Research report

Creatine increases hippocampal Na⁺,K⁺-ATPase activity via NMDA–calcineurin pathway

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A B S T R A C T

Achievements made over the past few years have demonstrated the important role of the creatine and phosphocreatine system in the buffering and transport of high-energy phosphates into the brain; however, the non-energetic processes elicited by this guanidine compound in the hippocampus are still poorly understood. In the present study we disclosed that the incubation of rat hippocampal slices with creatine (10 mM) for 30 min increased Na⁺,K⁺-ATPase activity. In addition, intrahippocampal injection of creatine (5 nmol/site) also increased the above-mentioned activity. The incubation of hippocampal slices with N-methyl-D-aspartate (NMDA; MK-801, 10 μM) and NMDA Receptor 2B (NR2B; ifenprodil, 3 μM) antagonists but not with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)/kainate antagonist (DNQX, 10 μM) and nitric oxide synthase inhibitor (NOS; 1-NAME, 100 μM), blunted the effect of creatine on Na⁺,K⁺-ATPase activity. Furthermore, the calcineurin inhibitor (cyclosporine A, 200 nM) as well as the Protein Kinase C (PMA, 100 nM) and Protein Kinase A (8-Br-cAMP, 30 μM) activators attenuated the creatine-induced increase of Na⁺,K⁺-ATPase activity. In addition, the incubation of hippocampal slices with creatine (10 mM) for 30 min increased calcineurin activity. The results presented here suggest that creatine increases Na⁺,K⁺-ATPase activity via NMDA–calcineurin pathway, proposing an putative underlying non-energetic role of this guanidine compound. However, more studies are needed to assess the contribution of this putative alternative role in neurological diseases that present decreased Na⁺,K⁺-ATPase activity.

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1. Introduction

Creatine (N-[aminoiminomethyl]-N-methyl glycine) is a guanidine compound synthesized in the kidneys, liver, pancreas, and brain or obtained from alimentary sources like meat and fresh fish (Wyss and Kaddurah-Daouk, 2000). Experimental and clinical findings indicate that creatine-induced high-energy phosphate maintenance protects against ATP depletion in a number of pathological conditions including Alzheimer’s (Barkl et al., 2001), Parkinson’s (Bender et al., 2006, 2008) and Huntington’s diseases (Ferrante et al., 2000; Hersch et al., 2006; Ryu et al., 2005); amyotrophic lateral sclerosis (Ellis and Rosenfeld, 2004; Rosenfeld et al., 2008; Shefter et al., 2004), and traumatic brain injury (Sullivan et al., 2000; Sakellaris et al., 2006; Scheff and Dhillon, 2004). Although there is the notion that the mechanisms of neuronal function improvement and neuroprotection exerted by creatine include enhanced energy buffering, a non-energetic neuromodulatory role for creatine has also been proposed (Persky and Brazeau, 2001). Creatine is not only synthesized and taken up by neurons, but that it is also released in an action-potential-dependent manner (Almeida et al., 2006). Furthermore, the bath application of creatine increases both amplitude and number of population spike in the stratum radiatum of the hippocampal CA1 subfield, an effect reverted by the selective NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) (Royes et al., 2008). In line with this view, creatine increases [3H]MK-801 binding to hippocampal membranes by 55% (Royes et al., 2008) and leads to spatial learning improvement, possibly by modulating...
polyamine binding site at the NMDA receptor (Oliveria et al., 2008). However, the downstream effectors of the creatine-induced modulation of the NMDA receptor are still unknown.

