotting membrane was assessed by reversible Ponceau S staining. After decolorization by rinsing twice in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl) for 5 min, the blot was blocked for 60 min with 4% fat-free milk powder in Tris-buffered saline and incubated with the primary antibodies (mouse monoclonal anti-BB-CK, 1 : 1000, de Sistermans *et al*. 1995 ; rabbit polyclonal anti-uMt-CK, 1 : 1000, Schlattner *et al*. 2002b; mouse monoclonal anti-MAP2, 1 : 500; Sigma, mouse monoclonal antivimentin, 1 : 1000; Sigma, mouse monoclonal anti-GFAP, 1 : 1000; Boehringer, Mannheim, Mannheim, Germany Chemicon, mouse monoclonal anti-α-tubulin, 1 : 20000; Boehringer) overnight at 4°C in the buffer solution. The blots were washed twice in Tris-buffered saline containing 0.2% Tween-20 (Merck) and subsequently incubated with an affinity purified peroxidase-coupled secondary antibody (donkey anti-mouse and anti-rabbit, 1 : 20 000 and 1 : 10 000, respectively, Jackson Immunoresearch) for 45 min at 20°C in Tris-buffered saline containing 4% milk powder. Blots were washed three times in 0.2% Tween-20 containing Tris-buffered saline 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). Membranes were reprobed with α-tubulin (α-Tubulin) as loading control. Protein content in the samples was determined according to the method of Bradford using the Bio-Rad™ protein assay (Bradford 1976).

Analysis of cell numbers and statistics

Cell counts were carried out under bright field illumination using a microscope (Axioplan; Zeiss, Oberkochen, Germany) connected to a digital image processing system (Lucia 4.50; Nikon, Tokyo, Japan) in six randomly chosen areas (0.8 mm^2) and cell density was calculated per mm^2. Only cells with distinct immunoreactivity, clear neuronal shape and a nucleus were counted as GABA or DARPP-32 positive neurons, respectively. Total neuronal cell number was assessed using phase-contrast microscopy on the same system.

For statistical analysis, a commercially available software package was used (STATISTICA 5.0; StatSoft, Tulsa, OK, USA). Experimental groups were compared by multivariate analysis of variance (MANOVA), followed by non-parametric post-hoc Mann–Whitney test. Differences were considered statistically significant at $p < 0.05$. Values are presented as mean ± SEM.

Results

Quantification and localization of CK isoenzymes

Both brain-type cytosolic CK (BB-CK) and ubiquitous mitochondrial CK (uMt-CK) were found to be expressed in E14 GE, i.e. at day of dissection and during the entire culturing period (Fig. 1a). Co-localization studies using the double immunolabelling technique revealed that GABA-ir neurons in the striatal cultures expressed BB-CK and uMt-CK (Fig. 1bh). Signals for both CK isoenzymes could also be detected in cells with glial morphology (data not shown).

Dose–response

In order to find the optimal concentrations of creatine to be used in our experiments, we performed a dose–response analysis (Table 1). Treatment of striatal cultures with creatine from DIV0 to 7 at concentrations of 0.25, 1, 5 and 20 mM, respectively, revealed that GABA-ir cell densities were already significantly higher using a dose of 1 mM as compared to controls ($p < 0.05$), while a dose of 0.25 mM failed to show any effects. The maximal effective dose was detected to be 5 mM.

Effects of chronic creatine treatment on survival and differentiation of striatal neurons

In chronically creatine-treated (5 mM) striatal cultures, the density of GABA-ir neurons was significantly higher compared with controls (Fig. 2a,c,d). A similar increase was also seen for DARPP-32-ir neurons (Fig. 2b). DARPP-32 is a protein expressed by mature striatal projection neurons.

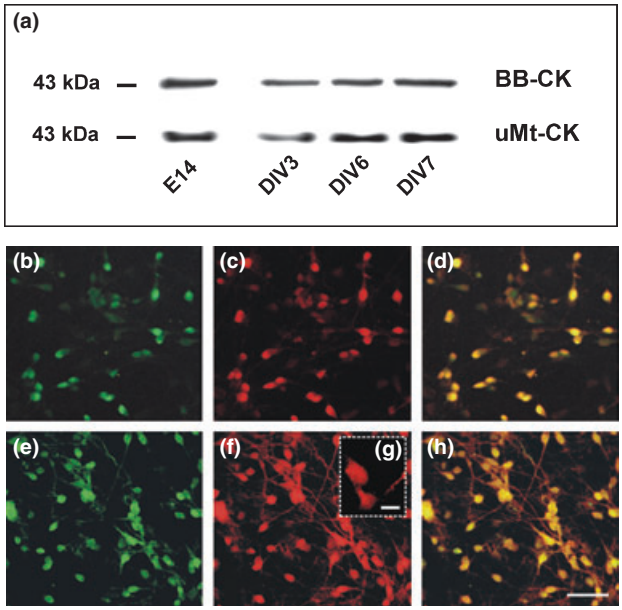

Fig. 1 BB-CK and uMt-CK expression in striatal cultures. Western blot analysis for BB-CK and uMt-CK in tissue of GE showed that both isotypes were present at day of dissection (E14) and in cultures grown for 3, 6 and 7 days *in vitro* (DIV) (a). Double immunofluorescence for GABA (b, e) and BB-CK (c) or uMt-CK (f) and corresponding merges (d, h) in untreated striatal cultures grown for 7 days. Note the punctuate pattern of uMt-CK expression in the neurons (insert, g). Scale bars: 50 μm (b–h) and 10 μm (g).

