Published in final edited form as:

Neurobiol Dis. 2012 August ; 47(2): 184–193. doi:10.1016/j.nbd.2012.03.037.

Creatine pretreatment protects cortical axons from energy depletion *in vitro*

Hua Shen* and Mark P. Goldberg**

Hope Center for Neurological Disorders and Department of Neurology, Washington University School of Medicine, 660 S. Euclid Avenue, Saint Louis, Missouri 63110, USA

Abstract

Creatine is a natural nitrogenous guanidino compound involved in bioenergy metabolism. Although creatine has been shown to protect neurons of the central nervous system (CNS) from experimental hypoxia/ischemia, it remains unclear if creatine may also protect CNS axons, and if the potential axonal protection depends on glial cells. To evaluate the direct impact of creatine on CNS axons, cortical axons were cultured in a separate compartment from their somas and proximal neurites using a modified two-compartment culture device. Axons in the axon compartment were subjected to acute energy depletion, an *in vitro* model of white matter ischemia, by exposure to 6 mM sodium azide for 30 min in the absence of glucose and pyruvate. Energy depletion reduced axonal ATP by 65%, depolarized axonal resting potential, and damaged 75% of axons. Application of creatine (10 mM) to both compartments of the culture at 24 h prior to energy depletion significantly reduced axonal damage by 50%. In line with the role of creatine in the bioenergy metabolism, this application also alleviated the axonal ATP loss and depolarization. Inhibition of axonal depolarization by blocking sodium influx with tetrodotoxin also effectively reduced the axonal damage caused by energy depletion. Further study revealed that the creatine effect was independent of glial cells, as axonal protection was sustained even when creatine was applied only to the axon compartment (free from somas and glial cells) for as little as 2 h. In contrast, application of creatine after energy depletion did not protect axons. The data provide the first evidence that creatine pretreatment may directly protect CNS axons from energy deficiency.

Keywords

creatine; energy depletion; ischemia; axonal injury; ATP; white matter; compartmental culture

Introduction

The mammalian brain has high and variable energy demands but low reserves and is vulnerable to the hypoxic/ischemic injury that deprives energy. Creatine (N-aminoiminomethyl-N-methylglycine) is a natural guanidino compound involved in

© 2012 Elsevier Inc. All rights reserved.

*Corresponding author. Current address: Department of Orthopaedic Surgery, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8233, Saint Louis, Missouri 63110, USA. Fax: 314-362-0334. shenhu@wudosis.wustl.edu.

**Current address: Department of Neurology and Neurotherapeutics, UT Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas TX 75390. mark.goldberg@utsouthwestern.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

bioenergy metabolism via creatine kinase (CK). CK catalyzes the reversible reaction that converts creatine and ATP into phosphocreatine (PCr) and ADP. PCr is generated at the sites of ATP production, and then diffuses to and accumulates at the sites of ATP consumption, where it reproduces ATP via localized CK independent of oxygen and glucose. Because creatine via CK/PCr system provides a read-to-release high energy phosphate pool at the sites of energy consumption, it has been proposed that creatine may not only play a particular essential role in brain energy transfer and buffering under normal conditions, but also replenish brain ATP during hypoxia/ischemia and therefore reduce brain injury (for reviews, see Adhihetty and Beal, 2008; Andres *et al*, 2008; Perasso *et al*, 2011; Wallimann *et al*, 1992; Wyss and Kaddurah-Daouk, 2000).

In agreement with this proposal, earlier *in vitro* studies have demonstrated the neuroprotective effect of creatine pretreatment against anoxic/hypoxic damage (Balestrino *et al*, 1999; Carter *et al*, 1995; Lipton and Whittingham, 1982; Luhmann and Heinemann, 1992) and oxygen and glucose deprivation (Okada and Yoneda, 1983) in adult brain slices as well as isolated CNS neurons (Zapara *et al*, 2004). The creatine mediated neuroprotection is creatine transporter dependent (Lunardi *et al*, 2006) and coupled with increased levels of PCr, reduced rates of ATP reduction (Balestrino *et al*, 1999; Balestrino *et al*, 2002; Carter *et al*, 1995; Lipton and Whittingham, 1982; Okada and Yoneda, 1983), and delayed membrane depolarization (Balestrino *et al*, 1999; Zapara *et al*, 2004). Despite these convincing *in vitro* findings, the *in vivo* effects of creatine on ischemic brain injury are controversial (Prass *et al*, 2007; Wick *et al*, 1999; Zhu *et al*, 2004). This controversy may be at least in part due to the poor penetration of creatine through the blood brain barrier (Perasso *et al*, 2003). It has been reported that, when applied intracerebroventricularly, creatine is neuroprotective in experimental cerebral ischemia (Balestrino *et al*, 2002; Lensman *et al*, 2006; Rebaudo *et al*, 2000). Recently, a creatine-derived compound has been developed, which may cross the blood-brain barrier independent of the creatine transporter (Lunardi *et al*, 2006) and protect neurons from experimental stroke via peripheral administrations (Perasso *et al*, 2009). Creatine may restore ATP, increase survival, and suppress seizures in hypoxic immature animals as well as adults (Holtzman *et al*, 1998; Holtzman *et al*, 1999).

Ischemic stroke damages not only central grey matter but also white matter. White matter injury is a major cause of functional disability in cerebrovascular disease (see review, Goldberg and Ransom, 2003). Despite considerable studies on the effects of creatine on CNS neurons, its action on CNS axons remains unclear. CNS axons express the creatine transporter (Dodd *et al*, 2010); a recent study has implied that exogenous creatine might increase local ATP production in axonal terminals (Lee and Peng, 2008), thus supporting the hypothesis that creatine may protect axons from ischemic/hypoxic injury as well. Nevertheless, it has been suggested that the creatine mediated-ATP production depends on neuron-glia interaction (Tachikawa *et al*, 2004). *In vitro* experiments in brain cell reaggregated cultures further implicated a glial mechanism involved in creatine protection of axonal growth under ammonium exposure (Braissant *et al*, 2008; Braissant *et al*, 2002).

To clarify the effects of creatine on CNS axons as well as glial dependency of creatine action, we cultured cortical axons in a separate compartment from their somas and proximal neurites using a modified two-compartment culture device. Axons in the axon compartment were subjected to energy depletion (an *in vitro* model of white matter ischemia) by removal of glucose and pyruvate from the culture medium and inhibition of mitochondrial electron transport chain with sodium azide. The effects of creatine treatment on axonal ATP and structural integrity were investigated. The finding from this study provided the first evidence that creatine directly protects CNS axons from energy depletion.

Materials and methods

Reagents

All reagents were obtained from Sigma-Aldrich (Saint Louis, MO) unless otherwise noted.

