ELSEVIER

Neurobiology of Disease

www.elsevier.com/locate/ynbdi
Neurobiology of Disease xx (2004) xxx–xxx

Neuroprotective mechanisms of creatine occur in the absence of mitochondrial creatine kinase

Peter Klivenyi,[a] Noel Y. Calingasan,[a] Anatoly Starkov,[a] Irina G. Stavrovskaya,[b] Bruce S. Kristal,[a,b,c] Lichuan Yang,[a] Bé Wieringa,[d] and M. Flint Beal[a,*]

[a] *Department of Neurology and Neuroscience, New York-Presbyterian Hospital, Weill Medical College of Cornell University, New York, NY 10021, USA*
[b] *Dementia Research Service, Burke Medical Research Institute, White Plains, NY 10605, USA*
[c] *Department of Biochemistry, Weill Medical College of Cornell University, New York, NY 10021, USA*
[d] *Department of Cell Biology, University Medical Center St. Radboud, NCMLS, University of Nijmegen, Geert Grooteplein 28, 6525 GA, The Netherlands*

Received 27 May 2003; revised 19 December 2003; accepted 23 December 2003

There is substantial evidence that creatine administration exerts neuroprotective effects both in vitro and in vivo. The precise mechanisms for these neuroprotective effects however are as yet unclear. We investigated whether creatine administration could exert neuroprotective effects in mice deficient in ubiquitous mitochondrial creatine kinase (UbMi-CK). UbMi-CK-deficient mice showed increased sensitivity to 1-methyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced dopamine depletion and loss of tyrosine hydroxylase (TH) stained neurons. Isolated mitochondria from these mice showed no alterations in calcium retention, oxygen utilization, membrane potential, or swelling in response to a calcium challenge. Creatine administration significantly increased brain concentrations of both creatine and PCr in the UbMi-CK knockout mice. Creatine administration to the UbMi-CK-deficient mice exerted significant neuroprotective effects against MPTP toxicity that were comparable in magnitude to those seen in wild-type mice. These results suggest that the neuroprotective effects of creatine are not mediated by an effect on UbMi-CK to inhibit the mitochondrial permeability transition, and are more likely to be mediated by maintenance of appropriate ATP/ADP and PCr/Cr levels.

Keywords: Creatine; Creatine kinase; MPTP; Phosphocreatine; Mitochondria

Several isoforms of creatine kinase are compartmentalized within cells to form a buffering system for cellular energy stores, which is thought to be important in tissues with high and fluctuating energy requirements such as brain and muscle. The creatine kinase system can shuttle high-energy phosphates (PCr) to sites of energy usage in the cell, such as ion pumps in organelles in the plasma membranes or dynamic cytoskeletal structures and molecular motors. Creatine may therefore exert neuroprotective effects by increasing PCr levels, and thereby providing extra energy for ion homeostasis and the functional and structural integrity of mitochondria. We and others showed that PCr levels are increased by creatine supplementation both in vitro and in vivo (Brewer and Wallimann, 2000; Ferrante et al., 2000; Holtzman et al., 1998; Matthews et al., 1998). Other neuroprotective effects of creatine may be due to increased glutamate reuptake (Xu et al., 1996), and scavenging of free radicals (Lawler et al., 2002).

Creatine exerts neuroprotective effects in vitro as well as in vivo. Creatine protects against both glutamate and β-amyloid toxicity in rat hippocampal neurons (Brewer and Wallimann, 2000). Creatine also protects against 3-nitropropionic and glutamate neurotoxicity in rat hippocampal and striatal neurons (Brustovetsky et al., 2001). We initially showed that creatine protects against both malonate and 3-nitropropionic acid (3-NP) striatal neurotoxicity in vivo (Matthews et al., 1998). We also found dose-dependent protection against MPTP toxicity (Matthews et al., 1999). Others found protection against traumatic brain injury (Sullivan et al., 2000). More recently, we found that creatine exerts neuroprotective effects in transgenic mouse models of both ALS and Huntington's disease (Andreassen et al., 2001; Ferrante et al., 2000; Klivenyi et al., 1999). Creatine also is protective in the wobbler mouse model of motor neuron disease (Ikeda et al., 2000). Phosphocreatine promotes recovery of ischemic heart tissue in vitro (Sharov et al., 1987), and improves survival of cultured myotubes deficient in dystrophin (Pulido et al., 1998).

A proposed mechanism of neuroprotection is inhibition of activation of the mitochondrial permeability transition pore (mPTP) that is linked to both necrotic and apoptotic cell death (Bernardi et al., 1998). Creatine can stabilize mitochondrial isoform of creatine kinase (Mi-CK) in an octomer that can inhibit activation of the mPTP (O'Gorman et al., 1997). Whether this is an important component of the neuroprotective effects of creatine in vivo however remains controversial (Brustovetsky et al., 2001; Sullivan et al., 2000). To resolve whether inhibition of mPTP plays a role in the neuroprotective effects of creatine, we examined the effects of creatine in ubiquitous mitochondrial creatine kinase (UbMi-CK)-deficient mice. Mice deficient in UbMi-CK are viable and fertile, and show no physical or behavioral deficits (Steeghs et

* Corresponding author. Neurology Department, New York Hospital-Cornell Medical Center, 525 East 68th Street, A5, New York, NY 10021. Fax: +1-212-746-8532.
E-mail address: fbeal@med.cornell.edu (M.F. Beal).
Available online on ScienceDirect (www.sciencedirect.com.)

0969-9961/$ - see front matter © 2004 Published by Elsevier Inc.
doi:10.1016/j.nbd.2003.12.014

al., 1995). If an interaction of creatine with Mi-CK to stabilize the MPTP plays an important role in the neuroprotective effects of creatine, then creatine feeding should not exert protective effects in UbMi-CK-deficient mice. In the present experiments, we therefore examined whether creatine protects against MPTP neurotoxicity in UbMi-CK-deficient mice.

Materials and methods

Experimental animals

Our experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of experimental animals. UbMi-CK knockout mice were generated as described (Steeghs et al., 1995). Briefly, a 7 kbp Sst1 DNA fragment was isolated from a genomic lambda FIX™ II phage library of mouse F1 hybrid [CBAxC57Bl/6] spleen DNA. The complete 4.8 kbp murine UbCKmit gene is encompassed within this genomic region. Starting from this subclone, a replacement-type vector for homologous recombination was constructed. From this DNA, the 0.6 kbp *Bam*HI–*Bgl*II fragment was deleted and replaced by a 1.1 kbp neomycin resistance (neo^r) cassette. This vector was introduced into the wild-type E14 ES cells by electroporation. E14 clones that were positive for homologous recombination were injected into recipient C57Bl/6 blastocysts and transferred into the uterus of pseudopregnant (C57Bl/6xCBA)F1 females. Resulting chimeric males were mated with C57Bl/6 females for 12 generations. Mice heterozygous for mutant UbMi-CK allele were intercrossed and null mutants were obtained. The mice were bred locally.

