Creatine is Neuroprotective to Retinal Neurons In Vitro But Not In Vivo

Paul Ikgan Sia,[1,2] John P. M. Wood,[1,2] Glyn Chidlow,[1,2] and Robert Casson[1,2]

[1]South Australian Institute of Ophthalmology, Royal Adelaide Hospital, Adelaide, South Australia, Australia
[2]Department of Ophthalmology and Visual Sciences, University of Adelaide, Adelaide, South Australia, Australia

Correspondence: Robert Casson, Ophthalmic Research Laboratories, Level 7 Adelaide Health and Medical Sciences Building, The University of Adelaide, North Terrace, Adelaide SA-5000, Australia; robert.casson@adelaide.edu.au.

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Purpose. To investigate the neuroprotective properties of creatine in the retina using in vitro and in vivo models of injury.

Methods. Two different rat retinal culture systems (one containing retinal ganglion cells [RGC] and one not) were subjected to either metabolic stress, via treatments with the mitochondrial complex IV inhibitor sodium azide, or excitotoxic stress, via treatment with N-methyl-D-aspartate for 24 hours, in the presence or absence of creatine (0.5, 1.0, and 5.0 mM). Neuronal survival was assessed by immunolabeling for cell-specific antigens. Putative mechanisms of creatine action were investigated in vitro. Expression of creatine kinase (CK) isoenzymes in the rat retina was examined using Western blotting and immunohistochemistry. The effect of oral creatine supplementation (2%, wt/wt) on retinal and blood creatine levels was determined as well as RGC survival in rats treated with N-methyl-D-aspartate (NMDA; 10 nmol) or high IOP-induced ischemia reperfusion.

Results. Creatine significantly prevented neuronal death induced by sodium azide and NMDA in both culture systems. Creatine administration did not alter cellular adenosine triphosphate (ATP). Inhibition of CK blocked the protective effect of creatine. Retinal neurons, including RGCs, expressed predominantly mitochondrial CK isoforms, while glial cells expressed exclusively cytoplasmic CKs. In vivo, NMDA and ischemia reperfusion caused substantial loss of RGCs. Creatine supplementation led to elevated blood and retinal levels of this compound but did not significantly augment RGC survival in either model.

Conclusions. Creatine increased neuronal survival in retinal cultures; however, no significant protection of RGCs was evident in vivo, despite elevated levels of this compound being present in the retina after oral supplementation.

Keywords: creatine, neuroprotection, retina, creatine kinase, retinal ganglion cell, culture, ischemia-reperfusion, excitotoxicity, oxidative stress

A number of common blinding diseases, such as glaucoma, diabetic retinopathy, and ischemic optic neuropathy, include bioenergetic compromise as a likely pathogenic component. At the molecular level such an energy failure can instigate a complex series of cellular events, including membrane depolarization, Ca^{2+} influx, oxidative stress, mitochondrial dysfunction, excitotoxicity and, even, ultimately, cell death.[1–3]

Bioenergetic-based neuroprotection refers to the concept of protecting injured and threatened neurons by strategies, including increasing their available energy supply, conserving their fuel reserves, improving their mitochondrial function, or augmenting their cellular energy buffering.[4] Enhancement of cellular energy metabolism has been attempted with variable success in a range of neurologic disorders, such as Alzheimer's disease,[5] Huntingdon's disease,[6] and Parkinson's disease.[7] More pertinently, this approach to neuroprotection has been targeted to the retina in glaucoma.[4] Nicotinamide, for example, which acts as a substrate for Complex I in the respiratory chain, and also acts as a free-radical scavenger and an inhibitor of poly-adenosine diphosphate (ADP)-ribose polymerase, attenuated ischemic injury to retinal ganglion cells (RGCs).[8] Another compound, coenzyme Q_{10}, a potent antioxidant and a component of the mitochondrial respiratory chain has been shown to be neuroprotective against in IOP-induced retinal ischemia-reperfusion injury.[9–11] Additional findings of relevance to bioenergetic neuroprotection of the retina are that short-term hyperglycemia, as well as local administration of glucose, attenuate RGC injury in models of experimental glaucoma, ischemia reperfusion, and chronic hypoperfusion.[12–15]

Creatine, a guanidine compound that occurs naturally in vertebrates, plays a central role in cellular adenosine triphosphate (ATP) buffering, in concert with its phosphorylated form, phosphocreatine. Creatine and phosphocreatine are interconverted by creatine kinase (CK), an enzyme family comprising separate isoenzymes based either in mitochondria or the cytoplasm. Mitochondrial CK converts creatine to phosphocreatine at the site of ATP production, with a concurrent production of ADP. Phosphocreatine has much greater intracellular mobility than adenine nucleotides and this compound is therefore more readily able to translocate from the mitochondrion to sites of energy use. Here, cytoplasmic CK catalyzes the donation of its phosphate group to ADP, thus reforming ATP and creatine. This cycle is responsible for buffering of ATP/ADP levels, aiding maintenance of a cellular

energy reserve and allowing the easy shuttling of fuel currency between mitochondrial sites of production and cytosolic sites of use.[16,17]

Creatine, therefore, possesses a variety of properties, which could contribute to a potential bioenergetic neuroprotective action, including being able to buffer intracellular energy reserves, stabilize intracellular calcium flux, inhibit mitochondrial permeability transition, and counteract oxidative stress build-up.[18,19] In fact, creatine supplementation has indeed been shown to exert neuroprotective effects in both in vitro and in vivo models of major neurodegenerative conditions, including models of Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis.[18,20] To date, there are no studies that have examined the direct neuroprotective efficacy of creatine in acute or chronic paradigms of RGC neurodegeneration. However, S-adenosyl-L-methionine, which is a biosynthetic precursor of creatine, was able to restore photoreceptor function in a rat model of retinal ischemia.[21] The present study aimed to assess the neuroprotective properties of creatine in different retinal models of neuronal compromise: retinal neurons in culture subjected to excitotoxic challenge or metabolic impairment and rat retinas subjected to excitotoxic or ischemic insults in vivo.

Materials and Methods

Experimental Design

There were two phases to the overall study as follows: (1) to assess the effect of creatine in culture preparations exposed to either energetic compromise with the mitochondrial complex IV inhibitor, sodium azide, or excitotoxic injury with the ionotropic glutamate receptor agonist, N-methyl-D-aspartate (NMDA); and (2) to assess putative neuroprotective effects of creatine in rat in vivo models of retinal ischemia reperfusion and NMDA-induced excitotoxicity.

Materials

General cell culture media and reagents, including fetal bovine serum (FBS), were obtained from Invitrogen (Mulgrave, Victoria, Australia). Culture vessels (flasks and plates) and polypropylene centrifuge tubes of all sizes were from Sarstedt Pty (Adelaide, South Australia, Australia). All other chemical reagents, except where noted, were from Sigma-Aldrich Chemical Company (Castle Hill, New South Wales, Australia).

Animals

This study was approved by the Animal Ethics Committees of SA Pathology/Central Adelaide Local Health Network and The University of Adelaide and conformed to both the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013) and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were obtained from the University of Adelaide, South Australia. For culture studies, Sprague-Dawley rat litters (2–4 days postpartum, average of 8–12 pups per litter) were obtained and for the in vivo experiments, adult Sprague-Dawley rats (8–10 weeks) were used.

Mixed Retinal Cell Culture

Rat retinal cell cultures comprising glia, photoreceptors, and neurons, but not, generally RGCs, were prepared from the pups via a trypsin- and mechanical-digest procedure.[22] After tissue dissociation, cells were dispensed onto 13-mm diameter borosilicate glass coverslips coated previously with poly-L-lysine (10 μg/mL, 15 minutes) in 24-well culture plates for immunocytochemical, fluorescent dye-labeling, or apoptotic analyses. Mean cell density at seeding was approximately 0.5×10^6 cells/mL. Subsequently, cultures were grown at 37°C in a humidified incubator with 5% CO_2 in growth medium (MEM containing 10% FBS, 91 mg/L gentamicin sulphate, 2.3 mg/L amphotericin B, and 25 mM glucose).

After 6 days in vitro, medium was changed and the cultures were incubated for 24 hours with either creatine (concentrations of 0.5, 1, and 5 mM) or standard medium (control group). To establish mitochondrial compromise, some of both the creatine-treated group and the control group were subjected to concurrent incubation for 24 hours with 1 mM NaN_3 or for 1 hour with 10 mM NaN_3. To establish excitotoxicity, after creatine pretreatment, cells were exposed to 200 μM NMDA plus 10 mM $CaCl_2$ for 24 hours. At the conclusion of the period of cell stress, cultures were fixed in 10% (wt/vol) neutral-buffered formalin in 0.1 M phosphate buffer, pH 7.4 (NBF) for immunocytochemical analysis. In some cases, incubations were carried out in the presence of the specific CK inhibitor, 2,4-dinitro-1-fluorobenzene (DNFB; 10 μM), to determine whether this enzyme was responsible for the observed actions of creatine.

Retinal Ganglion Cell–Containing Culture System

Retinal neuron cultures containing ganglion cells were essentially established as described previously.[23] Adult Sprague-Dawley rats were euthanized and eyes were enucleated and placed into PBS containing 100 U/mL penicillin/streptomycin. Retinas were removed and chopped into small pieces with dissecting spring scissors. Tissue pieces were incubated in activated papain (2 mg/mL papain, 0.4 mg/mL DL-cysteine, 0.4 mg/mL BSA, in Neurobasal medium) at 37°C for 20 minutes and then washed three times with Neurobasal medium, before being dissociated by gentle trituration through fire-polished Pasteur pipettes. Cell suspensions (1×10^6 cells/well in Tissue Medium: Neurobasal medium plus B27 nutrient supplement, 100 U/mL penicillin, 100 U/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 5 μg/mL insulin, 100 μg/mL transferrin, 100 μg/mL BSA, 60 ng/mL progesterone, 16 μg/mL putrescine, 40 ng/mL sodium selenite, 40 ng/mL L-thyroxine, 40 ng/mL tri-iodothyronine, 5 μg/mL forskolin, 50 ng/mL BDNF, 10 ng/mL ciliary neurotrophic factor, 10 ng/mL BFGF and 1% [vol/vol] BSA) were dispensed onto 13-mm borosilicate glass coverslips precoated sequentially with 5 μg/mL poly-D-lysine (overnight) and 1 μg/mL laminin (2 hours). Cells were cultured for 14 days before treatments were established, with half medium changes every 5 days.

After treatments had been carried out for the appropriate times, cultures were fixed for 10 minutes in NBF and cells were immunocytochemically labeled for dendrites (MAP2) and axons (tau); DAPI was applied to label cell nuclei. When quantification was undertaken, only tau-immunoreactivity (-IR) was determined.

Animal Models of Retinal Ischemia-Reperfusion and Excitotoxicity

For establishment of both retinal ischemia reperfusion and NMDA-induced excitotoxicity, rats were randomly assigned to either a treatment group, which received 2% oral creatine supplementation in their feed (20 g/kg) for 4 to 6 weeks prior to commencement of experiments, or to a control group, which received standard normal chow throughout the

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experiment ($n = 8$ control, $n = 7$ creatine for retinal ischemia; $n = 15$ control, $n = 18$ creatine for NMDA).

For establishment of retinal ischemia in rats, the left eye was cannulated with a 32-G needle attached to a reservoir containing sterile 0.9% (wt/vol) NaCl. The IOP was elevated to 120 mm Hg, which rendered the retina ischemic as evidenced by fundus pallor. This was maintained for 75 minutes. All rats were killed at 7 days following retinal ischemia and their eyes removed for analysis.

To produce retinal excitotoxicity, the left eyes of rats were subjected to an intravitreal injection (using a 32-G needle) of 10 nmol of NMDA (4-μL injected volume), under observation with a dissecting microscope. All rats were killed at 7 days following NMDA administration and their eyes removed for analysis. To assess the effect of creatine on neuronal apoptosis, separate groups of NMDA-treated animals ($n = 8$ control, $n = 8$ creatine) were killed at 8 hours following injection and their retinae processed for TUNEL labeling. The right eye in each animal was used as a control.

Electroretinography

Rats assigned to the retinal ischemia group had scotopic ERG readings recorded from both eyes before ischemia (baseline) and 3 and 7 days after reperfusion. To undertake this procedure, rats were dark-adapted overnight and anaesthetized briefly with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Pupils were dilated with 1% tropicamide. Eyes were further anaesthetized with 0.04% oxybuprocaine hydrochloride. Poly Gel lubricating eye gel (Alcon, Fort Worth, TX, USA) was applied to the cornea before applying the corneal electrode, reference electrode (connected to the ipsilateral ear), and ground electrode (on midline dorsal cephalad). ERGs were recorded after 12 hours of dark adaptation under general anesthesia and all experimental procedures were performed under dim red light. The rats were placed on a heating blanket to maintain body temperature at 37°C. A mixed rod-cone response was obtained using a photometrically calibrated 10-μs Ganzfeld flash with a 0.75-log flash intensity (cd·s/m^2). Signals were amplified (gain:1000) and acquired with a PowerLab 4/35 (ADInstruments, Bella Vista, NSW, Australia) bioamplifier and data acquisition system (sampling rate, 1000 Hz) using a band-pass filter between 0.3 and 500 Hz and analyzed with LabChart software (ADInstruments). The a-wave amplitude was measured from the prestimulus potential to its trough and the b-wave amplitude from the a-wave trough to the b-wave peak. Ten consecutive responses were recorded and averaged for each animal.

