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REVIEW ARTICLE

Creatine in mouse models of neurodegeneration and aging

T. Klopstock · M. Elstner · A. Bender

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Abstract The supplementation of creatine has shown a marked neuroprotective effect in mouse models of neurodegenerative diseases (Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis). This has been assigned to the known bioenergetic, anti-apoptotic, anti-excitotoxic and anti-oxidant properties of creatine. As aging and neurodegeneration share pathophysiological pathways, we investigated the effect of oral creatine supplementation on aging in 162 aged wild-type C57Bl/6J mice. The median healthy life span of creatine-fed mice was 9% higher than in their control littermates, and they performed significantly better in neurobehavioral tests. In brains of creatine-treated mice, there was a trend toward a reduction of reactive oxygen species and significantly lower accumulation of the "aging pigment" lipofuscin. Expression profiling showed an upregulation of genes implicated in neuronal growth, neuroprotection, and learning. These data showed that creatine improves health and longevity in mice. Creatine may, therefore, be a promising food supplement to promote healthy human aging. However, the strong neuroprotective effects in animal studies of creatine have not been reproduced in human clinical trials (that have been conducted in Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis). The reasons for this translational gap are discussed. One obvious cause seems to be that all previous human studies may have been underpowered. Large phase III trials over long time periods are currently being conducted for Parkinson's disease and Huntington's disease, and will possibly solve this issue.

Keywords Creatine supplementation · Animal models · Neurodegeneration · Parkinson's disease · Huntington's disease · Amyotrophic lateral sclerosis · Aging · Neuroprotection

T. Klopstock (✉)
Department of Neurology, Friedrich-Baur-Institute,
University of Munich, Ziemssenstr. 1, 80336 Munich, Germany
e-mail: tklopsto@med.lmu.de

M. Elstner · A. Bender
Department of Neurology, University Hospital Grosshadern
Campus, University of Munich, Marchioninistr. 15,
81377 Munich, Germany

Introduction

The mitochondrial theory of neurodegeneration and aging implies that reactive oxygen species (ROS), mitochondrial DNA (mtDNA) damage, and progressive respiratory chain dysfunction are mutually interacting in a vicious circle (Harman 1972; Bender et al. 2006b). Possible neuroprotective treatments may be targeted by reduction of ROS or enhancement of energy production. While the overexpression of antioxidant enzymes increases life span in several organisms (Schriner et al. 2005), this has not proved feasible in humans. Food supplemented with various antioxidants has so far failed to retard aging in mice (Lee et al. 2004) and men (Bjelakovic et al. 2007). Creatine is a natural ergogenic compound and is widely used by athletes as a food supplement to enhance muscular performance. It also has anti-apoptotic (O'Gorman et al. 1997), anti-excitotoxic (Xu et al. 1996), and direct antioxidative properties (Lawler et al. 2002), both in vitro and in vivo. Creatine meets all requirements proposed by the Committee to Identify Neuroprotective Agents in Parkinson's disease (CINAPS): (1) scientific rationale, (2) evidence of blood–brain barrier penetration, (3) adequate

safety data, and (4) efficacy in animal models (NINDS NET-PD Investigators 2006). This review summarizes data on creatine effects in mouse models of neurodegenerative diseases and aging.

Parkinson's disease

Parkinson's disease (PD) is characterized by progressive loss of dopamine neurons from the nigrostriatal pathway and by a slow onset of motor symptoms. Mitochondrial dysfunction and oxidative damage play a key role in the pathogenesis (Schapira 2008). While there is good symptomatic treatment for PD, neuroprotective approaches are urgently needed.

