

The effects of creatine supplementation on striatal neural progenitor cells depend on developmental stage

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Abstract Transplantation of neural progenitor cells (NPCs) is a promising experimental therapy for Huntington's disease (HD). The variables responsible for the success of this approach, including selection of the optimal developmental stage of the grafted cells, are however largely unknown. Supporting cellular energy metabolism by creatine (Cr) supplementation is a clinically translatable method for improving cell transplantation strategies. The present study aims at investigating differences between early (E14) and late (E18) developmental stages of rat striatal NPCs *in vitro*. NPCs were isolated from E14 and E18 embryos and cultured for 7 days with or without Cr [5 mM]. Chronic treatment significantly increased the percentage of GABA-immunoreactive neurons as compared to untreated controls, both in the E14 (170.4 ± 4.7 %) and the E18 groups (129.3 ± 9.3 %). This effect was greater in E14 cultures ($p < 0.05$). Similarly, short-term treatment for 24 h resulted in increased induction ($p < 0.05$)

of the GABA-ergic phenotype in E14 (163.0 ± 10.4 %), compared to E18 cultures (133.3 ± 9.5 %). Total neuronal cell numbers and general viability were not affected by Cr ($p > 0.05$). Protective effects of Cr against a metabolic insult were equal in E14 and E18 NPCs ($p > 0.05$). Cr exposure promoted morphological differentiation of GABA-ergic neurons, including neurite length in both groups ($p < 0.05$), but the number of branching points was increased only in the E18 group ($p < 0.05$). Our results demonstrate that the role of Cr as a GABA-ergic differentiation factor depends on the developmental stage of striatal NPCs, while Cr-mediated neuroprotection is not significantly influenced. These findings have potential implications for optimizing future cell replacement strategies in HD.

Keywords Creatine · Creatine kinase · GABA · Differentiation · Neuroprotection · Development

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Abbreviations

ATP	Adenosine triphosphate
BB-CK	Brain-type cytosolic isoform of CK
CK	Creatine kinase
CNS	Central nervous system
Cr	Creatine
CRT	Creatine transporter
Cx	Cortex
DAB	3,3'-Diaminobenzidine
DIV	Day <i>in vitro</i>
DMEM	Dulbecco's modified Eagle medium
FCS	Fetal calf serum
DMSO	Dimethylsulfoxide
E14	Embryonic day 14
E18	Embryonic day 18
GAA	Guanidinoacetate

GAMT	Guanidinoacetate methyltransferase
GE	Ganglionic eminence
GFAP	Glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HS	Horse serum
ir	Immunoreactive
Me	Mesencephalon
MTT	3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSC	Neural stem cell
NPC	Neural progenitor cell
PCr	Phosphocreatine
Rh	Rhombencephalon
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SC	Spinal cord
SDS	Sodium dodecyl sulfate
uMt-CK	Ubiquitous mitochondrial isoform of CK

Introduction

Huntington's disease (HD) is a hereditary neurodegenerative disorder characterized by degeneration of the GABAergic projection neurons in the striatum, leading to involuntary, uncontrollable choreiform movements of the body that coincide with progressive emotional and cognitive disturbances (Sanberg and Coyle 1984; Hefter et al. 1987). The underlying mutation of HD is a trinucleotide expansion of a polyglutamine tract in the huntingtin protein, inducing accumulation and aggregation of cleaved N-terminal fragments in the cytoplasm and nucleus (The Huntington's Disease Collaborative Research Group 1993). Compromised cellular energy metabolism due to reduced mitochondrial complex-II and complex-III activity has been attributed to neuronal degeneration in HD (Gu et al. 1996; Calabresi et al. 2001).

Transplantation of different types of neural stem cells (NSCs) and neural progenitor cells (NPCs) has been shown to be effective in animal models of HD (Deckel et al. 1983; Dunnett et al. 1988; Isacson et al. 1986; McBride et al. 2004; Roberts et al. 2006; Aubry et al. 2008; Kordower et al. 1997) and first clinical trials have demonstrated the feasibility and safety of this approach (Bachoud-Levi et al. 2000a, b; Freeman et al. 2000; Hauser et al. 2002; Gallina et al. 2010). Using ^{18}F -fluorodeoxyglucose positron emission tomography, a reduction in striatal hypometabolism in transplanted patients was reported (Gaura et al. 2004). At long-term follow-up, motor and cognitive improvement reached a plateau at 2 years after transplantation and then gradually declined in a pilot study (Bachoud-Levi et al. 2006). Another group reported prolonged clinical recovery

over a period of 5 years (Reuter et al. 2008). Neuropathological examination demonstrated graft survival 6 years after transplantation and differentiation towards striatal interneuron phenotypes. However, the grafts showed poor integration into the host striatum (Keene et al. 2007). A better understanding of the pathological changes underlying HD and the complex interactions between the grafted cells and the host are required to advance translational research in this field. Major limitations of this approach include difficulties in obtaining adequate NSC or NPC cell numbers, poor survival of transplanted cells, undirected differentiation in the host brain, and selection of appropriate patients. Factors that improve the survival of grafted cells and guide the differentiation of NSCs and NPCs towards specific neurotransmitter phenotypes hold a great potential for improving cell replacement techniques.

The creatine kinase (CK) isoenzyme family, along with their substrates creatine (Cr) and phosphocreatine (PCr), constitutes an important, phylogenetically ancient system for maintaining cellular energy reserves (Wallimann et al. 1998a, b). In the mammalian central nervous system (CNS), the brain-specific cytosolic isoform (BB-CK) and the ubiquitous mitochondrial isoform (uMt-CK) of CK are both co-expressed (Andres et al. 2008). In general, it is assumed that the capacity of neuronal cells to synthesize Cr is limited, and to rely on its supply from glial cells or from the blood stream (Tachikawa et al. 2004; Braissant et al. 2001; Hanna-al-Daher and Braissant 2016). Recent studies, however, demonstrated that this holds not true for a number of brain structures (Braissant et al. 2010). Therefore, for example, in the cortex and hippocampus, neurons contain very high levels of guanidinoacetate methyltransferase (GAMT), an enzyme critically involved in the synthesis of Cr. Moreover, shuttling of the intermediate guanidinoacetate (GAA) between cells has been described (Braissant et al. 2010; Hanna-al-Daher and Braissant 2016). Hence, Cr synthesis in the CNS is more complex than previously assumed.

Still, half of the daily needed Cr comes from the diet. Accordingly, Cr is actively relayed across the blood-brain barrier by a specific transporter (CRT) and subsequently taken up by neurons and glial cells (Andres et al. 2008). Supplementation of exogenous Cr supports the cellular adenosine triphosphate (ATP) metabolism by augmentation of the PCr pool (Woznicki and Walker 1979) and providing the substrate for a cellular energy shuttle responsible for coupling the sites of ATP production and consumption (Bessman and Geiger 1981; Wallimann et al. 1989; Brewer and Wallimann 2000). Elevated cellular Cr levels furthermore have been shown to provide antiapoptotic properties by stabilizing the mitochondrial membrane and inhibiting the mitochondrial permeability transition (O'Gorman et al. 1997; Dolder et al. 2003). Cr supplementation in humans

results in increased cerebral PCr levels (Matthews et al. 1998). Neuronal PCr reserves are reduced in HD patients and an inverse correlation with the clinical severity of the disorder has been postulated (Aronin et al. 1995). Accordingly, beneficial effects of Cr administration have been reported in the R6/2 transgenic mouse model of HD (Ferante et al. 2000; Dedeoglu et al. 2003).