Immunocytochemistry and Immunohistochemistry

Retinal cells in culture were fixed with 10% (wt/vol) NBF containing 1% (vol/vol) methanol for 15 minutes and then washed in standard PBS. Cells on coverslips were permeabilized with PBS containing 0.1% Triton X-100 (PBST-0.1%), followed by further washing in PBS and then blocking in PBS containing 3% normal horse serum (PBS-HS). After overnight incubation with primary antibodies against calretinin and γ-amino butyric acid (GABA; see Table 1 for antibody details), coverslips were incubated with biotinylated anti-mouse secondary antibody (1:250) plus AlexaFluor 488-conjugated anti-rabbit secondary antibody (1:250; Invitrogen), followed by streptavidin-conjugated AlexaFluor 594 (1:500) for 1 hour. Finally, cells on coverslips were mounted using anti-fade mounting medium (DAKO; Botany Bay, New South Wales, Australia) and examined under a confocal fluorescence microscope.

For in vivo experiments, all rats were killed by transcardial perfusion with physiologic saline. Whole eyes with optic nerve attached were removed and fixed with NBF for 24 hours at room temperature. For wholemount immunochemistry, posterior eye cups were dissected and each retina was prepared as a flattened wholemount via four relaxing incisions. Retinas were permeabilized with PBS containing 1% Triton X-100, blocked in PBST-1% containing 3% normal horse serum, then incubated overnight at 4°C in the same solution containing anti-Brn3a and anti-γ-synuclein primary antibodies (see Table 1). After washing with PBS, wholemounts were incubated with anti-goat Alexa Fluor 594 conjugate and anti-mouse Alexa Fluor 488 conjugate for 3 hours at room temperature, before rinsing in PBS and mounting using anti-fade mounting medium. Slides were examined under a confocal fluorescence microscope. Immunochemistry on transverse sections was achieved as described previously.[24] In brief, eyes were processed for routine paraffin-embedded sections, embedded sagitally, and 5-μm serial sections were cut. Sections were deparaffinized, endogenous peroxidase activity was blocked, and tissue subjected to high-temperature antigen retrieval. Sections were then blocked in PBS-HS and incubated overnight at room temperature with primary antibodies, followed by consecutive incubations with biotinylated secondary antibody and streptavidin-peroxidase conjugate. Color development was achieved using 3′,3′-diaminobenzidine. Sections were counterstained with hematoxylin, dehydrated, and mounted. Confirmation of the specificity of antibody labeling was judged by the morphology and distribution of the labeled cells, by the absence of signal when the primary antibody was replaced by isotype/serum controls, and by comparison with the expected staining pattern based on our own, and other, previously published results.

Electrophoresis and Western Immunoblotting

Tissue samples for electrophoresis were prepared from freshly killed adult Sprague-Dawley rats. Samples of heart, brain cortex, skeletal muscle, and retina were dissected and solubilized in homogenization buffer (20 mM Tris-HCl, pH 7.4; containing 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 50 μg/mL leupeptin, 50 μg/mL pepstatin A, 50 μg/mL aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) at a concentration of 10-mg tissue wet weight per 100 μL of buffer. An equal volume of sample buffer (62.5 mM Tris-HCl, pH 7.4, containing 4% [vol/vol] SDS, 10% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, and 0.002% [wt/vol] bromophenol blue) was then added and samples were heated to 80°C for 6 minutes. Electrophoresis was performed using 10% denaturing polyacrylamide gels for protein separation. After electrophoresis, proteins were transferred to polyvinylidine fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) for immunodetection. Membranes were blocked in tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, 140 mM NaCl plus 0.1% [vol/vol] Tween 20 [TBST] containing 5% [wt/vol] non-fat dried skimmed milk powder [TBST-NDSM]) before being incubated with the appropriate primary antibodies (see Table 1), diluted in TBST-NDSM for 3 hours at room temperature. Relevant biotinylated secondary antibodies (1:1000 for 30 minutes; Vector Laboratories, Inc., Burlingame, CA, USA) were applied followed by streptavidin horseradish peroxidase conjugate (1:1000 for 1 hour; Thermo Scientific Pierce Protein Biology, Waltham, MA, USA). Chromogenic detection of antibody labeling was achieved using 3-amino-9-ethylcarbazole. Reactions were stopped by immersion of membranes in 0.01% (wt/vol) sodium azide. Detection of histone H3 was assessed in all samples to normalize total protein levels. Labeled membranes were scanned with a conventional flat-bed scanner.

Table 1. Antibodies Used in the Study

Name	Cat *N*/Clone*	Host	Company	Dilution
Brn 3a	C-20*	Goat	Santa Cruz	1:3000, 1:600†
γ-Synuclein	CPTC-SNCG-1	Mouse	DSHB‡	1:40, 1:100†
Calretinin	Mab 1568	Mouse	Millipore	1:1000
GABA	A2052	Rabbit	Sigma-Aldrich	1:1000
Histone H3	D1H2*	Rabbit	CST	1:10000
Glutamine synthetase	610517	Mouse	BD Transduction	1:1000
CHX10	Ab 9016	Sheep	Millipore	1:10000
Neurofilament-light chain (NFL)	Mab 1615	Mouse	Millipore	1:5000
PKCα	MC5*	Mouse	Abcam	1:1000
RBPMS	ABN1362	Rabbit	Millipore	1:2000
S100	Mab 079-1	Mouse	Millipore	1:1000
Synaptophysin	A0010	Rabbit	Dako	1:2000
Syntaxin-1	HPC-1*	Mouse	Sigma-Aldrich	1:2000
CK-B	Ab 125114	Mouse	Abcam	1:500/1:2000
CK-M	C-4*	Mouse	Santa Cruz	1:2000
CK-MT1A	15346-1-AP	Rabbit	Proteintech	1:5000
CK-MT1B	Ab 204114	Rabbit	Abcam	1:2000

Antibody manufacturer locations: Santa Cruz (Dallas, TX, USA); Millipore (Burlington, MA, USA); Sigma-Aldrich (St. Louis, MO, USA); CST (Danvers, MA, USA); BD Transduction (San Jose, CA, USA); Abcam (Cambridge, UK); Dako (Santa Clara, CA, USA); Proteintech (Rosemont, IL, USA).

* Indicates clone number of cell line.

† Wholemount immunohistochemistry.

‡ From the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology (Iowa City, IA, USA).

TUNEL

TUNEL was performed according to previously described.[25] Retinal wholemounts were prepared as previously described. These were permeabilized with PBS containing 3% Triton X-100 for 30 minutes, treated with proteinase K (10 μg/mL in PBS; Sigma Aldrich) for 10 minutes at 37°C and blocked in PBST-1% containing 3% normal horse serum. The transferase reaction was performed by incubating retinal wholemounts in TdT buffer (30 mM Tris-HCl, pH7.2, containing 140 mM sodium cacodylate and 1 mM cobalt chloride) overnight at room temperature, with added 0.5 U/μL and 10 μM biotin-16-dUTP. Reaction was stopped with saline sodium citrate buffer (300 mM NaCl, 30 mM sodium citrate) for 2 × 15 minutes before blocking reaction with PBS-HS. For visualization, the wholemounts were incubated with streptavidin-Alexa Fluor 594 or 488 (1:500), as appropriate, for 3 hours before being mounted using anti-fade mounting medium.

Assessment of Cellular Levels of Peroxide in Culture

For determination of reactive oxygen species (ROS) levels in cultures, the ROS-Glo H_2O_2 Assay Kit (Promega, Madison, WI, USA) was used as per the manufacturer's protocol. Briefly, incubations of cells with creatine (pretreatment for 24 hours with 5 mM creatine) and nontreated controls (standard medium) were established as described above except that cultures were also incubated with H_2O_2 substrates simultaneously with sodium azide (1 mM) for 1 hour. H_2O_2 substrates react with H_2O_2 in the media to produce luciferin precursors. The medium in the 24-well plates was then transferred to 96-well plates and mixed with an equal amount of ROS-Glo Detection Solution (D-cysteine and luciferase; 50 μL each) for 20 minutes to produce luciferin molecules that react with luciferase to generate a luminescent signal, which is proportional to the H_2O_2 concentration. Luminescence was quantified by spectroscopy using a luminometric plate reader (Fluostar Optima; BMG Labtech, Mornington, Victoria, Australia). For quantification, luminescence readings (expressed as luminescence units) from three individual coverslips per data point were averaged.

Assessment of Cellular ATP Content

For determination of ATP levels in culture, a firefly bioluminescence assay kit from Sigma-Aldrich Chemical Company was used. Cell samples were obtained by removing culture medium and extracting cellular contents, including ATP, into hot (65°C) distilled water for 5 minutes to denature ATP-metabolizing enzymes. ATP levels were then determined in comparison with a standard curve using a luciferin-luciferase assay on a luminometer (Fluostar Optima).

Creatine Tissue Assay

Levels of creatine were assessed in retinal homogenates and in blood plasma using a kit supplied by Sigma-Aldrich (product #MAK079). Retinal samples were prepared by rapid homogenization in four volumes of cold kit assay buffer, followed by centrifugation at 13,000*g* for 10 minutes at 4°C because high concentrations of proteins can interfere with the assay, retinal supernatants, and blood serum samples were spun again on 10 kDa MW cut-off spin filters (VivaSpin 500; Sigma-Aldrich). Samples were then assayed as per kit instructions, in assay buffer, in the presence of creatinase, by incubating all components together for 1 hour at 37°C. Suitable controls and blanks were established (as per kit instructions) and readings obtained after colorimetric assessment at 570 nm using a Fluostar Optima automated microplate reader with its own associated software package (BMG LabTech Pty Ltd., Mornington, Victoria, Australia). Creatine concentrations were calculated against a creatine standard curve and data expressed as nanogram per microliter of supernatant/serum. Twelve animals were used to obtain assay data as follows: six animals had been fed on normal chow and six on creatine-supplemented diet for 4 weeks.

Quantification of Retinal Neuronal Survival

To quantify neuronal survival in retinal cultures, images were captured using an Olympus DP73 scientific grade (Olympus, Tokyo, Japan), cooled CCD camera mounted on an Olympus

BX61 microscope with an epifluorescence attachment. In the case of calretinin-IR and GABA-IR neurons, counting was undertaken manually as numbers per microscopic field using a cell counter. Quantification of tau-IR neurons in mixed cultures or RGC cultures was carried out using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Images were converted to grayscale, inverted, and threshold-adjusted. Positive tau-immunolabeling was thus represented as red pixels on a white background and this was quantified as proportion of each image/captured field that was labeled. Counting was undertaken for five random fields per coverslip and on six to eight individual coverslips per treatment. Counts were expressed as percentages of nontreated (without NMDA or NaN_3) cells. Culture data were analyzed for significance using 1-way ANOVA followed by Tukey multiple-comparison test.

For retinal wholemounts, in each of the four quadrants, three rectangular areas (central, mid, and peripheral) each at 1.2, 1.9, and 2.6 mm from the optic disc were analyzed, yielding 12 separate retinal areas for counting. Each rectangular area measured 1.40 × 1.08 mm. RGC counts for wholemounts were expressed as percent of control eyes; TUNEL counts were expressed as counts of positively labeled cells per observed retinal field. Quantification was performed by masked observers. Data from wholemounts were analyzed using Student's *t*-test (normal diet versus creatine). A P value of < 0.05 was considered significant.

Results

Effect of Creatine on Sodium Azide-Induced Neuronal Loss in Retinal Cultures Lacking Ganglion Cells

Incubation of mixed retinal cultures with creatine alone at different concentrations (0.5, 1, and 5 mM) had no detrimental effect upon the numbers of calretinin- and GABA-positive neurons (Supplementary Fig. S1). Investigation into whether creatine augmented neuronal survival during mitochondrial dysfunction was conducted in both acute and chronic settings, which was achieved by treatment with the mitochondrial complex IV inhibitor sodium azide for 1 (10 mM) or 24 hours (1 mM), respectively.

Treatment with 10 mM sodium azide for 1 hour led to significant losses of calretinin- (40.1 ± 4.8% survival relative to untreated controls) and GABA-positive (13.9 ± 3.0% survival relative to untreated controls) neurons in the retinal cultures. Loss of GABA-positive neurons was greater relative to calretinin-positive neurons. Pretreatment with creatine led to significantly increased numbers of calretinin-positive neurons across all three creatine concentrations (Fig. 1; $P < 0.01$, $n = 12$). For GABAergic neurons, only pretreatment with the highest concentration of creatine (5 mM) resulted in a statistically significant increase compared with azide treatment alone (Fig. 1; $P < 0.01$, $n = 13$).