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Isolated mitochondria measurements

Nonsynaptic mouse forebrain mitochondria were isolated by the Percoll gradient separation method as described (Sims, 1990). For each experiment, brains of two mice of each type were pooled. The isolation procedure was performed simultaneously and strictly in parallel with both wild-type and UbMi-CK-deficient mouse forebrain tissue. Animals of approximately 12 months of age were used. Membrane potential of isolated mitochondria was estimated using the fluorescence of safranine O (2.5 μM) with excitation and emission wavelengths of 495 and 586 nm, respectively (Starkov et al., 2002). Changes in the extramitochondrial medium-free Ca^{2+} were followed using the fluorescence of Calcium Green 5N (“Molecular Probes”, USA) with excitation and emission wavelengths of 506 and 532 nm, respectively. Mitochondrial swelling was estimated by a conventional method, from changes in light scattering of mitochondrial suspension using a fluorimeter set at 660 nm excitation and emission wavelengths with 10:5 nm excitation/emission slits and an 8% transmittance filter set over the photomultiplier window. For all fluorescence measurements, a Hitachi F4500 fluorescent spectrometer equipped with a water-thermostated cuvette holder and a stirrer was utilized.

Dopamine measurement

Control and UbMi-CK knockout mice were fed lab chow diets supplemented with 2% creatine, or a standard unsupplemented diet for 2 weeks before MPTP administration. MPTP (20 mg/kg, 5 ml/kg, i.p.) was administered four times at 2-h intervals to control, heterozygous, and homozygous knockout mice ($n = 12$/group). The animals were killed at 1 week, and both striata were rapidly dissected on a chilled glass plate and frozen at −70 °C. The samples were subsequently thawed in 0.4 ml of chilled 0.1 M perchloric acid and sonicated. Aliquots were taken for protein quantification using a spectrophotometric assay. Other aliquots were centrifuged, and dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were measured in supernatants by HPLC with electrochemical detection. Concentrations of dopamine and metabolites were expressed as ng/mg protein (mean ± SEM).

MPP^+ levels

To determine whether MPTP uptake or metabolism was altered, MPTP 20 mg/kg was administered intraperitoneally four times, 2 h apart, and mice were killed 90 min after the last injection ($n = 6$/group). MPP^+ levels were quantified by HPLC with UV detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid, and an aliquot of supernatant was injected onto a Brownlee aquapore X03-224 cation exchange column (Rainin, Woburn, MA). Samples were eluted isocratically with 90% 0.1 M acetic acid and 75 mM triethylamine HCl, pH 2.3, adjusted with formic acid and 10% acetonitrile.

Measurements of creatine, phosphocreatine, and ATP

Wild-type and homozygous Mi-CK knockout mice (10 mice per group) fed 2% creatine for 2 weeks were killed by the freeze-clamp procedure for measurements of creatine, phosphocreatine, ADP, and ATP in cerebral cortex as previously described (Matthews et al., 1998).

Histological analysis

Control and homozygous UbMi-CK knockout mice were fed lab chow diets supplemented with 2% creatine, or a standard unsupplemented diet for 2 weeks before MPTP administration. MPTP (20 mg/kg, 5 ml/kg, i.p.) was administered three times at 2-h intervals to control, heterozygous, and homozygous knockout mice ($n = 6$/group). After 1 week, the mice were deeply anesthetized with pentobarbital and perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate-buffered saline, pH 7.4. Brains were removed, postfixed for 2 h in the same fixative, and then placed in 30% sucrose overnight at 4 °C. Serial coronal sections (50 μm) were cut through the substantia nigra. Two sets consisting of eight sections each, 100 μm apart, were prepared. One set of sections was used for Nissl staining (cresyl violet). Another set was processed for tyrosine hydroxylase (TH) immunohistochemistry using avidin–biotin peroxidase technique. Briefly, free-floating sections were pretreated with 3% H_2O_2 in PBS for 30 min. The sections were incubated sequentially in (a) 1% bovine serum albumin (BSA)/0.2% Triton X-100 for 30 min, (b) rabbit anti-TH affinity purified antibody (Chemicon, Temecula, CA; 1:2000 in PBS/0.5% BSA) for 18 h, (c)

Table 1
Number of neurons in the substantia nigra pars compacta in mitochondrial creatine kinase knockout and control mice with or without MPTP treatment

Experimental group	Tyrosine hydroxylase	Nissl
Control/normal diet/PBS	9151 ± 460	10629 ± 396
Control/normal diet/MPTP	6326 ± 540**	6903 ± 265***
Control/creatine/MPTP	8085 ± 424$^{\#}$	9639 ± 547$^{\#\#\#}$
Knockout/normal diet/PBS	9305 ± 761	10848 ± 606
Knockout/normal diet/MPTP	6285 ± 134^{xx}	6778 ± 269^{xxx}
Knockout/creatine/MPTP	8052 ± 485^{+}	9512 ± 460^{++}

Creatine attenuates MPTP-induced neurodegeneration in the SNpc of wild-type controls and Mi-CK knockout mice. Neuronal counts (means ± SEM) were made using the optical fractionator method.
** $P < 0.01$, vs. control/normal diet/PBS.
*** $P < 0.001$, vs. control/normal diet/PBS.
$^{\#}$ $P < 0.05$, vs. control/normal diet/MPTP.
$^{\#\#\#}$ $P < 0.001$, vs. control/normal diet/MPTP.
^{xx} $P < 0.01$, vs. knockout/normal diet/PBS.
^{xxx} $P < 0.001$, vs. knockout/normal diet/PBS.
$^{+}$ $P < 0.05$, vs. knockout/normal diet/MPTP.
$^{++}$ $P < 0.01$, vs. knockout/normal diet/MPTP.

biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:500 in PBS/0.5% BSA) for 1 h, and (d) avidin–biotin–peroxidase complex (Vector; 1:500 in PBS) for 1 h. The immunoreaction was visualized using 3,3-diaminobenzidine tetrahydrochloride dihydrate (DAB) with nickel intensification (Vector) as the chromogen. All incubations and rinses were performed with agitation using an orbital shaker at room temperature. The sections were mounted onto gelatin-coated slides, dehydrated, cleared in xylene, and coverslipped. The numbers of Nissl-stained or TH-immunoreactive cells in the substantia nigra pars compacta (SNpc) were counted using the optical fractionator method in the Stereo Investigator (v. 4.35) software program (Microbrightfield, Burlington, VT).

Statistical analysis

Results are expressed as the mean ± SEM. Statistical comparisons were made using one-way ANOVA followed by Newman–Keuls post hoc tests, or two-way ANOVA for the data in Table 1.