We have previously shown that Cr supplementation resulted in induction of the GABA-ergic phenotype in cultured striatal NPCs and exerted neuroprotective properties (Andres et al. 2005a). Cr treatment might therefore offer a feasible approach to improve neuronal differentiation and survival in the context of cell-based regenerative strategies. However, there is evidence for age-dependent expression of CK in different types of neural tissues (Andres et al. 2008; Ducray et al. 2007), which might affect the efficacy of Cr supplementation of NPCs. In the current study, we therefore examined whether the Cr-mediated effects, including GABA-ergic cell fate determination, neuroprotection against a metabolic insult consisting of glucose and serum deprivation, and morphological differentiation, depend on the donor age of rat embryonic day 14 (E14) and embryonic day 18 (E18) striatal NPCs.

Materials and methods

Isolation and culture of neural progenitor cells

After induction with 4.5–5 % isoflurane (Attane™; Minrad Inc., Buffalo, NY, USA) in 75 % N₂O/20 % O₂, time-pregnant Wistar rats (Janvier, Le Genest, France) were anesthetized with ketamine (Ketapan™, 75 mg/kg body weight i. p.; Vétoquinol, Lure, France) and xylazine (Xylapan™, 5 mg/kg body weight i. p.; Vétoquinol). E14 and E18 fetuses (E0 = day of vaginal plug) were then removed by cesarean section. Ganglionic eminence (GE), the primordial striatal domains, and cortical (Cx) regions were dissected bilaterally under a microscope in cold Dulbecco's modified Eagle medium (DMEM; Gibco-BRL, Grand Island, NY, USA), as previously described (Andres et al. 2005a; Dunnett and Bjorklund 1992). In addition, mesencephalic (Me), rhombencephalic (Rh), and spinal cord (SC) tissue were dissected and stored for protein expression analysis according to standard protocols (Dunnett and Bjorklund 1992). GE and Cx explants were then washed two times in DMEM and mechanically dissociated in 2 ml culture medium by gently pipetting through a 1 ml sterile pipette. Cells were resuspended in 10 ml of medium and the undispersed tissue pieces were allowed to settle. The supernatant containing the individual cells was then transferred to a second tube, spun down at 1200 rpm for 4 min and resuspended in 2 ml of DMEM. The trypan blue dye

exclusion method was used to assess cell viability, which was always above 65 %. For immunocytochemistry and GABA uptake assays, cells were seeded at a density of 600 viable cells per mm² in 24-well plates (Falcon Multiwell 3047, Becton–Dickinson Biosciences, Bedford, MA, USA) containing sterile glass coverslips of 12 mm diameter (Assistant, Sondheim, Germany). For immunoblot analysis, NPCs were plated at a density of 600 viable cells per mm² in 6-well plates (Falcon Multiwell 3046, Becton–Dickinson). Culture wells were coated with a solution of poly-L-lysine (0.1 mg/ml; Sigma, Buchs, Switzerland) in 0.15 M sodium borate buffer, pH 8.3 (Sigma) over night and washed three times with sterile PBS before adding culture medium.

Cells were cultured in 24- and 6-well plates in 0.5 or 2.5 ml of medium consisting of 55 % DMEM, 32.5 % Hank's balanced salt solution (HBSS; Gibco-BRL), 1.5 % glucose (Sigma), 10 % fetal calf serum (FCS; Gibco-BRL) and 1 % 0.01 M HEPES (Merck, Darmstadt, Germany) at 37 °C in a 5 % CO₂ humidified atmosphere. Culture medium was changed every 24 h. Antibiotics/antimycotics (No. 061-05240 D; Gibco-BRL) were added during the first 3 days in culture. In cell culture medium containing 10 % FCS, the final concentration of FCS-derived Cr is in the range of 10–50 µmol/l, which corresponds to the values measured in previous experiments (Andres et al. 2005a).

All animal procedures were approved by the Veterinary Office of the Canton Berne, Switzerland, and the Animal Care and Use Committee at the University of Berne, Switzerland.

Experimental setup and treatment regimens

Wells containing E14 and E18 cultures were randomly assigned to the Cr-treated group or to the control group. Cultures were exposed to Cr monohydrate (Creapure™, AlzChem, Trostberg, Germany) at a final concentration of 5 mM from day in vitro (DIV) 0–7 ($n = 16$ for E14 and $n = 10$ for E18 cultures, 5 and 2 independent experiments, respectively), this treatment regimen is termed 'chronic' or from DIV6–7 ($n = 18$ for E14 and $n = 16$ for E18 cultures, 5 and 4 independent experiments) which is termed 'acute'. Cultures grown in standard medium served as controls. The concentration of 5 mM Cr was chosen based on dose–response experiments with E14 striatal NPCs, where this concentration was found to be most effective. The concentration of the added Cr was found to be stable over the interval between the medium changes (Andres et al. 2005a). For analysis of GABA-ir cell densities, morphological differentiation and BB-CK/uMt-CK co-localization studies with GABA and the astrocytic marker glial fibrillary acidic protein (GFAP), cultures were fixed at DIV7 and processed as described below. GABA uptake ($n = 8$, 2 independent

experiments per group) and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays ($n = 6$, 2 independent experiments per group) were carried out at DIV7. In addition, we analyzed GABA-ir cell densities, total neuronal cell numbers and MTT reduction activity at DIV10 in cultures treated with Cr from DIV6-7 that were grown for three further days in regular maintenance medium ($n = 6-12$, 2 or 3 independent experiments per group).

To investigate for possible neuroprotective effects of Cr against a metabolic insult involving serum and glucose deprivation, cultures were grown in regular culture medium until DIV6, when the medium was replaced for the rest of the culturing period, i.e., up to DIV7, with 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 (all from Sigma) and 5 mM HEPES (Merck) at pH 7.2, but lacking serum and glucose, as previously described (Cheng and Mattson 1994; Andres et al. 2005a). Additional controls were maintained in regular growth medium or in Locke's solution with 20 mM glucose added. Cr was added to the treatment groups from DIV6-7 at a concentration of 5 mM and cultures were fixed at DIV7 ($n = 10-12$, 3 independent experiments per group) or DIV10 ($n = 6-8$, 2 independent experiments per group).

For protein expression analysis, striatal and cortical NPCs were grown in culture as described above without Cr supplementation and processed for Western blotting at DIV0 (immediately after dissection), 3, 6, and 7. In addition, samples of mesencephalic, rhombencephalic and spinal cord tissue were also prepared for Western blotting immediately after dissection (2 independent experiments).

Western Blot analysis

Immunoblot analyses were carried out as described before (Andres et al. 2005a). Cultures were rinsed with sterile ice-cold HBSS (Invitrogen, Carlsbad, CA, USA), supplied with 160 μl of lysis buffer containing leupeptin (2 $\mu\text{l}/\text{ml}$), phenylmethylsulfonyl fluoride (10 $\mu\text{l}/\text{ml}$), beta-mercaptoethanol (1 mM), EDTA (0.2 mM) and Triton X-100 (1 $\mu\text{l}/\text{ml}$) (all from Sigma) in ice-cold HBSS. The tissue was detached with a cell scraper and the suspension immediately set on ice. Cells were sonicated for 3 min, the lysate centrifuged for 2 min at 18,000g, 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 % bromophenol blue, all from Sigma) added to 15 μl of the supernatant and incubated at 95 °C for 5 min. Protein content in the samples was determined according to the method of Bradford using the Bio-Rad™ protein assay (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed using a 12 % sodium dodecyl sulfate (SDS)-polyacrylamide separating gel

(Bio-Rad) with 1–10 μg of sample protein per lane. Highly purified BB-CK (Eder et al. 1999) and uMt-CK (Eder et al. 2000) protein standards at concentrations of 0.1 and 0.5 μg per lane served as positive controls. Proteins were blotted using the semidry technique on a 0.45 μm nitrocellulose membrane (BA85; Schleicher & Schüll, Dassel, Germany) 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 (all from Sigma). Sufficient transfer efficiency was checked by Coomassie Blue (Bio-Rad) staining of the gels for residual protein bands while total protein on the blotting membrane was assessed by reversible staining with Ponceau S reagent (Sigma). After decolorization by rinsing twice in TBS (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 TBS and incubated with the primary antibodies (mouse monoclonal anti-BB-CK, 1:1000 (Sistermans et al. 1995); rabbit polyclonal anti-uMt-CK, 1:1000 (Schlattner et al. 2002b); mouse monoclonal anti- α -tubulin, 1:20,000; Boehringer Mannheim, Mannheim, Germany) overnight at 4 °C. The blots were washed twice in TBS containing 0.2 % Tween-20 (Merck) and subsequently incubated with corresponding affinity purified peroxidase-coupled secondary antibodies (donkey anti-mouse and anti-rabbit, 1:20,000 and 1:10,000, respectively, Jackson ImmunoResearch, West Grove, PA, USA) for 45 min at 20 °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). Results are given as relative chemiluminescence intensities.

