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Research Report

Mitochondrial function and nitric oxide production in hippocampus and cerebral cortex of rats exposed to enriched environment

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ABSTRACT

Male rats (21 days) were assigned to enriched environment (EE) or to standard environment (SE) for 1 year. Oxygen consumption and the sensitivity to calcium induced mitochondrial permeability transition (MPT), through mitochondrial membrane potential ($\Delta\Psi_m$) and swelling, were determined in isolated hippocampal and cerebral cortex mitochondria. Mitochondrial H_2O_2 production rate, and NOS activity and expression associated with mitochondrial membranes were also assayed. Results showed that state 3 respiratory rate was increased by 80% in cerebral cortex mitochondria from EE rats and no changes were observed in hippocampal mitochondria after EE exposure. Calcium induced-swelling was 40% and 53% lower in hippocampal and cerebral cortex mitochondria from EE rats, as compared with SE rats. Calcium loading induced membrane depolarization in cerebral cortex mitochondria from EE rats but did not affect mitochondrial $\Delta\Psi_m$ in hippocampal mitochondria from EE animals, probably due to decreased H_2O_2 formation. NO production associated to mitochondrial membranes was increased by 195% in cerebral cortex mitochondria but decreased by 47% in hippocampal mitochondria from EE rats, as compared with SE rats. Western blot analysis from nNOS protein expression associated to mitochondrial samples revealed a similar pattern. Our results suggest that in hippocampus and cerebral cortex, EE exposure protects mitochondria against calcium-induced MPT maintaining a convenient membrane potential, which assures a continuous energy supply.

1. Introduction

Exposure to enriched environments has many positive effects on brain structure and function, including increased numbers of dendritic branches and spines, enlargement of synapses, increased glial numbers and improved performance in tests of spatial memory (Rosenzweig and Bennett, 1996).

Morphological, neurochemical and behavioural changes have been observed in the prefrontal cortex and the limbic areas in animals housed in an enriched environment. It has been

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observed that animals living in an enriched environment show an increased number of dopamine transporters in the prefrontal cortex. Also, changes in dendritic branching and spine density, and glutamate and GABA extracellular concentrations have been observed in hippocampus and/or nucleus accumbens, in animals living in enriched environment. It has been hypothesized that environmental enrichment can change the functional interactions between the prefrontal cortex and limbic areas through primary changes in the prefrontal cortex (Del Arco and Mora, 2009). Moreover, exposure of adult rodents to increased environmental complexity has been shown to induce neurogenesis in the hippocampus (Kempermann et al., 1998).

Nitric oxide (NO) is a small signal molecule that can act at the central nervous system as neurotransmitter and neuromodulator. NO has been proposed to act as a retrograde messenger in an increasing number of experimental models of plasticity, in the formation of long term memory and in spatial learning in rats. It has been observed that nNOS is enriched in regenerating axons, thus suggesting that NO seems to be necessary for growth of nerve fibers (Gonzalez-Hernandez and Rustioni, 1999).

Previous results from this laboratory have shown that rat exposure to enriched environments throughout whole life prevents the aging impairment of spatial cognition through NO-dependent mechanisms (Lores-Arnaiz et al., 2006). Also, increased cognitive performance together with an increased NOS activity and expression was observed in pre-pubertal rats after a short exposure to enriched environment but no differences were found in cognitive performance or in enzyme levels when they were exposed to different environments at a young adult age. Age, environment and training seem to modulate neuronal plasticity through NO-dependent mechanisms (Lores-Arnaiz et al., 2007).

Synaptic mechanisms of plasticity are calcium dependent processes that are affected by dysfunction of mitochondrial calcium buffering. Mitochondria outer membrane permeability is conferred by a family of porin proteins, also known as voltage-dependent anion channels (VDACs). Weeber et al. have shown that fear conditioning and spatial learning are disrupted in porin-deficient mice, demonstrating a functional role of mitochondrial porins and the permeability transition pore in learning and synaptic plasticity (Weeber et al., 2002).

Taking into account that mitochondria in synaptic terminals are subjected to relatively high metabolic demands and calcium fluxes, an adequate energy supply seems to be essential for neuronal plasticity.

The aim of this study was to evaluate the effect of enriched environment exposure on mitochondrial function and nitric oxide production in cerebral cortex and hippocampus mitochondria. As parameters of mitochondrial function and oxygen radical generation, mitochondrial respiratory function and hydrogen peroxide production were evaluated respectively in isolated cerebral cortex and hippocampal mitochondria after enriched environment exposure. The mitochondrial response to calcium-induced permeability transition was evaluated