Results

Brain mitochondria isolated from either wild-type or UbMi-CK-deficient mice were incubated in high ionic strength cytosol-mimicking medium and challenged with repeated pulses of 100 μM Ca^{2+}, and their responses were compared by several parameters relevant to Ca^{2+}-induced damage and the mPTP. Fig. 1A demonstrates that both types of mitochondria possess virtually identical membrane potential. After addition of ADP, the membrane potential transiently decreases in both types of mitochondria indicating the onset of oxidative phosphorylation (state 3). As shown in Fig. 1A, both the amplitude and the duration of the

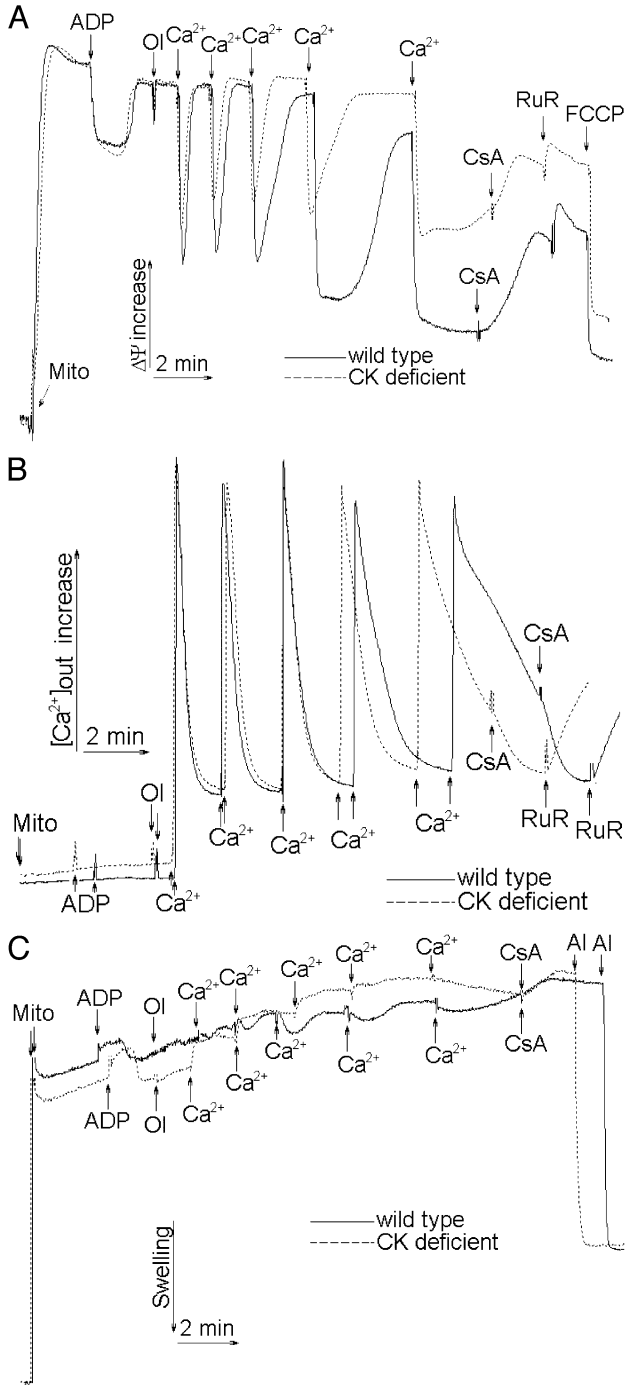

Fig. 1. Ca^{2+}-induced changes in membrane potential, Ca^{2+} uptake, and swelling of mouse brain mitochondria. Incubation medium was composed of 125 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 13.6 mM NaCl, 20 mM HEPES (pH 7.2), 0.2 mg/ml fatty-acid-free bovine serum albumin, 5 μM EGTA, 10 mM potassium succinate, and 1 μM rotenone; $t = 37\,^{\circ}C$. In A, the incubation medium was supplemented with 2.5 μM safranine O. In C, the incubation medium was supplemented with 0.1 μM Calcium Green 5N. Mitochondria were added at 0.125 mg/ml. (A) Membrane potential of mouse brain mitochondria estimated from safranine O fluorescence quenching as described in Materials and methods. Please note that the tracings are inverted upside down for the sake of visual consistency. (B) Ca^{2+} uptake in mitochondria estimated from changes in external Ca^{2+} concentration measured as described in Materials and methods, and (C) the swelling of mitochondria. Additions: Mito, mouse brain mitochondria; ADP, 100 μM ADP; Ol, 1 μg/ml oligomycin; Ca^{2+}, 100 μM $CaCl_2$; CsA, 1.1 μM cyclosporine A; RuR, 0.4 μM Ruthenium Red; FCCP, 100 nM FCCP; Al, 80 μg/ml alamethicin A. All tracings were produced in a single experiment representative of five experiments performed.

decrease in membrane potential are virtually identical in both types of mitochondria reflecting an equal efficiency of the oxidative phosphorylation system (mtATPase, ADP/ATP translocator, and Pi transporter) and energy-producing system (substrate transporters and mitochondrial respiratory chain). It is well known that change in the membrane potential is one of the most sensitive parameters reflecting the bioenergetic qualities of mitochondria (Beatrice et al., 1980; Jensen et al., 1986; Petit et al., 1990; Zanotti and Azzone, 1980). Fig. 1A indicates that in the absence of added Ca^{2+}, the bioenergetics of UbMi-CK-deficient mitochondria are not different from those of wild-type mitochondria. Addition of Ca^{2+} induced a transient decrease in membrane potential followed by its spontaneous restoration to the initial level reflecting the transport of Ca^{2+} into mitochondria that is an energy-hungry membrane potential dependent process (Gunter and Gunter, 2001; Gunter et al., 1994) (please note that an amplitude of a deflection of tracings immediately after an addition does not bear any relevant information as it merely reflects the insertion and removal of a pipettor's tip into a cuvette). Fig. 1A shows that up to the third addition of Ca^{2+}, there was no difference in membrane potential changes in both types of mitochondria. However, after the third Ca^{2+} pulse, a difference became apparent and consisted of a delayed restoration of membrane potential in wild-type mitochondria as compared to UbMi-CK mitochondria. After the fifth Ca^{2+} addition, membrane potential failed to spontaneously restore in wild-type mitochondria whereas the restoration was significantly delayed in UbMi-CK mitochondria. A specific inhibitor of MPTP cyclosporine A (Fournier et al., 1987; Zoratti and Szabo, 1995) promoted a partial restoration of membrane potential in both types of mitochondria indicating an activation of mPTP. The restoration of membrane potential was incomplete and was not further promoted by Ruthenium Red, a specific inhibitor of the mitochondrial Ca^{2+} uniporter (Gunter and Gunter, 2001; Gunter et al., 1994). This indicates that incomplete restoration of the membrane potential was not due to Ca^{2+}-induced Ca^{2+}-recycling (Gunter and Gunter, 2001; Gunter et al., 1994). Overall, these data imply that there is no significant difference between wild-type and UbMi-CK mitochondria in handling moderate amounts of Ca^{2+} (up to 2400 nmol/mg protein) whereas higher amounts (4000 nmol/mg protein) of Ca^{2+} induced permanent damage to bioenergetics, which was similar in both types of mitochondria (Fig. 1A). However, UbMi-CK mitochondria appear to be slightly more resistant to massive Ca^{2+} loading, since they demonstrate better restoration of membrane potential after the fifth Ca^{2+} addition.