DARPP-32 is the most commonly used medium spiny neuron marker (Ivkovic and Ehrlich 1999; Mohapel *et al*. 2005). Moreover, functional recovery in an animal model of HD has been correlated with the percentage of DARPP-32 positive cells in grafts of lateral GE (Nakao *et al*. 1996). In addition, creatine exposure resulted in an up-regulation (0.4-fold) of microtubule-associated protein-2, a general marker for neurons (Matus *et al*. 1986). No effects were detected on protein levels of vimentin, and glial fibrillary acidic protein (Fig. 3).

Interestingly, creatine treatment only showed a slight tendency for an increase in total number of neuronal cells in the cultures and did not alter measures of MTT reduction activity (data not shown).

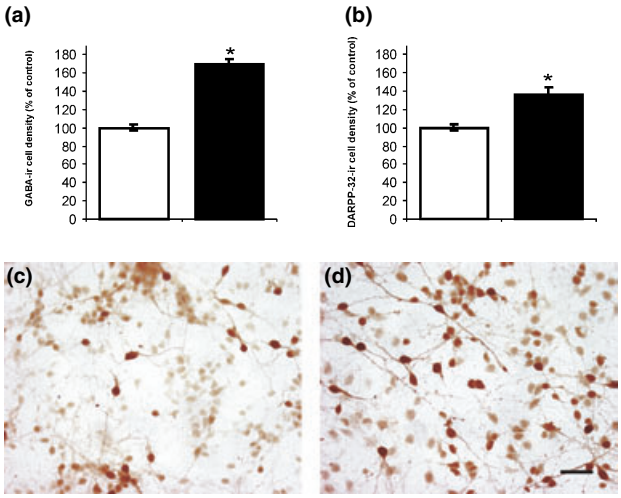

Fig. 2 Effects of chronic creatine treatment on cell densities of GABA-ir (a) and DARPP-32 (b) neurons in striatal cultures. Cells were grown for 7 days *in vitro* in the absence (open bars) or presence (filled bars) of creatine (5 mM). Data are presented as percentage of corresponding control values and are given as mean ± SEM. *Significant at $p < 0.05$. Representative photomicrographs of GABA-ir neurons in striatal cultures grown in the absence (c) and presence (d) of creatine (5 mM). Scale bar: 50 μm.

Effects of short-term creatine treatment on survival and differentiation of striatal neurons

Short-term treatment of striatal cultures with creatine (5 mM) from DIV6 to 7 or DIV5 to 7, respectively, resulted in significantly higher densities of GABA-ir neurons compared with controls and assessed at DIV7 (Fig. 4a). There was no significant difference between creatine-treated cultures from DIV6 to 7 and DIV5 to 7. Similarly, creatine administration from DIV6 to 7 and DIV5 to 7 resulted in a significant increase in GABA uptake (121.5 ± 7.3 and 119.8 ± 2.7%, respectively), compared with untreated control cultures (100 ± 2.0%; Fig. 4c). The total number of neuronal cells (Fig. 4b) and cell viability (Fig. 4d) were not significantly affected by creatine. Moreover, western blot analysis revealed an increase in the expression level of MAP2 after creatine exposure. A slight increase was also observed for vimentin expression after short-term creatine exposure. Similar to the results after chronic creatine treatment, no significant effects were observed on the level of GFAP (Fig. 5).

Table 1 Dose–response analysis of chronic creatine treatment on cell densities of GABA-immunoreactive neurons in striatal cultures

Creatine concentration in medium	Control	0.25 mM	1 mM	5 mM	20 mM
Cell density (cells/mm^2)	156.9 ± 4.0	159.2 ± 4.5	211.5 ± 6.5*	321.7 ± 19.4*	265.0 ± 13.1*
% of control	100.0 ± 2.3	100.5 ± 3.3	133.5 ± 4.8*	202.1 ± 9.3*	166.6 ± 5.7*

Cultures were grown for one week in the absence (control) or presence of various concentrations of creatine. GABA-ir cell densities were assessed as described in Materials and Methods. Data are given as mean ± SEM ($n = 6$–10; two independent experiments). *Significant at $p < 0.05$.

