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Research report

Extensive enriched environments protect old rats from the aging dependent impairment of spatial cognition, synaptic plasticity and nitric oxide production

S. Lores-Arnaiz [a,*], J. Bustamante [a], M. Arismendi [b], S. Vilas [b], N. Paglia [b], N. Basso [d], F. Capani [c], H. Coirini [c], J.J. López Costa [c], M.R. Lores Arnaiz [b]

[a] Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, C1113AAD Buenos Aires, Argentina
[b] Laboratory of Working Memory and Stress, School of Psychology, University of Buenos Aires, Buenos Aires, Argentina
[c] Cellular Biology and Neuroscience Institute "Prof. E. De Robertis", School of Medicine, University of Buenos Aires, Buenos Aires, Argentina
[d] Laboratory of Cardiovascular Physiopathology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

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In memory of Patricia Goldman-Rakic.

Abstract

In aged rodents, neuronal plasticity decreases while spatial learning and working memory (WM) deficits increase. As it is well known, rats reared in enriched environments (EE) show better cognitive performances and an increased neuronal plasticity than rats reared in standard environments (SE). We hypothesized that EE could preserve the aged animals from cognitive impairment through NO dependent mechanisms of neuronal plasticity. WM performance and plasticity were measured in 27-month-old rats from EE and SE. EE animals showed a better spatial WM performance (66% increase) than SE ones. Cytosolic NOS activity was 128 and 155% higher in EE male and female rats, respectively. Mitochondrial NOS activity and expression were also significantly higher in EE male and female rats. Mitochondrial NOS protein expression was higher in brain submitochondrial membranes from EE reared rats. Complex I activity was 70–80% increased in EE as compared to SE rats. A significant increase in the area of NADPH-d reactive neurons was observed in the parietotemporal cortex and CA1 hippocampal region of EE animals.
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1. Introduction

In 1988 Diamond et al. [14] found a plastic mechanism involved in working memory (WM), named *primed burst potentiation* (PBP), a low threshold kind of long-term potentiation, which they characterized as "... a long-term increase in CA1 population spike amplitude produced by physiologically patterned electrical stimulation of the hippocampal commissure" [15]. They also found that stress impairs spatial WM but not long-term memory (LTM) in rats previously trained in a 14-arm maze with external cues [16]. Accordingly, a previous work from this laboratory has shown stress impairment of WM but not of LTM in rats that had been trained in an eight-arm radial maze task with external cues and then exposed to a novel, predator-scented environment [12].

The effects of changes in the environment on learning and on neuronal tissue regeneration have been extensively studied by several laboratories, showing that neuronal connections and cortical maps are continuously remodeled by our experience, by learning and in response to brain lesions [24]. Recently, it was reported that enriched environments increased the neural stem cell pool and neurogenesis in the adult subventricular zone 5 weeks after a cortical stroke [30]. Other studies have shown that environmental and temporal contexts can independently, but similarly modulate taste aversion learning in rats [41].

As it was proved that WM involves plasticity mechanisms [16] we hypothesized that the exposition of rats to enriched environments (EE) [29,48] could reduce the cognitive impairment produced by stressors on the spatial WM. Results from this lab-

* Corresponding author. Tel.: +54 11 4508 3646; fax: +54 11 4508 3646.
E-mail address: slarnaiz@ffyb.uba.ar (S. Lores-Arnaiz).

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oratory have shown that rats reared in enriched environments for 3 months, trained in spatial WM and LTM and then exposed to three different stressors made significantly less WM errors than standard reared rats under any of the stressors. We also measured the same spatial WM and LTM tasks in animals treated with enalapril or losartan, in order to study the protective role of rennin–angiotensin system inhibition on the aging impairment of brain functions, and drug-treated animals showed a better cognitive performance than non-treated ones [3]. Recently, Boveris et al. [6] have shown that mitochondrial NOS activity is up-regulated during enalapril treatment [6].

In aging rodents, hippocampal electrophysiology [27] and plasticity [4] are impaired and cognitive deficits, particularly spatial learning and WM deficits, increase. Although previous reports showed a reduction of hippocampal neurons in aging [49], more detailed stereological studies did not reveal a loss of hippocampal and subicular neurons in aged rats [46]. Similar data have been obtained in hippocampus and neocortex in humans [45]. Therefore, it seems that the reduction in brain volume in aging is due mainly to reduction in the connections and surrounding tissues.

The discovery of the intercellular messenger nitric oxide (NO) stimulated new concepts of how synaptic plasticity can be induced in the nervous system. The activation of the NMDA receptor during glutamatergic neurotransmission increases intracellular Ca^{2+} concentrations activating neuronal nitric oxide synthase (nNOS). The nitric oxide–cGMP signaling pathway is implicated in an increasing number of experimental models of plasticity, in the formation of LTM and in spatial learning in rats [26]. As NO is a highly diffusible molecule, it may act not only in the postsynapsis but in the presynapsis as a retrograde messenger as well [55]. In addition, it was demonstrated that NO may stimulate neurotransmitter release [9,21,40,50].

