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Prenatal stress induces alterations in cerebellar nitric oxide that are correlated with deficits in spatial memory in rat's offspring

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ABSTRACT

Prenatal stress (PS) has been linked to abnormal cognitive, behavioral and psychosocial outcomes in both animals and humans. Since PS has been shown to induce a cerebellar cytoarchitectural disarrangement and cerebellar abnormalities that have been linked to an impairment of behavioral functions, the aim of the present work was to investigate whether the exposure to PS in a period in which the cerebellum is still immature can induce behavioral deficits in the adult and whether this alterations are correlated with changes in nitric oxide (NO) and cellular oxidative mechanisms in offspring's cerebellum. Our results show impairments in spatial memory and territory discrimination in PS adult rats. PS offspring also displayed alterations in cerebellar nitric oxide synthase (NOS) expression and activity. Moreover, a correlation between spatial memory deficits and the increase in NOS activity was found. The results found here may point to a role of cerebellar NO in the behavioral alterations induced by stress during early development stages.

1. Introduction

During the prenatal period animals are vulnerable to factors, such as maternal stress, that can disrupt homeostasis and therefore affect development. Recent research in humans has related psychosocial work stress during pregnancy to postnatal consequences, namely the decrease in birth weight and gestational time (Lee et al., 2011). Moreover, animal studies and preliminary human studies have linked PS with alterations in development and behavioral disorders, and suggest that the effects of PS can persist long after birth (Vallee et al., 1997; Zuena et al., 2008). In fact, prenatal maternal stress can have long lasting effects on the offspring immune system and in neurocognitive functions (Ruiz and Avant, 2005).

Many studies have associated different models of prenatal stress (PS) with deficits in offspring's development. This study suggests that PS could predispose rats to behavioral abnormalities such as increased anxiety, greater tendency to drug addiction and depressive-like behavior (Weinstock, 2001; Maccari et al., 2003; Mueller and Bale, 2006; Vallee et al., 1997). There has also been reported an alteration in circadian rhythms (Van Reeth et al., 1998). Behavioural studies have shown that PS offspring exhibit increased exploratory behavior and increased locomotor activity (Vallee et al., 1997; Van den Hove et al.,2005) as well as impairments in learning and memory performance (Wu et al., 2007). Deficits in social behavior are found in several neuropsychiatric disorders with a presumed developmental origin (Patin et al., 2005).

The cerebellum (CE), which has traditionally been related to motor functions, is currently being redefined as a structure with a much broader functionality. Human and animal research models have involved the cerebellum in cognitive functions, such as attention, perception, language and particularly working memory and spatial orientation. Animal models of cerebellar injuries of genetic origin or artificially induced, have demonstrated the role of the cerebellum in spatial orientation (Martin et al., 2003, 2004). It has also been suggested that the cerebellum is involved in emotional behavior and motivation, the latter evaluated as motivation to explore new environments in animals in which cerebellar Purkinje and granular cells were injured (Caston et al., 1998). The studies of Bobeé et al. (2000) describe the role of the cerebellum in spatial cognition, suggesting that the cerebellar vermis is involved in motor control, attentional capabilities, territory discrimination and emotional behavior. Passot et al. (2012) have recently shown that cerebellar deficits may impact on the exploration–exploitation balance during spatial navigation. Moreover, Caceres et al. (2009) in a model of cerebellar citoarchitecture disarrangement suggest that the changes in spatial memory found by them might be CE dependent. PS can produce long lasting changes in cerebellar granule cell layer structure and function, such as a decrease in the number of synapses (Ulupinar and Yucel, 2005). It has been suggested

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therefore that stress critically affects the development of neural communications and the establishment of networks (Ulupinar and Yucel, 2005; Ulupinar et al., 2006). To assess spatial memory performance, a full baited radial maze was chosen, since it is one of the most suitable tasks to measure spatial working memory abilities as well as the procedural strategies used (Leggio et al., 2006). In addition, the radial maze is neither aversive nor stressful (Hodges,1996; Belzung et al., 2001; Mandolesi et al., 2001). Success in solving the task depends on how well the rat remembers the last visited (and rewarded) arm of the maze.

Nitric oxide (NO) is one of the most versatile bioactive molecules identified. It is synthesized by nitric oxide synthase (NOS), which catalyzes the conversion of L-arginine into citrulline and NO, in the presence of oxygen and nicotinamide adenine dinucleotide 2′-phosphate, reduced form (NADPH) (Dawson and Dawson, 1996). Three different isoforms of NOS has been identified: neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II) and endothelial NOS (eNOS, type III). All three isoforms are expressed in the central nervous system (CNS). NO has a role as a neurotransmitter in the central nervous system (CNS) (Garthwaite et al., 1988; Dawson and Snyder, 1994) and has been involved not only in physiological but also in pathological processes (Dawson and Dawson, 1994; Yun et al., 1997). In addition, it plays an important role in the control of neural activity by diffusing into neurons and participates in learning and memory processes (Chen et al., 1997). It has been demonstrated the NO plays an important role in long term potentiation (LTP) in hippocampus (Hawkins et al., 1998) and long term depression (LTD) in cerebellum (Lev-Ram et al., 1997). NOS-containing neurons are found in many brain loci, including the cerebral cortex, cerebellum, hippocampus and hypothalamus. A high concentration of nNOS is present in cerebellar neurons, particularly in basket and granule cells and the pattern of localization provides a cellular basis for the involvement of NOS activity in the deleterious effects of different noxa on cerebellar function (Coyle and Puttfarken, 1993; Dawson and Dawson, 1996; Ikeda et al., 2001).