Immunocytochemistry

After rinsing three times in 0.1 M PBS, cultures were fixed in 0.1 M PBS containing 4 % paraformaldehyde (Sigma) for 20 min at room temperature. Cultures were then rinsed and preincubated in 0.1 % Triton X-100 (Sigma) in PBS plus 10 % horse serum (HS, Gibco-BRL) for 30 min, washed in PBS and incubated overnight at 4 °C with primary antibodies (rabbit polyclonal anti-GABA, 1:5,000, Sigma Immunochemicals, St. Louis, MO, USA) in 0.1 M PBS containing 0.1 % Triton X-100 and 2.5 % HS. Wells were then rinsed and subsequently incubated with corresponding biotinylated secondary antibodies (goat anti-rabbit or horse anti-mouse, 1:200; Vector Labs, Burlingame, CA, USA) in PBS containing 2.5 % HS for 30 min. After rinsing and incubation with an avidin-peroxidase-complex (1:150; Vector Labs) for 45 min, specifically bound antibodies were visualized with a metal-enhanced

3,3'-diaminobenzidine (DAB) substrate kit (No. 34065; Pierce, Rockford, IL, USA) for 3–5 min. Cultures were then rinsed in PBS and mounted on glass slides (Superfrost™, Menzel-Glaeser, Braunschweig, Germany) using Aquamount™ medium (BDH Laboratory Supplies, Poole, UK).

BB-CK and uMt-CK Co-localization with GABA and GFAP

Cultures were processed for immunocytochemistry 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, 1:5,000; Sigma Immunochemicals or mouse monoclonal anti-GFAP, 1:1000; Chemicon, Temecula, CA, USA, as well as rabbit polyclonal anti-uMt-CK, 1:500 (Schlattner et al. 2002b) and mouse monoclonal anti-GABA, 1:100; Sigma Immunochemicals, or mouse monoclonal anti-GFAP, respectively) in 0.1 M PBS containing 0.1 % Triton X-100 and 2.5 % HS. Following three washes in PBS, cultures were incubated with PBS containing 2.5 % rabbit serum for 30 min and subsequently with appropriate secondary antibodies (Alexa Fluor™ 488 and 555, Molecular Probes, Eugene, OR, 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, Wetzlar, Germany).

MTT reduction assay

The MTT reduction assay was performed as previously described (Mosmann 1983; Rubinstein et al. 1990). Cultures were incubated with 250 µg MTT (Sigma) in 500 µl sterile PBS at 37 °C for 4 h. The medium was then aspirated and 500 µl dimethylsulfoxide (DMSO; Sigma) were added to resolve the resulting MTT-formazan product. After transfer into standard photometric cuvettes and dilution with 500 µl DMSO, the absorbance at 540 nm was determined against DMSO in the linear range of the absorption curve using a photospectrometer (Uvikon 810; Kontron Instruments, Zurich, Switzerland).

GABA uptake assay

The GABA uptake assay was performed as described before (Andres et al. 2005a). Briefly, cultures were rinsed twice with 5 mM glucose and 0.5 mM ascorbic acid (Sigma) in sterile PBS and preincubated for 5 min at 37 °C with 250 µl of incubation solution (5 mM glucose, 10 µM aminooxyacetic acid and 1 mM β-alanine, all from

Sigma) in PBS. After addition of [³H]-GABA (TRK527, 94 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a final concentration of 50 nM, the cultures were 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 5 washes with ice-cold PBS. The cultures were set on ice, mechanically lysed by trituration in 0.5 M NaOH and ³H uptake was measured by liquid scintillation counting (Ultima Gold™; Packard Instruments, Meridan, CT, USA).

Analysis of cell numbers

Cell numbers were determined using a microscope (Axioptan; Carl Zeiss, Feldbach, Switzerland) with a CCD camera connected to a digital image processing system (Lucia 4.50; Nikon, Tokyo, Japan). DAB-positive cells were counted in six randomly chosen areas (sized 0.8 mm²) per well and cell density was calculated per mm². Only cells with distinct immunoreactivity, clear neuronal shape and a nucleus were counted as GABA-positive neurons. Phase-contrast microscopy was used to assess for the total neuronal cell numbers.

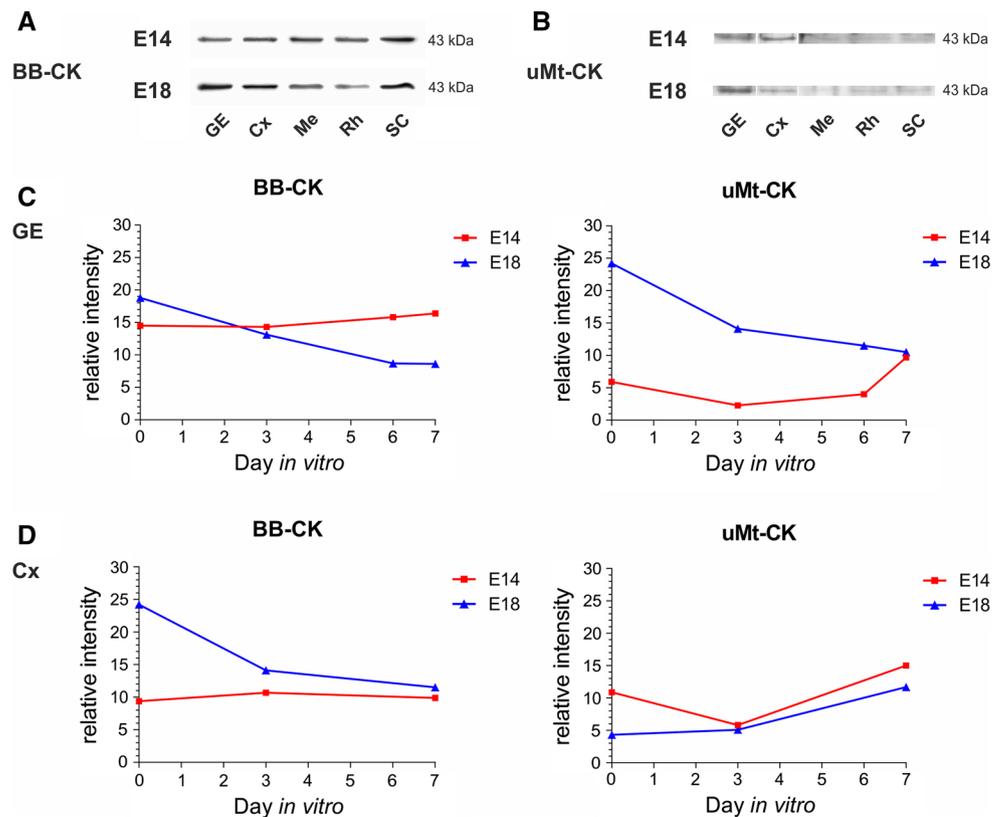
Analysis of neuronal morphology

For quantitative analysis of neurite outgrowth and morphological complexity in neurons from E14 and E18 Cr-treated cultures and from corresponding untreated controls, digital images of randomly selected fields from GABA-immunostained cultures ($n = 12$ per well) were acquired using a bright field microscope (Olympus BX51; Olympus Optical Co., Tokyo, Japan) with a CCD camera (DP72; Olympus) connected to a digital image processing system (Cell*F V3.4; Olympus Soft Imaging Solutions, Munster, Germany). Cells were analyzed using automatic high-throughput neurite tracing software (HCA Vision V1.6.5, CSIRO Mathematical and Information Sciences, North Ryde, Australia) as described before (Andres et al. 2011). Parameters assessed included soma size, average length of all neurites per neuron, number of branching points, and estimated area of influence of each cell.