In the chronic paradigm of metabolic challenge, treatment with 1 mM sodium azide for 24 hours resulted in a loss of calretinin- (28.3 ± 1.8% survival relative to untreated controls) and GABA-positive (2.9 ± 0.8% survival relative to untreated controls) neurons. Pretreatment with creatine led to significantly increased numbers of calretinin-positive counts across all creatine concentrations (Fig. 2; $P < 0.05$, $n = 3$). GABA-immunoreactive neurons were more drastically affected by 24 hours of sodium azide, and pretreatment with creatine failed to elicit a significant preservation when analyzed by ANOVA followed by Tukey multiple-comparison test (0.5 mM creatine, $P = 0.78$; 1 mM creatine, $P = 0.42$, 5 mM creatine $P = 0.06$), although less conservative tests such as Dunnett's post hoc test did reveal a significant protection at the highest concentration of creatine (5 mM, $P = 0.04$).

Loss of GABA immunoreactivity is not necessarily indicative of neuronal viability, as it may simply represent diminished or altered content of their major cellular neurotransmitter. In order to better assess actual survival of retinal neurons in the context of metabolic dysfunction, we quantified the number of tau-immunoreactive neurons in azide-treated cultures in the presence or absence of creatine (Fig. 3). Labeling for the microtubule-associated protein and pan-neuronal marker, tau, persists until neurons have died. Treatment with 250 μM, 500 μM or 1 mM sodium azide for 24 hours resulted in an increasing loss of tau-positive neurons (25.0 ± 1.3%, 10.3 ± 1.6%, 3.8 ± 0.2% survival relative to untreated controls, respectively). Pretreatment with creatine led to significantly increased numbers of tau-positive cells across the three azide concentrations (71.3 ± 2.4%, 33.8 ± 1.7%, 17.1 ± 1.4% survival relative to untreated controls, respectively, $P < 0.001$, $n = 10$).

Effect of Creatine Kinase Inhibition on Sodium Azide-Induced Neuronal Loss in Mixed Retinal Cultures

Having established that creatine administration promotes survival of retinal neurons in culture, we next investigated whether the mechanism of action of creatine was mediated through creatine kinase, the action of which instigates the cellular creatine-phosphocreatine energy-buffering system. The results showed that when the CK inhibitor, DNFB, was present, the protection by creatine against sodium azide–induced neuronal toxicity was completely abolished (Table 2). This was the case for both calretinin- and GABA-positive neurons and for both the acute (1 hour) and chronic (24 hour) paradigms. DNFB, furthermore, had no toxic effect over a 24-hour period on either calretinin- or GABA-positive neurons when applied to cultures alone (Table 2).

Effect of Creatine on Sodium Azide-Induced ROS Production in Mixed Retinal Cultures

Creatine itself had no effect on levels of ROS in retinal cultures (Fig. 4A). In contrast, a 1-hour sodium azide treatment produced no change in the level of detectable ROS when applied at 0.1 mM (95.7 ± 1.6 of control), a significant increase in the level of ROS when applied at 1 mM (127.1 ± 0.6% of control; $^{*}P = 0.03$) and a reduction in the level of ROS when applied at 10.0 mM (75.1 ± 3.1% of control). However, the reduction in detectable ROS levels when sodium azide was applied at 10.0 mM likely reflects the widespread death of cells caused at this concentration (Fig. 1). Pretreatment with creatine (5 mM) was able to cause a significant reduction in azide-stimulated ROS levels both when this latter compound was applied at 0.1 mM (69.3 ± 5.2% of control; $^{*}P = 0.037$) and at 1.0 mM (74.6 ± 8.2% of control; $^{***}P < 0.001$). Creatine did not affect the levels of ROS detected after treatment with 10.0 mM azide.

Effect of Creatine on Sodium Azide-Induced Reduction in ATP Content in Mixed Retinal Cultures

Incubation of retinal cultures with sodium azide (10 mM) for 1 hour caused a reduced level of ATP compared with untreated controls (Fig. 4B; 55.5 ± 13.2%, $n = 3$). Pretreatment with creatine (5 mM) had no significant protective effect on the

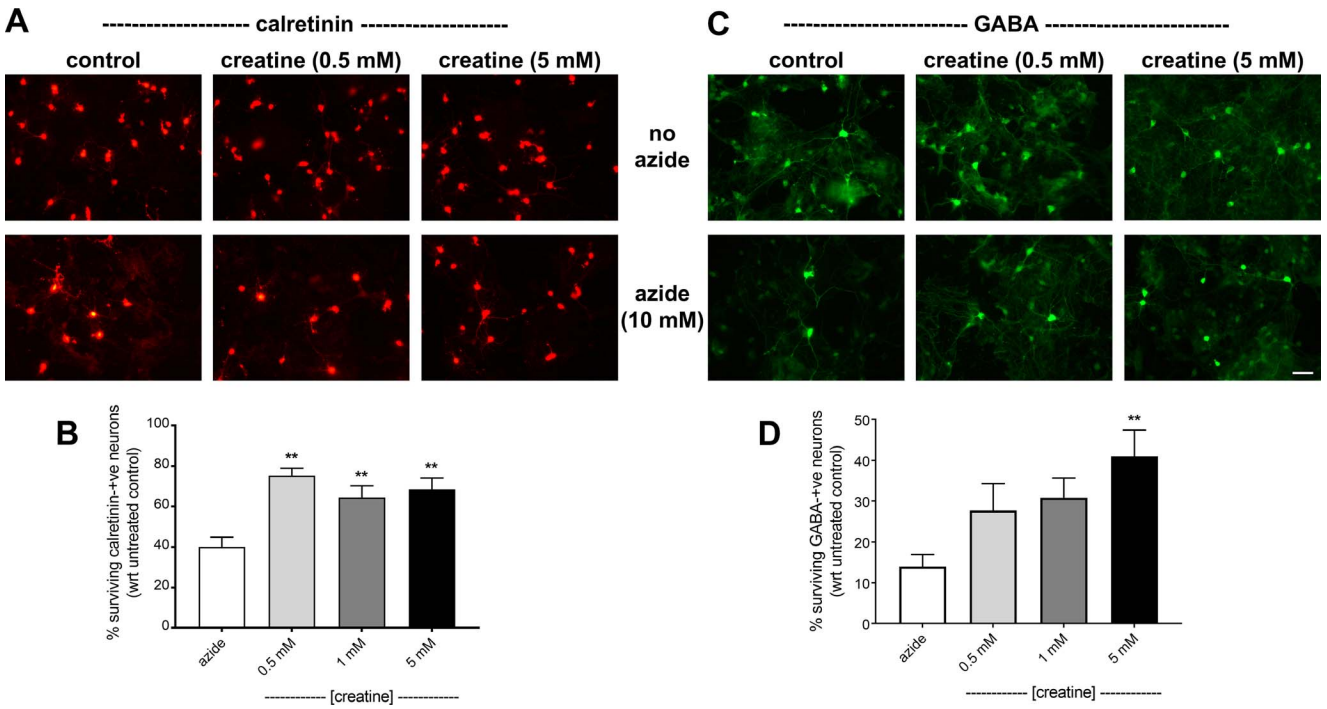

FIGURE 1. Effect of creatine on neuronal survival in mixed rat retinal cultures following an acute metabolic insult (sodium azide, 10 mM, 1 hour). (**A**, **B**) Calretinin-immunoreactive neurons. (**C**, **D**) GABA-immunoreactive neurons. No significant difference was detected in neuron survival after treatment with different creatine concentrations in the absence of sodium azide. In contrast, pronounced calretinin- and GABA-positive cell loss was observed in the sodium azide–treated group (**A**, **C**, respectively). Pretreatment of neurons with creatine for 24 hours elicited significant protection of both calretinin-immunoreactive neurons (**A**, **B**, 0.5, 1, 5 mM creatine) and GABA-immunoreactive neurons (**C**, **D**, 5 mM creatine). $^{*}P < 0.01$ by 1-way ANOVA followed by Tukey multiple-comparison test ($n = 6$–13). *Scale bar*: 20 µm.

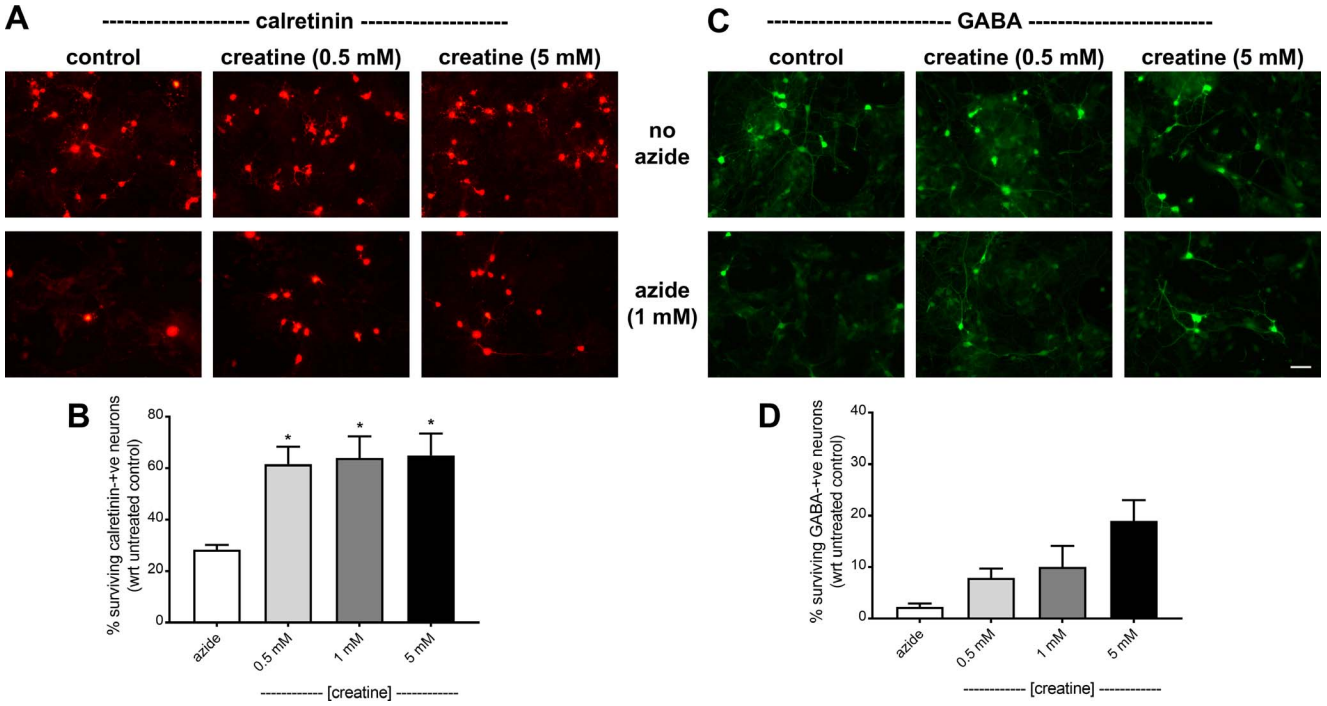

FIGURE 2. Effect of creatine on neuronal survival in mixed cultures following a chronic metabolic insult (sodium azide, 1 mM, 24 hours). (**A**, **B**) Calretinin-immunoreactive neurons. (**C**, **D**) GABA-immunoreactive neurons. No significant difference was detected in neuron survival after treatment with different creatine concentrations in the absence of sodium azide. In contrast, pronounced calretinin- and GABA-positive cell loss was observed in the sodium azide–treated group (**A**, **C**, respectively). Pretreatment of neurons with creatine for 24 hours elicited a significant protection of calretinin-immunoreactive neurons (**A**, **B**, 0.5, 1, 5 mM creatine). $^{*}P < 0.05$ by 1-way ANOVA followed by Tukey multiple-comparison test ($n = 3$–4). GABA-immunoreactive neurons were more drastically affected by 24 hours of sodium azide and pretreatment with creatine failed to elicit a significant preservation when analyzed by Tukey multiple-comparison test (**D**). *Scale bar*: 20 µm.

