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Short Communication

Neurochemical correlates of differential neuroprotection by long-term dietary creatine supplementation

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Abstract

Dietary supplementation with creatine has proven to be beneficial in models of acute and chronic neurodegeneration. We report here data on the neurochemical correlates of differential protection of long-term creatine supplementation in two models of excitotoxicity in rats, as well as in the mouse model for ALS (G93A mice). In rats, the fall in cholinergic and GABAergic markers due to the excitotoxic death of intrinsic neurons caused by intrastriatal infusion of the neurotoxin, ibotenic acid, was significantly prevented by long-term dietary supplementation with creatine. On the contrary, creatine was unable to recover a cholinergic marker in the cortex of rats subjected to the excitotoxic death of the cholinergic basal forebrain neurons. In G93A mice, long-term creatine supplementation marginally but significantly increased mean lifespan, as previously observed by others, and reverted the cholinergic deficit present in some forebrain areas at an intermediate stage of the disease. In both rats and mice, creatine supplementation increased the activity of the GABAergic enzyme, glutamate decarboxylase, in the striatum but not in other brain regions. The present data point at alterations of neurochemical parameters marking specific neuronal populations, as a useful way to evaluate neuroprotective effects of long-term creatine supplementation in animal models of neurodegeneration.

Theme: Disorders of the nervous system
Topic: Neurotoxicity

Keywords: Rat; Transgenic mice; Neurodegeneration; Neuroprotection; Cholinergic neuron; GABAergic neuron

Long-term dietary supplementation with creatine has been proposed to be neuroprotective in both acute and chronic neurodegenerative diseases. Neuroprotective effects of creatine have been demonstrated in some animal models of neurotoxicity/excitotoxicity but not in others [23–25], in traumatic brain and spinal cord injury [17,31] and in ischemia [38]. Furthermore, attenuation of disease symptoms and increased lifespan through dietary creatine supplementation has been described in animal models of human neurodegenerative diseases [3]. In addition to recent results obtained in a mouse model of Huntington disease in which dietary creatine supplementation delayed motor symptoms and neuronal atrophy [8], several reports have dealt with mouse models of amyotrophic lateral sclerosis (ALS). In these mice (G93A strain), long-term dietary supplementation with creatine delayed motor neuron death and onset of motor symptoms and increased survival [19,37], even if no significant beneficial effects were reported regarding muscle function [9]. Clinical trials based on creatine administration to ALS patients have not demonstrated, so far, any significant beneficial effect [3,10,29]. In these trials, however, the human equivalent dose extrapolated from animal studies was likely underestimated and new trials, based on increased daily creatine administration, are awaited.

Surprisingly, reports on dietary creatine supplementation in animal models of neurodegenerative injuries and diseases have not dealt, so far, with the derangement of neuro-

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chemical parameters related to degenerating neurons and with their rescue through the dietary manipulation. This information is very relevant, because specific alterations of neurochemical parameters are one of the most reliable markers to evaluate the extent of neural damage. Furthermore, this approach may be potentially able to put in evidence differential responses to insults and protection of different neuronal populations present in the same brain region. We report here data on the neurochemical correlates of differential protection of long-term creatine supplementation in two models of excitotoxicity in rats, as well as in the mouse model for ALS (G93A mice). To quantitatively evaluate neuronal damage and protection granted by the dietary supplementation, we have measured neurochemical parameters, whose alterations are established markers for the degeneration of specific types of neurons in the experimental models adopted for the present study [5–7].