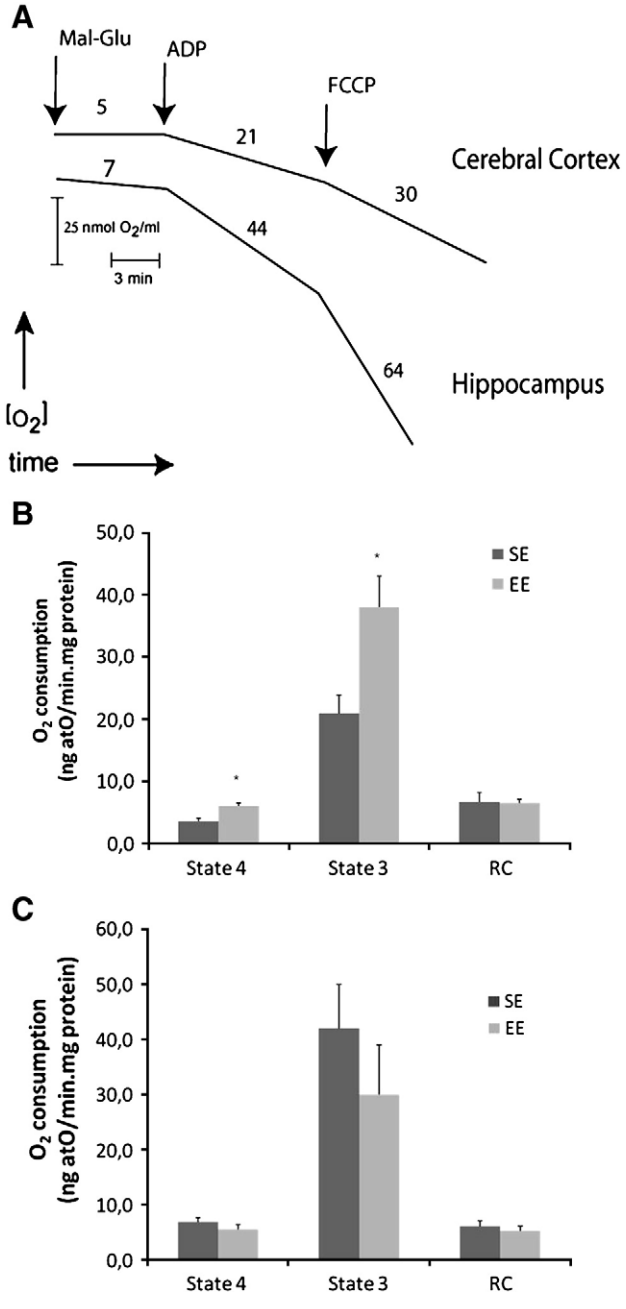

Fig. 1 – A) A typical polarographic recording of oxygen uptake in state 3 and state 4 in the presence of malate plus glutamate together with FCCP response is shown for brain cortex and hippocampal mitochondria. O_2 and time are indicated in the respective arrows and the corresponding scale bars (nmoles O_2/ml and time in min) are shown. Numbers over the records correspond to slope values of oxygen consumption in the different conditions, expressed in natgO/min.mg protein. B) Bar graph quantification of state 4 and state 3 oxygen consumption rates and respiratory controls of cerebral cortex mitochondria from SE or EE animals, in the presence of malate and glutamate or Mal-Glu plus 1 mM ADP. Bars represent mean values ± SEM of 4–6 mitochondrial samples. *$p < 0.05$, significantly different from its corresponding control (EE vs SE condition). C) Bar graph quantification of state 4 and state 3 oxygen consumption rates and respiratory controls of hippocampal mitochondria from SE or EE animals, in the presence of malate and glutamate or Mal-Glu plus 1 mM ADP. Bars represent mean values ± SEM of 4–6 mitochondrial samples. *$p < 0.05$, significantly different from its corresponding control (EE vs SE condition).

through swelling and mitochondrial membrane potential analysis. Finally, the effect of enriched environment on NOS activity and expression associated to mitochondrial membranes was also assayed.

2. Results

2.1. Mitochondrial respiration

Mitochondrial preparations from cerebral cortex and hippocampus were highly metabolically active as indicated by the high rates of oxygen uptake in the presence of ADP or the uncoupling agent FCCP. They exhibited respiratory control ratios above 6 indicating the presence of a well-preserved inner mitochondrial membrane. Addition of 1 μM FCCP increased state 3 respiratory rate by 45–55% both in cerebral cortex and hippocampal mitochondria. A typical register of oxygen consumption in state 4, state 3 and uncoupled state 3 is shown in Fig. 1A.

Malate-glutamate-dependent oxygen consumption rate was measured in state 4 (resting or controlled respiration) and in state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis) and the respiratory control ratios were calculated (Boveris et al., 1999).

Cerebral cortex and hippocampal mitochondria from SE animals showed respiratory controls of 6.6±1.5 and 6±1 respectively, and did not change significantly after enriched environment exposure, thus revealing a well preserved mitochondrial integrity and function (Fig. 1B and C). Previously our laboratory has reported data of succinate-dependent respiration and RC for hippocampal mitochondria of 3.1±0.1 (Lores-Arnaiz et al., 2005). Using malate-glutamate as respiratory substrates, this report shows RC even higher (6-7), which are in the same range as those obtained by Navarro et al. (2008), being much higher than the ones obtained after Ficoll gradient purification procedures (Clark and Nicklas, 1970).

State 4 and state 3 respiratory rates were increased by 69% and 80% in cerebral cortex mitochondria from EE rats, as compared with SE rats (Fig. 1B). No changes were observed in oxygen consumption in hippocampal mitochondria from EE animals as compared with SE rats (Fig. 1C).

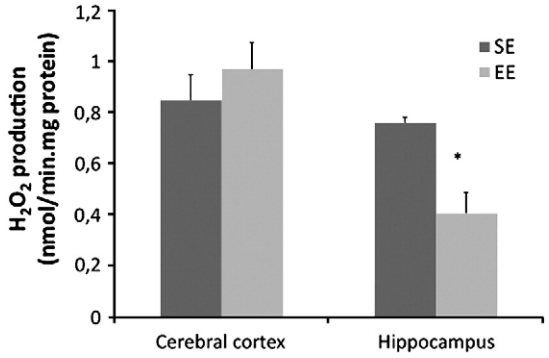

Fig. 2 – H_2O_2 production rates of cerebral cortex and hippocampal mitochondria from SE or EE animals. Bars represent the mean ± SEM of 3 individual mitochondrial samples. *$p<0.05$, significantly different from control (EE vs SE condition).