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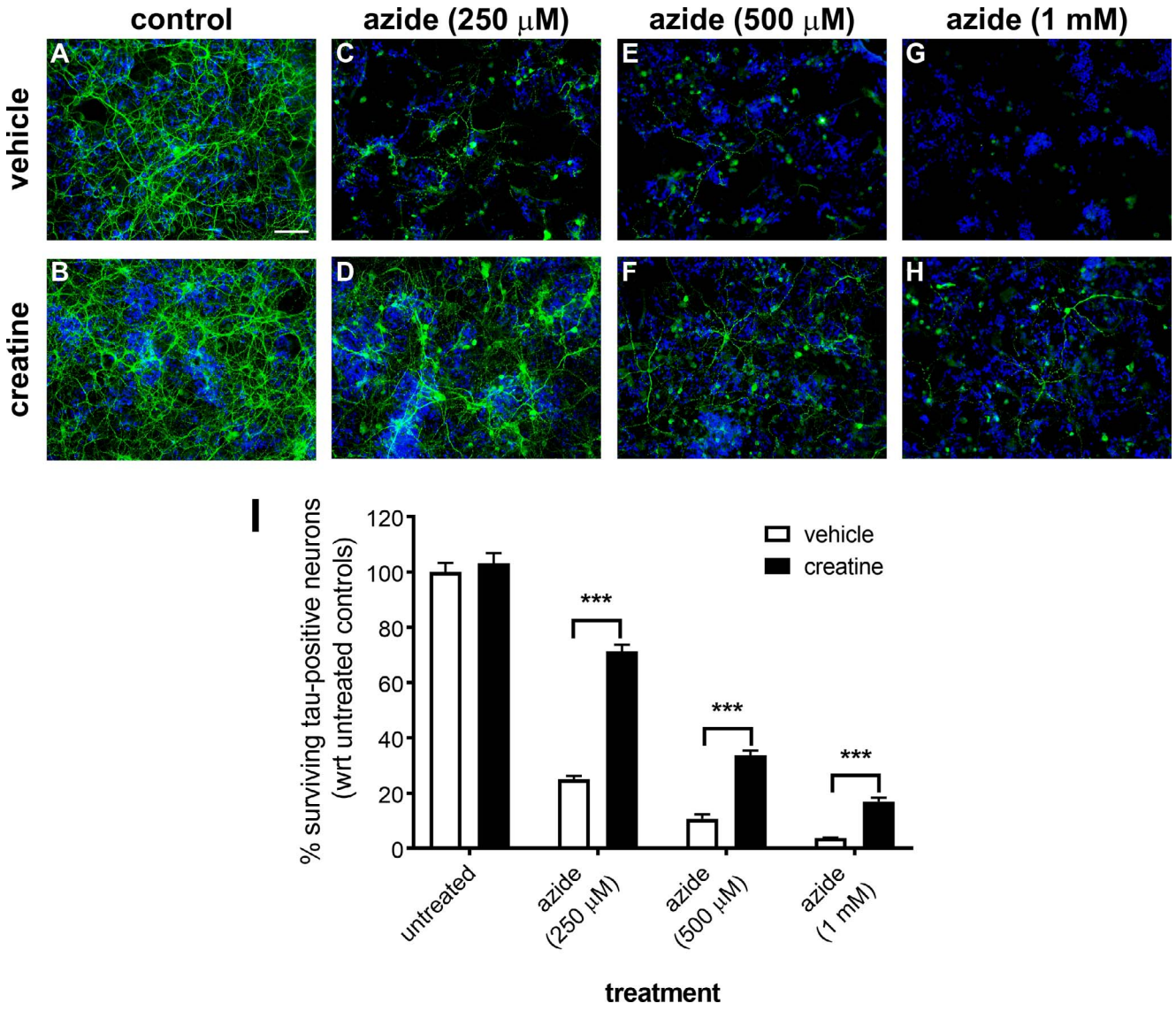

Figure 3. Effect of creatine on survival of tau-positive neurons in mixed rat retinal cultures following a chronic metabolic insult (sodium azide, 24 hours). No significant difference was detected in neuron survival after treatment with creatine in the absence of sodium azide (**A**, **B**, **I**). In contrast, an increasing amount of tau-positive cell loss was detected in the 250-μM, 500-μM, and 1-mM sodium azide–treated groups (**C**, **E**, **G**, **I**). Pretreatment of neurons with creatine for 24 hours elicited a significant protection of tau-immunoreactive neurons (**D**, **F**, **H**, **I**). $^{***}P < 0.001$ by Student's unpaired *t*-test with Tukey correction ($n = 10$). *Scale bar*: 40 μm.

Table 2. Effect of Creatine Kinase Inhibition on Protection of Neurons in Culture

Treatment	% GABA-Positive Neurons	% Calretinin-Positive Neurons
24-hr treatment		
Control	100.0 ± 6.9	100.0 ± 9.6
1 mM Azide	5.3 ± 2.0	32.3 ± 3.5
Azide + 5 mM creatine	26.7 ± 3.6*	53.7 ± 3.3*
10 μM DNFB	102.5 ± 7.1	99.7 ± 5.9
Azide + creatine + DNFB	4.7 ± 1.8†	27.1 ± 5.1†
1-hr treatment		
Control	100.0 ± 8.5	100.0 ± 9.8
10 mM Azide	18.4 ± 4.2	44.4 ± 4.7
Azide + 5 mM creatine	38.2 ± 5.4*	71.1 ± 7.7*
DNFB	102.9 ± 9.8	102.0 ± 8.3
Azide + creatine + DNFB	16.7 ± 3.4†	35.6 ± 4.2†

* $P < 0.05$ compared with azide-treated neurons.

† $P < 0.05$ compared with neurons treated with azide plus creatine when comparing groups via 1-way ANOVA plus Tukey's post hoc analysis. For all treatments, $n = 10$.

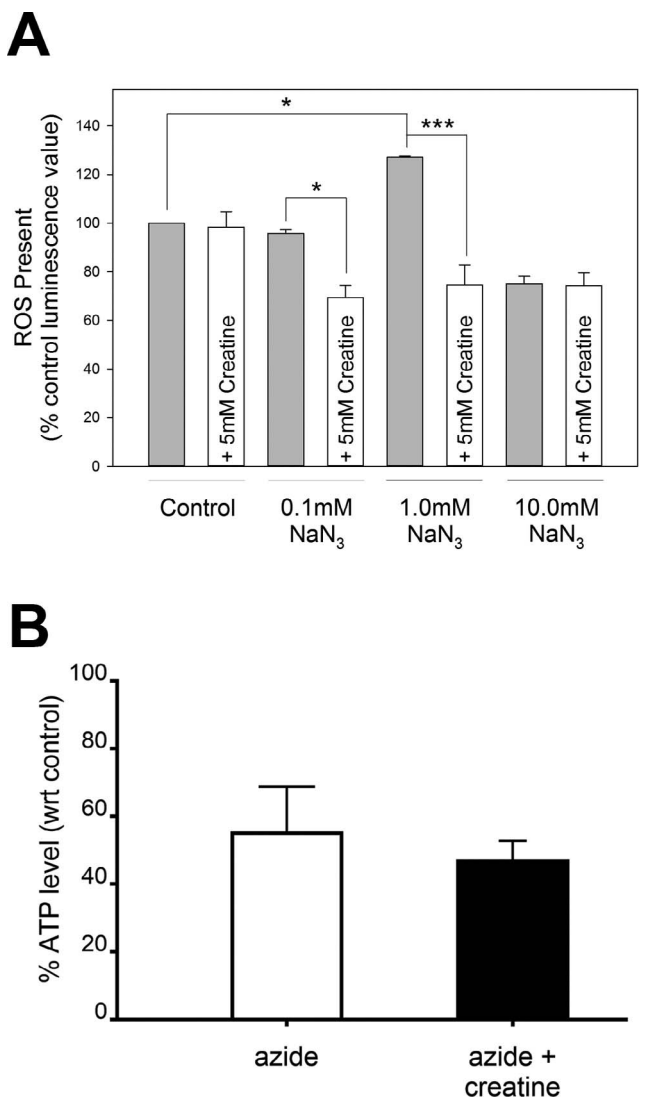

FIGURE 4. Effect of creatine on ROS production and ATP content in mixed retinal cultures subjected to an acute metabolic insult (sodium azide, 1 hour). (**A**) Sodium azide (1 mM) treatment induced an increase in the level of H_2O_2 in rat retinal cultures; at 0.1 mM azide produced no change in ROS, and at 10.0 mM azide produced a decrease in ROS, likely resulting from the death of cells caused at this concentration. Coincubation of azide (0.1 or 1.0 mM) with creatine (5 mM) resulted in significantly reduced H_2O_2 levels ($^*P < 0.05$ and $^{***}P < 0.001$, respectively, by 1-way ANOVA plus post hoc Tukey's HSD text; $n = 4$). (**B**) Sodium azide (10 mM) treatment induced a decrease in the level of ATP in rat retinal cultures relative to untreated controls. Coincubation with creatine (5 mM) had no effect on the level of ATP as altered by azide ($P = 0.59$ by Student's unpaired t-test, $n = 3$).

azide-stimulated reduction in ATP content (47.2 ± 5.5%, $P = 0.59$; $n = 3$).

Effect of Creatine on Excitotoxic Death of Retinal Neurons in Mixed Culture

Having determined that creatine was able to protect retinal neurons in culture from stress derived from mitochondrial dysfunction, we sought to examine whether this compound was protective in a more general sense (i.e., could it protect against a stressor with a different molecular basis). We therefore tested whether creatine could prevent neuron loss as a result of excitotoxicity (Fig. 5), which was induced in retinal cultures by treatment for 24 hours with 200 μM NMDA in the presence of 10 mM $CaCl_2$. This excitotoxic challenge led to significant losses of calretinin-positive (40.0 ± 4.0% survival relative to untreated controls; Figs. 5A, 5B) and GABA-positive (23.9 ± 5.5% survival relative to untreated controls; Figs. 5C, 5D) neurons in the retinal cultures. These reductions could be completely blocked in the presence of MK801 (10 μM), confirming that the effect of NMDA was receptor-mediated (Figs. 5A–D). Creatine (5 mM) was able to reverse the effect of NMDA on both calretinin- (67.9% ± 6.8% survival relative to untreated controls, $P < 0.05$, $n = 10$; Figs. 5A, 5B) and GABA-immunoreactive (83.9% survival relative to untreated controls ±8.9%, $P < 0.001$, $n = 10$; Figs. 5C, 5D) neurons. Creatine did not produce any significant protection of neurons at the lower concentrations tested (Figs. 6A–D).

Effect of Creatine on Survival of Neurons in Cultures Containing RGCs

Following identification of the protective influence of creatine against retinal neurons in vitro, cultures containing retinal ganglion neurons were established and examined to determine whether positive effects were specific to the cells present in the former system or whether they encompassed different classes of retinal neurons. Comparison of the two systems showed that, as expected, the major class of neuron group present in the mixed cultures was the amacrine cell (positive labeling for the amacrine cell-specific marker, in the retina, syntaxin-1) while the alternate cultures did, indeed, contain ganglion cells (as shown by positive labeling for RBPMS, neurofilament light chain and γ-synuclein; double labeling also shown for tau-immunoreactivity and γ-synuclein; Supplementary Fig. S2). Assessment of the effect of creatine specifically on neurons in cultures containing RGCs after exposure to sodium azide or NMDA was therefore subsequently undertaken (Fig. 6). Untreated cultures were generally replete with cells expressing neurites, including dendrites and axons (Figs. 6A, 6H), and this was unaffected by the presence of creatine alone (Figs. 6B, 6I, 0.5 mM creatine; Figs. 6C, 6J, 5 mM creatine). After exposure to either 500 μM sodium azide (Fig. 6D) or 200 μM NMDA (plus 10 mM $CaCl_2$; Fig. 6K) for 24 hours, however, there were less cells (i.e., less observable perikarya), less obvious axons, and less dendrites. However, the coincubation with creatine partially preserved all of these parameters after treatment with azide (0.5 mM creatine, Fig. 6E; 5 mM creatine, Fig. 6F) or NMDA (0.5 mM creatine, Fig. 6L; 5.0 mM creatine, Fig. 6M). Quantification of these effects demonstrated that creatine at both 0.5 and 5.0 mM produced statistically significant protection against sodium azide toxicity (Fig. 6G) and NMDA (Fig. 6N).