Animals

C57BL/6J0laHsd mice (Harlan laboratories, Inc., Blackthorn, UK) were housed and bred at the animal facility of the Washington University School of Medicine. All experiments were conducted in accordance with the guidelines established by the National Academy of Sciences and overseen by the Animal Studies Committee at Washington University in St. Louis.

Modified two-compartment culture device

Cortical axons and their somas with proximal neurites were cultured in separate compartments using a modified two-compartment culture device (Figure 1A; Ivins *et al*, 1998; Underhill and Goldberg, 2007). The device was assembled by attaching a hemisected cylinder of Teflon tubing (10 mm in inner diameter, 5 mm in height) to a 35 mm glass bottom (glass area, 23 mm in diameter) tissue culture dish (World Precision Instruments, Inc., Sarasota, FL), precoated with 100 µg/ml poly-L-lysine and 4 µg/ml laminin (BD Biosciences, Bedford, MA), via high vacuum grease (Dow Corning, Midland, MI). The hemisected Teflon tubing was then enclosed with a #1 rectangular glass coverslip (9 × 22 mm, Bellco Glass Inc., Vineland, NJ) with vacuum grease. The inside (white area in Figure 1A) and the outside (light grey area in ure 1A) of the resulting enclosure were used as the cell and the axon compartment, respectively. The gap between the bottom edge of the coverslip and the surface of the culture dish (dark grey area in Figure 1A) was sealed with a mixture of collagen/matrigel (BD Biosciences) containing 1 mg/ml collagen, 33% matrigel, and 200 ng/ml Netrin (R&D Systems, Minneapolis, MN) in Minimal Essential Medium (pH 7.4, adjusted with 1 N NaOH), which is axon- but not cell-permeable. The assembled device was incubated for 1 h at 37 °C to allow the collagen/matrigel seal to gel. To prevent the collagen/matrigel seal from drying out during the incubation, 50 µl of neuronal culture medium containing 2% B27 (Life Technologies, Grand Island, NY) and 0.5 mM glutamine (Life Technologies) in Neurobasal Medium (Life Technologies) was added to each side of the coverslip. After the collagen/matrigel seal gelled, 1.5 ml of the neuronal culture medium was added to the axon compartment only to examine whether there was a leak between the cell and the axon compartment. The devices with fluid accumulation in the cell compartment were discarded, while those without leak were used for the subsequent compartmental culture.

Compartmental culture of cortical axons

Dissociated cortical neurons were isolated from C57Bl/6J0laHsd mice at embryonic day 15 and suspended in equal volumes of the neuronal culture medium described above. The cell suspension was then plated on the cover of a tissue culture dish as droplets (about 10 µl per drop) and cultured upside-down for about 5 h to reaggregate. The resulting cortical aggregates were cut into small pieces, 300 – 400 µm in size. Approximately 3 – 5 of such cortical aggregates were plated in the neuronal culture medium in the cell compartment of the modified two-compartment culture device described above. The fluid level in the cell compartment was set up about 3 mm higher (forming a convex meniscus) than that in the axon compartment. No significant fluid flow between the two compartments was found for at least 2 days, as no equalization of fluid level between the two compartments was detectable. Typically, neurons extended their axons into the axon compartment after 3 – 5 days *in vitro* (DIV), whereas no cells were present in the axon compartment. Every 3 – 4

days, half of the neuronal culture medium in both compartments was replaced with the axonal culture medium containing 2% B27 and 0.5 mM GlutaMAX™ (Life Technologies) in Neurobasal A medium (Life Technologies). The culture was used for experiments at 12 – 14 DIV.

Energy depletion and drug treatments

Energy depletion was achieved by temporarily exposing the axons in the axon compartment to the mitochondrial complex IV inhibitor sodium azide (6 mM) in Neurobasal A medium without glucose and pyruvate (Life Technologies) for 30 min to inhibit both oxidative phosphorylation and glycolysis. At the end of energy depletion, axons in the axon compartment were thoroughly washed three times with PBS and further cultured in the axon culture medium for either 4 h or 24 h. Sham wash and vehicle controls were included in all experiments.

For the general creatine treatment, creatine was given 24 h prior to energy depletion by replacing half of the culture medium with the fresh axon culture medium supplemented with 20 mM creatine (final concentration, 10 mM) in both cell and axon compartments. The same concentration of creatine was maintained in the culture during and after energy depletion. In local creatine pretreatment, only the axons in the axon compartment received 10 mM creatine either before or after energy depletion as illustrated in Figure 6A.

To prevent axonal depolarization caused by sodium influx via tetrodotoxin (TTX)-sensitive sodium channels during energy depletion, axons in the axon compartment were treated with TTX (1.5 μ M) from 10 min prior until the end of energy depletion. This concentration was chosen for sufficient inhibition of axonal depolarization. The 10 min pretreatment was used to ensure that TTX was at the site of action before axons were depolarized.

Immunostaining and quantification of axonal injury

At the end of experiments, the axon culture was fixed with 2.56% paraformaldehyde and 0.1% glutaraldehyde in a cytoskeleton stabilization buffer containing 20 mM PIPES (pH 6.5), 2.2 mM NaH₂PO₄, 0.8 mM KH₂PO₄, 254 mM NaCl, 10 mM KCl, 4 mM MgCl₂, 11 mM Glucose, and 2 mM EGTA at 37°C for 15 min. The culture was then washed twice with TBS buffer containing 100 mM Tris-HCl (pH 7.4) and 150 mM NaCl, followed by two washes with PBS. After permeabilization with 0.125% Triton X-100 in PBS for 10 min, the axons in the axon compartment were treated with 10% normal goat serum in PBST (0.05% Triton X-100 in PBS) for 30 min and stained with Tuj1 monoclonal antibodies (1:1000 dilution; Covance, Emeryville, CA) at 4 °C overnight. Tuj1 recognizes type III beta-tubulin, a component of neuronal microtubule. After three washes with PBST, the axons were further incubated with Cy3-labeled goat anti-mouse antibodies (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature, and then washed three times with PBST and once with PBS. The Tuj1-stained axons in the axon compartment were imaged consecutively from the proximal cell-axon compartmental boundary to the axonal terminals with a Nikon Eclipse TE300 using a Plan Apo 10X/0.45 objective. Montages were assembled with Adobe Photoshop CS2 software (Adobe Systems Incorporated). The integrity of axons was analyzed at five defined regions of interest (ROIs) by counting the intact axons in each of the five ROIs and adding the counts together. The ROIs were defined by five parallel lines equal spaced by 0.42 mm and ranging 1.26 mm to 2.94 mm from the top of each montage (dashed lines in Figure 1A). An intact axon was defined as no beading and no fragmentation. The axon counting was performed blindly to the treatments using a multiline selection tool of MetaMorph 6.1 (Universal Imaging, West Chester, PA). The data are shown as percentage of control.