Statistical evaluation

A commercially available software package was used for statistical analysis (GraphPad Instat™ 3.06, GraphPad Software, San Diego, CA, USA). Statistical comparisons were carried out using multivariate analysis of variance (MANOVA), followed by nonparametric post hoc Mann–Whitney test, or Student's *t* test when appropriate. Differences were considered statistically significant when $p < 0.05$. Values are presented as mean ± SEM.

Fig. 1 Expression of creatine kinases in the developing rat brain and in cultured NPCs. Western blot demonstrating expression of BB-CK, which was found in all investigated regions of the E14 and E18 central nervous system (CNS), including cortex (Cx), ganglionic eminence (GE), mesencephalon (Me), rhombencephalon (Rh), and spinal cord (SC) (a). Correspondingly, uMt-CK expression was detected in all CNS regions derived from E14 and E18 embryos (b). BB-CK (left panels) and uMt-CK (right panels) levels quantified on Western blots at day of dissection (DIV0) and in cell culture up to DIV7 demonstrated a tissue-specific expression pattern in striatal (e) and cortical (f) NPCs ($n = 2$ per time point) (color figure online)



Results

Brain-specific cytosolic and mitochondrial creatine kinases are ubiquitously co-expressed in E14 and E18 CNS tissue and in cultured striatal and cortical NPCs

BB-CK (Fig. 1a) and the uMt-CK isoenzyme (Fig. 1b) were found to be expressed in all investigated regions of E14 and E18 CNS tissue, including ganglionic eminence (GE), cortex (Cx), mesencephalon (Me), rhombencephalon (Rh), and spinal cord (SC). Semi-quantitative analyses of BB-CK and uMt-CK expression using Western blots at day of dissection (=day in vitro (DIV) 0) and during the culturing period up to DIV7 demonstrated a tissue-specific expression pattern in striatal (Fig. 1c) and cortical (Fig. 1d) NPCs. While expression of BB-CK was stable in E14 striatal and cortical NPCs, there was a tendency for a decrease in E18 NPCs during the culturing period (Fig. 1c, d, left panels). uMt-CK expression showed a trend for a slight increase between DIV0-7 in E14 striatal and cortical cultures, while in E18 NPCs, uMt-CK gradually decreased in striatal and increased in cortical cultures (Fig. 1c, d, right panels). GABA-ergic cells at DIV7 expressed both BB-CK and uMt-CK (Fig. 2a). In E18 cultures, both GABA-expressing neurons and glial fibrillary acidic protein (GFAP)-immunoreactive (-ir) astrocytes in striatal cultures co-localized with BB-CK and uMt-CK (Fig. 2b).

Effects of chronic creatine treatment on GABA-ergic differentiation of striatal neurons

After chronic Cr exposure [5 mM] from DIV0-7, the density of GABA-ir neurons in striatal cultures was higher as compared to controls in both the E14 ($170.44 \pm 4.67 \%$, $p < 0.01$) and E18 ($129.30 \pm 9.32 \%$, $p < 0.05$) groups (Fig. 3). However, this effect was significantly less pronounced in cultures derived from E18 embryos ($p < 0.05$). No significant effect of Cr treatment was observed on the total neuronal cell numbers between Cr-treated cultures and controls in the E14 (n.s.) and E18 (n.s.) groups.

Effects of short-term creatine exposure on differentiation and survival of striatal NPCs

Short-term treatment of NPC cultures with Cr [5 mM] from DIV6-7 resulted in significantly higher GABA-ir neuron densities in both the E14 ($162.97 \pm 10.37 \%$, $p < 0.01$) and the E18 ($133.27 \pm 9.51 \%$, $p < 0.05$) groups at DIV7, as compared to untreated controls (Fig. 4a). In line with the observations made with chronic Cr exposure, this effect was more pronounced in the E14 group ($p < 0.05$).

The total number of neuronal cells was not affected by Cr treatment in both the E14 ($103.02 \pm 7.05 \%$, $p > 0.05$) and the E18 group ($104.10 \pm 13.92 \%$, $p > 0.05$), and did not differ between E14 and E18 cultures ($p > 0.05$).

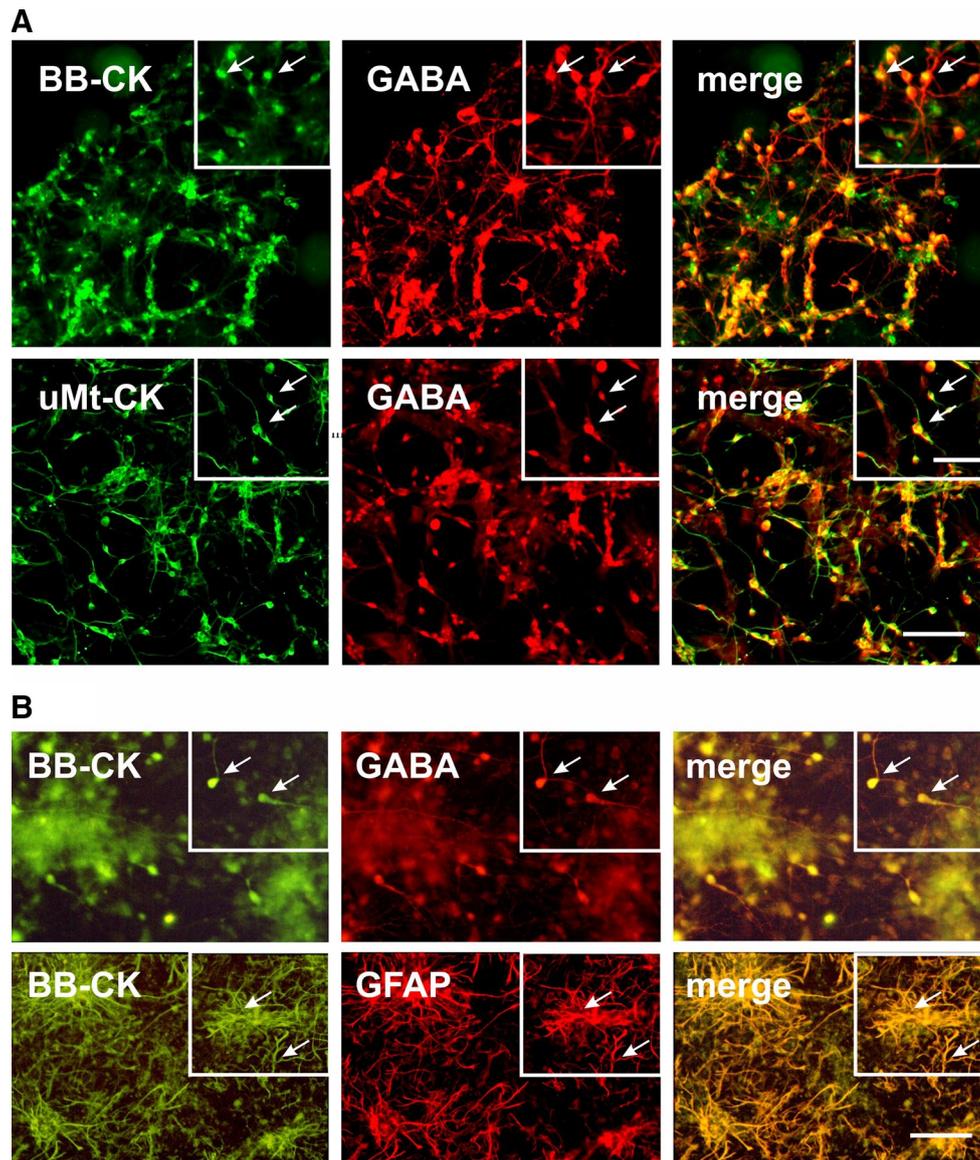


Fig. 2 Representative digitalized photomicrographs of double immunolabeling co-localization of BB-CK or uMt-CK (green) and GABA (red) in E14 striatal cultures at DIV7 (a). All GABA-ir neurons showed marked co-localization (arrows). Similarly, BB-CK (green)