Expression of Creatine Kinase Isoforms in the Retina

Having ascertained that creatine prevented death of retinal neurons in vitro from both metabolic and excitotoxic insults, via a mechanism dependent upon CK, we next sought to examine whether this compound was neuroprotective in the in vivo setting. Prior to conducting these experiments, however, it was imperative to characterize the cellular distribution of CK in the retina, because the presence of a cellular creatine-phosphocreatine system is essential in order for any supplemental creatine to be able to provide energy buffering for neurons. To date, the distributions of CK isoforms in the rat retina is unknown. CK is an enzyme family comprising two major isoenzyme subclasses, the cytosolic isoforms (brain CK, CK-B; muscle CK, CK-M) and the mitochondrial isoforms (CK-MT1A and CK-MT1B).[19]

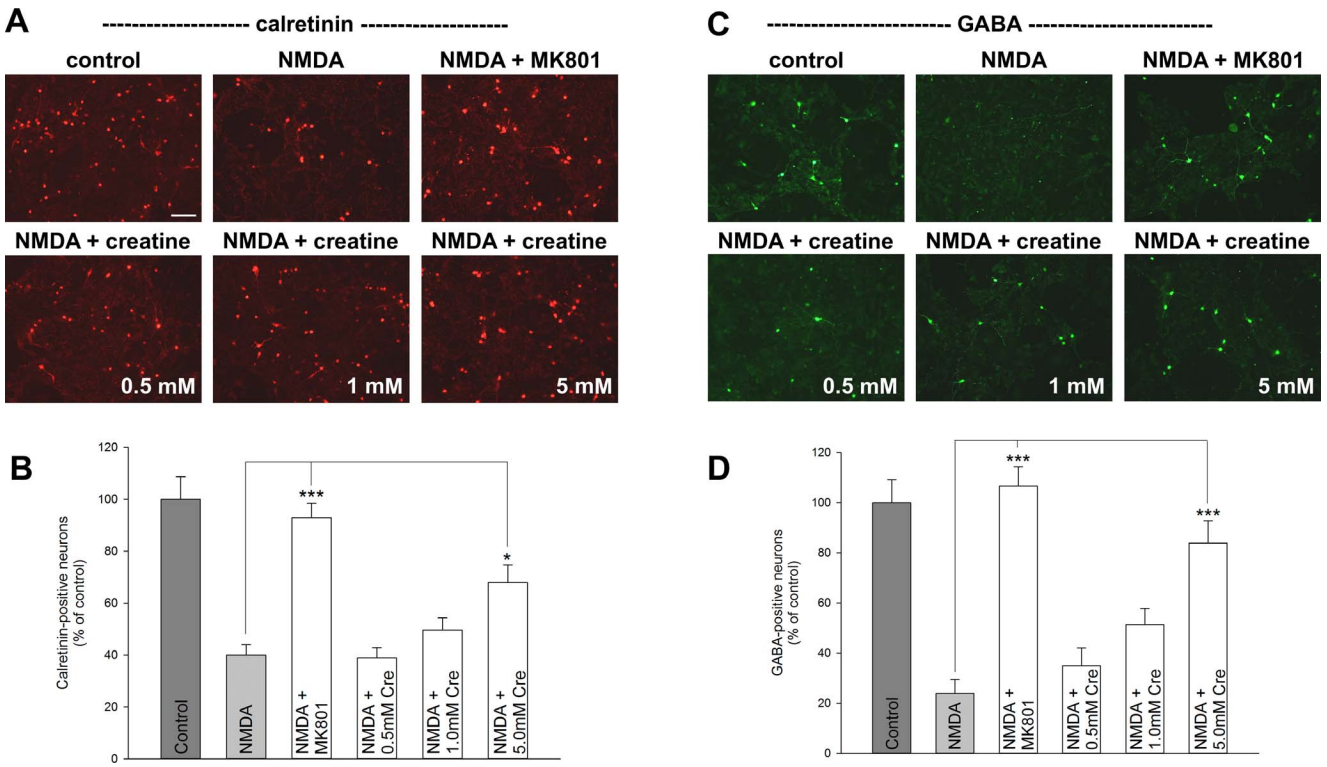

Figure 5. Effect of creatine on neuronal death induced by treatment with NMDA (200 μM) in mixed rat retinal cultures. (**A**, **B**) The number of calretinin-positive neurons was markedly reduced by NMDA treatment, which could be reversed entirely by MK801 (10 μM) and partially by creatine (5 mM). Creatine at 0.5 or 1 mM had no significant effect on negating cell death. (**C**, **D**) The number of GABA-immunoreactive neurons were also reduced by NMDA treatment. Again, this was completely reversed with MK801 and partially reversed by creatine (5 mM). Creatine at 0.5 or 1 mM had no significant effect on protecting these neurons. $^{***}P < 0.001$, $^{*}P < 0.05$, when compared with NMDA-treated values, by 1-way ANOVA followed by Tukey multiple-comparison test ($n = 10$ determinations for each). *Scale bar*: 40 μm.

By immunohistochemistry, the cytosolic form CK-B was widely distributed throughout the different cell types of the retina, as expected (Fig. 7A). Serial dilution of the antibody, however, revealed that this isoenzyme was most abundant within macroglial cells, namely Müller cells and astrocytes (Fig. 7B). This pattern of labeling was essentially consistent throughout the central nervous system, with CK-B preferentially localized to astrocytes of the optic nerve (Fig. 7C) and brain (Fig. 7D). Double immunolabeling in the retina with S100 (Figs. 7E, 7F) confirmed the predominant glial (Müller cell and astrocyte) localization of CK-B. In agreement with a glial localization, CK-B did not colocalize with the synaptic protein, synaptophysin, and was thus not enriched in neuronal synapses (Figs. 7G, 7H). The cytosolic isoenzyme CK-M was highly expressed in extraocular muscle (Figs. 7I, 7J), but the only other location expressing this enzyme in the retina was the basolateral surface of the nonneural RPE (Figs. 7K, 7L).

Of the mitochondrial isoforms of CK, CK-MT1A was present, as expected, throughout heart muscle (Figs. 7P, 7X), which expresses a large number of mitochondria. In the retina, CK-MT1A was expressed throughout the optic nerve head, notably in prelaminar axons (Fig. 7M), and in all layers of the retina excluding the outer nuclear layer (Figs. 7N, 7O). Labeling was particularly dense in photoreceptor segments, in both plexiform layers and in perikarya in the ganglion cell layer (Fig. 7O), which were proven to be RGCs via double labeling with Brn3a (Fig. 7Q). In the inner nuclear layer, CK-MT1A was notable in a discrete population of cells (Fig. 7N). Double-labeling experiments showed this labeling to be within the cell bodies of a population of bipolar cells, as CK-MT1A colocalized with the pan-bipolar cell marker Chx10 in some cells (Fig. 7S). There was, however, no obvious colocalization of labeling in any cell bodies in the inner nuclear layer with the rod bipolar cell marker, PKCα (Fig. 7T), which likely suggests that the only bipolar cells that express CK-MT1A in their perikarya are cone bipolar cells. Interestingly and furthermore, there was no discernible colocalization of CK-MT1A with the Müller cell–specific protein, glutamine synthetase, in cell bodies, end-feet, or processes (Fig. 7R). This clearly shows that CK-MT1A is not expressed at detectable levels in Müller cells. Results for the alternative mitochondrial CK, CK-MT1B, in the retina, mirrored that of CK-MT1A, except that labeling was less abundant. All locations were the same as CK-MT1A, however, as shown in the optic nerve head (Fig. 7U), retina (Fig. 7V), RGCs (Fig. 7W), and cardiac muscle (Fig. 7X).

By Western blotting (Fig. 7Y), each of the four CK antibodies recognized a major protein of the expected molecular weight in positive-control tissue extracts, namely heart and brain (CK-MT1A and CK-MT1B), brain (CK-B), and skeletal muscle (CK-M). In rat retinal extracts, CK-B and CK-MT1A were abundant, CK-MT1B was detectable but at a lower level, whilst CK-M was undetectable.

Assessment of Creatine Levels in Retina and Blood Plasma

After oral supplementation of creatine in feedstock for 4 weeks (Fig. 8), a statistically significantly elevated level of this additive was present in both retina (18.9 ± 0.9 ng/μL in treated versus 11.9 ± 0.9 ng/μL in control) and blood plasma (16.6 ± 2.3 ng/μL in treated versus 2.3 ± 1.5 ng/μL in control).

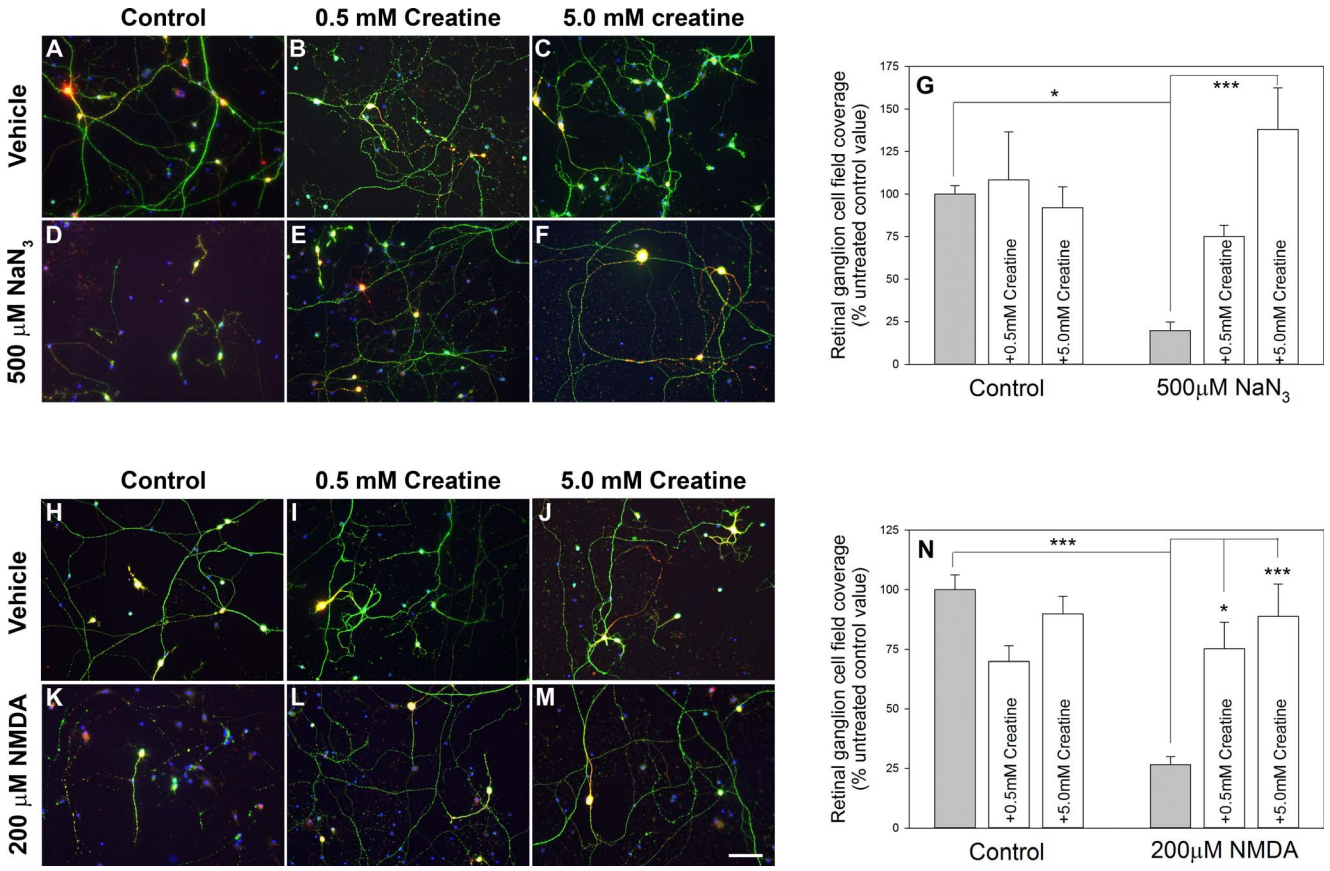

FIGURE 6. Effect of creatine against NaN_3- (**A–G**) and NMDA-induced (**H**, **N**) toxicity to rat retinal neurons in cultures containing RGCs. Assessment was made by immunocytochemical labeling (**A–F**, **H–M**) of cultured neurons with MAP2 (*red* labeling; dendrites), tau (*green* labeling; axons), and DAPI (*blue* labelling; nuclei). Quantification of effects was achieved using ImageJ (**G**, **N**). A 24-hour treatment with NaN_3 (500 μM) induced widespread damage to neurons, including loss of dendrites, destruction/shortening of axons, and reduction in numbers of cells (**D**). This was significantly prevented by creatine at both 0.5 and 5.0 mM (**E–G**). A 24-hour treatment with 200 μM NMDA was also extremely damaging to cultured neurons, again causing loss of dendrites, reduction of axons, and loss of whole cells (**K**, **N**). Damage to cells was significantly reduced in the presence of both 0.5 and 5.0 mM creatine (**L–N**). $^{***}P < 0.001$, $^{*}P < 0.05$, when compared with treated values, by 1-way ANOVA followed by Tukey multiple-comparison test ($n = 6$ determinations for each). *Scale bar*: 40 μm.

Effect of Creatine on Excitotoxicity-Induced RGC Loss In Vivo

Treatment with NMDA resulted in a significant loss of both Brn3a- (34.9 ± 5.4% survival relative to untreated control eyes) and γ-synuclein-labeled (36.1 ± 5.5% survival relative to untreated control eyes) RGCs after 7 days (Fig. 9). Rats that underwent prophylactic treatment with creatine showed a tendency to higher RGC counts (Brn3a-labeled RGCs, 43.9 ± 5.2% survival relative to untreated control eyes; γ-synuclein-labeled RGCs, 43.6 ± 4.5% survival relative to untreated control eyes), but the results did not reach statistical significance for either marker in any of the central, mid, or peripheral regions analyzed (Brn3a: $P = 0.43$, $P = 0.22$, $P = 0.15$; γ-synuclein: $P = 0.35$, $P = 0.30$, $P = 0.34$; $n = 15$–18, respectively).