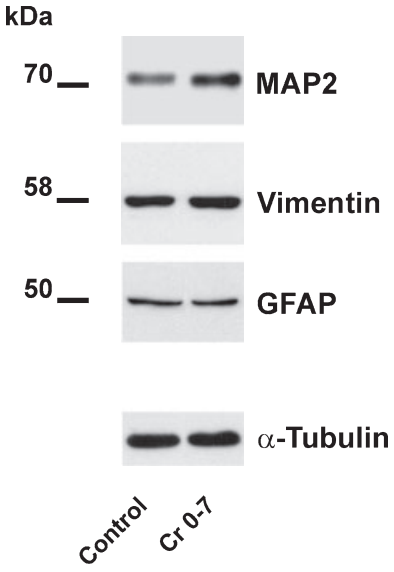

Fig. 3 Western blot analysis for microtubule-associated protein 2 (MAP2), vimentin and glial fibrillary acidic protein (GFAP) in striatal cultures grown for one week *in vitro* without (Control) or with creatine (5 mM) (Cr 0–7). Note an up-regulation in the expression of MAP2. Membranes were reprobed with α-tubulin (α-Tubulin) as loading control.

Higher GABA-ir cell densities are still observed when short-term creatine exposed cultures were maintained for a further three days period without creatine, i.e. as assessed at DIV10 (Fig. 6a). Creatine exposure from DIV6–7 not only increased cell densities of GABA-ir neurons at DIV10, but also resulted in a modest but significant increase in densities of NeuN-ir neurons (100.0 ± 3.7 and 114.3 ± 4.2% for controls and creatine-treated cultures, respectively; mean ± SEM; $n = 11$; three independent experiments), a marker developmentally first observed after neurons have become post mitotic. Again, and in parallel to the outcome of chronic treatment, creatine exposure from DIV6 to 7 did not result in significant alterations of the total numbers of neuronal cells in the cultures and measures of the MTT assays, as analyzed at DIV10 (Figs 6b,c).

Effects of creatine treatment against a metabolic insult

Serum and glucose deprivation from DIV5 to 7 resulted in a marked and significant decrease in the overall number of GABA-ir neurons in striatal cultures evaluated at DIV7 (Fig. 7a). Concurrent creatine treatment was able to fully protect against the loss in GABA-ir cell densities. The creatine-induced increase in GABA-ir cell densities was significantly more prominent in the absence of serum and glucose compared with control medium-containing cultures (2.3-fold vs. 1.5-fold). The metabolic insult also resulted in a robust and significant decrease in MTT reduction activity estimated at DIV7. In contrast to the outcome seen with GABA-ir cell densities, concurrent creatine treatment during the period of the metabolic insult failed to protect against a loss in MTT reduction capacity (Fig. 7b). Interestingly, measures of the MTT assay partially recovered (to ≈ 80% of control levels) when cultures were kept for an additional three days in maintenance medium, i.e. by DIV10 (data not shown).

Effects of creatine treatment against a neurotoxic insult induced by 3-NP

Exposure of striatal cultures to the neurotoxin 3-NP (10 mM) for two days (DIV5–7) resulted in significantly lower

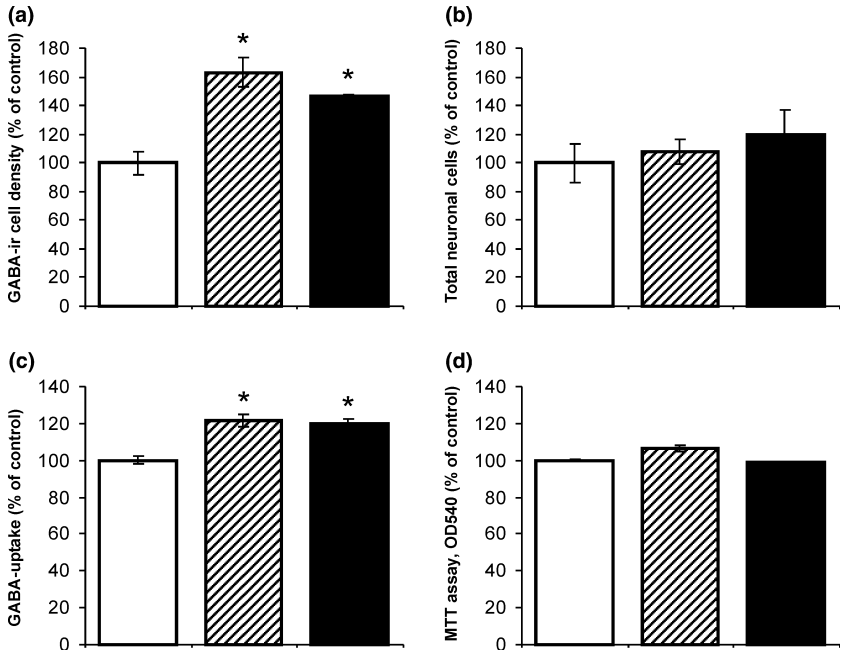

Fig. 4 Effects of short-term treatment with creatine (5 mM) from DIV6 to 7 (hatched bars) and DIV5 to 7 (filled bars) on cell density of GABA-ir neurons (a), total number of neurons (b), GABA uptake (c) and measures of the MTT assay (d) in striatal cultures grown for 7 days *in vitro* (DIV). Untreated cultures served as controls (open bars). Data are given as mean ± SEM and are expressed as percentage of the corresponding controls. *Significant at $p < 0.05$ compared with control.

