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Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats

Bárbara Aisa, Rosa Tordera, Berta Lasheras, Joaquín Del Río, Maria J. Ramírez*

Department of Pharmacology, School of Medicine, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain

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Summary
Exposure to early stressful adverse life events may increase vulnerability to psychopathology in adult life. There are important memory disturbances in stress-related psychiatric disorders. Therefore, there is much interest in understanding the mechanisms responsible for interactions between stress and cognition. Male Wistar rats that experienced 3-h daily separations from the dam during the first 3 weeks of life (maternal separation, MS) showed in adulthood a depressive-like behaviour in the forced swimming test, increased hypothalamic–pituitary–adrenal (HPA) axis responsiveness to stressors and elevated CRF mRNA in the paraventricular nucleus of the hypothalamus (PVN). In the hippocampus of MS rats, there was a lower glucocorticoid receptor density. MS produced significant learning impairments both in the Morris water maze and in the novel object recognition test (NORT). The glucocorticoid receptor antagonist mifepristone and the β-adrenoceptor antagonist propranolol were able to completely reverse the increased immobility time in the forced swimming test and the memory deficits in the NORT observed in MS rats. Our data support the hypothesis that elevated secretion of glucocorticoids may be associated to behavioural and cognitive deficits in MS rats. The stress hyperresponsiveness observed in MS rats could be attributed, at least in part, to an impaired feedback sensitivity mediated by hippocampal glucocorticoid receptors. It can also be suggested the possible involvement of the noradrenergic system in cognitive impairments mediated by glucocorticoids in the MS model.

1. Introduction

Depression is a common mental disorder, and currently a major public health concern. Although to date the underlying neurobiology of depression remains elusive, there is an increasing evidence implicating stress in brain disturbances

*Corresponding author. Tel.: +34 948425600; fax: +34 948425649.
E-mail address: mariaja@unav.es (M.J. Ramírez).

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thought to underlie certain forms of depression or particular components of the depressive syndrome (Kessler, 1997; Kendler et al., 1999; Van Praag, 2004). There are important memory disturbances in stress-related psychiatric disorders (Bremner and Narayan, 1998; Bremner et al., 2003), and therefore, there is much interest in understanding the molecular mechanisms responsible for interactions between stress and cognition. The hypothalamic–pituitary–adrenal (HPA) axis is an essential component of an individual's capacity to cope with stress and in fact, a hyperactivity of the HPA axis is observed in the majority of patients with depression (review by Arborelius et al., 1999; De Kloet et al., 2005). Stress stimulation of the axis starts when corticotropin releasing factor (CRF) released by the paraventricular nucleus of the hypothalamus (PVN) stimulates the release of corticotropin (ACTH) from the anterior pituitary, which in turn, stimulates secretion from the adrenal cortex. Many of the behavioural consequences of stress are thought to be mediated by the activation of the glucocorticoid receptor by stress-induced high levels of glucocorticoid hormones (De Kloet et al., 1998; Oitzl et al., 2001; Roozendaal et al., 2006a) and subsequent alteration in gene expression (see review by Berton and Nestler, 2006).

It is well documented that corticosteroids modulate learning and memory processes in animals and humans in a complex manner. A transient increase in circulating corticosteroids associated with a learning task has been shown to facilitate memory consolidation (Roozendaal et al., 1999; Buchanan and Lovallo, 2001; Abercrombie et al., 2003). However, it has also been shown that acute stress produces a deficit in hippocampal long-term potentiation (Foy et al., 1987), and the elevation of circulating corticosterone concentrations by systemic administration of corticosterone or glucocorticoid receptor agonists exerts an inhibitory influence on learning and memory retrieval (Bodnoff et al., 1995; Roozendaal et al., 2003; Roozendaal et al., 2004b, c). It has been suggested that the effects of glucocorticoids on memory depend on noradrenergic activation of the amygdala and interactions of the amygdala with other brain regions, mainly hippocampus and frontal cortex (review by Roozendaal et al., 2006b).

There is compelling evidence that exposure to early stressful adverse life events may increase vulnerability to psychopathology in adult life. In fact, individuals who experience early trauma, such as parental loss, sexual abuse or physical assault in childhood, present an increased risk for suffering depression later in life (Heim and Nemeroff, 2001). Based on these arguments, it has been shown that prolonged periods (>1 h) of maternal separation (MS) during the first weeks of life result in animals with behavioural and neuroendocrine signs of elevated stress reactivity as adults (Anisman et al., 1998; Ladd et al., 2000; Lehman and Feldon, 2000; Ploj et al., 2003). In addition to an increase in immobility time in the Porsolt forced swimming test, anhedonia, and an enhanced anxiety-like behaviour, MS animals exhibit a dysfunction of the HPA axis reactivity to stress and therefore, the MS model in rat is considered nowadays as a robust model of enhanced stress responsiveness and depressive-like behaviour (Ladd et al., 2000; Van den Hove et al., 2005).

