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Creatine improves health and survival of mice

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Abstract

The supplementation of creatine (Cr) has a marked neuroprotective effect in mouse models of neurodegenerative diseases. This has been assigned to the known bioenergetic, anti-apoptotic, anti-excitotoxic, and anti-oxidant properties of Cr. As aging and neurodegeneration share pathophysiological pathways, we investigated the effect of oral Cr supplementation on aging in 162 aged C57Bl/6J mice. Outcome variables included "healthy" life span, neurobehavioral phenotyping, as well as morphology, biochemistry, and expression profiling from brain. The median healthy life span of Cr-fed mice was 9% higher than in control mice, and they performed significantly better in neurobehavioral tests. In brains of Cr-treated mice, there was a trend towards 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 show that Cr improves health and longevity in mice. Cr may be a promising food supplement to promote healthy human aging.

Keywords: Creatine; Aging; Oxidative stress; Mitochondria; Lipofuscin; Life span

1. Introduction

The mitochondrial theory of aging implies that reactive oxygen species, mitochondrial DNA (mtDNA) damage, and progressive respiratory chain dysfunction are mutually interacting links in a vicious circle (Harman, 1972; Bender et al., 2006). Although the overexpression of antioxidant enzymes increases life span in several organisms, this is not feasible in humans (Schriner et al., 2005). Food supplemented with various antioxidants on the other hand has so far failed to retard aging in mice (Lee et al., 2004). Creatine (Cr) is a natural ergogenic compound and is widely used by athletes as a food supplement to enhance muscular performance. It also has

Abbreviations: Cr, Creatine; mtDNA, mitochondrial DNA; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; COX, cytochrome-c-oxidase

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anti-apoptotic (O'Gorman et al., 1997), anti-excitotoxic (Xu et al., 1996), and directly anti-oxidative properties (Lawler et al., 2002), both in vitro and in vivo. Marked Cr-mediated neuroprotection was found in rodent models of neurodegenerative diseases, in particular Parkinson and Huntington disease (Matthews et al., 1999; Andreassen et al., 2001). Since aging and neurodegeneration share pathophysiological pathways (Beal, 2005), we hypothesized that Cr may also exert an anti-aging effect in wild-type mice. To test this hypothesis, "healthy" life span, neurobehavioral functioning, and aging-associated changes of biomarkers were examined in 162 female wild-type C57BL/6J mice fed a standard rodent diet ad libitum with ($n = 81$) or without ($n = 81$) 1% Cr beginning at age of 365 ± 2 days.

2. Materials and methods

2.1. Mice and animal welfare

Inbred C57BL/6J were bred and kept at the GSF animal facilities in isolated ventilated cages type II at a temperature of 20–24 °C, humidity of 50–60%, 20 air exchanges per hour and a 12/12-h light/dark cycle. They were tested for microorganisms in 12-week intervals according to the FELASA Guidelines to the level required for rederived mice (Nicklas et al., 2002). Mice were provided with a standardized mouse diet (1314, Altromin, Germany) and drinking water ad libitum until the age of 12 months. Starting at the age of 365 ± 2 days, 81 mice were fed a standard rodent diet containing 1% Cr and 81 mice were fed an equicaloric standard diet. From the age of 2 years on, i.e. after 1 year of food supplementation, mice were checked daily for their health status. The end of the experiment for a given mouse was defined as either spontaneous death (measured as life span) or signs of severe illness (measured as healthy life span), not compatible with animal welfare regulations (impaired ambulation, rapid or shallow breathing rate, haunched posture, ruffled fur, anorexia, diarrhea, ulcerative dermatitis, loss of weight, neoplasms). Sacrificed mice were dissected and representative samples of brain, muscle, and kidney were taken from each animal, fixed in buffered 4% formalin and processed for routine histology. To compare the neurobehavioral fitness at a well-defined and comparable age, 27 Cr-treated and 30 control mice were phenotyped after 1 year of dietary supplementation (at 2 years of age) and sacrificed thereafter. Mice husbandry and all animal procedures were in accordance with the Animal Care and Use Regulations of the GSF and with German Legislation.

