1506
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PhD Course in Life Sciences, Health and Biotechnologies

Curriculum in Health and Exercise Science

XXXI CYCLE

CREATINE SUPPLEMENTATION AND NEURAL PLASTICITY IN CNS DEVELOPMENT

SSD BIO/09

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Academic Year: 2017/2018

Index

Chapter 1

CREATINE METABOLISM AND PLEIOTROPIC EFFECTS

This chapter provides a review about creatine, including its biosynthesis and transport, as well as its metabolism and functions. An excursus on the creatine kinase isoforms and the importance of their different locations in subcellular sites will be described, as well as creatine and phosphocreatine effects as spatial and temporal buffers, and as metabolic regulators. Besides these roles, other creatine effects have been observed, suggesting a "pleiotropic" effect, which might be crucial in normal Central Nervous System development and function.

1.1 Creatine biosynthesis

Creatine (Cr, α-*N*-methylguanidino acetic acid) is a nitrogenous organic acid discovered in 1832 by Michel Eugène Chevreul, and chemically identified by Justus von Liebig in 1847. It can be taken up from the diet, especially from meat and fish, and endogenously synthesized in the liver, pancreas, kidneys, and brain. Endogenous synthesis involves the amino acids glycine, arginine and methionine, and two enzymes: arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). In the first step of Cr biosynthesis, AGAT transfers a guanidinic group from arginine to glycine, yielding guanidinoacetic acid (GAA) and ornithine. The second step, catalyzed by GAMT, consists in the transfer of a methyl group from S-adenosylmethionine (SAM) to convert GAA into Cr, also releasing S-adenosylhomocysteine (SAH) (Fig. 1). The first reaction occurs mainly in the kidney and pancreas, where elevated AGAT activity is found, while the second step is mainly located in the liver where there is elevated GAMT activity. Subsequently, Cr, about 1 g/d acquired from the diet and 1 g/d endogenously synthesized, is distributed through the blood to organs characterized by high and fluctuating energy demands such as the muscles and brain. Here, a transporter Na^+-Cl^-dependent, CT1, allows Cr transit into the cells (Salomons and Wyss 2007). CT1 is the most expressed transporter, although another isoform, CT2, has been found primarily in the testes. 95% of Cr stores are located in the skeletal muscle, while the remaining 5% is distributed in the brain, liver, kidneys, and testes. It is possible to find Cr in two forms: the phosphorylated form (Phosphocreatine) which represents 60% of the stores, and the free form representing the other 40%. Finally, the Cr pool in an average 70 kg young male is around 120-140 g, however it varies between individuals depending on the skeletal muscle fiber type and quantity of muscle mass (Cooper et al. 2012).

Once inside the cell, Cr is phosphorylated through the reversible reaction catalyzed by creatine kinase (CK): Phosphocreatine (PCr) + MgADP + H^+ ↔ Creatine (Cr) + MgATP and acts primarily as a temporal and spatial ATP buffer. In particular, Cr is phosphorylated to generate a pool of PCr that could be used during energy demand to yield ATP from ADP. Then, Cr and PCr are subject to a spontaneous, non-enzymatic conversion to creatinine and phosphocreatinine, which are excreted through urine. This conversion amounts to a significant drop in the cellular Cr/PCr pool, with a constant daily turnover of about 1.5-2% of Cr (Salomons and Wyss 2007).

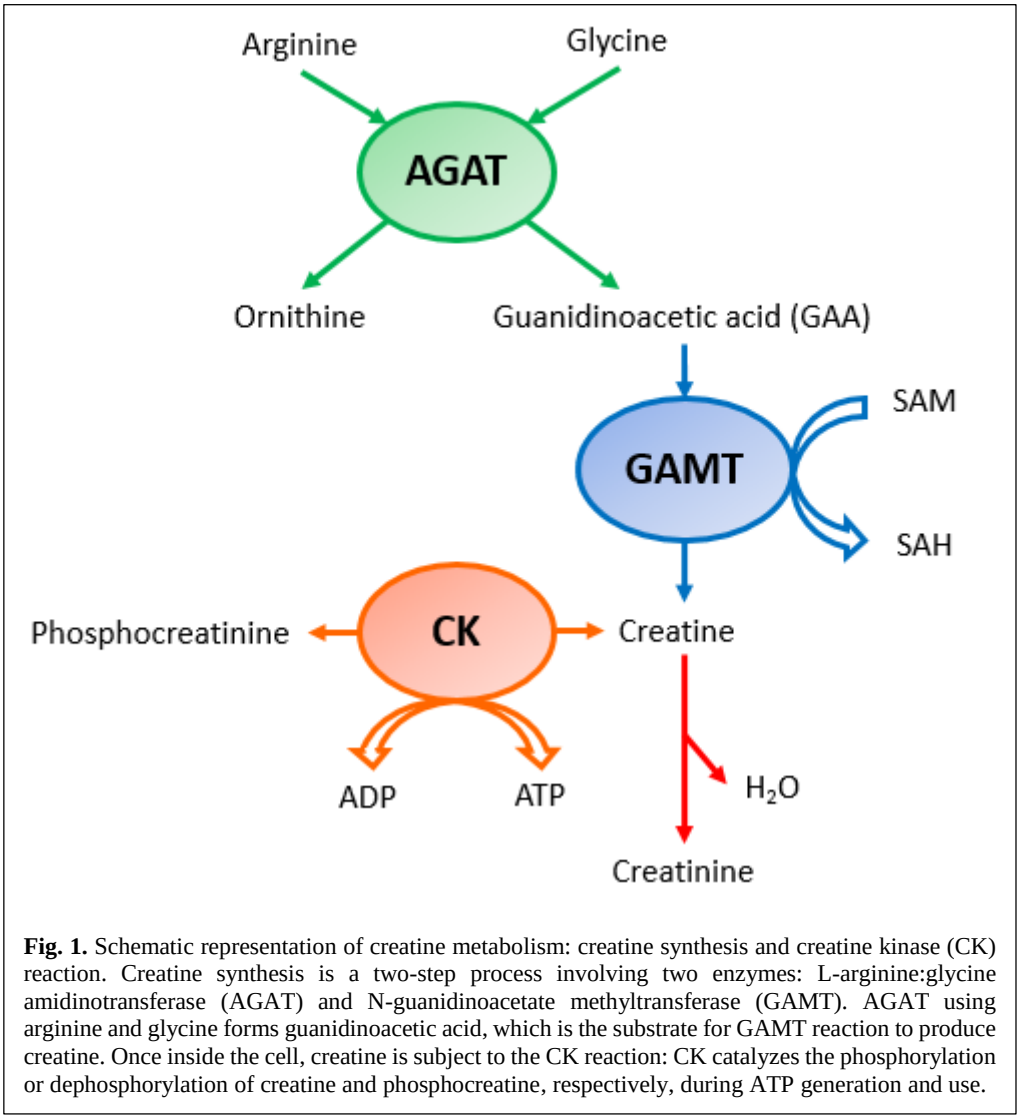

Fig. 1. Schematic representation of creatine metabolism: creatine synthesis and creatine kinase (CK) reaction. Creatine synthesis is a two-step process involving two enzymes: L-arginine:glycine amidinotransferase (AGAT) and N-guanidinoacetate methyltransferase (GAMT). AGAT using arginine and glycine forms guanidinoacetic acid, which is the substrate for GAMT reaction to produce creatine. Once inside the cell, creatine is subject to the CK reaction: CK catalyzes the phosphorylation or dephosphorylation of creatine and phosphocreatine, respectively, during ATP generation and use.

1.2 Creatine transporters

The physiological importance of Cr transport was first recognized with respect to muscle where high concentrations of PCr are demanded for muscle contractions. Cr biosynthesis occurs mostly in other organs, thus about 2 g/day of Cr must be taken up by skeletal muscle to compensate for losses due to irreversible conversion to creatinine. A specific plasma membrane Cr transporter, CT1 (also called SLC6A8, CRT, CRTR, CreaT), is required for the cellular uptake of Cr.

CT is a member of solute carrier family 6 (SLC6), a large family of membrane transporters that mediate the translocation of a range of solutes across plasma membranes, through the co-transport of sodium and chloride down their electrochemical gradients. Regarding this solute carrier family, the transporters for neurotransmitters such as γ-aminobutyric acid (GABA), norepinephrine, and subsequently, dopamine and serotonin were the first to be discovered. Later, transporters for solutes like Cr were also included in this family (Salomons and Wyss 2007). Another transporter, the monocarboxylate transporter 12 (MCT12, also called SLC6A12), has recently been shown to be involved in both the uptake and efflux of Cr. This transporter is also a member of solute carrier family 6 and is predominantly found in the kidneys and retina (Abplanalp et al. 2013).

CT allows Cr to cross the membrane in a saturable, sodium/chloride-dependent and highly specific manner. In more detail, the transport of Cr through the CT is saturable because of Cr K_m and Cr concentrations in plasma. For example, rat and human brain CTs are characterized by an apparent K_m of 29 μM and 15 μM (Saltarelli et al. 1996, Sora et al. 1994), respectively, and these K_m values are 10-40 fold lower than serum Cr concentration (528 μM in rats and 58 μM in humans) (Marescau et al. 1986). Therefore, it is obvious from the concentration of Cr in plasma that CTs work close to saturation, suggesting that the number of transporters in the cell membrane may limit Cr storage. Moreover, research has shown that CT is Na^+-dependent, underlying that other cations such as lithium, choline or N-methylglucamine cannot replace sodium. In addition, CT is also Cl^--dependent. This is an important consideration as not all SLC6 transporters are dependent on chloride. It appears that CT exchange 2 Na^+ and 1 Cl^- for each Cr molecule. Finally, CTs are highly specific for Cr and neither creatinine nor phosphocreatine are substrates. The key features for substrate specificity are a carboxyl group and a guanidino group, separated by no more than 2–3 carbon atoms (Salomons and Wyss 2007).

The fundamental feature of CT and other transporters is how they are able to recognize their substrates and translocate them across the membrane. The structure of a member of the SLC6 transporter family was first provided through the study of the crystal structure of a bacterial leucine transporter ($LeuT_{Aa}$) (Yamashita et al. 2005). $LeuT_{Aa}$ is Na^+-dependent, but not Cl^--dependent, thus its structure could not provide information on the Cl^- binding site. The structure of $LeuT_{Aa}$ supports the two gate theory for transporters: exposure of the binding site to the outside of the cell enables the substrate to bind; closure of the external gate traps the substrate within the membrane; opening of an internal gate allows the release of the substrate into the cell. Although $LeuT_{Aa}$ is only 20-25% identical to the mammalian SLC6 transporters, the conservation of residues in SLC6 transporters at key positions and the topology of $LeuT_{Aa}$ make this bacterial SLC6 homologue a valuable model for mammalian CT and other SLC6 transporters (Salomons and Wyss 2007).

CT is important for normal brain development function as mutations in the CT gene (*SLC6A8*) result in X-linked mental retardation, associated with cerebral creatine deficiency, severe speech and language delay, epilepsy, autistic behavior, and increased urinary Cr/creatinine ratio (Salomons and Wyss 2007). Moreover, studies investigating CT structure and function are essential to understand the optimal Cr supplementation that may efficiently increase the PCr pool in the cell.

1.3 Creatine kinase

The creatine kinase enzyme (CK) was discovered by Lohmann in 1934 and the CK reaction was found in 1936, but its physiological importance was understood only later. In the last few decades, discussion has arisen concerning the role of the Cr/CK system in the distribution and intracellular transport of high-energy phosphates, the importance of which is related to cellular diffusion distances. The presence and the localization of different CK isoforms at various ATP sources and sinks provide the elements for a spatial and temporal ATP buffering and energy transport pathway, the so-called CK shuttle or PCr circuit. In 1966, Bessman and Fonyo were the first to propose this shuttle concept, which implicates cellular compartmentation of the reactants of the CK reaction (Salomons and Wyss 2007).

In higher organisms there are three CK isoforms, cytoplasmic, mitochondrial and flagellar (Cyt-, Mt- and FlgCKs), each targeted to different micro-compartments in the cell. They are found in different forms, in particular, CytCKs are dimers while typical MtCKs exist as both dimers and octamers, with the latter predominating under physiological conditions. FlgCKs are “contiguous trimers” consisting of three fused, complete CK domains in a single polypeptide chain. Furthermore, four CK genes coding for five different isoenzymes have been identified. Two genes for CytCK encode for muscle (M-CK) and brain (B-CK) isoforms, which assemble into active homo-dimeric (MM-CK, BB-CK) and hetero-dimeric (MB-CK) enzymes in the cytosol. MM-CK is expressed at high levels in muscle, representing between 85% and 95% of total CK activity. MB-CK is a transitional isoform, which is present in immature stages of skeletal muscle and, in low amounts in the heart. BB-CK is expressed at high levels in the brain and various other tissues in adult mice. Expression of the other isoenzymes of CK, ubiquitous (uMt-CK) and sarcomeric mitochondrial CK (sMt-CK), is also cell-type dependent. uMt-CK is mostly co-expressed with BB-CK (Salomons and Wyss 2007). Recent studies indicate that in the brain, BB-CK is preferentially expressed in astrocytes and inhibitory neurons, while mitochondrial CK seems to be restricted primarily to neurons (Tachikawa et al. 2004). The expression of sMt-CK is confined to muscle. uMt-CK and sMt-CK isoforms occur as dimeric or octameric enzymes and reside between the inner and outer membranes of mitochondria. Mitochondrial isoforms of CK were discovered in Klingenberg’s laboratory in 1964, shortly after the discovery of the cytosolic MM- and BB-CK isoforms.

In summary, mitochondrial and cytosolic CKs differ in regard to their organellar locations, and their form, dimeric and mainly octameric, respectively, as well as for the fact that the octameric mitochondrial CK behaves as a peripheral membrane protein, interacting with different protein and lipid components of the mitochondrial membranes (Salomon and Wyss 2007). Each isoform has a specific role as mitochondrial CKs

consume ATP to produce PCr to export it to the cytoplasm, while cytoplasmic CKs control energy demand through PCr consumption for local ATP production at subcellular sites of high-energy demand (Hanna-El-Daher and Braissant 2016).

1.4 The Creatine/Phosphocreatine/Creatine Kinase system and pleiotropic effects of Creatine

Cr has pleiotropic effects mainly through the functions of the enzyme CK and its high-energy product PCr. Many studies have demonstrated molecular, cellular, organ and somatic effects of the Cr/PCr/CK system, in particular for cells and organs with high and intermittent energy demands such as skeletal, cardiac and smooth muscle, the kidneys, the Central Nervous System (CNS) and neuronal cells, retina photoreceptor cells, spermatozoa and sensory hair cells of the inner ear. The Cr/PCr/CK system acts primarily as an immediately available temporal energy buffer, a spatial energy buffer or intracellular energy transport system (the Cr/PCr/CK energy shuttle or circuit), and as a metabolic regulator. Indeed, the Cr/PCr/CK energy circuit allows the shuttling of high-energy phosphates between sites of ATP production (glycolysis and mitochondrial oxidative phosphorylation) and sites of ATP utilization (ATPases), counteracting diffusion limitations in cells (Wallimann et al. 2011).

High intracellular ATP concentration and ATP hydrolysis would increase the ADP, Pi and H^+ content, leading to acidification of the cytosol. This would negatively interfere with ATPases activity such as the ATPase related with myofibrillar acto-myosin, and consequently, muscle contraction, and many other cellular processes. The phosphagens system, including PCr together with its corresponding kinase, CK, solves this problem and allows for the immediate replenishment of ATP stores. For example, during muscle contraction the CK reaction uses PCr to resynthesize ATP, removing ADP and H^+ as products of hydrolysis of ATP, so that the net product of ATPases combined with the CK reaction is liberation of Pi as a metabolic signal. Thus, CK acts not only as an energy buffer but also as a metabolic regulator (Wallimann et al. 2011).

The presence of different CK isoforms and their specific subcellular locations are the most relevant features of the Cr/PCr/CK system and the associated cellular functions. The Cr/PCr/CK shuttle exerts its spatial energy buffering role through functionally coupled, subcellular CK micro-compartments located where ATP is produced and where it is consumed. In fact, CK is the enzyme of a reversible reaction, leading either to the increase of the PCr pool or the re-synthesis of ATP, depending on the subcellular location and needs. Studies have shown important features of structure/function relationships and molecular physiology of CK that has allowed an understanding of the Cr/PCr/CK system and its eminent physiological role (Wallimann et al. 2011).

1.4.1 Temporal and spatial energy buffering role

The temporal and spatial energy buffering function of the Cr/PCr/CK shuttle is represented in Fig. 2. Cr acquired from the diet or via endogenous synthesis may cross the membrane through CTs. Inside the cell, Cr is converted into PCr through the action of cytosolic CKs (CK-c, (Fig. 2-C)), CKs coupled to glycolysis

(CK-g, (Fig. 2-B)) or MtCKs coupled to oxidative phosphorylation (MtCK, (Fig 2-A)). In a resting cell, the total Cr pool is subdivided in approximately two-thirds [PCr] and one-third [Cr] and in a high ATP/ADP ratio. Thus, the functional micro-compartments, specifically the glycogenolytic/glycolytic CK-g microcompartments (Fig. 2-B) and the MtCK microcompartment connected to oxidative phosphorylation and energy channeling reactions inside the mitochondria (Fig. 2-A), represent the ATP/PCr-generating side of the circuit (Schlattner et al. 2006). The result of the coupling of CK to glycolytic enzymes and adenine nucleotide translocator (ANT) in the mitochondria is the generation of a large PCr pool (up to 30 mM) available for temporal or spatial energy buffering.

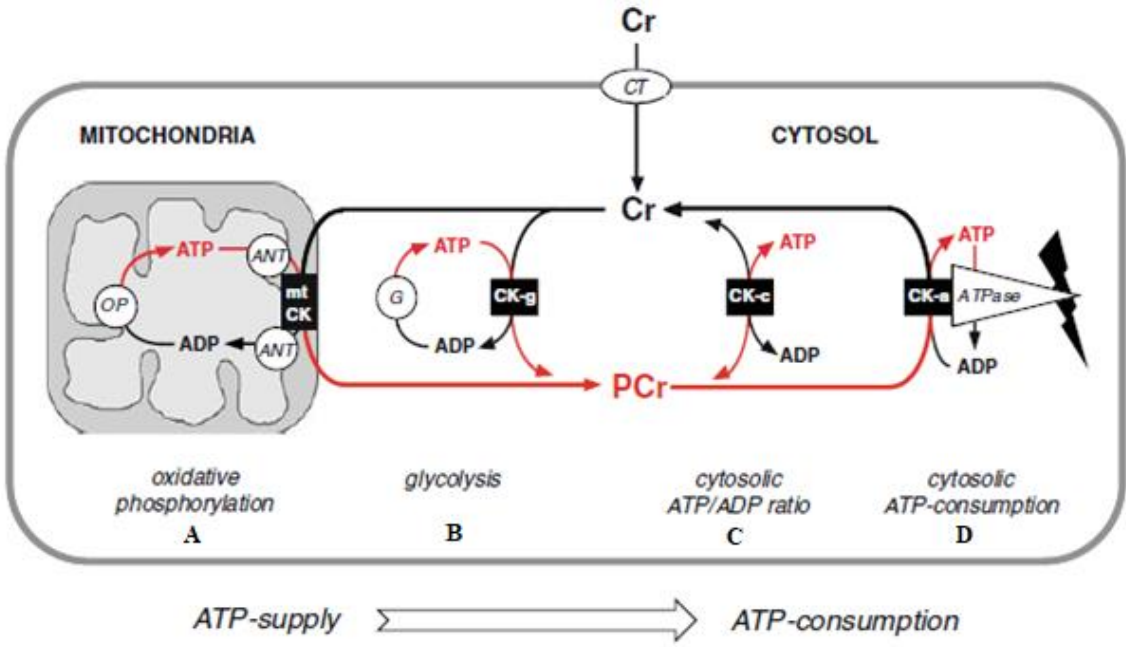

Fig. 2. The temporal and spatial energy buffering of the CK/PCr system. Creatine enters the cells trough Cr transporters (CTs). Inside the cell, PCr, Cr and ATP/ADP are balanced by a soluble fraction of cytosolic CK isoforms (CK-c, see (C)). Another portion of cytosolic CK (CK-g, see (B)) is specifically associated with glycolytic enzymes (G), which accepts glycolytic ATP, while mitochondrial CK isoforms (MtCK, see (A)) is connected to adenine nucleotide translocator (ANT), thus accepting ATP exported from the mitochondrion and generated by oxidative phosphorylation (OP). The PCr generated creates a pool of PCr up to 30 mM, which is available for temporal or spatial energy buffering. Another portion of cytosolic CK (CK-a, see (D)) specifically linked with subcellular sites of ATP utilization also forms tightly coupled microcompartments that regenerate the ATP utilized by the ATPase. The CK/PCr energy shuttle, trough highly diffusible PCr and Cr, connects subcellular sites of ATP production (glycolysis and mitochondrial oxidative phosphorylation) with subcellular sites of ATP utilization (ATPases).

Figure adapted from Wallimann et al. 2011

Other CKs are specifically associated with ATPases that hydrolyze ATP (CK-a, (Fig. 2-D)), such as the myofibrillar acto-myosin ATPase, the SR Ca^{2+}-ATPase, and the plasma membrane Na^{+}/K^{+}-ATPase. These functional micro-compartments, where CK regenerates ATP by using the PCr stores, represent the ATP/PCr-consuming side of the CK/PCr system (Fig. 2-D). In details, the MtCK isoform is specifically located in the mitochondrial intermembrane space with preferential access to ATP generated by oxidative phosphorylation

via ANT of the mitochondrial inner membrane. Thus, the ATP produced by mitochondria, is phosphorylated by MtCKs into PCr, which then moves to the cytosol. The ATP generation in this site leads to the refill of PCr pool in oxidative tissues, such as in the muscle after contraction.

The cytosolic PCr store is built up by CK using ATP predominantly from oxidative phosphorylation (Fig. 2-A) as in the heart, or from glycolysis (Fig.2-B) plus oxidative phosphorylation (Fig.2-A) as in skeletal muscle. PCr is then used to buffer global cytosolic (Fig. 2-C) and local (Fig.2D) ATP/ADP ratios. This represents the temporal buffer function of the system. The importance of the spatial and temporal energy buffering is evident in cells that are polarized, such as spermatozoa, retina photoreceptor cells, and sensory hair cells located in the inner ear, where the Cr/PCr/CK shuttle solves the problem of the limited diffusion capability in long distances of ATP and ADP (Wallimann et al. 2011).

1.4.2 Cytosolic Creatine Kinase associated with glycolysis function

Cytosolic CKs are coupled with glycolytic enzymes (Fig. 2-B) that are involved in ATP synthesis, such as pyruvate kinase (PK), or with the principal regulatory enzyme of glycolysis, phosphofructokinase (PFK), which is regulated through a negative feedback mechanism by ATP increase. The CK-PFK interaction depends on pH and it is stronger at lower pH than at neutrality. This feature is physiologically relevant because during muscle contraction and concomitant glycolysis the intracellular pH may rapidly decrease. Thus, the localization of CKs in this site is crucial for two reasons: (a) through removing glycolytic ATP from this compartment, CK prevents the inhibition of glycolysis by negative feedback regulation via ATP accumulation, and (b) through reducing the depletion of the PCr pool during contraction using the glycolytic ATP (Wallimann et al. 2007).

1.4.3 Mitochondrial Creatine Kinase function

1.4.3.1 Metabolite channeling in the mtCK microcompartment

MtCKs are first assembled into dimers that rapidly associate into octamers, which is the favored form. The mtCK octamers then directly bind to acidic phospholipids in the mitochondrial membranes (Fig. 3), preferentially to cardiolipin of the inner mitochondrial membrane (Schlattner et al. 2004), and in a calcium dependent way directly to VDAC (Schlattner et al. 2001). ANT and MtCK both have affinity for cardiolipin, and are thus located in cardiolipin patches, where they may functionally interact. MtCK is situated in two sites of the mitochondria: (a) in the so-called mitochondrial contact sites (Fig. 3), where MtCK binds both to the inner and outer membrane and it functionally connected with ANT and VDAC and (b) in the cristae (not shown) connected with inner membrane and ANT only. In the figure 3, black arrows represent the preferred or exclusive substrate and product fluxes.

According to this representation, ATP synthesized by oxidative phosphorylation through the F_0F_1-ATPase can cross the inner membrane through ANT in exchange for ADP. This ATP may either move to the cytosol directly via the outer membrane VDAC or used by MtCK to phosphorylate Cr, yielding PCr that exit the mitochondrion via VDAC. Subsequently, ADP generated from the MtCK reaction is accepted by ANT and immediately transported back into the matrix to be re-phosphorylated. In cristae, only ATP/ADP transfer is promoted through direct channeling to the MtCK active site, while Cr and PCr must diffuse along the cristae space to reach VDAC (not shown; Schlattner et al. 2006; Wallimann et al. 2007).

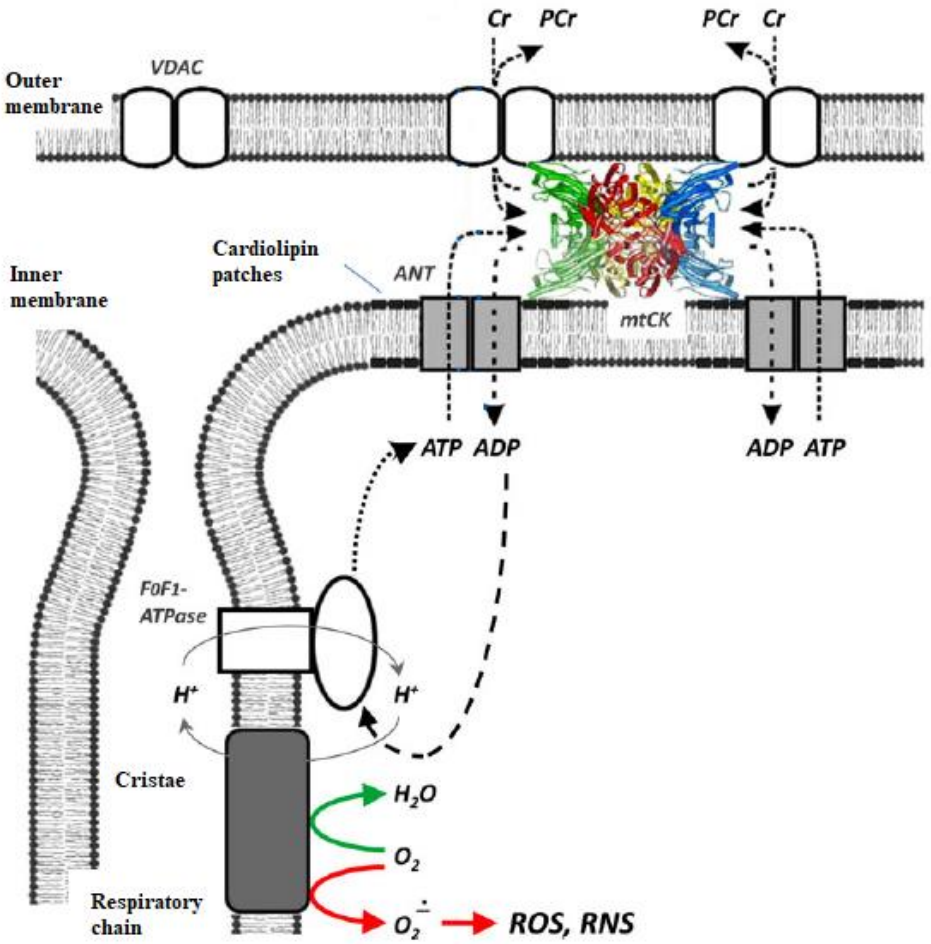

Fig. 3. Mitochondrial MtCK functions for high-energy metabolite channeling. In cells depended on oxidative metabolism, respiration (green arrow), ATP generation and ATP export through the inner membrane of the mitochondrion via adenine nucleotide transporter (ANT) are tightly coupled to phosphorylation of ATP into PCr through MtCK and transfer of PCr into the cytosol through the voltage-dependent anion channel (VDAC) in the outer membrane (black arrows). Creatine stimulates respiration by favoring constant provision of ADP to the matrix (black arrows), that also decreases ROS/RNS generation in the mitochondrion (red arrows) and inhibits mitochondrial permeability transition. The tight coupling of substrate and product fluxes (black arrows) allows the so-called channeling of ''high-energy'' metabolites, with PCr being the one released into the cytosol, and ATP/ADP being mainly recycled within the mitochondrion.