2.2. H_2O_2 production rate

H_2O_2 production was measured in cerebral cortex and hippocampal mitochondria from SE and EE rats, using malate-glutamate as mitochondrial substrates. Control H_2O_2 production rate was approximately 0.7–0.9 nmol/min.mg protein for cerebral cortex and hippocampal mitochondria (Fig. 2). A 46% decrease in H_2O_2 production rate was observed in hippocampal mitochondria, while it remained unchanged in cerebral cortex mitochondria (Fig. 2).

2.3. Calcium-induced mitochondrial permeability transition (MPT)

In this study, the response to calcium-induced MPT was evaluated by swelling assays and by mitochondrial membrane

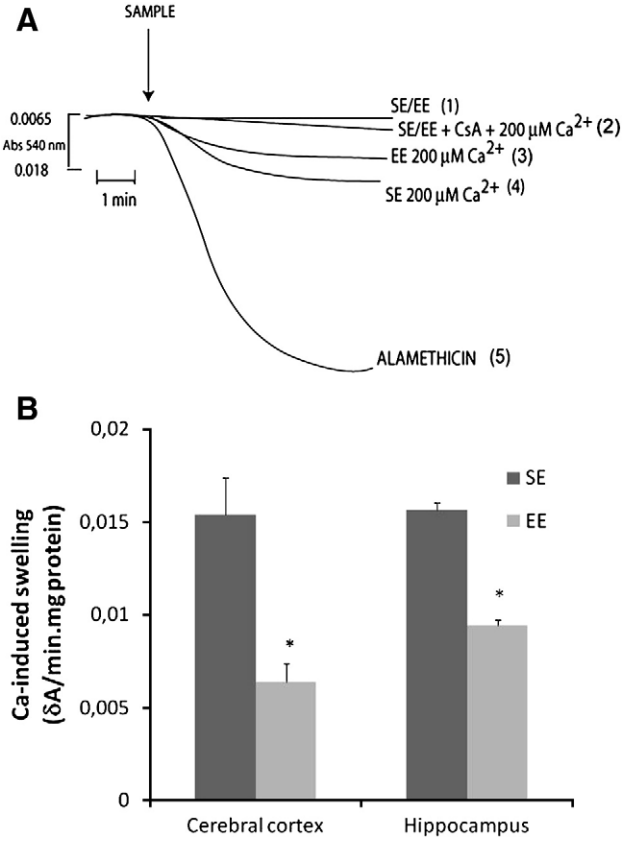

Fig. 3 – A) A typical swelling experiment showing the decrease in absorbance at 540 nm of brain cortex mitochondria from SE and EE animals in the following conditions: untreated, treated with 200 μM Ca^{2+}, pretreated with 2 μM CsA before 200 μM Ca^{2+}, or with 10 μg/ml alamethicin. B) Quantification of calcium-induced swelling in cerebral cortex and hippocampal mitochondria from SE or EE rats. Bars represent mean values of the difference in absorbance change (δ540 nm/min mg protein) in the presence of 200 nmol Ca^{2+}/mg protein minus the absorbance change in the absence of calcium from three different mitochondrial samples. *$p<0.05$, significantly different from control (EE vs SE condition).

potential determinations. Previous results from this laboratory have shown that 200 μM calcium is able to induce MPT in mouse cerebral cortex mitochondria. In this study, the MPT response to calcium overload of cerebral cortex and hippocampal mitochondria from SE and EE animals was analyzed by incubating mitochondrial samples with 200 μM calcium. A typical swelling experiment is shown in Fig. 3A, showing spontaneous swelling and the effect of 200 μM calcium for cerebral cortex mitochondria from SE and EE animals. In all cases, 5 min of pre-treatment with 2 μM Cyclosporin A (CsA) before calcium addition was able to inhibit swelling. Unspecific and maximal mitochondrial swelling was registered after incubation with 10 μg/ml alamethicin. Fig. 3B shows the swelling results corresponding to the differences in the absorbance change (δ540 nm/min mg protein) in the presence of 200 nmol Ca^{2+}/mg protein minus the corresponding absorbance change in the absence of calcium in cerebral cortex and hippocampal mitochondrial samples from each environmental condition.

In SE rats, swelling was similar for both cerebral cortex and hippocampal mitochondria (Fig. 3B). Meanwhile, calcium-induced swelling was 59% and 40% lower in cerebral cortex and hippocampal mitochondria from EE rats respectively, as compared with SE rats (Fig. 3B).

Mitochondrial membrane potential was not significantly affected by enriched environment exposure, as compared with