2.2. Neurobehavioral tests

The modified Hole Board test was specifically developed for behavioral screening of lab rodents (Ohl et al., 2001). It allows the comprehensive analysis of a range of behavioral parameters known to be indicative of dimensions such as locomotor activity, exploratory behavior, arousal, emotionality, memory, and social affinity in a single short test. Here it was carried out in a modified version, adapted to German Mouse Clinic (GMC) hygiene and workflow requirements (Gailus-Durner et al., 2005). The test apparatus consisted of a box (150 cm × 50 cm × 50 cm) which was divided into a test arena (100 cm × 50 cm) and a group compartment (50 cm × 50 cm) by a transparent PVC partition (50 cm × 50 cm × 0.5 cm) with 111 holes (1 cm diameter) staggered in 12 lines to allow group contact. A board (60 cm × 20 cm × 2 cm) with 23 holes (1.5 cm × 0.5 cm) staggered in three lines with all holes covered by movable lids was placed in the middle of the test arena. The area around the board was divided into 12 similarly sized quadrants by lines taped onto the floor of the box. Both box and board were made of dark grey PVC. All lids were closed before the start of a trial. For each trial, an unfamiliar object (a blue plastic tube lid, similar in size to the metal cube) and a familiar object (metal cube), which had been placed in the animals' home cages 3 days and removed one day before testing, were placed into the test arena, in the corner quadrant diametrical to the starting point, with a distance of 2 cm between them. The illumination levels were set at approximately 150 lx in the corners and 200 lx in the middle of the test arena.

At the beginning of the experiment, an animal was placed individually into the test arena and allowed to explore it freely for 5 min, during which the animal's behavior was recorded by a trained observer blind to the treatment group with a hand-held computer. The animals were always placed into the test arena, facing the board diagonally. Behaviors scored included line crossings, rearings, board entries, hole exploration, object exploration, grooming, defecation and immobility. Data were analyzed by using the Observer 4.1 Software (Noldus, Wageningen). Additionally, a camera was mounted 1.20 m above the center of the test arena, and the animal's track was videotaped and its locomotor path analyzed with a video-tracking system (Ethovision 2.3, Noldus, Wageningen). To assess object recognition memory, the unfamiliar object exploration was calculated as a function of total object exploration time ("object recognition index"): unfamiliar object exploration time − familiar object exploration time/(unfamiliar + familiar object exploration times).

The SHIRPA protocol (Smithkline Beecham, MRC Harwell, Imperial College, the Royal London hospital phenotype assessment) is a rapid, comprehensive, and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in mice (Irwin, 1968). We carried out 23 test parameters that contribute to an overall assessment of muscle, lower motor neuron, spinocerebellar, sensory and autonomic function. Statistical analysis was done by one-way ANOVA for quantitative parameters and by chi-square test for qualitative data.

Grip strength analysis was used to measure the muscle strength in the forelimbs of the mice (Gailus-Durner et al., 2005). The animals grasp a horizontal metal bar while being pulled by their tail. A sensor allows measurements of up to

600 ponds. Five trials within one minute were performed for each mouse and these values were used as statistical variables in subsequent analysis.

Rotarod analysis was performed to measure motor coordination, balance and motor learning ability (Gailus-Durner et al., 2005). On the first day, the mice were habituated to the device (TSE, Bad Homburg, Germany) in two 180 s sessions at constant speeds of 12 and 20 rpm. In the motor coordination performance test on the second day, mice exerted four trials with accelerating speed from 4 to 40 rpm. The mean latency to fall off the rotarod was recorded.