Effect of Creatine on Excitotoxicity-Induced RGC Apoptosis In Vivo

While creatine did not reduce the overall level of neuronal death that occurred 1 week after excitotoxic challenge, it is conceivable that supplementation may have delayed the onset of neuronal loss. Thus, we analyzed whether creatine-treated rats displayed fewer apoptotic nuclei in retinal wholemounts than rats fed a normal diet, at a time point at which it is known that apoptosis is widespread in the retina after NMDA injection (8 hours).[26] In NMDA-treated animals, numerous TUNEL-positive nuclei were detected throughout the retina (Fig. 10; images shown are from midregion of retinas). The TUNEL-positive nuclei typically colocalized with shrunken Brn3a-positive cells (Fig. 10). Quantification of TUNEL-positive cells revealed no differences between the normal diet (288 ± 66 cells) and creatine-fed animals (270 ± 50 cells; $P = 0.39$, $n = 8$).

Effect of Creatine on Ischemia-Reperfusion-Induced RGC Loss In Vivo

Finally, we investigated whether creatine supplementation protected against ischemia reperfusion–induced injury in the retina, induced via acute elevation of IOP above systolic blood pressure for 75 minutes. ERG traces recorded from rats at 7 days after ischemia reperfusion showed a substantial loss of the b-wave, and a moderate decrease in the a-wave, when compared with baseline (Fig. 11). Treatment with creatine did not significantly preserve either the b-wave amplitude (0.15 ± 0.02 normal diet versus 0.15 ± 0.04 creatine diet; $P = 0.93$) or the a-wave amplitude (0.16 ± 0.03 normal diet versus 0.15 ± 0.03 creatine diet; $P = 0.89$).

Retinal ischemia reperfusion caused a significant loss of both Brn3a- (30.4 ± 3.8% survival relative to untreated control

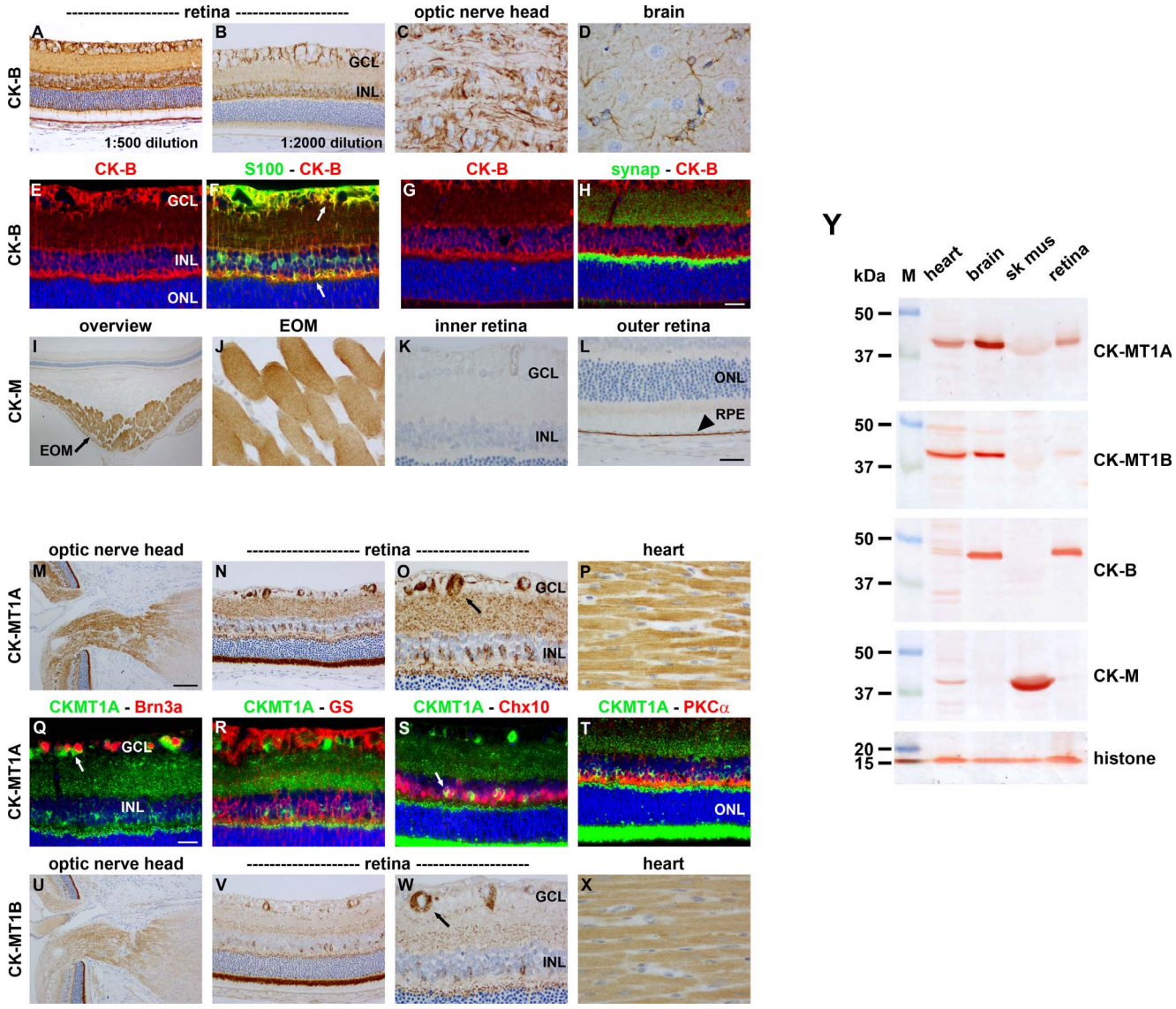

Figure 7. Expression of creatine kinase isoenzymes in rat retinal tissue. (**A–L**) Representative images of cytosolic creatine kinase isoenzymes in rat retina, optic nerve, extraocular muscle, and brain, as determined by immunohistochemistry. CK-B was widely distributed throughout the different cell types of the retina (**A**). Serial dilution of the antibody, revealed that this isoenzyme was most abundant within Müller cells and astrocytes of the retina (**B**), and astrocytes of the optic nerve (**C**) and brain (**D**). CK-B co-localized with the Müller cell/astrocyte S100 in the retina (**E**, **F**, *arrows*). In contrast, CK-B did not obviously colocalize with the synaptic marker synaptophysin in either plexiform layer (**G**, **H**). CK-M was highly expressed in extraocular muscle (**I**, **J**), but not within the neural retina. CK-M did, however, localize to the RPE (**K**, **L**). *Black scale bar*, (**I**) = 250 µm; (**A**, **B**) = 50 µm; (**C**, **D**, **J**, **K**, **L**) = 25 µm. *White scale bar*, (**E–H**) = 20 µm. (**M–X**) Representative images of ubiquitous mitochondrial creatine kinase immunolabeling in rat retina, optic nerve, and heart. In the optic nerve, CK-MT1A was expressed in prelaminar axons (**M**). In the retina, CK-MT1A labeling was intense in photoreceptor segments, in both plexiform layers, and in perikarya in the ganglion cell layer (**N**, **O**). CK-MT1A labelled cardiac muscle (**P**). Double-labeling immunofluorescence revealed colocalization of CK-MT1A with the RGC marker Brn3a (**Q**), but not with the Müller cell marker glutamine synthetase (**R**). In addition, CK-MT1A colocalized with the pan-bipolar cell marker Chx10 (**S**), but not obviously with the specific rod bipolar cell marker PKCα (T). CK-MT1B displayed a similar distribution to CK-MT1A in the retina, except that labeling was less abundant (**U–X**). *Black scale bar*, (**M**, **U**) = 100 µm; (**N**, **V**) = 50 µm; (**O**, **P**, **W**, **X**) = 25 µm. *White scale bar*, (**Q–T**) = 20 µm. (**Y**) CK isoform expression, as analyzed by Western immunoblot, in retinal extracts, and extracts of heart, brain, and skeletal muscle. Molecular weight markers were used to determine the size of detected gel products. For all proteins analyzed, a major band of the expected molecular weight is apparent in the relevant positive control tissues (see below), confirming the specificity of each antibody for its intended target in the rat. Histone H3 (15 kD), loading control; CK-MT1A (40 kD), heart, brain; CK-MT1B (40 kD), heart, brain; CK-B (45 kD), brain; CK-M (40 kD), skeletal muscle. In retinal extracts, a band of the expected molecular weight is observed for CK-MT1A and CK-B, a fainter band is apparent for CK-MT1B, while CK-M was undetectable. EOM, extraocular muscle; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

eyes) and γ-synuclein-labeled (31.2 ± 3.1% survival relative to untreated control eyes) RGCs after 7 days (Fig. 12). Rats that underwent prophylactic treatment with creatine showed a slight tendency to higher RGC counts Brn3a- (32.6 ± 7.3% survival relative to untreated control eyes) and γ-synuclein labeled (35.6 ± 6.9%, survival relative to untreated control eyes), but the results did not reach statistical significance for either marker in any of the central, mid, or peripheral regions analyzed (Brn3a: $P = 0.39$, $P = 0.79$, $P = 0.67$; γ-synuclein: $P = 0.15$, $P = 0.49$, $P = 0.65$; $n = 7$–8).

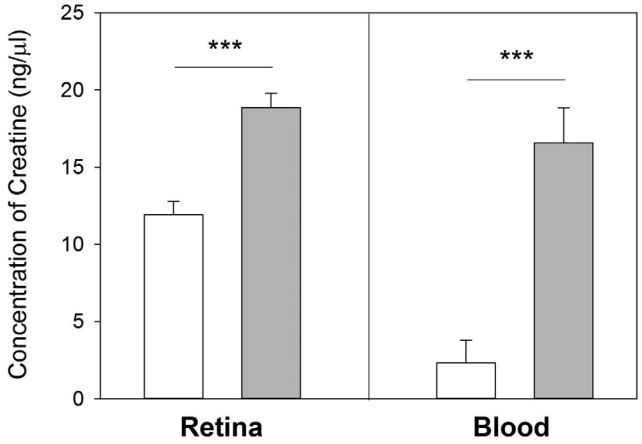

FIGURE 8. Assessment of creatine levels in blood plasma and within retinal tissue after dietary supplementation as 2%-enriched feed for 4 weeks. After 4-weeks supplementation in feed, creatine levels were significantly elevated in both blood plasma and within retinal extracts. ***$P < 0.001$ when compared with treated values, by unpaired Student's *t*-test analysis ($n = 6$ determinations for each).

DISCUSSION

Creatine is Neuroprotective Against Mitochondrial Stress and Excitotoxicity In Vitro

In this study, we conducted experiments to assess whether creatine was able to protect retinal neurons in cultures lacking and cultures containing RGCs from insults that detrimentally affect cellular metabolism or stimulate excitotoxicity. Initial studies employed a model of metabolic dysfunction induced by using sodium azide to compromise mitochondrial oxidative phosphorylation. We have previously determined in rat retinal cultures that sodium azide reliably induces neuronal loss via oxidative stress, mitochondrial membrane disruption, and energetic dysfunction.[22] In the present study, sodium azide caused significant neuronal loss when applied in both acute and chronic paradigms to mixed cultures. It also caused a marked degeneration and loss of neurons when applied to cultures enriched in RGCs for 24 hours. When creatine was added prophylactically to mixed cultures, a significant and concentration-dependent neuronal protection was observed when assessing numbers of both calretinin- and GABA-positive