Mouse studies

Among various surgical, toxic and genetic approaches to model PD in animals, one neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has become the most widely used method in the last decades. MPTP is converted by monoamine oxidase B to 1-methyl-4-phenylpyridinium (MPP1), which blocks complex I of the mitochondrial electron transport chain leading to depleted striatal ATP concentrations. This pathogenic cascade led to the hypothesis that strategies to improve oxidative phosphorylation or to buffer ATP depletion might prove effective in attenuating MPTP neurotoxicity. In a pivotal paper, Matthews et al. (1999) found that oral supplementation with either 1% creatine or 1% cyclocreatine for 2 weeks prior to MPTP administration produced significant protection against MPTP-induced neuronal loss and dopamine depletion in mice. With regard to combination therapies, the combination of creatine with the cyclooxygenase 2 (COX-2) inhibitor rofecoxib (Klivenyi et al. 2003) as well as the combination of creatine and coenzyme Q10 (Yang et al. 2009) produced additive neuroprotective effects. These results suggest that combinations of therapies targeting different disease mechanisms may be a useful neuroprotective strategy for PD.

Clinical trials

The success of creatine in these experimental studies led to a few clinical trials in PD. In a futility trial, creatine (10 g per day) was shown to reduce the UPDRS (Unified Parkinson's Disease Rating Scale) scores in 67 early PD patients and could not be rejected as futile based on the 30% DATATOP futility threshold (NINDS NET-PD Investigators 2006). We conducted a double blind, placebo-controlled clinical pilot study on the effect of oral creatine on clinical and single photon emission computed tomography (SPECT) variables of PD progression (Bender et al. 2006a). Sixty patients with clinical and SPECT findings compatible with PD were enrolled and received oral creatine (loading dose of 20 g daily for 6 days, followed by 2 g daily for 6 months and 4 g daily for the remainder) or placebo over 2 years. Creatine treatment had neither significant effect on dopamine transporter SPECT, the primary endpoint, nor on total UPDRS. Among secondary endpoints, creatine improved patient mood and led to a smaller dose increase of dopaminergic therapy. These two trials, together with the excellent safety profile of creatine (Bender et al. 2008b) prompted the National Institutes of Health in 2007 to issue a large phase III clinical neuroprotection trial for PD, with a goal of recruiting 1,720 participants randomized to 10 g of creatine or placebo. This study is currently recruiting participants (http://www.parkinsontrial.ninds.nih.gov).

Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative condition, clinically characterized by chorea, dementia, and personality changes. It starts mostly in midlife and inexorably leads to death after a mean survival time of 15–20 years. At present, there is no effective treatment. HD is caused by expansion of a CAG triplet repeat leading to polyglutamine expansions in huntingtin, a protein of still unknown function. Glutamate excitotoxicity and impaired cellular energy metabolism have been implicated in HD pathogenesis, leading to the hypothesis that augmenting intracellular energy levels may ameliorate the neurodegenerative process.

Mouse studies

A breakthrough in HD research was the development of transgenic mouse models that represent the human disease much better than the formerly used toxic models (systemic administration of the respiratory chain complex II inhibitors 3-nitropropionic acid or malonate). Transgenic mice express exon 1 of the human HD gene containing a markedly expanded CAG repeat under the control of the human HD promoter. These mice develop a progressive neurological disorder with irregular gait, stereotypic movements, resting tremor, and epileptic seizures, and show loss of brain and body weight. Since creatine had produced significant neuroprotection in the toxic rat model (Matthews et al. 1998), Ferrante et al. (2000) placed transgenic mice vs. wild-type littermates on either unsupplemented diets or diets supplemented with 1, 2, or 3% creatine at 21 days of age. The mean survival time increased from 97.6 ± 0.7–106.6 ± 0.5 days with 1%

creatine ($p < 0.0001$), to 114.6 ± 0.9 days with 2% creatine ($p < 0.0001$), and 101.9 ± 1.0 days with 3% creatine ($p < 0.0002$). Moreover, creatine supplementation significantly improved body weight and motor performance, delayed the onset of diabetes, and slowed development of brain atrophy, loss of striatal neurons and formation of huntingtin-positive aggregates (Ferrante et al. 2000). Similar effects were obtained in another transgenic mouse model of HD in which transgenic mice expressed a cDNA encoding a 171 amino acid N-terminal fragment of huntingtin containing 82 CAG repeats (Andreassen et al. 2001a). In both studies, creatine supplementation started at a pre-symptomatic stage (at weaning, 21 days), which is obviously not reflecting the situation in patients and was, therefore, criticized. Importantly, however, it could be shown that creatine administration starting after onset of clinical symptoms, at 6 and 8 weeks of age, also significantly improved motor performance and survival in the R6/2 transgenic mouse model of HD (Dedeoglu et al. 2003).