2.3. Determination of 8-OHdG levels in brain tissue

DNA extraction employing the chaotropic Na-Iodide method was chosen in order to minimize artificial post mortem 8-OHdG generation. From each brain tissue sample 225 mg were mechanically disrupted in liquid nitrogen and further processed using a DNA extraction kit (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) following the suppliers protocol. In order to prevent the iron-dependent Fenton reaction, which constitutes a major source of reactive oxygen intermediates, desferrioxamine mesylate was added to the primary extraction buffer at a concentration of 100 μM. DNA solution was adjusted to a concentration of 50 μg/ml with TE buffer (1 mM EDTA in 10 mM Tris–HCl, pH 8.0), digested with 6 units of nuclease P1 in 10 mM sodium acetate buffer at 37 °C for 2 h (final concentration 1 mg/ml) and subsequently incubated with 2 units of alkaline phosphatase (7 μl, 200 units/ml) in 40 mM Tris buffer at 37 °C for 1.5 h. The solution was then purified by ultrafiltration with a molecular weight cut-off at 10,000 Da (Millipore Ultra free C3LGC Millipore GmbH, Schwalbach, Germany) and centrifuged at 15,000 × *g* for 30 min. Levels of 8-OHdG level were determined with a quantitative sandwich enzyme-linked immunoabsorbent assay kit (high-sensitivity 8-OHdG check, GENTAUR, Brussels, Belgium) using a microplate ELISA-reader.

2.4. Histochemistry of brain samples

Cryostat sections (10 μm) were cut from coronal orientated brains ($n = 5$ per group; age 671–731days). Sequential assay of cytochrome-c-oxidase (COX) and succinate dehydrogenase (SDH) activities identified COX-deficient cells and muscle fibres in positive controls.

2.5. Long range PCR

The DNA was extracted from brain as described in the determination of 8-OHdG levels. For the extraction from skeletal muscle a DNA extraction kit (QIAamp DNA Micro Kit) was used. Long extension PCR of the entire 16.3 kb mtDNA was performed using the Expand Long Template PCR System (Roche) and the following pairs of primers: mLXfor1 (14520–14545), mLXrev1 (14207–14183), mLXfor2 (5685–5707), mLXrev2 (5423–5401), mLXfor3 (13644–13667), mLXrev3 (12333–12313). Final concentrations were 0.3 μM primers, 5.0 μl Ultra Pure BSA, 2.5 μl supplied 10xdNTP Mix, 5.0 μl Expand Long Template Buffer 3, 3.75 U Expand Long PCR Enzyme mix/reaction, 2.5 μl DMSO, and 2.0 μl of DNA lysate in a final volume of 50 μl with the addition of sterile redistilled H_2O. The PCR profile was one cycle at 93 °C for 2 min, 25 cycles consisting of 93 °C for 10 s, 59 °C for 30 s and 68 °C for 15 min + 10 s/cyle for the last 15 cycles, and one final extension of 7 min at 68 °C.

2.6. Real time PCR

DNA was extracted from laser-microdissected (Leica LMD) CA2-hippocampus using a DNA extraction kit (QIAamp DNA Micro Kit). DNA from brain and muscle was extracted as described before. Real time PCR was performed using a ready-to-use hot start reaction mix (TaqMan Universal Master Mix, Applied Biosystems) on a LightCycler-System (Roche) using specific Probes and primers (MRTND1for (3306–3331), mRTND1rev (3382–3355), mRTND1probe (3333–3352), mRTND4for (11136–11159), mRTND4rev (11210–11188), mRTND4probe (11186–11162)). 5.0 μl of DNA lysate from hippocampal CA2-neurons, skeletal muscle and brain were amplified separately with primer/probe combinations as previously described (He et al., 2002).

2.7. Lipofuscin staining

Unstained deparaffinized transversal brain sections (8 μm) were mounted in Rotihistokit (Roth, Germany). Fluorescence microscopy examinations were performed on a Zeiss AxioVert 200 M microscope (Zeiss, Germany) equipped with an AxioCamHRC using a rhodamine filter. To avoid differences due to the well known strong fading-effect of lipofuscin autofluorescence, slices were not exposed to light and fluorescence was only used during the acquisition process following a strict protocol concerning the sequence of acquisition, microscope and camera settings. Digital image analysis was done using Image-Pro Plus Version 5.0 (Media Cybernetics, USA).