NMDA receptors stimulation leads to the activation of Na⁺,K⁺-ATPase (Marcaida et al., 1996; Munhoz et al., 2005; Bersier et al., 2008), a key enzyme involved in the transmembranal transport of sodium and potassium which plays a pivotal role in the cellular ionic gradient maintenance (Skou and Esmann, 1992) and may be modulated by a complex and not completely understood phosphorylation cascade of regulatory proteins. PKA activators, such as forskolin and Sp-5,6-DCI-cBMP5 – as well as the PKC activator phorbol 12,13-dibutyrate – significantly reduce Na⁺,K⁺-ATPase activity in neurons or COS cells (Cheng et al., 1999, 1997a,b; Nishi et al., 1999a). On the other hand, α-adrenergic as well as NMDA receptor activation increases Na⁺,K⁺-ATPase activity through the activation of the calcium-dependent protein phosphatase 2B, calcineurin (Aperia et al., 1992; Marcaida et al., 1996). The glutamate-induced calcineurin activation counteracts the PKC-mediated phosphorylation of Na⁺,K⁺-ATPase leading to a decrease of pump activity (Marcaida et al., 1996).

In this research we take into account that creatine may play a putative role as a neuromodulator in the brain (Almeida et al., 2006). In this sense, previous works from our group suggest that creatine may modulate brain NMDA receptors (Royes et al., 2008; Oliveira et al., 2008). Furthermore, in the same way, there are data in literature showing that glutamatergic agonists increase Na⁺,K⁺-ATPase activity in cultured neurons (Inoue et al., 1999). Therefore, we decided to investigate whether creatine alters Na⁺,K⁺-ATPase activity in rat hippocampal slices. Moreover, given that PKA, PKC and calcineurin are major downstream kinases/phosphatases involved in the Na⁺,K⁺-ATPase activity regulation (Cheng et al., 1999; Nishi et al., 1999a,b), we also investigated the effect of creatine on calcineurin activity and whether those pathways are involved in the effect exerted by creatine on Na⁺,K⁺-ATPase activity.

2. Materials and methods

2.1. Animals and reagents

Adult male Wistar rats (250–300 g) maintained under controlled light and environment (12 h light/dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to food and water were used. Animal utilization reported in this study was conducted in accordance with the policies of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised in 1996) and with the Institutional and National regulations for animal research. All efforts were made to reduce the number of animals used, as well as to minimize their suffering.

Phorbol 12-myristate 13-acetate (PMA) was dissolved in 100% ethanol and then diluted with acSF (artificial cerebrospinal fluid) in such a way that the ethanol concentration did not exceed 0.006%. 6,7-Dinitroquinoxaline-2,3-(1H,4H)-dione (DNQX) was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted with acSF so that DMSO concentration would not exceed 0.003%. Creatine, creatinine, 3-guanidinopropionic acid (3-GPA), tetrodotoxin (TTX), N-nitro-l-arginine methyl ester (l-NNAME, 50, 100 and 1000 μM) were stored in aliquots at −20 °C and then diluted in acSF. 30 mM Tris-HCl buffer, pH 7.4, 10 μM DNQX, 10 μM of theophylline, and 50 μg of protein in the presence or absence of ouabain (1 mM); summung up to a final volume of 250 μL. The reaction was started by the addition of adenosine triphosphate to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 50 μL of 60% (v/v) trichloroacetic acid. Saturated substrate concentrations were used and the reaction was lineal with protein (1 mg/mL) and time (30 min). Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as described by Fiske and Subbarow (1925), using KH₂PO₄ as a reference standard. Specific Na⁺,K⁺-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol Pi/mg protein/min.

In a separate set of experiments, we investigated whether some Na⁺,K⁺-ATPase α isoforms are selectively modulated by creatine. For this purpose, we used a classical pharmacological approach based on the isoform-specific sensitivity to ouabain (Nishi et al., 1999a). We determined whether creatine increased ouabain-sensitive ATPase activity using 3 μM or 4 μM ouabain (so as to inhibit only the Na⁺,K⁺-ATPase isoforms containing α₂ and α₃ subunits, or to inhibit every isoform, respectively). Furthermore, Mg²⁺-ATPase activity was measured in the presence of 3 μM ouabain, according to Munhoz et al. (2005).