Male Wistar rats form Harlan Italy were divided into two groups at 40 days of age. The first group was fed ad libitum with standard diet. The second group received the same diet supplemented with 2% creatine. Rats underwent surgical operation at 70 days of age and were then allowed to survive for additional 30 days, continuing to be fed with the normal or the supplemented diet during all these periods. For surgery, rats were anesthetized with ether, fixed to a David Kopf stereotaxic instrument and holes were drilled in the skull at the appropriate stereotaxic coordinates. Two different models of excitotoxic neurodegeneration were adopted to test possible neuroprotection from creatine supplementation. In the first model, rats received unilateral injections of the neurotoxin, ibotenic acid (IBO, 7 μg/1 μl saline), into the left striatum at the following coordinates [28]: 1 mm in front of bregma, 3 mm lateral, 5 mm below the dura. This treatment results in widespread degeneration of striatal neurons and in particular of the cholinergic and GABAergic neuronal population present in this brain region [5]. In the second model, IBO (5 μg/1 μl saline) was unilaterally injected in the area of the nucleus basalis magnocellularis, where cholinergic neurons providing most of the cholinergic input to the neocortex are localized [26,35], at the following coordinates: 1 mm behind bregma, 2.7 mm lateral, 7.8 mm below the dura. The excitotoxic lesion of these neurons results in fall of cholinergic parameters in cortical areas as a consequence of the degeneration of cholinergic terminals [4,6]. Injections were performed slowly (about 3 min followed by 2 min during which the needle was left in place), using a 10 μl Hamilton syringe operated by a micrometric device. In both cases, the contralateral (right) side of the brain received an equivalent injection of saline and served as control for the excitotoxic lesion. After 30 days of recovery, rats were killed by decapitation, the brains were rapidly removed and sliced with a Sorvall tissue chopper. Samples of the striata (for striatum-injected animals) obtained from two consecutive slices comprised between levels bregma 1.2 and bregma 0.2 [28], or the fronto-parietal cortex (for nucleus basalis magnocellularis-injected animals) obtained from three slices comprised between levels bregma 1.2 and bregma −1.3 [28], from the two brain sides were separately microdissected under the stereomicroscope, frozen in dry ice and stored at −80 °C until assayed. This procedure, which has been used for many years in our lab [5–7], allows reproducible dissections of equivalent samples from different animals without compromising reliability of the neurochemical determinations indicated below. Samples were homogenized in 0.32 M sucrose, added with 0.5% Triton X-100 (final concentration) and the whole homogenate was used to assay the activity of the cholinergic marker, choline acetyltransferase (ChAT) [12] and of the GABAergic marker, glutamate decarboxylase (GAD) [13], as well as sample protein content [22]. ChAT activity was assayed by incubating aliquots of the homogenates containing known amount of protein, for 10–30 min at 37 °C in the presence of choline (8 mM) and ^{14}C-acetylCoA (0.2 mM, NEN, specific activity 51.6 mCi/mmol) and adding eserine (0.1 mM) to block acetylcholinesterase. The labeled acetylcholine formed through the enzymatic reaction was extracted by Kalignost (0.5% in acetonitrile), brought to the organic phase of a scintillation cocktail (Instafluor, Packard), counted and expressed as μmol formed/unit protein weight. GAD activity was assayed by incubating aliquots of the same samples for 1 h at 37 °C in the presence of ^{14}C-glutamate (21.6 mM, NEN, specific activity 45 mCi/mmol), trapping the CO_2 evolved by the enzymatic reaction with hyamine hydroxyde, counting and expressing the activity as μmol of CO_2 formed/unit protein weight. We knew from previous experiments that glial proliferation consequent to the lesion does not alter the protein content of the affected regions [33].

Transgenic mice, G93A, carrying a high number of copies of the mutated human Cu/Zn superoxide dismutase (SOD1) gene (glycine/alanine substitution at codon 93) [16] were purchased from Jackson Laboratories under the strain designation B6SJL-TgN (SOD1)^{1GUR}. The colony was maintained by crossing male transgenic mice with females of the B6EiC3Sn strain. This allows to maintain both transgenic and wild type mice on the same genetic background and, therefore, to avoid effects not directly related to the specific mutation investigated. To identify transgenic mice, genotyping was performed at 30 days of age by extracting DNA from tails with Dneasy Tissue kit (Qiagen, GmbH, Germany) and using the PCR protocol suggested by Jackson Laboratories. Starting from 40 days of age, wild type and G93A transgenic mice were divided in groups, composed of approximately the same number of males and females, which received standard diet or diet supplemented with 2% creatine. When 110 days old, an age at which disease symptoms are manifested in G93A mice and cholinergic deficit is evident not only in the degenerating segments of the spinal cord, but also in some forebrain regions [7], wild type mice and part of the G93A ones were killed by decapitation. Samples from the lumbar spinal cord, the hippocampus, the olfactory cortex and the striatum [14] were rapidly collected in parallel by two experienced operators and stored in the