demonstrated a distinct co-localization (arrows) with GABA-ir neurons as well as GFAP (red) in E18 striatal cultures at DIV7, while high contents of BB-CK in astrocytes can be seen. Scale bars 100 μ m (overview) and 50 μ m (insets) in a; 50 μ m in b (color figure online)

(Fig. 4b). Accordingly, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity, representing general cell viability, was not affected by Cr supplementation in both the E14 (99.25 ± 2.21 %, $p > 0.05$) and the E18 group (103.25 ± 4.16 %, $p > 0.05$) group. MTT reduction activity was furthermore not different between the E14 and E18 groups ($p > 0.05$) (Fig. 4c).

A significant increase in GABA-ir cell densities was still present when NPCs exposed to Cr from DIV6-7 were maintained in culture for 3 further days without Cr, as assessed at DIV10, both in E14 (154.80 ± 14.31 %, $p < 0.05$) and E18 cultures (128.31 ± 7.83 %, $p < 0.05$).

We still observed a tendency towards a more pronounced effect on E14 NPCs ($p > 0.05$) (Fig. 4d). Short-term Cr treatment from DIV6-7 did not affect total neuronal cell numbers (E14: 103.81 ± 6.91 %, E18: 98.14 ± 11.2 %, both $p > 0.05$) (Fig. 4e) and MTT reduction activity (E14: 101.04 ± 0.77 %, E18: 120.06 ± 8.70 , both $p > 0.05$) (Fig. 4f), as assessed at DIV10.

Effects of creatine exposure on GABA uptake

Cr administration from DIV6-7 resulted in a significant increase in GABA-uptake in both E14 (121.5 ± 7.3 %, $p < 0.05$) and E18 cultures (118.5 ± 10.5 %, $p < 0.05$).

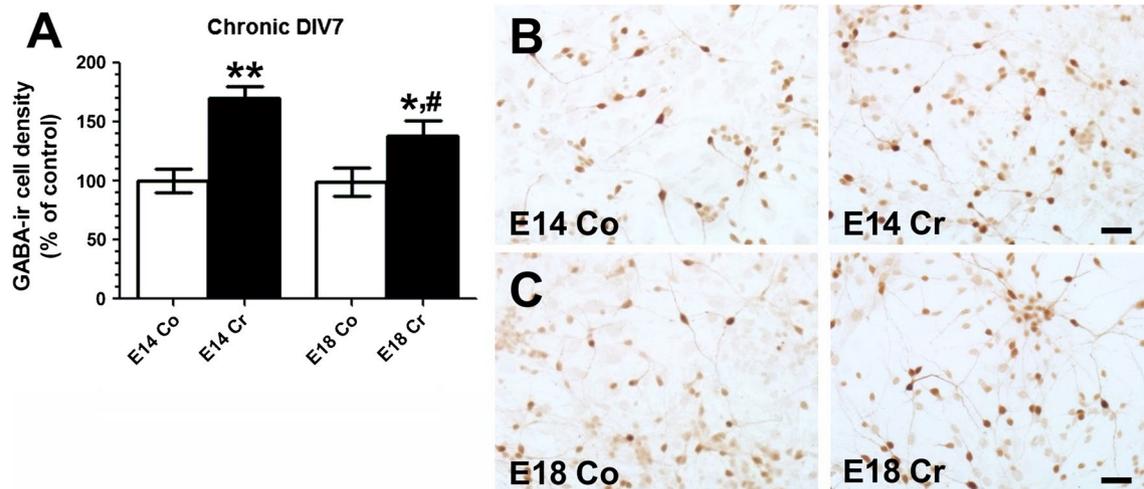


Fig. 3 Effects of chronic creatine supplementation on GABA-ergic cell densities in E14 and E18 cultures at DIV7, demonstrating a more pronounced induction of the GABA-ergic phenotype in E14 NPCs (**a**). Representative photomicrographs of untreated controls (Co) and creatine-exposed (Cr) cultures derived from E14 (**b**) and E18 embryos (**c**) stained for GABA, depicting a marked increase of

GABA-ergic neurons. Data are given as mean \pm SEM and are presented as percentage of corresponding controls. ** $p < 0.01$, * $p < 0.05$ versus corresponding control values, # represents significance at $p < 0.05$ between E14 Cr and E18 Cr. Scale bars 50 μ m (color figure online)

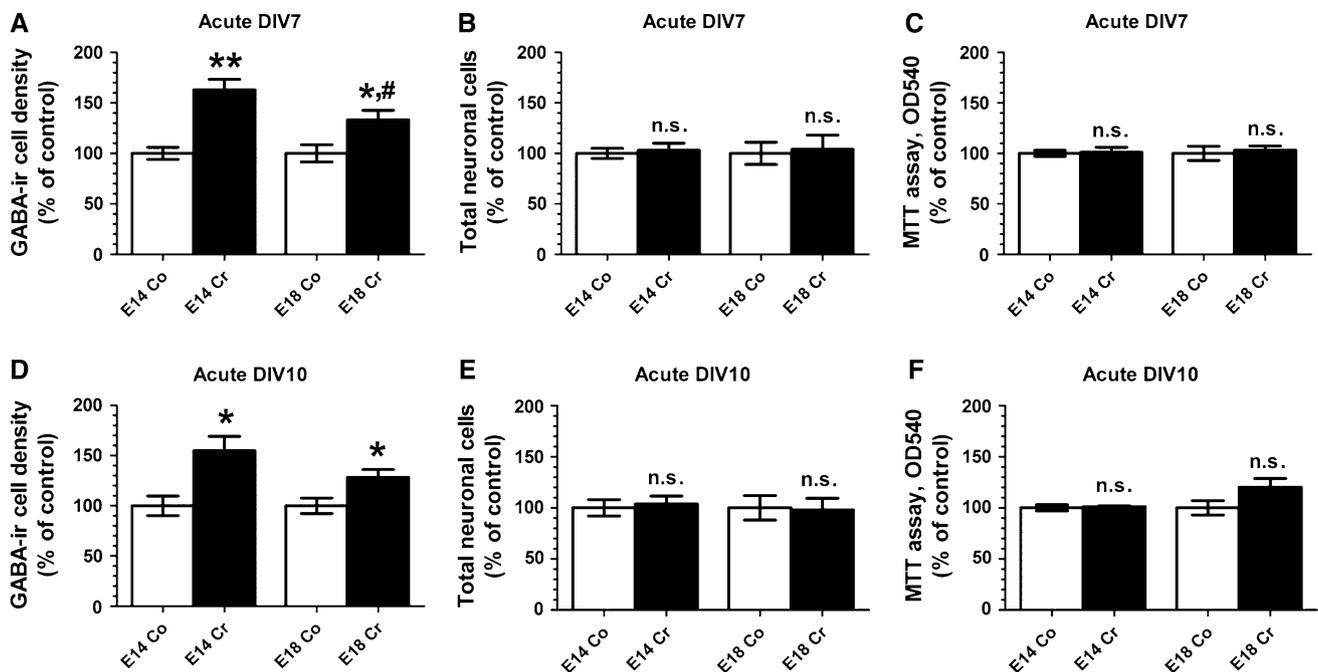


Fig. 4 Effects of acute creatine (Cr) treatment during 24 h on GABA-ergic cell densities in E14 and E18 cultures at DIV7, demonstrating superior induction of the GABA-ergic fate in E14 cells (**a**). Total neuronal cell numbers (**b**) and measurements of the MTT assay (**c**) were not affected by Cr exposure. GABA uptake was enhanced after Cr treatment in both E14 and E18 cultures, but did not differ between the E14 and E18 groups. After 3 further days in culture without Cr (DIV10), GABA-ergic cell numbers were still higher in treated