Figure adapted from Wallimann et al. 2011

Functional linking of ANT to MtCK leads to a saturation of MtCK with ATP released by ANT, and simultaneously to a locally high ATP/ADP ratio in the proximity of MtCK. Together with cytosolic Cr, crossing the outer membrane through VDAC, these conditions promote the generation of PCr from ATP by

MtCK without losing energy content. All these reactions together are an instructive example of functional coupling and metabolite channeling. The active ATP/ADP transfer allowed by MtCK promotes ATP synthesis by the F_0F_1-ATPases and, thus, proper functioning of the respiratory chain, preventing the superoxide and reactive oxygen species (ROS) levels increasing (Schlattner et al. 2011). The intricate functional coupling of MtCK to the ANT, that leads to a saturation of the ANT on the outer site of the inner membrane with ADP, which then is transported back into the matrix to be re-phosphorylated by the F_0F_1-ATPase, efficiently connects substrate oxidation to ATP synthesis. This complex functional coupling, by avoiding futile electron transfer, would potentially also lower the production of free oxygen radicals (ROS). In fact, mitochondrial respiration in the presence of Cr needs low ADP concentration (μM) to be fully stimulated, while lacking of Cr and relatively high concentration of ADP (mM) are needed for a comparable respiratory rate. This important phenomenon is called "creatine-stimulated respiration".

Finally, Cr shows an indirect antioxidant effect by significantly reducing the ROS production in mitochondria, as well as elevating and preserving the mitochondrial membrane potential (Meyer et al. 2006). These and other Cr effects that go beyond cell bioenergetics may represent the basis for the interesting neuroprotective role that has been discovered recently (Andres et al. 2008).

1.4.3.2 MtCK and mitochondrial membranes

Both mitochondrial isoforms of CK, sMt-CK and uMt-CK octamers, are associated with the phospholipids of the mitochondrial membranes, in particular cardiolipin. The capacity of MtCK to bind to and cross-link the inner and outer membranes explains the contact site formation between the two membranes and the resulting mechanical stabilization of mitochondria. Furthermore, recent studies have shown that MtCK, once bound to cardiolipin containing membrane vesicles, is able to create cardiolipin molecules clusters around its molecular surface (Epand et al. 2007b) and, if cross-linked to another membrane vesicle, MtCK is able to facilitate lipid exchange between the inner and outer membranes (Epand et al. 2007a). This might be important for the structure and physiology of these membrane contact sites and the pre-apoptotic process of mitochondrial permeability transition pore (MPTP) function.

As previously mentioned, MtCK is located in two strategically important sites: in the intermembrane and the cristae space. In the periphery of mitochondria, MtCK is part of a protein complex that is included in the so-called MPTP complex. Although the molecular structure of the pore has not been clarified, it appears to involve ANT-1 isoform, VDAC and MtCK. MPTP opening is an early event in apoptosis, leading to swelling of mitochondria and the release of apoptosis-inducing factors that can be initiated by elevation of extra-mitochondrial [Ca^{2+}] (Azzolin et al. 2010). Experiments in mice in which MtCK is absent or overexpressed have shown inducing or protection from apoptotic events, respectively. In these studies, Cr is comparable to antiapoptotic agents showing the role of Cr not only in stimulating mitochondrial respiration, but also in preventing apoptosis beginning in the mitochondria (Brdiczka et al. 2006). This might explain some of the cell-related protective effects observed with Cr supplementation against hypoxia, liver toxins and tumor

necrosis factor-induced apoptosis. Moreover, PCr is able to bind to and protect biological membranes (Saks et al. 1996; Tokarska-Schlattner et al. 2003, 2005), and this is probably also true for the PCr produced by MtCK in mitochondria that bind to mitochondrial membranes and stabilize against swelling. Thus, MtCK together with Cr seems to exert cell protection not only through improving cellular energetics, but also by energy-independent effects that also prevent and reduce apoptosis.

1.5 Creatine and Central Nervous System

The brain, which constitutes only about 2% of body mass, may account for up to 20% of total energy consumption. Thus, a high rate of ATP synthesis is necessary to maintain normal Central and Peripheral Nervous System function, including the electrical membrane potentials, ions gradients, Ca^{2+} homeostasis, and signaling activities (Braissant et al. 2010). In this scenario, the Cr/PCr/CK system plays an important role during rapid changes in ATP demands that occur for physiological activities of neurons, in particular through functionally coupling sites where ATP is generated and where it is consumed. In fact, the diffusion distances may be longer in neurons, and sites of high-energy consumption are often localized at remote locations from the soma, i.e., synapses. The importance of this energy buffer system in energy metabolism (Fig. 4) is evident in the brain where Cr is depleted, which is characterized by disruption of neuronal functions such as loss of hippocampal mossy fiber connection (Zandt et al. 2004), and changes in mitochondrial structure showing intramitochondrial uMt-CK-rich inclusion bodies (Wyss and Wallimann 1994). This last feature is typical for several clinical diseases, such as encephalomyopathies and mitochondrial myopathies.

The importance of the Cr/PCr/CK system is also demonstrated by experiments using CK knockout mice or through depletion of brain Cr using pharmacological agents, where structural modifications as well as cognitive and behavioral aspects are seriously affected. In humans, Cr deficiency syndromes, involving either its biosynthesis or transport, have been recently discovered. Patients suffering from these disease show a lack of cerebral Cr, and exhibit multiple and critical neurological symptoms, such as developmental and speech delay, epileptic seizures, autism and severe mental retardation. This evidence suggests a significant physiological relevance of Cr/PCr/CK system for normal brain function (Andres et al. 2008). It is important to note that the CNS synthesizes its own Cr and low amounts of Cr is supplied from the periphery due to the limited permeability of the blood-brain barrier, as shown in detail in next chapters. Finally, in the brain, the Cr/PCr/CK system shows some peculiarities, with several studies on the variable expression of BB-CK and uMt-CK isoforms in glial and neuronal cells demonstrating close metabolic collaboration between these cell types for brain energetics (Kasischke et al. 2004). In fact, Cr through the CK reaction has been proposed to play the metabolic role in an intricate neuron-glial shuttle (Tachikawa et al. 2004).

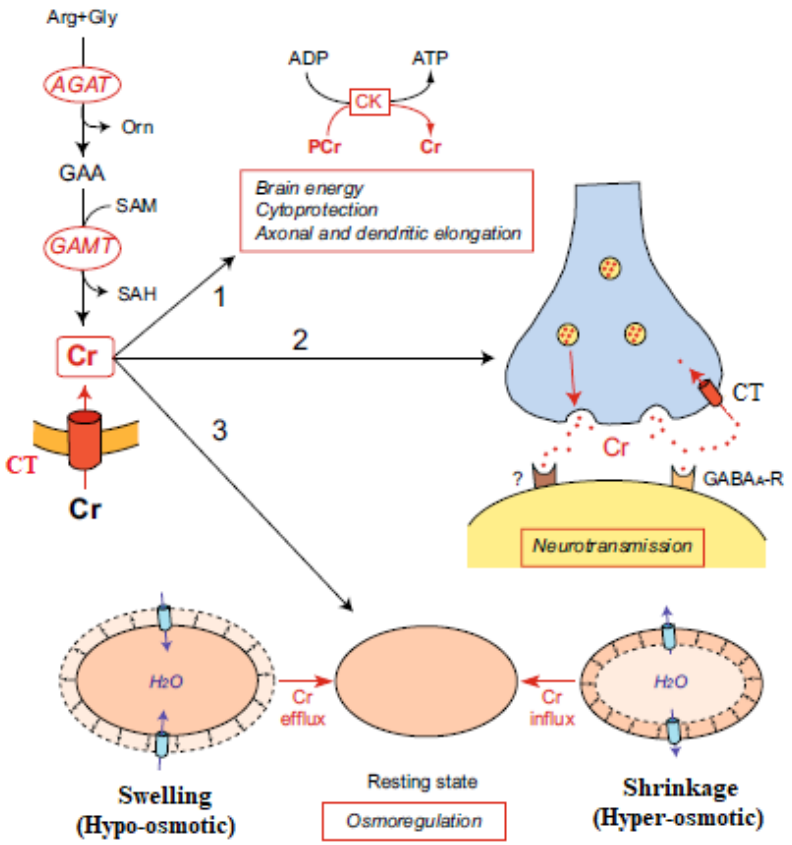

Fig. 4. Creatine functions in the Central Nervous System. (1) Creatine allows ATP regeneration and high-energy phosphate shuttling through the Cr/PCr/CK system, and it is linked to basic cellular functions, namely maintaining membrane potential, ions gradients, Ca^{2+} homeostasis and, as a consequence, plays a role in cytoprotection as well as axonal and dendritic elongation. (2) Creatine is suggested to play a role in neurotransmission as it can be synthesized in neurons, released in an action potential-dependent manner, and it is a partial agonist of postsynaptic $GABA_A$-R. Moreover, CT is expressed in the presynaptic element, probably for re-uptake and recycling of creatine. (3) Creatine represents one of the main CNS osmolytes (Cr efflux in hypo-osmotic conditions, and Cr taken up in hyper-osmotic conditions).

Figure adapted from Hanna-El-Daher and Braissant 2016.

The effect of Cr on the CNS goes beyond a role in cellular energy metabolism and in enhancing the cellular energy status. Indeed, recent findings have shown that Cr acts also as a direct antiapoptotic and antioxidant molecule, assuming additional importance in CNS functions. As a direct antiapoptotic molecule, as mentioned previously, Cr together with the action of uMt-CK in mitochondria prevents MPTP opening, an early event in apoptosis (Dolder et al. 2003). Furthermore, Cr supplementation has shown antioxidant effects through a mechanism involving a direct scavenging of ROS (Sestili et al. 2006), or by reducing the production of ROS generated by mitochondrion. This reducing of ROS production is an indirect effect achieved through the stimulation of mitochondrial respiration by Cr (Kay et al. 2000) that allows an efficient recycling of ADP inside mitochondria by uMt-CK, leading to tight coupling of mitochondrial respiration with ATP synthesis and suppression of ROS formation (Meyer et al. 2006). Protective effects of Cr were shown also against UV stress in keratinocytes and on human skin (Lenz et al. 2005). Moreover, it has been found that Cr normalizes mutagenesis of mitochondrial DNA and its functional consequences produced by UV irradiation of skin cells

(Berneburg at al. 2005). This evidence denotes the role of Cr in preventing and reducing the generation of ROS that may cause cell damage and inactivation of CK, and through this effect Cr may have important role also in CNS function and protection. In fact, recent experiments *in vivo* and *in vitro,* using rat brains and CNS cells respectively, have demonstrated the antioxidant role of Cr in the CNS (Ireland et al. 2011; Sartini et al. 2012).

An interesting study has shown a neuroprotective role of Cr through improved reperfusion rather than changes in the bioenergetics status in a mouse model of stroke (Prass et al. 2007), and in *in vivo* and *in vitro* rodent models of ischemia/anoxia, Cr perfusion before ischemia/anoxia reduced the volume of damaged brain tissue and led to neurological improvement (Hanna-El-Daher and Braissant 2016). Cr was also proposed as neuroprotective for several neurodegenerative disease such as Parkinson's or Huntington's disease (Lensman et al. 2006; Beal 2011; Cunha et al. 2014), however, the first therapeutic Cr supplementation trials appeared rather disappointing. This is probably due to the limited Blood-Brain barrier (BBB) permeability for Cr, and suggests to find new ways to facilitate Cr uptake by CNS. Finally, the Cr/PCr/CK system is essential during brain development to provide enough ATP for growing axons and dendrites at their growth cone extremities, and Cr was found to have pro-differentiation/trophic actions on maturing neuroblasts, suggesting an importance also in CNS development (Sartini et al. 2012).

In recent years, several studies have suggested that Cr may play roles in the brain neurotransmission systems (Fig.4). *In vivo* and *in vitro* studies have demonstrated that Cr is able to act on GABAergic neurons as partial agonists of post-synaptic $GABA_A$ but not $GABA_B$-receptors (Koga et al. 2005). Moreover, Cr was shown to be released in an action-potential dependent manner within organotypic cultures of rat brain slices, while synaptosomes prepared from the brain of young and adult rats were able to take up Cr (Almeida et al. 2006; Peral et al. 2010). This strongly indicates that Cr may even act as a true neurotransmitter, involving a recapture/recycling system through CT expression in axonal terminals (van de Kamp et al. 2013). Finally, Cr may also act as an osmolyte and was found to protect cells from hyper-osmotic shock *in vitro* (Alfieri et al. 2006) and *in vivo* (Bothwell et al. 2002; Braissant et al. 2011), suggesting Cr as one of the main brain cell osmolytes.

Chapter 2

CREATINE SUPPLEMENTATION

An excursion on creatine supplementation will be provided in this chapter. First, early studies about creatine supplementation as an ergogenic aid in sport will be reported. Then, recently, because of its pleiotropic properties, including antiapoptotic, antiexcitotoxic, antioxidant, and neuroprotective effects, creatine supplementation has also been investigated in the clinical field. Therefore, the focus in the second part of the chapter will be on creatine administration in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis, and Huntington's disease, and in inborn errors of metabolism, namely mitochondrial encephalomyopathies, hyperammonemia, and cerebral creatine deficiency syndromes. The latter focus represents important diseases to investigate and clarify the importance of creatine on brain function and development. If creatine were so fundamental for Central Nervous System development, supplementation would be important during pregnancy to prevent and treat hypoxic-ischemic brain injury as long as it is safe. Thus, in the last part of this chapter, the safety of creatine supplementation will be addressed.

2.1 Creatine supplementation in sport

Cr is mostly known as an ergogenic aid in sport. Cr supplementation increases the content of Cr in muscle tissue. Muscle Cr uptake appears to be positive correlated with exercise performance, because it enables a more rapid ATP re-synthesis, especially between resistance training sets. Cr supplementation combined with heavy resistance training produces a significant increase in physical performance, namely in maximum (1RM) or endurance strength, as well as in fat free mass, and muscle morphology. However, it should be clarified that there are some contradictory studies that show no increase in exercise performance, although, some of these have not used an efficient Cr supplementation protocol or have recruited more non-responders (Cooper et al. 2012). Furthermore, studies have shown that Cr supplementation may enhance recovery after high intensity exercise, as well as improving injury prevention, thermoregulation, rehabilitation, and concussion and spinal cord neuroprotection.

The most common form of Cr used in studies is creatine monohydrate (CM), with the most effective supplementation protocol consisting of 5 g of CM (or about 0.3 g/kg body weight) four times daily for 5-7 days. This supplementation protocol may enhance muscle Cr and PCr by 20-40%, considering that in a normal diet muscle Cr stores are already 60-80% saturated. After this supplementation-loading period leading

the muscle Cr pool to be fully saturated, a supplementation-maintaining period by ingesting 3–5 g/day is commonly used to preserve Cr stores. The concomitant carbohydrate or carbohydrate and protein together with CM have been demonstrated to be more effective in Cr retention. An ingestion of 3 g/day of CM for 28 days is also generally used in the literature. However, through using this method it is possible that the muscle Cr pool will increase only gradually in comparison to the more rapid loading method. This would result in a lengthening of the time necessary for Cr stores to be saturated, and in a reduced effect on exercise performance and training adaptations. Once muscle Cr stores are elevated, it usually takes 4–6 weeks for them to return to baseline. Furthermore, muscle Cr level after the end of supplementation does not fall below baseline, suggesting that potential for long-term suppression of endogenous Cr synthesis does not seem to occur (Kreider et al. 2017).

Cr supplementation plays its ergogenic role mainly through optimizing training adaptations in power/strength athletes or by reducing the recovery time in sprinters. Depending on the increase in muscle PCr, athletes show improvements in high intensity and repetitive exercise by 10-20% after Cr loading (Kreider et al. 2003). These ergogenic effects can be evident in adolescents, young adults and old individuals, as well as in both men and women, although with different magnitudes. Recently, there is a growing amount of literature that has investigated the long-term effects of Cr supplementation, in particular examining the muscle mechanisms affected, such as satellite cell proliferation, myogenic transcription factors and insulin-like growth factor-1 signaling, suggesting a role of Cr supplementation in muscle anabolism (Cooper et al. 2012).

2.1.1 Resistance exercise

In young healthy males supplemented with CM and trained using resistance exercise, a reduction was found in myostatin serum level, which is a muscle growth inhibitor (Saremi et al. 2010). Combined Cr, protein, and carbohydrate supplementation together with heavy resistance training improved 1RM, lean body mass, fiber cross sectional area and contractile proteins in trained young males (Cribb et al. 2007). This study was the first to show such improvements in body composition at the cellular and sub cellular level in resistance trained participants due to Cr supplementation. Another study found that soon after the Cr loading period (5 days) there was an increase of 250%, 45% and 70% for collagen mRNA, glucose transporter 4 (GLUT4) and Myosin heavy chain IIA, respectively (Deldicque et al. 2008). The researchers suggested that Cr in addition to a single session of resistance exercise might favor an anabolic environment by inducing changes in gene expression after only 5 days of supplementation. Cr supplementation in addition to heavy resistance training also increases muscle insulin like growth factor (IGF-1) concentration (Burke et al. 2008). Thus, despite some controversial results, it appears that Cr supplementation combined with heavy resistance training amplifies performance improvements in maximum and endurance strength as well as in muscle hypertrophy, stimulating production of muscle IGF-I and muscle protein synthesis (Cooper et al. 2012).

2.1.2 Anaerobic exercise

Regarding the role of Cr supplementation in predominantly anaerobic exercise, Cr increases neuromuscular performance in short duration, anaerobic, intermittent exercises. These exercises could be divided into activities lasting less than 30 seconds that primarily use the ATP/PCr energy system, and anaerobic endurance exercise lasting from 30-150 seconds, in which the primary energy system is glycolysis. Exercises less than 30 seconds in duration have shown enhancements mainly in the number of repetitions, but also in the total work carried out, weight lifted, time, force production, cycle ergometer revolutions per minute and power. The mechanism responsible could be related both to an increased Cr pool, allowing rapid and greater ATP synthesis, and an enhanced reuptake of Ca^{2+} into the sarcoplasmic reticulum by the action of the Ca^{2+}-ATPase pump, potentially leading to a faster production of force through quicker detachment of the acto-myosin bridges. Thus, the effects of Cr supplementation on multiple high intensity short duration bouts (<30 s) could be due to reduced fatigue symptoms. In anaerobic endurance exercise lasting from 30-150 seconds, Cr supplementation improves both work and power. However, it seems that Cr is more effective in short duration exercise bouts rather than longer bouts (Cooper et al. 2012).

2.1.3 Aerobic exercise

Some studies on predominantly aerobic exercise provide evidence of Cr supplementation effectiveness. Endurance activities lasting more than 150 seconds depend mainly on oxidative phosphorylation and it seems that there is an inverse relationship between the ergogenic potential of Cr supplementation and the increase in exercise duration. Nevertheless, Cr supplementation was suggested to cause a change in substrate utilization during aerobic activity possibly leading to an increase in steady state endurance performance. However, results are controversial as while some studies demonstrated decreased blood lactate accumulation and increased lactate threshold, others found no differences between supplemented and control groups. Furthermore, the content of glycogen limits the onset of fatigue and performance in exercise lasting more than 1 hour. Cr supplementation might enhance muscle glycogen accumulation and GLUT4 expression when combined with glycogen depleting exercise. Cr alone is not able to increase muscle glycogen, but a combination of Cr and carbohydrate supplementation leads to higher muscle glycogen stores (Cooper et al. 2012).

2.1.4 Other Creatine supplementation effects in sport

Cr supplementation may negatively affect range of motion (ROM). This could be due to increasing intracellular water content resulting in greater muscle stiffness and resistance to stretch, or enhanced muscle cell volume affecting neural outflow from the muscle spindles. However, active ROM was measured

immediately after the loading phase, so the effect of Cr on ROM after several weeks of a maintenance phase should also be investigated (Sculthorpe et al. 2010).

Other effects rather than ergogenic may benefit athletes involved in high intensity intermittent and endurance sport. For example, Cr may enhance recovery, reduce the risk of injury, and help individuals recover from injuries at a faster rate (Kreider et al. 2017). CM supplementation may also help athletes recover from intense training. As previously mentioned, Cr can amplify the increased muscle glycogen loading. Glycogen replenishment is important to promote recovery and prevent overtraining during high intensity training periods. Thus, CM supplementation might help to maintain optimal glycogen level for athletes who are involved in sport where significant glycogen depletion occurs during training and/or performance. Evidence also suggests that Cr supplementation may reduce muscle damage and/or enhance recovery from intense exercise. For example, Cooke et al. (2009) investigated the effects of Cr supplementation on muscle force recovery and muscle damage following intense exercise. The results from this study showed higher isokinetic (+10%) and isometric (+21%) knee extension strength during recovery and lower CK level (-84%) in the days after high intensity exercise in participants supplemented with Cr. In general, studies have found that Cr enhances recovery by reducing inflammatory and muscle damage markers, such as CK, prostaglandin E2, and tumor necrosis factor α, and by reducing the increase of lactate dehydrogenase after fatiguing exercise or intermittent anaerobic sprint exercise. Therefore, there is a strong evidence that CM supplementation could be used to enhance glycogen loading; to experience less inflammation and/or muscle enzyme efflux following intense exercise; and to tolerate high volumes of training (Kreider et al. 2017).

Regarding injury prevention role, CM seems to be safe because reported incidence of musculoskeletal injuries, dehydration, muscle cramping, gastrointestinal upset, and renal dysfunction in studies appears only anecdotally. On the contrary, researchers have reported that Cr users experienced significantly less incidences of muscle cramping, heat illness/dehydration, muscle tightness, muscle strains, and total injuries compared to athletes who did not use Cr. Furthermore, due to the effect of Cr on promoting gains in muscle mass and improving strength, there has been interest in examining the effects of Cr supplementation on muscle atrophy rates as a result of limb immobilization and during rehabilitation (Kreider et al. 2017). For example, Hespel and colleagues (2001) investigated the effect of Cr supplementation (20 g/day down to 5 g/day) on atrophy rates and rehabilitation outcomes in subjects who had their right leg casted for two weeks. During the 10 weeks rehabilitation phase, subjects performed three sessions per week of knee extension rehabilitation. Results from this study showed greater enhancement in the cross-sectional area of muscle fiber (+10%) and peak strength (+25%) after the rehabilitation period in the supplemented group, suggesting an effect of Cr in hypertrophy. Indeed, these changes were associated with an increase of myogenic regulating factor 4 and myogenic protein expression. Moreover, another study showed that CM supplementation counteracts the decline in muscle GLUT4 protein level that occurs during immobilization and promotes the increase of GLUT4 protein content during subsequent rehabilitation training in healthy subjects (Op't Eijnde et al. 2001). Collectively, these findings suggest that Cr supplementation is able to reduce the level of muscle atrophy and detrimental effects on muscle associated with immobilization, while also promoting greater gains in strength during rehabilitation.

2.2 Creatine supplementation in clinical field

2.2.1 Neurodegenerative diseases

Physiopathology of neurodegenerative diseases is related to a loss of distinct neuron population. Neuronal loss or dysfunction could lead to several neurological diseases whose phenotype depends on the neuronal loss localization and on neurons degeneration velocity. Common processes in neurodegeneration are protein aggregation, transcriptional dysregulation, excitotoxicity, oxidative stress, impaired energy metabolism, and mitochondrial dysfunction. These processes are involved in disorders such as Huntington's disease (HD), Parkinson's disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Alzheimer's disease (AD).

In particular, impaired energy metabolism together with mitochondrial dysfunction are thought to play an important role in the pathogenesis and progression of these neurodegenerative diseases as a primary and/or secondary mechanism in the neuronal death cascade (Beal 2011). While other mechanisms, namely protein aggregation and altered transcription, may amplify the bioenergetics dysfunction, the impaired energy metabolism may cause pro-apoptotic signaling, oxidative damage, excitotoxicity, and compromised nuclear and mitochondrial DNA repair (Fig 5). Therapeutic strategies that act on mitochondrial dysfunction and on impaired energy metabolism may be useful in the treatment of neurological diseases. Therefore, in the last few decades, CM supplementation to improve the global cellular bioenergetics and as a neuroprotective agent has been investigated in different neurodegenerative disorders (Salomons and Wyss 2007).

As already mentioned in the first chapter, Cr and its metabolite, PCr, play fundamental role in the cell. First, they are important in spatial and temporal buffering of high-energy phosphates leading to improved bioenergetics defects, but Cr may also indirectly benefit other pathophysiological mechanisms by improving cellular homeostasis. Cr counteracts energy depletion by connecting cellular sites of ATP consumption and production. ATP produced by the CK reaction using PCr is the major source of energy in the brain and is tightly coupled to Cr and PCr level within the cell. Therefore, CM supplementation may help to prevent reduced energy stores and to improve neuronal function through enhancing Cr level. Cr is also involved in regulating glycolysis, in stabilizing the mitochondrial, octameric form of CK, and in inhibiting the MPTP, whose opening is associated with both apoptotic and necrotic cell death mechanisms. Another potential neuroprotective mechanism of Cr supplementation is the ability of PCr to stimulate synaptic glutamate uptake and thus to decrease the accumulation of extracellular glutamate and the subsequent excitotoxicity. Cr has also been demonstrated to act as a direct antioxidant, scavenging ROS that may further potentiate mitochondrial dysfunction (Salomons and Wyss 2007; Sartini et al. 2012). Interestingly, the neuroprotective effects of Cr may be independent from mitochondrial CK. Brustovetsky and colleagues showed that Cr had no effect on the MPTP in isolated brain mitochondria (Brustovetsky et al. 2001). In addition, Beal and colleagues reported that Cr supplementation in mice deficient in uMt-CK increased brain level of Cr and PCr, suggesting that the neuroprotective effects of Cr are the result of the maintenance of PCr and ATP level and are not due to the inhibition of the MPTP (Klivenyi et al. 2004a). Of great interest is the fact that oral CM supplementation (20

g/d over 4 weeks) in healthy human volunteers is able to significantly increase (8.7% overall) the Cr pool in the brain (Dechent et al. 1999).

2.2.2 Mitochondrial involvement in neurodegeneration

Mitochondria are organelles that play a critical role in regulating the energy status of the cell through oxidative phosphorylation, yielding ATP; they are important especially in tissues with large ATP requirements such as heart, skeletal muscle, and brain. Thus, the primary role of mitochondria is to supply and regulate energy for the cell. However, mitochondria are also involved in controlling processes such as excitotoxicity and apoptosis that lead to cell death (Fig. 5). Mitochondrion is the principal source of ROS production within cells. It has been demonstrated that several toxins that specifically target the electron transport chain of mitochondria, namely 3-nitropropionic acid, 1-methyl-4-phenyl-2,3,6-tetrahydropyridine, rotenone, and malonate are able to reproduce the phenotype of HD or PD. In fact, the finding that 1-methyl-4-phenyl-2,3,6-tetrahydropyridine toxicity was related with impairment of complex I function of the electron transport chain indicates that mitochondria may be involved in the pathogenesis of PD (Beal 2011).

The involvement of this organelle in apoptosis is evident by the fact that it contains several proapoptotic proteins, which, after being released into the cytosol, result in cell death. These proapoptotic proteins include cytochrome c and apoptosis inducing factor, endonuclease G, SMAC/Diablo, and OMI/HTRA2, modulated by proteins of the Bcl2/Bax family. In particular, Bcl2 localized in the outer mitochondrial membrane prevents the release of proapoptotic factors, while Bax enhances their release. The MPTP is crucial in releasing these factors and the following cell death pathways onset. The MPTP might be formed and opened by several triggers, such as an increase in calcium concentration, a reduction in membrane potential, an increase in inorganic phosphate, a reduction in adenine nucleotides or an elevation of ROS (Beal 2011).

Mitochondrial oxidative phosphorylation involves a series of electron transfers within the inner mitochondrial membrane. The inefficient transfer of electrons may result in the generation of unstable and potentially damaging ROS, leading to mitochondria to be considered as the primary source of ROS within the cell. It has been estimated that approximately 2% of all oxygen is converted into ROS. The production of mitochondrial ROS is thought to initiate early apoptotic triggering events. In fact, they can directly induce cytochrome c dissociation from the inner mitochondrial membrane and cause subsequent release into the cytosol. In addition, ROS can directly act to facilitate MPTP opening. Antioxidant enzymes including manganese superoxide dismutase, glutathione peroxidase, and phospholipid hydroperoxide glutathione peroxidase limit the accumulation of ROS within the matrix. ROS can also indirectly affect the apoptotic pathways through activation of mitogen-activated protein kinases (MAPKs) and of several redox sensitive transcription factors involved in the expression of anti- and pro-apoptotic gene expression (Beal 2011).

Oxidative stress is a common feature of most neurodegenerative diseases, and the mitochondria as the main source of ROS production is the possible target of therapeutic strategies to address such diseases. In this

scenario, CM supplementation and its neuroprotective effects may represent an important tool to prevent or treat these neurodegenerative diseases. Cr has been shown to be effective in a variety of animal and cellular models of neurodegenerative diseases including AD, PD, ALS, and HD. Such neuroprotective effects involve the bioenergetics, antiapoptotic, antiexcitotoxic, antioxidant properties of Cr. However, the strong evidence found in animal studies is not properly replicated in human clinical studies including PD, HD, and ALS patients (Bender and Klopstock 2016).