2.5. Creatine kinase enzymatic activity assay

After the incubation period, the medium was discarded and slices were gently homogenized (7–10 strokes) in creatine kinase assay buffer. Briefly, creatine kinase activity assays were performed on hippocampal slices homogenates (6 mg/mL) using a commercial creatine kinase kit according to the manufacturer’s protocol (Enzo Life Sciences, kit no. BML-AK804). Reactions were stopped after 30 min, and absorption was read spectrophotometrically at a wavelength of 620 nm. Creatine kinase was expressed in nmol Pi/mg protein/min.
2.6. In vivo experiments

To determine whether the creatine-induced increase on Na⁺,K⁺-ATPase activity also occurred in vivo, animals were anesthetized with Equithesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, and 11% ethanol, 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Two cannulae were inserted 1 mm above the CA1 region of the dorsal hippocampus, bilaterally (coordinates relative to bregma: AP 4 mm, ML 3 mm, V 2 mm from the dura) under stereotaxic guidance (Paxinos and Watson, 1986). Chloramphenicol (200 mg/kg, i.p.) was administered immediately before the surgical procedure. Three days after the surgical procedure, animals were injected with creatine (5 mM, 0.5 μL) and 30 min thereafter animals were sacrificed. The hippocampi were rapidly removed and gently homogenized (7–10 strokes) in ice-cold 10 mM Tris–HCl buffer, pH 7.4 and Na⁺,K⁺-ATPase activity measured as described above. The dose of creatine used in this set of experiments was chosen based on previous studies (Oliveira et al., 2008) and is comparable to the concentrations of creatine used in the in situ experiments.

2.7. Protein determination

The protein content was colorimetrically determined by the method of Bradford (1976) using bovine serum albumin (1 mg/mL) as a standard.

2.8. Statistical analyses

Data were analyzed by a t test, one- or two-way ANOVA, and post hoc analyses were carried out by the Student-Newman-Keuls test, when appropriate. Correlation analysis was carried out using the Pearson’s correlation coefficient. A probability of \( P < 0.05 \) was considered significant. All data are expressed as mean ± SEM.

3. Results

Fig. 1A shows the effect of creatine (0, 0.1, 1 or 10 mM) on Na⁺,K⁺-ATPase activity in rat hippocampal slices. Statistical analysis disclosed that creatine (10 mM) increases Na⁺,K⁺-ATPase activity in rat hippocampal slices [\( F(3,32) = 2.99; P = 0.05 \), Fig. 1A]. Fig. 1B shows the effect of creatine (10 mM) on Na⁺,K⁺-ATPase activity of purified plasma membranes from hippocampal slices. In purified plasma membranes from hippocampal slices creatine also increase Na⁺,K⁺-ATPase activity [\( t(8) = 4.055; P < 0.05 \), Fig. 1B] in spite of the higher specific activity. To determine the effect of creatine in a cell free system, we added creatine directly to hippocampal homogenates. In this experimental condition, creatine (10 mM) did not alter the Na⁺,K⁺-ATPase enzyme activity [\( F(1, 8) = 0.47; P > 0.05 \), Fig. 1C]. Using a classical pharmacological approach based on the isoform-specific sensitivity to ouabain concentration (Nishi et al., 1999a), we investigated whether some α isoforms of Na⁺,K⁺-ATPase are selectively activated by creatine. The results presented in this report suggest that the stimulatory effect of creatine on Na⁺,K⁺-ATPase activity is specific for α2/3 isoform, since the effect of creatine on the enzyme activity was not observed in the presence of 3 μM ouabain (a concentration that inhibits α2/3, but not α1 isoforms). Furthermore, such creatine effect was extended to Mg²⁺-ATPase [\( F(7, 40) = 231.2; P < 0.05 \), Fig. 1D].

Statistical analysis also disclosed that the intra-hippocampal injection of creatine (5 nmol/site) increased Na⁺,K⁺-ATPase activity by 21% in the hippocampus [\( F(1, 18) = 7.03; P < 0.05 \), Fig. 1F], confirming the results obtained in situ. To test whether the creatine-induced increase in Na⁺,K⁺-ATPase activity may be mediated by trans-cellular creatine transport and for its metabolite (creatine), hippocampal slices were incubated with increasing concentrations of creatine (0, 0.1, 1 or 10 mM) and 3-GPA (10 mM). In our experimental conditions, creatine [\( F(3, 20) = 0.75; P > 0.05 \) (Table 1) had no effect on Na⁺,K⁺-ATPase activity. Accordingly, statistical analysis disclosed that the creatine-induced Na⁺,K⁺-ATPase activity increase was not attenuated by 3-GPA [\( F(3, 36) = 2.21; P < 0.05 \), Fig. 2A].