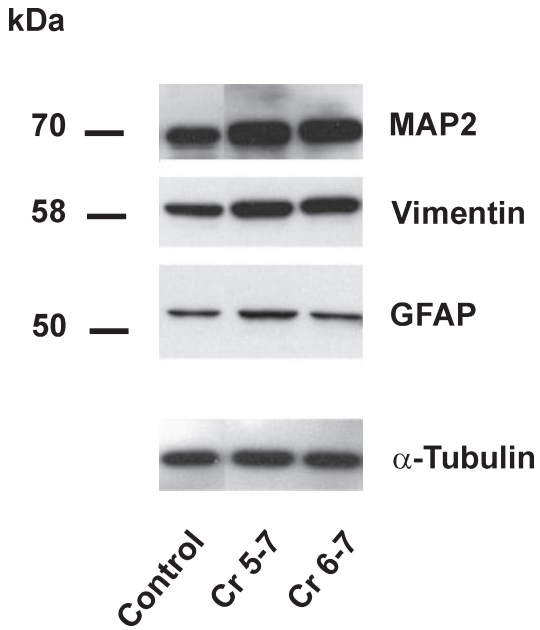

Fig. 5 Western blot analysis for MAP2, vimentin and glial fibrillary acidic protein in striatal cultures grown for 7 days *in vitro* (DIV). Creatine (5 mM) was present either from DIV5 to 7 (Cr 5–7) or DIV6 to 7 (Cr 6–7). Untreated cultures served as controls (Control). Membranes were reprobed with α-tubulin (α-Tubulin) as loading control.

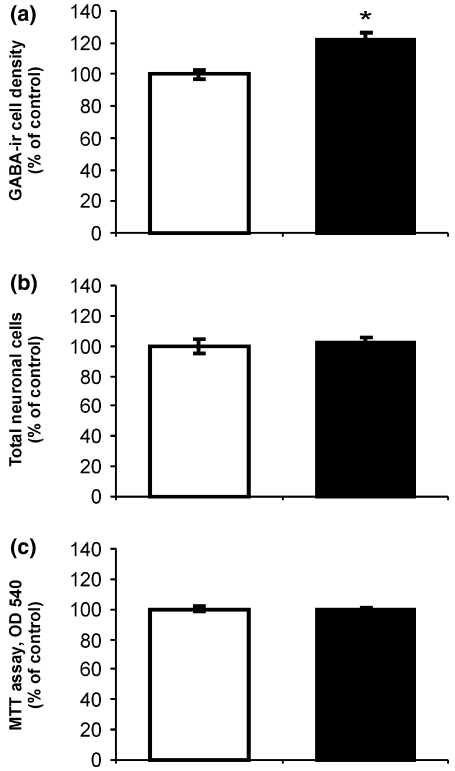

Fig. 6 Effects of a short-term creatine treatment on GABA-ir cell densities (a), total number of neuronal cells (b), and on measures of the MTT assay (c) in striatal cultures grown for 10 days *in vitro* (DIV). Cultures were exposed to creatine (5 mM) (filled bars) or regular maintenance medium (Control; open bars) for 24 h at DIV6 and thereafter maintained three further days in standard conditioning medium. Data are given as mean ± SEM and are expressed as percentage of the corresponding controls. *Significant at $p < 0.05$ compared with control.

GABA-ir and DARPP-32-ir cell densities compared with untreated controls and assessed at DIV7 (Fig. 8). Creatine administration exerted a distinct protection against 3-NP toxicity with significantly higher cell densities of GABA-ir and DARPP-32-ir neurons, compared with cultures not exposed to creatine (Fig. 8).

Creatine-mediated effects are independent of transcriptional and translational processes

In order to investigate whether transcriptional and/or translational processes were involved in the creatine-induced differentiation of GABA-ergic cells, striatal cultures were exposed to the specific inhibitors actinomycin D (3 μM) or cycloheximide (10 μM) from DIV6 to 7 in the absence or presence of creatine (5 mM). Both, inhibition of transcription (Fig. 9a) and translation (Fig. 9b) resulted in significantly lower densities of GABA-ir neurons, compared with untreated control cultures. However, addition of creatine still resulted in significantly increase densities of GABA-ir neurons, comparable with that observed in the absence of actinomycin D or cycloheximide (Fig. 9).

Extracellular Ca^{2+} is not required for creatine-induced differentiation

Calcium deprivation in the medium from DIV6 to 7 resulted in a significant reduction in density of GABA-ir neurons in the cultures (Fig. 10). We observed that concurrent creatine administration significantly protected against the attenuation of GABA-ir neuron density (Fig. 10). Similarly to when cultures were exposed to a metabolic insult (vide supra), the increase in densities of GABA-ir neurons caused by creatine treatment in the absence of Ca^{2+} was significantly higher compared with the effects seen in medium containing Ca^{2+} and as assessed in parallel positive control experiments (100.0 ± 4.9 and $176.5 \pm 7.3\%$, and 100.0 ± 3.8 and $135.9 \pm 3.0\%$, in the absence and presence of extracellular calcium for control and creatine treatment, respectively; mean ± SEM; $n = 7–8$; two independent experiments).