Determination of membrane potential of cortical axons

The changes in membrane potential of cortical axons ($\Delta\Psi_{\text{axon}}$) were determined with a slow voltage-sensitive anionic fluorescent dye, bis(1,3-dibutylbarbituric acid) trimethine oxonol (Dibac₄(3); Molecular Probes, Eugene, OR; Braeuner *et al*, 1984; Epps *et al*, 1994). The dye was loaded to the axons in HCSS buffer containing 12 mM HEPES (pH 7.4), 1 mM NaH₂PO₄, 140 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂, and 5.5 mM glucose at a final concentration of 1 μ M. After the axons were incubated for 1 h at room temperature on the stage of a Zeiss LSM 5 PASCAL Vario laser scanning confocal microscope equipped with a 40X/1.2 water immersion lens, the Dibac₄(3) fluorescence in these axons was excited at 488 nm with 1% power and collected via band-pass filter (505–530 nm) at 1 min intervals for 10 min at baseline, 30 min during energy depletion, and 5 min during potassium depolarization. For energy depletion, the axons in the axon compartment were washed three times with HCSS buffer without glucose (HCSS-0-glucose) and then treated with 6 mM sodium azide in HCSS-0-glucose buffer supplemented with 1 μ M Dibac₄(3) in the presence or absence of 1.5 μ M TTX for 30 min. At the end of energy depletion, the axons were washed three times with HCSS and then depolarized with 50 mM KCl. Average fluorescence intensities of Dibac₄(3) (F) were analyzed with ImageJ 1.38x software (Wayne Rasband, National Institutes of Health, USA). The changes in $\Delta\Psi_{\text{axon}}$ were evaluated as the ratio of F/F₀. F₀ denotes the average fluorescence intensities of Dibac₄(3) at baseline.

Measurement of axonal ATP

At the end of energy depletion, axons in the axon compartment were washed with PBS and immediately dissolved in 50 μ l of ice-cold lysis buffer containing 0.1 M Tris-HCl (pH 7.8), 1% Triton X-100, and 5 mM EDTA. The levels of axonal ATP were determined by luciferase and its substrate luciferin using an ATP Determination Kit (Molecular Probes) according to the manufacturer's instructions and normalized by beta-actin contents in the respective samples. The beta-actin content in each axon lysate was determined by the dot-blot method with monoclonal anti-beta-actin antibodies (1:1000 dilution) followed by Peroxidase-conjugated anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories). Recombinant beta-actin (ProSci Incorporated, Poway, CA) was used in each blot to determine the standard curve.

Statistics

All data were collected from at least three independent experiments unless otherwise specified and are shown as mean $\pm$ SEM. Statistics was performed with the Student's t-tests for the data with a normal distribution and the Mann-Whitney rank sum tests for the data with a nonnormal distribution using SigmaStat 3.50 software (Systat Software, Inc., Chicago, IL). The area under the curve (AUC) during 30 min energy depletion was computed via the Area Below Curves macro of SigmaPlot 10.0 (Systat Software, Inc.).

Results

Compartmental culture of cortical axons

To spatially separate CNS axons from their somas and proximal neurites, a modified two-compartment culture device (Figure 1A) was established based on previous designs (Ivins *et al*, 1998; Underhill and Goldberg, 2007). With this device, the somas of cortical neurons together with their proximal neurites were restrained in the cell compartment (white area in Figure 1A), while their axons were allowed to penetrate through the collagen/matrigel seal (dark grey area in Figure 1A) and grow in the axon compartment (grey area in Figure 1A). Several modifications were made to improve the axon growth in the axon compartment, which included replacing the CNS axon-impermeable vacuum grease with the CNS axon-

permeable collagen/matrigel as the seal of the compartmental barrier, adding the axon attractant Netrin to the collagen/matrigel seal, and plating larger size cortical aggregates instead of microexplants in the cell compartment. Compared with the previous design (Figure 1, Underhill and Goldberg, 2007), these modifications dramatically increased the number of axons in the axon compartment by 10-fold and lengthened the axons from several hundred micrometers to two to three millimeters (Figure 1B). Meanwhile, the replacement of the barrier seal also effectively reduced the cell and/or fluid leak at the cell-axon compartmental boundary by 30%, a major hurdle in compartmental culture. Of note, in the modified device, cortical axons were cultured on optical quality glass instead of conventional thick polystyrene surface, which made it possible for imaging experiments required for objectives with shorter working distances and larger numerical apertures.

Creatine treatment reduces structural damage of cortical axons caused by energy depletion

To evaluate if in addition to neurons, creatine might also protect CNS axons from ischemic/hypoxic injury, cortical axons in the axon compartment were deprived of energy by temporarily inhibiting both oxidative phosphorylation and glycolysis with the complex IV inhibitor sodium azide (6mM) in the absence of glucose and pyruvate for 30 min. Sodium azide has been used in the *in vitro* models of ischemic/anoxic brain injury at concentrations in the range of 1 to 10 mM for up to 60 min (Jørgensen *et al*, 1999; Malek *et al*, 2003; Varming *et al*, 1996). We chose 30 min treatment to induce acute energy deprivation. The concentration of sodium azide used in this study was predetermined to induce irreversible axonal damage (supplemental Figure 1 and data not shown). Creatine (10 mM) was administered to both the cell and axon compartments at 24 h prior to, during, and after energy depletion. We chose to add creatine to both compartments, because this approach is more relevant to delivery of exogenous creatine *in vivo* and has broader applications. Axonal injury was evaluated by Tuj1 staining at 4 h and 24 h after energy depletion, respectively. As shown in Figure 2A, energy depletion alone damaged cortical axons. Axon beading was evident at 4 h after energy depletion. By 24 h, many axons were further severed. Only 43% of axons at 4 h and 25% of axons at 24 h after energy depletion remained intact (Figure 2B). In contrast, no or only mild axonal injury was detected in the creatine-treated axons at 4 h or 24 h after energy depletion, respectively (Figure 2A). Quantitative analysis revealed that the creatine treatment increased intact axons by 2.8-fold at 24 h after energy depletion (Figure 2B), thus dramatically reducing CNS axonal injury caused by energy depletion.