Since the cerebellum is rich in NOS-containing neurons and sensitive to the effects of PS, we investigated the influence of prenatal restraint stress on adult cerebellar NOS activity, and the relationship between cerebellar NOS activity and behavioral performance.

2. Materials and methods

2.1. Animals and experimental groups

Nulliparous female Wistar rats of three months of age, weighting aproximately 250 g, were purchased from School of Pharmacy and Biochemistry, University of Buenos Aires. The animals were housed in an animal room on a light–dark cycle of 12:12 h and room temperature was held constant at 22 ± 2 °C. Tap water and comercial food were available *ad libitum*. After their arrival, the female rats were housed (three per cage) to synchronize their estrous cycle. Females were then placed with a sexually experienced male for a night (the following day the appearance of a vaginal plug was determined, being designated as day 0 of gestation), after which they were housed individually in Plexiglas cages. Pregnant females rats were then randomly assigned into two experimental groups: prenatal restraint stressed (PS) or control (C). Dams from C group were left undisturbed and dams from PS group were exposed to a restraint stress procedure (n = 10 in each group). All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996 and approved (Res. CD N°2962/10) by the Institutional Committee for the care and use of laboratory animals (CICUAL), School of Medicine, University of Buenos Aires. The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.2. Prenatal restraint stress protocol

Pregnant Wistar rats were subjected to a daily immobilization stress model in which animals were placed inside a transparent plastic device (7 cm diameter, 18 cm length), from day 14 of pregnancy until delivery (Maccari et al., 2003. A strict schedule was maintained for the treatment, performing three 45 min (min) immobilization periods per day, at 9, 12 and 15 h.

Male and female offspring were weaned 21 days after birth, and only male offspring from litters containing 10–12 pups with a comparable number of males and females were used for the experiments. Given that many of the long-term behavioral alterations caused by prenatal stress, as well as the structural alterations in brain areas controlling behavior, are gender specific (Reznikov et al., 1999; Rhees et al., 1999; Bowman et al., 2004; Tobe et al., 2005; Weinstock, 2007; Mueller and Bale, 2006; Zuena et al., 2008) and that the estrous and hormonal cycles could interfere with the behavioral parameters analyzed, we only used males. We found no differences in body weight and pup number between C and PS. A maximum of two male pups were taken from each litter, to avoid litter effect. After weaning, male rats from each experimental group (control or PS) were housed in groups of three and maintained under the same environmental conditions until the experiments were started. Animals from different postnatal ages (PN) were used (PN 7, 15 or 90 days for biochemical studies and PN 90 for behavioral studies).

2.3. Drugs

Most of the drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA). nNOS antibody was obtained from BD Company (Miami, USA) and ^{14}C-Arginine was from PerkinElmer Life and Analytical Science (Boston, MA, USA). Dowex AG 50WX-8 was purchased from Bio Rad. All other reagents were provided by Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Behavioural evaluation

Nine to ten PN 90 animals per group (control and PS) were used for behavioral evaluation.

2.4.1. Radial arm maze (RAM)

For spatial working memory evaluation a radial arm maze test, adapted from Caceres et al. (2009), was used. All eight arms were baited. Rats had to learn to visit each arm, only once per session. During the tests, animals would therefore have to remember which food reward shad already been eaten. In order to orient themselves in space, animals would have to look up ward: for these reasons, several extramaze cues were placed around the walls of the behavioral room. *Apparatus*: A custom-made, automated, eight-arm metallic radial maze was used. It consisted of eight identical arms (40 × 10 cm), radiating from a circular platform (20 cm in diameter), with 35 cm high surrounding walls. Each arm was equipped with two infrared diodes located in both lateral walls, at 5 cm from the end of the arm and placed 3 cm above the floor. The sequence of photo cell beam interruptions was monitored so that each time the animal crossed the infrared diodes, a computer connected to the apparatus would receive a signal and compute that the rat had successfully entered the arm, providing an on-line display of the animal's location. An opaque cylinder (20 cm diameter × 26 cm high) was placed over the central platform. Training procedures were done between 10 and 12 a.m. The RAM was situated in a

room measuring 2.6 × 2.3 m. This area had extra maze posters (70 × 60 cm) on each of the three walls to provide visual cues, consisting of vertical stripe lines, and black and white squares or circles (Hodges, 1996).