Nitric oxide is synthesized from L-arginine, in the presence of NADPH and O_2, by a series of isoenzymes of the family of nitric oxide synthases: neuronal NOS, inducible NOS (iNOS) and endothelial NOS (eNOS) [28].

Recent studies gave evidence of the existence of a NOS in rat liver mitochondrial inner membrane, the mitochondrial NOS (mtNOS) [18,19,52], which was identified as nNOS α [17]. Concerning brain, mtNOS activity able to produce NO upon supplementation with the required substrates was reported in rat and mouse mitochondria [31,34,35].

Being WM a function weaken by aging, the present experiment was planned to study WM performance and plasticity in aged animals, in order to ascertain if EE preserve animals from the degree of impairment suffered by standard environments (SE) reared rats and if this advantage was associated with a different degree of synaptic plasticity, mainly through NO dependent mechanisms. We conducted a long life study, in which animals were trained and assessed in spatial WM and LTM several times during their lifetime. Cyotosolic and mitochondrial NOS activity and expression were determined both in EE and SE reared rats. NADPH-diaphorase histochemical technique was carried out to demonstrate specifically NO producing neurons in fixed tissue. Considering that the aging process is associated with a reduction in complexes I and IV of the mitochondrial respiratory chain [32,42,43] and that NO plays an important role as a modulator of mitochondrial respiration [8], it seemed relevant to measure the effect of extensive enriched environment on the activity of the respiratory complexes.

2. Materials and methods

2.1. Subjects

Male and female Sprague–Dawley 27 months old rats (Veterinarian School Vivarium, University of Buenos Aires) were used. Animals were randomly assigned to one of the two rearing conditions when they arrived at our laboratory, being 21 days old. Half of the animals were kept in enriched big cages (condition 1) and the other half in standard wire cages (condition 2) during all its life. Males and females were assigned to different cages, both in the standard as in the enriched condition. All the animals were maintained in a 12-h light:12-h dark cycle (lights on at 8 a.m.) and fed with laboratory rat chow (Gilardoni, Buenos Aires, Argentina), fixing their training weight levels within 90% of their pre-training ad libitum feeding weights. Water was available ad libitum all the time in either environment. All subjects were group housed (five rats per enriched cage and three to four per standard cage).

2.2. Enriched and standard environments

In condition 1, animals were housed in cages of 50 cm × 98 cm × 54 cm furnished with a large cord pending from the top of the cage, two inclined rambles, suspended bridges, a wooden nest, three horizontal walkways. Three wooden made galleries and a truck toy with moving parts were located on the cage ground [29]. The four walls of each cage were of interweave metal wires allowing the animals to move on 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 remained in the cage for 24 h, until their replacement.

In condition 2, animals were housed in cages of 29 cm × 21 cm × 34 cm without any other object than food cages and water bottles.

2.3. WM and LTM training in the maze

Animals were firstly exposed to 7 days of handling and 7 days of shaping. The first training in spatial WM and LTM started when the animals had 90 days of life and 68 days of exposition to one of the two environmental conditions. We adopted the procedure employed by Diamond et al. [16], adapting their proceedings to our simpler task. The acrylic maze had eight arms (each arm measures 76 cm × 10 cm) radiating from a central circular platform (70 cm in diameter) which rested 60 cm above the floor on a table. There were four opaque plastic doors that allowed the experimenter to open or close the arms. The experimenter always occupied the same place within the view of the subjects. A food circular well was placed at the end of each arm (2.5 in. diameter × 1.5 cm deep). Eight distinct visual cues – both plane and round ones – surrounded the maze, which was uniformly illuminated.

From the third week animals were trained in spatial WM and LTM tasks in the eight-arm maze once a day. A single FL was placed in the food well in each of the four baited arms and each subject were run by itself. Subjects were allowed to explore the maze for up 10 min or until the four FL were eaten. An arm selection was noted when the rat passed its hindquarters over the entry of the arm.

The WM task had two phases: the acquisition phase and the election phase. During the acquisition phase two of the four arms were opened—a different set of two randomly selected baited arms was opened every day. Rats were removed from the maze after they consumed the two Froot Loops (FL). A short delay between the two phases (30 s) made possible to clean the maze with a lemon-scented liquid to prevent odor cues. When the animals were returned to the maze, the election phase started and all the arms were opened, giving the animals the opportunity to explore the maze for up to 5 min or until the two remaining FL were eaten. The maze was moved every day to a new position by means of a rotating mechanism to prevent rats of using intra maze cues. WM errors were

only counted during the election phase: one entry into one of the four non baited arms was counted as a LTM error; one entry into one of the two previously baited arms or a re-entry into one of the baited arms just visited was counted as a WM error [16]. A WM standard performance was set at a maximum of one error in every 5 consecutive training days [16].

When animals reached 27 months of age, the remaining rats from the enriched environments ($N = 15$) and from the standard one ($N = 14$) were given a new training period to assess its WM performances.