deep freezer for subsequent determination of ChAT activity. The remaining G93A mice were used to assess survival and were therefore maintained under the different diets until euthanized when they reached the final stage of the disease, characterized by complete limb paralysis and inability to move and feed. Statistical analysis was carried on through Student's t test or analysis of variance (one way ANOVA) followed by post-hoc Bonferroni's test. The number of animals used for each experiment is indicated in figure legends. Experiments were performed in accordance with the Italian and European Community law on the use of animals for experimental purposes and were approved by the University of Bologna bioethical committee. Throughout the study, animals were kept under veterinary surveillance for their comfort and health.

The dose of creatine supplementation used for present experiments was the same recently adopted for studies of neuroprotection in rats and mice, including G93A mice [8,19,24,25]. Creatine is transported to body organs, including the brain, where it is assumed by cells through an active membrane transport system [18]. Long-term (4–8 week) oral administration of creatine, resulting in average intake similar to the one produced by our diet, increased by 15–30% total creatine and phosphocreatine concentration in brain of rats and mice [18]. Rats fed with normal or with creatine-enriched diet did not show any significant difference in daily food intake and body weight increase. Unilateral intrastriatal injection of ibotenic acid in rats resulted in large depletion of markers for cholinergic and GABAergic striatal neurons (Fig. 1), reflecting the widespread excitotoxic death of these neurons, as previously shown [5]. Long-term feeding with creatine-enriched diet was remarkably neuroprotective towards the excitotoxic lesion. In the IBO-injected striatum, the marker for cholinergic neurons, ChAT, was decreased by 63% as compared to the contralateral side in normally fed rats, while the decrease was only 32% in rats fed with creatine-enriched diet (Fig. 1A). Similarly, the marker for GABAergic neurons, GAD, was decreased by 68% in the IBO-injected striatum of normally fed rats, while it was significantly less reduced (−49%) in rats fed with creatine-enriched diet (Fig. 1B). Interestingly, while creatine supplementation did not affect, per se, the striatal ChAT level, the GABAergic marker was significantly increased (+35%) in the saline-injected striatum of rats fed with creatine-enriched diet (Fig. 1B). The levels of enzymatic activities measured in saline injected striata were not different from those present in sham-operated animals assayed in parallel (Figs. 1A, B).

In a different model of excitotoxicity, IBO injection in the nucleus basalis magnocellularis (NBM) with degeneration of cholinergic basal forebrain neurons and consequent decrease of cholinergic innervation to the cortex [4,6], dietary creatine supplementation was not neuroprotective, as in both groups of rats a similar decrease of ChAT activity in the ipsilateral cortex was recorded (Fig. 2).

In G93A SOD1 mutant mice, the progression of the ALS-like disease can be monitored by the decrease of the