Procedure: At the beginning of each session, the animal was placed in the central platform inside the opaque cylinder for 10 s. Then, the cylinder was removed and the session started, with the rat being able to explore freely. When the rat entered all eight arms, or if the maze had not been completed in 15 min, the session was considered finished. This training lasted for 5 consecutive days. The maze was cleaned with ethanol 70% between each rat to minimize olfactory intra-maze cues. Chocolate cereal was used as reward (Caceres et al., 2009).

Data analysis: Rats' performances were evaluated by measuring different spatial and exploratory parameters. *Exploratory parameters:* Total time needed to complete the maze for each rat at each session was recorded. *Spatial parameters*: Number of errors (reentry to an already entered arm before completing the maze) and sum of error number (sum of 5 sessions) were determined.

2.4.2. Territory discrimination

This test was adapted from Patin et al. (2005), and consists in quantifying the animal's ability to enter and explore a novel territory (unknown) while it has access to its own one. Territory is defined as a familiar place in which the animal lives regularly. A compartment occupied by an animal during 24 h can be considered as its territory (Hamidou, 1994). *Apparatus:* a custom made box was utilized. The box had two compartments (A and B), separated by a third, smaller, one. The A compartment represented the territory of the tested animal in which it had stayed for 24 h and the B compartment the unknown territory, in which another rat had stayed for 24 h too. The test started when the animal was put into the starting box, with the walls which connected to the other two compartments having been removed, so that the rat could explore freely. Its behavior was recorded during 15 min. Testing took place under ambient laboratory lighting conditions, between 10 and 12 a.m. A camera was mounted vertically above the arena to record the animal's behavior. We evaluated how much time the animal spent in the territory in which it had stayed for 24 h (own) and in the new one (unknown), were the other rat had stayed.

2.5. Biochemical evaluation

For biochemical studies the brains were removed by decapitation at different postnatal ages (PN 7, 15 or 90 days). The cerebella were dissected out on an ice-cold glass petri dish. Tissues were immediately weighed out, used or stored −80 °C for biochemical determinations.

2.5.1. NOS enzymatic activity determination

NOS activity was determined by measuring the conversion of [U-^{14}C] arginine to [U-^{14}C] citrulline as described by Bredt and Snyder (1990). Briefly, the cerebella were pre-incubated in prewarmed 50 mM HEPES buffer, pH 7.4 and equilibrated with 5% CO_2 in O_2. Then CE were homogenized by sonication in 1 mL of medium containing 20 mM HEPES (pH 7.4), 1 mM dithiothreytol (DTT), 1 mM leupeptin and 0.2 mM phenylmethanesulphonyl fluoride (PMSF). An aliquot of the sample was incubated at 37 °C for 30 min in 5% CO_2 in O_2 in the presence of 0.45 mM $CaCl_2$ and [U-^{14}C] arginine (0.5 μCi). In addition, the same buffer without $CaCl_2$ and containing 1 mM EDTA was added to another aliquot of homogenate for iNOS activity determination. The reaction was stopped by quick ice-cold cooling and the samples were centrifuged at 14,000*g* for 10 min at 4 °C. Supernatants were passed through to 2 mL Dowex AG 50 WX-8 (sodium form) columns. [^{14}C] citrulline was eluted with 2 mL of distilled water and quantified by liquid scintillation counting. Calcium dependent activity was estimated by subtracting the activity in the absence of calcium from the activity in the presence of this ion (Zorrilla Zubilete et al., 2005).

2.5.2. Western blot

The cerebella were homogenized by sonication in lysis buffer (1:2 w/v) containing DTT 1 mM, PMSF 0.2 mM, Leupeptine 1 μM, Octylphenyl-polyethylene glycol (Igepal) 0.5%. The homogenates were centrifuged at 12,000*g* for 5 min at 4 °C. An aliquot from the supernatant was taken for protein determination by Bradford technique. For western blot analysis, lysate aliquots containing 50 μg of protein were denatured by the addition of loading buffer (Tris–HCl 50 mM pH 6.8, SDS 1%, glycerol 10%, bromophenol blue 0.05% y 2-β-mercaptoethanol 140 mM) and subsequent boiling for 3 min. The samples were then separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, UK) using a Bio-Rad Transblot Mini II (100 V constant voltage for 1 h at 4 °C). Proteins on PVDF membranes were visualized by reversible staining with Ponceau-S solution (Sigma, Co., MO, USA) and washed in tris-buffered saline (TBS). Membranes were blocked at 4 °C in milk buffer (5% non fat dry milk in TBS Tween 0.1%) and then incubated overnight at 4 °C with polyclonal antibodies for nNOS. The mouse polyclonal anti-nNOS was used at 1:1000 dilution in milk buffer (5% non fat dry milk in TBS Tween 0.1%). Subsequently, membranes were incubated for 1 h at room temperature with 1:2000 anti-mouse IgG–horseradish peroxidase-conjugated secondary antibodies (Abcam Inc.). After washing with TBS Tween 0.1%, membranes were analyzed by enhanced chemiluminescence (Amersham Biosciences, UK). The optical density (OD) of the bands on films was determined by quantitative densitometry with a computerized image processing system (UVP Labworks and Image J software). After stripping, the membranes were probed with anti β-actin antibody, to normalize the results.