2.4. Biochemical studies

After their last training animals were sacrificed and biochemical studies were conducted to assess expected differences in synaptic plasticity between groups—enriched males, enriched females, standard males and standard females.

2.4.1. Isolation of brain mitochondria

Male and female Sprague–Dawley rats (250–300 g) were killed by decapitation and the brains were immediately excised. Brains were weighed 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 700 × *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 obtained pellet, washed and resuspended in the same buffer, consisted of intact mitochondria able to carry out oxidative phosphorylation. The supernatant obtained after 8000 × *g* centrifugation was called "cytosolic fraction". The operations were carried out at 0–2 °C. Submitochondrial membranes were obtained from mitochondria by twice freezing, thawing and homogenizing by passing the suspension through a 15/10 hypodermic needle [5]. Further mitochondrial purification was performed by Ficoll gradient [11] for Western blot analysis. Mitochondrial pellets were washed with isolation buffer and dialyzed against it at 4 °C for 4 h. 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 [38].

2.4.2. Western blotting and chemiluminescence

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) or with the carboxy terminus of (R-20) (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 [5,10]. Densitometric analysis of nNOS (amino terminus) and nNOS (carboxy terminus) bands was evaluated through the NIH Image 1.54 software (Wayne Rasband for Macintosh). All experiments were performed in triplicate.

2.4.3. Nitric oxide synthase activity

NO production was measured in brain 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 [5].

2.4.4. Respiratory complexes activity

Complexes I and III: NADH:ubiquinone oxidoreductase (complex I) was assayed according to a modification of the method described by Whitfield et al. [56] in the presence and absence of 5 μM rotenone to measure complex III or both complexes. The reaction mixture consisted in buffer 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl and 1 mM EDTA, pH 7.4, 80 μM coenzyme Q_1, 2 mM NaN_3 and 0.1 mM NADH. The decrease in absorbance at 340 nm was monitored for 2 min and calculated from linear plots using a combined extinction coefficient for Q_1 and NADH of $6.81\ mM^{-1}\ cm^{-1}$.

Succinate dehydrogenase (complex II) was determined by a spectrophotometric method based on the utilization of phenazine methosulphate as the immediate electron acceptor and 2,6-dichlorophenolindophenol (DCPIP) as terminal acceptor. The decrease in the absorbance of DCPI was followed at 600 nm in a reaction medium containing 100 mM phosphate buffer, pH 7.4, 8 mM succinate, 1 mM KCN, 0.8 mM phenazine methosulphate, 60 μM DCPIP and 0.2–0.5 mg/ml mitochondrial protein, at 30 °C [2].

Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome *c* [58].

2.4.5. Drugs and chemicals

L-Arginine, catalase, coenzyme Q_1, dithiothreitol, EDTA, mannitol, NADH, NADPH, L-NNA, oxyhemoglobin, sodium azide, sucrose, superoxide dismutase and Trizma base, were purchased from Sigma Chemical Co. (St. Louis, Missouri). Other reagents were of analytical grade.

2.4.6. Statistics

Results are expressed as mean values ± S.E.M. Student's *t* test was used to analyze the significance of differences between paired groups. ANOVA followed by Scheffé tests were used to analyze differences between mean values of more than two groups.

2.5. Anatomical studies

2.5.1. Tissue processing

Sprague–Dawley male rats (250–300 g) of 27 months of age from EE or SE conditions were used. Animals were anaesthetized i.p. with chloral hydrate (350 mg/kg) and perfused transcardially with 50 ml of 0.9% NaCl solution containing 50 IU of heparin followed by 200 ml of a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were dissected out and were postfixed in the same fixative solution during 2 h followed by overnight immersion in a solution containing 5% sucrose in 0.1 M phosphate buffer. Coronal tissue sections (50 μm thick) were obtained using an Oxford vibratome.

2.5.2. Histochemical procedure

Tissue was processed with the NADPH-d histochemical method [54]. Briefly, free floating sections were incubated for 1 h at 37 °C in a solution containing 0.1% β-NADPH (1 mg/ml) and 0.02% (0.2 mg/ml) nitroblue tetrazolium chloride diluted in 0.1 M phosphate buffer (pH 7.4) with 0.3% Triton X-100 (all reagents from Sigma). Negative controls of the technique were obtained by incubating floating sections in a reaction mixture without β-NADPH. In order to avoid differences in NADPH-diaphorase reactivity due to the staining procedure, experimental (EE) and control sections (SE) were processed simultaneously using the same solution batch. Sections were mounted on gelatin-coated glass slides and coverslipped with a PBS:glycerol mixture (1:3). Sections were observed and photographed using a Zeiss Axiophot light microscope.