In this study we hypothesized that (i) neonatal MS would lead to cognitive deficits in adulthood; (ii) such an effect could be dependent on a dysfunction of the HPA axis, specifically to a hypersecretion of corticosterone. To test these hypotheses, Wistar rats that experienced 3-h daily separations from the dam during the first 3 weeks of life were tested in adulthood for HPA axis responsiveness, depressive-like behaviour, presence of cognitive deficits and the effect of the glucocorticoid receptor antagonist mifepristone on behavioural performance in MS rats. Furthermore, the involvement of the noradrenergic system in corticosterone-mediated effects will be also investigated.

2. Material and methods

2.1. Animals

All the experiments were carried out in strict compliance with the recommendations of the EU (DOCE L 358/1 18/2/1986) for the care and use of laboratory animals. Timed-pregnant Wistar rats were provided on gestation day 16 from Charles River Laboratories (Portage, MI, USA), individually housed in a temperature ($21\pm1\,^{\circ}$C) and humidity ($55\pm5\%$) controlled room on a 12-h light/dark cycle with food and water freely available.

2.2. Maternal separation

All litters were born within a 2-day period. As previously described (Huot et al., 2001; Ladd et al., 2004), on postnatal day (PND) 2, all pups were sexed and randomly assigned to the control group (animal facility rearing, AFR), pups were only briefly manipulated to change the bedding in their cages once weekly, or the separation group (MS), pups separated from their dam for 180 min from PNDs 2–21 inclusive. Before manipulation of the MS pups, each dam was removed from her home cage and placed in an adjacent cage and then, the pups were removed as complete litters, placed in an empty cage with standard bedding material and transferred to an incubator in an adjacent room. To compensate for the mother's body heat, the temperature of the incubator was adjusted to the age of the neonates: $32\pm0.5\,^{\circ}$C (PNDs 2–5), $30\pm0.5\,^{\circ}$C (PNDs 6–14) or $28\pm0.5\,^{\circ}$C (PNDs 15–21). Rats were weaned on PND 23 and only males were chosen for the present work. All subsequent experiments were performed in adulthood (60–75 days).

2.3. Experimental design and drug treatments

Different subsets of rats were used for the measurement of each of the behavioural or biochemical parameters studied. Animals from different litters were distributed over each of the experiments. It was also used a different subset of animals in which a forced swimming test of 15 min was used as an acute stressor.

Administration of mifepristone (10 mg/kg, SC) or propranolol (2 mg/kg, SC) took place 60 min before the behavioural testing. Mifepristone and propranolol were dissolved in saline.

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2.4. Behavioural test

For all behavioural testing, observers were blind to either the rearing condition or drug treatment. In a pilot set of experiments, the performance of a vehicle-injected group was compared to non-injected controls, and no differences were found in any of the behavioural test studied. A different animal cohort was used for carrying out each of the different test.

2.5. Locomotor activity

Horizontal locomotor activity was measured for 30 min in an open field, which consisted of nine square arenas ($43 \times 51 \times 45\,cm^3$) made of black wood, using a video tracking system (Ethovision 3.0, Noldus Information Technology B.V., The Netherlands), in a softly illuminated room. Tracking system was set to determine the position of the animal five times per second. Total path length (cm) was analysed.

2.6. Forced swimming

As described by Porsolt et al. (1977), two swimming sessions were conducted: an initial 15-min pretest followed 24 h later by a 5-min test. Rats were placed individually in a vertical Plexiglas cylinder (height: 45 cm, diameter: 19 cm) filled with 28–30 cm of 26 °C water. Immobility was considered as rats floating passively, making only small movements to keep its nose above the surface.

2.7. Sucrose intake

The protocol for sucrose preference was adapted from D'Aquila et al. (1997). At the start of sucrose intake testing, all animals were first trained to drink 1% sucrose solution for 24 h. Test consisted of presenting two bottles on the rat's cage (water and 1% sucrose) following a 20-h period of food and water deprivation, and allowing the rat to drink freely for 1 h. The position of the two bottles (right/left) was varied randomly across the animals. Sucrose and water consumption were corrected with the animals' weight.