Clinical trials

Unfortunately, this neuroprotective effect could not be replicated in the recent clinical trials. An open-label trial (13 patients, 10 g per day of creatine for 12 months) reported unchanged UHDRS (Unified Huntington's Disease Rating Scales) motor scores and neuropsychological test results after 12 months of therapy (Tabrizi et al. 2003) and a rather stable course at follow-up after 24 months (Tabrizi et al. 2005). This may indicate stabilization of the disease, but as expected, this open-label trial could not solve the issue. Verbessem et al. (2003) (41 HD patients, 5 g creatine per day for 12 months) found high safety and tolerability of creatine, but no significant differences in motor and cognitive function in a placebo-controlled double-blind trial. This may be because the treatment period (maximum 1 year) is rather short for impact on clinical endpoints. On these grounds, other trials employed surrogate markers as endpoints. In a randomized, double-blind, placebo-controlled study in 64 HD subjects, 8 g/day of creatine administered for 16 weeks was well tolerated and safe. Serum and brain creatine concentrations increased in the creatine-treated group and returned to baseline after washout. Serum 8-hydroxy-2′-deoxyguanosine levels, an indicator of oxidative injury to DNA, were markedly elevated in HD and reduced by creatine treatment (Hersch et al. 2006). In our studies, we employed 1H-MRS as a surrogate marker and conducted an open clinical pilot study on the effect of 8–10 weeks of creatine supplementation on brain metabolite levels and clinical symptoms in 20 HD patients (Bender et al. 2005). We found in 1H-MRS a 15.6% decrease of unresolved glutamate (Glu) + glutamine (Gln); (Glu + Gln = Glx; $p < 0.001$) and a 7.8% decrease of Glu ($p < 0.027$) after creatine treatment. *N*-acetylaspartate tended to fall ($p = 0.073$), whereas total creatine, choline-containing compounds, glucose, and lactate remained unchanged. There was no effect on clinical rating scales. This cortical Glx and Glu decrease may be explained by creatine enhancing the energy-dependent conversion of Glu–Gln via the Glu–Gln cycle, a pathway known to be impaired in HD. Since Glu-mediated excitotoxicity is presumably pivotal in HD pathogenesis, these results indicate a therapeutic potential of creatine in HD. Thus, long-term clinical trials are warranted. A large placebo-controlled double-blind phase III trial of high-dose creatine (up to 40 g) over a period of 36 months in 650 HD patients is currently recruiting patients (CREST-E; cf. http://www.clinicaltrials.gov).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder affecting both upper and lower motor neurons, leading to widespread paralysis and death 2–5 years after disease onset (Pastula et al. 2010; Shaw 2005). Pathogenic mechanisms seem to include oxidative stress, glutamate excitotoxicity, impaired mitochondrial function, and aberrant protein folding (Turner and Talbot 2008). Familial ALS accounts for 5–10% of all ALS cases and is caused by a variety of genetic mutations, the most common in copper/zinc superoxide dismutase (SOD1), a cytosolic enzyme responsible for scavenging oxidative stress in the cell (Hervias et al. 2006). Currently, riluzole (a glutamate antagonist and sodium channel blocker) is the only approved disease-modifying medication for ALS, modestly improving survival by approximately 2–4 months (Lacomblez et al. 1996).