2.5.3. Image analysis

The cell soma of NADPH-d reactive neurons from cerebral cortex and CA1 hippocampus of experimental and control animals were measured by using a computerized image analyzer (VIDAS-Kontron, Zeiss). Randomly selected images were obtained by a light microscope and were transferred by an attached video camera to the interactive image analysis system. The images were digitized into an array of 512 × 512 pixels being the resolution of each pixel 256 gray levels. The areas of NADPH-d reactive neurons from sections of experimental and control groups were measured.

2.5.4. Statistical analysis

Brains from three animals of each condition were processed. At least, 5 randomly selected fields of the cortex and hippocampus of 10 sections per animal

were analyzed. Values were expressed as mean ± S.E.M. Results were evaluated using one-way analysis of variance (ANOVA) and comparisons among groups were made by Fisher and Bonferroni–Dunnet test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Behavioral results

Standard environment condition significantly lowered the animals' longevity, being this effect more marked for males than for females. At the end of the last training, 15 of the 20 enriched reared animals survived, while only 9 of the 20 standard rats survived. From the original 10 standard females, 8 survived at the beginning of the last training. None of the 15 enriched trained animals – 7 males and 8 females – died during the last experiment.

Following the procedure used by Diamond et al. [16], we count every 5 consecutive training days during which the animals have a maximum of one WM error in 5 days of WM successful performance or, in the Diamond words, 5 days during which the animals remained in the assumed criterion.

Enriched rats kept WM criterion – fixed in a maximum of one error in every 5 days – during significantly more days (mean = 10 ± 1) than standard rats (mean = 5 ± 1) (Mann–Whitney *U* test, $p = 0.014$). In order to appropriately compare WM performance in both groups we calculated the percentage of days in which enriched and standard rats kept WM criterion over the total number of training days. A significant increase (66%) was observed in WM performance in enriched rats as compared with standard rats (Fig. 1A).

Only three animals never achieved WM criterion in all the training days: two standard females and one enriched male.

We also counted the total number of training days in which the animals kept WM criterion, i.e., all the days with 0 WM errors, even if its last set did not fulfill another set of 5 days. Enriched animals also had a higher number of days with 0 WM errors (57%) than the standard ones (Fig. 1A).

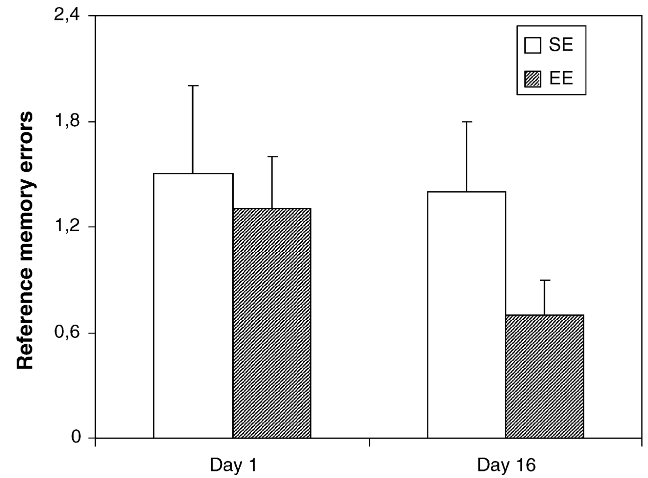


Fig. 2. Reference memory errors for enriched and standard rats from days 1 to 16 of training. Bars represent mean values from 15 to 14 rats, ±S.E.M.

Enriched rats significantly reduced their election running times by 25% at the radial maze between the 1st and the 16th day of training. No significant differences were found between the election running times of the 1st training day and those of the 16th training day for standard rats (Fig. 1B).

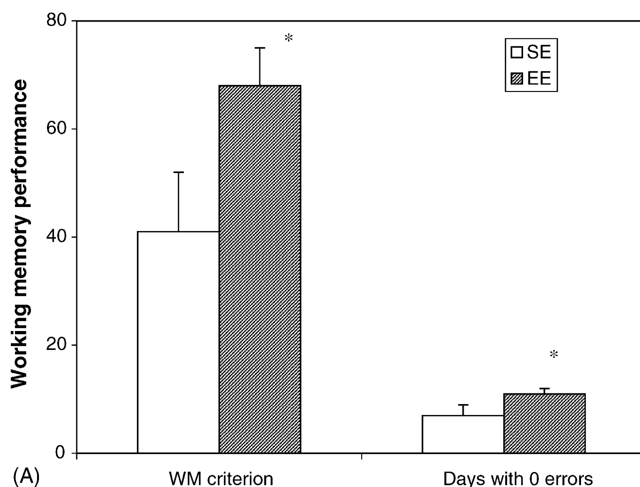
No significant differences were found between the number of reference memory errors at the 1st and at the 16th training day in any environmental condition during the same period (Fig. 2).

Having the old enriched animals clearly reduced their maze times during reference memory task without increasing their number of errors, it is possible to assert that they showed a learning performance during those days while the standard ones did not.