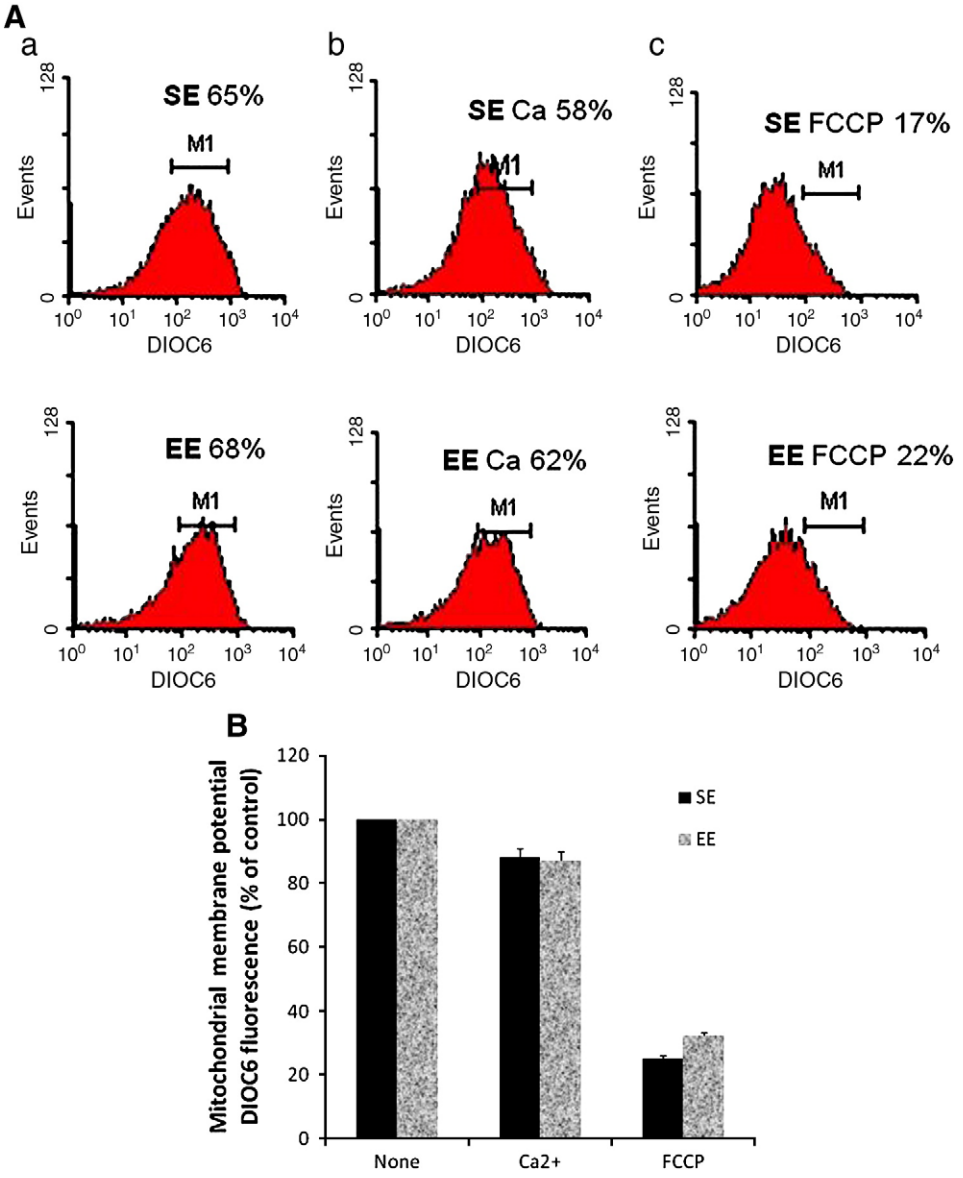

Fig. 4 – A) Histograms of relative fluorescence intensity (FL-1) which indicate mitochondrial membrane potential of cerebral cortex mitochondria from SE or EE animals, in three different experimental conditions: (a) unloaded, (b) 200 nmol Ca^{2+}/mg protein, and (c) 0.5 μM FCCP. Each histogram represents a typical experiment, which was performed in triplicate. B) Bars graph of mitochondrial membrane potential ($\Delta\psi_m$) expressed as % of control of cerebral cortex mitochondria from SE or EE animals in three different experimental conditions: unloaded, loaded with 200 nmol Ca^{2+}/mg protein or with 0.5 μM FCCP. Bars represent mean values ± SEM.

control values from SE rats (Figs. 4 and 5). Calcium loading induced similar mitochondrial membrane depolarization in cerebral cortex from both SE and EE animals (Figs. 4A, b).

In hippocampal mitochondria from SE rats, calcium was able to induce 15% depolarization. However, calcium loading did not affect significantly mitochondrial membrane potential in hippocampal mitochondria from EE animals. In fact, mitochondrial membrane potential was 12% higher in hippocampal mitochondria from EE animals as compared with SE rats, after calcium loading (Fig. 5A, b and 5B).

The mitochondrial uncoupler FCCP was used as positive control for mitochondrial depolarization. Both in cerebral cortex and hippocampal mitochondria, significant mitochondrial depolarization (70–90%) was observed after FCCP treatment (Figs. 4 and 5).

2.4. *NO production*

Control NO production was 0.43±0.06 nmol/min.mg in cerebral cortex submitochondrial membranes, while mean control value for hippocampus was 1.6±0.2 nmol/min mg. NO production was increased by 195% in cerebral cortex submitochondrial membranes from EE rats, as compared with control SE animals. In hippocampal submitochondrial membranes NO production was decreased by 47% in EE rats, as compared with control SE animals (Fig. 6).