2.8. Expression profiling

Total RNA was isolated using standard protocols (RNeasy, Qiagen, Germany) from brain hemispheres of six mice fed ad libitum on 1% Cr supplemented pellets for 6 months and seven mice fed on equicaloric pellets without Cr (Altromin). For RNA expression profiling, 5 μg of each RNA sample were labeled using the one-cycle target labeling kit and hybridized to 13 Gene Chip Mouse Genome 430 2.0 Arrays (Affymetrix UK Ltd.). Genewise tests for significantly regulated transcripts between Cr-treated and untreated mice were performed in the statistical programming language R with BioConductor (Gentleman et al., 2004) using the

dChip and the Robust Multichip Average (RMA) probeset summaries (Bolstad et al., 2003; Li and Wong, 2001). Data are log-transformed and normalized with a lowess-smoother operating on MA-scale against the mean of the reference group. Transcripts are called significantly regulated if the corresponding p-value of the Welch's t-test is below the 5% FDR significance level (Benjamini and Hochberg, 1995).

2.9. Statistical methods

Life span data were analyzed for significant differences by the log rank test. A two-sided Mann–Whitney U-test was employed to test for differences in maximal life spans between groups. For this purpose, the 10% longest lived animals of each group were compared (Schriner et al., 2005). For all other parameters, a Kolmogorov–Smirnov test was used to determine the type of data distribution. Serum lactate and brain lipofuscin content were thus evaluated by two-sided t-tests. Results of the modified hole board test were reported as mean ± S.E.M. and statistically analyzed by two-sided Mann–Whitney U-test and Student's t-test, respectively. The accepted level of significance was $P < 0.05$. Brain 8-OHDG content between groups was compared by a two-sided Mann–Whitney U-test, as were the results of the neurological phenotyping. A Chi-square test was used to compare incidences of tumors, renal damage, and dermatitis. All analyses were performed with the SPSS 12.0 software package for windows (SPSS Inc., USA).

3. Results

3.1. Cr increases health and lifespan

Cr levels in serum increased in the Cr-fed group after 3 (245.5 ± 184.5 versus 73.5 ± 14.4 μmol/l in Cr- and control mice, respectively; $p = 0.003$) and after 6 months (385.6 ± 237 versus 84.2 ± 19.1 μmol/l; $p = 0.0001$) of supplementation as a proof of actual gastrointestinal Cr ingestion and resorption. Mean "healthy" life span (i.e. age at which mice were classified as suffering from disease) was higher in Cr-fed mice (613 ± 84 days) than in their littermates (563 ± 95 days), corresponding to a 9% increase ($p < 0.05$; Fig. 1). The effect on maximum life span was less pronounced

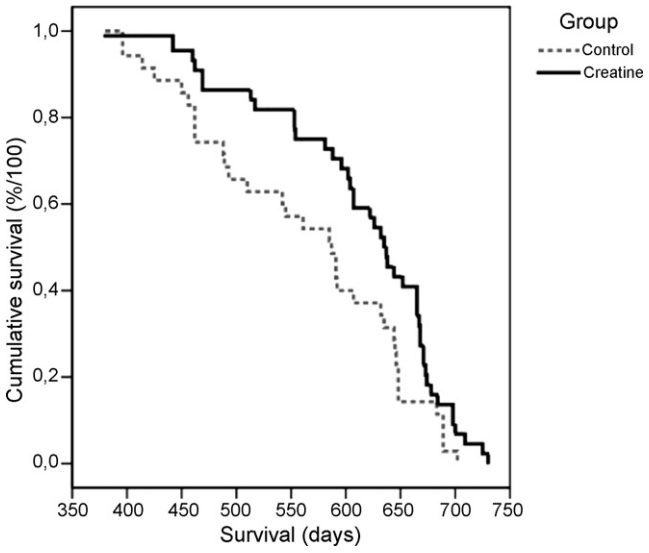

Fig. 1. Kaplan–Meier survival curves for female mice on standard diet or diet supplemented with 1% creatine. Mean life span is 9% higher in Cr-fed mice.