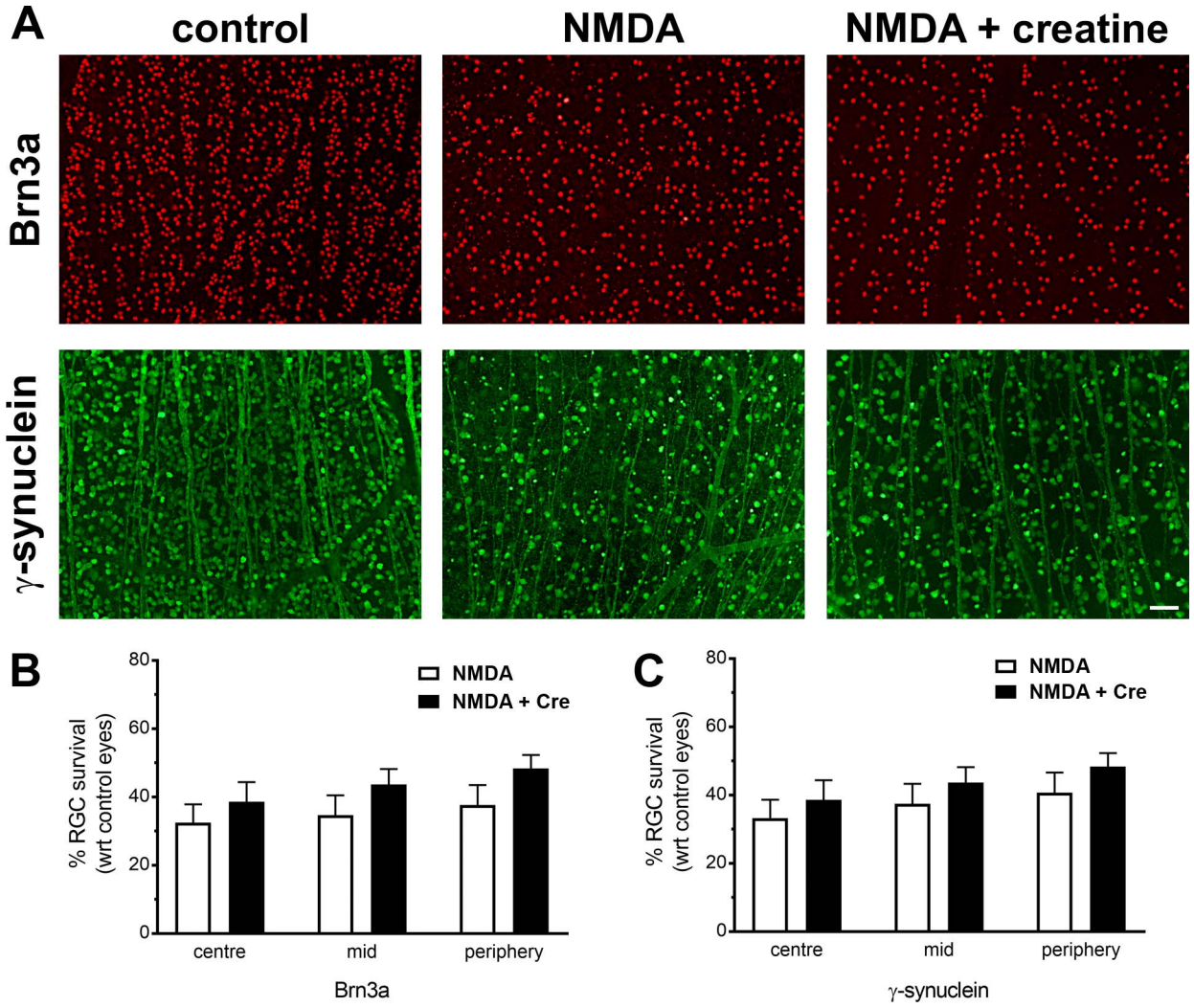

FIGURE 9. Effect of creatine supplementation on RGC survival in retinal wholemounts following NMDA-induced excitotoxicity. Intravitreal injection of NMDA (10 nmol) caused substantial decreases in the numbers of Brn3a- (**A**, **B**) and γ-synuclein-positive (**A**, **C**) RGCs after 7 days. Rats that underwent prophylactic treatment with creatine showed a tendency to higher RGC counts, but the results did not reach statistical significance for either marker in any of the central, mid, or peripheral regions analyzed (Brn3a: $P = 0.43$, $P = 0.22$, $P = 0.15$; γ-synuclein: $P = 0.35$, $P = 0.30$, $P = 0.34$; by Student's unpaired *t*-test, where $n = 15$–18). *Scale bar*: 100 µm.

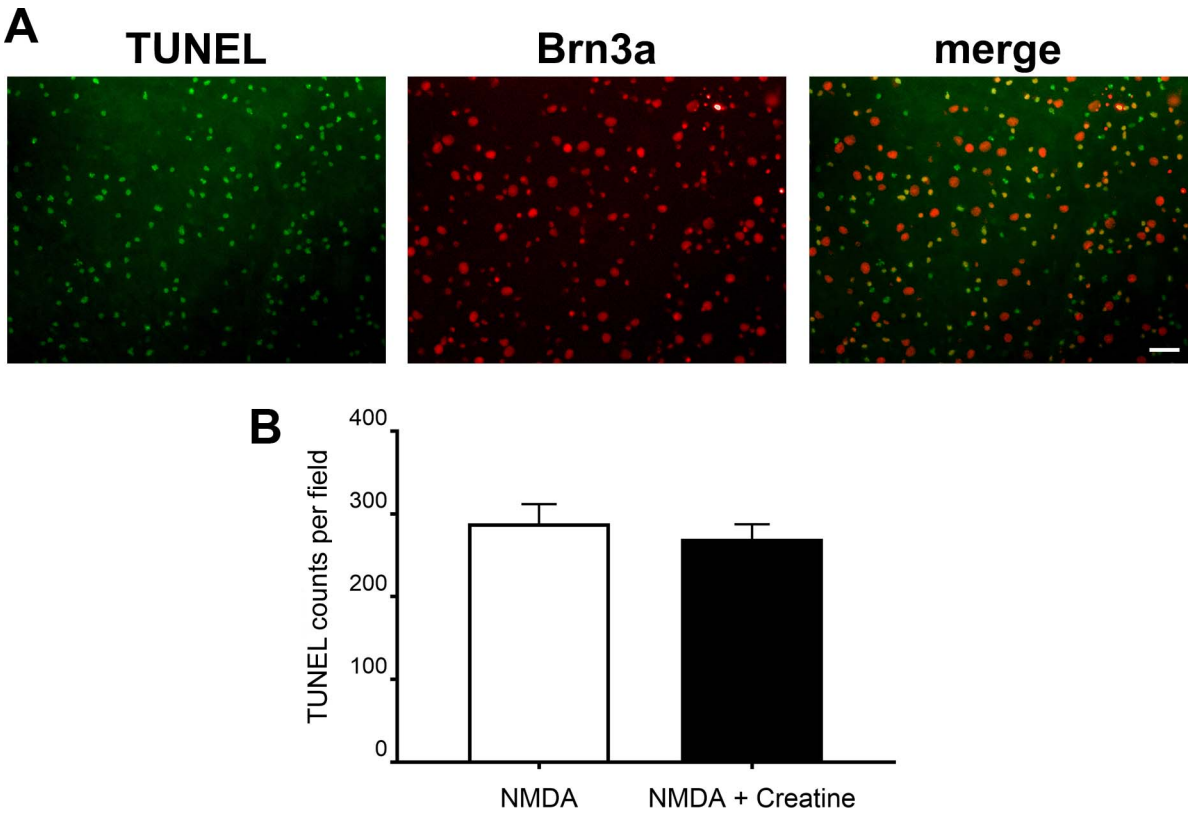

FIGURE 10. Effect of creatine supplementation on TUNEL labeling in retinal wholemounts following NMDA-induced excitotoxicity. (**A**) Double-labeling immunofluorescence of TUNEL labelling and Brn3a in retinal wholemounts analyzed 8 hours after intravitreal injection of NMDA; images represent midpoint regions of the retina. (**B**) NMDA treatment induced a marked increase in TUNEL labeling, which was not counteracted by prophylactic treatment with creatine ($P = 0.39$, by unpaired Student's *t*-test; $n = 8$). *Scale bar*: 50 μm.

cells. Both calretinin and GABA are reliable markers, which label distinct neuron sets in rat retinal cultures, both predominantly label-specific populations of amacrine cells.[27,28] In order to confirm a more generic protective effect to neurons, we also labeled cultures with the pan-neuronal marker, tau. In agreement with calretinin- and GABA-labeled neurons, we detected a clear neuroprotective effect of creatine to tau-labeled neurons subjected to sodium azide–induced metabolic compromise. Subsequent to these studies we also showed that creatine was able to proffer a significant protective effect to RGCs when present in enriched cultures. The neuronal protections observed in our culture systems were consistent with other in vitro studies, which have demonstrated positive effects of creatine in paradigms of central nervous system neurodegenerative diseases,[18,29–32] including against sodium azide–induced metabolic dysfunction in cortical axons.[22] We also found that creatine was able to protect retinal neurons in both mixed cultures and RGC-containing cultures from an excitotoxic challenge, as previously demonstrated by Brewer and colleagues.[30] Interestingly, although superficially manifest via distinct pathways—with excitotoxicity largely brought about by uncontrolled stimulation of intracellular calcium increases, and mitochondrial compromise largely brought about by ATP synthesis abridgement—these two insults must share a common mechanism of toxicity. It is this pathway or process that must be blocked by the action of creatine.

Mechanisms of Protection

Having shown in our in vitro studies that creatine could protect neurons in different preparations from different insults; mechanistically, we showed that it was also able to reduce oxidative stress. The presented data were in agreement with previously proposed mechanisms for creatine in studies related to models of other neurodegenerative diseases.[18,29–45] We did not, however, detect that creatine was able to elevate the level of cellular ATP. Previous studies have not consistently detected higher ATP levels in cultured neurons treated with creatine, but they have suggested a role for creatine in neuroprotection as an energy buffer. In 2007, Prass and colleagues[43] found an augmented cerebral blood flow and a 40% reduction in infarct volume in creatine-fed mice in a rodent model of cerebral ischemia, but no significant increase in ATP content. Conversely, Wilken and colleagues[46] treated rat dams with anoxia for 30 minutes and found that in creatine-fed animals subjected to the same insult, histologic brain slices of their pups showed a significantly higher level of ATP. Brewer and colleagues[30] incubated hippocampal neurons with creatine and showed protection against glutamate-induced excitotoxicity, but did not find a significantly higher ATP level in the neuronal tissues.[30] Their study did, however, show that in the early stages of glutamate exposure, the phosphocreatine-to-ATP ratio was significantly higher in creatine-treated neurons, although the creatine level itself was lower. They proposed that this change in creatine/phosphocreatine ratio was due to initial conversion of new intracellular creatine to phosphocreatine with the consumption of ATP (and rise in phosphocreatine). Thus, creatine likely acted as an energy buffer instead of an immediate energy supply. Furthermore, in models of cellular injury, energy consumption may easily exceed the ability of the cell to regenerate high-energy phosphates, hence explaining the lack of elevation of ATP content in our own and other studies. An elegant study by Walsh et al.[17] provided evidence of a key role

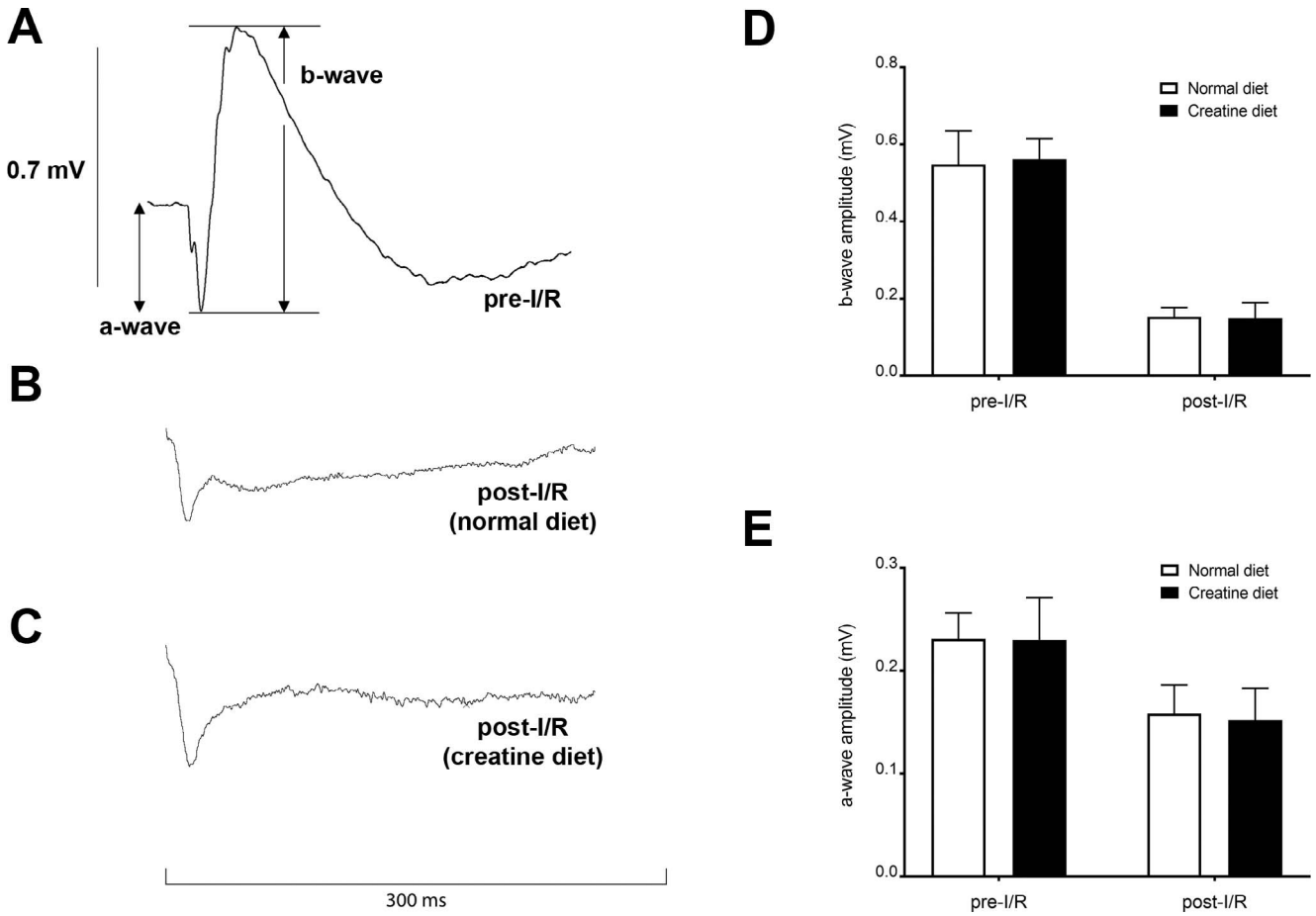

Figure 11. Representative ERGs recorded prior to- (**A**), and 7 days after 75 minutes of high IOP-induced retinal ischemia reperfusion (**B**, **C**). Ischemia reperfusion caused decreases in the a- and b-wave amplitudes (**D**, **E**). Rats that underwent prophylactic treatment with creatine showed no preservation of a- or b-wave amplitudes (**D**, **E**). I/R, ischemia reperfusion.

for both creatine and phosphocreatine in regulation of metabolism. They showed that creatine augmented mitochondrial respiration, which is stimulated by elevated cellular ADP, but interestingly they also showed that phosphocreatine had an opposite effect (i.e., it reduced the ADP-induced stimulation of mitochondrial metabolism). These data inform us that rather than being purely involved in energy buffering and phosphate shuttling, the creatine/phosphocreatine ratio can directly affect the rate of mitochondrial production of ATP. Taking all of this information into account, the lack of an increased ATP level in our study may well reflect the fact that creatine-stimulated mitochondrial respiration, which contributed toward neuroprotection, but that this increased mitochondrial output ultimately reflected elevated phosphocreatine rather than ATP.