Effects induced by creatine treatment involve the MAPK and PtdIns3K downstream pathways

Because inhibitors of transcription and translation did not affect creatine-induced increases in GABA-ir cell densities, we addressed the role of selective inhibitors of PtdIns3K (LY294002) and MAPK (PD98059). We investigated these inhibitors, because both PtdIns3K-Akt and MAPK- extracellular signal-regulated kinase (MAPK-ERK) pathways

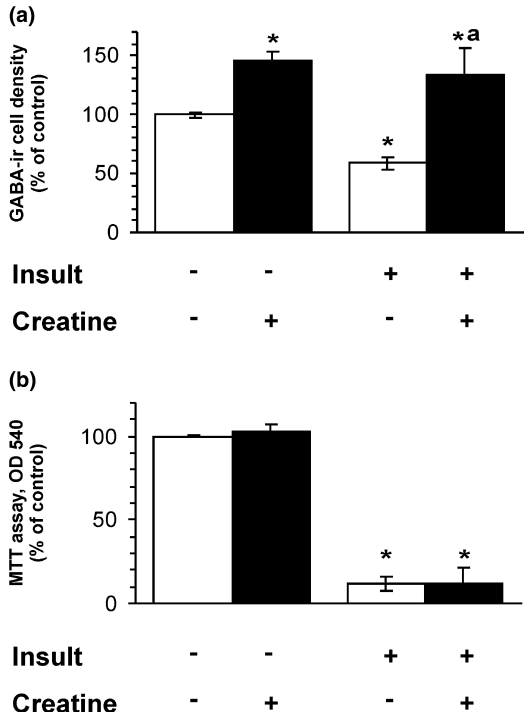

Fig. 7 Effects of creatine treatment on GABA-immunoreactive cell densities (a) and measures of the MTT assay (b) in striatal cultures exposed to a metabolic insult. Cells were grown for 7 days *in vitro* (DIV). Cultures in the metabolic insult groups underwent serum and glucose deprivation from DIV5 to 7 in the absence (open bars) or presence of creatine (5 mM) (filled bars). Controls were exposed to standard growth medium for the same time in the absence (control, open bars) or presence of creatine (filled bars). Data are given as mean ± SEM and are expressed as percentage of the untreated controls. *Significant at $p < 0.05$ versus standard growth medium controls. [a]Significant at $p < 0.05$ compared with corresponding control.

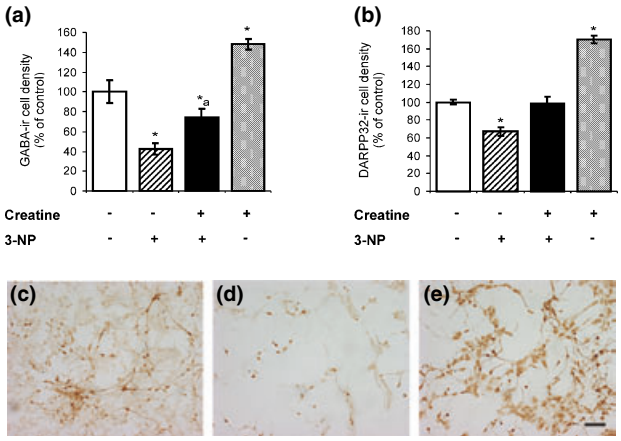

Fig. 8 Effects of creatine treatment on cell densities of GABA-ir (a) and DARPP-32-ir (B) neurons in striatal cultures exposed to 3-NP. Cultures were grown for 7 days *in vitro* (DIV) and exposed to 3-NP (10 mM) from DIV5 to 7 in the absence (hatched bars) or presence of creatine (5 mM) (filled bars). Controls were exposed to standard growth medium for the same period in the absence (control, open bars) or presence of creatine (grey bars). Values are presented as mean ± SEM and expressed as percentage of the untreated controls. *Significant at $p < 0.05$ versus standard growth medium controls. [a]Significant at $p < 0.05$ compared to corresponding control. The lower panel shows representative photomicrographs of GABA-ir neurons in control cultures (c) and cultures exposed to 3-NP in the absence (d) or presence (e) of creatine. Scale bar: 50 μm.

have been implicated to play an important role in the differentiation and survival of neuronal cells (Kruttgen *et al*. 2003; Chin *et al*. 2004). We found that in the presence of either of the two inhibitors the increase of GABA-ir cell densities induced by creatine exposure was significantly attenuated (by ≈ 50%). Concomitant treatment with PD98059 and LY294002 was significantly more effective than single inhibitor exposure in preventing acquisition of the GABA-ergic phenotype due to creatine administration (Fig. 11).