but still significant (716 ± 14 days versus 692 ± 7 days for the top 10% longest lived animals of each group; $p < 0.05$). Standardized post mortem analysis revealed no differences in the incidences of neoplasms (24% versus 27% in Cr-fed and control mice, respectively), renal damage (92% versus 83%), or dermatitis (47% versus 44%), which are common pathological findings in this inbred mouse line.

Prolonged life in Cr-fed animals was accompanied by prolonged health. While tests like the SHIRPA protocol, rotarod, or grip strength analysis showed no group differences, Cr-treated mice exhibited improved object recognition memory ($p < 0.05$; Fig. 2A), a decreased latency to initiate exploration of the novel environment ($p < 0.01$; Fig. 2B), and a trend toward increased forward locomotor activity (modified Hole Board Test, $p = 0.05$; Fig. 2C).

3.2. Cr promotes discreet changes of biomarkers for aging and energy status

Several aging- and energy-related biomarkers were examined. Serum lactate, a global marker of aerobic metabolism, was significantly lower in Cr-fed mice than in controls after

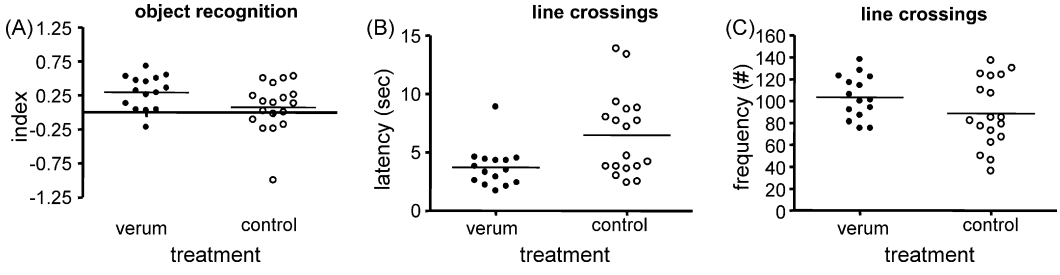

Fig. 2. Cr-fed mice showed better object recognition memory ($p < 0.05$, A), a decreased latency to initiate exploration of the novel environment ($p < 0.01$, B), and a trend toward increased forward locomotor activity (modified Hole Board Test, $p = 0.054$, C).