2.5.3. Real-time PCR (qPCR)

2.5.3.1. RNA isolation for real time qPCR. PS or C rat pups were killed (as described above) at different ages (7, 15 and 90 days), the cerebellum was separated from the brain and immediately snap frozen in liquid nitrogen and stored at −80 °C. Each sample was then transferred into a sterile polypropylene centrifuge tube containing 1 ml of TRIzol solution (Invitrogen, Carlsbad, CA). Samples were homogenized for 10 s at room temperature, and incubated in the TRIzol solution for 10 min. Two hundred microlitres of chloroform were added to the sample and thereafter shaken vigorously. Each sample was incubated 5 min at room temperature and centrifuged at 12,000*g* for 15 min at 4 °C. Five hundred microlitres of isopropanol were added to the supernatant to precipitate RNA. Ten minutes later the sample was centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in 100 μl diethylpyrocarbonate (DEPC)-treated water. RNA was first treated with DNAse to eliminate any contaminating genomic DNA before the conversion of RNA to cDNA. In brief, RNA was placed into a 200 μl PCR tube to which the following solutions were added: 10 μl of 10× RQ1 buffer (Promega Corporation), 1.5 μl of RNAse-Free DNAse I (6.8 units, Promega), and nuclease-free H_2O to a final volume of 100 μl. The tube was placed into a thermocycler (Biometra) and incubated at 25 °C for 10 min. The resulting purified and concentrated RNA was quantified by fluorometry using a Nanodrop Spectrophotometer (Model 1000, Thermo Scientific). Total RNA integrity and size distribution were analyzed by denaturing agarose gel electrophoresis.

2.5.3.2. Retrotranscription and RT-PCR. Total RNA was reverse transcribed using oligo (dT) 12–18 nucleotide primers and Moloney

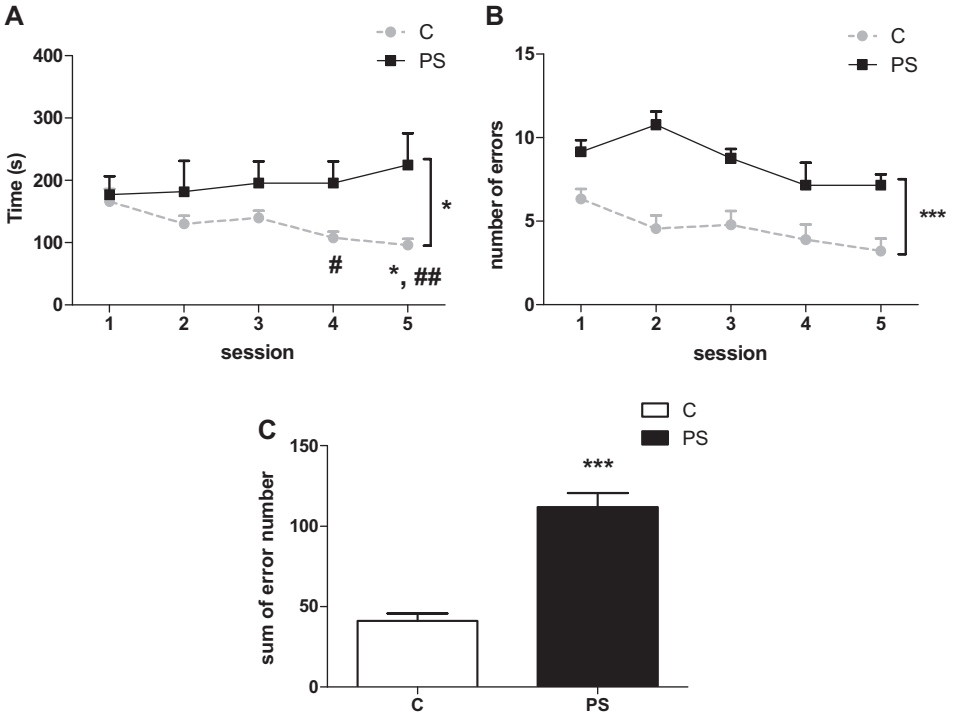

Fig. 1. PS induces alteration in spatial memory Performance of control (C) and prenatally stressed (PS) rat's offspring in the spatial memory task. (A) Total time needed to complete the radial arm maze (RAM) for adult life PN90 rats .(B) Number of errors in 5 consecutive trials, one per day. (C) Sum of errors computed on the 5 training sessions for C (open bar) and PS (full bar) rats. The values are presented as mean ± SEM (n = 9–12 rats per group). $^{*}p < 0.05$ respect to first session (in the same group) and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ respect to C respectively (in the same session).