An important finding in our study was that the positive effect of creatine in culture against azide-induced toxicity was abolished in the presence of a CK inhibitor. Because creatine-phosphocreatine cycling via CK action is thought to be the major means by which energy buffering and intracellular shuttling is achieved,[19] this may seem self-evident. However, inhibiting CK, per se, would not necessarily affect any direct stimulation of metabolism by creatine, nor would it affect any antioxidative role for this compound. Further, because mitochondrial ATP synthesis is drastically limited by azide treatment, then there would be inadequate phosphocreatine to shuttle to the cytoplasm. What is likely is that in the azide-toxicity model, electron transport complex inhibition results in a stimulation of nonmitochondrial (glycolytic) respiration via the Warburg effect,[47] a conclusion supported by our earlier work in which we showed that glucose counteracts azide toxicity to neurons in retinal cultures.[22] Thus, the action of creatine in the azide-induced toxicity model must, first, be manifest in the cytoplasm and, second, be manifest through the action of a nonmitochondrial CK. Therefore, we postulate the importance of the cytoplasmic isoform of CK, acting independently of the mitochondrial enzyme. This conclusion is given indirect support by our finding that retinal neurons express both mitochondrial and cytoplasmic CK isoforms, but retinal macroglia, including Müller cells and astrocytes, only express a cytoplasmic form of CK (CK-B). These data together suggest that neuronal tissues, including the retina, may, in fact, not use the classically described intracompartmental cellular creatine-phosphocreatine shuttle.

Creatine Supplementation is Not Neuroprotective in Models of Ischemia and Excitotoxicity In Vivo

Having observed protection in vitro, via a CK-dependent mechanism, we next investigated whether creatine was able to protect retinal neurons in vivo from excitotoxic and ischemia-reperfusion injuries. Prior to conducting these experiments, however, we sought to characterize the distributions of the CK isoforms in the rat retina, which were hitherto unknown. Our results revealed, as highlighted above, that retinal neurons, notably photoreceptors, bipolar cells, and RGCs, particularly express mitochondrial CK isoforms, but retinal macroglia,

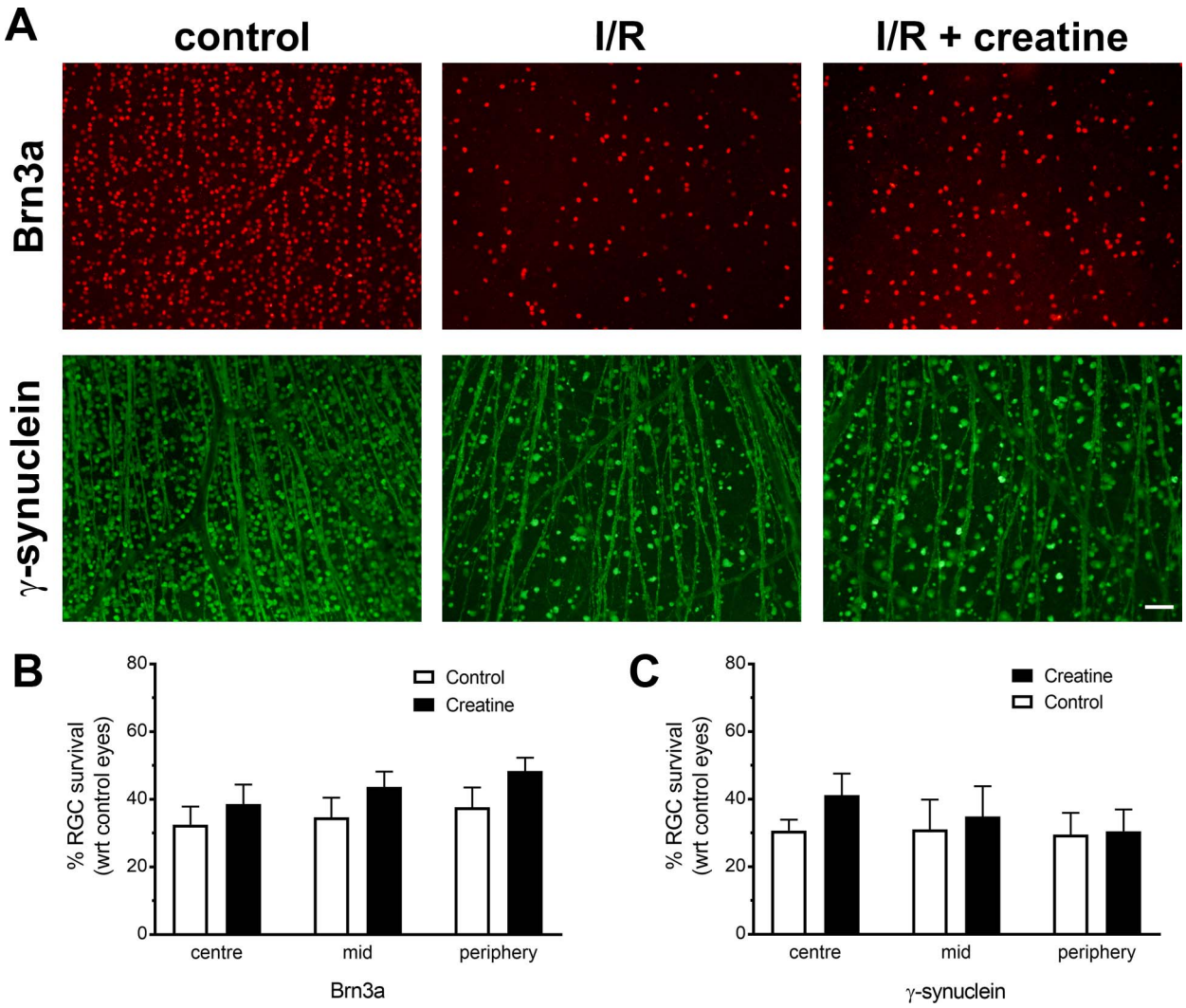

Figure 12. Effect of creatine supplementation on RGC survival in retinal wholemounts following ischemia-reperfusion injury. High IOP-induced retinal ischemia (75 minutes) caused substantial decreases in the numbers of Brn3a- (**A**, **B**) and γ-synuclein-positive (**A**, **C**) RGCs after 7 days. Rats that underwent prophylactic treatment with creatine showed a tendency to higher RGC counts, but the results did not reach statistical significance for either marker in any of the central, mid, or peripheral regions analyzed (**B**, Brn3a: $P = 0.39$, $P = 0.79$, $P = 0.67$; **C**, γ-synuclein: $P = 0.15$, $P = 0.49$, $P = 0.65$; by Student's unpaired *t*-test, where $n = 7$–8). *Scale bar*: 100 µm.

including Müller cells and astrocytes, only express a cytoplasmic form of CK (CK-B). The results are in complete agreement with previous studies in rodent and human brain, which have shown that mitochondrial CK is expressed exclusively in neurons, with cytoplasmic CK-B being predominantly localized to astrocytes.[48,49] The intense labeling of RGCs for mitochondrial CKs would suggest that the intracellular creatine-phosphocreatine shuttle plays a key role in regulating energy metabolism in mitochondria in these cells in vivo. This is an important finding as both the NMDA-induced excitotoxicity and high IOP-induced retinal ischemia-reperfusion models of injury primarily affect the RGC population.[2,50–54]

To assess RGC survival following excitotoxic and ischemic injuries, we supplemented the feed of test animals with a 2% creatine-enriched diet for 4 weeks prior to initiating insults. After this time, it was noted that retinal and blood plasma levels of creatine were significantly elevated after oral supplementation, proving this additive was available within the tissue after insults had been promulgated. In order determine RGC protection in in vivo situations, these cells were immunolabeled for two reliable, well-characterized markers, Brn3a and γ-synuclein.[55,56] Surprisingly, feeding rats a 2% creatine-enriched diet did not result in any significant protection against loss of RGCs in either model, although there was a trend of higher numbers of RGCs in both studies. With each series of experiments, comparisons of the numbers of remaining RGCs were undertaken after 7 days of treatment, a time point at which approximately 65% of RGCs had been lost. In the case of NMDA-induced excitotoxicity, cell death is known to be rapidly induced within a few hours,[26] thus, it was considered conceivable that creatine may have delayed, but not prevented, the onset of cell death, such that the effect was no longer evident at the 7-day time point of analysis. Accordingly, we also analyzed the effect of creatine on NMDA-induced RGC death by quantifying the extent of TUNEL labeling at 8 hours, a time point known to be near to peak of apoptosis.[57] The results showed no difference in the numbers of TUNEL-positive RGCs between rats fed normal chow and those on a creatine-enriched diet, data that are consistent with the quantification of RGC numbers at 7 days.

Our negative results are in contrast to those of Malcon and colleagues,[33] who treated rats with 1% creatine for 1 week

before and after subjecting them to intracerebral injection of NMDA and showed a significant reduction in volume of NMDA-induced striatal lesions (~20%) in the creatine-treated brains compared with controls. Conversely, in a recent study, intracerebral β-amyloid injections were performed on Wistar rats before feeding these animals on a diet containing 2% (wt/wt) creatine for a total of 6 weeks. At the end of the experiment, several tests, including a measure for learning and memory retrieval as well as TUNEL staining on histologic sections, did not show any significant difference between the creatine-treated and control groups.[58]

Discrepancy Between In Vitro and In Vivo Findings

Mitochondrial compromise via sodium azide, excitotoxicity, and ischemia-reperfusion all lead to elevated cellular oxidative stress levels and increased apoptosis.[22] It is interesting that although creatine was able to protect neurons in vitro, protection was not evident in vivo. One putative explanation for this discrepancy is that the in vivo models of RGC injury were too drastic, such that the resultant toxicity overwhelmed any minor protection afforded by creatine. Both high IOP-induced retinal ischemia and NMDA-induced retinal excitotoxicity are acute models of retinal damage, rather than slow-progressing, chronic diseases, such as glaucoma. If creatine is neuroprotective, it would arguably be more evident in a model where damage is less pronounced and of a more protracted duration. However, previous experiments have demonstrated neuroprotective efficacy of compounds in these models (e.g., betaxolol[59]), meaning that protection of neurons is possible in these systems. Another possible explanation for the lack of neuroprotective effect seen in the animal models is that the prophylactic treatment regime of creatine, as applied via the oral route, may not provide a sufficient dose of this compound in the retina. However, the dosing regimen used herein (2% wt/wt in chow) is similar to that employed in other neuroprotection studies in the brain, and assaying for creatine levels after supplementation clearly showed elevated levels of this additive in the retina. In a Parkinson model pretreatment of mice with 1% creatine (wt/wt in chow) for 2 weeks prior to N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine injections almost completely abolished deleterious effects to dopaminergic neurons.[39] Similarly, in studies in a rodent model of amyotrophic lateral sclerosis, a dose-dependent increase in survival compared with placebo (reduction in neuronal cell death) was observed with 1% to 2% of creatine (in chow), when started within 1 to 2 months postpartum.[34] Interestingly, Moxon-Lester and colleagues[21] showed that retinal ischemia in rats leads to rapid and prolonged downregulation of creatine transporter-1, with eventual recovery by day 10 postischemia. Creatine transporters are preferentially localized to photoreceptor inner segments and neurons in the inner retina, including amacrine and RGCs, and are required for cellular uptake of creatine from the blood.[60] Therefore, the lack of protection in our ischemia-reperfusion experiment may be explained by inadequate uptake of creatine into vulnerable cells throughout the postinsult period because of creatine transporter downregulation.

Conclusion

In conclusion, prophylactic creatine treatment provided neuroprotection to retinal neurons in culture by mechanisms mediated via CK that likely involved reducing oxidative stress. Yet, creatine was not protective to RGCs in models of excitotoxicity and ischemia reperfusion in vivo, under the conditions that were employed here. This discrepancy needs to be further explored and validated in future studies; for example, in chronic animal models of RGC damage, for instance, experimental glaucoma models that induce slow progressive loss of RGCs.

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