The changes in membrane potential were paralleled by Ca^{2+} fluxes as shown in Fig. 1B. This figure shows that mitochondria efficiently and rapidly remove exogenously added Ca^{2+} upon moderate loading. At higher Ca^{2+} loading, mitochondrial Ca^{2+} uptake became much slower whereas the mPTP inhibitor cyclosporine A promoted the uptake (Fig. 1B). Ruthenium Red stimulated the release of Ca^{2+} back into the incubation medium. Once again, it is clearly seen that UbMi-CK mitochondria exerted relatively better handling of a massive Ca^{2+} load than wild-type mitochondria.

Opening of mPTP is frequently associated with a large amplitude changes in light scattering of a mitochondrial suspension, which is caused by swelling of the mitochondrial matrix, and therefore represents a convenient way of assessing mPTP activation in vitro (Beatrice et al., 1980; Zoratti and Szabo, 1995). Fig. 1C shows that addition of ADP induced a transient increase in light scattering of mitochondria that correlated with changes in the membrane potential (Fig. 1A). These changes are most likely related to the well-known effect of changes in the mitochondrial matrix aggregation state induced by oxidative phosphorylation (so-called "orthodox" to "condensed" state transition) (Hackenbrock, 1981; Mannella et al., 2001). Ca^{2+} additions did not induce any appreciable swelling in either type of mitochondria. Instead, Ca^{2+} caused a moderate increase in light scattering. This is a well-known and frequently observed phenomenon (Andreyev and Fiskum, 1999; Zoratti and Szabo, 1995) that most likely reflects changes in matrix density of mitochondria induced by accumulation of Ca^{2+}-phosphate precipitate (Kristian et al., 2002). However, a slow swelling-like response was observed after the fifth Ca^{2+} addition in both types of mitochondria, which was always blocked and reversed by cyclosporin A. Although a similar slow decrease in light scatter-

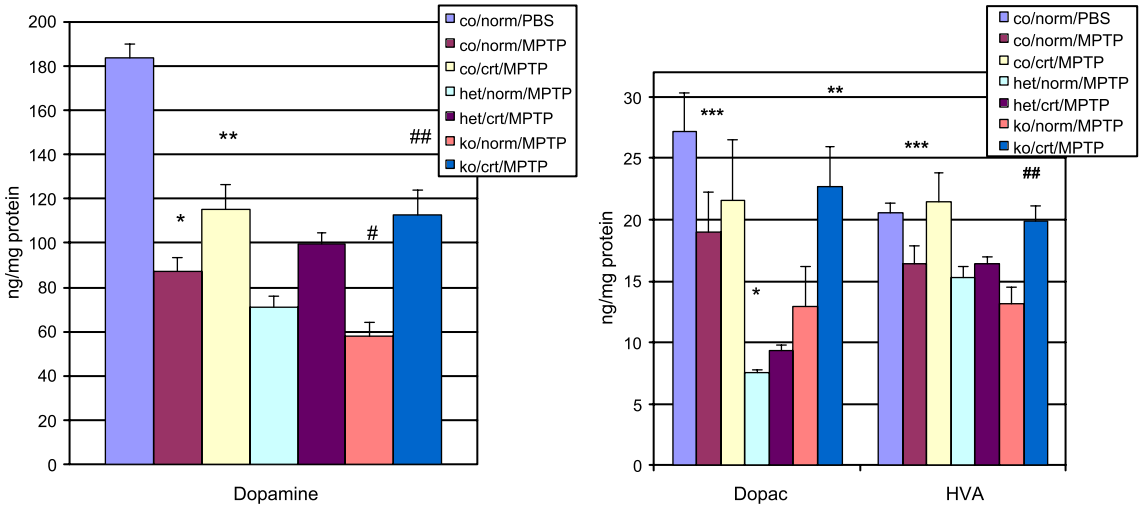

Fig. 2. Effects of MPTP on dopamine, DOPAC, and HVA in wild-type control, UbMi-CK heterozygous knockout, and homozygous knockout mice. *$P <$ 0.001, as compared to PBS-treated animals; **$P < 0.05$, as compared to MPTP-treated wild-type mice, ***$P < 0.05$, as compared to PBS-treated wild-type mice, #$P < 0.05$, as compared to MPTP-treated wild-type mice, ##$P < 0.002$, as compared to MPTP-treated homozygous knockout animals.

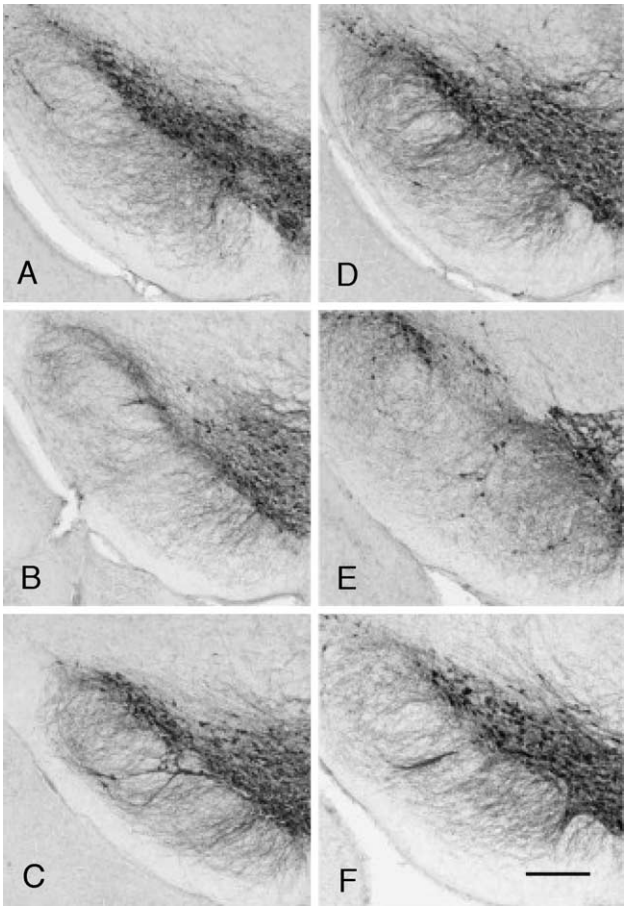

Fig. 3. Photomicrographs of TH-immunostained sections through the substantia nigra pars compacta of wild-type controls (A–C) and UbMi-CK knockout mice (D–F) showing TH-positive neurons following treatment with PBS (A, D), MPTP (B, E), and MPTP and dietary creatine (C, F). Scale bar, 200 μm.

ing was reproducibly observed in all experiments, the amplitude and the velocity of that swelling were inconsistent and varied in both types of mitochondria. As expected, an addition of a pore-forming peptide alamethicin induced similar large amplitude swelling of both types of mitochondria thus indicating their equal ability to swell (Sansom, 1993). Similar findings were made with a four-channel respiration system (Zhu et al., 2002).