2.5. *Western blot*

Brain NOS associated to mitochondrial membranes has been previously identified by Western blot analysis mainly as nNOS

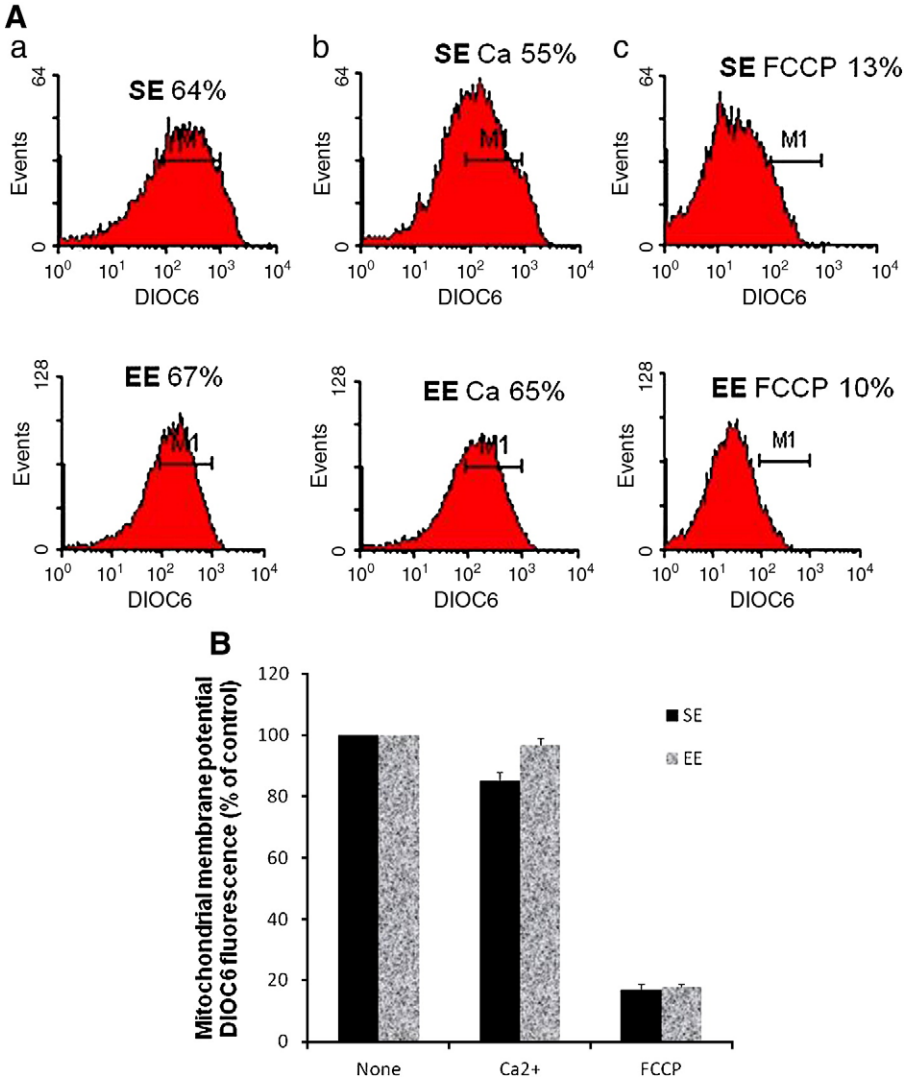

Fig. 5 – A) Histograms of relative fluorescence intensity (FL-1) which indicate mitochondrial membrane potential of hippocampal mitochondria from SE or EE animals, in three different experimental conditions: (a) unloaded, (b) 200 nmol Ca^{2+}/mg protein, (c) 0.5 μM FCCP. Each histogram represents a typical experiment, which was performed in triplicate. B) Bars graph of mitochondrial membrane potential ($\Delta\psi_m$) expressed as % of control of hippocampal mitochondria from SE or EE animals in three different experimental conditions: unloaded, loaded with 200 nmol Ca^{2+}/mg protein or with 0.5 μM FCCP. Bars represent mean values ± SEM. * $p<0.01$, significantly different from its corresponding control in the same experimental condition (EE vs SE, Ca^{2+}-loaded).

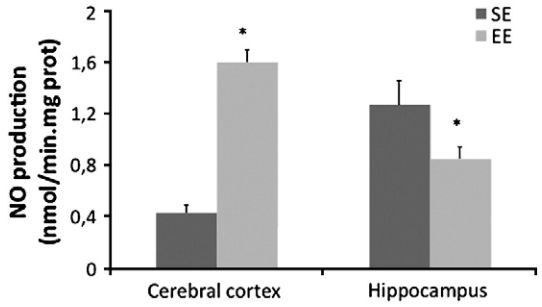

Fig. 6 – NO production of cerebral cortex and hippocampal submitochondrial membranes from SE or EE animals. Bars represent the mean ± SEM of 3 individual mitochondrial samples. *$p<0.05$, significantly different from control (EE vs SE condition).

and also but to a lesser extent as eNOS (Lores-Arnaiz et al., 2004b). In this study, Western blot analysis identified a NOS protein expression, in association with submitochondrial membranes from cerebral cortex and hippocampus, with a molecular mass of 147 kDa reacting with anti-nNOS (Fig. 7). The ratio of nNOS/cyt oxidase expression was 30% higher in preparations obtained from cerebral cortex of EE rats, as compared with SE rats, as shown by densitometric analysis (Fig. 7).

Hippocampal ratio of nNOS/cyt oxidase expression was 84% lower in association with submitochondrial membranes obtained from EE animals, as compared with hippocampal samples from SE rats (Fig. 7).

3. Discussion

It has been extensively described that neurons can respond to proper stimuli by forming new synapses and by elaborating dendritic processes (Sapolsky, 2003). Mitochondria are believed to concentrate in these subcellular regions which present high metabolic requirements. For instance, they accumulate in the vicinity of active growth cones of developing neurons (Morris and Hollenbeck, 1993). It has been also described that environmental enrichment can induce changes in the hippocampus, which include increased dendritic arborisation, synapse density and number of gial cells. Housing animals in an enriched environment, has been shown to increase the survival of newborn neurons and to improve performance in hippocampus-dependent learning tasks (Brown et al., 2003, van Praag et al., 2000).

