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MATERNAL CREATINE SUPPLEMENTATION AFFECTS THE MORPHO-FUNCTIONAL DEVELOPMENT OF HIPPOCAMPAL NEURONS IN RAT OFFSPRING

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Abstract—Creatine supplementation has been shown to protect neurons from oxidative damage due to its antioxidant and ergogenic functions. These features have led to the hypothesis of creatine supplementation use during pregnancy as prophylactic treatment to prevent CNS damage, such as hypoxic–ischemic encephalopathy. Unfortunately, very little is known on the effects of creatine supplementation during neuron differentiation, while *in vitro* studies revealed an influence on neuron excitability, leaving the possibility of creatine supplementation during the CNS development an open question. Using a multiple approach, we studied the hippocampal neuron morphological and functional development in neonatal rats born by dams supplemented with 1% creatine in drinking water during pregnancy. CA1 pyramidal neurons of supplemented newborn rats showed enhanced dendritic tree development, increased LTP maintenance, larger evoked-synaptic responses, and higher intrinsic excitability in comparison to controls. Moreover, a faster repolarizing phase of action potential with the appearance of a hyperpolarization were recorded in neurons of the creatine-treated group. Consistently, CA1 neurons of creatine exposed pups exhibited a higher maximum firing frequency than controls. In summary, we found that creatine supplementation during pregnancy positively affects morphological and electrophysiological development of CA1 neurons in offspring rats, increasing neuronal excitability. Altogether, these findings emphasize the need to evaluate the benefits and the safety of maternal intake of creatine in humans.

Key words: hippocampus, neuronal maturation, LTP, neuron excitability, pregnancy.

INTRODUCTION

Creatine (N-[aminoiminomethyl]-N-methyl glycine, Cr) is a guanidine compound synthesized from glycine, arginine and S-adenosylmethionine in kidney, liver, pancreas and brain, but it can be also obtained through diet (Wyss and Kaddurah-Daouk, 2000; Braissant and Henry, 2008), especially from meat and fish. Currently, Cr is well known to be an ergogenic supplement able to enhance muscle performance, but the use of oral Cr supplementation is now being extended in medical field in order to prevent or treat a number of neurodegenerative disorders such as Parkinson's disease, Huntington's chorea and amyotrophic lateral sclerosis (Matthews et al., 1998, 1999; Klivenyi et al., 1999; Malcon et al., 2000; Pastula et al., 2012; Parashos et al., 2014; Rosas et al., 2014).

Conversely, defects in Cr metabolism heavily affect cognitive development in humans (Stökler et al., 2007), and Cr deficiency syndromes related to the enzyme AGAT or GAMT shortage significantly improve upon Cr supplementation started during the neonatal phase (Battini et al., 2006; Schulze et al., 2006; Schulze and Battini, 2007), confirming the Cr importance in neuronal development. Consistently, a number of recent works suggest that *in vitro* neuronal differentiation is affected by Cr (Ducray et al., 2007a,b; Sartini et al., 2012).

Moreover, Cr, by virtue of its pleiotropism, is also involved in a variety of processes relevant to brain physiology and homeostasis such as acid–base balance, post-ischemic recovery of protein synthesis, cerebral perfusion and vasodilation, preservation of mitochondrial function, stabilization of lipid membranes, prevention of lipid peroxidation and antioxidant actions (Sestili et al., 2006, 2011; Ireland et al., 2011). Furthermore Cr is known to interact with the benzodiazepine site of the $GABA_A$ receptor, an effect which might counteract glutamate excitotoxicity and hence may protect the preterm and term fetal brain from the effects of birth hypoxia (Ireland et al., 2011).

Taken together, this wealth of evidences indicates that Cr plays important roles in brain development, function and protection and, accordingly to these, there is a recent growing interest about the possible use of Cr supplementation during human pregnancy, in order to

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Abbreviations: APs, action potentials; ACSF, artificial cerebrospinal fluid; C, capacitance; fEPSPs, field excitatory postsynaptic potentials; GSH, glutathione; HFS, high-frequency stimulations; IR, input resistance; PBS, Phosphate Buffer Saline; PPR, Paired-pulse ratio; PSCs, Postsynaptic currents; RMP, resting membrane potential.