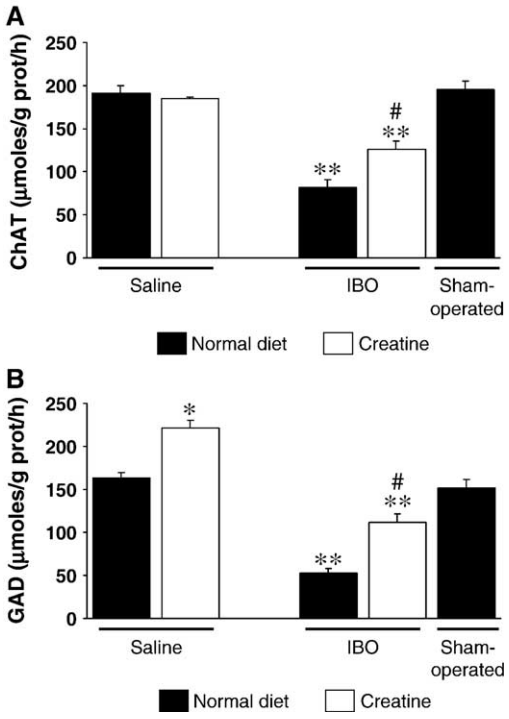

Fig. 1. Effect of long-term creatine supplementation on neuroprotection of striatal cholinergic and GABAergic neurons from ibotenic acid excitotoxic insult. Bars are the mean ± SE of 7 animals per group. (A) ChAT activity in the striatum of IBO-injected rats fed with normal- or creatine-supplemented diet, compared to the saline-injected striatum or to the striatum of sham-operated rats. (B) GAD activity assayed in the same samples. $^{**}P < 0.001$ vs. respective saline-injected striata; $\#P < 0.01$ vs. the striatum of normal diet-fed rats. Bonferroni's test after ANOVA.

cholinergic marker, ChAT, not only in the affected regions of the spinal cord where cholinergic motor neurons degenerate, but also in some forebrain areas receiving cholinergic innervation from basal forebrain cholinergic neurons [7]. Creatine supplementation results in increased survival of G93A mice and in delay of some disease-related symptoms [19,37] and it may, therefore, favorably affect the loss of the cholinergic marker. In the present experiment, 2% creatine supplementation starting at the age of 40 days resulted in a small, but significant, increase in the survival of transgenic mice (mean lifespan of normally fed G93A mice: 132.4 ± 2.6 days; mean lifespan of creatine-supplemented G93A mice: 142 ± 3 days; $n = 7$, $P < 0.05$, Student's t test). Some G93A mice were sacrificed at the age of 110 days, together with age-matched wild type littermates, as at this stage of progression the disease is already characterized by significant decrease of ChAT activity in the spinal cord, as well as in some forebrain areas [7]. The levels of ChAT activity in wild type mice were not changed by the different dietary regimen in any of the regions examined and, accordingly, data from these animals were pooled together. In the lumbar spinal cord, ChAT activity was similarly decreased in G93A mice, either fed with normal or creatine-supplemented diet, compared to wild type animals (Fig. 3A). In the olfactory cortex and the hippocampus of the same mice, instead, the decrease in ChAT

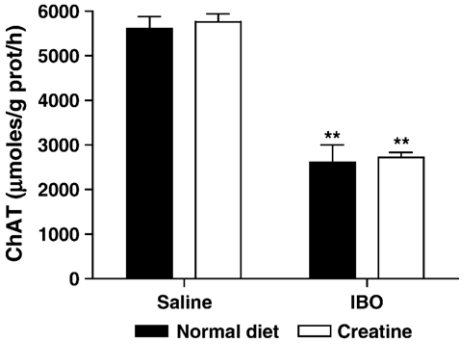

Fig. 2. Lack of effect of long-term creatine supplementation on the degeneration of the basal forebrain-neocortex cholinergic system. Bars are the mean ± SE of 7 animals per group. $^{**}P < 0.001$ vs. corresponding samples of saline-injected rats. Bonferroni's test after ANOVA.

activity present in G93A mice at 110 days of age was completely counteracted by the long-term dietary supplementation with creatine (Figs. 3B, C). Interestingly, also in mice, creatine supplementation increased GAD activity in the striatum, but not in the hippocampus of both wild type and transgenic mice (Fig. 4).