The effects of administration of MPTP in wild-type controls and UbMi-CK knockout mice are shown in Fig. 2. The dose of MPTP we used (4 × 20 mg/kg) produced a significant dopamine depletion of 52% in wild-type controls on unsupplemented diet ($P < 0.001$). The same dose of MPTP produced a 61% depletion of dopamine in heterozygous UbMi-CK knockout mice that was not statistically significant as compared to MPTP-treated wild-type mice, and a significant 70% dopamine depletion in homozygous UbMi-CK knockout mice ($P < 0.05$). Supplementation with 2% creatine produced significant protection against MPTP-induced depletion of dopamine both in wild-type mice (38% protection) and in homozygous UbMi-CK knockout mice (98% protection; $P < 0.05$ and $P < 0.001$, respectively). This dose of MPTP produced a significant depletion of HVA in wild-type controls. There was a trend toward decreased levels of HVA in the UbMi-CK knockout mice on an unsupplemented diet, but this did not reach statistical significance. Creatine supplementation produced significant protection against MPTP-induced depletion of HVA in both the wild-type mice as well as in the homozygous UbMi-CK knockout mice ($P < 0.05$ and $P < 0.001$, respectively). The increased sensitivity to MPTP was not caused by an alteration in metabolism of MPTP to MPP^+ because striatal MPP^+ levels did not significantly differ between the wild-type and the UbMi-CK knockout mice at 90 min after MPTP administration (controls, 96.7 ± 17.1 ng/mg protein; UbMi-CK knockout, 109.6 ± 20.8 ng/mg protein).

Creatine significantly attenuated the MPTP-induced neurodegeneration in both the wild-type controls and UbMi-CK knockout mice. In wild-type controls, MPTP significantly reduced the numbers of TH-immunostained and Nissl-stained neurons in the substantia nigra pars compacta (SNpc) compared with PBS-treated mice by 31% ($P < 0.01$) and 35% ($P < 0.001$), respectively (Table 1, Fig. 3). In UbMi-CK knockout mice, MPTP produced a 32% reduction of TH-positive neurons ($P < 0.01$, vs. PBS) and 38% decrease ($P < 0.001$, vs. PBS) in the number of Nissl-stained neurons in SNpc. In MPTP-treated wild-type controls, 2% dietary creatine treatment significantly increased the numbers of surviving TH-positive neurons ($P < 0.05$) and Nissl-stained cells ($P < 0.001$) compared with the unsupplemented groups. In UbMi-CK knockout mice, creatine

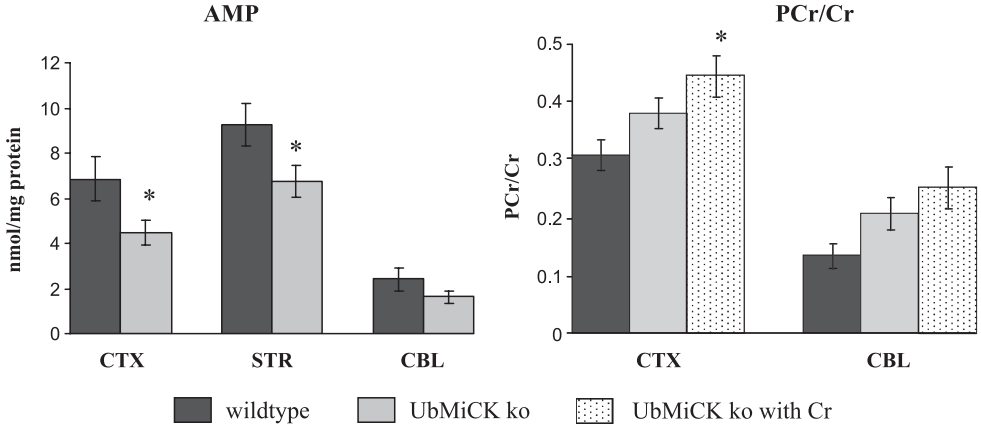

Fig. 4. AMP levels and effects of creatine supplementation for 2 weeks on PCr/Cr concentrations in wild-type (wt) and UbMi-CK-deficient mice. PCr/Cr concentrations were significantly increased in the UbMi-CK mice after creatine supplementation (*$P < 0.001$).

supplementation significantly attenuated the MPTP-induced loss of TH-immunoreactive neurons ($P < 0.05$) and Nissl-stained cells ($P < 0.01$) to a comparable extent.

The effects of creatine supplementation on PCr/Cr levels in cerebral cortex and cerebellum are shown in Fig. 4. There were no significant changes in ADP, ATP in the wild-type mice as compared to UbMi-CK-deficient mice at baseline (data not shown). AMP levels were significantly reduced in both cerebral cortex and striatum of UbMi-CK-deficient mice. Creatine supplementation significantly increased concentrations of creatine and PCr in cerebral cortex of UbMi-CK-deficient mice, and there was a similar trend in cerebellum.

Discussion

Mi-CK is part of a complex of proteins that form an efficient, tightly coupled multienzyme energy channel, which generates and transports PCr to the cytoplasm. The creatine transporter has recently been localized to the inner mitochondrial membrane and there is evidence that there may be subcellular compartmentalization of Cr/PCr pools that are not fully in equilibrium (Walzel et al., 2002a,b). Substantial evidence supports a direct functional coupling of creatine kinase with Na^+/K^+ ATPase, neurotransmitter release, and in maintenance and restoration of ion gradients before and after depolarization (Dunant et al., 1988; Hemmer and Wallimann, 1993). High energy turnover and high creatine kinase concentrations are found in brain regions that are rich in synaptic connections (Kaldis et al., 1996) and creatine kinase flux correlates with brain activity as measured by EEG, as well as the amount of 2-deoxyglucose found in the brain (Sauter and Rudin, 1993). Creatine kinase appears to be coupled directly or indirectly to energetic processes required for calcium homeostasis (de Groof et al., 2002; Steeghs et al., 1997; Wallimann et al., 1992). PCr is essential for normal calcium regulation by the sarcoplasmic reticulum (de Groof et al., 2002; Yang and Steele, 2002). Creatine pretreatment delays increases in intracellular calcium produced by 3-nitropropionic acid (3-NP) in astrocytes in vitro (Deshpande et al., 1997).

There is an extensive literature showing neuroprotective effects of creatine both in vitro and in vivo. Creatine increases PCr levels in cultured neurons and protects against glutamate, 3-NP, and β-amyloid toxicity (Brewer and Wallimann, 2000; Brustovetsky et al., 2001). In hippocampal slices, creatine administration increased PCr levels, delayed synaptic failure, and ameliorated neuronal damage produced by anoxia (Carter et al., 1995; Sharov et al., 1987; Whittingham and Lipton, 1981). Creatine administration to rat pups for 3 days increased brain PCr levels, decreased hypoxia-induced seizures and death, and enhanced PCr and ATP recoveries after hypoxia (Holtzman et al., 1998).