3.2. Biochemical results

3.2.1. Nitric oxide synthase activity

Nitric oxide synthase activity was measured in brain cytosolic fractions and in submitochondrial membranes at rates of 0.2–0.5 nmol NO/(min mg protein), in the same range of values



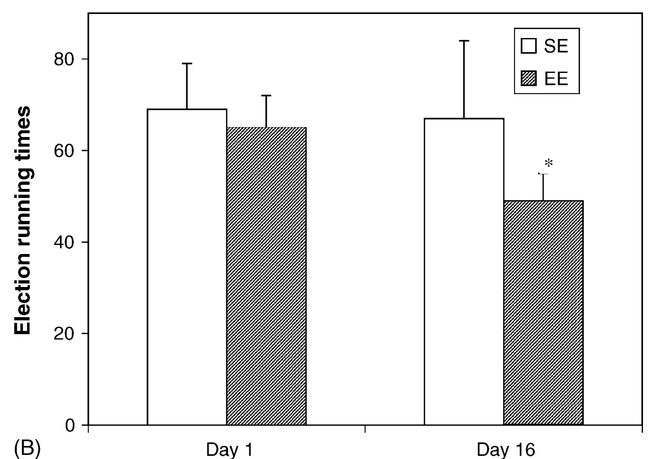


Fig. 1. Working memory performance for enriched and standard reared rats, evaluated as: (A) criteria days number, and days with 0 WM errors and (B) election running times from days 1 to 16 of training. Bars represent mean values from 15 to 14 rats, ±S.E.M. $^{*}p < 0.02$; $^{**}p < 0.005$.

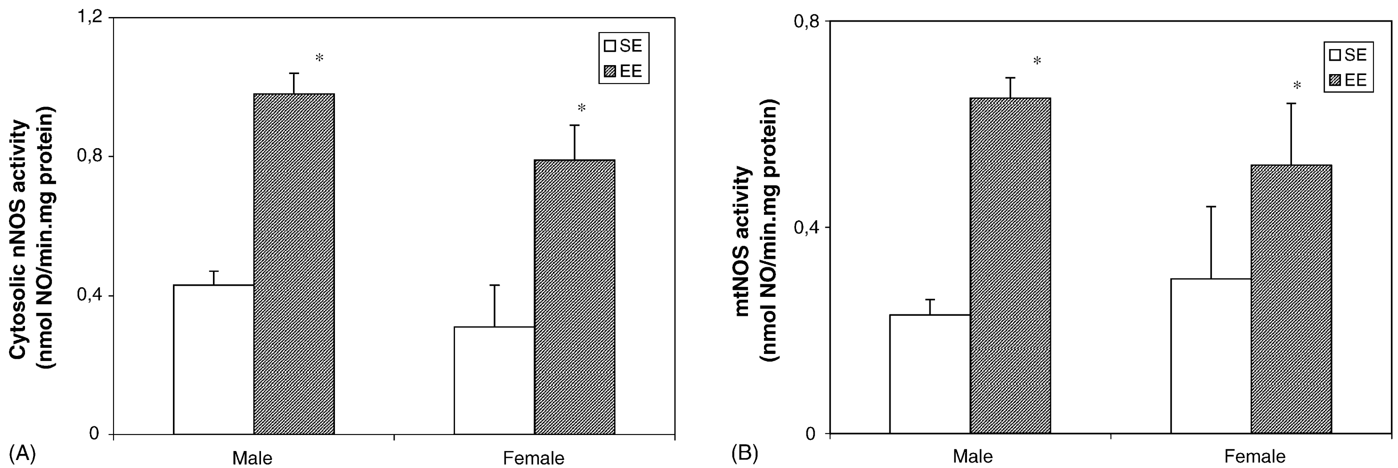


Fig. 3. Effect of enriched environment on: (A) cytosolic NOS activity and (B) mtNOS activity, of male and female rats. Bars represent mean values from 4 to 6 rats, ±S.E.M. $^{*}p<0.05$.

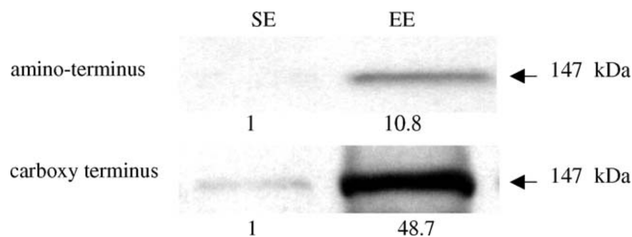


Fig. 4. Western blot analysis of the mtNOS of rat brain mitochondrial membranes from standard and enriched reared rats. nNOS antibodies against amino and carboxy terminus were from Santa Cruz Laboratories (Santa Cruz, CA, USA).

of the previously reported rates of NO production in brain tissue: 0.5–1.6 nmol/(min mg protein) [31,34,35].

Cytosolic NOS activity was 128 and 155% higher in male and female rats from enriched environments, as compared with control male and female rats, respectively (Fig. 3A). mtNOS activity was 183 and 73% higher in male and female rats from enriched environments as compared with control animals (Fig. 3B).