Creatine suppresses axonal depolarization caused by energy depletion

It was interesting to understand how creatine protected CNS axons from energy depletion. Previous studies have shown that oxygen and/or glucose deprivation that dissipates cellular ATP may compromise $\text{Na}^+\text{-K}^+\text{-ATPase}$ and depolarize both CNS neurons (Balestrino *et al*, 2002; Balestrino 1995) and axons (Leppanen and Stys, 1997a). A causal link between the anoxic depolarization and neuronal damage has been suggested (Balestrino *et al*, 2002). CK is both structurally and functionally associated with $\text{Na}^+\text{-K}^+\text{-ATPase}$ (for review, see Andres *et al*, 2008; Wallimann *et al*, 1992). Balestrino and coworkers have reported that creatine treatment may increase intracellular PCr, delay anoxic depolarization, and therefore prevent neuronal damage (Balestrino *et al*, 1999; Balestrino 1995). To test if this machinery is also applied to the CNS axons, the action of creatine on $\Delta\Psi_{\text{axon}}$ during energy depletion was evaluated using a slow voltage sensitive anionic fluorescent dye Dibac₄(3). The dye has a first order fluorescence response to the voltage change across the plasma membrane (Braeuner *et al*, 1984; Epps *et al*, 1994) and has been used to detect the changes in plasma membrane potential in various studies (Düssmann *et al*, 2003; Piller *et al*, 1998; Reichert *et al*, 2001). As seen in Figure 3A, in resting cortical axons, Dibac₄(3) fluorescence intensity

(DFI) was stable for at least 1 h and increased two-fold upon depolarization with 50 mM KCl (Figure 3A, $n = 31$). Energy depletion increased DFI by 1.7-fold in less than 10 min (open circles in Figure 3B, $n = 35$), and this increase persisted thereafter, indicating a sustained axonal depolarization caused by energy depletion. Interestingly, the DFI of the creatine-treated axons was also increased during the first 5 min of energy depletion at a rate similar to that of the creatine-nontreated axons, but this increase was then reversed, thus reporting a gradual repolarization response instead (filled squares in Figure 3B, $n = 41$). As a dye loading and imaging control, at the end of energy depletion, all axons were thoroughly washed and subsequently exposed to 50 mM KCl, and a depolarization response was confirmed in all axons evaluated (data not shown). Quantitative analysis revealed that the AUC for the DFI curve of the creatine-treated axons was significantly smaller than that of the creatine-nontreated axons during energy depletion ($P < 0.001$). Thus, creatine treatment effectively suppresses the axonal depolarization caused by energy depletion.

Creatine prevents axonal ATP loss caused by energy depletion

To verify if creatine improved $\Delta\Psi_{\text{axon}}$ during energy depletion via its ability to buffer cellular ATP, the levels of axonal ATP were evaluated immediately after energy depletion. As shown in Figure 4, energy depletion reduced axonal ATP by 65%. Creatine treatment alone did not alter axonal ATP level; however, when applied in combination with energy depletion, this treatment did prevent axonal ATP loss, thus supporting a causal link between the creatine-mediated energy homeostasis and membrane potential improvement.

Preventing sodium influx during energy depletion reduces axonal depolarization and structural damage

We next asked if inhibition of membrane depolarization is sufficient to protect axons from energy depletion. Given axonal depolarization caused by metabolic inhibition is largely dependent on sodium influx via TTX-sensitive sodium channels (Leppanen and Stys, 1997a), we applied TTX (1.5 μM) to the axon compartment and monitored the changes in $\Delta\Psi_{\text{axon}}$ with DIBAC₄(3). As seen in Figure 5A, the TTX treatment induced a small and transient hyperpolarization followed by a greatly suppressed depolarization during energy depletion (comparing the DFI curve denoted by open circles in Figure 3B; AUC, $P = < 0.001$; $n = 35$). Subsequent evaluation of axonal structure with Tuj1 staining at 24 h after energy depletion confirmed that the TTX treatment also significantly reduced axonal injury by 45% (Figure 5B and 5C). Thus, retaining membrane potential is adequate for reducing axonal damage caused by energy depletion.

Local creatine pretreatment but not post-treatment protects axons from energy depletion

Axonal degeneration has been found as an autonomous process (Finn *et al*, 2000; Ikegami and Koike, 2003; Perry *et al*, 1990; Wang *et al*, 2005). In the human CNS, axons may extend over one meter away from their somas, and neurite ATP can be synthesized locally and independently from soma (Tolkovsky and Suidan, 1987). It would be interesting to know if the creatine-provided protection persists when administered only to the axons in the absence of somas and glial cells and if creatine needs to be applied prior to energy depletion. To answer these questions, different creatine-treatment strategies were compared as illustrated in Figure 6A. The results show that even when added exclusively to the cell-free axon compartment for as little as 2 h and then removed during and after energy depletion, creatine provided potent axonal protection against energy depletion, increasing intact axons by 2.6-fold at 24 h after energy depletion. In contrast, application of creatine to the axons in the axon compartment after energy depletion for up to 24 h failed to rescue axons (Figure 6B and 6C). Creatine treatment alone to the axons in the axon compartment for over 48 h had no influence on axon integrity and growth (Figure 6B and 6C). Thus, a local and brief creatine pretreatment but not post-treatment protects CNS axons from energy depletion.

Discussion

Using a modified two-compartment culture device, this study explored the direct effect of creatine on the cultured CNS axons exposed to energy depletion. The findings revealed that, in addition to neurons, creatine protects CNS axons as well. The axonal protection offered by creatine is preventive, independent of glial cells, and linked to the ability of creatine to buffer axonal ATP and improve membrane potential during energy depletion.

Compartmental culture of the mouse CNS axons

Several compartmental culture devices have been developed in the past to culture axons and their somas in separate compartments. The original Campenot chamber was designed for culturing rat sympathetic axons (Campenot, 1977). Unlike sympathetic axons, mouse CNS axons are typically much finer and weaker in culture and unable to cross the thick Teflon compartmental barrier in the Campenot chamber and grow into designated compartments. To solve the problem, a two-compartment culture device has been developed (Ivins *et al*, 1998; Underhill and Goldberg, 2007), which uses a thin coverslip as compartment barrier (~200 μm). The development makes it possible to culture mouse CNS axons compartmentally but yet faces some other problems including frequent leakage between compartments, limited penetration of axons through the seal of compartmental barrier, and nondirectional axon growth.

To overcome these problems, we replaced silicon grease with collagen/matrigel as the barrier seal that secures the coverslip barrier to the culture surface. The collagen/matrigel seal has several advantages over the silicon grease. First, it is applied as liquid and gels later, thus sealing better. Second, the gelled collagen/matrigel seal forms porous architecture. The pore size can be easily controlled by adjusting the concentration of collagen to allow axons but not their soma to penetrate. Third, the collagen and matrigel themselves are extracellular matrix molecules, supporting cell adhesion and axon growth on one hand (Thompson and Pelto, 1982; Tonge *et al*, 1997); on the other hand they are used to deliver the axon attractant Netrin (Kennedy *et al*, 1994), promoting directional axon growth toward the axon compartment. This together with other modifications made in this study have dramatically increased the number and length of axons in the axon compartment and reduced the occurrence of leakage between the cell and the axon compartment.