Discussion

In rat E14 striatal cultures, we found both brain-type cytosolic (BB-CK) and ubiquitous mitochondrial (uMt-CK) creatine kinase to be expressed at high levels and were able to demonstrate colocalization in GABA-ergic cells. Therefore, we can assume that the phosphocreatine/creatine kinase system is operational in these cells and that exogenous creatine addition should be effective in improving their metabolic state.

We demonstrated that chronic creatine treatment at a concentration of 5 mM from DIV0 to 7 resulted in a significant increase in the number of GABA-immunoreactive neurons in our cultures. Correspondingly, a significant increase in the number of DARPP-32-ir neurons was observed, while the total neuronal cell number and general viability, as assessed by the MTT formazan assay, were not affected. The unchanged total neuronal cell numbers in combination with a significantly higher percentage of GABA-ir cells strongly points to an induction of a differentiation process towards the GABA-ergic phenotype. This conclusion is also supported by increased expression levels of MAP2 after chronic and short-term creatine treatment, which may indicate an induction of neuronal cell differentiation and maturation. We did, however, not study the effects of creatine exposure on the morphological differentiation, e.g. number and length of processes of MAP2 positive neurons. Furthermore, the observation of a higher cell density of DARPP-32-ir neurons provides evidence that creatine is able to promote the maturation of medium spiny neurons (Ivkovic and Ehrlich 1999).

Interestingly, analysis of GABA-ir cell numbers in cultures treated from DIV6 to 7 and maintained for further 3 days in culture medium lacking creatine revealed a persisting effect

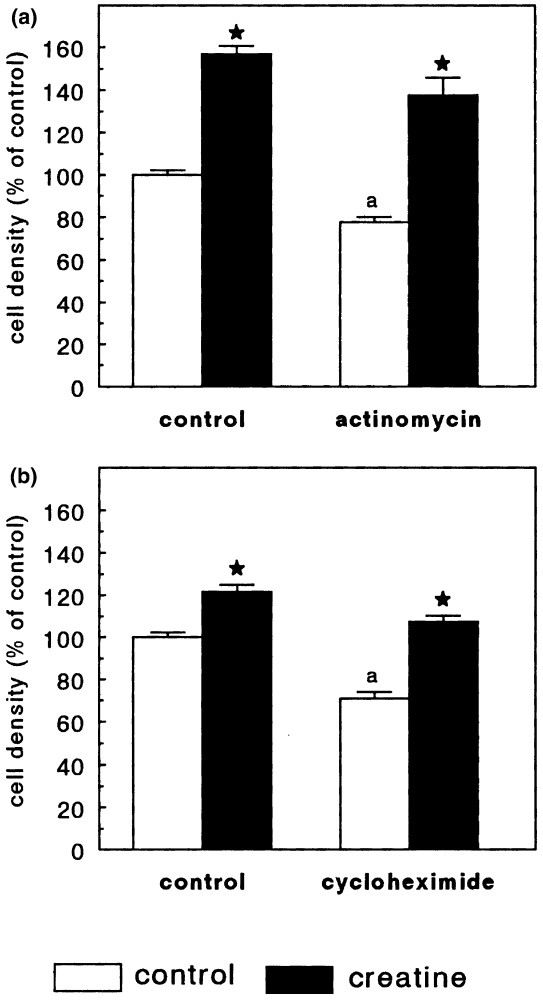

Fig. 9 Effects of creatine treatment on cell densities of GABA-ir neurons in striatal cultures exposed to actinomycin (a) or cycloheximide (b). Cultures were grown for 7 days *in vitro* (DIV) and exposed to actinomycin D (3 μM) (a) or cycloheximide (10 μM) (b) from DIV67 in the absence (open bars) or presence of creatine (5 mM) (filled bars). Data are presented as percentage of corresponding control values and are given as mean ± SEM. *Significant at $p < 0.05$ versus corresponding controls. [a]Significant at $p < 0.05$ versus untreated controls.

on GABA-ir cell numbers. This indicates that a subset of neurons was permanently shifted to the GABA-ergic phenotype or that an accelerated maturation of neurons occurred which were already committed to the GABA-ergic phenotype. The effect at DIV10, however, was less pronounced as compared to the outcome seen at DIV7. Ongoing cell proliferation, differentiation and maturation of neurons in the cultures likely contributed to the less prominent effect of creatine treatment detected at DIV10. The overall higher GABA-ir and total neuronal cell densities in the cultures at this latter time point favour this view. Nevertheless, we cannot exclude that the smaller creatine effect was due to differentiated cells that more likely die at prolonged culture times.