In the present study, we showed that such increase was attenuated by a voltage-gated sodium channel blocker (TX, 0.1 μM) [\( F(3, 24) = 6.56; P < 0.05 \), Fig. 2B], NMDA antagonist (MK-801, 10 μM) [\( F(3, 20) = 5.75; P < 0.05 \), Fig. 2C] and the selective antagonist for NR2B subunit (ifenprodil, 3 μM) [\( F(3, 28) = 6.42; P < 0.05 \), Fig. 2D].

Table 1

<table>
<thead>
<tr>
<th>Treatment (μM)</th>
<th>Na⁺,K⁺-ATPase activity (nmol P/mg protein/min)</th>
<th>Creatinine</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td>aCSF</td>
<td>72.38 ± 4.81</td>
<td></td>
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<tr>
<td>0.1</td>
<td>79.32 ± 4.74</td>
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<tr>
<td>1</td>
<td>78.14 ± 4.79</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>80.88 ± 2.17</td>
<td></td>
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</tbody>
</table>

Data are mean ± SEM for n = 6 in each group.

However, statistical analysis disclosed that the stimulatory effect exerted by creatine on Na⁺,K⁺-ATPase activity was not reverted by co-incubation with AMPA/kainate antagonist (DNQX, 10 μM) [\( F(3, 16) = 0.12; P > 0.05 \), Fig. 2E]. In addition, although the Na⁺,K⁺-ATPase activity is regulated by NMDA–NOS pathway (Munhoz et al., 2005), the results presented in this report showed that a non-specific isoform NOS inhibitor (L-NAME, 100 μM) did not alter the studied enzyme activity [\( F(3, 24) = 0.82; P > 0.05 \), Fig. 2F].

The results presented in this report showed that slices preincubated with activator of PKC (PMA; 100 mM) and PFA (8-Br-cAMP; 30 μM) attenuated the creatine-induced increase in Na⁺,K⁺-ATPase activity [\( F(3, 32) = 4.79; P < 0.05 \) and \( F(3, 20) = 4.47; P < 0.05 \), Fig. 2G and H, respectively]. In addition, statistical analysis disclosed that the incubation with cyclosporin A (200 mM) attenuated the stimulatory effect of creatine on Na⁺,K⁺-ATPase activity [\( F(3, 20) = 7.08; P < 0.05 \), Fig. 2I]. In line of this view, we demonstrated that hippocampal slices incubated with creatine had higher both Na⁺,K⁺-ATPase [\( F(1, 8) = 7.86; P < 0.05 \), Fig. 3A] and calcineurin [\( F(1, 8) = 24.16; P < 0.05 \), Fig. 3B] activities. Moreover, correlation analysis (Pearson’s) disclosed that Na⁺,K⁺-ATPase activity positively correlates with calcineurin activity (\( r = 0.874; P < 0.05 \), Fig. 3C).

4. Discussion

In the current study we showed that the incubation with creatine (10 mM) increases Na⁺,K⁺-ATPase activity in rat hippocampal slices homogenates and plasma membranes and that this effect seems to be α2/3 isoform-specific, taking into account the isoform-specific sensitivity to ouabain, according to our pharmacological approach. Furthermore, the stimulatory effect of this guanidine compound on Na⁺,K⁺-ATPase activity was evidenced in vivo, after intrahippocampal injection, but not in hippocampal homogenates of naive animals incubated directly with creatine, indicating that it requires an intact cellular system. Regarding this point, it is also possible that tissue homogenizing disrupts critical anchoring protein linkages between NMDA receptors, kinases, and Na⁺,K⁺-ATPase, resulting in the uncoupling of enzyme modulatory components. The results presented in this report disclosed that NR2B-containing NMDA receptors, voltage-gated Na⁺ channels and subsequent calcineurin pathway activation are involved in the stimulatory effect exerted by this guanidine compound. Our results also evidenced pharmacologically that the increase of Na⁺,K⁺-ATPase activity elicited by creatine is independent of its uptake. Besides, such effect possibly involves a mechanism independent of changes in the bioenergetic status of the hippocampus, since creatine needs to be transported into the cell to be phosphorylated by creatine kinase and thus to exert its role as an energy buffer.