The modified two-compartment culture device is inexpensive, achievable in any biology lab, and suitable for a wide variety of studies of CNS axons *in vitro*. In addition to the axonal cytoskeleton, ATP, and membrane potential investigated in this study, the device has been successfully used in studying axon-oligodendrocyte interaction as well as imaging mitochondrial trafficking and free calcium accumulation in axons.

Jeon and colleagues recently described a microfluidic device for culturing CNS axons in micron-scale (Taylor *et al*, 2005). The device, now commercially available, is made from elastic polydimethylsiloxane (PDMS). Compared with our modified two-compartment culture device, the microfluidic device demands less time in device assembly and has advantage in transilluminated light microscopy; however, it also has its own limitations. Bulk PDMS contains low molecular weight non-crosslinked oligomers. These non-crosslinked oligomers compromise the hydrophilicity of PDMS, which are required for assembling and filling the microfluidic device with liquid (Lee *et al*, 2003). In addition, participation of these oligomers on the culture surface and into culture medium may impair cell growth and attachment (Lee *et al*, 2004; Millet *et al*, 2007; our unpublished observation). It has been reported that porous PDMS absorbs small molecules. Considering the high surface area to volume ratio of the microfluidic device, the absorption can significantly change solution concentration, thus potentially modifying experimental

outcome (Toepke and Beebe, 2006). The micro-feature of the microfluidic device saves experimental material and space but also limits the scale of culture. Thus, the microfluidic and our modified two-compartment culture devices complement each other and provide valuable tools for studying CNS axons *in vitro*.

Energy depletion induced axonal injury

In mammalian unmyelinated axons, all or nearly all of the axonal energy derived from oxygen consumption is utilized by $\text{Na}^+\text{-K}^+\text{-ATPase}$ to generate sodium and potassium gradients across plasma membrane that give rise to the resting membrane potential (Ritchie, 1967). Consistently, this study has shown that deprivation of axonal ATP up to 65% by energy depletion is sufficient to induce membrane depolarization and subsequent irreversible structural damage in cortical axons. The depolarization response found in these unmyelinated CNS axons is in agreement with those described in the myelinated CNS axons (Leppanen and Stys, 1997a and 1997b). Likely, energy depletion disrupts the ion gradients, and the disrupted ion gradients in turn lead to axon degradation, as blocking sodium influx with TTX during energy depletion not only suppressed membrane depolarization but also protected axons (Figure 5). In concert with this idea, earlier studies in the myelinated (Stys *et al*, 1992; Tekkk and Goldberg, 2001) and unmyelinated axons (Underhill and Goldberg, 2007) have indicated that energy deprivation induced structural and functional damage are associated with the sodium overload and adverse free calcium accumulation in axons, and the latter may inappropriately activate calcium-dependent enzymes, which eventually destroy axonal structure and function (for reviews see Stirling and Stys, 2010; Stys, 2005). Taken together, current results emphasize that energy homeostasis is crucial for the integrity of axonal structural and function.

Creatine-mediated axonal protection against energy depletion

This study revealed that creatine pretreatment is sufficient to protect axons against energy depletion. Although energy depletion initially depolarized both the creatine-treated and the creatine-nontreated axons at a similar rate, the creatine-treated axons were soon gradually repolarized toward the resting potential. The repolarization response was coupled with ATP retention in axons without altering the baseline ATP level. Likely, energy depletion triggered ATP reproduction from PCr, which in turn fueled $\text{Na}^+\text{-K}^+\text{-ATPase}$ to improve resting membrane potential. The axonal PCr may be either generated locally in axons or diffused from connecting somas. Current findings favor the local mechanism, as axonal protection persisted even when creatine was applied exclusively to the axons (Figure 6B and 6C). However, given axons were not physically separated from their somas in our culture model, we cannot rule out the possibility that soma-derived PCr might also contribute to the total axonal PCr pool. Further analyses of PCr and ATP levels in intact axons and their somas as well as transected axons after local creatine treatment are necessary to better understand the mechanism of creatine-mediated energy buffering and axonal protection.

Unlike in axon culture, creatine delays neuronal anoxic depolarization in brain slice (Balestrino *et al*, 1999). This discrepancy may result from different energy deprivation- and tissue culture- model used. For example, brain slice contains both neurons and glial cells, while our axon culture is unmyelinated and devoid of glial cells. In the absence of glia-provided insulation and substrates for ATP synthesis, axons are more prone to energy depletion. Hypoxia only affects ATP synthesis via oxidative phosphorylation, while energy depletion inhibits both oxidative phosphorylation and glycolysis. Together energy depletion might have caused a sudden and sharp energy decrease in axons before ATP could be regenerated from PCr, while the energy reduction by hypoxia in brain slice might have been more gradual and therefore compensated by PCr-derived ATP, as there was a latency of anoxic depolarization in control brain slice but not in axon culture (comparing the open

circles in Figure 3B versus control in Figure 3 by Balestrino *et al*, 1999). Alternatively, because the synthesis of neurite ATP is soma-independent (Tolkovsky and Suidan, 1987), the different effects of creatine on neurons and axons may reflect a difference in energy metabolism between the two tissues. Nevertheless, in either case, creatine treatment results in membrane potential improvement and subsequently tissue protection, implicating a shared mechanism of creatine-mediated protection against energy deficiency in neurons and axons.

Using the modified two-compartment culture device, we demonstrated that the creatine-provided axonal protection is independent of glial cells (Figure 6B and 6C). However, the axonal protection by creatine demands a pretreatment. This may explain the insufficient axonal protection by creatine cotreatment against ammonium exposure in neuron-enriched culture (Braissant *et al*, 2008; Braissant *et al*, 2002). Nevertheless, it is possible that glial cells might further potentiate creatine protection, as they may increase cellular pool of creatine on one hand (Braissant *et al*, 2008; Braissant *et al*, 2002), and on the other hand astrocytes may provide energy to axons in form of lactate (Brown and Ransom, 2007).

In addition to buffering high-energy phosphates, creatine has been suggested in inhibiting mitochondrial permeability transition as a substrate of the mitochondrial isoform of CK (O’Gorman *et al*, 1997; Dolder *et al*, 2003). Nevertheless, some others were not able to prove it (Brustovetsky *et al*, 2001; Klivenyi *et al*, 2004). Creatine has also been reported as a direct anti-oxidant (Lawler *et al*, 2002; Sestili *et al*, 2006). If reactive oxygen species are generated during energy depletion and contribute to axonal damage, and creatine acts as an anti-oxidant, one may expect that creatine is required during energy depletion, which is controversial with our observations that creatine pretreatment was axonal protective; and extending the creatine pretreatment beyond energy depletion did not provide additional protection to axons. Hence, although we cannot exclude the possibility that other mechanisms are involved in the creatine-mediated axonal protection, current findings are better in agreement with the role of creatine in maintaining high-energy phosphates.