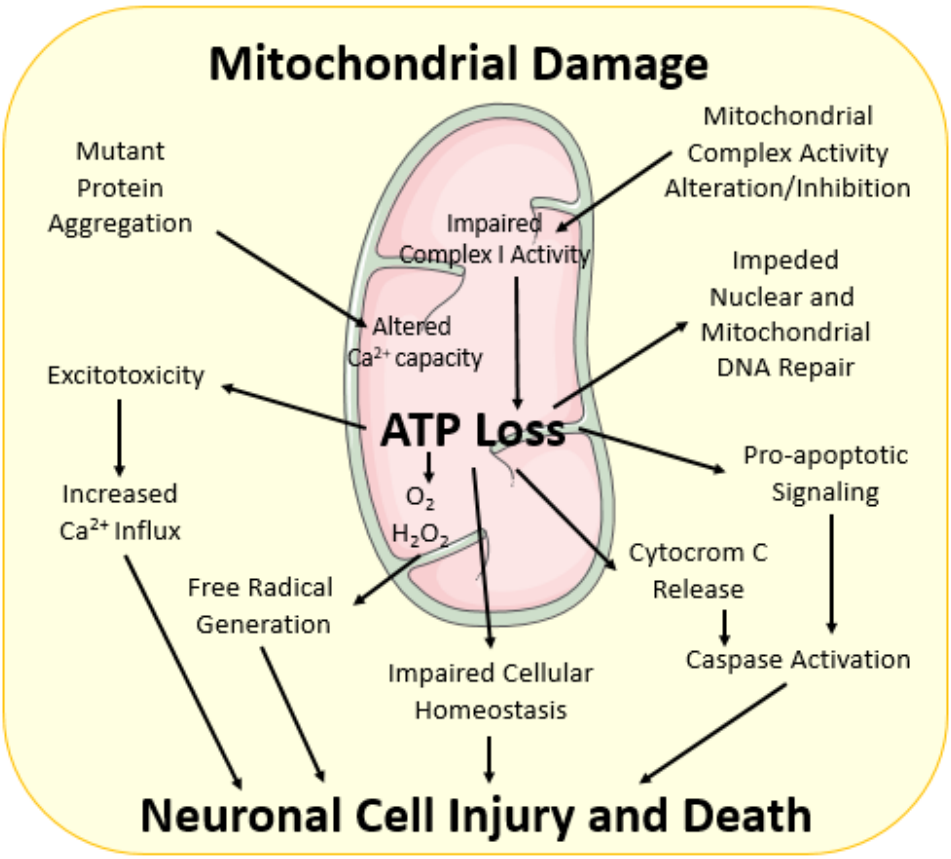

Fig. 5. Mitochondrion-related neuronal dysfunction in neurodegenerative diseases. Mutant protein aggregation can disrupt the mitochondrial membrane potential and alter Ca^{2+} influx induced by excitotoxins. Furthermore, respiratory enzyme activities impairment might cause reduction of ATP synthesis and increase of ROS level. In addition, ROS generation can cause further damage to cellular macromolecules through oxidation, nitrosylation, and peroxidation that may contribute to neuronal injury and death. Moreover, the cytochrome c release from the damaged/dysfunctional mitochondria onsets the apoptotic cascade and the caspases activation, which result in neurons death. Impaired energy metabolism can onset pro-apoptotic signaling, impair cellular homeostasis, and counteract nuclear and mitochondrial DNA repair. These pathological mechanisms are further able to exacerbate mitochondrial damage and energy loss. Creatine administration may improve intracellular high-energy phosphate stores, ameliorating different cellular functions and neurons survival.

Figure adapted from Salomons and Wyss 2007.

2.2.3 Alzheimer's disease

Alzheimer's disease (AD) is a common neurodegenerative disorder, which leads to progressive dementia. This disease is characterized by the loss of neurons, particularly of the cholinergic system, as well

as deposition of extracellular amyloid plaques and intracellular neurofibrillary tangles (Andres et al. 2008; Querfurth and La Ferla 2010). It has been shown that the earliest detectable defects in AD patients are induced by impaired energy metabolism and mitochondrial electron transport chain dysfunction, and interestingly such defects often precede the amyloid deposition. In addition, there is strong evidence for increased oxidative damage to proteins and DNA, as well as to mitochondrial DNA (Bonda et al. 2010). AD brain homogenates showed a marked decrease in CK activity, which may be related to oxidative stress (Aksenov et al. 2000). This is because the CK enzyme presents highly sensitive cysteine residues that can be easily modified by an oxidative insult. Pettegrew et al. (1994) reported that AD patients have reduced level of brain PCr at the onset of symptoms, and decreased oxidative metabolism in later stages. In addition, in a transgenic model of AD, increased Cr deposits were found (Gallant et al. 2006). The most plausible explanation for this Cr deposition is that the oxidative stress associated with AD, resulting in impairment of both cytosolic and mitochondrial CK, reduces the generation of PCr from Cr, thereby enhancing the cytosolic Cr level.

Furthermore, it has been suggested that the amyloid precursor protein directly interacts with and binds to mitochondrial CK (Li et al. 2006). Brewer and Wallimann (2000) demonstrated that Cr supplementation protects against oxidative mediated conversion of octameric MtCK to the dysfunctional dimeric MtCK. Therefore, increased Cr level following supplementation may protect the CK system and delay the inactivation of CK induced by ROS, which occurs in AD patients (Aksenov et al. 2000). Another possible mechanism underling the neuroprotective effect of Cr is related to the AMPK signaling activated by Cr supplementation shown in skeletal muscle cells (Ceddia and Sweeney 2004). The activation of the AMPK pathway is crucial in regulating mitochondrial content and function in a PGC-1α dependent pathway (Zong et al. 2002). If Cr may improve mitochondrial content and/or function by activating AMPK, this is potentially beneficial. This is particularly the case since a reduction in PGC-1α level has been reported in post-mortem brain tissue of AD subjects, which correlates with elevating numbers of neurofibrillary tangles, as well as pathologic and clinical grade prior to death (Qin et al. 2009).

2.2.4 Parkinson's disease

Parkinson's disease (PD) is characterized by a degeneration of dopaminergic neurons resulting in the clinical phenotype of progressive bradykinesia, rigidity, tremor, and gait disorders. Several molecular mechanisms contribute to induce dopaminergic neuronal loss and dysfunction in PD. There is substantial evidence that mitochondrial dysfunction plays a role in the pathogenesis of this disease (Beal 2009). Studies showed that there is a decreased complex I activity of the mitochondrial electron transport chain in the substantia nigra of post-mortem tissue of PD patients, and there is also lower complex I activity in platelets of patients with early PD. Furthermore, toxins including 1-methyl-4-phenyl-2,3,6-tetrahydropyridine and rotenone specifically inhibit complex I of the electron transport chain, leading to a depletion of ATP and increasing the importance of the impaired energy metabolism in PD physiopathogenesis (Beal 2011).

These toxins cause selective loss of dopaminergic neurons and if chronically administered they result in the development of α-synuclein aggregates, a pathologic feature of PD. 1-methyl-4-phenyl-2,3,6-tetrahydropyridine is converted in 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B. MPP^+ enters into neurons through the dopamine transporter and subsequently it is accumulated within the mitochondria, where it blocks complex I of the electron transport chain and causes increased production of ROS. A number of genetic causes of PD are also correlated with mitochondrial dysfunction. These genetic causes include nuclear genes that codify for α-synuclein, parkin, DJ-1, PINK1, LRRK2, and Omi (Thomas and Beal 2007). It is interesting to note that defects in parkin and PINK1 are involved in a critical pathway, which utilizes autophagy to remove damaged mitochondria. The depolarization of mitochondria, which occurs with uncoupling agents, causes the expression of PINK1 on the membrane of the mitochondria (Narendra et al. 2010). Subsequently PINK1 is recognized by parkin, which ubiquinates the mitochondrion leading to autophagy of the damaged mitochondria. Thus, because of the important role of mitochondrial impairment and oxidative damage in the pathogenesis of PD, Cr supplementation has been hypothesized as a neuroprotective treatment.

Several studies using Cr supplementation as a neuroprotective agent in PD have been conducted both in animals as well as in humans. The first study showed Cr to be strongly neuroprotective in a toxic mouse model for PD, targeting mitochondrial function (Matthews et al. 1999). 1% Cr supplementation for 2 weeks before 1-methyl-4-phenyl-2,3,6-tetrahydropyridine injection was able to abolish its deleterious effects on dopaminergic neurons. A second study reported additive neuroprotective effects, when Cr was combined with the cyclooxygenase 2 (COX-2) inhibitor rofecoxib (Klivenyi et al. 2003). However, it is important to note that, in 2004, rofecoxib has been withdrawn from the market after controversial results about safety, because it has been showed to increase the risk of cardiovascular events (Juni et al. 2004). Subsequently, the combination of Cr with coenzyme Q10 was found to be more neuroprotective, leading to less lipid peroxidation damage and less α-synuclein accumulation within dopaminergic substantia nigra neurons in mice (Yang et al. 2009). Following these encouraging results in animal models, human investigations began.

The first randomized placebo-controlled clinical trial of oral Cr supplementation (up to 4 g/day) over 2 years in 60 PD patients was negative for primary outcomes. However, positive results were found in measures for mood and behavior, and Cr-treated patients increased their dopamine dosage to a lesser degree in comparison to control group over the 2 years (Bender et al. 2006). At the same time, a futility randomized placebo-controlled clinical trial using 10 g of Cr per day for 12 months and involving 200 early PD patients showed that Cr could not be rejected as futile with regard to slowing clinical disease progression (NINDS NET-PD Investigators 2006). A meta-analysis of the 194 patients of the two pilot or Phase II trials reported no effect on either motor function or activities of daily living, however determined that data were insufficient to draw a firm conclusion (Xiao et al. 2014). In 2007, a multicenter Phase III double-blind neuroprotection randomized placebo-controlled clinical trial was conducted by the National Institute of Neurological Disorders and Stroke (NINDS). A total of 1741 patients with early and stable PD were recruited, and received either 10 g of Cr per day or placebo (Kieburtz et al. 2015). This study that should have lasted 5 years was terminated for

futility, because no differences in any of the five individual outcome measures (activities of daily living, ambulatory capacity, disease-specific health problems, cognitive functioning, and overall outcome) nor in the aggregate measure were observed. There were also no differences in secondary outcome measures (levodopa equivalent daily dose, depression, cognition, or quality of life). A recent clinical trial investigating Cr supplementation combined with coenzyme Q10 in 75 PD patients for 18 months revealed that the cognitive decline determined by the Montreal Cognitive Assessment (MoCA) was reduced in the treated group compared to the placebo group. However, there was no significant treatment effect on motor symptoms, as quantified by the Unified Parkinson's Disease Rating Scale (Li et al. 2015).

2.2.5 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by degeneration of motor neurons in the spinal cord and of corticospinal neurons in the motor cortex (Hervias et al. 2006), leading to muscle weakness, atrophy, and spasticity. In some cases, genetic abnormalities causing ALS were found, for example mutations in copper zinc superoxide dismutase, a cytosolic enzyme responsible for scavenging and reducing ROS in the cell (Peters et al. 2015). With this knowledge, a transgenic mouse model was developed with the G93A mutation in SOD, characterized by progressive loss of motor neurons, and increasing weakness. A fraction of SOD1 is localized on the outer mitochondrial membrane, where it may interfere with the function of Bcl2, a neuroprotective antiapoptotic protein, and VDAC (Beal 2011). Some of the pathological mechanisms in ALS include mitochondrial dysfunction, glutamate-mediated excitotoxicity, and oxidative stress, which are potential targets for Cr-mediated neuroprotection.

Oral Cr supplementation induced dose-dependent improvement of motor performance, and extended survival in the G93A mice (Klivenyi et al. 1999). Furthermore, Cr administration reduced the loss of neurons in both the substantia nigra and the motor cortex, and limited the oxidative damage. Other studies using Cr supplementation combined with other potentially neuroprotective agents in the same animal model showed a comparable but not additive neuroprotective effect of Cr compared to the standard clinical ALS drug, riluzole (Snow et al. 2003). In contrast, the combination of minocycline and Cr as well as the combination of Cr with the COX-2 inhibitors celecoxib and/or rofecoxib, produced additive neuroprotective effects (Zhang et al. 2003; Klivenyi et al. 2004b).

Due to this neuroprotective evidence, Cr supplementation (with doses of 5 or 10 g/day) was investigated in three human randomized placebo-controlled trials. However, these trials did not show any beneficial effect of Cr on survival and/or disease progression in patients with ALS (Shefner et al. 2004; Groeneveld et al. 2003; Rosenfeld et al. 2008). This was confirmed by a systematic Cochrane collaboration meta-analysis covering these three individual randomized placebo-controlled trials (Pastula et al. 2012). One phase II randomized placebo-controlled trial (http://www.clinicaltrials.gov, NCT01257581) using 30 g of Cr or one of two different tamoxifen doses was completed, however results are not published yet. Results from

humans studied are disappointing, suggesting that the SOD transgenic mouse may not be predictive of therapeutic outcome in patients with sporadic ALS.

2.2.5 Huntington's disease

Huntington's disease (HD) also known as Huntington's chorea because of an abnormal ''dancelike'' body movements (called chorea), is an inherited autosomal dominant progressive neurologic disorder. This disease is characterized by impaired coordination, as well as cognition and psychiatric abnormalities. The cause of HD is identified in trinucleotide repeat (CAG) in the huntingtin gene, resulting in an expansion of a polyglutamine stretch in the huntingtin protein. The huntingtin protein is ubiquitously expressed in both the nervous system and peripheral tissues. Possible mechanisms with which mutant huntingtin is thought to induce toxic effects to neural tissue are transcription dysregulation, proapoptotic signaling, oxidative injury, excitotoxicity, inflammation, malfunctioning proteolysis, metabolic dysfunction, and mitochondrial dysfunction (Beal 2011).

Cr administration was studied both in toxic (3-nitropropionic acid or malonate, inhibitors of the respiratory chain complex II) as well as in genetic HD animal models. In toxic models, oral Cr produced lower lesion volume and higher brain ATP and PCr level (Matthews et al. 1998). The transgenic mouse models of HD are considered more suitable models to study human disease. In these models, Cr administered prior to the development of the phenotype showed significant neuroprotection, improvement in motor performance, and higher overall survival (Ferrante et al. 2000; Andreassen et al. 2001a). More relevant to application in human HD is that the same effect was obtained when Cr was used after symptom onset (Dedeoglu et al. 2003). So far, two phase II randomized placebo-controlled trials of Cr in human manifest and one in premanifest HD have been published. The first study using 5 g/day of Cr for 1 year had no effect on clinical outcome measures in 41 HD patients (Verbessem et al. 2003). A higher dose of 8 g of Cr per day was given to 64 HD patients for 4 months. Cr seemed to cross the BBB, and was well tolerated (Hersch et al. 2006). While there was no effect on clinical outcomes, Cr was able to reduce elevated serum level of 8-hydroxy-2'-deoxyguanosine (8OH2'dG), a marker of oxidative stress. The following PRECREST trial recruited 64 patients in a premanifest state or at 50% genetic risk of HD, and involved supplementation of either up to 30 g of Cr per day or placebo, with a double-blind protocol for 6 months (Rosas et al. 2014). Diarrhea and nausea occurred more often under this high-dose Cr regime supplementation, but Cr was well tolerated and proved to be safe. Cr slowed the rate of region specific brain atrophy in repeated MRI scans, but did not show any effect on secondary clinical outcome measures. The Cr Safety, Tolerability, and Efficacy in HD (CRESTE) phase III trial then set out to recruit 650 early symptomatic HD patients to receive up to 40 g of Cr per day for up to 4 years. However, the study was terminated for futility after recruiting 553 participants, because there were no positive Cr effects in slowing functional loss in HD patients, even at this very high Cr doses. Side effects, mainly gastrointestinal, were significantly more frequent in participants using Cr. Serious adverse events, including deaths, were more

common in the placebo group. However, it is interesting to note that subgroup analysis suggested that men and women might respond differently to Cr supplementation (Hersch et al. 2017).

2.2.7 Creatine supplementation in neurodegenerative diseases conclusions

In conclusion, prominent results from animal studies on neurodegenerative disease are not properly translated to human studies. This is especially true for Cr administrated in AD, PD, and ALS patients, while some positive results were found in human studies using HD patients. Common speculative reasons for failed neuroprotection trials are that Cr was administrated too late in the course of the disease or at low dosage, that trials are not long enough, or that the wrong outcome measures were chosen. In the case of Cr in PD, animal models might be not suitable to draw firm conclusions for human PD. In these animal models, Cr was usually administered before the application of toxins, suggesting the use of a different animal model in the future. Furthermore, it could be interesting to understand if there is a gender response to Cr supplementation in PD patients, given that rodent models of depression suggest a therapeutic Cr gender effect, favoring female animals (Allen et al. 2012). In addition, the comparison between vegetarian and non-vegetarian patients in the PD phase III trial would be an interesting subgroup analysis. It was suggested that healthy vegetarian women had improved memory functions compared to omnivores upon supplementation of 20 g of Cr for 5 days (Benton and Donohoe 2011). However, Cr seems to not provide neuroprotection in PD (Bender and Klopstock 2016). Furthermore, an interesting study by Lowe et al. (2015) showed a different distribution of CTs in the human brain. The CT is hardly identified in the striatum and the dopaminergic substantia nigra neurons, the cell population that predominantly dies in human PD. Thus, it might be that Cr supplementation effects depend on brain area and cell type. However, data showed no difference in CT distribution between rodents and humans (Mak et al. 2009). This does not explain the discrepancy between the promising preclinical data and the overall disappointing results of randomized controlled human trials.

Also in the case of ALS, Cr seems not to be neuroprotective in human studies. The reasons for this could be low dosage or the late timing of Cr administration in the course of the disease. Regarding the Cr dosage, 10 g of Cr per day used in human trials represent still only 10% of the dose used in rodent models (Bender et al. 2006). In the typical mouse studies, brain Cr increased by 21 ± 3.8%, while in the human randomized trials, increases between only 7.5 and 13% could be obtained (Ferrante et al. 2000; Atassi et al. 2010; Hersch et al. 2006; Lyoo et al. 2003). In the case of timing of Cr administration, neuroprotective Cr effects were demonstrated in rodent models, where Cr supplementation commenced weeks prior to injection of the neurotoxin (Matthews et al. 1998, 1998; Yang et al. 2009). This study design seems not to be suitable for human studies, where patients show an established disease, as this may be too late for Cr to exert significant neuroprotective effects. This is why the recent work by Rosas and colleagues (2014) is so noteworthy, because they found a way to administer Cr to premanifest HD patients. Indeed, they reported significantly decreased brain atrophy rates in the Cr group (30 g day for 6 months) compared to the placebo group. If this were to translate into clinical patient benefit in the long-term (III phase study), it would be

important for other neurodegenerative diseases, such as PD or ALS, to have recognized early biomarkers or genetic testing for high disease risk or early disease activity, so that Cr supplementation could begin as early as possible. However, in genetic HD animal models, Cr was still neuroprotective, even when administrated after the onset of disease-related symptoms (Dedeoglu et al. 2003). At least in animals, it seems not a prerequisite for Cr to be given prior to the onset of the pathophysiological processes. Finally, Cr does not easily cross the BBB, thus, it could be useful to study other chemical forms of Cr rather than CM, such as cyclocreatine or PCr-magnesium-complex-acetate, or other administration strategy like intrathecal administration. This latter solution is far from practical in the context of neurodegenerative disease though, but would possibly be an alternative in states of critical acute brain energy failure, such as in global cerebral ischemia or traumatic brain injury.

In summary, Cr has failed as a neuroprotective treatment in neurodegenerative diseases with the exception that it may be beneficial in premanifest HD patients when given at high doses, which will have to be further examined in a phase III trial. Furthermore, Cr could be considered a neuroprotective prophylactic strategy in conditions with imminent neurological damage. In fact, Cr was recently shown to prevent neuropsychological deficits in healthy volunteers under experimental oxygen deprivation (Turner et al. 2015).

2.3 Inborn errors of metabolism

2.3.1 Mitochondrial encephalomyopathies and hyperammonemia

Mitochondrial encephalomyopathies are a heterogeneous group of diseases characterized by a broad range of biochemical and genetic mitochondrial defects, as well as variable types of inheritance. Mitochondrial myopathy, encephalopathy, lactic acidosis with stroke-like episodes (MELASs) syndrome are the most common maternally inherited mitochondrial disorders (Andrea et al 2008). While the exact cause of specific neuronal death is unknown, mechanisms associated with neuronal energetic deficiency and subsequent lactic acidosis, and NMDA receptor-mediated excitotoxicity, have been implicated. Therapeutic strategies have been adopted as the result of single case studies, and they are based on the use of antioxidants, respiratory chain substrates, and cofactors such as Cr and coenzyme Q10 (Salomons and Wyss 2007).

In the few clinical trials using MELAS patients, Cr administration showed a normalization of seizures and an improved vocabulary. Notably, Cr supplementation resulted in a reversal of the paracrystalline intramitochondrial inclusions in the muscle, which were shown to consist mainly of crystallized uMt-CK. In Leigh syndrome (LS) patients suffer from characteristic focal necrotizing lesions in one or more regions of the CNS, including the brainstem, thalamus, basal ganglia, cerebellum, and spinal cord. The clinical symptoms of this disorder depend on which areas of the CNS are involved. The most common underlying cause for LS is a defect in oxidative phosphorylation. In a recent case report, oral Cr supplementation was shown to improve behavioral and physiological functions of a child suffering from LS (Komura et al. 2003). Rodriguez and co-workers (2007) reported that a combination therapy (CM, coenzyme Q10, and lipoic acid) favorably influenced surrogate markers of cellular energy dysfunction in an inhomogeneous population of

patients with mitochondrial cytopathies. The outcome of this randomized, double-blind, placebo-controlled, crossover study design suggests that targeting the final common pathway of mitochondrial dysfunction positively influences the course of the diseases.

Another inborn error that could benefit from Cr supplementation is the one regarding ammonia metabolism. Inborn errors of ammonia metabolism, such as urea cycle deficiencies and organic acidemias, can lead to Cr depletion in developing brain cells, likely through interference with Cr transport and synthesis pathways. Interestingly, it has been shown *in vitro* that treating developing brain cells exposed to ammonium with Cr protects them from axonal growth inhibition due to ammonium exposure, which may be one of the irreversible effects of hyperammonemia on CNS development (Braissant et al. 2002). Cr administration to hyperammonemic neonates and children may therefore represent a method to protect the developing CNS from some of the deleterious effects of ammonium (Andres et al. 2008).

2.3.2 Cerebral creatine deficiency syndromes

In the last two decades, a new group of inborn errors that affect the biosynthesis and the transport of Cr, namely the cerebral creatine deficiency syndromes (CCDSs) has been discovered. These CCDSs include two autosomal recessive disorders that affect Cr biosynthesis, the arginine:glycine amidinotransferase (AGAT) deficiency and guanidinoacetate methyltransferase (GAMT) deficiency, and an X-linked defect that affects the creatine transporter (CT deficiency). These disorders are characterized by cerebral Cr deficiency as detected *in vivo* by 1H magnetic resonance spectroscopy (MRS) of the brain, and specific abnormalities in metabolites of Cr metabolism in plasma and urine. Specifically, in urine and plasma, abnormal guanidinoacetic acid (GAA) level is found in AGAT deficiency (reduced GAA) and in GAMT deficiency (increased GAA), while CT deficiency is characterized by an elevated Cr/creatinine ratio in the urine of males. Mental retardation, expressive speech and language delay, autistic like behavior and epilepsy are common features of CCDSs, however in GAMT deficiency, the neurotoxicity of elevated GAA level leads to a more severe phenotype. Cr biosynthesis deficit treated with Cr may show a partial remission of the symptomatology, or a total prevention in case of early diagnosis or presymptomatic treatment. On the contrary, an effective treatment for CT deficiency it is not still available. The discovery of these inborn diseases is important for understanding the physiological functions and pharmacological potential of Cr (Salomons and Wyss 2007).

In two Italian sisters affected by AGAT deficiency, CM supplementation was able to induce almost the complete normalization of cerebral Cr level, as well as an improvement in psychomental development (Bianchi et al. 2000). In their younger brother, the disease was diagnosed prenatally and Cr supplementation was started at the age of 4 months, due to the inefficiency of breast-feeding in enhancing Cr level in the child. Growth and psychomotor development were normal compared to his sisters (Battini et al. 2006). This suggests that early neurological sequelae might be preventable by early treatment.

In GAMT deficiency, oral CM supplementation was able to increase cerebral Cr up to 90% in a 24 months-old German boy, followed by improvements in extra-pyramidal movement disorder and head nodding. However, other symptoms such mental retardation, speech development and autism-like behavior were not ameliorated by Cr supplementation. One reason for the incomplete clinical improvement seems to be the accumulation of GAA, which is still compromised following Cr supplementation. A possible solution to the accumulation of GAA could be represented by Cr supplementation combined with dietary restriction of arginine and low doses of ornithine or combined with high ornithine dosage. Arginine is the immediate precursor of GAA, namely the substrate of AGAT reaction, while ornithine is the competitive inhibitor of AGAT. Thus, increasing level of Cr together with reducing GAA accumulation improves clinical symptoms in such patients. This was true especially for autistic-like behavior, movement disorder, and some cognitive ability, while speech delay and general cognitive development remained poor or only slightly improved. The experience with the only patient diagnosed at birth and treated at a pre-symptomatic stage of the syndrome suggests that early treatment might widely prevent neurological manifestations (Salomons and Wyss 2007).

Finally, in CT deficiency syndrome, in contrast to GAMT and AGAT deficiency, GAA level was normal and oral Cr supplementation was not effective in restoring cerebral Cr level (Cecil et al. 2001). The main biochemical hallmark of CT deficiency syndrome is an increased urinary Cr/creatinine ratio in males (Stöckler-Ipsiroglu and Salomons, 2006). In addition to affected males who have a hemizygous mutation in CT, 80 females having a heterozygous mutation (carriers) have also been identified. However, diagnosis is more complicated in females, and clinical symptoms involve learning disabilities and therapy-resistant epilepsy. The cause of CT deficiency is still unknown, but it is interesting to note that GAA is a competitive inhibitor of CT. Therefore, due to the absence of a functional CT, GAA absorption could be compromised. This might interfere with GAA methylation and, therefore, with the Cr biosynthesis. Currently, despite there is not an effective therapy, supplementation with high doses of arginine and glycine, which are the primary substrates for Cr biosynthesis, combined with high doses of CM is being investigated. In addition, alternative strategies that facilitate Cr transport into the brain either by modified transport via carrier molecules (e.g., peptides) or by supplementation with suitable Cr analogs might be developed (Salomons and Wyss 2007).

Diagnostic work-up in CCDS patients is generally commenced only when symptoms are manifested. If these symptoms are due to an irreversible brain impairment, the reason why Cr supplementation is generally not efficacious is evident. On the other hand, when treatment can be initiated prior to the occurrence of irreversible damage, clinical symptoms may be entirely and permanently prevented. In fact, the diagnosis of CCDSs could precociously occur, in prenatal or neonatal periods, through 1H-MRS that reveals the cerebral Cr deficit or through measures of urinary GAA concentration or urinary Cr/creatinine ratio (Salomons and Wyss 2007).

2.4 Creatine supplementation: safety and side effects

There is a growing interest about Cr supplementation both in healthy and clinical populations. However, to develop a therapeutic agent it is important to assess its safety. Although Cr seems to be well tolerated by most participants in short-term studies, some anecdotal reports and case-reports suggest that Cr supplementation may lead to various side effects, such as muscle cramping, gastrointestinal discomfort and renal dysfunction. It is important to say that when a drug, food or supplement is considered safe this does not mean the product is harmless, but that the benefits outweigh the risk of side effects in that population. Thus, Cr would be clinically useful if the positive effects, including increased muscle strength and reduced fatigue, outweighed the possible side effects, such as muscle cramping.

In the last few decades, several studies examining Cr safety were conducted especially in animals. However, data from animal models may not be directly applicable to the effects of Cr in humans. Additionally, the appearance of side effects seems to be species-specific as shown by Tarnopolsky et al. (2003) in a study that compares mice and rats. In this study, CM supplementation had caused hepatitis in mice (0.025 and 0.05 $g \cdot kg^{-1} \cdot day^{-1}$), but not in rats (2% weight/weight). To date, Cr appears to be safe because randomized control studies have not found clinically significant deviations from normal values in renal, hepatic, cardiac or muscle function. However, a number of side effects were anecdotally reported in some case reports, including muscle cramping, muscle damage and rhabdomyolysis, dehydration, renal dysfunction, and gastrointestinal, hepatic and cardiovascular problems, raising some doubts about Cr supplementation safety.