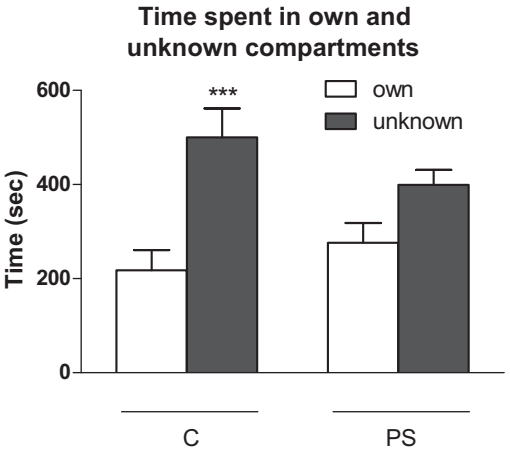

Fig. 2. Territory discrimination. Time spent in the own territory (open bars) and in the unknown territory (full bars), for PN90 C and PS male rats. Data are expressed as mean ± SEM. n = 10 animals per group. Significant difference between time spent in own and unknown territory at $^{***}p < 0.001$.

Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen) in a final volume of 20 μl at 42 °C for 50 min. Real-time qPCR was performed using 1 μL of cDNA for each reaction (equivalent to 50 ng of total RNA), 12.5 μL of SYBR Green/ROX qPCR master mix from Biodinamycs, 12.5 μl of ddH_2O and 2.5 μl of rat gene-specific primers for β-actin or nNOS, all from IDT (Integrated DNA Technologies, USA) in a final volume of 25 μL. All experiments were performed in triplicate. The PCR reaction mixture was run in the Rotor-Gene TM 6000 Real Time PCR-System (Corbett, Life Science). The thermal cycling conditions included an initial denaturizing step at 95 °C for 10 min, 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s, followed by melting curves to differentiate non-specific primer-dimmers and specific amplicon. Each cDNA sample was tested for target genes of interest and a housekeeping gene. To verify that the reagents were not contaminated with DNA, or that the primers do not produce a signal by dimmer formation, a no-template control sample was included for each primer set. Amplicons were analyzed by 4% agarose gel electrophoresis. β-Actin was used as a reference gene. Primers were designed from rat-specific sequences: β-actin forward primer, 5′-TGAGCGCAAGTAC TCTGTGTGGAT-3′, and reverse primer, 5′-TAGAAGCATTTGC GGTGC ACGATG-3′ (NM_031144). nNOS forward primer, 5′-CTGCAAAGCC CTAAGTCCAG-3′, and reverse primer, 5′-AGCAGTGTTCCTCTCC TCC-3′ (NM_052799).

2.5.3.3. Quantitative PCR data Analysis. Analysis was performed throughout the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001). The threshold cycle (CT) is defined as the PCR cycle number at which the fluorescence intensity crosses a manually determined threshold value, at a level where the fluorescent signal is appreciably above the background level but is still in the early exponential phase of amplification. Each of the 4 samples in each group was assayed in triplicate, the resulting CT values were averaged and the standard deviation (SD) calculated (rotor-gene software version 1.7). Next, for each pool of RNA the difference in the averaged CT values (CTavg) for a gene of interest (nNOS) and β-actin for a given experimental group was calculated and defined as ΔCT for each gene of interest (e.g.: $\Delta CT_{control} = CTavg_{nNOS} - CTavg_{\beta\text{-actin}}$). The ΔCT value for each of four pools for each experimental group was then averaged and the SD calculated. For each gene of interest the average ΔCT value for the control group was subtracted from the average ΔCT value of the experimental group (PS) resulting in the ΔΔCT (e.g.: $\Delta\Delta CT = \Delta CT_{PS} - \Delta CT_{Control}$). The error associated to ΔΔCT was equivalent to the standard deviation of the mean of the ΔCT for the experimental group. The data are

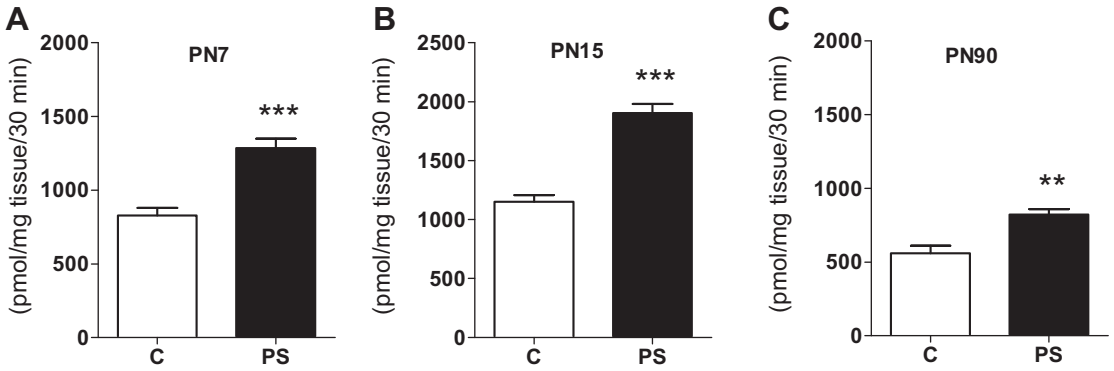

Fig. 3. Cerebellar NOS activity at 7, 15 and 90 postnatal days (PN) (A), (B) and (C) respectively. C: control (open bars) and PS: prenatally stressed animals (full bars). ^{14}C-citrulline production is expressed as pmol/g tissue/30 min. Values are expressed as mean ± SEM. $n = 6$ animals per group. $^{*}p < 0.05$, $^{***}p < 0.001$.