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reduce the prenatal risk of neurological disorders and the perinatal morbidity and mortality (Dickinson et al., 2014). It is important to consider that brain alone accounts for more than 20% of total body energy consumption (Kreis et al., 2002), and it is interesting to note that from early pregnancy up to its later stages endogenous Cr synthesis in the developing brain is very limited, leaving the maternal Cr as the main source for most of fetal life (Braissant et al., 2007; Ireland et al., 2009). Consequently, developing CNS might be exposed to the risk of Cr deficiency in case of premature birth or placental insufficiency (Weis et al., 2011; Sartini et al., 2012). Because its high levels of unsaturated fatty acids, high rate of oxygen consumption and high availability of "free" redox-available iron (Siddappa et al., 2002), brain of surviving infants can be left with severe damages (for an extensive review see Dickinson et al., 2014) as a result of chronic hypoxia-induced oxidative stress (Kaandorp et al., 2012). Thus, taking into account the pleiotropic role of Cr (ergogenic, antioxidant, lipid membrane stabilizer, Sestili et al., 2011; Beal, 2011), it is conceivable that maternal Cr supplementation might represent a powerful tool to prevent or reduce neurological disorders associated with the above described fetal and perinatal risks. Moreover it is important to consider that most perinatal morbidity and demise still occurs in underdeveloped areas of the world where millions of newborn babies suffer from birth hypoxia each year with an elevated number of death or severe disability (Lawn et al., 2005, 2007) in survivors. Related to this serious problem, the availability of an inexpensive tool easy to use as Cr supplementation, could represent an efficient mean of prevention (Dickinson et al., 2014).

However, despite its popularity as a nutritional supplement, its long-term use in healthy adults and its potential application during human pregnancy, the effect of Cr supplementation in functional and structural neuronal differentiation is still poorly studied. In our previous work (Sartini et al., 2012), we have shown the protective role of Cr supplementation on *in vitro* neuron development, but also the influence on neuron excitability, leaving the maternal Cr supplementation as safely treatment during pregnancy an open question.

Considering the great importance of hippocampal neurons in learning processes and their well-known ability to generate functional and structural synaptic plasticity varying their intrinsic excitability, we use this *in vivo* model to address the question of whether Cr supplementation during pregnancy affects neuron development of neonatal rats, using electrophysiological, morphological and biochemical approaches.

EXPERIMENTAL PROCEDURES

Animals and supplementation protocol

Sprague–Dawley albino rats (Charles River, Italy) were used in this study. Animal care and experimentations were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All the experiments were approved by animal subjects review board of the University of Urbino. All efforts were made to minimize the number of animals used and their suffering.

Virgin female and male rats (weighing 200–250 g) were housed with free access to food and water and maintained at an ambient temperature of 22 ± 1 °C with a 12-h light and 12-h dark cycle (lights on at 6 a.m. and off at 6 p.m.). After mating, female rats were randomly divided in two groups: 1. control group (CTRL; $n = 3$) drinking tap water; 2. supplemented group (TREAT; $n = 3$) drinking tap water in which creatine (Fluka, Sigma–Aldrich, Italy) was dissolved (1 g/100 ml; Ipsiroglu et al., 2001) from the eleventh day of pregnancy to the day before delivery, due to the expression of Cr membrane transporters at this time point (Braissant et al., 2005). Pups from CTRL and TREAT groups were weighed daily throughout the experimental period. For the present experimental purposes, CTRL and TREAT offspring were killed at various developmental ages from postnatal day 0 (P0, day of birth) to weaning (P21).

Biochemical assays

P0 pups from different CTRL and TREAT litters were used ($n = 10$ for each group) for measuring levels of creatine and reduced glutathione (GSH) in brain tissue. Pups were anesthetized by hypothermia and killed by decapitation; brains were then quickly removed, immediately frozen in liquid nitrogen and stored at −80 °C.

Brain tissues from control or Cr supplementation groups were homogenized with a glass potter in PBS (Phosphate Buffer Saline, Sigma–Aldrich, Italy) and the homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatants were used for measuring creatine, GSH and total protein levels.

Brain creatine levels were colorimetrically detected at 570 nm using the Creatine Assay Kit (Abnova, DBA, Italy), following the manufacturer's instruction, and a BioRad Microplate Reader (Model 680). The same tissues were used to evaluate the levels of GSH, as a marker of oxidative stress: GSH levels were spectrophotometrically determined at 412 nm by the method of Sedlak and Lindsay (1968) with slight modifications (Sestili et al., 2006). Total protein content of brain tissues was assayed spectrophotometrically at 595 nm according to Bradford's method (1976).

Electrophysiological experiments

Male pups from different CTRL and TREAT litters, aged from P14 to P21 ($n = 10$ for each group), were used to obtain slices for performing electrophysiological recordings in the CA1 subfield of the hippocampus, as previously described (Ambrogini et al., 2004; Betti et al., 2011): briefly, pups were anesthetized with ketamine (65 mg/kg b.w.), killed by decapitation and brains were quickly removed to prepare six hundred-micrometer-thick brain slices. Following a slice equilibration period in the recording chamber, field potential and whole-cell patch-clamp recordings were carried out. Analysis of electrophysiological data was performed offline using a