2.4.1 Muscle dysfunction

Muscle dysfunction following Cr supplementation reported by popular media comprises cramps, muscle damage and rhabdomyolysis. Increased muscle Cr stores subsequent to Cr supplementation is associated with increments in total body water (Powers et al. 2003) and enhanced compartment pressure (Hile et al. 2006). This leads to speculation that Cr supplementation could cause muscle dysfunction. However, the effect of Cr supplementation on mild and severe markers of skeletal muscle dysfunction have been studied in cross-sectional studies, clinical trials, and case studies, showing no increase in dysfunction after supplementation. Although some researchers reported muscle cramping in Cr users (common loading/maintenance Cr dosage), no control groups were used in these studies, indicating that the relationship between Cr and muscle cramps cannot be determined from these experiments (Greenwood et al. 2000; Juhn et al. 1999). A more recent study showed that Cr supplementation (21.6 g/day of CM for 7 days) does not affect plasma sodium and potassium, or dehydration level following exercise, and thus it does not increase muscle cramping (Watson et al. 2006).

Collectively, clinical studies of the interactions between Cr supplementation and fatiguing exercise indicate that Cr supplementation does not amplify muscle damage. In particular, Cr supplementation does not decrease strength and ROM, and does not increase muscle soreness, serum CK or lactate dehydrogenase activity (Rawson et al. 2001, 2007). On the contrary, Cr administration might protect muscle from damage

during certain types of stressful exercise (Santos et al. 2004). It is important to note that serious adverse events associated with severe muscle damage, for example rhabdomyolysis, which may occur infrequently (1 in 10,000 exposures), are difficult to detect in the small clinical trials typically conducted on Cr supplementation involving less than 50 participants. Few cases of rhabdomyolysis in Cr users have been reported in the literature. However, these are singular cases, that might be due to higher and prolonged supplementation dosage (25 g/d for 5 years) (Robinson 2000), to a combination with other supplements (e.g. ephedrine, natural diuretics) (Kuklo et al. 2000; Sandhu et al. 2002) or to Cr supplemented after knee surgery (Sheth et al. 2006). Thus, it is unclear what role the Cr supplementation plays in inducing rhabdomyolysis.

Cr is often incorrectly correlated with dehydration. In fact, Cr supplementation (25 g/d of Cr for 7 days and 5 g/d for the remaining 21 days) is able to enhance total body water (Powers et al. 2003) and is more correctly considered as a hyper-hydrating agent. It has been suggested that Cr supplementation may increase intracellular water, reducing available water for heat loss through sweat evaporation. However, Powers et al. (2003) demonstrated that Cr supplementation did not affect fluid distribution. In addition, other studies demonstrated that hyper-hydration induced by Cr administration does not impair thermoregulatory or metabolic responses to prolonged exercise in the heat. In fact, Cr may attenuate thermoregulatory responses and prevent heat-related injuries and performance decrements (Kilduff et al. 2004).

2.4.2 Renal Dysfunction

Cr and its major metabolite, creatinine, are excreted by the kidney. Creatinine is used as an important clinical marker for renal function. In particular, creatinine clearance is an indicator of glomerular filtration rate. Generally, creatinine clearance in studies is calculated from a single serum creatinine sample and the subsequent use of the Cockcroft-Gault equation. However, the use of serum creatinine as a marker for renal function requires several assumptions. To use serum creatinine as a marker for renal function, the daily anabolic production of Cr should be constant as well as the conversion of Cr to creatinine. However, during Cr supplementation, the first requirement is violated because the supraphysiologic doses consumed far exceed daily endogenous synthesis, while the second condition is preserved, although serum creatinine tends to rise due to increased Cr stores (Salomons and Wyss 2007).

Clinical studies investigating changes in serum creatinine following Cr supplementation have found serum creatinine either does not change or increases, but remains in the normal range. The possible increment of serum creatinine raises some concern because it is assumed this increase might indicate reduced kidney function. However, studies that investigate both serum and urine creatinine to estimate renal function in healthy individuals and patients have found no effect of Cr supplementation on kidney function (Salomons and Wyss 2007). As of yet, only three case studies have reported subjects with renal dysfunction during CM supplementation (Koshy et al. 1999; Pritchard and Kalra 1998; Revai et al. 2003). However, in all of these studies the patients either had previous renal disease (i.e., glomerulosclerosis with relapsing nephrotic

syndrome) or had ingested 4 to 100 times the recommended daily dose of Cr for prolonged periods of time with or without other anabolic agents (Salomons and Wyss 2007). 2007).

2.4.3 Other possible side effects

Other problems anecdotally related to Cr supplementation are gastrointestinal distress and diarrhea. However, studies using recommended dosages of Cr have not reported such complaints. There is one case study related to Cr ingestion and cardiac dysfunction, in which a 30 year-old vegetarian male developed diarrhea and cramps after one month of Cr supplementation, and subsequently changed to a different Cr supplement and developed palpitations and dyspnea (Kammer 2005). It is unknown what role Cr played in this case.

Some doubts about the safety of Cr supplementation linked to the related production of metabolites. Cr can be converted to formaldehyde and hydrogen peroxide through a minor metabolic pathway. Formaldehyde has the potential to cross-link proteins and DNA leading to cytotoxicity. Thus, there is some concern about possible cytotoxicity following Cr supplementation, particularly after a study conducted by Yu and Deng (2000) found an increase in urine formaldehyde after Cr supplementation. However, markers of protein or DNA cross-linking, or measures of oxidative stress, were not investigated. Poortmans et al. (2005), examining urinary methylamine, formaldehyde and formate following 21 g/d for 14 days Cr administration, found increments in both methylamine and formaldehyde. This increase was below the upper-limit of normal (Salomons and Wyss 2007).

The data collected so far suggest that there are few adverse effects associated with Cr supplementation when administrated at recommended doses. Muscle dysfunction, impaired thermoregulation or dehydration, gastrointestinal- and renal-related side effects are anecdotally reported with Cr supplementation, but data do not support this. Furthermore, information regarding the safety of Cr supplementation is available from relatively small clinical studies lasting short periods, and examining healthy volunteers. There is a lack of evidence from studies involving patients, larger numbers of subjects, long periods of time, and doses above recommendations. Finally, it is important to include placebo controls in future studies to assess the possible increased risk of side effects caused by Cr supplementation.

Chapter 3

CREATINE SUPPLEMENTATION DURING PREGNANCY

Creatine supplementation during pregnancy will be discussed in this chapter. The pleiotropic effect of creatine could represent an important and economic tool in order to prevent and treat hypoxic-ischemic events that might occur pre-, peri-, and post-natally. In the context of maternal creatine supplementation, it is relevant to understand that the embryonic CNS is more reliant on maternal creatine supply. Therefore, before addressing the creatine supplementation during pregnancy, the differences between adult and developing CNS regarding Cr synthesis and transport will be described.

3.1 Creatine and the Central Nervous System

To better understand how Cr supplementation could be important and effective during pregnancy, it is necessary to explain the differences between the adult and the embryonic CNS in relation to the different expression of the Cr transporter (CT1) and the Cr biosynthesis enzymes (AGAT and GAMT). Cr synthesis occurs mainly in the kidneys, pancreas and liver, however, the brain is known to also express the Cr transporter and biosynthesis enzyme during development and adulthood. The expression of AGAT, GAMT and CT1 in different brain cells varies during CNS development. For this reason, the capacities to take up Cr from the periphery and endogenous synthesis are different between the embryonic and the adult CNS. In particular, the embryonic CNS is more dependent on Cr provisions from periphery, likely from maternal origin, than endogenous synthesis, while the adult CNS depends more on endogenous Cr synthesis because of the limited permeability of the BBB (Braissant et al. 2001). The importance of Cr for CNS function and development was made evident by the discovery of the cerebral Cr deficiency syndromes. In fact, as already mentioned in the second chapter, in these inborn diseases, in which the expression of AGAT, GAMT or CT1 is absent, the main clinical symptoms are related to CNS impairment. Patients with AGAT and GAMT deficiency can be treated with high doses of Cr supplementation, with a partial restoration of the brain Cr pool being reached within months, while Cr supplementation in CT1 deficiency patients is ineffective (Salomons and Wyss 2007).

In the CNS, the Cr/PCr/CK system plays critical functions in maintaining high energy levels essential for CNS development and function, achieved through ATP re-synthesis and buffering (Braissant 2012). In particular, studies demonstrated that Cr is important in the migration of growth cones, in dendritic and axonal growth, Na^+/K^+-ATPase activity, neurotransmitters release, membrane potential maintenance, Ca^{2+}

homeostasis and ions gradients restoration (Salomons and Wyss 2007). Other studies have also demonstrated that Cr acts as a neuromodulator and neurotransmitter and, finally, that it is one of the main cellular osmolytes (Hanna-El-Daher and Braissant 2016).

In mammals, half of Cr is acquired from the diet and half is synthesized in the periphery through the dissociated expression of the first enzyme AGAT in the kidney, where GAA is released for the second enzyme GAMT expressed in the liver. While it had long been thought that brain Cr was of peripheral origin, AGAT and GAMT are well expressed in the CNS, suggesting that the brain is able to produce its own Cr. Furthermore, the expression of CT1 occurs only in microcapillary endothelial cells at BBB, but not in astrocytes. This is especially the case in their feet lining the BBB, suggesting a limited permeability for peripheral Cr through the BBB, and that the brain could be more dependent on endogenous synthesis of Cr (Braissant 2012). However, despite the restricted efficiency of CNS Cr uptake from the periphery, demonstrated *in vivo* both in rodents (Perasso et al. 2003) as well as in patients with AGAT and GAMT deficiency supplemented with Cr (Schulze 2005), Cr (and GAA) can cross barriers (Tachikawa and Hosoya 2011). However, it is well known that this transfer of peripheral Cr into the brain is limited. Oral Cr supplementation in healthy human volunteers with approximately 60 times the daily Cr requirement leads to a very modest 5% to 10% enhancement in their total cerebral Cr (Cr+PCr) after one month of supplementation (Dechent et al. 1999).

The brain is separated from the blood through different barriers, which firmly regulate exchanges between the periphery and CNS. The two most important barriers are the BBB and the blood-cerebrospinal fluid barrier (BCSFB). In particular, the BBB, the largest barrier between the periphery and the brain (Fig. 6A), consists of non-fenestrated microcapillary endothelial cells (MCEC) linked together through tight and adherent junctions that preclude paracellular diffusion of molecules between the blood and CNS. The tight regulation of exchanges through the BBB is allowed by specific transporters on luminal and abluminal sides of MCEC and through a very limited pinocytosis. Furthermore, other structures limit and strongly regulate these exchanges, such as cells adjacent to the abluminal side of MCEC (namely pericytes which cover approximately 32% of the microcapillary surface; and astrocytic feet which cover more than 98% of the surface made by MCEC and pericytes) and a specific extracellular matrix which sheathes MCEC, pericytes and astrocytic feet and restricts molecular diffusion between MCEC and CNS. BCSFB (Fig. 6B) is located in the lateral, third and fourth ventricles and is characterized by unique apical tight junctions between the choroid plexus epithelial cells (CPEC), which are responsible for the secretion of cerebrospinal fluid and also express specific transporters allowing regulated exchanges between the cerebrospinal fluid and blood (Braissant 2012). In physiological conditions, the adult CNS is able to synthesize Cr and has a limited supply from the periphery; however, in the embryonic CNS there is a different situation depending on the different expression of CT1 and Cr biosynthesis enzymes. This difference between adult and embryonic CNS might clarify the importance and effectiveness of Cr supplementation during pregnancy.

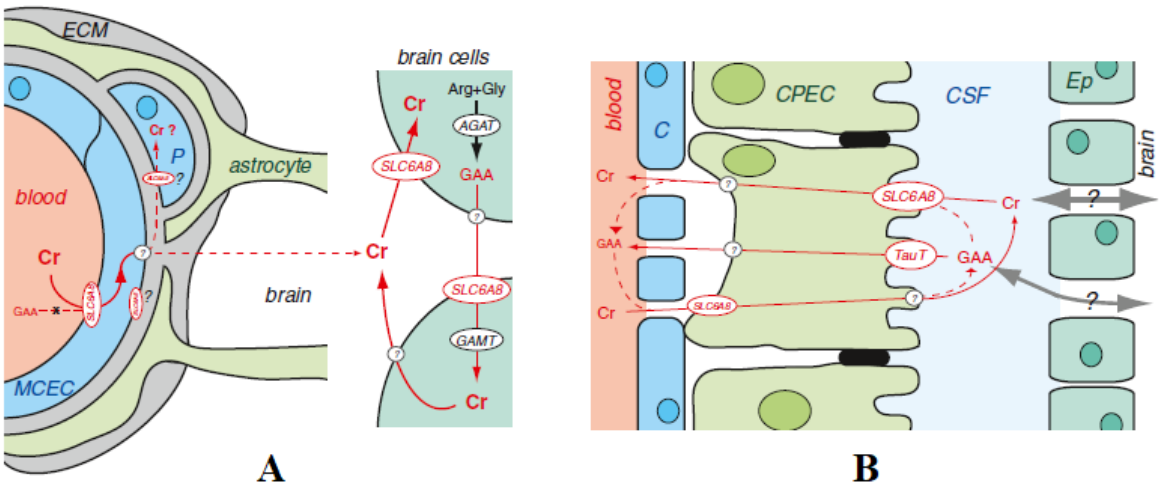

Fig. 6. (A) Transport of creatine at the blood-brain barrier. Cr is taken up by the CNS from the periphery through CT1 (SLC6A8 in the figure) expressed on MCEC at the BBB, but in limited amounts due to the absence of CT1 in the surrounding astrocytes, as well as the difficult diffusion of Cr through the ECM surrounding the BBB. Therefore, the brain needs its own synthetic pathway to ensure sufficient levels of Cr, and does so by expressing AGAT and GAMT. (B) Transport of creatine at the blood-cerebrospinal fluid barrier (BCSFB). CPEC are able to take up Cr both from the blood and CSF. Arg: arginine; AGAT: arginine:glycine amidinotransferase; Cr: creatine; ECM: extracellular matrix; GAA: guanidinoacetate; GAMT: guanidinoacetate methyltransferase; Gly: glycine; MCEC: microcapillary endothelial cells (BBB); P: pericyte; SLC6A8: Cr transporter (CT1); CPEC: choroid plexus epithelial cells (BCSFB); CSF: cerebrospinal fluid; Ep: ependymal epithelium.

Figure adapted from Braissant 2012

3.1.1 AGAT, GAMT and CT1 expression in the adult Central Nervous System

The adult mammalian CNS is able to synthesize Cr and has a limited supply of Cr from the periphery due to the expression of AGAT and GAMT in brain, as well as the limited expression of CT1 along the barriers. Specifically, AGAT is expressed in all the principal CNS structures, with notably high levels in the telencephalon and cerebellum (Braissant et al. 2005). This enzyme is expressed in all the main cell types of the brain, namely neurons, astrocytes and oligodendrocytes. In the barriers, AGAT is expressed by microcapillary endothelial cells (MCEC) and the astrocytes contacting them (at the BBB), as well as by the choroid plexus and ependymal epithelia (Braissant et al. 2001). GAMT is also expressed among the principal structures of the adult mammalian brain, in particular in the telencephalon (mainly in the corpus callosum and hippocampus), pons nuclei and cerebellum. Furthermore, GAMT is expressed in neurons, oligodendrocytes and astrocytes, and while it is not expressed in MCEC, it is present in the astrocytes that contact them (at the BBB), as well as in the choroid plexus and ependymal epithelia (Braissant et al. 2001; Tachikawa et al. 2004). Recently, it was found that AGAT and GAMT, in most areas of the rat brain, seem to be expressed in a dissociated manner, being rarely co-expressed within the same cell (Braissant et al. 2010). This dissociated AGAT and GAMT expression suggests the transfer of GAA from AGAT- to GAMT-expressing cells through CT1 to complete Cr synthetic pathway (Braissant 2012; Hanna-El-Daher and Braissant 2016). Because CT1 appears to be essential for Cr biosynthesis, the low cerebral Cr level found in CT1 deficiency patients despite normal AGAT and GAMT in CNS might be explained. *In vivo*, mouse and rat CNSs can obtain Cr from the circulation against the concentration gradient, but this uptake of Cr through the BBB seems almost inefficient.

CT1 is present in the main regions of the adult mammalian brain. In rats and mice, CT1 is found in neurons and oligodendrocytes, and is expressed by MCEC but not by astrocytes sheathing them (except for a very few in the cerebellum (Mak et al. 2009)), in contrast to the expression of AGAT and GAMT. CT1 is also found in the choroid plexus and ependymal epithelia. Thus, in the mature CNS, Cr can only cross the BBB only through the limited space occupied by MCEC, while it cannot cross the BCSFB (Braissant 2012).

Two strikingly coherent patterns of AGAT, GAMT, and CT1 expression are found in different brain structures. In the first pattern, present in most brain areas, high amount of cells do not express either AGAT, GAMT or CT1 (20-50%), the proportion of cells that express CT1 only (Cr "users") remains below 15%, while AGAT and GAMT are not co-expressed. In these structures, separation of AGAT and GAMT expression might facilitate the fine tuning of GAA and Cr synthesis, in particular for the two main roles of Cr in cellular energy and neuromodulation/neurotransmission, while the efficient uptake of Cr and GAA by brain cells might be essential to maintain their extracellular concentrations in low ranges, thereby allowing neuromodulation/neurotransmission by Cr while avoiding the toxic effects of GAA (Braissant et al. 2010; van de Kamp et al. 2013). It is noteworthy that the dissociated expression of AGAT and GAMT in the CNS (at the intercellular level) appears organized as in the periphery, where it may also support tight regulation of Cr synthesis. In the second pattern, specific brain areas such as the hippocampus, cerebellar Purkinje neurons or hypothalamic nuclei present large amounts of cells that co-express AGAT + GAMT (40-60%), and large amounts of cells that express CT1 (45-65%). This suggests that these neuronal layers permanently require high Cr levels, in line with their high CK activity (Hanna-El-Daher and Braissant, 2016).

Interestingly, the observed presence of GAMT and CT1 mRNAs in neuronal processes (axons and dendrites) suggests that GAMT and CT1 transcripts might be transferred along axons and dendrites in order to allow CNS cells to translate these proteins at their required locations. This would permit the cell to react quickly to instantaneous peripheral demands of, or the re-uptake of, Cr. This process is crucial 1) in CNS development during synaptogenesis or growth cone migration which has been demonstrated to be connected directly with CK (mammals) or arginine kinase (insects) activity, and 2) in adulthood for the described function of Cr as a central neuromodulator (Salomons and Wyss 2007).

3.1.2 AGAT, GAMT and CT1 expression in the developing Central Nervous System

During embryogenesis, the fetal requirements for Cr are partially supported by Cr actively transported from the mother to the fetus (Ireland et al. 2008). However, AGAT, GAMT and CT1 are also well expressed during vertebrate embryogenesis, including in the CNS (Braissant et al. 2005; Wang et al. 2007; Ireland et al. 2009). Nevertheless, the low levels of AGAT, GAMT and CT1 (GAMT in particular) during the initial developmental stages suggests that in contrast with the adult brain, the fetal CNS predominantly depends on external Cr sources, either from the fetal periphery or of maternal origin. This can be correlated with CT1 expression in the whole fetal brain at early stages, with particularly high levels in the periventricular area and

choroid plexus, the principal metabolic exchange areas of the developing CNS before microcapillary angiogenesis and differentiation of the BBB occur (Braissant et al. 2005; Braissant 2012).

Recently, studies about AGAT, GAMT and CT1 gene expression in the developing embryonic mammalian CNS have allowed understanding of the difference between the embryonic and adult CNS regarding Cr transport and biosynthesis. AGAT and CT1 are widely expressed in rat brains at embryonic day 12.5 (E12.5), while GAMT appears later, and only in scarce regions of the developing brain: at E15.5 GAMT is identified only in striatum and pons, and only at E18.5 it is found that in the neocortex, striatum, hippocampus, pallidum and spinal cord. An interesting expression pattern was shown in the two structures responsible for exchanges between the periphery and the CNS, the choroid plexus and MCEC that build the BBB. CT1 is highly expressed in the choroid plexus (E15.5 and E18.5), but is absent from MCEC (E18.5), a situation reversal in comparison to adulthood (Braissant et al. 2001). AGAT is not present in the choroid plexus but is found in MCEC at E18.5, while GAMT is not present in both structures throughout the entirety of embryonic development (Salomons and Wyss 2007).

Furthermore, it is important to underline the different roles of BCSFB and BBB between the embryonic and adult CNS. The BCSFB develops earlier in comparison to the BBB, and is therefore the first structure responsible for exchanges between the periphery and CNS during development (Fig. 7). Cr uptake by the CNS from the periphery is likely made possible by the much higher levels of CT1 expression in the CPEC (BCSFB) during development than in the mature brain, as well as by the very high expression of CT1 in fetal ependymal cells and the periventricular zone, as shown in rats (Braissant et al. 2005) (Fig. 7). In contrast, CT1 is not detectable at the BBB in the first stages of MCEC development (Braissant et al. 2005), while it is expressed in mature MCEC (Braissant et al. 2001; Ohtsuki et al. 2002) (Fig. 7).

The better ability of the developing CNS to obtain Cr from the periphery is evident in pre-symptomatic treatments of AGAT and GAMT deficiencies that seems to entirely prevent the phenotype of these syndromes. The discovery of these diseases suggests that Cr plays a critical role in the development of CNS higher cognitive functions, such as speech acquisition, during the first months and years after birth, and that Cr supplementation before irreversible damage occurs might prevent clinical symptoms of AGAT and GAMT deficiencies permanently. As described above, early Cr supplementation in postnatal stages and during the first years after birth may also promote Cr entry into the brain, at stages when the BBB is not as firmly regulated as in more mature stages, and when CT1 expression on the BBB and BCSFB may still largely promote entry of peripheral Cr into the brain (Braissant et al. 2005; Ireland et al. 2009), in contrast to adulthood (Braissant et al. 2001) (Fig. 7).

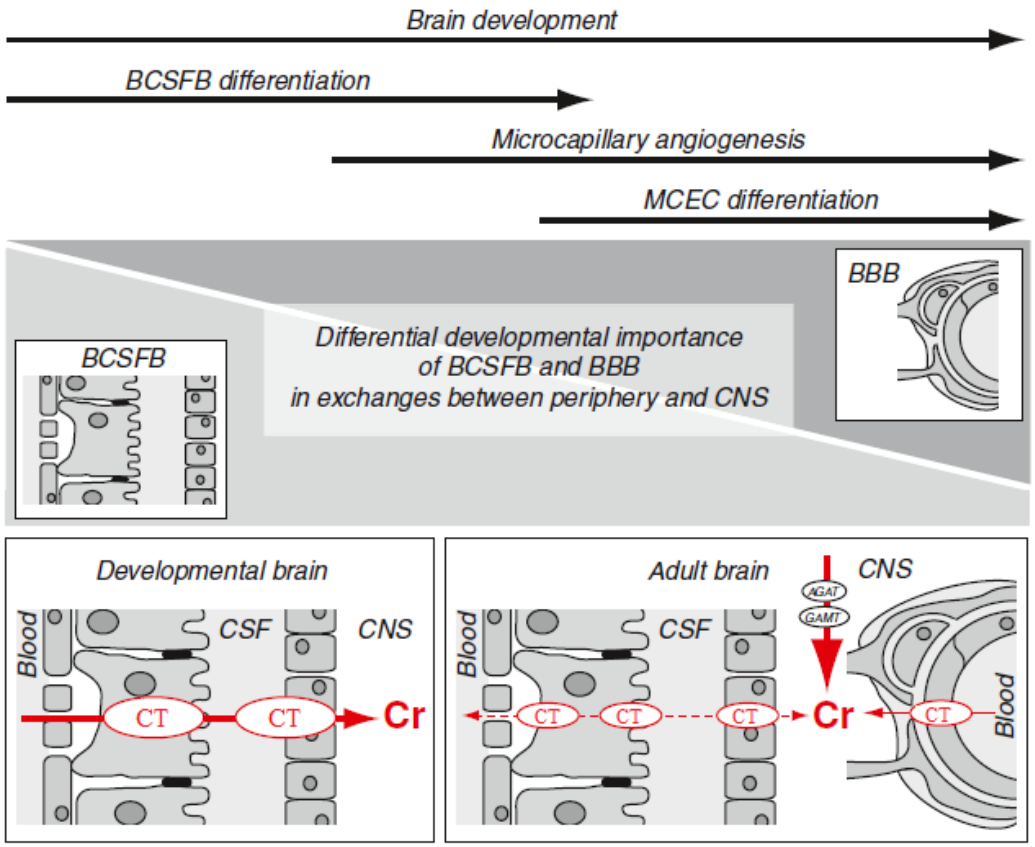

Fig. 7. Transport of creatine at blood-cerebrospinal fluid barrier (BCSFB) and blood-brain barrier (BBB) in the developmental versus the adult brain. Fetal and perinatal CNS may predominantly depend on Cr supply from the periphery. This is probably made possible during development by the high expression of CT in CPEC at BCSFB as well as in fetal ependymal cells and periventricular zone. In contrast, Cr is supplied to the mature brain by its limited uptake from the periphery, predominantly at BBB, as well as by endogenous synthesis. AGAT: arginine:glycine amidinotransferase; CNS: central nervous system; Cr: creatine; CSF: cerebrospinal fluid; GAMT: guanidinoacetate methyltransferase; MCEC: microcapillary endothelial cells (BBB); CT: Cr transporter.

Figure adapted from Braissant 2012

In conclusion, AGAT, GAMT and CT1 are extensively present in the brain, both during development and in adulthood. Most probably because of the absence of CT1 on the astrocytic feet that sheath microcapillaries, the adult CNS shows a limited capacity to obtain Cr from the periphery, as has been demonstrated *in vivo* both for mice and humans. Thus, the adult CNS seems to depend more upon endogenous Cr synthesis than on Cr uptake from the circulation. Differently, GAMT is expressed only at the end of the fetal CNS development, while CT1 is present on the first exchange structures between the CNS and periphery, namely the ependymal epithelia and choroid plexus. Thus, the embryonic CNS might be more dependent on Cr supply from the periphery than on endogenous synthesis, at least in the first stages of development. Therefore, even if human studies have demonstrated a limited increase of cerebral Cr following supplementation, it could be hypothesized that Cr administration during pregnancy results in a higher efficacy and a higher transfer of Cr to the brain.

3.2 The need for a treatment that decreases the probability of perinatal morbidity and mortality

Premature birth and neonatal hypoxic-ischemic encephalopathy (HIE) are the main areas in obstetric and neonatal medicine characterized by the lack of an effective prophylactic treatment. Morbidity and mortality are known to be higher for neonates born pre-term. Indeed, infants that survive to the premature birth may experience severe, life-long CNS disabilities such as cerebral palsy, occurring not only as a consequence of prematurity, but also from co-existing obstetric problems, namely intrauterine infections, chronic fetal hypoxia, or the difficulties that arise during resuscitation of the newborn (Dickinson et al. 2014a).

Maternal corticosteroid supplementation for impending preterm birth has been established to significantly decrease the risk of neonatal decease, respiratory distress, cerebroventricular hemorrhage, and necrotizing enterocolitis, and to limit the necessity for neonatal respiratory support and intensive care (Roberts et al. 2006). Prenatal magnesium sulfate supplementation has also been demonstrated to decrease the risk of cerebral palsy if provided to women immediately before the preterm birth (Doyle et al. 2009). Trials using xanthine oxidase inhibitor allopurinol supplementation during pregnancy as a tool for protecting the fetal brain from oxidative stress induced by hypoxia were conducted (Kaandorp et al. 2012). However, these therapies are provided relatively late, and only when it is obvious that preterm labor will occur, or when the fetus is already clearly under severe hypoxia.

Most perinatal deceases occur in the Third World environment. The World Health Organization estimates that approximately 9 million newborns experienced hypoxia during birth each year, and it is estimated that 1.2 million of these cases culminates in death (29% of global neonatal deaths), with similar numbers of newborns developing severe disability. In addition to cerebral palsy, cognitive and behavioral dysfunctions and psychiatric diseases including autism and schizophrenia occur often in infants born from pregnancies characterized by obstetric complications such as prematurity, fetal growth restriction, or birth hypoxia. In developing an effective prophylactic treatment for preterm birth and birth-related HIE, the greatest challenge is related to the difficulty of predicting their occurrence (Dickinson et al. 2014a). Thereby, strategies that prevent rather than rescue perinatal brain damage are necessary.

Head cooling or total body cooling are the most encouraging and generally used clinical treatments for HIE, however the need for immediate application following the onset of HIE is one of several limitations. In fact, if applied in the 6 hours immediately after birth, hypothermia can reduce mortality, and significantly limits the incidence of neurodevelopmental disabilities. A multi-center review established that no additional side effects are related to the use of hypothermia treatment, although adverse outcomes have been reported when hypothermia was applied to healthy term infants (Gluckman et al. 2004). Thus, the prolonged period prior to assessing the neonate as being “suitable for treatment” reduces the efficiency of this intervention. In this scenario, it is important to find a treatment that could be safe for both mother and fetus, which could also be administrated before hypoxia-ischemia occurrence.