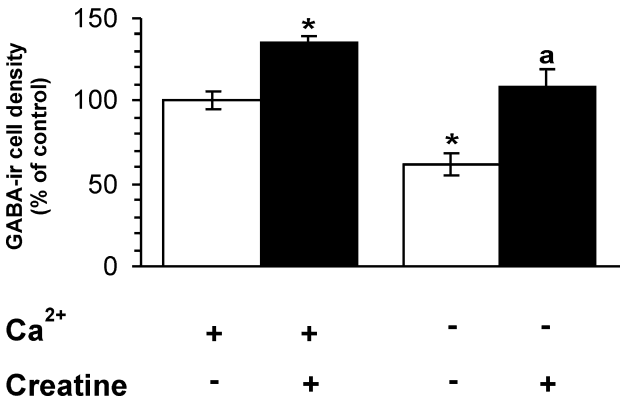

Fig. 10 Effects of creatine treatment on cells densities of GABA-ir neurons in striatal cultures undergoing calcium deprivation. Cultures were grown for 7 days *in vitro* (DIV) and exposed to Ca^{2+}-free Locke's medium containing 20 mM glucose from DIV6–7 in the absence (open bars) or presence of creatine (5 mM) (filled bars). Cultures that were not calcium deprived served as controls. Data are presented as percentage of untreated control values and are given as mean ± SEM. *Significant at $p < 0.05$ versus standard growth medium controls. [a]Significant at $p < 0.05$ versus corresponding control.

According to previous reports (Cheng and Mattson 1994), serum and glucose deprivation resulted in a significant lower number of GABA-ir neurons and in significantly decreased MTT reduction activity in our cultures, as assessed at DIV7. As presented here, concurrent creatine addition to the culture medium during the period of insult significantly protected against loss of GABA-ir cells, whereas general cell viability at DIV7 was found unchanged. We consider that the decrease in MTT reduction may represent lowered mitochondrial activity due to impaired energy metabolism rather than a decrease of viable cells in the cultures, given the recovery seen at DIV10. Interestingly, it has been reported (Cheng and Mattson 1994) that glucose deprivation resulted in increased intracellular calcium levels, an observation that may explain the stronger increase in GABA-ergic cell densities by creatine compared with the outcome seen in glucose containing media (see below).

Administration of the irreversible succinate dehydrogenase inhibitor 3-NP results in death of GABA-ergic neurons that closely mimics HD neuropathology (Beal *et al.* 1993; Brouillet and Hantraye 1995; Greene *et al.* 1998), with depletion of cellular energy reserves as an initiating step. The finding that creatine provided significant neuroprotection against 3-NP toxicity supports the contention that creatine administration might be successful in improving cellular energy metabolism of striatal cells and might therefore be beneficial for slowing the course of HD. In line with this hypothesis are two recent reports where significant protection against 3-NP by high doses of creatine (2% creatine w/w in the dry animal food) has been found in the adult rat (Matthews *et al.* 1998; Shear *et al.* 2000). A

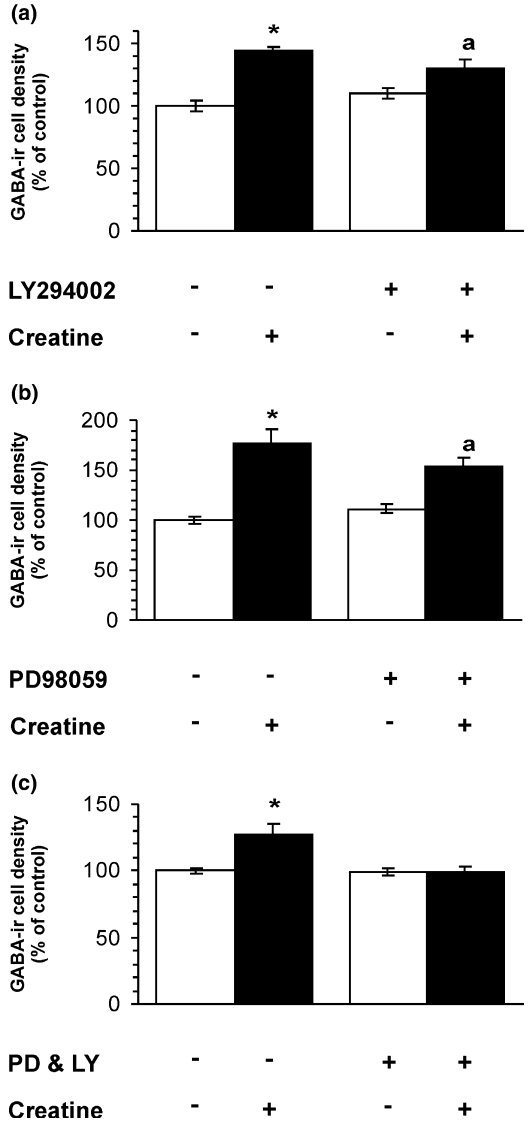

Fig. 11 Effects of creatine treatment on cell densities of GABA-ir neurons in striatal cultures exposed to the PtdIns3K inhibitor LY294002 (a), the MAPK inhibitor PD98059 (b) or both (c). Cultures were grown for 7 days *in vitro* (DIV) and exposed to LY294002 (50 μM), PD98059 (50 μM) or both from DIV6–7 in the absence (open bars) or presence of creatine (5 mM) (filled bars). Data are presented as percentage of untreated control values and are given as mean ± SEM. *Significant at $p < 0.05$ versus all groups. [a]Significant at $p < 0.05$ versus corresponding controls.

first pilot study with HD patients ingesting creatine at much lower amounts (5 g/day) did not improve functional, neuromuscular and cognitive status (Verbessem *et al*. 2003). However, long-term studies with higher doses of creatine are needed to see whether progression of disease could be halted by creatine.