reported as fold change, which was calculated as $2^{-\Delta\Delta CT}$. This assumes doubling of products every amplification cycle or 100% amplification efficiency. Standard deviations (SD) from triplicates were propagated by using standard methods and confidence intervals were calculated as: $2^{-(DDCT \pm propagated\ SD)}$ (Livak and Schmittgen, 2001). The significance of the ΔΔCT values for each age was evaluated with a Student t test (InStat software) with a $p < 0.05$ considered to be statistically significant.

2.6. Statistical analysis

Significant differences between C and PS groups were evaluated. For the radial arm maze test, a two way ANOVA (2 groups (C and PS) × 5 session) with repeated measures test was used (Winer et al., 1991). When interaction was significant, simple effects analysis (F) was made. For total time analysis, as the variablés distribution of probability was not gaussian, a LOG10 transformation was performed. For territory discrimination test, a one way ANOVA was used. For NOS activity, western blot and qPCR, a Student's *t* test was used, as only two means were compared (each individual age was analyzed separately, no comparisons between different ages were performed). For *post hoc* comparisons Bonferronís Multiple Comparison Test or Tukey test were utilized. The data were expressed as mean values ± standard mean error (±SEM). The significance levels were fixed at levels of probability $p < 0.05$. Pearson's correlation test was used to examine the degree of correlation between behavioral data and neurochemical results.

3. Results

3.1. Effect of PS on behavior

3.1.1. Effect of PS on spatial learning

Animals from C and PS group were evaluated in adult life at PN90 in a RAM task. The results from two way ANOVA for total time show an interaction between session and group ($F_{4,76} = 2.69$, $p = 0.037$). Simple effects analysis showed a significant decrease in the time needed to complete the maze in C rats as expected, when comparing the first and the last session ($F_{4,76} = 2.66$, $p = 0.039$). In contrast, no differences were found between sessions in PS rats, showing an impaired learning ($F_{4,76} = 0.66$, $p = 0.617$). Simple effects analysis also shows a decrease in total time in session 4th ($F_{1,76} = 5.12$, $p = 0.027$) and 5th ($F_{1,76} = 10.93$, $p = 0.0015$) in C respect to PS rats. When we analyzed the number of errors, we found a main effect of group, showed in Fig. 1B (Two way ANOVA with repeated measures, $F_{1,76} = 55.10$, $p < 0.0001$ for group). There was also an effect of session, thus showing a decrease