3.2.1 Creatine as a therapy for the third trimester

Cr is not yet used in human pregnancy, but positive results from animal experiments have shown that maternal Cr supplementation protects the fetal CNS, diaphragm, and kidneys against hypoxic damage at term (Cannata et al. 2010; Ellery et al. 2013; Ireland et al. 2011). This suggests that Cr administered in the second or third trimester of human pregnancy could provide protection to all pregnant women against the risk of prenatally or perinatally acquired brain damage, similar to the manner in which folate is nowadays utilized to prevent defects of the neural tube during pregnancy. Cr supplementation is also different from treatments such as hypothermia and magnesium sulphate by providing protection to numerous organs against the physiological challenge during the transition from fetal to newborn life. Cr and its pleiotropic effects are hypothesized to benefit several fetal tissues where vasoconstriction, oxidative stress, or glutamate toxicity might occur, in addition to the main role of preserving mitochondrial function and ATP buffering (Dickinson et al. 2014a). Pregnancy is a condition characterized by high metabolic demands with extra nutritional requirements by the mother, and even in healthy pregnancy, there are increased levels of oxygen and nitrogen free radicals, a key source of which is the placenta (Myatt et al. 2004). Fetal tissues, particularly the developing CNS, are vulnerable to oxidative stress especially if infection or inflammatory processes are present. Thus, an antioxidant agent may be useful to protect the fetus against oxidative stress, especially in late pregnancy.

Another reason for considering the use of Cr supplementation during pregnancy relates to the possibility that preterm newborns might experience Cr depletion because endogenous synthesis is not yet fully developed. It seems that the fetus is dependent on Cr supply from the mother. In humans, and probably in most omnivore species, Cr may cross the placenta through active transport; in fact, it seems to first accumulate in the placenta before subsequently diffusing down a concentration gradient into fetal circulation (Davis et al. 1978; Koszalka et al. 1972; Miller et al. 1977). However, it is unclear when the capacity for Cr synthesis develops in the fetus. Studies using early spiny mice demonstrate that the reno-hepatic axis of Cr synthesis develops late in gestation (Ireland et al. 2009), and if this occurs also in human pregnancy it would indicate that infants born prematurely have an under-developed capacity for Cr synthesis, and potentially representing an increased risk of becoming Cr deficient. As already mentioned in the second chapter, the fact that the fetus depends on maternal Cr supply is suggested by the knowledge that newborns diagnosed with cerebral Cr deficiency develop signs of neurological dysfunction in the days or weeks following birth, suggesting that Cr deficiency was not present before birth, even if the ability to synthesize Cr on their own during pregnancy was absent. It is also important to investigate the capacity for reabsorption of Cr from glomerular filtrate in neonates, because preterm birth is often associated with renal dysfunction. Urine composition, such as urinary lactate/creatinine ratio, has been measured to assess the risk of developing HIE in neonates, however the ability of the kidney to retain Cr in preterm newborns has not yet been investigated (Dickinson et al. 2014a).

The occurrence of birth before the major organs are sufficiently developed to meet the postnatal life demands is a crucial problem. Therefore, the Cr status of premature neonates needs close attention. In human premature births, the heart, lungs, kidneys, and brain are immature. In these cases, respiratory and cardiovascular support is often required, and resuscitation procedures usually provide further risk of hypoxic-

ischemic injury to the brain. It is clear that acute hypoxic event leads to depletion of ATP and PCr in human infants and children, confirming animal studies and confirming that Cr supplementation should be studied as a treatment to prevent and treat hypoxic-ischemic SNC damage at birth (Dickinson et al. 2014a).

3.2.2 Creatine as a clinically important and safe treatment

Higher intracellular Cr/PCr stores following CM supplementation or subcutaneous injections have been shown to be neuroprotective in numerous animal models of neurodegenerative diseases and acute CNS damage. Furthermore, Cr supplementation has recently been shown to improve cognitive function in normal and elderly people (Benton et al 2011; Rawson and Venezia 2011), and cognitive and motor skills in sleep-deprived subjects (Cook et al. 2011).

The relevant statutory authorities in the EU (European Medicines Agency), UK (Medicine & Healthcare Products Regulatory Agency), USA (Federal Drug Administration) and Australia (Therapeutic Goods Administration) have yet to classify Cr, CM and creatine phosphate, and they have also not been assigned to a category for administration in pregnancy. While long-term Cr supplementation in adult humans seems to be safe, some concerns could arise before recommending use in human pregnancy, as detailed studies have yet to be conducted. In a human study, Bohnhorst et al. (2004) demonstrated that although 14 days of oral Cr supplementation was ineffective to treat apnea in premature infants, it was well tolerated by the premature neonates with no side effects noted. So far, no evidence has found effects upon body weight or body composition modifications in mothers and fetuses after Cr supplementation. Nevertheless, given that pregnancy is usually linked with fluid balance changes in women, some concerns could arise, as Cr is an osmolyte, and in very high concentrations may be linked to enhanced cellular water uptake, aggravating fluid shifts during pregnancy (Dickinson et al. 2014a).

Understanding the neuroprotective effects of Cr to treat and prevent brain damage in the adult is important in order to appreciate how it might be supplemented to prevent or attenuate fetal and neonatal CNS injury. Indeed, many adult neuropathologies in which Cr has been demonstrated to be beneficial also include the primary mechanisms of damage induced by hypoxia-ischemia in the immature CNS, namely mitochondrial dysfunction, impaired energy metabolism, excitotoxicity and oxidative stress. However, it is important to consider that Cr supplementation may represent a multi-organ protective agent for the fetus and neonate in contrast to the other current therapies, namely magnesium sulphate or head cooling.

The “pleiotropic” effects of Cr go beyond the spatial-temporal buffering of ATP, and are summarized as follows:

1) Acid-base balance role: the rephosphorylation of ADP utilizes a proton (H^+), reducing the intracellular acidity during hypoxic events (Walliman et al. 2011). Besides this, Cr has the ability to scavenge free radicals (Lawler et al. 2002; Sestili et al. 2006). Therefore, the Cr/PCr system has the capability to modulate variations of intracellular acid-base balance that can arise during events of acute hypoxia in a direct antioxidative manner.

2) Antioxidant actions: perhaps associated with this effect on H^+ accumulation, Cr has been shown to have an antioxidant effect (Lawler et al. 2002; Sestili et al. 2006). Furthermore, Ireland and colleagues (2011) showed that maternal Cr supplementation from mid-pregnancy prevented the increase of malondialdehyde (product of lipid peroxidation) levels caused by intrapartum hypoxia in spiny mouse pup brains.
3) Post-ischemic recovery of protein synthesis: Cr pre-treatment in the oxygen-glucose deprived adult rat (Carter et al. 1995) and fetal guinea pig hippocampal slices (Berger et al. 2004) provides a faster recovery of the decreased protein synthesis that occurs before neuronal cell loss in the post-ischemic brain, resulting in a more positive histological outcome.
4) Improved cerebral vascular function: Cr pre-treatment provides quicker recovery of cerebral blood flow during reperfusion following middle cerebral artery occlusion, probably due to larger dilator responses to extra-luminal K^+ and acidosis (Prass et al. 2007).
5) Interaction with the benzodiazepine receptor: Cr may bind to the $GABA_A$ receptor, which was demonstrated in chick brains (Koga et al. 2005). Furthermore, animals supplemented with Cr showed increased GABAergic activity in some brain areas (Pena-Altamira et al. 2005). The anti-excitatory effect of the enhanced $GABA_A$ receptor activity is probably protective for the developing CNS.
6) Promoting the uptake of glutamate: The re-uptake of glutamate occurs in an ATP-dependent manner by synaptic vesicles, and Cr therefore acts to maintain the ATP required. Furthermore, PCr has been demonstrated to promote the uptake of glutamate in synaptic vesicles, and might account for the neuroprotective function of Cr against glutamate toxicity in neuronal cell culture (Brewer et al. 2000).
7) Stabilization of lipid membranes: PCr might interact with phospholipid membranes to stabilize them and prevent their permeabilization (Tokarska-Schlattner et al. 2012).

3.3 Protective functions of Creatine against Hypoxic-Ischemic events at birth

Circa 4 per 1000 live term born have experience of hypoxia-ischemia at birth, and depending on its severity and duration, 4-8% of these infants will die. Newborns that survive are typically subject to several health problems, caused by irreversible damage to organs such as the brain, kidneys, heart and lungs. The brain is the main organ affected by hypoxia-ischemia, with large percentage of survivors suffering brain damage followed by lifelong effects including mental and physical disability, cerebral palsy and seizures. However, other systemic complications of hypoxia-ischemia, namely acute kidney, muscle, and heart dysfunctions should be recognized and treated for overall homeostasis and thus survival (Dickinson et al. 2014a). Studies in the early spiny mouse demonstrated that 5% Cr supplementation commenced from half-way through pregnancy resulted in a 10-30% increase in Cr levels in fetal tissues (including heart, kidney, liver, brain and muscle) and a 2-fold enhancement in the placenta at term (Ireland et al. 2008). The increased amount of Cr in the fetal liver at term might act as an additional Cr store available for the newborn that could be, for the reasons mentioned above, at risk of Cr depletion.

It is important to consider that Cr supplementation does not impair protein expression of the enzymes AGAT and GAMT in the neonatal kidneys and liver, which are required for postnatal Cr synthesis (Dickinson et al. 2013). Furthermore, Cr administration during normal pregnancy seems to not have side effects on the mother or newborn in terms of health status or body composition (Ellery et al. 2016). However, no data are available regarding the effects of Cr during pregnancy where fetal growth restriction is present. Animal studies using spiny mice have demonstrated protective effects of Cr administration on brain structures (Ireland et al. 2011), postnatal behavior (Ireland et al. 2008), the diaphragm (Cannata et al. 2010), and kidney structure and function (Ellery et al. 2013) following asphyxial birth, suggesting that Cr may also protect preterm infants in their premature passage from fetal to neonatal life.

Cr and PCr are critical in maintaining intracellular ATP and therefore allow cells to extend mitochondrial function and counteract the initial metabolic collapse due to hypoxia-ischemia. Cr might also act against several secondary responses to hypoxia-ischemia, for example through reduction of oxidative stress and promotion of the post-ischemic recovery of protein synthesis. However, it should be note that acting in the first phase of hypoxia-ischemia may already prevent secondary responses to the hypoxic event.

A limitation found in adult studies investigating the neuroprotective effects of Cr is the slow transfer of exogenous Cr into the brain, influenced by limited BBB permeability and by CT working close to saturation, and leading to the awareness that long-term, high doses of Cr are required to significantly increase Cr levels in the brain. Indeed, early studies in mice (Zhu et al. 2004) and humans (Dechent et al. 1999) demonstrated that pre-treatment with Cr required several weeks or months before effectively increasing cerebral Cr content and acting as a neuroprotective agent against cerebral ischemia (Zhu et al. 2004). However, other studies demonstrated effective supplement protocols of shorter duration (one or two weeks), which successfully enhanced the amount of Cr in the human brain (Pan et al. 2007; Braissant et al. 2005). Nevertheless, Cr seems to reach the immature brain with greater ease through the higher expression of CT1 in endothelial cells of the choroid plexus, allowing the developing CNS to acquire peripheral Cr more so than the mature brain.

In the postnatal rat brain, a subcutaneous injection of Cr at day 10, but not at day 20 produced a significant enhancement of the PCr/NTP (nucleoside triphosphate) ratio, and this was able to increase the recovery of the cerebral PCr/NTP ratio within 2 h following hypoxia (Holtzman et al 1998). Furthermore, Cr administered before hypoxic insult reduced brain edema and the incidence of severe cystic cerebral infarction after hypoxic-ischemia in 7 days old rats (Berger et al. 2004). Therefore, it must be considered that Cr supplemented to women even for only several weeks in late pregnancy is likely to enhance the resistance of the fetal CNS to oxygen deprivation or acidemic/hypercapnia during the birth, or if poor ventilatory efforts by the newborn require resuscitative procedures immediately after birth (Dickinson et al. 2014a).

3.4 Which obstetric population could benefit from Creatine supplementation?

The potential benefit of Cr supplementation during pregnancy may be crucial for several situations during pregnancy that lead to preterm birth, and therefore to high risk of HIE, such as preeclampsia and

gestational diabetes mellitus. Preeclampsia is a common syndrome of pregnancy that occurs after 20 weeks of gestation, characterized by new-onset hypertension together with maternal end-organ dysfunction and/or intrauterine fetal growth restriction, and represents a disease associated with nearly 50% of preterm births. With an incidence of around 5% of all pregnancies, preeclampsia shows significantly elevated oxidative stress levels within the maternal-fetal unit. Gestational diabetes mellitus is another common human pregnancy disorder affecting around 7% of women, and is characterized by enhanced oxidative stress and higher risk of preterm birth. These pregnancy conditions along with many others, namely cervical incompetence, preterm premature rupture of the membranes (PPROM), partial placental abruption and placenta praevia, and fetal growth restriction, might benefit from Cr supplementation. These conditions can lead to fetal hypoxia, hypoglycemia and activation of the fetal hypothalamic-adrenal axis, causing not only fetal growth restriction and preterm delivery but also, potentially, fetal death or stillbirth. Fetus intrauterine death will always involve mitochondrial energy impairments. To reduce perinatal morbidity and mortality, therapeutic interventions such as bed rest, low dose aspirin, or conventional nutrient supplementation are used with limited success (Dickinson et al. 2014a). Cr supplementation could be used as a conservative treatment and it would not interfere with other clinical practices. However, while a trial of Cr administration for neuroprotection of the human fetus at term has been conducted (Dickinson et al. 2014b), no other clinical trials of Cr supplementation in human pregnancy exist. Uterine smooth muscle contains very low Cr levels, and ATP and PCr are quickly consumed by uterine activity. Given that uterine activity in late gestation may be uncoordinated and labors sometimes “fail to progress”, Cr supplementation during pregnancy might also decrease the caesarean section rate (Dickinson et al. 2014a).

Chapter 4

EXPERIMENTAL STUDY

4.1 Aim of the study

The brain utilizes large quantities of ATP to preserve normal function, involving the maintenance of electrical membrane potentials, ions gradients, Ca^{2+} homeostasis, and signaling activities (Braissant et al. 2010). In this scenario, Cr/PCr/CK system plays an important role during rapid changes in ATP demands that occur due to physiological activities of neurons, in particular functionally coupling sites where ATP is generated (mitochondria and glycolysis) and where it is consumed (ATPases). Furthermore, following the discovery of cerebral Cr deficiency syndromes, Cr is well known to play a critical role in CNS development, as Cr administration to these pre-symptomatic patients prevents neurological symptoms, such as developmental and speech delay, epileptic seizures, autism and severe mental retardation (Braissant et al. 2011).

In addition to an ergogenic role, other effects, including antiapoptotic, antioxidant (Sestili et al. 2006), and neuroprotective effects (Beal 2011; Sartini et al. 2012) might be important for proper CNS function and development (Wallimann et al. 2011; Sestili et al. 2016). A study has shown a neuroprotective role of Cr through the improvement of reperfusion rather than a changing of the bioenergetics status in a mouse model of stroke (Prass et al. 2007). Furthermore, in *in vivo* and *in vitro* rodent models of ischemia/anoxia, Cr perfusion before ischemia/anoxia occurrence reduced the volume of damaged brain tissue, and led to neurological improvement (Hanna-El-Daher and Braissant 2016). Cr was also proposed as a neuroprotective treatment for several neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease, as well as Amyotrophic lateral sclerosis (Lensman et al. 2006; Beal 2011; Cunha et al. 2014) characterized by mitochondrial impairments and oxidative stress. However, the first therapeutic Cr supplementation trials appeared rather disappointing, probably due to the limited BBB permeability for Cr, which suggests a need to find new means to facilitate Cr uptake by the CNS. Finally, the Cr/PCr/CK system plays an essential role during brain development by providing enough ATP for growing axons and dendrites at their growth cone extremities, and Cr was found to have pro-differentiation/trophic actions on maturing neuroblasts, suggesting an importance also in CNS development (Sartini et al. 2012).

Due to the pleiotropic effects of Cr upon correct brain function, development, and protection, there is growing interest surrounding the possible use of Cr supplementation during human pregnancy. The reason is that Cr may represent an important and economic tool in order to prevent and treat CNS damage in case of

pre-, peri-, and post-natal hypoxic-ischemic events caused by premature birth or placental insufficiency (Dickinson et al. 2014a; Ellery et al. 2016). Maternal Cr supplementation could be important and effective in enhancing cerebral Cr of the fetus, because during fetal life, the brain is largely dependent upon the external source of Cr, which is likely of maternal origin (Braissant et al. 2007; Ireland et al. 2009).

Little is known on the effects and safety of Cr supplementation on neuron differentiation, and our *in vitro* study has shown a positive effect of Cr in protecting developing neuroblasts from oxidative stress (Sartini et al. 2012), a status closely associated to peri-natal hypoxia-ischemia, which is a severe condition for the developing CNS. However, Cr supplementation induced a significant and dose-dependent anticipation of Na^+ and K^+ current expression, and a higher excitability, expressed as number of spikes following depolarization, suggesting that Cr administration may affect neuron excitability (Sartini et al. 2012), leaving the safety of Cr supplementation during CNS development as an open question. Moreover, in our *in vivo* study, maternal Cr supplementation at low dosage (1% of Cr in drinking water) was demonstrated to affect the morpho-functional development of hippocampal neurons in neonatal rats at postnatal day 14-21 (P14-21), showing enhanced dendritic tree development, higher intrinsic excitability, larger evoked-synaptic responses and increased Long Term Potentiation (LTP) maintenance (Sartini et al. 2016). Since these results were observed at P14-21, thus weeks after maternal Cr supplementation (administration ceased one day before delivery), we hypothesized the possibility that the faster morphological and functional maturation observed in the hippocampus of the pups may be the results of long-lasting modifications in neuronal development triggered during fetal life. Because of this evidence, the long-term effects of maternal Cr supplementation on developing (P14-21) and adult brains (P60-70), using electrophysiological, morphological, and calcium imaging approaches have been investigated in this study. Moreover, considering that LTP is one of the crucial cellular mechanisms involved in learning processes, the ability of spatial learning using the Morris Water Maze has been also studied.

4.2 Materials and Methods

4.2.1 Animals and supplementation protocol

Sprague-Dawley albino rats (Charles River, Italy) were used in this study. The handling and care of animals were carried out in conformity with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All the experimental procedures were approved by animal subjects review board of the University of Urbino and were designed to minimize the number of animals used and their suffering.

Virgin male and female rats (weighing 200-250 g) were housed in pairs 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=4) drinking tap water; 2. Supplemented group (TREAT; n=4) 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. This Cr supplementation period was decided because of the expression of Cr membrane transporters occur at E12.5 in the embryonic CNS (Braissant et al. 2005). Daily weight of rats from CTRL and TREAT groups was monitored during the experimental period. In order to verify the effect of the hyperexcitability found in the previous study (Sartini et al. 2016) upon the risk of the induction of epileptiform activity, the CTRL and TREAT offspring were killed from postnatal day 14 to 21 (P14-21); to address the effects of maternal Cr supplementation upon the hippocampus of adult progeny, the CTRL and TREAT offspring were killed at P60-70.

4.2.2 Electrophysiological experiments

Male pups from different CTRL and TREAT litters, aged from P14 to P21 and adult male rats from different CTRL and TREAT litters, aged from P60 to P70, were used to obtain hippocampus slices for performing electrophysiological recordings in the CA1 subfield. Animals (CTRL, *n* = 19; TREAT *n* = 18) were killed by decapitation after being anesthetized with intraperitoneal injection of ketamine anesthetization (65 mg/kg body weight). The brain was quickly removed and incubated in chilled oxygenated solution containing in millimolar: 110 choline Cl^-, 2.5 KCl, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 20 dextrose, 1.3 Na^+ ascorbate, 0.6 Na^+ pyruvate, 5.5 kinurenic acid (pH = 7.4; 320 mosM). Afterwards, the brain was divided into two hemispheres, each of which was prepared for cutting. Hippocampal transversal slices (400 µm thick) were obtained from each hemisphere by vibrating microtome (Campden Instruments, USA) and allowed to recover at least 1 hour in an interface recovery apparatus with artificial cerebrospinal fluid (ACSF) containing in millimolar: 125 NaCl, 2.5 KCl, 1.3 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.3 Na^+ ascorbate, 0.6 Na^+ pyruvate, 10 dextrose, equilibrated with a 95% O_2-5% CO_2 gas mixture (pH = 7.4; 320 mosM), before electrophysiological recordings. Each slice was then transferred into a recording chamber where it was continuously superfused throughout electrophysiological recordings with oxygenated ACSF at a rate of 3 ml/min (Ambrogini et al. 2004; Betti et al. 2011). Here, following an equilibration period, field

potential and whole-cell patch-clamp recordings were carried out. Electrophysiological traces were offline analyzed using a WinWCP software (Strathclyde Electrophysiology Software, John Dempster, University of Strathclyde, UK).

4.2.2.1 Field potential recordings

In order to investigate the role of maternal Cr supplementation in the generation of epileptiform activity in the hippocampus of the progeny, we used one of the most extensively studied models of epilepsy, providing blocking synaptic inhibition. Thus, to induce spontaneous epileptiform-like discharges, the hippocampal slices obtained from rats at P14-21 of both groups were perfused with ACSF, containing bicuculline methiodide (BMI, 50 μM; Sigma), a $GABA_A$ receptor antagonist, and 4-Aminopyridine (50 μM, Sigma), a K^+ channel blocker, in the recording bath. Bipolar stimulating, connected with A385 stimulus isolator (World Precision Instruments, USA), and recording electrodes, filled with ACSF, were placed respectively in the Schaffer collaterals and in the CA1 cell body layer of the hippocampus. Field potential recordings were carried out under visual guidance using a Zeiss Axioskop microscope (Carl Zeiss International, Italy) equipped with an infrared video camera connected to a monitor. The parameters analyzed to verify the epileptic threshold were the latency of the first interictal event and the frequency of interictal events.

The effect of maternal Cr supplementation on adult synaptic plasticity was investigated by evaluating the ability to elicit LTP in the Schaffer collaterals-CA1 pathway. Recording and bipolar stimulating electrodes were prepared, filled with ACSF, and placed in the stratum radiatum of CA1 at approximately 300 μm of distance between them. Slices showing extracellular field excitatory postsynaptic potentials (fEPSPs) of at least 1 mV in amplitude were used to perform the field potential recordings. Before LTP induction protocol application, input-output relationship between fiber volley amplitude and fEPSP slope was constructed increasing Schaffer Collateral stimulation intensity applying to the slice square pulses of current (500 μs in duration, from 0 to 140 μA, steps of 20 μA) with A385 stimulus isolator (World Precision Instruments, USA). Then, test pulses at 30-s of intervals were applied to elicit baseline responses; afterward, Schaffer collaterals were stimulated using 3 stimulus patterns every 5 seconds, each one characterized by 10 trains of 100 Hz applied for 0.1 s separated by an interval of 1.2 s; the fEPSP was then monitored by recordings for 40 min. fEPSP slope (between 10% and 80% of the max) was analyzed and taken as measures of synaptic strength; values were normalized to the mean value obtained over the last 15 min of the baseline period and expressed as a percentage of this baseline value (Betti et al. 2011).

4.2.2.2 Patch-clamp recordings

To investigate the effects of maternal Cr supplementation on the electrophysiological properties of CA1 pyramidal cells, patch-clamp recordings in whole-cell configuration were carried out under visual guidance using a Zeiss Axioskop microscope (Carl Zeiss International, Italy) equipped with an infrared

videocamera connected to a monitor. Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, USA) using a vertical puller (model PP-830 Narishige, Japan) and had 3-5 MΩ tip resistance. These patch-clamp electrodes were filled with internal solution contained in millimolar: 126 potassium gluconate, 8 NaCl, 0.2 EGTA, 10 HEPES, 3 Mg_2ATP, 0.3 GTP, and biocytin (Sigma; 0.2%), for subsequent neuron morphology analysis (pH = 7.2; 290 mosM) (Ambrogini et al. 2004; Betti et al. 2011).The electrode resistance in the bath ranged from 3 to 5 MΩ; the junction potential between internal and external solutions was not corrected. Somas to be recorded were identified in CA1 pyramidal cell layer based on their typical shape using an Axopatch-200B amplifier (Axon Instruments, USA) and WinWCP software (Strathclyde electrophysiology software whole cell analysis V 3.2.9, John Dempster, University of Strathclyde, UK). Resting membrane potential (RMP), input resistance (IR), capacitance (C) and cell excitability were determined as following described. Cell access was obtained in the voltage clamp mode and RMP was measured immediately upon break-in. Input resistance and capacitance were calculated in response to a 100-ms, 5-mV hyperpolarizing pulse. In order to evaluate cell excitability, membrane potential in response to depolarizing current pulses (10 pA steps) was measured; if RMP was different from -70 mV, a holding current was delivered to bring the membrane potential to approximately -70 mV before the delivery of current pulses (Ambrogini et al. 2004). The amplitude and shape of the first elicited action potential, and the relationship between depolarizing injected current and percent of cell that elicit action potential were evaluated. The Afterhyperpolarization (AHP) was triggered using a depolarizing current step of 1 s able to induce maximum firing rate. A bipolar stimulating electrode pulled from theta capillary (World Precision Instruments) and filled with ACSF solution was placed in the stratum radiatum approximately 300 µm from the recorded cell. Postsynaptic potentials (PSPs) were recorded in current clamp mode and were evoked with an interstimulus frequency of 0.033 Hz. Stimulus intensity was adjusted at the lowest intensity (ranging from 100 to 200 µA) that evoked the maximal response. During PSC recordings, cells were held at a membrane potential of -70 mV, if not otherwise specified (Ambrogini et al. 2004; Betti et al. 2011). Tiple stimuli with an interstimulus interval of 50 ms were delivered every 20 s to the Schaffer collateral inputs and the amplitude of the three excitatory postsynaptic potentials was measured. In order to evaluate the effect of prenatal Cr supplementation on resting membrane potential, we considered RMP of all neurons and recordings were rejected only if the initial series resistance was >30 MΩ, if the series resistance measured at the end of the experiment had changed (±5 MΩ) or if DC offset exceeded 5 mV after withdrawal from the cell.

4.2.3 Morphological analysis

The same slices used for performing patch-clamp recordings, in which neurons were filled by the recording electrode with biocytin, were fixed with paraformaldehyde (4% PFA in PBS; Sigma-Aldrich, Italy) overnight at room temperature. Then, after several rinses with PBS, slices were incubated with streptavidin-conjugated CY3 (Amersham, Italy; 1:500 in 0.01 M phosphate buffer saline-PBS, pH 7.4, containing 0.1% Triton X-100) overnight at room temperature; afterwards, slices were rinsed several times in PBS and mounted

on slides by antifading medium (Vector, DBA, Italy). Morphological reconstruction of each labeled pyramidal cell was performed using Leica TCS-SL confocal microscope, equipped with Argon and He/Ne laser sources. Morphological analysis was carried out on neurons without clear dendritic cutting at the slice surface. The total length of basal and apical dendritic trees of CA1 pyramidal cell was assessed using the image analysis software NeuronJ. Sholl concentric ring analysis was applied to evaluate dendritic tree complexity; concentric rings were superimposed to reconstructed cells and dendritic crossings were counted along both the basal (negative number) and apical (positive number) dendrites (Betti et al. 2011).

4.2.4 Calcium Imaging

Calcium imaging recordings in whole-cell configuration were performed at RT and the experiments were carried out under visual guidance using a Zeiss Axioskop microscope (Carl Zeiss International, Italy) equipped with a 40X water immersion objective and the Orca Flash 4.0 CCD camera (C11440, Hamamatsu, Japan). All recordings were performed on CA1 pyramidal neurons using an Axopatch-200B amplifier (Axon Instruments, USA) and WinFluor software (Strathclyde Imaging Software V 3.8.7, John Dempster, University of Strathclyde, UK). Patch electrodes were filled with an intracellular solution containing in millimolar: 126 potassium gluconate, 8 NaCl, 10 HEPES, 3 Mg_2ATP, 0.3 GTP (pH = 7.2; 290 mosM), and Fluo-4 Pentapotassium Salt (100µM, Sigma), used for evaluating cellular calcium changes.

Fluorescence images (600 X 600 pixels) were acquired at 10Hz frequency using a FITC excitation filter of 450-490 nm and fluorescence values were expressed as ΔF/F where F is the fluorescence at resting condition: the ROI (10 X 10 pixels) was placed on cell body and the background fluorescence was sampled in a region far from the loaded cell. We minimized the photobleaching illuminating the slice with minimal light intensity. Fluorescence evaluation started immediately after break-in. During the loading phase F_0 and the calcium transient in response a single action potential has been monitored: the single action potential was evoked in current clamp mode every 30 s by means of brief current pulse (2 ms) and the neurons were held at about -70 mV to avoid spontaneous action potentials. The steady-state was reached after about 15 minutes. Moreover, once the cells were full loaded with Fluo-4, fluorescence changes in response to high frequency stimulations (20-100 Hz, 600 ms) were evaluated.