We found that creatine administration increased brain PCr concentrations and produced significant neuroprotection against striatal lesions produced by the mitochondrial toxins 3-nitropropionic acid and malonate (Matthews et al., 1998), and MPTP neurotoxicity (Matthews et al., 1999). Creatine significantly extended survival, improved motor performance, protected against loss of spinal cord motor neurons (Klivenyi et al., 1999), and reductions in Mi-CK activity in a transgenic mouse model of ALS (Wendt et al., 2002).

In two transgenic mouse models of Huntington's disease, creatine administration significantly improved survival, slowed the development of brain atrophy, and delayed the formation of huntingtin-positive aggregates (Andreassen et al., 2001; Ferrante et al., 2000). Using magnetic resonance spectroscopy, we showed that creatine administration increased brain creatine levels 21.3% and delayed decreases in *N*-acetylaspartate concentrations (Andreassen et al., 2001; Ferrante et al., 2000). Creatine administration in a model of traumatic brain injury reduced the extent of cortical damage by 36% in mice and 50% in rats (Sullivan et al., 2000).

A potential neuroprotective mechanism of creatine is to stabilize the mPTP that is linked to both necrotic and apoptotic cell death (Bernardi et al., 1998). The mPTP is a large pore in the inner mitochondrial membrane that is activated by increases in mitochondrial calcium, free radicals, and decreases in reduced pyridine nucleotides. The mitochondrial isoform of creatine kinase (Mi-CK) is located at contact sites between the inner and outer membranes where it is associated with porin (Brdiczka et al., 1998; Schlattner et al., 2001). Mi-CK can convert intramitochondrially produced ATP to PCr directly, which then gets transported to sites of energy consumption. The mitochondrial isoform is also coupled to oxidative phosphorylation via the adenine nucleotide transporter, and creatine stimulates respiration by increasing mitochondrial ADP (Kay et al., 2000). It was suggested that creatine converts UbMi-CK from a dimer to an octomer, which then stabilizes the mPTP (Dolder et al., 2003; O'Gorman et al., 1997). Creatine prevents increases in respiration produced by Ca^{2+} with atractylate, an activator of the mPTP. Furthermore induction of the mPTP was inhibited in cortical mitochondria of rats fed with creatine and then subjected to traumatic brain injury (Sullivan et al., 2000). Studies of isolated brain mitochondria, however, found no effect of creatine on the mPTP, and creatine supplementation for 2 weeks before isolation of brain mitochondria also showed no effect on the MPTP (Brustovetsky et al., 2001).

An important issue therefore is how much of the neuroprotective effects of creatine can be attributed to inhibition of the mPTP, versus maintenance of cellular PCr and ATP levels, which are critical to cellular homeostasis, synaptic transmission, and restoration of ionic gradients. We therefore examined whether creatine could exert neuroprotective effects in mice deficient in UbMi-CK. Mi-CKs are encoded by two nuclear genes. Sarcomeric Mi-CK is expressed in skeletal muscle fibers and cardiac cells, while UbMi-CK is expressed in brain, kidney, intestinal epithelial cells, smooth muscle, and sperm. Mice deficient in either isoform have been generated (Boehm et al., 1996; Steeghs et al., 1995, 1997). Surprisingly, these mice show no overt phenotypic abnormalities.

We found that mice deficient in UbMi-CK have normal ADP and ATP levels at baseline, consistent with studies using phosphorous NMR spectroscopy (Kekelidze et al., 2001). Phosphorous NMR studies showed no differences in brain PCr levels in UbMi-CK knockout mice compared to wild-type mice (Kekelidze et al., 2001). We found that the UbMi-CK mice have reduced AMP levels suggesting that increased adenylate kinase activity may contribute to maintaining ATP levels. The UbMi-CK-deficient mice showed a greater depletion of dopamine than wild-type mice after MPTP administration, suggesting that under conditions that stress mitochondria, compensation is not fully adequate. Similarly, they show greater seizure-induced decreases in PCr and ATP (Kekelidze et al., 2001).

In the present study, isolated brain mitochondria from UbMi-CK-deficient mice did not show any abnormalities in calcium uptake, capacity, and retention, membrane potential, or mitochon-

drial matrix volume changes in response to a calcium challenge. Differences between wild-type and UbMi-CK mitochondria were insignificant and fall within the typical variability of experiments with isolated mitochondria. Overall, these data strongly suggest the absence of any alterations to Ca^{2+}-dependent mPTP regulation or elevated sensitivity to Ca^{2+}-induced damage inherently present in mitochondria isolated from UbMi-CK-deficient mice.

If the neuroprotective effects of creatine are due to an interaction of creatine with UbMi-CK to prevent the MPTP, one would expect that creatine would not be neuroprotective in the brains of mice deficient in the UbMi-CK. In the present experiments, creatine administration produced significant protection against MPTP neurotoxicity in UbMi-CK-deficient mice as assessed biochemically and histologically. The neuroprotective effects were comparable to those seen in wild-type mice. Furthermore creatine administration significantly increased brain concentrations of both creatine and PCr. These results strongly suggest that the neuroprotective effects of creatine are not mediated by an effect on UbMi-CK to inhibit the mPTP.

Our results are congruent with the recent studies of Brustovetsky et al. (2001) who found that creatine had no significant effects on the mPTP of isolated brain mitochondria, or in brain mitochondria isolated from rats fed with 2% creatine for 2 weeks. These results suggest that the primary neuroprotective effects of creatine are likely to be mediated by maintenance of brain PCr and ATP levels. Creatine administration increases both skeletal muscle and brain PCr levels in man (Dechent et al., 1999; Wiedermann et al., 2001) and it improves exercise performance (Jacobs et al., 2002; Jones et al., 2002). These and other findings suggest that creatine administration is a promising strategy for the treatment of neurodegenerative diseases (Tarnopolsky and Beal, 2001).

Acknowledgments

The secretarial assistance of Sharon Melanson and Greta Strong is gratefully acknowledged. Dr. Boris Krasnikov is thanked for assistance in analyzing data from the four-channel system. This work was supported by grants from NINDS grant NS39258, NIA grants AG20729 and AG12992, The Department of Defense and The Parkinson's Disease Foundation, The Hereditary Disease Foundation, the Huntington's Disease Society of America, and NIA AG14390 [Director Blass, Projects 2 (PI Kristal) and 5 (PI Beal)].

References

Andreassen, O.A., Dedeoglu, A., Ferrante, R.J., Jenkins, B.G., Ferrante, K.L., Thomas, M., Friedlich, A., Browne, S.E., Schilling, G., Borchelt, D.R., Hersch, S.M., Ross, C.A., Beal, M.F., 2001. Creatine increase survival and delays motor symptoms in a transgenic animal model of Huntington's disease. Neurobiol. Dis. 8, 479–491.