The creatine concentrations chosen for concentration/response curve were based on previous data. It was reported that exogenous creatine at high concentration (25 mM) protects in vitro brain slices from hypoxic damage (Kass and Lipton, 1986). Incubation of hippocampal slices with different concentrations of creatine (0.5, 1, 10, 25 mM) results in a dose-dependent increase in intracellular
phosphocreatine, reaching a plateau with roughly 10 mM creatine, a condition associated with delayed anoxic depolarization (Balestrino et al., 1999). Such creatine concentration range exerts excitatory action on hippocampal slices (Royes et al., 2008).

We showed here that creatine (10 mM) induced an increase on \( \alpha_{2/3} \) Na\(^+\),K\(^+\) ATPase activity, by a classical pharmacological approach based on the isoform-specific sensitivity to ouabain, without to employ specific antibodies for enzyme isoforms. Therefore, our discussion is based only in our pharmacological data. In the brain, \( \alpha_2 \) and \( \alpha_3 \) isoforms are found in glial and neuronal cells (in same order), contributing to membrane potential generation control, K\(^+\) re-uptake after depolarization (Lecuona et al., 1996; Mobasher et al., 2000; Peng et al., 1997). In this sense, Na\(^+\),K\(^+\) ATPase is a crucial enzyme involved in the control of neuronal excitability and its inhibition may be associated with several neurological disorders. In line of this view, it has been demonstrated experimentally that the Na\(^+\),K\(^+\) ATPase inhibitor ouabain increases Ca\(^{2+}\) entry into brain slices (Fujisawa et al., 1965), and causes electrophysiographically recorded seizures in mice (Jammes et al., 1995), glutamate release by reversal of the Na\(^+\)-dependent transporter (Li and Stys, 2001), and cell death in rat hippocampi (Lees et al., 1990). Furthermore, a mutation in the \( \alpha_3 \) isoform – that reduces Na\(^+\),K\(^+\) ATPase activity about 42% in mice brain – was associated with increased hyperexcitability in the central nervous system, suggesting that Na\(^+\),K\(^+\) ATPase may be involved in control of epileptogenic activity (Clapcote et al., 2009). In this sense, a mutation in the \( \alpha_2 \) isoform has been related with sporadic hemiplegic migraine and epileptic seizures in humans (Gallanti et al., 2008). Considering such fact, it is plausible to propose that the stimulation of \( \alpha_{2/3} \) Na\(^+\),K\(^+\) ATPase elicited by creatine may be a parallel mechanism involved in the neuroprotective effects of this compound in neurological diseases.