To summarize, this study has shown that creatine pretreatment may directly protect CNS axons from acute energy depletion, an *in vitro* model of ischemic stroke. The axonal protection of creatine is correlated with its ability to buffer ATP in axons. Our findings suggest a novel therapeutic potential of creatine supplement in reduction of white matter injury in people with high risk of stroke. Further study is yet to be performed to prove its efficacy *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Krzysztof L. Hyrc for helpful discussion and technical advice, Karen S. Bequette for taking care of the animal colonies, and Rosmy M. George for her technical assistance. This work was supported by NIH Neuroscience Blueprint Interdisciplinary Center Core Grant P30 NS057105 to Washington University and NIH P01 NS032636 and NIH R01 NS036265 to MPG.

Abbreviations used

AUC	Area under the curve
CNS	central nervous system
CK	creatine kinase

DFI	Dibac ₄ (3) fluorescence intensity
Dibac₄(3)	bis(1,3-dibutylbarbituric acid) trimethine oxonol
DIV	days <i>in vitro</i>
$\Delta\Psi_{\text{axon}}$	membrane potential of axon
PCr	phosphocreatine
PDMS	polydimethylsiloxane
ROI	region of interest
TTX	tetrodotoxin

References

- Adhihetty PJ, Beal MF. Creatine and its potential therapeutic value for targeting cellular energy impairment in neurodegenerative diseases. *Neuromol Med.* 2008; 10:275–90.
- Andres RH, Ducray AD, Schlattner U, Wallimann T, Widmer HR. Functions and effects of creatine in the central nervous system. *Brain Res Bull.* 2008; 76:329–43. [PubMed: 18502307]
- Balestrino M. Pathophysiology of anoxic depolarization: new findings and a working hypothesis. *J Neurosci Methods.* 1995; 59:99–103. [PubMed: 7475257]
- Balestrino M, Rebaudo R, Lunardi G. Exogenous creatine delays anoxic depolarization and protects from hypoxic damage: dose-effect relationship. *Brain Res.* 1999; 816:124–30. [PubMed: 9878706]
- Balestrino M, Lensman M, Parodi M, Perasso L, Rebaudo R, Melani R, et al. Role of creatine and phosphocreatine in neuronal protection from anoxic and ischemic damage. *Amino Acids.* 2002; 23:221–9. [PubMed: 12373542]
- Braeuner T, Huelser DF, Strasser RJ. Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes. *Biochim Biophys Acta.* 1984; 771:208–16. [PubMed: 6704395]
- Braissant O, Henry H, Villard AM, Zurich MG, Loup M, Eilers B, et al. Ammonium-induced impairment of axonal growth is prevented through glial creatine. *J Neurosci.* 2002; 22:9810–20. [PubMed: 12427837]
- Braissant O, Cagnon L, Monnet-Tschudi F, Speer O, Wallimann T, Honegger P, et al. Ammonium alters creatine transport and synthesis in a 3D culture of developing brain cells, resulting in secondary cerebral creatine deficiency. *Eur J Neurosci.* 2008; 27:1673–85. [PubMed: 18380667]
- Brown AM, Ransom BR. Astrocyte glycogen and brain energy metabolism. *Glia.* 2007; 55:1263–71. [PubMed: 17659525]
- Brustovetsky N, Brustovetsky T, Dubinsky JM. On the mechanisms of neuroprotection by creatine and phosphocreatine. *J Neurochem.* 2001; 76:425–34. [PubMed: 11208905]
- Campenot RB. Local control of neurite development by nerve growth factor. *Proc Natl Acad Sci U S A.* 1977; 74:4516–9. [PubMed: 270699]
- Carter AJ, Mueller RE, Pschorn U, Stransky W. Preincubation with creatine enhances levels of creatine phosphate and prevents anoxic damage in rat hippocampal slices. *J Neurochem.* 1995; 64:2691–9. [PubMed: 7760049]
- Dodd JR, Birch NP, Waldvogel HJ, Christie DL. Functional and immunocytochemical characterization of the creatine transporter in rat hippocampal neurons. *J Neurochem.* 2010; 115:684–93. [PubMed: 20731764]
- Dolder M, Walzel B, Speer O, Schlattner U, Wallimann T. Inhibition of the mitochondrial permeability transition by creatine kinase substrates. Requirement for microcompartmentation. *J Biol Chem.* 2003; 278:17760–6. [PubMed: 12621025]
- Düssmann H, Rehm M, Kögel D, Prehn JH. Outer mitochondrial membrane permeabilization during apoptosis triggers caspase-independent mitochondrial and caspase-dependent plasma membrane potential depolarization: a single-cell analysis. *J Cell Sci.* 2003; 116:525–36. [PubMed: 12508113]