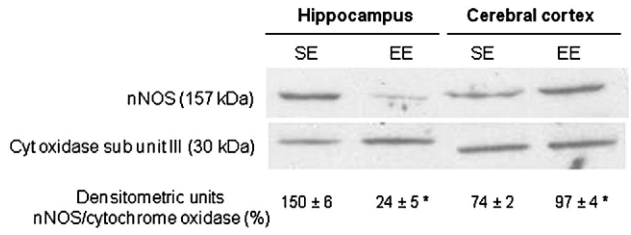

Fig. 7 – Representative western blots analysis of nNOS of cerebral cortex and hippocampal sumitochondrial membrane fractions from SE and EE animals. Mitochondrial encoded cytochrome oxidase (COX) subunit III was used as loading control. Densitometric analysis was performed and values below blots represent the ratio of nNOS/COX (means ± SEM, $n=4$) of cerebral cortex and hippocampal submitochondrial membranes. Western blots were performed in triplicate.

The focus of this paper was to evaluate the effects of enriched environment (EE) on mitochondrial functioning and free radical generation in two different brain areas: cerebral cortex and hippocampus, and to propose that this condition could be beneficial for cellular metabolism, contributing to maintain mitochondrial integrity and physiology. There are no studies in the literature linking the consequences of changes in mitochondrial function in terms of bioenergetic capacity and free radical generation caused by enriched environment in cerebral cortex and hippocampus.

Results from our laboratory have shown that long-term exposure of rats to enriched environment prevents the aging impairment of cognition, neuronal plasticity and brain nitric oxide production (Lores-Arnaiz et al., 2006). In a recent study we have also shown that a brief exposure to enriched environment at an early age increases brain nNOS activity and expression (Lores-Arnaiz et al., 2007). In both studies, enriched environment exposure induce an increase in mitochondrial and cytosolic NOS activity and expression in whole brain. In agreement with our previous findings, we now present evidence of an increased NO production and increased nNOS expression in association with mitochondrial membranes of cerebral cortex of rats exposed to enriched environment.

Pham et al. (2002) have described that early life experience can have long-term consequences on both the neurochemical and the behavioural parameters later in life, being such changes at least partly mediated by endogenous trophic factors. It has been postulated that BDNF has the ability to modify energy metabolism. In this study, we show increased state 4 and state 3 oxygen consumption rates in cerebral cortex mitochondria from rats exposed to enriched environment, as compared with animals kept in standard environments. These results are in agreement with previous observations from El Idrissi & Trenker (1999), showing that the addition of BDNF to cerebellar granule cells increased mitochondrial activity. Recent studies by Markham et al. have also shown that BDNF improved mitochondrial respiratory coupling in a concentration-dependent manner, the effect being selective for complex I of the mitochondrial respiratory chain and dependent on a synaptosomal component (Markham et al., 2004). Also, we have previously reported increased complex I activity in association with an improvement in cognitive performance in brain mitochondria from rats after enriched environment exposure, reflecting that plasticity requires a considerable energy demand (Lores-Arnaiz et al., 2006; Lores-Arnaiz et al., 2007).

Sirevaag and Greenough (1988) have reported differential expression of coordinated vascular and cellular metabolic processes in rats reared in complex, social and individual housing environments. It is possible that the increased oxygen consumption rate observed in EE rats could be a consequence

of an increased capillary density in these tissue regions, rather than to an intrinsic improvement in the electron transfer chain.

In contrast with observations in cerebral cortex, NOS activity and expression associated to hippocampal submitochondrial membranes were decreased in EE rats. NO can contribute to the establishment of plasticity under physiologically relevant conditions by selectively increasing the probability of LTP induction or by inhibiting long-term depression (Malen and Chapman, 1997). However, subsequent investigations have suggested that the role of NO in hippocampal LTP is limited. One important aspect, the experience of stress, can have adverse effects on neural plasticity. The hippocampus displays remarkable structural plasticity and exposure to excessive levels of glucocorticoids compromises its plastic potential. In fact, it has been observed that chronic stress suppresses plasticity in CA3 pyramidal cells of rat hippocampus (Stewart et al., 2005). In particular, 21-day administration of stress levels of corticosterone decreased LTP in the dentate gyrus (Pavlides et al., 1993), and co-administration of corticosterone and phenytoin led to a significant reduction in nNOS messenger RNA levels in hippocampal interneurons (Reagan et al., 1999). In our study, it is possible that some level of stress could be induced by EE, impairing hippocampal plasticity through a decreased NOS activity and expression.

Mitochondrial H_2O_2 production rate, although not affected by EE exposure in cerebral cortex mitochondria, was significantly decreased in hippocampal mitochondria from EE animals. Taking into account that mitochondrial respiratory rates were not modified in hippocampal mitochondria after EE exposure, the decrease in H_2O_2 production would not be due to alterations in mitochondrial respiratory chain, but caused by changes in other mitochondrial sources of H_2O_2, such as monoamine oxidase.

Synaptic mechanisms of plasticity are calcium-dependent processes that are affected by dysfunction of mitochondrial calcium buffering. Mitochondrial permeability transition has been recently been shown to play a role in learning and synaptic plasticity in mice (Weeber et al., 2002). Recent reports have proposed a role for the MPT in neuronal calcium regulation (Levy et al., 2003). We have exposed brain cortex and hippocampal isolated mitochondria to calcium concentrations high enough to induce MPT, which are above the normal cytosolic level. Excitotoxic conditions and NMDA receptor overactivation induce large cytosolic Ca^{2+} elevations, strong enough to trigger mitochondrial overload and dysfunction (Stanika et al., 2008). In addition, in a cell system even a minor disturbance in cellular Ca^{2+} homeostasis could result in mitochondrial calcium accumulation sufficient to promote Bax-mediated MPT. In the absence of Bax (double knockout cells) the amount of Ca^{2+} required to induce MPT is much higher (Bustamante et al., 2005). Thus, in a system such as isolated mitochondria, characterized by the absence of the different mitochondrial messengers, the Ca^{2+} concentrations necessary to induce MPT are higher.