Although it is believed that the mechanism underlying creatine-induced neuronal function improvement and neuroprotection involves enhanced energy storage in a variety of experimental models of neurological disease (Klein and Ferrante, 2007; Magni et al., 2007; Royes et al., 2003, 2006), a direct neuromodulatory role for creatine has also been proposed (Persky and Brazeau, 2001). In this context, it has been shown that creatine is not only synthesized and taken up by neurons, but also released in such a manner that it depends on the action potential of the brain cells (Almeida et al., 2006). This guanidino compound also augments cerebral blood flow after stroke (Prass et al., 2007) and reduces inhibitory GABA and glycine responses in mice neurons in cell culture (De Deyn and Macdonald, 1994). Recently, experimental findings from our group suggest that creatine not only seems to be involved in energy metabolism but may also play an important role in the early consolidation of spatial learning in hippocampus with participation of polyamines binding site at the NMDA receptor (Oliveira et al., 2008). Accordingly, the incubation of hippocampal slices with MK-801 and ifenprodil, respectively selective antagonists for NMDA and NMDA/NR2B subunit receptors attenuated the creatine-induced enzyme activity increase, suggesting that this effect is dependent of NMDA/NR2B activation. Furthermore, although the glutamate-NMDA–NOS–cGMP–PKG pathway is known to stimulate \( \alpha_{2/3} \) Na\(^+\),K\(^+\) ATPase activity (Munhoz et al., 2005), in our experimental conditions we did not find this pathway involved in the stimulatory effect of creatine on Na\(^+\),K\(^+\) ATPase.
In this context, considering that another intracellular pathway could be involved in the present stimulatory effect of creatine, we decided to investigate the involvement of calcineurin and PKA/PKC pathways. This particular experiment data showed that the stimulatory effect exerted by creatine was attenuated by incubation with cyclosporin A (a calcineurin inhibitor). Furthermore, the increase of calcineurin activity as well as the positive correlation between Na⁺,K⁺-ATPase and calcineurin activity in hippocampal slices incubated with creatine reinforces the assumption that creatine stimulates Na⁺,K⁺-ATPase by increasing calcineurin activity. These findings agree with previous observations showing that the glutamate activation of NMDA receptors followed by calcineurin leads to stimulation of Na⁺,K⁺-ATPase in cerebellar neurons in culture (Marcaida et al., 1996). Furthermore, it has been proposed that the stimulation of the α-adrenergic receptor activates the phosphatase calcineurin, leading to an increase of Na⁺,K⁺-ATPase activity in renal tubular cells (Aperia et al., 1992). In the same way, calcineurin-induced Na⁺,K⁺-ATPase dephosphorylation is the mechanism proposed by which norepinephrine induces Na⁺,K⁺-ATPase increase in rat brain (Mallick et al., 2000). In the present study we disclosed that the incubation with the PKC activator (PMA) or the PKA activator (8-Br-cAMP) attenuated the stimulatory effect exerted by creatine on Na⁺,K⁺-ATPase. These data presented here leads us to speculate that the increase in Na⁺,K⁺-ATPase activity can occur in two ways: directly, by the activation of calcineurin and the counteraction of the PKC-mediated phosphorylation of the Ser-23 in the α subunit (Bertuccio et al., 2003, 2007); or indirectly, by dephosphorylation of the phosphorylation site for PKA (Thr-34) in the dopamine and cyclic AMP-regulated phosphoprotein (Nishi et al., 1999b). However, it is merely an speculation and further studies are needed to clarify this point.

Summing up, we provide, for the first time, pharmacological evidences of a putative non-energetic pathway by which creatine stimulates Na⁺,K⁺-ATPase activity in rat hippocampal slices.

Fig. 2. Effect of 3-GPA (10 mM, A), TTX (0.1 μM, B), MK-801 (10 μM, C), ifenprodil (3 μM, D), DNQX (10 μM, E), l-NAME (100 μM, F), PMA (100 nM, G), 8-Br-cAMP (30 μM, H) and cyclosporin A (200 nM, I) on the creatine-induced (10 mM) increase in Na⁺,K⁺-ATPase activity of rat hippocampal slices. Data are mean ± SEM for n = 5–9 in each group. *A significant difference compared with all groups.
Furthermore, the results presented in this report suggest that at least some of these effects are mediated by NR2B-containing NMDA receptors, voltage-gated Na⁺ channels and subsequent calcineurin pathway activation. The activation of this phosphatase, in turn, counteracts the PKC- and PKA-mediated Na⁺,K⁺-ATPase phosphorylation, leading to the stimulation of α₂δ Na⁺,K⁺-ATPase activity. Therefore, in addition to its role as a precursor of energy-rich compounds that maintains nearly all cellular function and induces neuronal function improvement, the Na⁺,K⁺-ATPase activity increase induced by creatine suggests an underlying non-energetic role of this guanidine compound in neuronal plasticity and possibly in several neurological diseases. However, more studies are required to assess the contribution of this alternative role in protection exerted by creatine in neurological diseases that present decreased Na⁺,K⁺-ATPase activity.

**Conflict of interest**

The authors declare that they have no conflicts of interest.

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**References**