Knowledge of the molecular mechanisms by which neuronal cells react in response to increased creatine levels is important for rational creatine supplementation on these cells. Despite intense research efforts, the precise mechanisms underlying the various effects of creatine supplementation is not known, although several possibilities have been suggested (Friedman and Roberts 1994; Wallimann and Hemmer 1994; Wallimann *et al*. 1999; Brewer and Wallimann 2000; Brustovetsky *et al*. 2001).

We first examined a possible role of calcium homeostasis. Spontaneously occurring calcium spikes play an important role in neuronal development (Gu *et al*. 1994; Spitzer 1991, 1994, 1995; Gu and Spitzer 1995, 1997), especially for the differentiation of the GABA-ergic phenotype (Spitzer *et al*. 1993; Watt *et al*. 2000). Cellular calcium homeostasis involves functional Ca^{2+}-ATPase and Ca^{2+} exchangers as well as Na^{+}/K^{+}-ATPase activity (Carafoli 1987). Because the function of the Na^{+}/K^{+}-ATPase, and in particular that of the Ca^{2+}-ATPase, are high energy-consuming processes, the maintenance of cellular calcium homeostasis is intimately related to ATP utilization and synthesis (Hemmer and Wallimann 1993). Strong evidence has been found for a functional coupling of BB-CK with the plasma membrane-bound Na^{+}/K^{+}-ATPase (Lim *et al*. 1983; Wallimann *et al*. 1985), so the creatine/phosphocreatine shuttle is likely to be a major component of the neuronal Ca^{2+} reconstitution mechanism (Hemmer and Wallimann 1993; Wallimann and Hemmer 1994). However, the effect of creatine on the number of GABA-ir neurons persisted in cultures kept in Ca^{2+}-depleted culture medium, so this mechanism may not significantly contribute to creatine-induced differentiation processes in striatal cultures. However, we cannot exclude that calcium release from other cells in the cultures, e.g. glial cells, and/or intracellular calcium stores compensated for the missing Ca^{2+} in the media. Given the finding of a robust increase in GABA-ir cell densities in the absence of extracellular Ca^{2+}, this hints at a combination of neuroprotective and differentiation inducing effects of creatine. This notion is also supported by the observations made for glucose and serum deprivation, which resulted in significant higher GABA-ir cell densities as compared to untreated cultures (see above).

Inhibitors of transcription and translation did not affect the induction of the GABA-ergic phenotype by creatine in our cultures, suggesting that neither process was involved. Nevertheless, the drugs used caused a significant loss in GABA-ir cells due to their neurotoxic properties. In the presence of inhibitors, the protective potential of creatine was even more pronounced as seen by the number of GABA-ir cells. Therefore, GABA-ergic differentiation must be triggered by mechanisms other than transcription and translation. In this respect, it is interesting to note that changes in protein levels are not necessarily associated with changes in mRNA levels, but rather by regulation of protein stability (Rimvall *et al*. 1993).

To further understand the mechanisms of creatine-induced differentiation processes, we investigated the possible implication of MAPK and PtdIns3K signal pathways. PtdIns3K is reported to be involved in neuronal survival (Encinas *et al*. 1999) and neurite outgrowth (Kita *et al*. 1998). The MAPK pathway has emerged as a major contributor to plasticity in vertebrates. MAPKs are a family of serine–threonine kinases that include the ERKs among its members. ERKs are strongly activated by mitogens and, in the CNS, by neurotrophins and neurotransmitters. These kinases are major effectors of signal transduction from the cell surface to the nucleus and are implicated in cell growth and differentiation (Dominguez *et al*. 2004). The effects of short-term creatine treatment on GABA-ir cell numbers were significantly attenuated by the MAPK inhibitor PD98059 and the PtdIns3K inhibitor LY294002. Moreover, we observed that concomitant treatment with both inhibitors prevented the acquisition of the GABA-ergic phenotype due to creatine exposure. Hence, our study suggests that downstream MAPK and PtdIns3K activity is involved in creatine-induced differentiation processes towards the GABA-ergic phenotype and that creatine seems to directly affect these cell signaling pathways. This notion is supported by the observation that a 10 min treatment of striatal cultures with creatine resulted in increased levels of phosphorylated Akt (Andres, Ducray and Widmer, unpublished data). We assume, however, that additional mechanisms contributed to the observed effects of creatine, since either of the two inhibitors did not fully block the induction of the GABA-ergic phenotype by creatine.

Our findings may have significant implications for cell-replacement strategies for the treatment of HD. The induction of the GABA-ergic phenotype in neuronal precursors by creatine may result in higher numbers of functionally active cells in the grafts. In addition, the differentiation-promoting properties of creatine may also be of importance in cell fate decision of stem cells. Taken together, the observed protective effects of creatine against metabolic and neurotoxic insults seen here might contribute additionally to better neuronal survival in the context of neurodegenerative disorders.

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