In our study, calcium was able to induce mitochondrial depolarization in cerebral cortex and hippocampal mitochondria from rats exposed to SE and also in cerebral cortex mitochondria from EE rats. However, no significant depolarization was observed in hippocampal mitochondria from EE animals. The calcium ability to induce swelling was also studied in these conditions. As expected, a lower mitochondrial swelling (40%) was observed in cerebral cortex and hippocampal mitochondria from EE animals as compared with the levels of mitochondrial swelling in SE animals (59%). These results led us to state that mitochondria from animals submitted to EE are less prone to induce mitochondrial permeability transition (MPT).

Our results suggest that cerebral cortex and hippocampal mitochondria from EE rats can handle calcium overload more efficiently than mitochondria from SE rats, probably by presenting an improved calcium buffering capacity. In hippocampal mitochondria, the low levels of H_2O_2 found in EE rats in our study, could be associated with the resistance to MPT induced by high Ca^{2+} concentrations, as observed by the decreased swelling and depolarization after calcium overload. Other mechanisms seem to be involved in the resistance to calcium-induced MPT observed in cerebral cortex mitochondria from EE rats. In the brain, different subregions show different vulnerability to ischemia-reperfusion damage. Also, calcium sensitivity of the MPT can vary in mitochondria from different species and tissues, perhaps as a result of the differences in their content of MPT modulators (Jurkowitz and Brierley, 1982). Also, the mitochondrial heterogeneity between the different cell populations (glia, neurons, endothelial cells) and from different cellular compartments (synaptic and non synaptic) could have contributed to the different results obtained for the two studied areas.

Both pro and antiapoptotic effects of NO have been described depending on cell type, NO levels and cellular environment. Antiapoptotic effects are associated with low NO levels (10 nM to 1 μM) coming from endogenous sources, while higher NO levels (>1 μM) are associated with PTP opening (Brookes et al., 2000). Although changes in NO production after enriched environment exposure are observed both in hippocampal and cerebral cortex mitochondria, these NO levels are still within the range of physiological levels, being produced by the nitric oxide synthase associated to mitochondria. Therefore, the different susceptibility of mitochondria from SE or EE animals to undergo MPT does not seem to be related with changes observed in NO levels.

There is increasing evidence to suggest that in aging and in Alzheimer disease, damaged synaptic mitochondria might not satisfy the high energy demands required at the synapses, which might lead to impaired neurotransmission and, ultimately, to cognitive failure (Reddy and Beal, 2008). So, an adequate mitochondrial functioning seems to be essential for synaptic activity; improvement of mitochondrial capacity as found after enriched environment would prevent neurons from age-dependent impairment of cognition.

The results of this study suggest that both in hippocampus and cerebral cortex, EE exposure protects against calcium-induced mitochondrial permeability transition, assuring mitochondrial integrity and continuous energy supply for the maintenance of neuronal and glial function. Further studies will help to elucidate the possible mechanisms involved in the improvement of mitochondrial function after enriched environment exposure and to evaluate how mitochondrial physiology can exert a relevant role in the interactions between cerebral cortex and hippocampus.

4. Experimental procedure

4.1. Experimental design

Male Sprague-Dawley – SD – rats of 21 days old from the Veterinarian School Vivarium — University of Buenos Aires (n=48) were randomly assigned to enriched or standard housing condition during 1 year. Following arrival at the lab all the animals were maintained in a 12-hour light–dark cycle (lights on at 8 a.m.); food and water were available *ad libitum* all the time in either environment. All subjects were group housed (6 rats per enriched cage and 3–4 per standard cage).

All efforts were made to minimize animal discomfort and to reduce the number of animals used.

4.2. Enriched and standard environments

In the enriched condition, animals were housed in cages of 50×98×54 cm, furnished with a large cord pending from the top of the cage, two inclined rambles, suspended bridges, a wooden nest and three horizontal walkways. Two wooden-made gallery entries and a truck toy with moving parts were located on the cage ground. The four walls of each cage were made of interwoven metal wires allowing the animals to move in all directions. Every day, five different objects selected from a set of one hundred safe toys were provided to every enriched cage before the animals were trained. The toys in the cage were renewed every 24 h (Kolb, 1998).

In the standard condition, animals were housed in cages of 29×21×34 cm without any other object than food cages and water bottles.