Mouse studies

Derived from SOD1 mutations being the most frequent cause of human familial ALS, transgenic mice carrying 23 copies of the human SOD1G93A gene have evolved as the standard murine ALS model. These mice show early mitochondrial swelling and vacuolization as well as altered electron transport chain enzyme activities (Wong et al. 1995; Gurney et al. 1994) corresponding to similar mitochondrial abnormalities observed in postmortem spinal cords of ALS patients (Hervias et al. 2006). Impaired mitochondrial function eventually leads to reduced ATP levels, increased oxidative stress and increased mitochondria-initiated apoptosis and may thus contribute to motor neuron cell death. Therefore, buffering cellular energy with exogenous creatine supplementation might exert neuroprotective effects and provide an effective treatment paradigm for ALS.

In fact, oral administration of creatine in this SOD1 mouse model starting at 70 days of age protected against the loss of neurons in both the motor cortex and in substantia nigra, reduced the level of oxidative damage, produced a dose-dependent improvement in motor performance and extended survival from 143.7 ± 2.3 to 157.2 ± 2.8 days with 1% creatine ($p < 0.05$) and to 169.3 ± 4.7 days with 2% creatine ($p < 0.001$) (Klivenyi et al. 1999). These findings were confirmed by the same group treating mice with creatine 2% starting at 4 weeks of age (Andreassen et al. 2001b). Again, survival increased significantly from 133.6 ± 2.5 in the regular fed to 153.2 ± 2.8 in the treated mice ($p < 0.001$). The motor performance in SOD1 mice on unsupplemented diets started to decline at age 110 days, and 2% creatine treatment delayed the onset of motor deficits by approximately 15 days.

With regard to combination therapies, the effects of creatine supplementation in SOD1 mice were found to be similar to the effects of riluzole, whereas a combination of the two was not better than either alone (Snow et al. 2003). In contrast, the combination of minocycline (a tetracycline antibiotic with neuroprotective properties) and creatine (Zhang et al. 2003) as well as the combination of creatine with the cyclooxygenase 2 (COX-2) inhibitors celecoxib or rofecoxib (Klivenyi et al. 2004) produced additive neuroprotective effects. These results suggest that combinations of therapies targeting different disease mechanisms may be a useful strategy in the treatment of ALS. A more recent study, however, was unable to reproduce the positive effects of either creatine, minocycline or even riluzole (Scott et al. 2008) highlighting, primarily, the dangers of confounding variables and low animal numbers in mouse trials (see below).

Clinical trials

Shortly after creatine was shown to prolong survival in animal models of ALS, clinical trials in human subjects were commenced. While a small study on creatine first showed promise in slowing ALS progression (Mazzini et al. 2001), three larger randomized, double-blind, placebo-controlled studies in 175, 104 and 107 patients, respectively, were unable to show any benefit of creatine on survival or functional measures (Rosenfeld et al. 2008; Shefner et al. 2004; Groeneveld et al. 2003). A recent systematic Cochrane review pooled the data from these three well-designed clinical trials involving a total of 386 ALS patients randomized to either creatine 5–10 g per day or placebo. Creatine was well tolerated, but did not have a statistically significant effect on survival, ALS functional rating scores or forced vital capacity (Pastula et al. 2010).

Analogous to the mouse studies, combining agents with different mechanisms of action may be superior to monotherapies in treating ALS. A futility trial indicated that a combination of creatine and celecoxib may be preferable to a combination of creatine and minocycline for further evaluation (Gordon et al. 2008).

In summary, creatine in doses of 5–10 mg/day did not have a significant beneficial effect on survival or progression of typical ALS. If creatine were to be researched again in the future, we would suggest starting it much earlier in the disease course (if possible), using higher doses, or combining it with other drugs.

Aging

Since aging and neurodegeneration share pathophysiological pathways (Beal 2005), creatine may also exert an anti-aging effect.