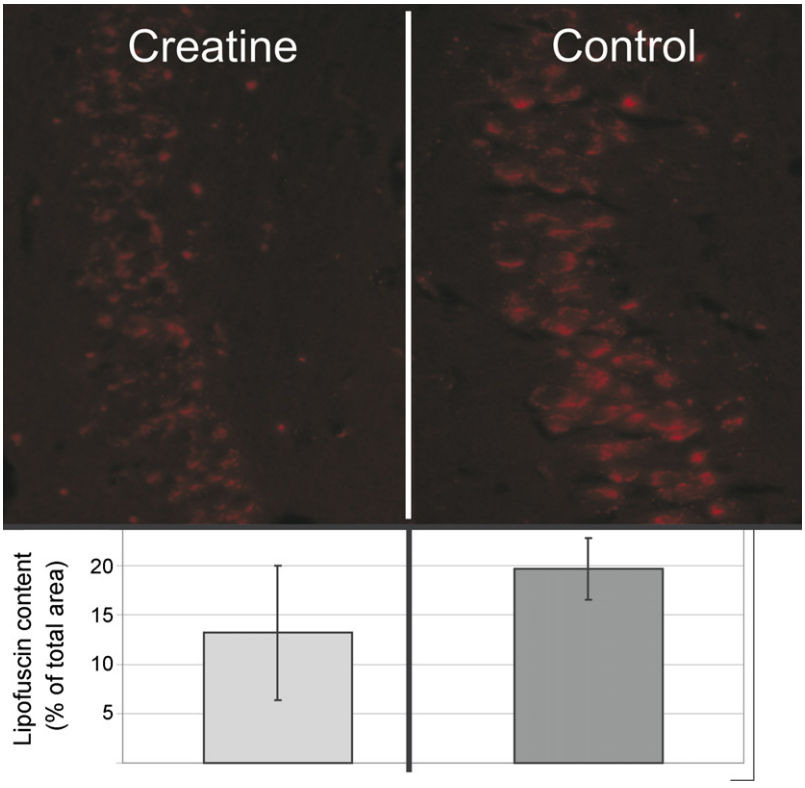

Fig. 3. Lipofuscin autofluorescence shows a trend ($p = 0.06$) towards less lipofuscin in the CA2 region of the hippocampus of aged Cr mice (left) as compared to age-matched controls (right).

1 year of therapy (3.23 mmol/l versus 4.11 mmol/l; $p < 0.05$). Accumulation of the "aging pigment" lipofuscin (visualized by autofluorescence) in the hippocampus trended to be lower in Cr-fed mice than in controls ($p = 0.06$, Fig. 3). The level of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of oxidative damage to DNA, trended to be lower in the brain of Cr-fed mice, even though this difference failed to reach significance ($n = 8$ Cr-mice: 7.13 ± 1.76; $n = 8$ placebo mice: 9.12 ± 2.97 pmol/mg; $p = 0.08$). Deletions of the mtDNA as well as COX-negative cells accumulate with aging in post-mitotic tissues such as brain and muscle in humans and mice (Bender et al., 2006; Melov et al., 1997). Contrary to previous findings, we were not able to detect abnormal levels of these two markers in the hippocampus or in muscle of 2-year-old mice (neither in controls nor in the Cr group) with long extension and real time PCR or histochemical staining, respectively.

3.3. Cr is associated with changes in differential gene expression

To identify regulated transcripts in the brain of Cr-fed versus control mice, expression profiles were analyzed with Gene Chip Mouse Genome Arrays. The dChip and RMA analysis tools characterized 453 and 555 genes, respectively, as significantly regulated, with an intersection of 56 genes (Table S1). In gene ontology (GO) terms, the most significant results were related to behavior, neurogenesis, energy pathways, and protein biosynthesis (Table S2). Compared with published expression profiles, there was some overlap with the genes regulated in mice on caloric restriction, e.g. growth factors such as *Bdnf*, *Ndn*, *Hgf*, and *Tgfb*2 (Lee et al., 1999). In several instances, genes known to be regulated in the aging mouse brain were reversely regulated in Cr-fed mice (Table S3) (Jiang et al., 2001). The systematic analysis of cocitations among regulated genes (Bibliosphere 5.02) suggested links to pathways such as *Wnt*, insulin, hypoxia, or DNA damage signaling (Supplementary data, Fig. S1).

4. Discussion

We have chosen a long-term model of dietary supplementation to examine changes in longevity, neurological function, biomarkers of aging, and gene expression promoted by the common "lifestyle" supplement Cr. Unlike most other life span studies, we have introduced healthy life span as main outcome variable as there is general consent that a gain of healthy lifetime is what is desirable in human aging. Cr increased healthy life span in mice by 9%. This seems rather modest compared to the increased life span achieved by caloric restriction and genetic models of longevity (Weindruch, 1996; Rogina and Helfand, 2004; Schriner et al., 2005). Yet, from a practical point of view, nei-