In each neuron unperturbed calcium transient, intracellular calcium concentration at resting condition $[Ca^{2+}]_0$ and during a single action potential $\Delta[Ca^{2+}]_{AP}$ were calculated according to Maravall et al. 2000. Calcium concentration was obtained using equation 1:

$$\frac{\Delta[Ca^{2+}]}{K_d} = \frac{F_{max}}{F_0}\left(1 - {R_f}^{-1}\right)\frac{\Delta F/F}{((\Delta F/F)_{max} - \Delta F/F)\cdot(\Delta F/F)_{max}} \quad (1)$$

where, $\Delta[Ca^{2+}]$ is the transient in calcium concentration, K_d is the Fluo-4 dissociation constant (345 nM), F_{max} is the fluorescence measured at saturation, F_0 is the fluorescence during resting condition, R_f is the dynamic range of the indicator (we considered a value of 85 as reported for Fluo-4 in Maravall et al. 2000), $\Delta F/F$ is the rise in fluorescence divided by the resting fluorescence, $(\Delta F/F)_{max}$ is the $\Delta F/F$ at saturation. F_{max} was measured in every cell using high frequencies action potentials trains (from 20 to 100 Hz) to obtain maximal $\Delta F/F$ values.

The calcium concentration at resting condition $[Ca^{2+}]_0$ was determined using equation 2:

$$\frac{[Ca^{2+}]_0}{K_d} = \frac{1 - {R_f}^{-1}}{(\Delta F/F)_{max}} - {R_f}^{-1} \tag{2}$$

The buffer capacity of the indicator (K_B) was calculated using equation 3:

$$K_B = \frac{K_d \times [B]_T}{(K_d + [Ca^{2+}]_0) \times \left(K_d + [Ca^{2+}]_{peak}\right)} \tag{3}$$

where $[B]_T$ is the Fluo-4 concentration (in nM) and $[Ca^{2+}]_{peak}$ is the calcium concentration at the peak of the calcium transient.

In both experimental groups the endogenous buffer capacity (K_S) was evaluated back-extrapolating the relationship between the reciprocal of calcium transient amplitude during a single action potential ($1/\Delta[Ca^{2+}]_{AP}$) and the exogenous buffer capacity (K_B). The data obtained were fitted with a straight line and the intercept of this line with the x-axis provide an estimation of endogenous buffer capacity (K_S = -(1 + x intercept)).

4.2.5 Behavioral experiments: Morris Water Maze

P60-70 CTRL and TREAT male rats (n = 6) were tested for spatial learning using the paradigm of Morris Water Maze. The apparatus consisted of a black circular swimming pool 1.65 m wide and 60 cm high, half-filled with water at temperature of 21 ± 1°C, in which an escape black platform 10 cm in diameter was placed, submerged 1.5 cm below the water surface and kept in the same position at 15 cm from pool wall (Betti et al. 2011). To guide rat escape behavior in locating the goal (platform), three different extramaze cues were used. To accustom the rat to the water, a pretraining session without platform was carried out. In this session the swimming speed of rats was also evaluated and it resulted not different between groups. Then, the training protocol was applied consisting in two sessions per day (8 a.m. and 2 p.m.) of four trials each (60 s with inter-trial time of 60 s), over 4 consecutive days, for a total of 7 sessions. The time taken to reach the platform (escape latency) and the path length covered by the rats to get to the platform were recorded (Video tracking

system; Smart-BS, 2biological Instruments, Italy) and considered as parameters to evaluate learning. Moreover, in order to obtain a more reliable measure of learning, the day after the end of training, the rats performed the probe task by removing the platform from the pool and tracking the swim-path for 1 min. To evaluate the result of this test, the maze was virtually divided in four quadrants, one of which was centered on the position formerly occupied by the platform (goal quadrant); the time spent in the goal quadrant and in the others was calculated.

4.2.6 Statistical analysis

Data were expressed as mean ± SEM. Differences between the experimental groups were statistically evaluated by appropriately applying Student's t-test or χ square or Two-Way repeated measures ANOVA followed by Sidack's *post hoc* test. The relationship between fEPSP slope and fiber volley amplitude was evaluated using linear regression. For all analyses the significance threshold was established at $p = 0.05$.

4.3 Results

The ponderal growth curves of pregnant rats supplemented with Cr and of control were not different (data not shown). Furthermore, Cr supplementation did not affect the survival rate, weight of the pups at birth and litter size, and no evident teratogenic effect was observed in TREAT offspring. Lactating pups showed no significant intergroup differences in ponderal growth throughout the considered experimental period (data not shown); the timing of major maturational steps, i.e. hair coat development, eye opening and onset of movement control did not show differences between groups.

4.3.1 Field potential recordings at P14-21

Maternal Cr supplementation did not improve the probability of the epileptiform activity induction in CA1 of the offspring at P14-21 after perfusion with synaptic inhibition blockers, bicuculline methiodide and 4-Aminopyridine. The epileptic threshold was comparable in both groups due to a similar latency of the first interictal event and frequency of interictal events, as shown in Fig. 8 and Fig. 9, respectively.

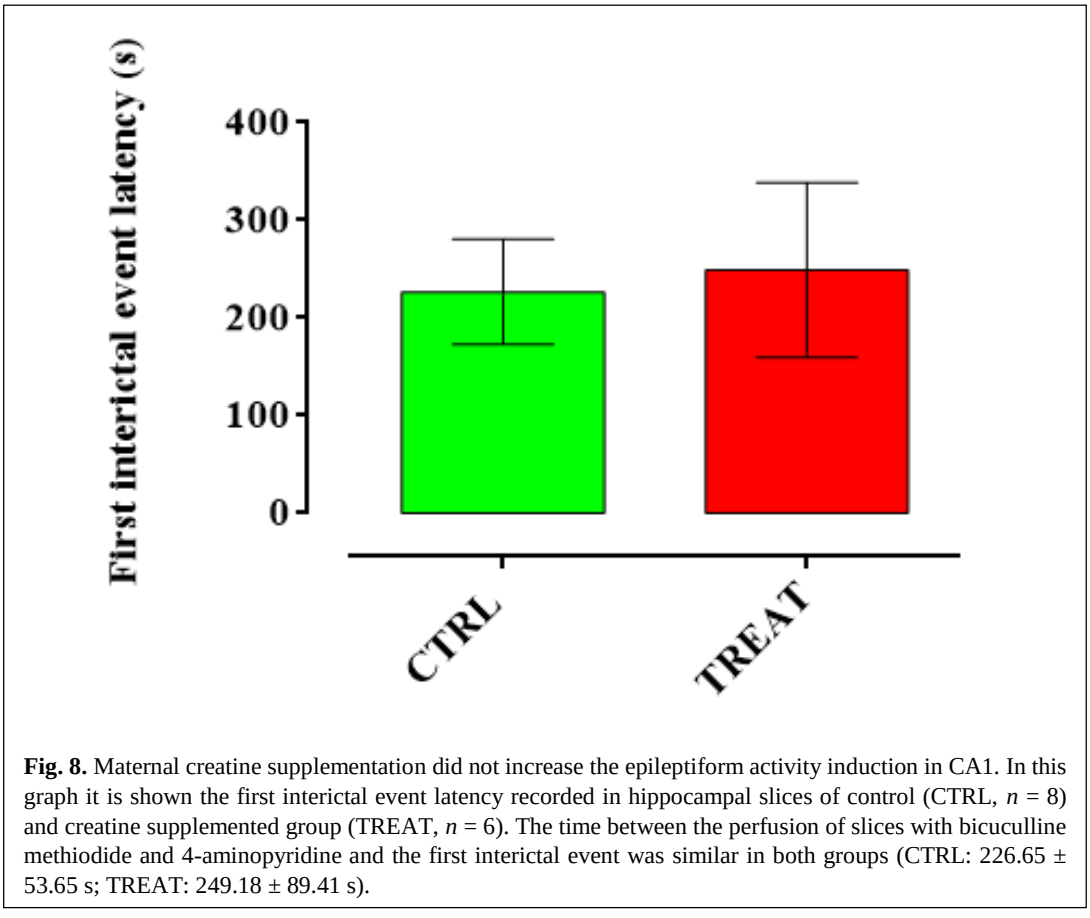

Fig. 8. Maternal creatine supplementation did not increase the epileptiform activity induction in CA1. In this graph it is shown the first interictal event latency recorded in hippocampal slices of control (CTRL, n = 8) and creatine supplemented group (TREAT, n = 6). The time between the perfusion of slices with bicuculline methiodide and 4-aminopyridine and the first interictal event was similar in both groups (CTRL: 226.65 ± 53.65 s; TREAT: 249.18 ± 89.41 s).

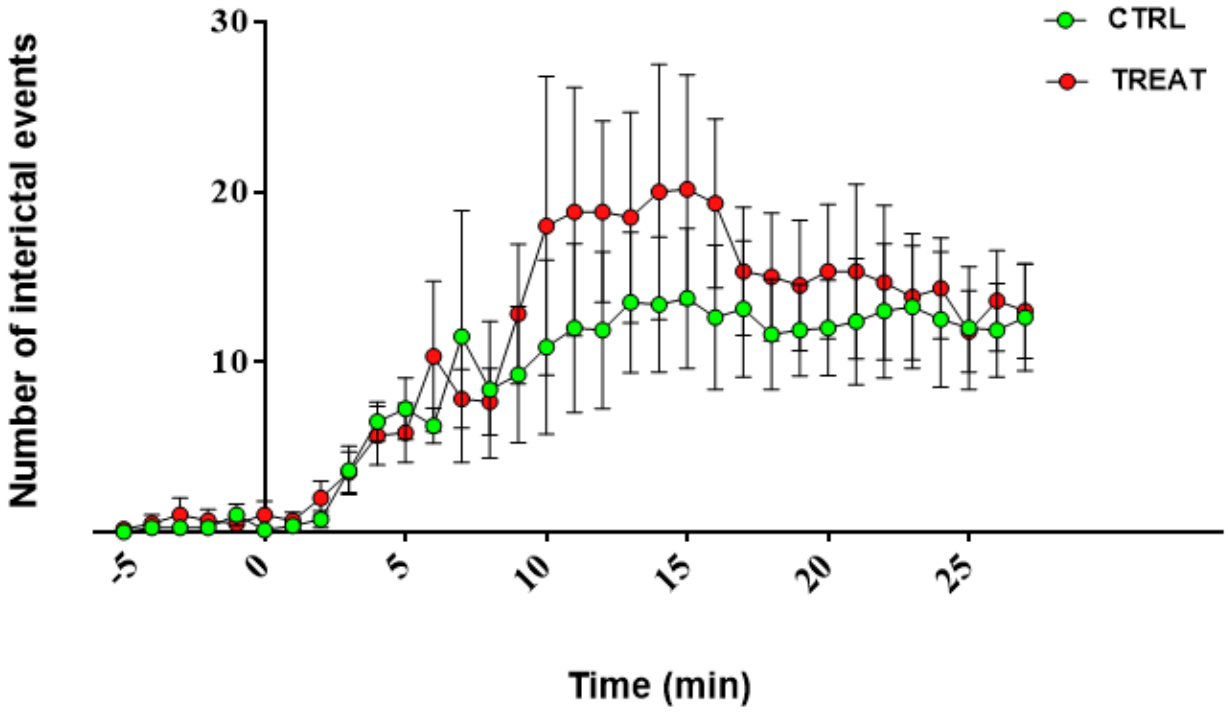

Fig. 9. Frequency of interictal events in slices perfused with bicuculline methiodide and 4-aminopyridine. Field potential recordings in hippocampal slices showed no significant difference in the number of interictal events for each minute following the perfusion with synaptic inhibition blockers between both groups. CTRL, *n* = 8 slices; TREAT, *n* = 6 slices.

-4.3.2 Whole-cell analysis at P60-70

Electrophysiological characteristics of CA1 pyramidal neurons were recorded in whole-cell at P60-70. Membrane passive properties (IR and C) and resting membrane potential (RPM) of CA1 pyramidal cells were not significantly different in the TREAT group compared to the CTRL group (Table 1).

	Number	RPM (mV)	IR (MΩ)	C (pF)
CTRL	25	-67.2 ± 2.9	140.9 ± 7.8	183.4 ± 10.4
TREAT	28	-63.1 ± 2.9	137.9 ± 8.8	171.3 ± 9.0

Table 1. Electrophysiological characteristics of CA1 pyramidal neurons: membrane passive properties (IR and C) and resting membrane potential (RPM).

Following, the neuronal firing evoked by somatic injection of increasing steps of depolarizing currents was investigated (Fig. 10A), and results demonstrated that CA1 neurons of offspring born from supplemented

rats had a greater, but not significant enhancement of action potentials frequency in comparison to the control group (Fig. 10B).

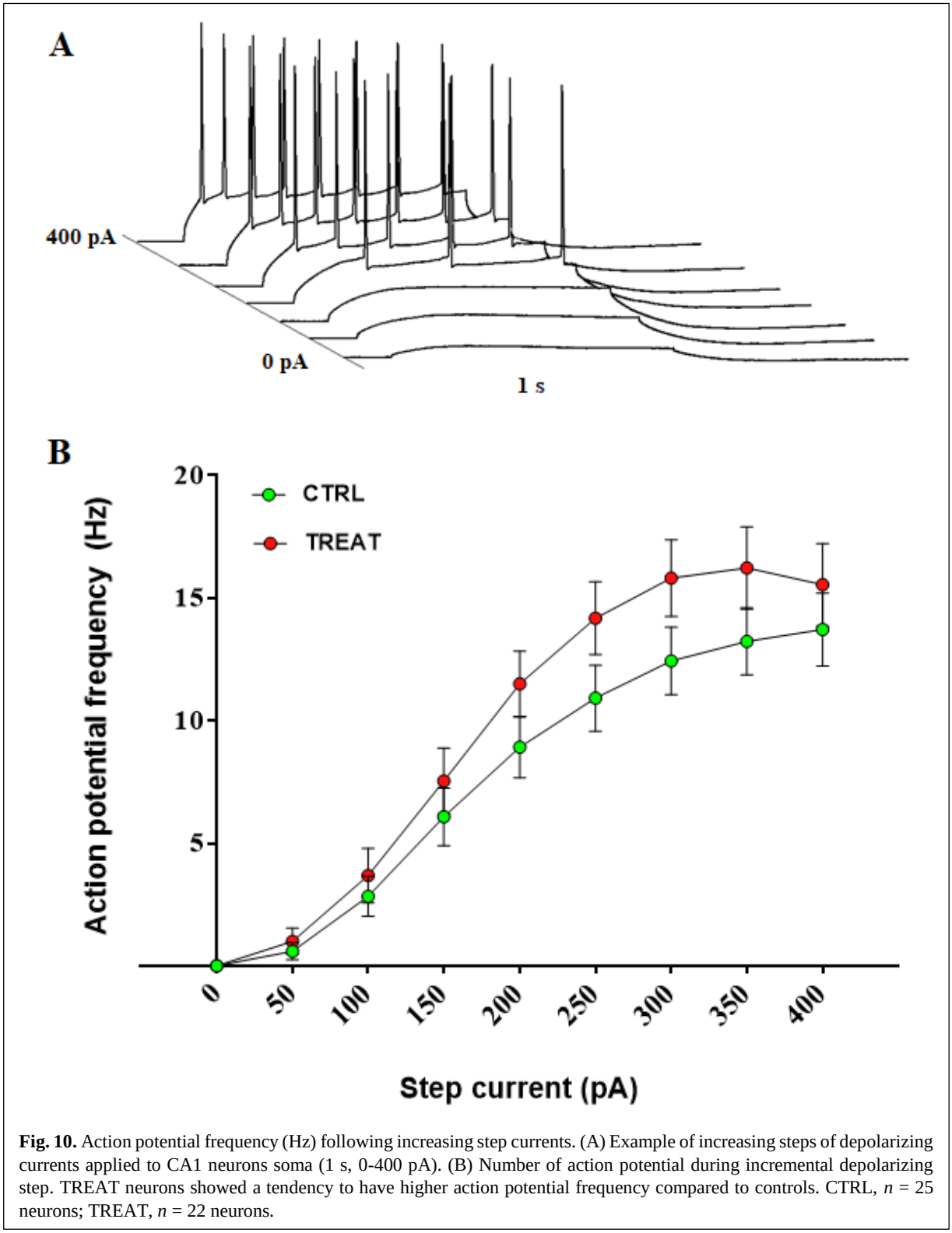

Fig. 10. Action potential frequency (Hz) following increasing step currents. (A) Example of increasing steps of depolarizing currents applied to CA1 neurons soma (1 s, 0-400 pA). (B) Number of action potential during incremental depolarizing step. TREAT neurons showed a tendency to have higher action potential frequency compared to controls. CTRL, *n* = 25 neurons; TREAT, *n* = 22 neurons.

However, although not significant, there was a tendency in TREAT cells to exhibit higher excitability during step by step incremental stimulation current, and this was confirmed by analyzing the percentage of neurons that expressed action potentials at different current injections (Table 2). In particular, using a physiological range of stimulation (150 pA), TREAT neurons showed a significantly higher probability to generate action potentials (91 vs. 64%, $p<0.05$, Table 2). In addition, the complete excitability (100% of neurons expressing action potentials) was reached using a lower step of current intensity in Cr treated group (200 pA vs. 350 pA, Table 2).

pA	**% CTRL**	**% TREAT**
0	0	0
50	12	18
100	44	50
150	64	91 *p<0.05
200	88	100
250	92	100
300	96	100
350	100	100

Table 2. Percentage of neurons that expressed action potentials at different current injections. Higher number of TREAT neurons showed action potentials after a physiological range of stimulation (91 vs. 64%, *Student's *t*-test: $p < 0.05$) and reached complete excitability at lower step of current intensity (CTRL: 350 pA; TREAT: 200 pA).

Then, analyzing the input resistance (IR) in these cells, it could be observed that neurons from both groups with higher IR generated action potentials with lower stimulation intensity (<150 pA). Interestingly, neurons from CTRL group generating action potentials with current steps greater that 150pA showed a significantly lower IR compared to the CTRL neurons generating action potentials with less amount of stimulation (164.91 ± 10.78 MΩ vs 122.11 ± 8.12 MΩ, $p<0.05$, Fig. 11), while there was no difference in the TREAT group, that showed tendentially higher IR with stimulation higher than 150 pA compared to the control group (Fig. 11). This might explain why lower stimulations were sufficient to excite the 100% of TREAT cells compared to control.

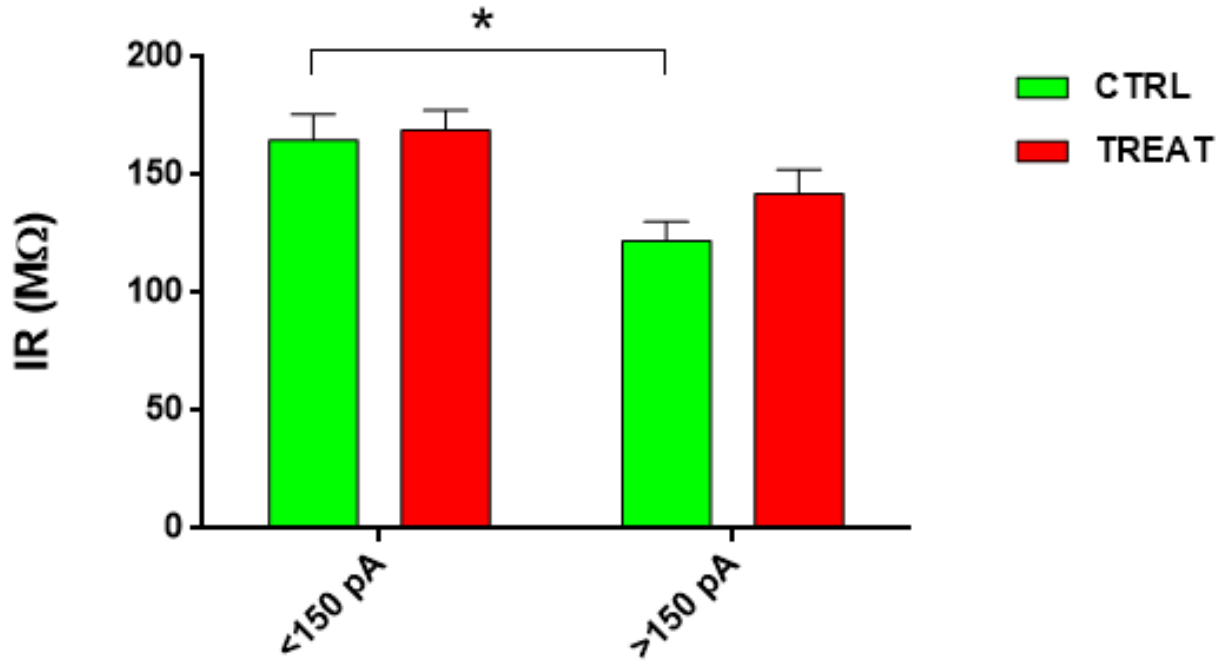

Fig. 11. Input resistance (IR) in CA1 neurons at different current injections. IR was higher in neurons that generated action potentials at lower stimulation intensity (<150 pA) compared to stimulation greater than 150 pA in both groups. Significant difference was observed in CTRL group, between neurons that generated action potentials at current step lower than 150 pA and neurons that generated action potentials with higher stimulation (164.91 ± 10.78 MΩ vs. 122.11 ± 8.12 MΩ, Two-way ANOVA, *Sidack's *post hoc*: $p < 0.05$). CTRL, $n = 25$ neurons; TREAT, $n = 22$ neurons.

Examining the first action potential elicited by incremental current injections, the action potential waveform was similar in both groups (Fig. 12A). This was confirmed by no significant differences in characteristic measures of the action potential, namely the action potential threshold, half-width, rate of rise and T90% (Table 3). However, cells from the TREAT group showed a significantly reduced action potential amplitude (110.2 ± 3.9 vs. 118.6 ± 2.4 mV, $p < 0.05$, Fig. 12B).

	Number	Threshold (mV)	Amplitude (mV)	Half-Width (ms)	Rate of Rise (mV/ms)	T90% (ms)
CTRL	25	54.0 ± 1.5	118.6 ± 2.4	1.7 ±0.1	310.5 ± 15.8	2.6 ± 0.1
TREAT	28	55.6 ± 1.3	110.2 ± 3.9	1.8 ± 0.0	269 ± 19.0	2.6 ± 0.1

Table 3. Action potential measures. There were no differences in threshold (mV), half-width (ms), rate of rise (mV/ms), and T90% (ms) between CTRL and TREAT neurons.

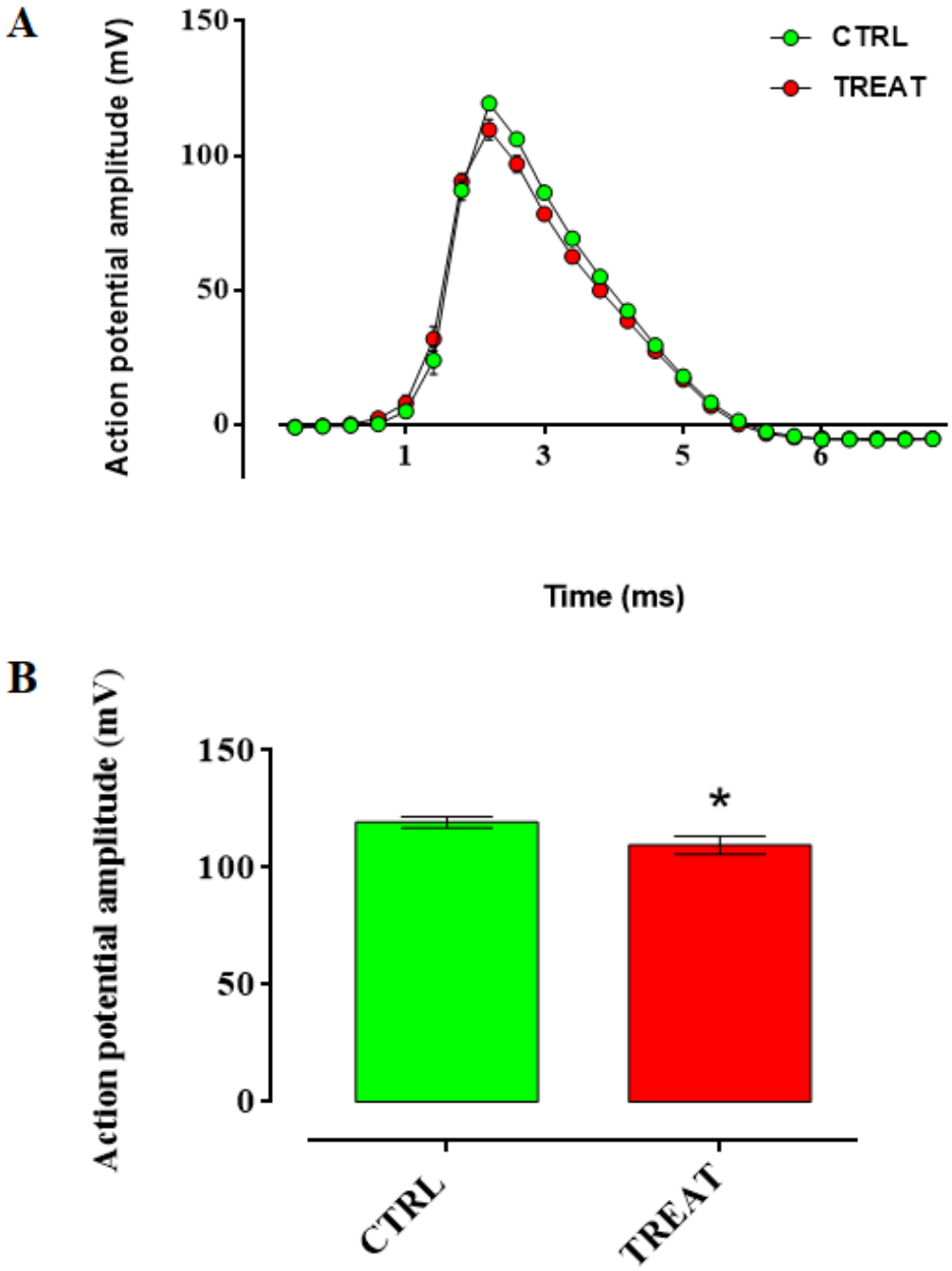

Fig. 12. First action potential elicited by incremental current injections. (A) Action potential waveform similar in both groups. (B) Action potential amplitude significantly lower in TREAT neurons compared to CTRL neurons. Values are expressed as mean ± SEM. *Student's *t*-test: $p < 0.05$. CTRL, $n = 25$ neurons; TREAT, $n = 28$ neurons.

Furthermore, we verified how many neurons showed AHP at maximum action potential frequency. As shown in Fig. 13B, greater number of TREAT cells exhibited AHP compared to CTRL group (12 vs. 48%, *p < 0.05).

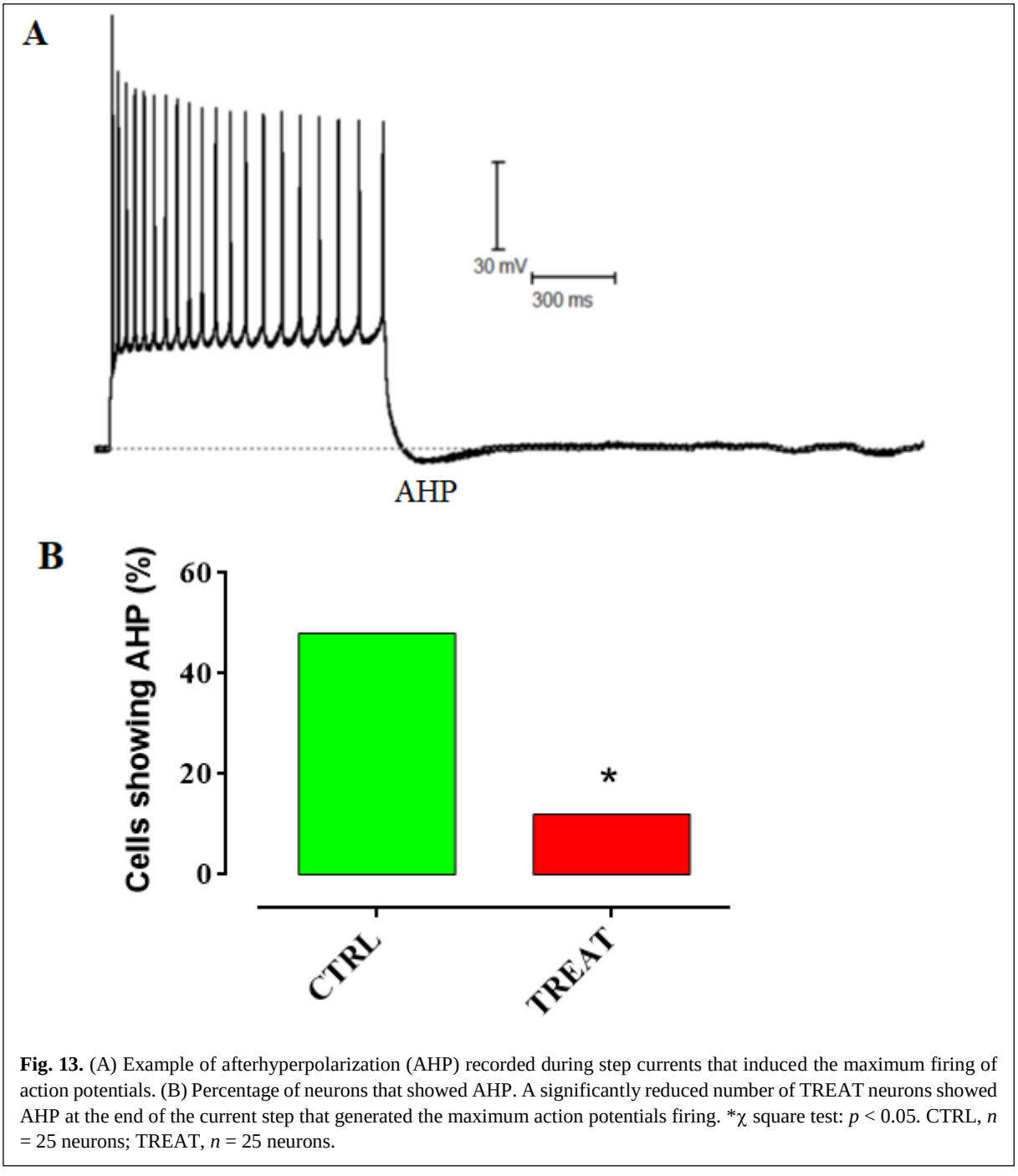

Fig. 13. (A) Example of afterhyperpolarization (AHP) recorded during step currents that induced the maximum firing of action potentials. (B) Percentage of neurons that showed AHP. A significantly reduced number of TREAT neurons showed AHP at the end of the current step that generated the maximum action potentials firing. *χ square test: $p < 0.05$. CTRL, n = 25 neurons; TREAT, n = 25 neurons.