Mouse studies

We examined "healthy" life span, neurobehavioral functioning, and aging-associated changes of biomarkers in 162 female wild-type C57BL/6J mice fed a standard rodent diet ad libitum with ($n = 81$) or without ($n = 81$) 1% creatine beginning at the age of 365 ±2 days (Bender et al. 2008a). Mean "healthy" life span (i.e., age at which mice were classified as suffering from disease) was higher in creatine-fed mice (613 ± 84 days) than in their littermates (563 ± 95 days), corresponding to a 9% increase ($p < 0.05$). The effect on maximum life span was less pronounced, but still significant (716 ± 14 days vs. 692 ± 7 days for the top 10% longest lived animals of each group; $p < 0.05$). This 9% increase in healthy life span of our mice seems rather modest compared with the increased life span achieved by caloric restriction and genetic models of longevity (Rogina and Helfand 2004; Weindruch 1996; Schriner et al. 2005). Yet, from a practical point of view, neither of these two approaches is likely to be directly applied to human aging. Natural dietary supplements such as creatine could on the other hand be realistically tried in human aging, since they are easily applicable and have a favorable safety profile.

Prolonged life in creatine-fed animals was also accompanied by prolonged health (Bender et al. 2008a). In our study, while tests like the SHIRPA protocol, rotarod, or grip strength analysis showed no group differences, creatine-treated mice exhibited improved object recognition memory ($p < 0.05$), a decreased latency to initiate exploration of the novel environment ($p < 0.01$), and a trend toward increased forward locomotor activity (modified Hole Board Test, $p = 0.05$). The level of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of oxidative damage to DNA, tended to be lower in the brain of

creatine-fed mice, but with low n values this difference failed to reach significance ($n = 8$ creatine-mice: 7.13 ± 1.76; $n = 8$ placebo mice: 9.12 ± 2.97 pmol/mg; $p = 0.08$). Accumulation of the "aging pigment" lipofuscin (visualized by autofluorescence) in the hippocampus tended to be lower in creatine-fed mice than in controls ($p = 0.06$). It is not clear whether the lower brain lipofuscin in our creatine mice is due to reduced lipofuscin production or accelerated removal. We propose, however, that creatine by its antioxidative effects lowers free radical production and consecutively lipofuscin generation. Gene expression profiling from brain showed that a number of genes in creatine-fed mice are (1) reversely regulated compared with the regulation data published on aging mice and (2) concordantly regulated as in mice on caloric restriction. The biological processes most significantly affected include behavior, neurogenesis, energy pathways, and protein biosynthesis. Several up-regulated genes, such as *Bdnf*, promote the survival of neurons and protect against excitotoxic, oxidative, and metabolic insults (Mattson et al. 2002). During caloric restriction, the level of *Bdnf* is significantly increased by a factor of 1.4–1.6 in mouse brain (Duan et al. 2001). In our creatine-fed mice, *Bdnf* was 1.27 times higher than in controls. Similarly, the glial high affinity glutamate transporter *Slc1a3* was up-regulated in creatine-fed mice by a factor of 1.92. Impairment of the transport of glutamate into glial cells increases excitotoxicity and cell death, and is a pathogenic factor in both Alzheimer and Huntington disease (cf. above).

Overall, creatine had only discrete effects on life span and other biomarkers of aging. Yet, taken together, the results all point in the same general direction of a beneficial creatine-effect on aging and neurobehavioral functioning even though some of the experiments only reached the level of 'trends' in terms of statistical analysis. In this context, it is noteworthy; however, that our experiments were carried out on female mice of the most widely used inbred strain. Extended life span in different experimental settings has been shown to be gender-related with male sex being a factor associated more likely with longevity. Using female mice, we might have obscured more significant changes (Burger and Promislow 2004). Also, we have not used a model of accelerated senescence, but have relied on normal aging in order to make more relevant assumptions. Models of accelerated animal aging are likely to produce greater differences upon intervention, but they do not reflect normal aging (Nadon 2006). In conclusion, long-term creatine supplementation leads to an increase in healthy life span in mice and promotes changes in biochemical and genetic markers associated with aging, which is overall compatible with a discreet anti-aging effect of creatine.