3.3. Western blot analysis of nitric oxide synthases

Brain mtNOS has been previously identified by Western blot analysis mainly as nNOS and also but to a lesser extent as eNOS [36]. In this study, Western blot analysis identified an important amount of NOS, with a molecular mass of 147 kDa, reacting with anti-nNOS antibodies directed both against the carboxy terminus and the amino terminus of the protein (Fig. 4). The amount of brain mitochondrial nNOS protein detected by Western blot analysis using nNOS antibodies against amino or carboxy terminus groups was 10.8 and 48.7 times higher in preparations obtained from rats kept in enriched environments, as compared with control rats, as shown by densitometric analysis (Fig. 4).

3.3.1. Respiratory complexes activity

Activity of complex I was found 73% increased in male rats from enriched environments, as compared with the controls. An increase of 80% in complex I activity was observed in brain submitochondrial membranes from female rats kept in enriched environments, as compared with control rats. No differences were observed between rats from enriched environments and controls in the activity of respiratory complexes II–IV (Table 1).

3.4. Anatomical results

The observation of the parietotemporal cortex from both EE and SE reared animals showed large multipolar neurons scattered within the gray matter as well as a reactive fibres throughout layers II–VI (Fig. 5A and B). This is in agreement with previous description of the piriform, cingulate and frontal cortex [54]. However NADPH-d reactive neurons observed in the cortex of EE reared animals showed bigger somas and longer and thicker processes (Fig. 5B and D). Cell area quantification showed a highly significant increase (50%) of the area of NADPH-d reactive neurons in the parietotemporal cortex of animals maintained in an enriched environment versus control animals ($p<0.01$) (Fig. 7).

Table 1
Activity of mitochondrial respiratory chain enzymatic complexes in brain submitochondrial membranes of SE reared rats and EE reared rats

	Male rats		Female rats	
	SE	EE	SE	EE
Complex I (nmol/(min mg protein))	14.3 ± 1.5	24.8 ± 0.8	18.7 ± 1.8	33.6 ± 8.3
Complex II (nmol/(min mg protein))	27.9 ± 3.5	32.3 ± 3.4	35.4 ± 4.1	34.4 ± 4.2
Complex III (nmol/(min mg protein))	20.8 ± 1.6	21.4 ± 2.1	23.7 ± 1.9	24.9 ± 3.9
Complex IV (nmol/(min mg protein))	131 ± 10	146 ± 12	126 ± 13	105 ± 5

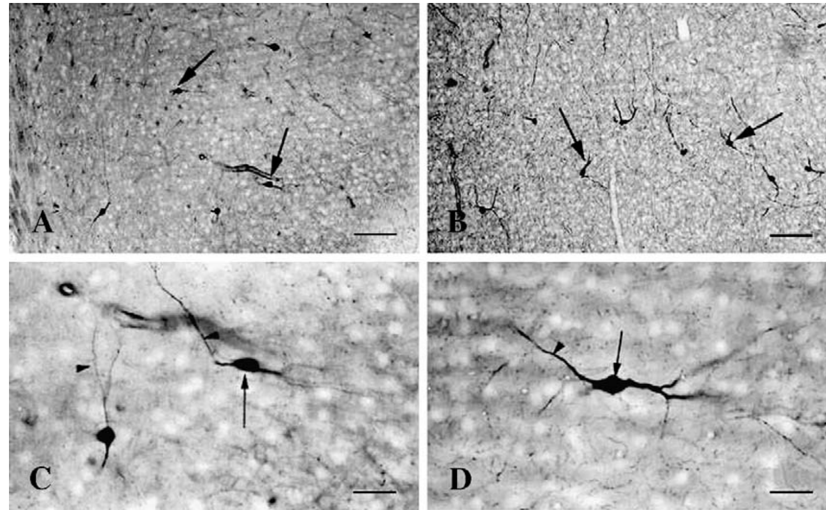


Fig. 5. Cortical sections of control animals (A and C) and animals reared in an enriched environment (B and D) stained with the NADPH-d histochemical technique. Multipolar reactive neurons (arrows) may be observed scattered in the different laminas of the cortex of control and enriched reared animals (A and B). Observe the increase of the soma size and the thicker and longer dendrites of the neurons from EE reared animals (compare B with A). A higher magnification of different NADPH-diaphorase reactive cortical neurons (C and D) shows in detail the increase of soma size (arrow) as well as longer and thicker dendrites (arrow heads) of a cortical neuron from an EE reared animal compared with neurons from a SE animal (arrows) (compare D with C). Scale bar: (A and B) 50 μm; (C and D) 20 μm.

The hippocampus (CA1) of control animals showed only a few small NADPH-d reactive cells in the pyramidal cell layer (Fig. 6A). However, sections from EE reared animals showed that NADPH-d reactive neurons in pyramidal cell layer were bigger and had longer processes (Fig. 6B and D). Some intensely stained neurons with long dendrites were also observed in the stratum radiatum in EE reared animals (Fig. 6B). Cell area quantification showed a significant increase (35%) of the area of NADPH-d reactive hippocampal neurons of rats maintained in EE versus control rats reared in SE ($p < 0.05$) (Fig. 7).