cultures (**d**), while neuronal cell numbers (**e**) and MTT activity (**f**) remained unaffected. Acute Cr exposure also resulted in increased GABA uptake in both the E14 and the E18 groups (**g**). Data are given as mean \pm SEM and are presented as percentage of corresponding controls. ** $p < 0.01$, * $p < 0.05$, versus corresponding control values, *ns* not significant, # represents significance at $p < 0.05$ between E14 Cr and E18 Cr

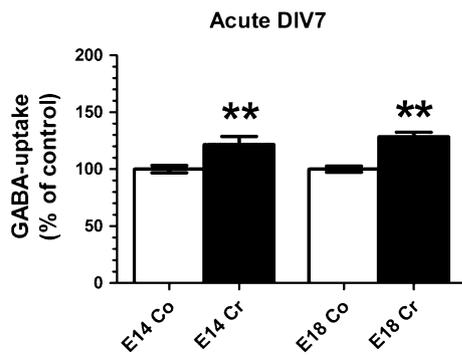


Fig. 5 Acute Cr exposure during 24 h resulted in increased GABA uptake in both the E14 and the E18 groups. Data are given as mean \pm SEM and are presented as percentage of corresponding controls. ** $p < 0.01$, versus corresponding control values

$p < 0.01$) and E18 cultures (128.4 ± 4.0 %, $p < 0.01$), as compared to untreated controls. This increase did not significantly differ between E14 and E18 cultures ($p > 0.05$) (Fig. 5).

Neuroprotective effects of creatine supplementation

A metabolic insult induced by serum and glucose deprivation was used to investigate the neuroprotective potential of Cr treatment. Serum and glucose deprivation from DIV6-7 resulted in a significant decrease in the number of GABA-ir cells, as assessed at DIV7, both in E14 (60.18 ± 8.65 % compared to controls, $p < 0.01$) and E18 cultures (96.51 ± 11.26 % compared to controls, $p < 0.05$). This effect was significantly less pronounced in E18 NPCs ($p < 0.05$), indicating that these cultures were more robust against a metabolic challenge. Concurrent Cr exposure from DIV6-7 significantly protected both E14 (129.66 ± 12.08 % compared to controls, $p < 0.05$) and E18 cultures (135.92 ± 16.29 % compared to controls, $p < 0.05$)

against the loss in GABA-ir cell numbers (Fig. 6a). As reported before (Andres et al. 2005a), Cr treatment resulted in induction of the GABA-ergic phenotype also during the metabolic insult, therefore even higher GABA-ir cell densities were observed in Cr-treated cultures undergoing serum and glucose deprivation than in untreated controls kept in standard conditioning medium.

A reduction of total neuronal cell numbers after glucose and serum deprivation was observed in both the E14 (70.16 ± 10.93 %, $p < 0.05$) and the E18 group (72.30 ± 9.08 %, $p < 0.05$). However, there was no significant influence of Cr supplementation on this parameter in both the E14 (96.18 ± 10.32 %, $p > 0.05$) and the E18 group (85.37 ± 9.68 %, $p > 0.05$) (Fig. 6b).

Glucose and serum deprivation further resulted in a markedly decreased MTT reduction activity at DIV7 in both E14 (12.24 ± 2.03 %, $p < 0.01$) and E18 cultures (18.36 ± 2.99 %, $p < 0.01$), which did not differ between E14 and E18 NPCs ($p > 0.05$). Cr treatment didn't protect significantly against the decrease in MTT reduction capacity in both the E14 (12.07 ± 4.39 %, $p > 0.05$) and the E18 group (15.53 ± 2.18 %, $p > 0.05$) (Fig. 6c).

When assessed at DIV10 after three additional days in standard conditioning medium without Cr supplementation, a significant difference between Cr-treated cultures and controls was still present in both the E14 (124.42 ± 14.05 %, $p < 0.05$) and the E18 group (132.11 ± 17.14 %, $p < 0.05$), with no significant difference between E14 and E18 cells ($p > 0.05$). No significant effects of Cr treatment were observed on total neuronal cell numbers (E14: 107.92 ± 15.13 %, E18: 117.12 ± 19.59 %, as compared to corresponding controls, both $p > 0.05$). As compared to DIV7, MTT reduction activity showed a distinct recovery in both the E14 (81.78 ± 6.81 %) and the E18 group (72.86 ± 8.47) at DIV10. At this time point, no significant effects of Cr were observed in both the E14 (83.21 ± 1.86 %) and the E18 group (70.27 ± 6.02).

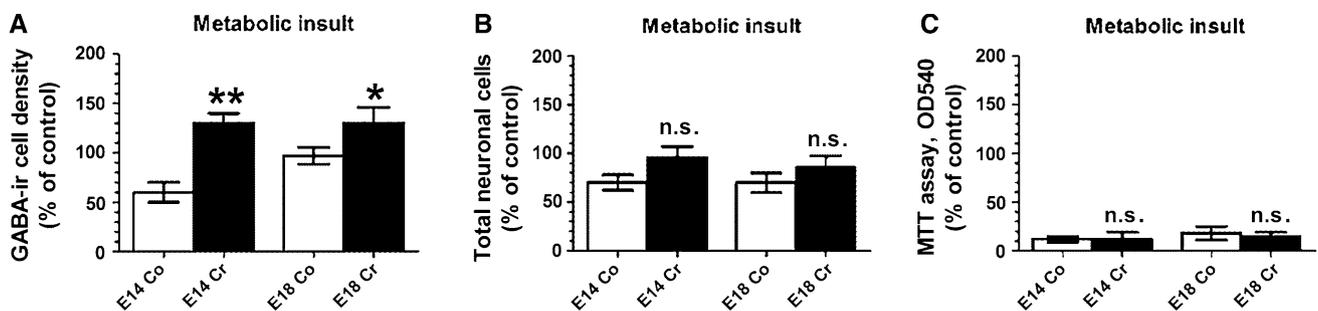


Fig. 6 Effects of creatine on GABA-ergic cell densities (a), total neuronal cell numbers (b) and measures of the MTT assay (c) in striatal cultures undergoing a metabolic insult induced by serum and glucose deprivation from DIV6-7. Cultures were analyzed at DIV7. Creatine induced solid neuroprotection on GABA-ergic cells in both

E14 and 18 cultures, but no difference on the efficacy of the drug was observed between the two age groups. Data are given as mean \pm SEM and are presented as percentage of corresponding controls without serum and glucose deprivation. ** $p < 0.01$, * $p < 0.05$, versus corresponding control values, ns not significant