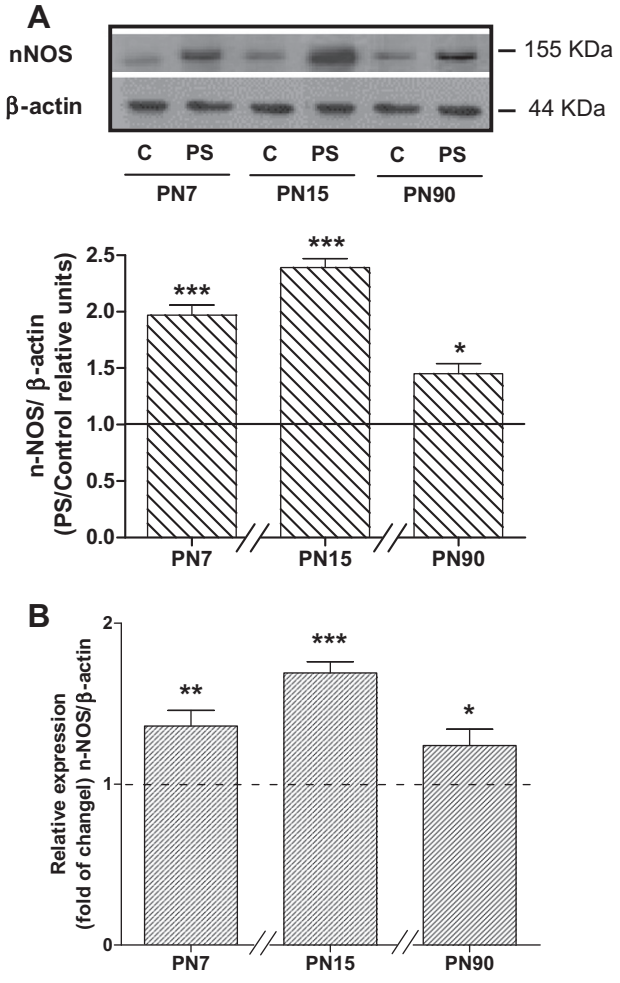

Fig. 4. Expression of nNOS in the cerebellum of control and PRS rats. (A) Immunoblots for cerebellar nNOS. Result are expressed as the ratio of the optical density (OD) of the nNOS band and the b-actin for PS animals relative to the OD control animals, at 7, 15 and 90 postnatal days (PN) that were processed separately. Values are expressed as means ± S.E.M. ($n = 7$ rats per group). $^{*}p < 0.05$, $^{***}p < 0.001$. (B) nNOS mRNA expression in the cerebellum evaluated by real time quantitative qPCR. Data are normalized against β-actin levels and expressed as relative fold changes compared to the control group. $n = 5$ animals per experimental group. nNOS mRNA at PN7, PN15 and PN90 were processed separately, when compared with each respective control, was significantly increased. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

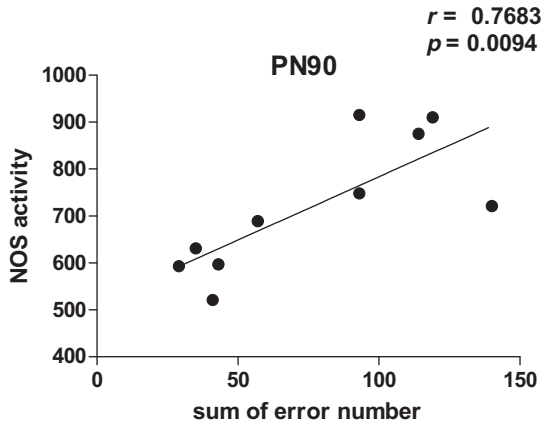

Fig. 5. Pearson correlation between sum of number errors and the total NOS activity (A), $r = 0.7683$, $p = 0.094$. Correlation between rank of first error and total NOS activity in adult rats (B), $r = -0.6552$, $p = 0.0398$ were performed in adult rats.

in the number of errors between the first and the last session ($F_{4,76} = 3.84$, $p = 0.0068$), which would indicate that both C and PS rats acquired the task. There was no interaction between treatment and session ($F_{4,76} = 1.06$, $p = 0.38$). Overall, results from this part show that both C and PS animals reduced the number of errors throughout the sessions, but controls committed fewer errors than PS rats. When the sum of the errors computed on the 5 training days for each animal was compared between groups, we found a significant increase in this parameter in PS animals at PN90 ($t_8 = 7.082$, $p = 0.0001$) (Fig. 1C). Altogether, we can conclude that adult PS rats needed more time to complete the RAM task, and that this would have been caused by an increase in the visits to arms already visited before ending the task.

3.1.2. Territory discrimination

In this task we found that C rats stayed in the unknown compartment more than twice the time they spent in the known one ($F_{3,36}$: 7.616, $p < 0.05$; Bonferroni *post hoc* test, $T = 4.363$, $p < 0.001$). For PS rats, there was no significant difference between the time the animals spent in the known and unknown compartment, showing no preference for either one (Bonferroni *post hoc* test, $T = 1.896$, $p > 0.05$) (Fig. 2).

3.2. Effect of PS on cerebellar nitric oxide

3.2.1. PS induces an increase in cerebellar NOS actvity

The production of ^{14}C-citrulline in cerebellum homogenates for C and PS animals, expressed as pmol in 30 min of reaction, is shown in Fig. 3. The assay was performed to evaluate short- (PN7, PN15) and long-term (PN90) alterations in NOS activity. The figure shows that PS animals presented a significant increase in total NOS activity at PN7 ($t_{11} = 5.451$, $p = 0.0002$) (Fig. 3A), PN15 ($t_{10} = 7.936$, $p < 0.0001$) (Fig. 3B) and P90 ($t_{11} = 4.261$, $p = 0.0013$) (Fig. 3C). No significant differences were found between C and PS groups when EDTA was added to the incubation medium, therefore, there were no differences in the activity of calcium-independent iNOS isoform between both groups (data not shown). NOS activity was inhibited in the presence of the inhibitor L-NAME for all ages studied, thus showing that citrulline production was indeed due to NOS activity (data not shown).

3.2.2. PS leads to long term increases in cerebellar nNOS levels

We examined the expression of nNOS isoforms in the cerebellum at different postnatal ages (PN7,15 and 90) in control and PS animals by immunobloting. The blot revealed a 155 kDa band wich corresponds to nNOS (Fig. 4A). PS exposure increased the steady-state leves of nNOS protein levels in the cerebellum at PN7 ($t = 7.204$, $p < 0.001$), PN15 ($t = 11.7$, $p < 0.001$) and PN90 ($t = 3.102$, $p < 0.05$). Optic density (OD) values for each band were relativized to the OD values of β-actin for each lane. The results are presented as the ratio between the value of optic density (OD) for PS animals and the value of OD for control rats.