Clinical trials

There are no randomized trials investigating the anti-aging effects of creatine supplementation in humans. Taking the results from mouse studies, such a human trial would be justified and of high interest. However, for reliable results it would need another long-term study (minimum 10 years) employing death as a very clear primary endpoint in a large cohort. Obviously, funding for such a trial is extremely difficult to obtain.

Creatine—lost in translation?

Taken together, creatine has shown an exceptionally strong neuroprotective effect in many animal studies, but the few published controlled clinical trials on neurodegenerative disorders supplemented with up to 10 g creatine per day for up to 2 years have been rather disappointing. Why is creatine more effective in mice than in men and does this invalidate the mouse as an appropriate model?

First, the validity of the standard murine models has been questioned. For example, the SOD1 mouse may not be an appropriate model of sporadic ALS, but just a model of familial ALS, or strictly spoken just a model of the human G93A SOD mutation. On the other hand, while criticism on the validity of specific animal models may very well be justified, the mere abundance of positive creatine effects across various rodent models argues against bias due to inappropriateness of the model. Creatine is neuroprotective in transgenic as well as toxic models, in rat models as well as mouse models of various genetic backgrounds, and also in models of rather slow disease progression (PD, HD, ALS, aging) or those with acute onset, as in ischemic stroke (Zhu et al. 2004) and traumatic brain injury (Sullivan et al. 2000). A closely related explanation may be, that creatine for unknown reasons just has diverse effects on different species as was suggested some years ago (Tarnopolsky et al. 2003; Kreider 2003). Second, the common approach of initiating treatment prior to the onset of symptoms in mice does not reflect the human situation (Benatar 2007). In future trials, timing of treatment relative to disease onset and relative to age should resemble that of human studies, to make the results comparable. Third, there may be a problem of dosage. Most creatine neuroprotection studies in rodents have administered food containing 1–2% creatine. Considering the normal daily food intake of mice (4 g/day) in relation to their body weight (30 g), this represents a dose of 1.3 g of creatine per kg body weight. 10 g/day of creatine applied in the high-dose human studies is equivalent to only 0.13 g per kg body weight for a 75 kg adult, translating into a 10-fold underdosing. On the other hand,

spectroscopic studies have shown significant increases in total brain creatine of 13.3% ± 5.9 in mice on a 2% creatine diet and of 8.7% ± 3.7 in humans after 20 g of oral creatine for 4 weeks (Lyoo et al. 2003). Fourth, study design in mice may be insufficient. In the murine ALS studies, for example, computer modeling revealed that non-ALS deaths, low-copy transgenics (leading to longer survival), and (epi) genetic background causing littermate clustering are major confounding variables that were not adequately corrected for in published mouse studies (Scott et al. 2008). Recommendations for improved study design in the SOD1 mouse model include correction for these confounds, a cohort size of at least 24 litter-matched gender-balanced mice, denying knowledge of animal treatment to technicians and investigators, and application of more appropriate statistical tests (Cox proportional hazards model rather than *t* test/ANOVA for survival statistics). In fact, retesting of several compounds using this optimized study design found no survival benefit in the SOD1 mouse for creatine nor for any other compound (Scott et al. 2008) thus resembling the findings in human trials.

Taken together, creatine remains a promising neuroprotective agent against neurodegeneration and aging, but its potential in human disease may only be revealed by large multicenter studies with higher creatine doses over an adequate observation period. Fortunately, such trials are currently being conducted by the NIH for Parkinson's disease and Huntington's disease. The results of these trials will be of utmost importance to judge the future benefit of creatine as a neuroprotective agent.

Conflict of interest T. Klopstock received free creatine supplements for the described mouse studies and human trials as well as a travel grant from the creatine manufacturer AlzChem Trostberg GmbH.

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