Andreyev, A., Fiskum, G., 1999. Calcium induced release of mitochondrial cytochrome *c* by different mechanisms selective for brain versus liver. Cell Death Differ. 6 (9), 825–832 (Sep.).

Beatrice, M.C., Palmer, J.W., Pfeiffer, D.R., 1980. The relationship between mitochondrial membrane permeability, membrane potential, and the retention of Ca2+ by mitochondria. J. Biol. Chem. 255 (18), 8663–8671 (Sep. 25).

Bernardi, P., Basso, E., Colonna, R., Costantini, P., Di Lisa, F., Eriksson, O., Fontaine, E., Forte, M., Ichas, F., Massari, S., Nicolli, A., Petronilli, V., Scorrano, L., 1998. Perspectives of the mitochondrial permeability transition. Biochim. Biophys. Acta 1365, 200–206.

Boehm, E.A., Radda, G.K., Tomlin, H., Clark, J.F., 1996. The utilization of creatine and its analogues by cytosolic and mitochondrial creatine kinase. Biochim. Biophys. Acta 1274, 119–128.

Brdiczka, D., Beutner, G., Ruck, A., Dolder, M., Wallimann, T., 1998. The molecular structure of mitochondrial contact sites. Their role in regulation of energy metabolism and permeability transition. BioFactors 8, 235–242.

Brewer, G.J., Wallimann, T.W., 2000. Protective effect of the energy precursor creatine against toxicity of glutamate and β-amyloid in rat hippocampal neurons. J. Neurochem. 74, 1968–1978.

Brustovetsky, N., Brustovetsky, T., Dubinsky, J.M., 2001. On the mechanisms of neuroprotection by creatine and phosphocreatine. J. Neurochem. 76, 425–434.

Carter, A.J., Muller, R.E., Pschorn, U., Stransky, W., 1995. Preincubation with creatine enhances levels of creatine phosphate and prevents anoxic damage in rat hippocampal slices. J. Neurochem. 64, 2691–2699.

Dechent, P., Pouwels, P.J., Wilken, B., Hanefeld, F., Frahm, J., 1999. Increase of total creatine in human brain after oral supplementation of creatine-monohydrate. Am. J. Physiol. 277, R698–R704.

de Groof, A.J.C., Fransen, J.A.M., Errington, R.J., Willems, P.H.G.M., Wieringa, B., Koopman, W.J.H., 2002. The creatine kinase system is essential for optimal refill of the sarcoplasmic reticulum Ca^{2+} store in skeletal muscle. J. Biol. Chem. 277, 5275–5284.

Deshpande, S.B., Fukuda, A., Nishino, H., 1997. 3-Nitropropionic acid increases the intracellular Ca^{2+} in cultured astrocytes by reverse operation of the Na^{+}–Ca^{2+} exchanger. Exp. Neurol. 145, 38–45.

Dolder, M., Walzel, B., Speer, O., Schlattner, U., Wallimann, T., 2003. Inhibition of the mitochondrial permeability transition by creatine kinase substrates. J. Biol. Chem. 278, 17760–17766.

Dunant, Y., Loctin, F., Marsal, J., Muller, D., Parducz, A., Rabasseda, X., 1988. Energy metabolism and quantal acetylcholine release: effects of botulinum toxin, 1-fluoro-2,4-dinitrobenzene, and diamide in the *Torpedo* electric organ. J. Neurochem. 50, 431–439.

Ferrante, R.J., Andreassen, O.A., Jenkins, B.G., Dedeoglu, A., Kuemmerle, S., Kubilus, J.K., Kaddurah-Daouk, R., Hersch, S.M., Beal, M.F., 2000. Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. J. Neurosci. 20, 4389–4397.

Fournier, N., Ducet, G., Crevat, A., 1987. Action of cyclosporine on mitochondrial calcium fluxes. J. Bioenerg. Biomembr. 19 (3), 297–303 (Jun.).

Gunter, T.E., Gunter, K.K., 2001. Uptake of calcium by mitochondria: transport and possible function. IUBMB Life 52 (3–5), 197–204 (Sep.–Nov.).

Gunter, T.E., Gunter, K.K., Sheu, S.S., Gavin, C.E., 1994. Mitochondrial calcium transport: physiological and pathological relevance. Am. J. Physiol. 267 (2 Pt 1), C313–C339 (Aug.).

Hackenbrock, C.R., 1981. Energy-linked condensed-orthodox ultrastructural transformations in mitochondria. Chemotherapy 27 (Suppl. 2), 21–26.

Hemmer, W., Wallimann, T., 1993. Functional aspects of creatine kinase in brain. Dev. Neurosci. 15, 249–260.

Holtzman, D., Brown, M., O'Gorman, E., Allred, E., Wallimann, T., 1998. Brain ATP metabolism in hypoxia resistant mice fed guanidinopropionic acid. Dev. Neurosci. 20, 469–477.

Ikeda, K., Iwasaki, Y., Kinoshita, M., 2000. Oral administration of creatine monohydrate retards progression of motor neuron disease in the wobbler mouse. Amyotroph. Lateral Scler. Other Mot. Neuron Disord. 1, 207–212.

Jacobs, P.L., Mahoney, E.T., Cohn, K.A., Sheradsky, L.F., Green, B.A., 2002. Oral creatine supplementation enhances upper extremity work capacity in persons with cervical-level spinal cord injury. Arch. Phys. Med. Rehabil. 83, 19–23.

Jensen, B.D., Gunter, K.K., Gunter, T.E., 1986. The efficiencies of the component steps of oxidative phosphorylation: II. Experimental determination of the efficiencies in mitochondria and examination of the

equivalence of membrane potential and pH gradient in phosphorylation. Arch. Biochem. Biophys. 248 (1), 305–323 (Jul.).

Jones, A.M., Carter, H., Pringle, J.S., Campbell, I.T., 2002. Effect of creatine supplementation on oxygen uptake kinetics during submaximal cycle exercise. J. Appl. Physiol. 92, 2571–2577.

Kaldis, P., Hemmer, W., Zanolla, E., Holtzman, D., Wallimann, T., 1996. 'Hot spots' of creatine kinase localization in brain: cerebellum, hippocampus and choroid plexus. Dev. Neurosci. 18, 542–554.

Kay, L., Nicolay, K., Wieringa, B., Saks, V., Wallimann, T., 2000. Direct evidence for the control of mitochondrial respiration by mitochondrial creatine kinase in oxidative muscle cells in situ. J. Biol. Chem. 275, 6937–6944.

Kekelidze, T., Khait, I., Togliatti, A., Benzecry, J.M., Wieringa, B., Holtzman, D., 2001. Altered brain phosphocreatine and ATP regulation when mitochondrial creatine kinase is absent. J. Neurosci. Res. 66, 866–872.

Klivenyi, P., Ferrante, R.J., Matthews, R.T., Bogdanov, M.B., Klein, A.M., Andreassen, O.A., Mueller, G., Wermer, M., Kaddurah-Daouk, R., Beal, M.F., 1999. Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. Nat. Med. 5, 347–350.