Moreover, evoked synaptic response (excitatory postsynaptic potential, EPSP, and inhibitory postsynaptic potential, IPSP) amplitude were not significantly different between CTRL and TREAT group (Fig. 14). However, it is important to note that TREAT group tended to have a lower EPSP amplitude (p = 0.08) compared to control, suggesting a lower synaptic response. In addition, triple pulse facilitation was similar in both group (Fig. 15), suggesting that the probability of neurotransmitter release from synaptic terminals was not affected by maternal Cr supplementation. Taking together, these results showed a reduced basal synaptic transmission in the TREAT group compared to control.

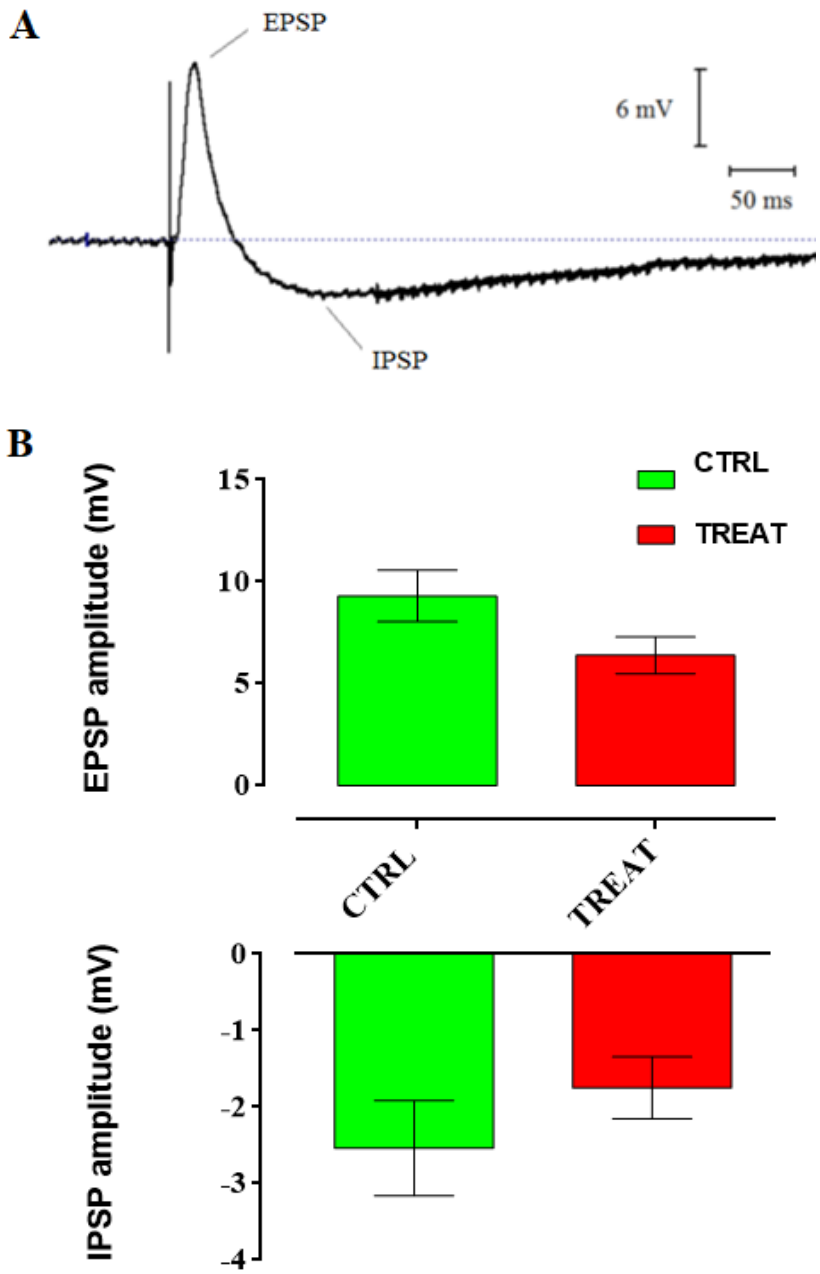

Fig. 14. Postsynaptic potential (ESPS and IPSP) amplitude. (A) Example of postsynaptic response recorded after stimulation of Schaffer collaterals. (B) EPSP and IPS amplitude. TREAT neurons tend to have reduced EPSP amplitude. Student's *t*-test: $p = 0.08$). CTRL, $n = 22$ neurons; TREAT, $n = 17$ neurons.

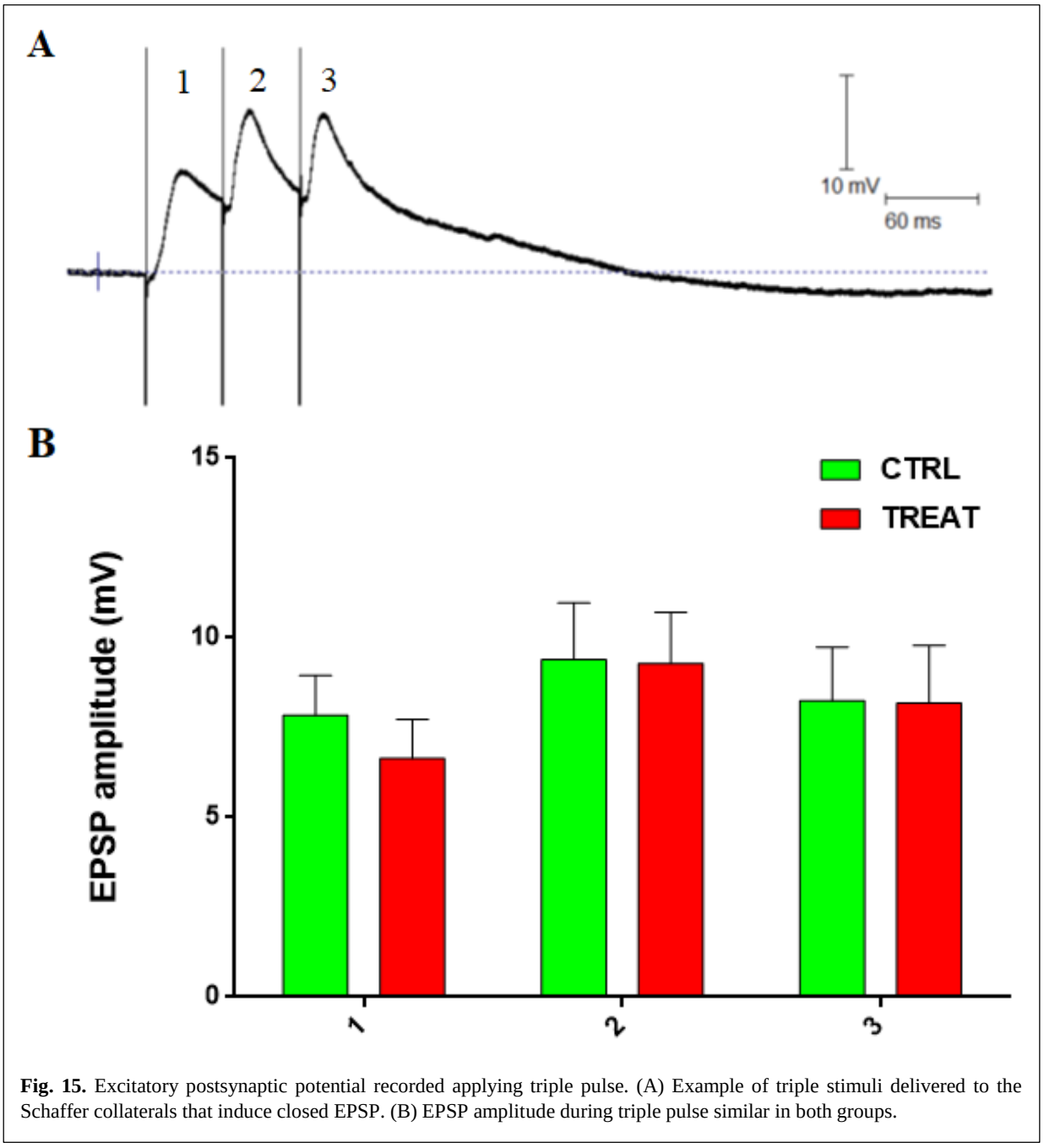

Fig. 15. Excitatory postsynaptic potential recorded applying triple pulse. (A) Example of triple stimuli delivered to the Schaffer collaterals that induce closed EPSP. (B) EPSP amplitude during triple pulse similar in both groups.

4.3.3 Field potential recordings at P60-70

High frequency stimulation (HFS) of the Schaffer collaterals, applied after a 15 minutes baseline, induced a robust LTP in both groups. In slices from TREAT adult rats, after an initial depression, the fEPSP was increased and stabilized at higher values in comparison to the control group at least up to 40 min post HFS, suggesting an enhanced LTP maintenance (Fig. 16). This finding indicates that Cr supplementation during fetal life positively affected LTP of CA1 neurons of adult rats, confirming the results previously obtained in pups at P14-21 (Sartini et al. 2016).

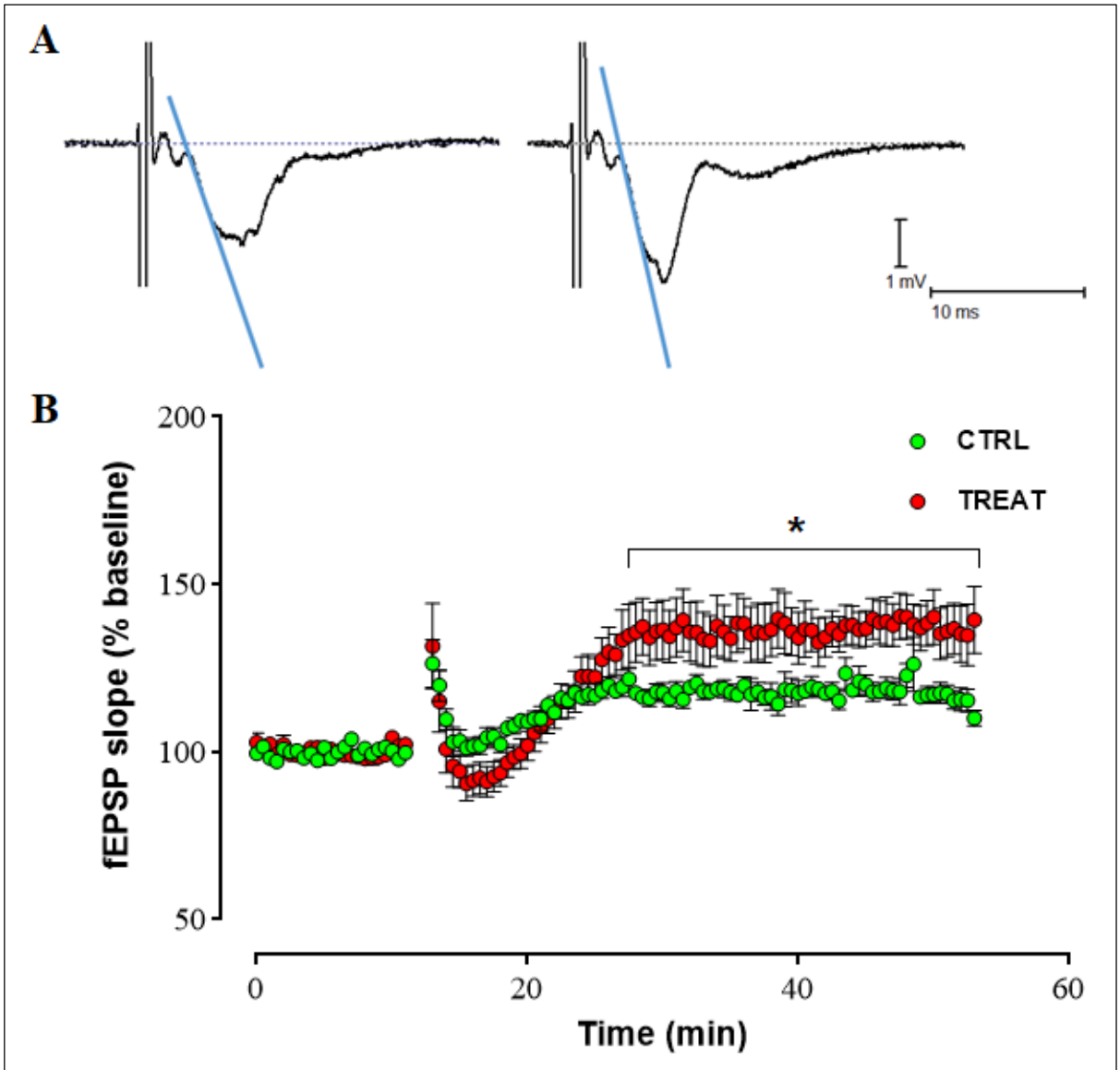

Fig. 16. Maternal creatine supplementation is associated with LTP improvement in CA1 neurons of adult rats. (A) fEPSP recorded during baseline and after high frequency stimulation; (▬) Slopes of fEPSPs. (B) Slope (between 10% and 80% of max) of the fEPSP was analyzed as measures of synaptic strength; values were normalized to the mean value obtained over the last 15 minutes of baseline period and express as a percentage of this baseline value. TREAT neurons exhibited a greater LTP compared to controls; Two-way ANOVA: $F(1,25) = 4.886$, $p = 0.037$. CTRL, $n = 11$ slices; TREAT, $n = 16$ slices.

Then, postsynaptic excitability was tested by input/output curves, in which the fEPSP slopes were measured in response to single electrical stimuli of increasing magnitude. In Fig. 17, the ratio between the amplitude of the presynaptic fiber volley (input) and the slope of the postsynaptic fEPSPs (output) were plotted. It can be observed that neurons of TREAT group showed a significantly higher fiber volley amplitude and a lower fEPSP slope compared to the CTRL neurons, suggesting a greater number of activated synapses but with reduced output intensity.

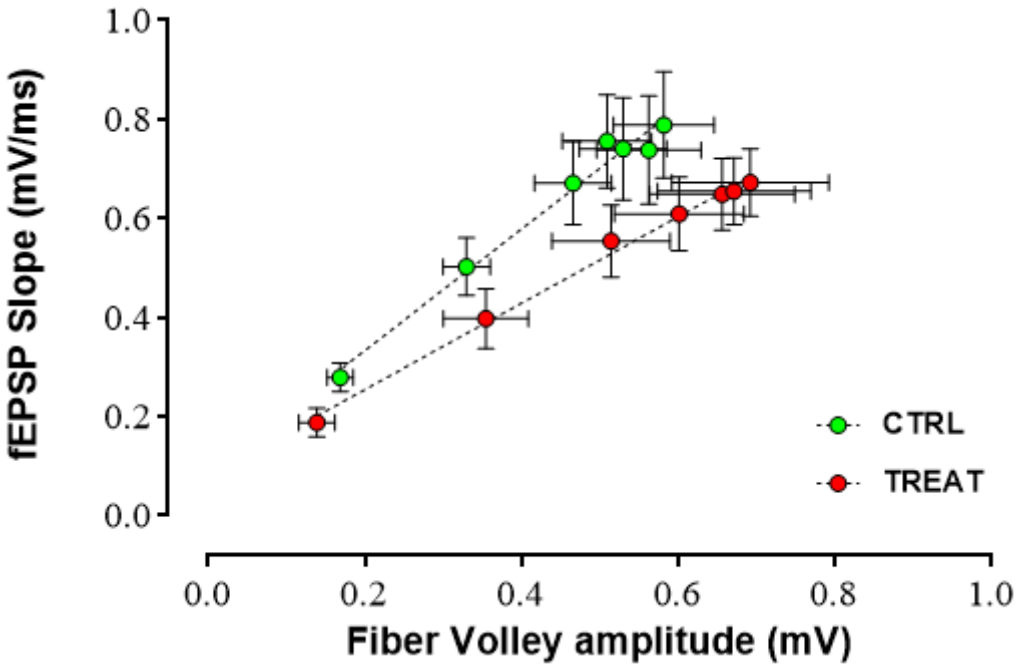

Fig. 17. Input/output curves. The fEPSP slopes vs. increasing presynaptic fiber volley amplitudes were plotted. Data were fit to a linear regression. The slope obtained in rats born by dams supplemented with creatine (0.87 ± 0.08, $r^2 = 0.99$) was significantly different ($p < 0.01$) compared to control rats (1.23 ± 0.09, $r^2 = 0.98$). CTRL, $n = 11$ slices; TREAT, $n = 16$ slices.

4.3.4 Morphological analysis

In the previous study, we found that maternal Cr supplementation affects cell morphology in rats at P14-21 (Sartini et al. 2016). In particular, CA1 pyramidal neurons filled with biocytin during whole-cell recordings from TREAT rats showed greater total dendritic length and global complexity of dendritic arborization. In order to evaluate whether these morphological modifications were maintained in adult life, dendritic length and complexity of dendritic arborization were analyzed also at P60-70 (two months after the end of Cr treatment). Results showed no differences in apical, basal, and total dendritic lengths between both groups. Moreover, Sholl analysis revealed that the number of basal and apical dendritic crossings along Sholl rings was similar in CA1 pyramidal cells of the two groups, suggesting that the morphological changes found at P14-21 are attenuated in adulthood.

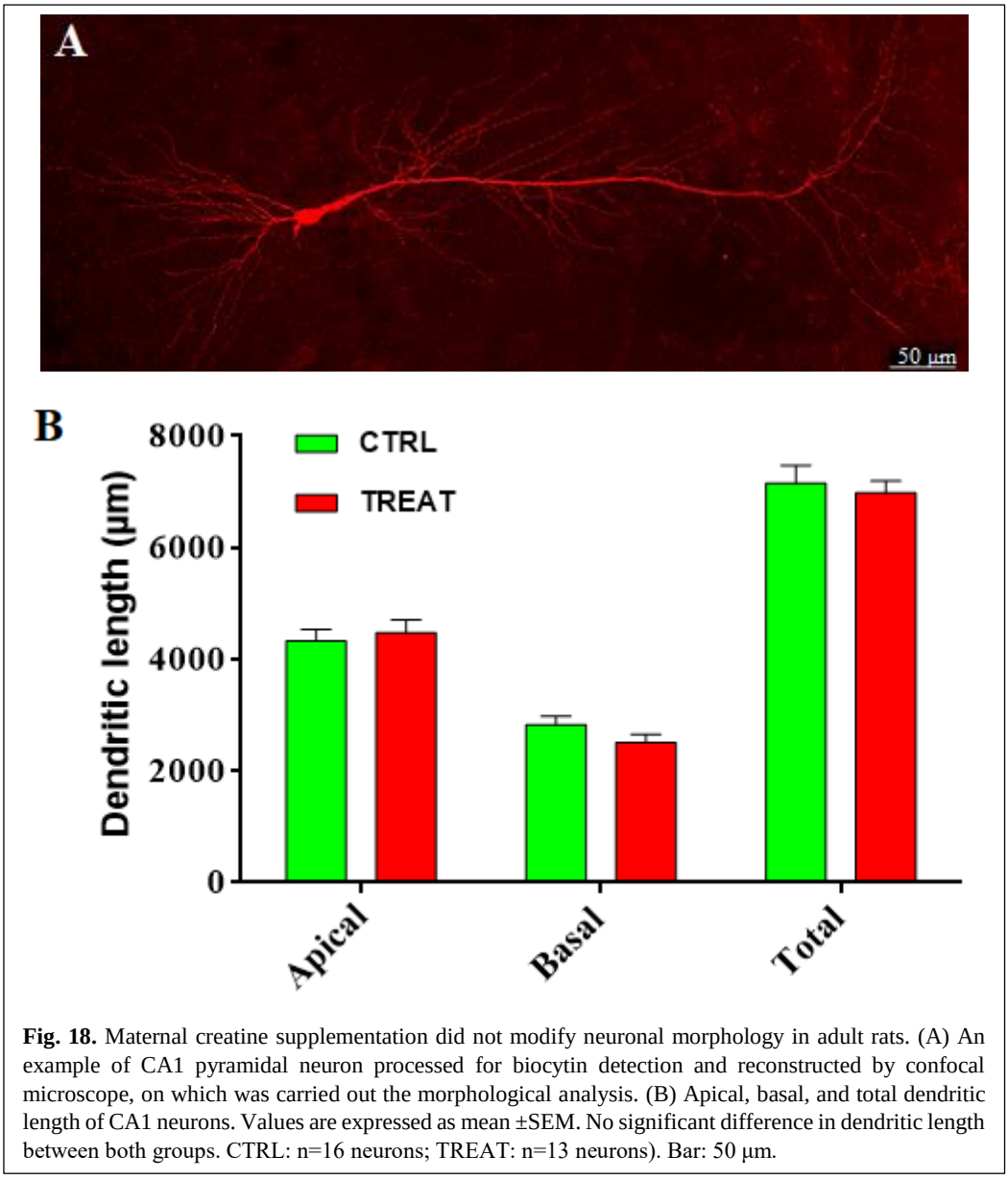

Fig. 18. Maternal creatine supplementation did not modify neuronal morphology in adult rats. (A) An example of CA1 pyramidal neuron processed for biocytin detection and reconstructed by confocal microscope, on which was carried out the morphological analysis. (B) Apical, basal, and total dendritic length of CA1 neurons. Values are expressed as mean ±SEM. No significant difference in dendritic length between both groups. CTRL: n=16 neurons; TREAT: n=13 neurons). Bar: 50 µm.

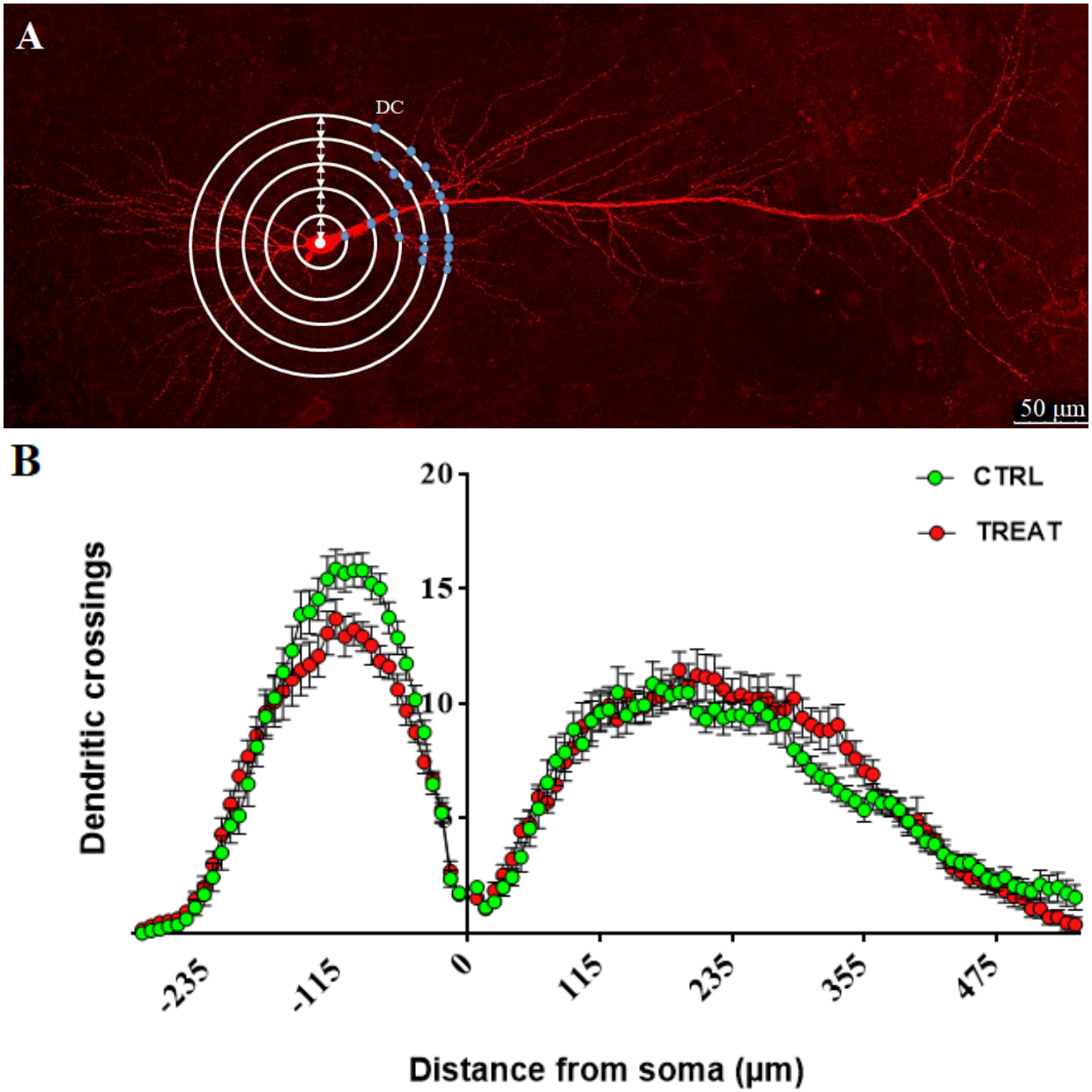

Fig. 19. Maternal creatine supplementation did not affect neuronal morphology in adult rats. (A) An example of CA1 pyramidal neuron processed for biocytin detection and reconstructed by confocal microscope, on which concentric Scholl rings from the soma and dendritic crossings (DC) were drawn; (B) Scholl analysis plot showing numbers of dendritic crossings along the Scholl rings as a function of distance from soma. Distributions of both basal and apical dendritic crossings did not result in significant differences between the two groups using Two-way ANOVA (basal: $F(1,27) = 1.662$, $p = 0.21$; apical: $F(1,27) = 0.4699$, $p = 0.50$). CTRL: n=16 neurons; TREAT: n=13 neurons). Bar: 50 µm.

4.3.5 Morris Water Maze

Analysis of data recorded during performances of CTRL and TREAT rats tested for spatial learning were collected in Fig. 20, Fig. 21. The time elapsed and the distance travelled to reach the hidden platform decreased over the sessions in both groups, indicating that the rats progressively became more efficient at locating the platform, thus escaping the water by learning the position of the platform relative to the visual cues (Fig. 20). However, comparing the learning curves between groups, there were no differences in the time (escape latency) and path length to reach the platform (Fig. 20).