4. Discussion

This work present evidence that EE prevent old rats from the aging dependent impairment of cognition and plasticity. Spatial WM deficits decrease in enriched rats, in association with an increased neuronal plasticity, as compared with standard reared rats.

Enriched animals showed better radial maze WM and LTM performances than the standard ones when young [37], but only WM performance advantage was maintained with

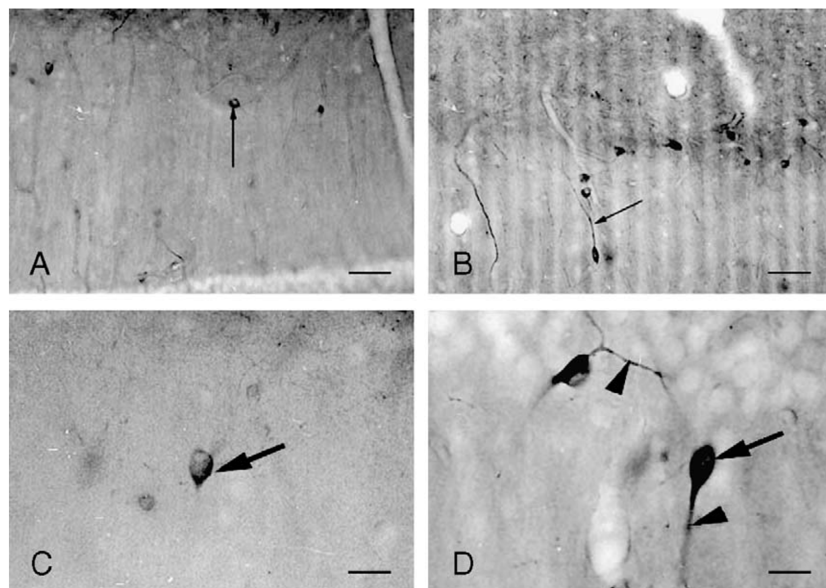


Fig. 6. Hippocampal sections (CA1 region) stained with the NADPH-diaphorase histochemical method. (A and C) SE animals and (B and D) EE animals. Very few small reactive pyramidal cells may be observed in hippocampal sections from control animal (arrow) (A). An increment in the area of cell somas and in the length of cell processes (arrows) may be observed in the sections of EE reared animals compared with the control sections from SE animals (compare B and A). A higher magnification of different NADPH-d reactive hippocampal neurons from CA1 shows in detail the increment of the cell soma size (arrow) and dendritic length (arrow head) (compare D with C). An increment in the intensity of NADPH-d reactivity may also be observed in sections EE reared animals compared with SE animals (compare D with C). Scale bar: (A and B) 15 μm; (C and D) 7.5 μm.

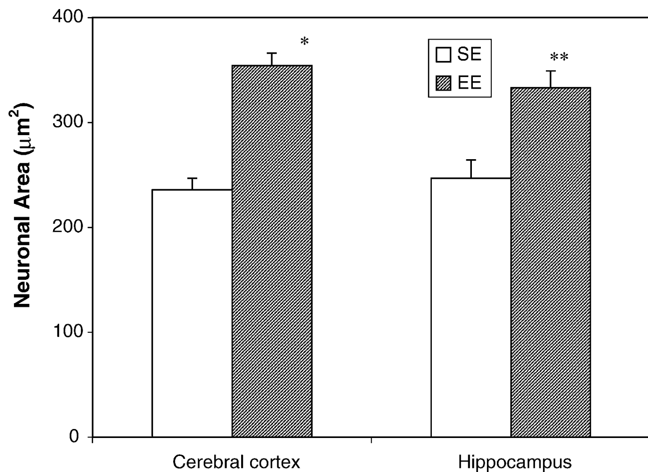


Fig. 7. Measurements of the area of NADPH-d reactive cortical and hippocampal neurons of animals reared in enriched environments (EE) and their controls (SE). Cerebral cortex: $N = 3$, $^{*}p < 0.01$, Hippocampus: $N = 3$, $^{**}p < 0.05$.

aging. Although spatial LTM acquisition needs of hippocampal integrity, retrieval of stored memories did not [16]. Particularly, when animals have repeated LTM training during a prolonged period, the involvement of the hippocampus in that kind of memory became minimal [16]. As old rats have been trained in LTM very often during their lifetime, the lack of their previous differences in their last training should not be related with hippocampal plasticity deficits in enriched animals.

Sex differences in plasticity could also be related with learning, because males are more skillful in spatial tasks. Being the use of distal visual cues the favorite strategy of males at the maze, it could be considered that the task was more appropriated for males than for females.