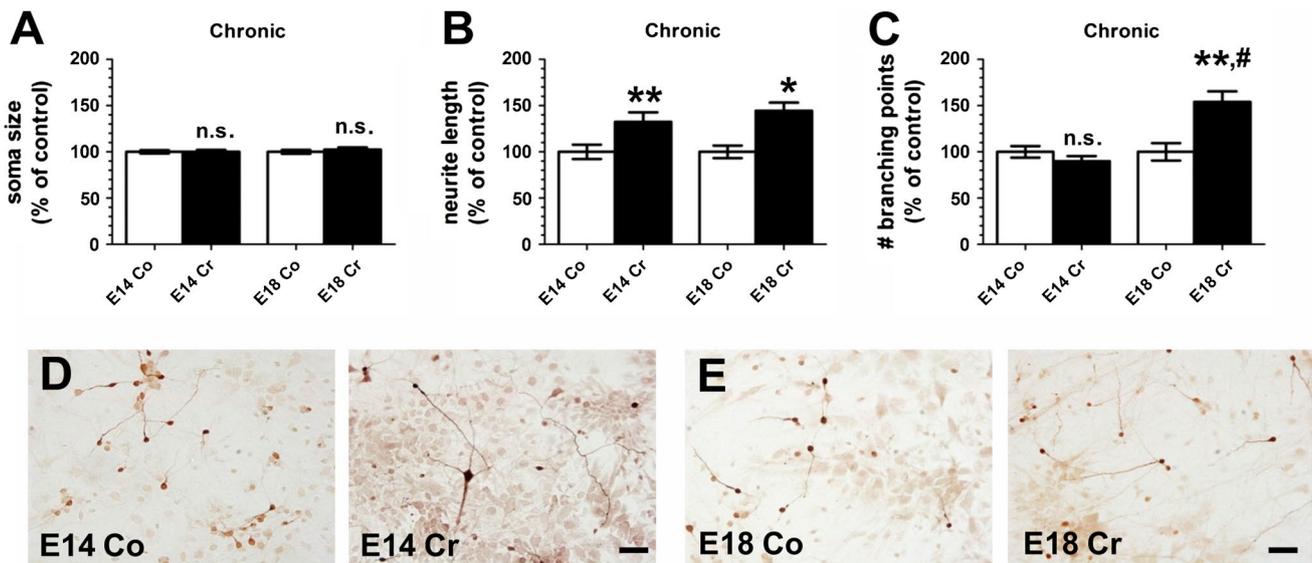


Fig. 7 Effects of creatine exposure on morphology of GABA-ergic neurons in cultured E14 and E18 striatal cultures. While soma size was not affected by creatine (Cr) exposure (a), average neurite length per neuron was found to be increased in both the E14 and E18 groups (b), and numbers of neuritic branching points were significantly higher after treatment in E14, but not in E18 cultures (c). Representative photomicrographs from creatine-treated (Cr) and untreated

(Co) E14 (D) and E18 (e) cultures immunohistochemically stained for GABA. Data are given as mean \pm SEM and are presented as percentage of corresponding controls. ** $p < 0.01$, * $p < 0.05$, versus corresponding control values, *ns* = not significant, # represents significance at $p < 0.05$ between E14 Cr and E18 Cr. Scale bars 50 μ m (color figure online)

Effects of creatine supplementation on morphological differentiation

Analysis of morphological differentiation revealed no difference in the soma size after chronic Cr exposure both in E14 (1.28 ± 2.05 %, $p > 0.05$) and E18 (3.55 ± 3.10 %, $p > 0.05$) GE cultures (Fig. 7a). Cr treatment promoted neurite outgrowth in both E14 (32.62 ± 10.50 %, $p < 0.05$) and E18 (48.16 ± 9.83 %, $p < 0.05$) cultures, with no significant differences between these groups (Fig. 7b, d, e). Interestingly, the number of neurite branching points was found to be significantly increased with Cr treatment in E18 (57.90 ± 12.0 %, $p < 0.05$), but not in E14 cultures (94.85 ± 7.14 %, $p > 0.05$) (Fig. 7c).

After a metabolic insult induced by glucose and serum deprivation, significantly decreased values for overall neurite length were observed in both the E14 (36.40 ± 16.33 %, $p < 0.05$) and the E18 group (28.08 ± 19.08 %, $p < 0.05$) at DIV7, with no significant difference between E14 and E18 cultures ($p > 0.05$). Cr supplementation resulted in a non-significant trend towards protection against this decrease in both the E14 (55.26 ± 18.84 %, $p > 0.05$) and the E18 group (45.36 ± 29.14 %, $p > 0.05$). Correspondingly, there was a marked decrease in the number of branching points after the insult in both the E14 (64.83 ± 24.91 %, $p < 0.05$) and the E18 group (57.80 ± 31.38 %, $p < 0.05$). There was no significant difference between the E14 and the E18

group ($p > 0.05$), Cr exposure did not protect against this decrease in E14 (53.19 ± 31.69 %, $p > 0.05$) and E18 cultures (60.05 ± 37.05 %, $p > 0.05$). No significant differences between E14 and E18 cultures after the metabolic insult were observed for soma size (104.23 ± 4.21 % and 101.81 ± 6.53 %, respectively, $p > 0.05$) and Cr treatment did not significantly affect these values (106.26 ± 7.71 % and 101.47 ± 5.30 %, respectively, $p > 0.05$).

Discussion

Cell replacement therapy using NSCs or NPCs has become a promising future avenue for the treatment of HD. However, the efficacy of this approach is limited by the necessity to obtain a sufficient amount of progenitor cells for transplantation, poor survival of grafted cells, difficulties in directing NPC differentiation towards the affected GABA-ergic phenotype, and suboptimal structural and functional graft integration in the host brain (Dunnett and Rosser 2007). Basic research needs to address these issues in order to successfully translate stem-cell-based therapies into future clinical applications.

Differentiation of NSCs and NPCs, requiring low energy resources, into the high-energy demanding neuronal lineage requires developmental changes of the cellular bioenergetic system. There is growing experimental evidence that the CK

phosphotransfer system plays a crucial role in cellular bioenergetics during neural development (Andres et al. 2008; Ducray et al. 2007; Braissant et al. 2005; Holtzman et al. 1993). Similar changes are observed during development of the embryonic heart, where beginning contractile activity in cardiomyocytes has been shown to be coupled with the establishment of the mitochondrial network and maturation of the CK phosphotransfer system (Chung et al. 2008). We have previously shown that in striatal NPCs isolated from E14 rat embryos, both BB-CK and uMt-CK isoforms of CK are co-expressed at high levels and co-localized with GABA-ergic cells after differentiation. We furthermore demonstrated that short- and long-term addition of Cr to the culture medium at a concentration of 5 mM promoted GABA-ergic differentiation without affecting general cell survival and resulted in neuroprotection (Andres et al. 2005a). Preliminary results on Cr supplementation in NSCs and NPCs have demonstrated that cellular ATP levels are significantly increased in Cr-exposed cells (Andres et al. 2010).

Cr supplementation is indeed a promising approach for improving cell-based therapies in HD given that the molecule is able to cross the blood–brain barrier (Ohtsuki et al. 2002), and has a low toxicity profile (Shao and Hathcock 2006). So far, three phase II studies have been carried out in HD patients, which showed no safety issues (Verbessem et al. 2003; Hersch et al. 2006; Rosas et al. 2014). Cr has recently been investigated as a possible neuroprotective drug in HD patients in a phase III trial (CREST-E study). However, the study had to be discontinued due to missing effects on clinical progression of HD (for review, see Bender and Klopstock 2016). Due to the proven clinical safety even at high dosages, not only pretreatment of NSCs and NPCs prior to transplantation, but also systemic administration to the patients after grafting would be a safe and feasible option for improving graft survival, differentiation and integration.

There is evidence that the pathological microenvironment in HD induces degeneration of grafted NPCs (Cicchetti et al. 2011). It might therefore be supposed that treatment regimens effective against HD-related neurodegeneration could be useful for improving long-term graft survival in HD patients.