3.2.3. nNOS mRNA expression in rat cerebellum

nNOS mRNA expression levels in the cerebellum were determined by real time qPCR and the data was normalized to the level of β-actin mRNA, a non-regulated reference gene (Fig. 4B). Data are expressed as fold change, where the data for the control group, the reference, was assigned 1-fold (no change or normative value) for PN7, PN15 and PN90 rats. There was a significant increase in the expression of nNOS mRNA in the cerebella at PN7 ($t = 25.49$, $p < 0.01$), PN15 ($t = 94.70$, $p < 0.001$) and PN90 ($t = 8.06$, $p < 0.05$).

3.3. Correlation analysis

As shown in Fig. 5, the Pearson's correlation test showed a positive correlation between the sum of error number and the cerebellar total NOS activity at PN90 rats ($r = 0.7683$, $p = 0.0094$).

4. Discussion

The results of the present study show deficiencies in spatial memory in adult animals which were correlated with alterations in nitric oxide levels in the cerebellum of prenatally stressed rats. Many studies have shown that environmental factors to which a mother is exposed during pregnancy influence brain development and subsequent functioning of offspring's CNS (Charil et al., 2010; King and Laplante, 2005). The behavioral consequences of prenatal stress cannot be explained by a single pathway. On the contrary, there seems to exist a great variety of mechanisms involved in the regulation of systems as complex as the nervous system (Kaiser and Sachser, 2005). Given that nitric oxide is an important mediator of neural function, in this work we wanted to evaluate the participation of cerebellar NO in the behavioral alterations induced by prenatal restraint stress. The hippocampus is one of the classic structures associated to cognitive processes. However, lately, the cerebellum has been redefined as a structure with a wide functionality (Le Marec et al., 1997). Certain investigations in humans and animal models involve the cerebellum in cognitive functions (Martin et al., 2003; Mandolesi et al., 2007; Manda et al., 2008). The cerebellum is one of the first brain structures to differentiate. However, it achieves its mature configuration many months after birth (Wang and Zoghbi, 2001). For this reason the cerebellum is especially vulnerable to developmental alterations under stress.

In this study, our results show that prenatal stress exposure induced long-lasting impairments in spatial memory performance. These impairments were observed in PS adult animals, 90 days post prenatal stress and without another exposure to stress during adult life. Thus, the offspring from stressed mothers showed a higher quantity of errors, along with longer total time in the spatial memory test. In the territory discrimination test we found that, while C animals spent more time in the unknown compartment, showing an innate preference for novelty, as expected (Poltirev et al., 1996), PS rats did not show a preference for either the known or the unknown one. This could suggest that PS rats have increased anxiety and therefore show an aversion to a novel environment. On the other hand, the results found here might implicate that PS rats could not differentiate the two compartments. Further studies are needed to find out which one of these two hypotheses would be more accurate. It is not always easy to dissociate motoric problems

from memory deficits displayed by animals with cerebellar damage. Therefore, CE must be considered a part of a large system involved in spatial memory that includes the frontal cortex, posterior parietal cortex, inferior temporal cortex, striatum, hippocampus and basal ganglia (Petrosini et al., 1998).

NO is an important regulator of the neural function and it possesses a principal role in synaptic plasticity, being neuroprotective at low levels and neurotoxic at higher concentrations (Colasanti and Susuki, 2000). At the same time, NO is a retrograde signaling molecule that might be involved in learning and memory processes conserved through evolution (Edwards and Rickard, 2007). Even though the effects of prenatal stress on cognitive alterations have been previously described, and the role of NO in this model has been briefly mentioned in our NO review (Rettori et al., 2009), its involvement has not been fully elucidated. In order to analyze if this model of prenatal stress induces changes in NO production, the activity of different NOS isoforms in cerebellum homogenates was analyzed, observing an increase in calcium-dependent NOS in prenatally stressed animals when compared to control animals. This increase in NO might drive to neurotoxicity mechanisms in the studied tissue, inducing long-term alterations in the circuits involved in memory processes. It is known that a high production of NO induces neurotoxicity by oxidative stress and that it is directly implied in neurodegenerative processes (Lipton, 1999; Iadecolo, 1997). When protein levels from stressed animals' offspring's cerebellum were determined, an increase in nNOS was found at different ages. At the same time iNOS expression did not vary in a significant manner in the analyzed tissue (data not shown). It is of interest to highlight that real time qPCR studies showed an increase in nNOS isoform expression in cerebellum from prenatally stressed animals in all the evaluated ages. Moreover, the increased cerebellar NOS activity correlated with a poor spatial memory performance in PS male rats.

Given the complexity and interactions present in the circuits involved in cognitive processes in the nervous system, the NO system cannot be thought to be the only factor involved in the alterations here observed. However, the results found here allow us to suppose that in this model NO might have an important participation in degenerative processes induced by stress during early development stages, which may be expressed later in life as an altered behavioral outcome.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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