Kristian, T., Weatherby, T.M., Bates, T.E., Fiskum, G., 2002. Heterogeneity of the calcium-induced permeability transition in isolated non-synaptic brain mitochondria. J. Neurochem. 83 (6), 1297–1308 (Dec.).

Lawler, J.M., Barnes, W.S., Wu, G., Song, W., Demaree, S., 2002. Direct antioxidant properties of creatine. Biochem. Biophys. Res. Commun. 290, 47–52.

Mannella, C.A., Pfeiffer, D.R., Bradshaw, P.C., Moraru I.I., Slepchenko, L.M., Loew, L.M., Hsieh, C.E., Buttle, K., Marko, M., 2001. Topology of the mitochondrial inner membrane: dynamics and bioenergetic implications. IUBMB Life 52 (3–5), 93–100 (Sep.–Nov.).

Matthews, R.T., Yang, L., Jenkins, B.G., Ferrante, R.J., Rosen, B.R., Kaddurah-Daouk, R., Beal, M.F., 1998. Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. J. Neurosci. 18, 156–163.

Matthews, R.T., Ferrante, R.J., Klivenyi, P., Yang, L., Klein, A.M., Mueller, G., Kaddurah-Daouk, R., Beal, M.F., 1999. Creatine and cyclocreatine attenuate MPTP neurotoxicity. Exp. Neurol. 157, 142–149.

O'Gorman, E., Beutner, G., Dolder, M., Koretsky, A.P., Brdiczka, D., Wallimann, T., 1997. The role of creatine kinase inhibition of mitochondrial permeability transition. FEBS Lett. 414, 253–257.

Petit, P.X., O'Connor, J.E., Grunwald, D., Brown, S.C., 1990. Analysis of the membrane potential of rat- and mouse-liver mitochondria by flow cytometry and possible applications. Eur. J. Biochem. 194 (2), 389–397 (Dec. 12).

Pulido, S.M., Passaquin, A.C., Leijendekker, W.J., Challet, C., Wallimann, T., Ruegg, U.T., 1998. Creatine supplementation improves intracellular Ca2+ handling and survival in mdx skeletal muscle cells. FEBS Lett. 439, 357–362.

Sansom, M.S., 1993. Alamethicin and related peptaibols—Model ion channels. Eur. Biophys. J. 22 (2), 105–124.

Sauter, A., Rudin, M., 1993. Determination of creatine kinase parameters in rat brain by NMR magnetization transfer: correlation with brain function. J. Biol. Chem. 268, 13166–13171.

Schlattner, U., Dolder, M., Wallimann, T., Tokarska-Schlattner, M., 2001. Mitochondrial creatine kinase and mitochondrial outer membrane porin show a direct interaction that is modulated by calcium. J. Biol. Chem. 276, 48027–48030.

Sharov, V.G., Saks, V.A., Kupriyanov, V.V., Lakomkin, V.L., Kapelko, V.I., Steinschneider, A., Javadov, S.A., 1987. Protection of ischemic myocardium by exogenous phosphocreatine: I. Morphologic and phosphorus 31-nuclear magnetic resonance studies. J. Thorac. Cardiovasc. Surg. 94, 749–761.

Sims, N.R., 1990. Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. J. Neurochem. 55, 698–707.

Starkov, A.A., Polster, B.M., Fiskum, G., 2002. Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax. J. Neurochem. 83, 220–228.

Steeghs, K., Oerlemans, F., Wieringa, B., 1995. Mice deficient in ubiquitous mitochondrial creatine kinase are viable and fertile. Biochim. Biophys. Acta 1230, 130–138.

Steeghs, K., Benders, A., Oerlemans, F., de Haan, A., Heerschap, A., Ruitenbeek, W., Jost, C., van Deursen, J., Perryman, B., Pette, D., Bruckwilder, M., Koudijs, J., Jap, P., Veerkamp, J., Wieringa, B., 1997. Altered Ca^{2+} responses in muscles with combined mitochondrial and cytosolic creatine kinase deficiencies. Cell 89, 93–103.

Sullivan, P.G., Geiger, J.D., Mattson, M., Scheff, S.W., 2000. Dietary supplement creatine protects against traumatic brain injury. Ann. Neurol. 48, 723–729.

Tarnopolsky, M.A., Beal, M.F., 2001. Potential for creatine and other therapies targeting cellular energy dysfunction in neurological disorders. Ann. Neurol. 49, 561–574.

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., Eppenberger, H.M., 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem. J. 281, 21–40.

Walzel, B., Speer, O., Boehm, E., Kristiansen, S., Chan, S., Clarke, K., Magyar, J.P., Richter, E.A., Wallimann, T., 2002a. New creatine transporter assay and identification of distinct creatine transporter isoforms in muscle. Am. J. Physiol.: Endocrinol. Metab. 283, E390–E401.

Walzel, B., Speer, O., Zanolla, E., Eriksson, O., Bernardi, P., Wallimann, T., 2002b. Novel mitochondrial creatine transport activity: implications for intracellular creatine compartments and bioenergetics. J. Biol. Chem. 277, 37503–37511.

Wendt, S., Dedeoglu, A., Speer, O., Wallimann, T., Beal, M.F., Andreassen, O.A., 2002. Reduced creatine kinase activity in transgenic amyotrophic lateral sclerosis mice. Free Radical Biol. Med. 32, 920–926.

Whittingham, T.S., Lipton, P., 1981. Cerebral synaptic transmission during anoxia is protected by creatine. J. Neurochem. 37, 1618–1621.

Wiedermann, D., Schneider, J., Fromme, A., Thorwesten, L., Moller, H.E., 2001. Creatine loading and resting skeletal muscle phosphocreatine flux: a saturation-transfer NMR study. MAGMA 13, 118–126.

Xu, C.J., Klunk, W.E., Kanfer, J.N., Xiong, Q., Miller, G., Pettegrew, J.W., 1996. Phosphocreatine-dependent glutamate uptake by synaptic vesicles. J. Biol. Chem. 271, 13435–13440.

Yang, Z., Steele, D.S., 2002. Effects of phosphocreatine on SR Ca(2+) regulation in isolated saponin-permeabilized rat cardiac myocytes. J. Physiol. 539, 767–777.

Zanotti, A., Azzone, G.F., 1980. Safranine as membrane potential probe in rat liver mitochondria. Arch. Biochem. Biophys. 201 (1), 255–265 (Apr. 15).

Zhu, S., Stavrovskaya, I.G., Drozda, M., Kim, B.Y.S., Ona, V., Li, M., Sarang, S., Liu, A.S., Hartley, D.M., Wu, D.C., Gullans, S., Ferrante, R.J., Przedborski, S., Kristal, B.S., Friedlander, R.M., 2002. Minocycline inhibits cytochrome *c* release and delays progression of amyotrophic lateral sclerosis in mice. Nature 417, 74–78.

Zoratti, M., Szabo, I., 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta 1241 (2), 139–176 (Jul. 17).