There is growing evidence that bioenergetic disturbances are involved in the pathogenesis of age-related diseases of the CNS (Slosman et al. 2001). Mitochondrial dysfunction and the production of reactive oxygen and nitrogen species (ROS, RNS) are common denominators of many neurodegenerative disorders. These conditions are often characterized by oxidative damage, poor energetic state of the cells, Ca^{2+} overload, and subsequent cell death (Schlattner et al. 2006). In general, CK isoenzymes are very sensitive to oxidation and oxidative stress related disorders, which leads to increased CK inactivation and compromised cellular energetics. Oxidative damage to uMt-CK furthermore impacts

the protection against mitochondrial permeability transition (Wallimann et al. 2007; Raha and Robinson 2000). Lack of BB-CK-generated ATP flux will diminish the efficiency of the many cerebral processes relying on this form of channeled energy supply. Animal models of BB-CK or Cr deficiency show characteristic deficits in the function of the brain and sensory organs (Schulze 2003). This is also observed in patients with Cr-deficiency syndromes or disorders with impaired BB-CK activity, such as AD and HD (Lin et al. 2013; Bürklen et al. 2006). Hearing loss in HD might be related to reduced BB-CK activity resulting from either ROS-/RNS-induced BB-CK damage or inhibition of BB-CK promoters by Huntingtin, leading to dysfunctional Ca^{2+} handling (Lin et al. 2011, 2013).

Recent research has reported on the presence of crystalline depots in the brains of patients suffering from AD and neurodegenerative diseases, and it has been shown that these deposits are constituted of crystalline Cr (Li et al. 2006). These findings might be explained by oxidative damage by ROS and RNS. Reduced ATP production by damaged mitochondria, as well as reduced CK activity, results in reduced transphosphorylation of Cr to PCr. Since Cr is less soluble than the electrically charged PCr molecule, it precipitates and accumulates in crystalline Cr deposits (Li et al. 2006; Kuzyk et al. 2010; Kutorasinska et al. 2013). When treating such conditions with stem cell-based therapies, it has to be considered that the pathological changes underlying the given disease continue to be present in the microenvironment of the newly grafted cells. Exogenous supplementation of Cr to the graft recipient might therefore be beneficial for improving these conditions and should be subject of further research.

Suboptimal host integration of grafted cells is another critical issue in neural transplantation. Since Cr treatment has been reported to promote axonal growth in NH_4Cl -exposed rat neurons (Braissant et al. 2002), this approach might also facilitate the establishment of functional connections between the host and the graft (Andres et al. 2011).

Since the expression of CKs in the developing central nervous system is dynamic (Ducray et al. 2007; Bourdelas et al. 2009) and the donor age best suitable for transplantation of striatal NPCs still has to be determined (Watts et al. 1997, 2000), we compared a possible dependency of Cr-mediated effects from the developmental stage in the present study. NPCs isolated from E14 (early stage) and E18 (late stage) rat embryos were grown in vitro as dissociated cultures. As previously described with E14 NPCs (Andres et al. 2005a), chronic Cr supplementation to the cell culture medium resulted also in higher numbers of GABA-ir neurons in E18 NPC cultures, as compared to untreated controls. However, the observed induction of differentiation towards the GABA-ergic fate was significantly more pronounced in E14 NPCs.

We next investigated whether acute Cr treatment for 24 h from DIV6-7 is sufficient to result in a robust induction of the GABA-ergic phenotype in E18 NPCs, as previously observed in cultures derived from E14 embryos (Andres et al. 2005a). We found a similar effect also in E18 NPCs. However it was less pronounced than in E14 cells. In both the E14 and the E18 group, the number of total neuronal cells and general viability, as assessed with the MTT assay, were not affected by Cr exposure, pointing towards a specific induction of the GABA-ergic neurotransmitter phenotype rather than a general neuroprotective or trophic effect.

As reported before with E14 striatal NPCs (Andres et al. 2005a), we found a persisting increase in GABA-ir cell densities at DIV10 in cultures supplemented with Cr from DIV6-7 and maintained for further 3 days in culture without Cr addition, indicating permanent induction of the GABA-ergic phenotype. In line with our results observed in E14 NPCs (Andres et al. 2005a), functional GABA uptake after Cr treatment was enhanced in E18 cultures. Given that the induction of the GABA-ergic phenotype in undifferentiated NPCs may result in a better outcome in cell replacement strategies in HD, Cr supplementation might be effective for improving this approach. However, there are important timing issues that have to be considered. Complete induction towards differentiated neurons is unwanted before transplantation, since it leads to poor survival of grafted cells and compromises graft integration into host neural circuitry. Therefore, it might be most beneficial to supplement NPCs with Cr immediately before grafting and/or to treat the recipient before and after transplantation, since Cr is able to cross the blood–brain barrier, at least in limited amounts.

A metabolic insult induced by serum- and glucose deprivation (Cheng and Mattson 1994) was used to compare possible neuroprotective effects of Cr between the E14 and the E18 group. We found that Cr provided protection against GABA-ergic cell loss in both groups and this effect was equal in E14 and E18 cultures. In addition to the lower numbers of GABA-ir cells observed after serum and glucose deprivation, the insult significantly decreased total neuronal cell numbers and MTT reduction activity, as assessed at DIV7, and Cr treatment did not affect these parameters.

Neuroprotective effects of Cr have been described in experimental models of neurological diseases, including HD (Matthews et al. 1998; Dedeoglu et al. 2003; Ferrante et al. 2000; Shear et al. 2000), Parkinson's disease (Andres et al. 2005c, b), Alzheimer's disease (Brewer and Wallimann 2000), amyotrophic lateral sclerosis (Klivenyi et al. 1999, 2004; Dupuis et al. 2004; Zhang et al. 2003), and ischemia (Prass et al. 2006; Berger et al. 2004; Adcock et al. 2002; Lensman et al. 2006), both in vitro and in vivo. In contrast to the observed effects on cell fate decision, our findings implicate that the developmental stage of the treated neural cells is not critical for Cr-mediated neuroprotection.

Cr exposure finally promoted morphological differentiation of NPC-derived neurons. While both neurite length and numbers of branching points were significantly higher after Cr exposure in E18 cultures, a similar effect on neurite length, but not branching points, was observed in the E14 group. The spatial configuration of the cellular phosphotransfer network becomes increasingly complex with ongoing development of neurons from undifferentiated NPCs. Cr supplementation might boost this process by providing more substrate for the transfer processes and increasing cellular energy reserves in the form of PCr as well. This might be particularly interesting in the context of improving graft integration in the host brain after transplantation. We also addressed possible effects of Cr supplementation on morphological parameters in cultures undergoing a metabolic insult. While we observed a non-significant trend towards a reduced decrease in neurite length both in E14 and E18 cultures after glucose and serum deprivation, Cr exposure did not affect soma size and the number of neuritic branching points, and there were no significant differences between the E14 and E18 groups. It might therefore be hypothesized that Cr does not promote neuronal complexity during acute metabolic stress.

In sum, the efficacy of Cr as a GABA-ergic differentiation factor depends on the developmental stage of striatal NPCs, while Cr-mediated neuroprotection is not significantly influenced by the developmental stage of the treated cells. These findings might be of importance for optimizing neuroregenerative strategies in HD and other neurological diseases, e.g. NSC or NPC transplantation. Future research is necessary to investigate the effects of Cr treatment subject to the developmental stage of human NSCs and NPCs.

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Compliance with ethical standards

Ethical statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals, as well as human embryonic tissues were approved and in accordance with the ethical standards of the institutions (Department of Neurosurgery, Inselspital, University of Berne, Swiss Federal Institute of Technology, Zurich, and Boston Children's Hospital, Harvard Medical School) at which the studies were conducted.

Conflicts of interest The authors declare no conflicts of interest.

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