- Epps DE, Wolfe ML, Groppi V. Characterization of the steady-state and dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol (Dibac4(3)) in model systems and cells. *Chem Phys Lipids*. 1994; 69:137–50. [PubMed: 8181103]
- Finn JT, Weil M, Archer F, Siman R, Srinivasan A, Raff MC. Evidence that Wallerian degeneration and localized axon degeneration induced by local neurotrophin deprivation do not involve caspases. *J Neurosci*. 2000; 20:1333–41. [PubMed: 10662823]
- Goldberg MP, Ransom BR. New light on white matter. *Stroke*. 2003; 34:330–2. [PubMed: 12574526]
- Holtzman D, Togliatti A, Khait I, Jensen F. Creatine Increases Survival and Suppresses Seizures in the Hypoxic Immature Rats. *Pediatr Res*. 1998; 44:410–4. [PubMed: 9727722]
- Holtzman D, Khait I, Mulkern R, Allred E, Rand T, Jensen F, et al. In vivo development of brain phosphocreatine in normal and creatine-treated rabbit pups. *J Neurochem*. 1999; 73:2477–84. [PubMed: 10582608]
- Ikegami K, Koike T. Non-apoptotic neurite degeneration in apoptotic neuronal death: pivotal role of mitochondrial function in neurites. *Neuroscience*. 2003; 122:617–26. [PubMed: 14622905]
- Ivins KJ, Bui ET, Cotman CW. Beta-amyloid induces local neurite degeneration in cultured hippocampal neurons: evidence for neuritic apoptosis. *Neurobiol Dis*. 1998; 5:365–78. [PubMed: 10069579]
- Jørgensen NK, Petersen SF, Damgaard I, Schousboe A, Hoffmann EK. Increases in $[Ca^{2+}]_i$ and changes in intracellular pH during chemical anoxia in mouse neocortical neurons in primary culture. *J Neurosci Res*. 1999; 56:358–70. [PubMed: 10340744]
- Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell*. 1994; 12:425–35. [PubMed: 8062385]
- Klivenyi P, Calingasan NY, Starkov A, Stavrovskaya IG, Kristal BS, Yang L, et al. Neuroprotective mechanisms of creatine occur in the absence of mitochondrial creatine kinase. *Neurobiol Dis*. 2004; 15:610–7. [PubMed: 15056469]
- Lawler JM, Barnes WS, Wu G, Song W, Demaree S. Direct antioxidant properties of creatine. *Biochem Biophys Res Commun*. 2002; 290:47–52. [PubMed: 11779131]
- Lee CW, Peng HB. The function of mitochondria in presynaptic development at the neuromuscular junction. *Mol Biol Cell*. 2008; 19:150–8. [PubMed: 17942598]
- Lee JN, Park C, Whitesides GM. Solvent compatibility of poly(dimethylsiloxane)-based microfluidic devices. *Anal Chem*. 2003; 75:6544–54. [PubMed: 14640726]
- Lee JN, Jiang X, Ryan D, Whitesides GM. Compatibility of mammalian cells on surfaces of poly(dimethylsiloxane). *Langmuir*. 2004; 20:11684–91. [PubMed: 15595798]
- Lensman M, Korzhhevskii DE, Mourouets VO, Kostkin VB, Izvarina N, Perasso L, et al. Intracerebroventricular administration of creatine protects against damage by global cerebral ischemia in rat. *Brain Res*. 2006; 1114:187–94. [PubMed: 16949559]
- Leppanen L, Stys PK. Ion transport and membrane potential in CNS myelinated axons. II. Effects of metabolic inhibition. *J Neurophysiol*. 1997a; 78:2095–107. [PubMed: 9325377]
- Leppanen L, Stys PK. Ion transport and membrane potential in CNS myelinated axons. I. Normoxic conditions. *J Neurophysiol*. 1997b; 78:2086–94. [PubMed: 9325376]
- Lipton P, Whittingham TS. Reduced ATP concentration as a basis for synaptic transmission failure during hypoxia in the in vitro guinea-pig hippocampus. *J Physiol (Lond)*. 1982; 325:51–65. [PubMed: 6286944]
- Luhmann HJ, Heinemann U. Hypoxia-induced functional alterations in adult rat neocortex. *J Neurophysiol*. 1992; 67:798–811. [PubMed: 1316953]
- Lunardi G, Parodi A, Perasso L, Pohvozcheva AV, Scarrone S, Adriano E, et al. The creatine transporter mediates the uptake of creatine by brain tissue, but not the uptake of two creatine-derived compounds. *Neurosci*. 2006; 142:991–7.
- Malek SA, Coderre E, Stys PK. Aberrant chloride transport contributes to anoxic/ischemic white matter injury. *J Neurosci*. 2003; 23:3826–36. [PubMed: 12736353]
- Millet LJ, Stewart ME, Sweedler JV, Nuzzo RG, Gillette MU. Microfluidic devices for culturing primary mammalian neurons at low densities. *Lab Chip*. 2007; 7:987–94. [PubMed: 17653340]

- O'Gorman E, Beutner G, Dolder M, Koretsky AP, Brdiczka D, Wallimann T. The role of creatine kinase in inhibition of mitochondrial permeability transition. *FEBS Lett.* 1997; 414:253–7. [PubMed: 9315696]
- Okada Y, Yoneda K. Effects of accumulation of phosphocreatine on the survival time of thin hippocampal slices from the guinea pig during deprivation of both oxygen and glucose. *Neurosci Lett.* 1983; 41:125–31. [PubMed: 6646512]
- Perasso L, Cupello A, Lunardi GL, Principato C, Gandolfo C, Balestrino M. Kinetics of creatine in blood and brain after intraperitoneal injection in the rat. *Brain Res.* 2003; 974:37–42. [PubMed: 12742622]
- Perasso L, Adriano E, Ruggeri P, Burov SV, Gandolfo C, Balestrino M. In vivo neuroprotection by a creatine-derived compound: phosphocreatine-Mg-complex acetate. *Brain Res.* 2009; 1285:158–63. [PubMed: 19523930]
- Perasso L, Spallarossa P, Gandolfo C, Ruggeri P, Balestrino M. Therapeutic use of creatine in brain or heart ischemia: available data and future perspectives. *Med Res Rev.* 2011 Dec 1. [Epub ahead of print]. 10.1002/med.20255
- Perry VH, Brown MC, Lunn ER, Tree P, Gordon S. Evidence that very slow wallerian degeneration in C57BL/Ola mice is an intrinsic property of the peripheral nerve. *Eur J Neurosci.* 1990; 2:802–8. [PubMed: 12106282]
- Piller SC, Jans P, Gage PW, Jans DA. Extracellular HIV-1 virus protein R causes a large inward current and cell death in cultured hippocampal neurons: Implications for AIDS pathology. *Proc Natl Acad Sci USA.* 1998; 95:4595–600. [PubMed: 9539783]
- Prass K, Royl G, Lindauer U, Freyer D, Megow D, Dirnagl U, et al. Improved reperfusion and neuroprotection by creatine in a mouse model of stroke. *J Cereb Blood Flow Metab.* 2007; 27:452–9. [PubMed: 16773141]
- Rebaudo R, Melani R, Carità F, Rosi L, Picchio V, Ruggeri P, et al. Increase of cerebral phosphocreatine in normal rats after intracerebroventricular administration of creatine. *Neurochem Res.* 2000; 25:1493–5. [PubMed: 11071368]
- Reichert SA, Kim-Han JS, Dugan LL. The mitochondrial permeability transition pore and nitric oxide synthase mediate early mitochondrial depolarization in astrocytes during oxygen-glucose deprivation. *J Neurosci.* 2001; 21:6608–16. [PubMed: 11517250]
- Ritchie JM. The oxygen consumption of mammalian non-myelinated nerve fibres at rest and during activity. *J Physiol.* 1967; 188:309–29. [PubMed: 6032203]
- Sestili P, Martinelli C, Bravi G, Piccoli G, Curci R, Battistelli M, et al. Creatine supplementation affords cytoprotection in oxidatively injured cultured mammalian cells via direct antioxidant activity. *Free Radic Biol Med.* 2006; 40:837–49. [PubMed: 16520236]
- Stirling DP, Stys PK. Mechanisms of axonal injury: internodal nanocomplexes and calcium deregulation. *Trends Mol Med.* 2010; 16:160–70. [PubMed: 20207196]
- Stys PK, Waxman SG, Ransom BR. Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na⁺ channels and Na⁺-Ca²⁺ exchanger. *J Neurosci.* 1992; 12:430–9. [PubMed: 1311030]
- Stys PK. General mechanisms of axonal damage and its prevention. *J Neurol Sci.* 2005; 233:3–13. [PubMed: 15899499]
- Tachikawa M, Fukaya M, Terasaki T, Ohtsuki S, Watanabe M. Distinct cellular expressions of creatine synthetic enzyme GAMT and creatine kinases uCK-Mi and CK-B suggest a novel neuron-glia relationship for brain energy homeostasis. *Eur J Neurosci.* 2004; 20:144–60. [PubMed: 15245487]
- Taylor AM, Blurton-Jones M, Rhee SW, Cribbs DH, Cotman CW, Jeon NL. A microfluidic culture platform for CNS axonal injury, regeneration and transport. *Nat Methods.* 2005; 2:599–605. [PubMed: 16094385]
- Tekkök SB, Goldberg MP. Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. *J Neurosci.* 2001; 21:4237–48. [PubMed: 11404409]
- Thompson JM, Pelto DJ. Attachment, survival and neurite extension of chick embryo retinal neurons on various culture substrates. *Dev Neurosci.* 1982; 5:447–57. [PubMed: 7160310]