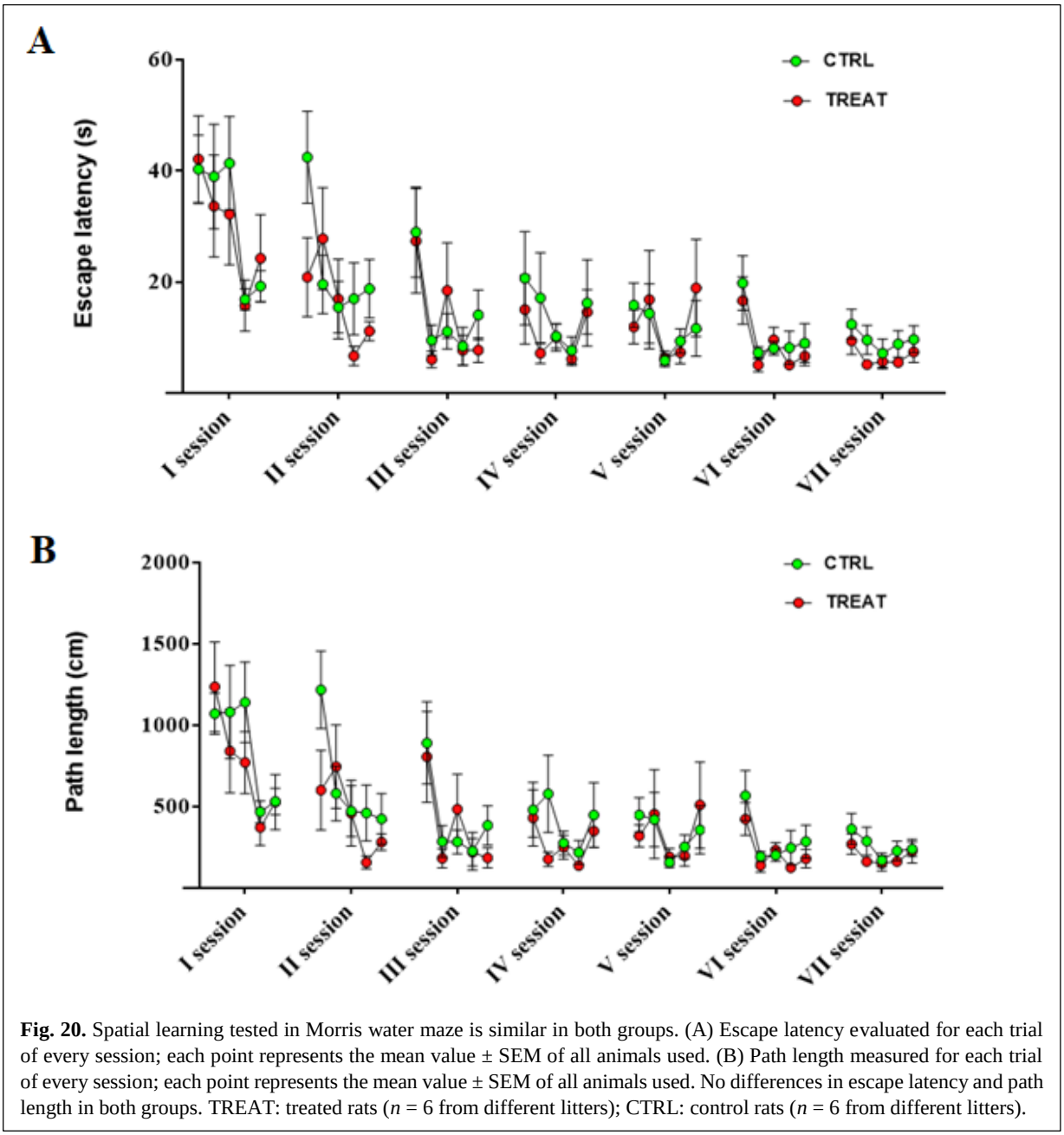

Fig. 20. Spatial learning tested in Morris water maze is similar in both groups. (A) Escape latency evaluated for each trial of every session; each point represents the mean value ± SEM of all animals used. (B) Path length measured for each trial of every session; each point represents the mean value ± SEM of all animals used. No differences in escape latency and path length in both groups. TREAT: treated rats (n = 6 from different litters); CTRL: control rats (n = 6 from different litters).

Moreover, no difference was found also in probe test, in which rats from both groups spent similar time in each quadrant, especially in the goal quadrant (5) (Fig. 21).

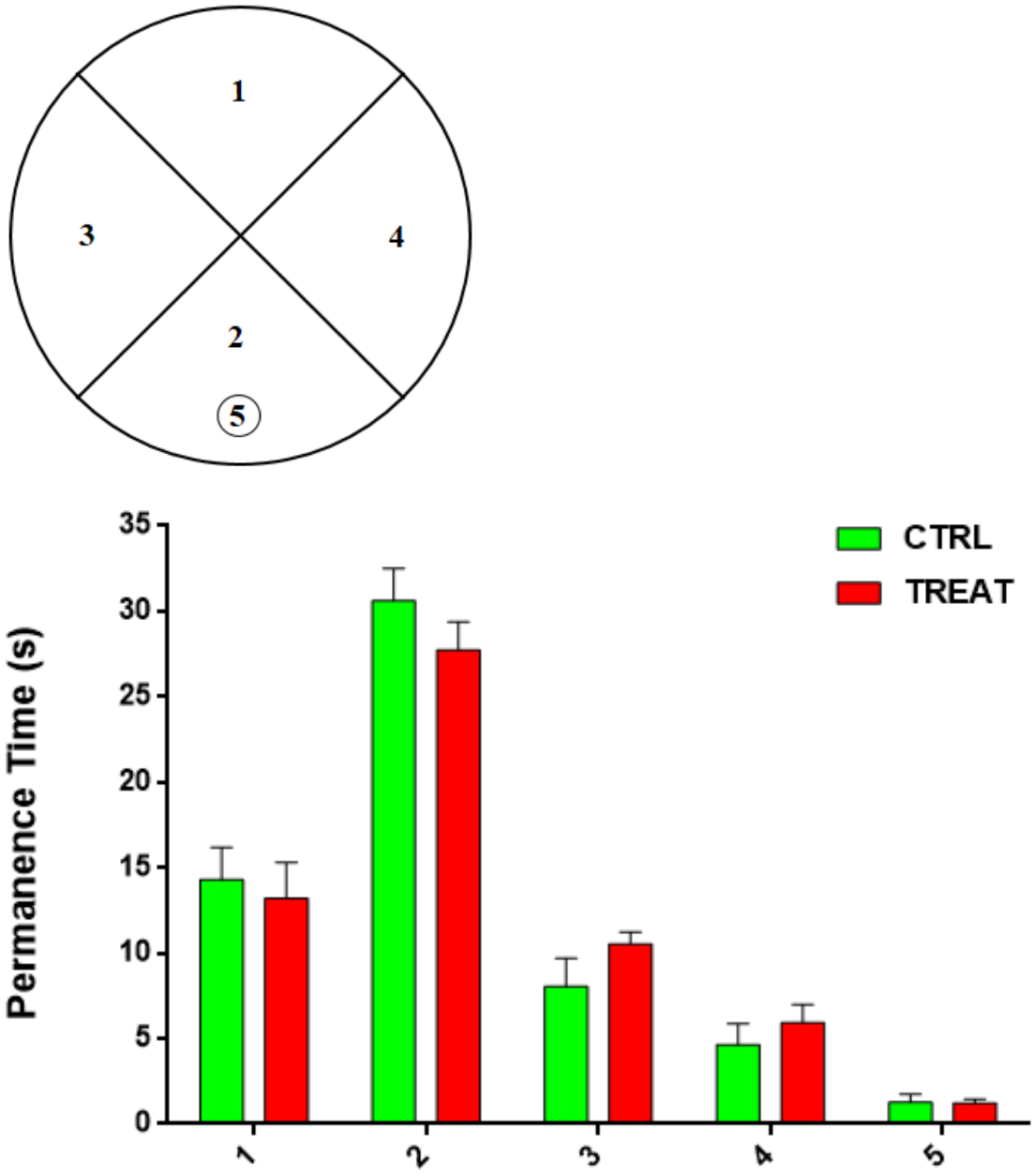

Fig. 21. Water Maze probe test, performed the day after the end of training, assessed as permanence time (s) of the animal in the goal quadrant (5), and in the other quadrants shown in the schematic draw of the pool. Values were expressed as mean ± SEM. During the one-minute probe test, adult rats from different TREAT and CTRL litters spent similar time in the goal quadrant (5) and in each quadrant. TREAT: treated rats (n = 6 from different litters); CTRL: control rats (n = 6 from different litters).

4.3.6 Intracellular calcium dynamics

No differences were found in resting Ca^{2+} concentrations ($[Ca^{2+}]_0$, endogenous total buffering capacity (Ks) (Fig. 22), and in calcium concentrations involved during a single action potential (Fig. 23A) evoked in CA1 neurons of the two groups. However, the recordings of Ca^{2+} concentration transients by Fluo-4 intensity revealed a significantly lower increase from resting values in neurons of Cr treated group, when stimulated at 20 Hz, a range of frequency easily reachable during the physiological hippocampal neuron activity (Fig. 23B).

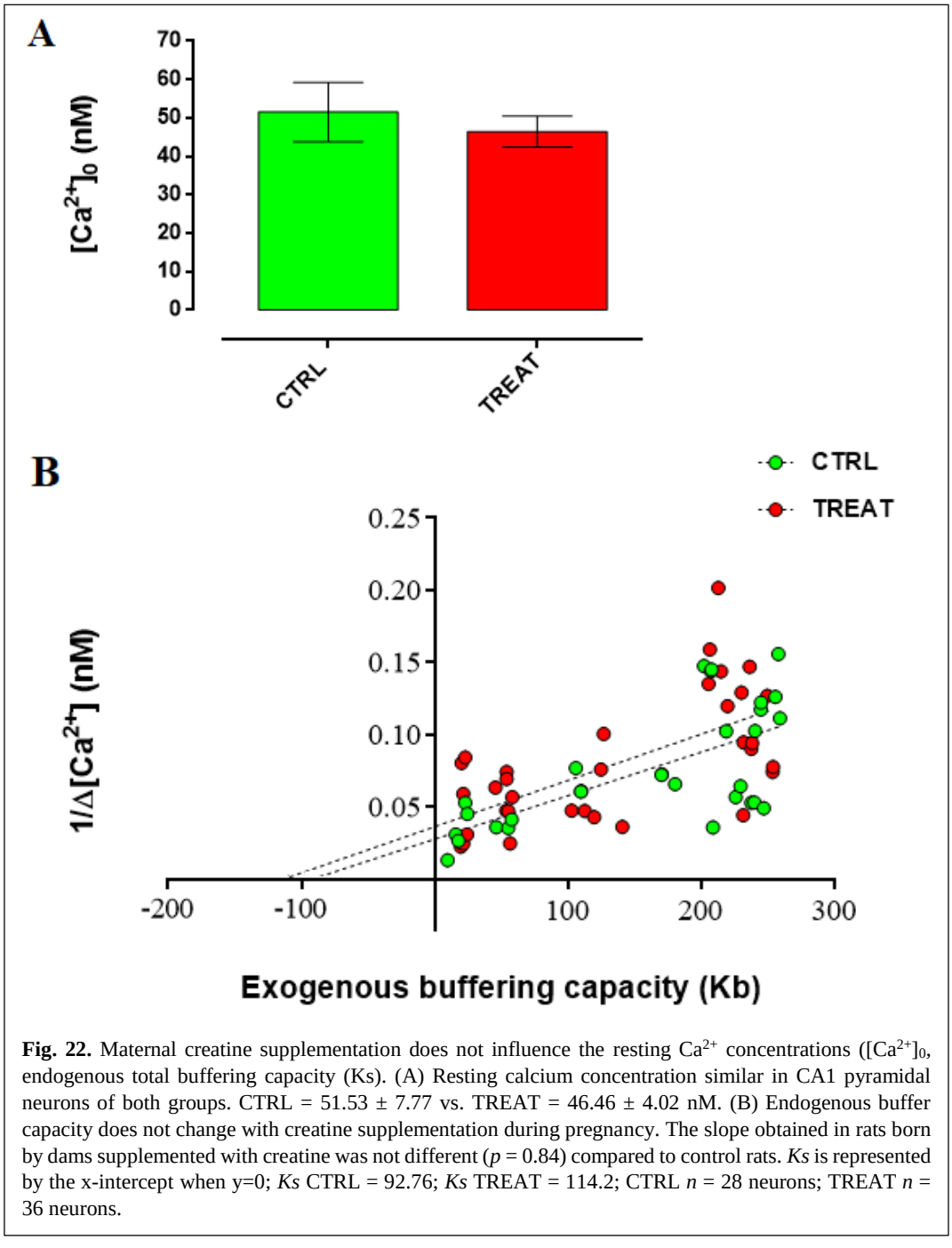

Fig. 22. Maternal creatine supplementation does not influence the resting Ca^{2+} concentrations ($[Ca^{2+}]_0$, endogenous total buffering capacity (Ks). (A) Resting calcium concentration similar in CA1 pyramidal neurons of both groups. CTRL = 51.53 ± 7.77 vs. TREAT = 46.46 ± 4.02 nM. (B) Endogenous buffer capacity does not change with creatine supplementation during pregnancy. The slope obtained in rats born by dams supplemented with creatine was not different (p = 0.84) compared to control rats. *Ks* is represented by the x-intercept when y=0; *Ks* CTRL = 92.76; *Ks* TREAT = 114.2; CTRL n = 28 neurons; TREAT n = 36 neurons.

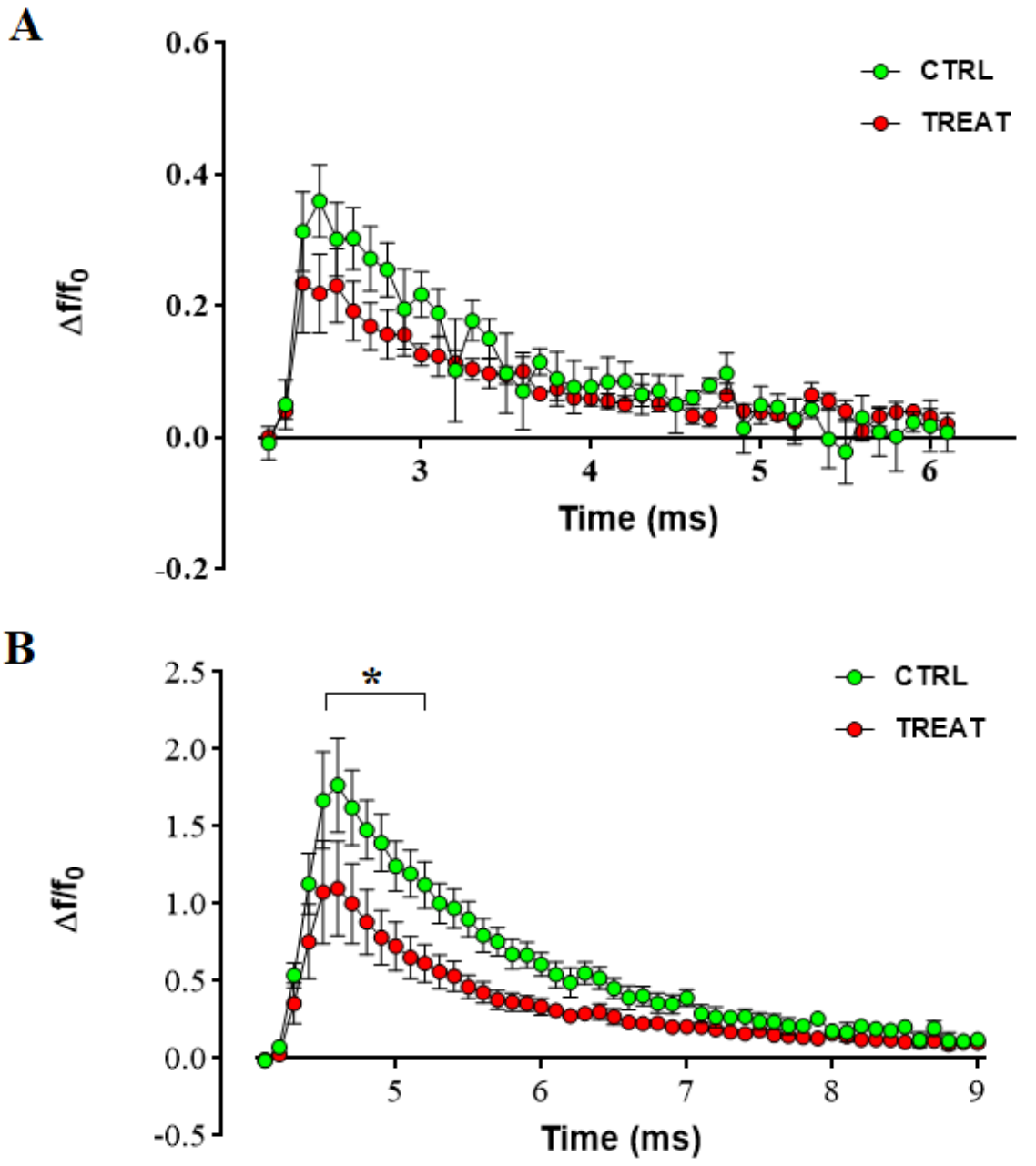

Fig. 23. Ca^{2+} concentration transient by Fluo-4 intensity during neuronal activity. (A) Ca^{2+} concentration transient during a single action potential did not differ between TREAT and CTRL neurons. (B) Maternal creatine supplementation affected calcium concentration transient elicited by 20 Hz stimulation. The ($[Ca^{2+}]_0$ increase was significantly lower in TREAT cells compared to controls. Two-way ANOVA: $F(1,11) = 4.988$, $p = 0.047$, *Sidack's *post hoc*, $p < 0.05$

4.4 Discussion

4.4.1 Epileptic Threshold

In this study, the effects of maternal Cr supplementation in CA1 pyramidal neurons of rat progeny was investigated. Due to a higher neuronal excitability and an enhanced hippocampal output found at P14-21 in previous work (Sartini et al. 2016), the risk of an epileptogenic focus has been evaluated in this study through inducing of spontaneous epileptiform-like discharges and analysis of the epileptic threshold at P14-21. This experiment was conducted because an area characterized by high excitability situated in a network in which the inhibitory charge is not completely developed (Ko et al. 2015) could enhance the probability of an epileptogenic focus establishment, especially when this condition occurs together with other inducing factors, such as high body temperature (DellaBadia et al. 2004). However, in other experimental models, although such models are not properly comparable to the design used in this study, Cr has rather been reported to be neuroprotective and to prevent seizures. Indeed various studies have shown (Ireland et al. 2008, 2011; Cannata et al. 2010; Ellery et al. 2013) that maternal Cr administration protects the fetal CNS and, in developing rats, suppresses seizures caused by hypoxia (Holtzman et al. 1998). In accordance with this evidence, our experiments demonstrated that maternal Cr supplementation does not improve the probability of epileptiform activity induction in CA1 of offspring at P14-21 after blocking of inhibition and enhancement of the excitability of the circuit. Both groups showed similar epileptic thresholds, as the latency of the first interictal event and the frequency of interictal events were not significantly different. This suggests that the higher excitability of CA1 found in the previous study at P14-21 does not lead to increased risk of an epileptogenic focus, at least when using this experimental setting.

4.4.2 Morphology of CA1 pyramidal neurons

The effects of maternal Cr supplementation on morphological features of CA1 pyramidal neurons has been evaluated in adult rat progeny (P60-70). Our previous results obtained in pups demonstrated increased neuritic tree development, including greater total dendritic length and global complexity of dendritic arborization of CA1 pyramidal neurons of Cr treated group (Sartini et al. 2016). In this study, results showed no significant differences in apical, basal, and total dendritic lengths between both groups. Furthermore, Sholl analysis revealed that the number of basal and apical dendritic crossings along the Sholl rings was similar in CA1 pyramidal cells of the two groups, suggesting that the morphological changes found at P14-21 progressively disappeared throughout the following weeks. These findings suggest that maternal Cr supplementation could anticipate the neuritic morphological development, as shown in P14-21 study, possibly because of a large availability of metabolic energy (Fukumitsu et al. 2015). This large metabolic energy would be able to favor cytoskeleton growth and dynamics, however this difference does not exist once neuron morphology has fully developed.

4.4.3 Intrinsic excitability

Functional features of CA1 pyramidal neurons have been also investigated in adult rat progeny, and, interestingly, electrophysiological results suggest that some fundamental functional changes found twenty days after birth (Sartini et al. 2016) could be detected also in adulthood, namely two months after the end of the maternal Cr supplementation. This might lead to a hypothesis that a number of mechanisms related to maternal Cr administration, which are triggered during fetal life, can be permanently operative in adult life.

First, the RMP and passive properties (IR and C) found were typical of CA1 pyramidal neurons of adult rats (Staley 1994; Milior et al. 2016) without significant differences between the two groups (Table 1). Furthermore, following somatic injection of increasing steps of depolarizing currents, CA1 neurons of offspring born from supplemented rats showed a greater, but not significant, increase of action potential frequency compared to the control group (Fig. 10). Nevertheless, in analyzing this finding from a different point of view, and thus examining the percentage of neurons that expressed action potentials at different current injection intensities (Table 2), some peculiarities can be observed. In particular, using a physiological range of stimulation (150 pA), TREAT neurons showed a significantly higher probability to generate action potentials. In addition, complete excitability (100% of neurons expressing action potentials) was reached using a lower step of current intensity in the Cr treated group (Table 2). These results confirm an enhanced excitability, with TREAT neurons seeming to be more excitable for the reason that they are able to respond to lower stimulation intensity.

The analysis of the input resistance (IR) of these cells, taking into account the Ohm law equation, $V = R \cdot I$, might clarify why lower stimulations were sufficient to excite 100% of TREAT cells compared to control cells. In fact, it could be observed that CA1 neurons from both groups with higher IR generated action potentials with lower stimulation intensity (<150 pA). Interestingly, neurons from the CTRL group that generated action potentials with current steps greater that 150pA showed significantly lower IR compared to the CTRL neurons, which generated action potentials with less intensity of stimulation, while there was no difference in the TREAT group, which tended to show higher IR with stimulation higher than 150 pA when compared to the control group (Fig. 11). Despite the fact that there was no difference in spike threshold (Table 3), the tendency of TREAT neurons to show greater IR compared to controls might suggest the existence of differences in passive ion channel expression and/or in their membrane trafficking, with the potential ability to increase membrane resistance and then the excitability of these neurons.

4.4.4 Long Term Potentiation and Calcium Dynamics

Considering the higher excitability found in whole-cell recordings, the CA3-CA1 pathway was studied. High frequency stimulation of Schaffer collaterals induced an increased LTP at P60-70 in CA1 neurons of the Cr treated group in comparison to controls (Fig. 16), as in previous study at P14-21 (Sartini et al. 2016). Furthermore, only a small percentage of these neurons showed AHP following maximum action potential frequency (medium afterhyperpolarization; King et al. 2015; Fig. 13). Regarding this AHP, however,

it is important to clarify that it was not possible to analyze the common measures of AHP, namely the amplitude and the area, because only three TREAT neurons showed AHP. The higher LTP in the TREAT group might be explained by a combination between presynaptic and postsynaptic activity. In particular, even if maternal Cr supplementation does not increase the presynaptic probability of neurotransmitter release as shown in whole-cell recordings using the triple-pulse stimulation, it enhances the number of synapses, although more weakly activated, as shown in the fiber volley amplitude/fEPSP ratio (Fig. 17). Thus, a greater number of synapses characterized by lower strength in the TREAT group, together with a similar presynaptic probability of neurotransmitter release, might explain the increased LTP through greater synapsis associativity and cooperativity, which are conditions that typically trigger LTP in hebbian CA3-CA1 synapses. Furthermore, TREAT neurons showing higher IR (Fig. 11) favor generation of action potentials, suggesting an enhancement of postsynaptic activity.

It is well known that inhibition of Ca^{2+} transients generated by the activity of voltage-gated L-channel (Disterhoft et al. 1996; Kumar and Foster 2002) and/or from intracellular stores (Kumar and Foster 2004) is able to reduce AHP via small-conductance Ca^{2+}-gated K^+ (SK) channels (Adelman et al. 2012; Berkefeld et al. 2010). Since LTP induction is facilitated by AHP reduction, and AHP might be related to different calcium handling (Kumar and Foster, 2004), calcium homeostasis in these cells was measured. Specifically measurements were taken of resting Ca^{2+} concentration, endogenous total buffering capacity, and Ca^{2+} transient during single action potential and during firing. No differences were found in resting Ca^{2+} concentrations and endogenous total buffering capacity (Fig. 22). However, in the Cr treated group, a tendency to develop lower Ca^{2+} transients during single action potentials was recorded; this tendency becomes significantly different when stimulated at 20 Hz, a range of frequency easily reachable during physiological hippocampal neuron activity, confirming the results of the literature. On the other hand, this lower Ca^{2+} transient intensity could be also a sufficient condition to explain the lower action potential amplitude of the same group (Fig. 5), as well as the lower AHP.

4.4.5 Spatial learning

Although underlying mechanisms are poorly understood, early life events producing subtle modifications in brain maturation can induce persistent behavioral and cognitive problems (Cannon et al. 2002), and the hippocampus, given its long-lasting period of maturation, may be especially vulnerable to early life events, such as stress (Fenoglio et al. 2006) or prenatal malnutrition (Morgane et al 2002). Thus, maternal Cr supplementation might affect some behavioral and cognitive tasks in the progeny. Considering that hippocampal LTP is thought to be one of the main mechanisms underlying learning processes, such as spatial learning, the Morris Water Maze, which is one of the hippocampus-dependent spatial learning tests for rodents, was used in this study. Adult rats of both groups showed similar performances in the Morris water maze; both groups of rats did acquire the task at the end of training because the time elapsed and the distance travelled to reach the hidden platform decreased over the sessions. However, there were no differences in time taken

(escape latency) or total path length to reach the platform (Fig. 20), which was also the case in the probe test (Fig. 21). These behavioral results are not consistent with the enhanced LTP found in the TREAT group and this might be due to various reasons. Firstly, the low number of animals used in the study (n=6 for each group) might have been partly responsible for the elevated variability found in both groups, although this amount was chosen to limit the use of animals in order to conform with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Furthermore, spatial learning might depend not only upon LTP but also on other mechanisms (McNamara and Skelton 1993). Finally, even though several authors have suggested that hippocampal LTP might be the neuronal basis for hippocampus-dependent learning, this was not proven in a conclusive manner (D'Hooge and De Deyn 2001). Indeed, some studies demonstrated that Morris Water Maze learning was still possible in animals where LTP mechanisms were blocked, and that Morris Water Maze performance can be impaired when LTP is increased (Cain 1997).

4.4.6 Possible mechanisms

It is interesting to consider that the majority of these findings at P60-70 imply direct or indirect ion-channel modulation by maternal Cr supplementation, likely initiated started during fetal life, and that the effects can be conserved long after birth. Mechanisms involved in this scenario are not the target of this thesis, and as such only speculations can be made in this regard. A number of previous *in vitro* and *in vivo* studies have reported the ability of Cr to modulate neurotransmitter receptors and ion-channels. For example, the activity of brain $GABA_A$ receptor is increased by intracerebroventricular Cr injection (Koga et al. 2005) and, in addition, Cr is known to interact with benzodiazepine site of these receptors (Ireland et al. 2011). Furthermore, the activation of adenosine A1 and A2 receptors by Cr provides an antidepressant effect (Cunha et al. 2015). Regarding ion channel modulation, Cr supplementation *in vitro* was seen to increase Na^+ and K^+ currents in spinal neurons (Sartini et al. 2012), possibly as a consequence of enhanced voltage gated ion-channel expression or membrane translocation, and to also accelerate the expression of the mature form of voltage-gated ion channels in rat CA1 neurons (Sartini et al. 2016). In this context it is also important to consider that neuron gene expression can be influenced by Cr via BDNF modulation (Allen et al. 2015) and that BDNF/TrKb signaling was shown to regulate ion channel membrane trafficking *via* phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB/Akt) cascade (Duan et al. 2012). Considering all of this information together, results in this study suggest that Cr supplementation during fetal life, a critical period for neuronal development, can affect rat CA1 neuron features, potentiating excitability and LTP for an extensive duration, weeks after the termination of supplementation.

4.5 Conclusion

Results from this experiment show that maternal creatine supplementation positively affects the morpho-functional features of CA1 pyramidal neuron of rat offspring. Some morpho-functional changes found in the creatine treated group in comparison to controls suggest an anticipation of neural development; other changes are maintained also in adulthood, two months after maternal creatine supplementation. In detail, the morphological modifications found two-three weeks after birth progressively disappear throughout the following weeks, suggesting that maternal creatine supplementation may anticipate the neuritic morphological development. Once fully developed, CA1 pyramidal neurons are similar in hippocampus of adult rats born from both supplemented and control dams. Electrophysiological results suggest that some fundamental functional changes found two-three weeks after birth (Sartini et al. 2016) could be identified also in adulthood. Thus, some mechanisms triggered during fetal life by maternal creatine administration can be permanently operative in adult life. It is this the case of the LTP. In fact, CA3-CA1 pathway of TREAT group shows a higher LTP maintenance in comparison to controls, likely related to a lower calcium transient during neural activity. This is an interesting finding considering that LTP is one of the main mechanisms underlying learning processes, thus, maternal Cr supplementation might improve behavioral and cognitive task in rat progeny through these permanent modifications. Furthermore, in our experimental setting Cr supplementation during pregnancy does not cause side effects in neonatal rats, because, despite of the higher excitability of CA1 found in the previous study (Sartini et al. 2016), it does not improve the risk of epileptiform activity induction in hippocampus of offspring at postnatal day 14-21. This finding might be encouraging to future human studies and to a possible use of Cr during pregnancy to prevent and treat CNS damage in case of pre-, peri-, and post-natal hypoxic-ischemic events caused by premature birth or placental insufficiency (Dickinson et al. 2014a; Ellery et al. 2016).

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