4.3. Isolation of mitochondria

Male Sprague-Dawley rats (250–300 g) were killed by decapitation and the brains were immediately excised. Brains were weighed, cerebral cortex and hippocampus were dissected and homogeneized in a medium consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl and 1 mM EDTA, pH 7.4, and homogenized at a ratio of 1 g brain/5 ml homogenization medium. Homogenates were centrifuged at 600×g for 10 min to discard nuclei and cell debris and the pellet was washed to enrich the supernatant that was centrifuged at 8000×g for 10 min. The resulting pellet, containing both synaptic and non-synaptic mitochondria, able to carry out oxidative phosphorylation, was washed and resuspended in the same buffer. The operations were carried out at 0–2 °C. Mitochondrial yield was estimated in approximately 50–100 mg mitochondrial protein/g brain tissue, both for hippocampus or for cerebral cortex. This estimation was based on the determination of the activity of the mitochondrial marker enzyme monoamine oxidase (MAO) both in total homogenates and in mitochondrial fractions. Measurement of mitochondrial marker enzyme MAO resulted in a recovery of 65% of the total mitochondrial pool. Submitochondrial membranes were obtained from mitochondria by twice freezing, thawing and homogenizing by passing the suspension through a 15/10 hypodermic needle (Lores-Arnaiz et al., 2004a). Further mitochondrial purification was performed by Ficoll gradient (Clark and Nicklas, 1970) for Western blot analysis. Submitochondrial membranes and purified mitochondria were less than 2% contaminated with cytosolic components according to β-actin Western blot analysis. Protein content was assayed by using the Folin phenol reagent and bovine serum albumin as standard (Lowry et al., 1951).

4.4. Mitochondrial respiration

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria) was used. Mitochondrial respiratory rates were measured in cerebral cortex and hippocampal mitochondria, in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate and 0.2% bovine serum albumin at 30 °C. Malate 6 mM and glutamate 6 mM were used as substrates to measure state 4 respiration and 1 mM ADP was added to measure state 3 respiration (Boveris et al., 1999).

4.5. Hydrogen peroxide production

Hydrogen peroxide generation was determined in intact cerebral cortex and hippocampal mitochondria (0.1–0.3 mg protein/ml) by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365–450 nm (λexc–λem) at 37 °C (Boveris, 1984). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 6 mM malate, 6 mM glutamate and 0.3 μM SOD. A calibration curve was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min.mg protein.

4.6. Mitochondrial permeability transition

Mitochondrial suspensions (0.5 mg/ml) from control and enriched-housed animals were incubated in MSH buffer supplemented with 5 mM malate, 5 mM glutamate and 1 mM phosphate. Mitochondrial permeability transition (MPT) was determined by swelling studies, measuring spectrophotometrically the decrease in absorbance at 540 nm (Azonne et al., 1984; Bustamante et al., 2005). The experiments were started by the addition of 200 nmol Ca^{2+}/mg protein, which correspond to 200 μM Ca^{2+} concentration. Five minutes pretreatment with 2 μM Cyclosporin A (CsA) before calcium addition was assayed in order to inhibit pore opening. Maximal mitochondrial swelling was registered after adding 10 μg/ml of the 20-residue channel-forming peptide alamethicin.

MPT was also evaluated by the determination of the mitochondrial membrane potential. Mitochondrial population with specific side scattering (SSC) and forward scattering (FSC) properties were analyzed; 20.000 events per sample were collected. Mitochondria were loaded with 30 nM of the potentiometric probe $DiOC_6$ during 20 min at 37 °C and immediately acquired by a FAC-SCAN flow cytometer equipped with a 488 nm Argon laser and a 615 nm red diode laser. In these conditions, toxic effects of this probe were not detected on mitochondrial respiration. Mitochondrial autofluorescence (no probe) was also measured as a loading

control. Data from the experiments were analyzed using the CellQuest software (Becton & Dickinson).

4.7. *Nitric oxide synthase activity*

NO production was measured in cerebral cortex and hippocampal submitochondrial membranes by following spectrophotometrically the oxidation of oxyhemoglobin to methemoglobin at 37 °C, in a reaction medium containing 50 mM phosphate buffer (pH 5.8), 1 mM $CaCl_2$, 50 μM L-arginine, 100 μM NADPH, 10 μM dithiothreitol, 4 μM Cu-Zn SOD, 0.1 μM catalase, 0.5–1.0 mg submitochondrial protein/ml and 25 μM oxyhemoglobin (expressed per heme group). The kinetics was followed at 577–591 nm ($\varepsilon = 11.2\ mM^{-1}cm^{-1}$) in a double-beam double-wavelength spectrophotometer (Boveris et al., 2002).

4.8. *Western blots*

Submitochondrial membranes (150 μg), in the presence of protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin), were separated by SDS-PAGE (7.5%), blotted onto a nitrocellulose membrane (Bio-Rad, München, Germany) and probed primarily with rabbit polyclonal antibodies (dilution 1:500) for nitric oxide synthase neuronal constitutive form (nNOS or NOS-1) reacting with the amino terminus (H-299) (Santa Cruz Biotechnology). Then, the nitrocellulose membrane was incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1:5000), followed by development of chemiluminescence with the ECL reagent (Santa Cruz Biotechnology) for 2–4 min (Bustamante et al., 2002). Mitochondrial encoded cytochrome oxidase (COX) subunit III was used as loading control. Densitometric analysis of nNOS (amino terminus) and cytochrome oxidase bands was evaluated through the NIH Image 1.54 software (Wayne Rasband for Macintosh) and expressed as the ratio of nNOS/cytochrome oxidase. All experiments were performed in triplicate.

4.9. *Drugs and chemicals*

ADP, L-arginine, Catalase, Dithiothreitol, EDTA, Glutamic Acid, Malic Acid, Mannitol, NADPH, N_ω-nitro-L-arginine, Haemoglobin, Scopoletin, Horseradish Peroxidase, Succinate, Sucrose, Superoxide Dismutase, Cytochrome c, Trizma Base, and Cyclosporin A were purchased from Sigma Chemical Co. (St. Louis, Missouri). Other reagents were of analytical grade.

4.10. *Statistics*

Results are expressed as mean values ± SEM. Student's *t* test was used to analyze the significance of differences between paired groups.

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