With the present report, we demonstrate differential response to dietary creatine supplementation in different types of acute excitotoxic insults as well as in an animal model of a human degenerative disease. In our model of excitotoxicity involving the degeneration of intrinsic striatal neurons, the neuroprotective effect of long-term creatine supplementation was similar to the one determined by different methods in similar types on neurodegenerative insults involving the striatum [23,24,38]. Our experimental approach allowed us to evaluate the relative damage, and the degree of protection granted by creatine, regarding different neuronal population of the striatal complex. Our present data suggest that one of the ways through which creatine is beneficial towards excitotoxic lesions, at least regarding the striatum, may be related to increased inhibitory activity at synaptic connections in the neuronal circuits involved in the excitotoxic mechanism. Theoretically, indeed, this may be the consequence of the increased activity of the GABA synthetic enzyme, GAD, in the striatum, which we have observed both in rats and mice. Our data on increased GAD activity in the striatum of creatine-supplemented rats may suggest that creatine supplementation has a role in favoring recovery of GABAergic neurons surviving the lesion rather than an actual neuroprotective role. This would not explain, however, the similarity of the results concerning striatal cholinergic neurons, whose neurochemical marker, ChAT, displays the same degree of protection of GAD, without undergoing any diet-related upregulation. Hypotheses regarding the mechanisms of neuroprotection from dietary creatine supplementation have been, so far, mainly focused on enhanced energy stores granted by increased phosphocreatine availability for ATP synthesis, stabilization of creatine kinase, protection of mitochondrial integrity and direct antioxidant activity [19,21,24,27,34,36–38]. These hypotheses are in agreement with the well established role of energy deficit/mitochondrial impairment in excitotoxic neurodegeneration [1,2,15,30,32] (see however the negative evidence concerning creatine kinase in a knockout mouse model) [20], and the mechanisms listed above likely contribute to the neuroprotective effect of creatine. It is, however, difficult to explain, on the sole basis of these hypotheses, why different types of excitotoxic insults are ameliorated or not by creatine administration and why different neuronal populations respond or not to the dietary supplementation. An example of the first case is given by a previous report demonstrating a protective effect of creatine against excitotoxic lesions caused by intrastriatal infusion of

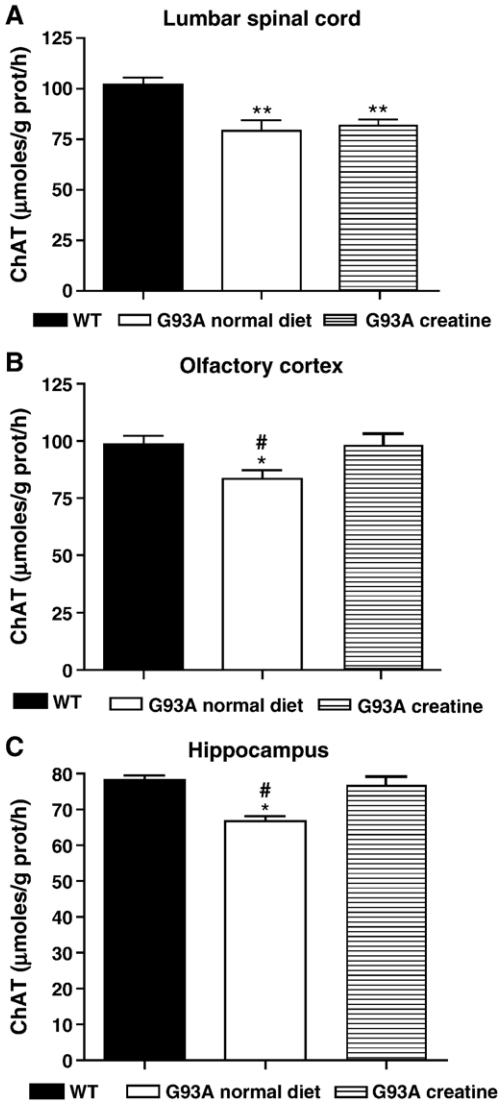

Fig. 3. Differential effect of long-term creatine supplementation on the cholinergic marker in the spinal cord and forebrain areas of G93A mice at 110 days of age. Bars are the mean± SE of 7 (G93A mice) or 10 (wild type mice) animals per group (A) ChAT activity in the lumbar spinal cord of wild type and transgenic mice. (B) ChAT activity in the olfactory cortex of wild type and transgenic mice. (C) ChAT activity in the hippocampus of wild type and transgenic mice. $^{*}P < 0.05$ vs. wild type mice; $^{\#}P < 0.05$ vs. creatine-supplemented G93A mice. Bonferroni's test after ANOVA.