It has been described that spatial learning in rats need NO–cGMP pathway integrity at the CA1 region of the hippocampus; particularly, NMDA/NO/cGMP plays a role in spatial WM. In fact, NO has been proposed to act as a retrograde messenger during LTP induction [25] and is thought to be necessary for growth of nerve fibers, since nNOS is enriched in regenerating axons [20]. Activation of NMDA receptors, needed for the acquisition of LTP or PBP induces nNOS activity with the consequent formation of NO. Deletion of the genes that encode nNOS and eNOS isoforms reduce the inducibility of LTP, and selective NOS inhibitors impair spatial learning [22].

In our study, rats kept in enriched environment had higher NOS activity both in cytosolic and mitochondrial compartments, giving evidence that support a role of NO in synaptic plasticity associated to the prevention of spatial cognition impairment during the aging process. Also, mtNOS protein expression was higher in brain mitochondria from enriched-housed rats than in standard environment rats, suggesting an up-regulation of the mitochondrial enzyme. Mitochondrial NOS activity appears to be regulated by different experimental conditions: it is inhibited by CNS drug treatment such as antipsychotics haloperidol [34] and chlorpromazine [35], and it is up-regulated in a model of experimental hypoxia [53]. Riobó et al. [47] recently recognized a different and complementary sequential expression of mtNOS and nNOS during rat brain development [47].

Results from this laboratory have shown that mtNOS expression and activity decrease with aging: rat brain mtNOS activity is 54% lower in 27 months rats as compared to 40 days old rats (data not shown). This is in agreement with previous data reported by Navarro et al. showing that brain mtNOS activity decreased almost linearly upon mice aging from 28 to 76 weeks [44]. The loss of NOS activity associated to the aging process may be prevented by enriched environment exposure thus contributing to a situation of higher NO production acting as a messenger for neuronal plasticity-related pathways.

Ultrastructural changes in NOS inmunoreactivity have been analyzed by histochemical studies using the NADPH-d method in different experimental situations in rat brain, such as perinatal asphyxia [33], and a model of serotonin depletion [51].

The distribution of nitrergic neurons from hippocampus and cortex of both EE and SE reared animals, revealed by the histochemical studies using the NADPH-d method is in agreement with previous descriptions of the literature [54]. The results obtained by the NADPH-d histochemical method with brain sections of rats reared in enriched environments showed a significant increase in the area of somas and dendritic processes of cortical and hippocampal neurons compared with those observed in brain sections of animals reared in standard environments.

In aging, the reduction in brain volume is now to be considered to be due mainly to reduction in connections and surrounding tissue. Stereological studies have shown that there is no loss of hippocampal and subicular neurons in aging rats; the age-related deficits in spatial memory seem to correlate with structural parameters other than the number of neurons in the hippocampus proper and the subiculum [46]. In our study, the increased area of somas and dendritic processes in cortical and hippocampal neurons of EE rats, probably reflects an increased number of spines and synapses.

As the NADPH-d histochemical method specifically shows NOS containing neurons in aldehyde-fixed tissue [7,13,23,54], it may be concluded that an increase of NO synthesis may be involved in the observed memory improvement in old animals reared in an enriched condition. These results are in agreement with biochemical determinations of NOS activity and expression.

Together, these results may suggest that NO could take part in mechanisms involved in improving the performance of spatial working memory in old rats reared in enriched environments. But we cannot discard that the improvement in the cognitive performance after exposure to enriched environment could be a consequence of the augmented number of neuronal spines and synapses, leading to an increased NO production.

Growing evidence indicates that changes in mitochondrial gene expression are involved in the developmental neuroplasticity. Yang et al. [57] reported the identification of a subset of mitochondrial genes that are regulated by visual experience in the first months of life: several genes (ATPase 6, cytochrome *b*, NADH dehydrogenase subunits 4 and 2) showed elevation in normal cats at 5 weeks and in dark-reared cats at 20 weeks (“plasticity genes”); others (NADH dehydrogenase subunits 3 and 5) showed the opposite (“anti-plasticity genes”) [57].

The aging process is associated with a reduction in complexes I and IV of the mitochondrial respiratory chain, as seen in aging rats and mice, whereas complexes II and III are unaffected [32,42,43]. Besides, evidence has been given of a decrease in complex I of the mitochondrial electron transfer complex in the substantia nigra of patients with Parkinson's disease [39] and of a decrease of NADH dehydrogenase (subunit 4) gene expression in the hippocampus, inferior parietal lobule and cerebellum of Alzheimer's disease patients [1].

Our results showing increased complex I activity in association with neuronal plasticity would support the idea that enriched environments contribute to prevent the loss of this respiratory complex activity and protect brain mitochondria from aging and neurodegenerative disorders-induced damage.

We conclude that long lasting environmental conditions, as the critical variable in significant differences between both groups of study, lead to an increased NO production and mediate a protective mechanism that preserves hippocampal function, spatial learning and memory in aged animals as well as rises longevity. It would be a question of the major interest to investigate the mechanisms by which longevity and learning and plasticity preservation are associated, as it seems to be the case in our experiment.

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