- Toepke MW, Beebe DJ. PDMS absorption of small molecules and consequences in microfluidic applications. *Lab Chip*. 2006; 6:1484–6. [PubMed: 17203151]
- Tolkovsky AM, Suidan HS. Adenosine 5'-triphosphate synthesis and metabolism localized in neurites of cultured sympathetic neurons. *Neurosci*. 1987; 23:1133–42.
- Tonge DA, Golding JP, Edbladh M, Kroon M, Ekström PE, Edström A. Effects of extracellular matrix components on axonal outgrowth from peripheral nerves of adult animals in vitro. *Exp Neurol*. 1997; 146:81–90. [PubMed: 9225741]
- Underhill SM, Goldberg MP. Hypoxic injury of isolated axons is independent of ionotropic glutamate receptors. *Neurobiol Dis*. 2007; 25:284–90. [PubMed: 17071096]
- Varming T, Drejer J, Frandsen A, Schousboe A. Characterization of a chemical anoxia model in cerebellar granule neurons using sodium azide: protection by nifedipine and MK-801. *J Neurosci Res*. 1996; 44:40–6. [PubMed: 8926628]
- Wang J, Zhai Q, Chen Y, Lin E, Gu W, McBurney MW, et al. A local mechanism mediates NAD-dependent protection of axon degeneration. *J Cell Biol*. 2005; 170:349–55. [PubMed: 16043516]
- Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM. 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*. 1992; 281:21–40. [PubMed: 1731757]
- Wick M, Fujimori H, Michaelis T, Frahm J. Brain water diffusion in normal and creatine-supplemented rats during transient global ischemia. *Magn Reson Med*. 1999; 42:798–802. [PubMed: 10502770]
- Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Phys Rev*. 2000; 80:1107–213.
- Zapara TA, Simonova OG, Zharkikh AA, Balestrino M, Ratuszniak AS. Seasonal differences and protection by creatine or arginine pretreatment in ischemia of mammalian and molluscan neurons in vitro. *Brain Res*. 2004; 1015:41–9. [PubMed: 15223365]
- Zhu S, Li M, Figueroa BE, Liu A, Stavrovskaya IG, Pasinelli P, et al. Prophylactic creatine administration mediates neuroprotection in cerebral ischemia in mice. *J Neurosci*. 2004; 24:5909–12. [PubMed: 15229238]

Figure 1.

Two-compartment culture of cortical axons. A, Schematic illustration of two-compartment culture of murine cortical axons. The culture device was assembled as described in the methods and materials. Cortical aggregates were plated in the cell compartment (white area). Collagen/matrigel seal with 200 ng/ml Netrin (dark grey area) was applied underneath the coverslip to block movement of cell bodies and allow axon growth toward the axon compartment (grey area). For clarity, only one cortical aggregate is shown in the illustration and not drawn to scale. Five predefined regions of interest (ROIs) for quantification of axon integrity are shown as dotted lines in the axon compartment. B, A representative montage image of Tuj1 stained cortical axons at 12 days *in vitro* corresponding to the boxed area in A. Bar, 200 μm .

Figure 2.

Creatine treatment reduces axonal injury caused by energy depletion. **A**, Representative images of cortical axons stained with Tuj1 antibodies at 4 h and 24 h after energy depletion. Creatine (10 mM) was applied to both the cell and axon compartments from 24 h prior to energy depletion until the end of the experiment. **B**, Quantification of the intact axons at 4 h and 24 h after indicated treatments. **, $P < 0.01$. Bar, 25 μ m in A.

Figure 3.

Creatine alleviates axonal depolarization caused by energy depletion. The change in membrane potential of resting cortical axons is monitored with a slow voltage sensitive dye, DIBAC₄(3). The rise of DIBAC₄(3) fluorescence corresponds to axonal depolarization. **A**, Resting axons. **B**, Axons exposed to energy depletion with or without creatine treatment. Creatine (10 mM) was given at 24 h prior to and during energy depletion.

Figure 4.

Creatine preserves ATP of axons exposed to energy depletion. Cortical axons were collected immediately after 30 min of energy depletion. The levels of axonal ATP were determined via a luciferin and luciferase mediated chemiluminescence reaction and normalized by beta-actin contents of respective samples. *, $P < 0.05$.

Figure 5.

Blockage of sodium influx via TTX-sensitive sodium channels suppresses axonal depolarization and subsequent structural damage caused by energy depletion. **A**, Membrane potential of cortical axon was determined by voltage sensitive dye Dibac₄(3). After baseline recording, axons were exposed to energy depletion in the presence of 1.5 μ M TTX. **B** and **C**, Representative images and quantification of cortical axons stained for Tuj1 at 24 h after indicated treatments. The data were obtained from two independent experiments. Bar, 25 μ m in B. **, $P < 0.01$; ***, $P < 0.001$. There was no significant difference between the control group and the energy depletion + TTX group.

Local creatine pretreatment but not post-treatment is sufficient to protect axons from energy depletion. **A**, Schematic illustration of different treatment strategies. Creatine (10 mM) was applied exclusively to the axon compartment during indicated periods. **B** and **C**, Representative images and quantification of Tuj1 stained cortical axons at 24 h after energy depletion. Bar, 25 μ m in B. ***, $P < 0.001$. There was no significant difference between the control group and either the creatine only group or the energy depletion + creatine pretreatment group.