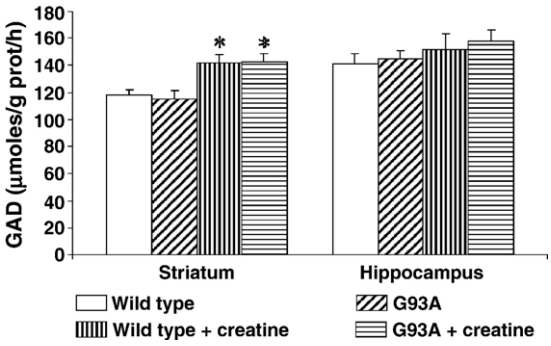

Fig. 4. Effect of long-term creatine supplementation on GAD activity in the striatum and hippocampus of wild type and G93A mice. Bars are the mean± SE of 7 (G93A mice) or 5 (wild type mice) animals per group. *$P < 0.05$ vs. corresponding normally-fed animals. Bonferroni's test after ANOVA.

NMDA, but not against kainic acid or AMPA, notwithstanding the similarity in the striatal lesion size [23]. The present results give an example of the second case, as we demonstrate here that long-term creatine supplementation protects striatal, but not basal forebrain, neurons against ibotenic acid excitotoxicity. These intriguing results suggest that, in addition to a general role of metabolic type, creatine supplementation may have differential effects related to the nature, the brain topography and the connectivity of different neuron types. In this respect, a possible effect on GABAergic system, which is suggested by our data on GAD activity increase in the striatum of creatine-supplemented rats, may be relevant in order to investigate at the neurochemical level these possible differences. Interestingly, indeed, a similar increase of GAD activity was not detected in the cortex of creatine-supplemented rats (data not shown). A specific effect of long-term creatine supplementation on GAD activity, apparently restricted to the striatum, also emerged from measurement of the enzymatic activity in transgenic and wild type mice. Researches are presently being carried on, to see whether increased potency of GABA synthesis actually results in increased GABA availability at synaptic sites and whether similar alterations can be detected in other brain regions.

Long-term creatine administration has been demonstrated to delay disease symptoms and to prolong mean lifespan in the G93A mouse model of ALS [19,37]. Concerning survival, we confirm here a small, but significant, beneficial effect of dietary creatine supplementation. Our new observation regards a primary neurochemical marker of the disease, i.e. the decrease of ChAT activity related to degeneration of the cholinergic motor neurons in the spinal cord, as well as an accessory neurochemical marker recently discovered by us [7], i.e. the decrease of ChAT activity in forebrain regions that are targets of the cholinergic neurons of the basal forebrain. As we have recently demonstrated that both these markers are significantly affected at an intermediate stage of the disease (110 days of age), we have used this stage to characterize protective effects of the creatine-supplemented diet. By using the neurochemical parameter to evaluate the effect of choline supplementation, no significant alteration in the cholinergic decrement associated with the disease was demonstrated in the lumbar segment of the spinal cord, while the decrease of cholinergic activity found in the two forebrain regions examined was completely reverted by the dietary supplementation. Previous reports have correlated the increased survival granted by creatine, which may vary in its extent likely depending on subtle differences among different founders of litters from the same strain ([19,37] and present results), with protection and delaying of motor neuron death [19]. This was, however, demonstrated at 120 days of age in a group of transgenic mice whose normal lifespan reached 143 days instead of the 126–132 days documented by others, including us ([7,11,37] and present results). It may be that a more rapid progression of the disease renders more difficult to put in evidence stages of partial protection concerning the primary targets of the neurodegenerative process, i.e. the spinal cord motor neurons and their distinctive neurochemical marker. Alternatively, this difference could be explained by the different way adopted to monitor spinal motor neuron death, i.e. direct cell counting vs. quantitative evaluation of the decrease of a specific neurochemical marker, as previous observation suggested that the matching between these two procedures is not absolute [7]. The complete reversion of the cholinergic impairment detected in forebrain areas of G93A mice at 110 days of age [7], on the other hand, demonstrates that other neurochemical correlates of the disease can be ameliorated by creatine supplementation, in parallel with the prolonged lifespan. Even if present data do not allow us to postulate a causal relationship among the two events, it is of interest the fact that also in G93A mouse model a neurochemical parameter, whose alterations characterize symptomatic stages of the disease [7], is kept at its normal levels by long-term dietary creatine supplementation.

Taken together, the present data emphasize the usefulness of studying alterations of neurochemical parameters marking specific neuronal populations, in evaluating neuroprotective effects of long-term creatine supplementation in animal models of acute and chronic neurodegenerative diseases.

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