ther of these two approaches is likely to be directly applied to human aging. Natural dietary supplements as Cr on the other hand could be realistically tried in human aging as they are easily applicable and have a favorable safety profile (Pline and Smith, 2005). The only other supplement recently shown to promote longevity in mammals is Vitamin E (Navarro et al., 2005). Given in a high dose, it led to increased life span in a senescence-accelerated mouse strain (CD-1/UCadiz; 40% in males, 14% in females). Moreover, Vitamin E-treated male mice showed better neuromuscular function and exploratory activity until 78 weeks of age. These clinical effects were paralleled by a less marked decline in brain mitochondrial function, including oxygen uptake, complex I and IV activities, and activities of antioxidant enzymes. Unfortunately, clinical trials with Vitamin E are less encouraging. Though there are some controversial data that it may slow down the pace of cognitive decline as well as the course of Alzheimer and Parkinson disease, two very large and thorough long-term studies showed that Vitamin E supplementation does not reduce the risk of cardiovascular disease, cancer and mortality (Lee et al., 2005), but may even increase the risk for heart failure (Lonn et al., 2005).

The increase in healthy life span in our animals was accompanied by favorable effects on neurobehavioral functioning, especially memory skills. The object recognition task employed here is a well-validated memory test in rodents (Bertaina-Anglade et al., 2006) and is a reasonable approximation to corresponding human tests (Flicker et al., 1987). Thus, the positive effect of Cr is of utmost importance for human aging, as age-associated memory impairment and cognitive decline are very frequent in the general elderly population (Park et al., 2003). In a Finnish prevalence study, over one-third of healthy 60–80-year-old adults had memory impairment (Koivisto et al., 1995) and the prevalence of definitive dementia approximates 30% in people over 90 years of age (Lobo et al., 2000). The associated socio-economic costs are tremendous.

Cr supplementation employs the same pathways as dietary restriction, the most effective anti-aging means in several species: gene regulation and suppression of oxidative stress. Low caloric intake probably causes a mild cellular stress response that triggers the expression of neural survival and plasticity genes (e.g. growth factors and chaperones) and suppresses oxidative stress (Zhu et al., 1999). Cr-fed mice showed a trend toward less 8-OHdG accumulation in the brain and significantly less aggregation of the "aging pigment" lipofuscin in the hippocampus. Lipofuscin granules are cellular deposits of organic and anorganic material that occur particularly in metabolically active postmitotic cells such as neurons. It originates primarily from the degradation of damaged organelles, such as mitochondria. It is considered to be a cellular marker of ageing in the nervous system. The most vulnerable area is the hippocampus. Lipofuscin levels in hippocampal regions of 16-month-old rats have been found 66-fold higher than for 4-month-old animals (Abd El Mohsen et al., 2005). Accumulating evidence suggests that lipofuscin may impair the functioning of other cellular processes, including the ubiquitin/proteasome pathway, finally leading to a "garbage catastrophe" (Gray et al., 2005). It is not clear whether the lower brain lipofuscin in our Cr mice is due to reduced lipofuscin production or accelerated removal. We propose, however, that Cr by its antioxidative effects lowers free radical production and consecutively lipofuscin generation.

Gene expression profiling from brain showed that a number of genes in Cr-fed mice are (i) reversely regulated compared to the regulation data published on aging mice and (ii) 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* was significantly increased by a factor of 1.4–1.6 in mouse brain (Duan et al., 2001). In our Cr-fed mice, *Bdnf* was 1.27-times higher than in controls. Similarly, the glial high affinity glutamate transporter *Slc1a3* was up-regulated in Cr-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. We have recently shown in patients with Huntington disease that Cr supplementation led to a decrease in brain glutamine and glutamate levels, which is in accordance with an anti-excitotoxic Cr effect (Bender et al., 2005).

Overall, Cr had only discreet effects on life span and other biomarkers of aging. Yet, taken together, the changes all point to the same general direction of a beneficial Cr effect on aging and neurobehavioral functioning even though some of our 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. By 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 be able to make more general assumptions. Models of accelerated animal aging are likely to produce greater differences upon intervention, yet they do not reflect normal aging (Nadon, 2006).

In conclusion, long-term Cr 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 Cr effect. As there are no real safety concerns, Cr might have the potential to contribute to healthy human aging.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2007.03.001.

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