2.8. Elevated plus maze

Rats were placed in the centre of a cross maze, facing an open arm, and allowed to explore the maze for 5 min. Two paws had to be inside the line indicating the entrance to an arms (Hogg, 1996), which signalled the start of the time spent in the specific arm. Time spent in the different arms of the maze was measured. The number of entries into the various arms of the maze was also determined, and served as a measure of the rat's locomotor activity.

2.9. Morris water maze

This memory task was performed as previously described (Diez-Ariza et al., 2003). The maze consisted of a black circular tank (140 cm diameter × 55 cm high) and filled with water (20–22 °C). A black invisible platform, 10 cm diameter, was positioned 1 cm below the water surface. A video camera was set above the centre of the pool and connected to a videotraction system (EthoVision; Noldus, The Netherlands). The pool was surrounded by white curtains marked with black geometric paintings, to enable the animals to learn the platform location. On the first day, each rat becomes habituated to the training environment for 60 s. In the acquisition phase (days 2–3), rats performed six training trials per day (120 s each) with the escape platform in a fixed position. Trials were started by placing the animals into the pool, close to and facing the wall at starting points designed as north, east, south, west, north, east. Time spent to reach the platform (latency); the swim path length (distance swam) and the swim speed (cm/s) were recorded. The results on time to reach the platform are not shown as, in all cases, parallel with distances swam. On day 4, (retention phase) a single transfer test was performed, in which the platform was removed from the tank. The animal was allowed to free swim for 60 s in search of the platform. The distance swam in the quadrant 3 (Q3) where the platform was previous located and the rest of quadrants were measured.

2.10. Object recognition

The object recognition test was adapted from Ennaceur and Delacour (1988). The open field consisted of a square open field (65 cm × 65 cm × 45 cm) made of black wood. On the previous day to the experiment, animals were familiarized with the field during 30 min. During the first trial of the experiment, two objects similar in shape, size, color, texture, etc., equidistant from the sides (10 cm) were placed within the chamber. The animal was placed into the centre of the open-field and allowed to freely explore for 5 min. It was considered that the animal was exploring the object when the head of the rat was oriented toward the object with its nose within 2 cm of the object. One hour later a second trial took place, in which one object was replaced by a different one, and exploration was scored for 5 min. In order to eliminate olfactory stimuli, chamber and objects were cleaned after testing each animal. To avoid preference for one of the objects, the order of the objects was balance between testing animals. Results were expressed as percentage of time spent with the novel object with respect to the total exploration time (discrimination index).

2.11. Tissue and blood collection

Rats were sacrificed by decapitation between 0800 and 1000 h. AFR animals were taken immediately from their home cages to collect tissue and blood. When the acute stressor was used, rats were sacrificed immediately after the 15 min stressor was finished. Brains were removed and dissected on ice to obtain the hippocampus, including the ventral and dorsal parts, and frontal cortex (for noradrenaline determinations and western blotting experiments), or frozen immediately at −40 °C in isopentane, and stored at −80 °C until sectioning (for CRF mRNA determinations). In a subset of experiments, a group of MS (and corresponding AFR controls) pups were sacrificed at the end of the

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separation period (PND 21). Trunk blood was collected into EDTA tubes, centrifuged at 1250*g* (15 min, 4 °C), and plasma was frozen until corticosterone and ACTH levels were determined.

2.12. Plasma corticosterone and ACTH determinations

Plasma corticosterone (30 μl) was determined using a commercially available enzymeimmunoassay kit (IDS OC-TEIA, USA) and ACTH was assayed in 200 μl plasma samples using the Allegro® HS-ACTH radioinmmunoassay kit (Nichols Institute, San Juan Capistrano, CA, USA), as previously described (e.g. Huot et al., 2001).

2.13. Noradrenaline determination

Noradrenaline content in the hippocampus and frontal cortex was measured using high performance liquid chromatography (HPLC) with electrochemical detection (Waters Spheribor® 5μ ODS2 4.6 × 150 mm). The mobile phase consisted of 80:20 (v/v) mixture of buffer (KH_2PO_4 0.05 M, pH 3) and methanol; the mixture was filtered and degassed through a 0.22 μm nitrocellulose membrane (Millipore, UK). Noradrenaline content was calculated by comparing with a 1 ng standard. The limit of detection was 1 pg/10 μl.

2.14. In situ hybridization for CRF mRNA

Coronal brain sections (15 μm thick) were serially cut with a cryostat at the level of paraventricular hypothalamus nucleus (−0.3 mm relative to bregma) according to the atlas of Paxinos and Watson. The sections were thaw-mounted on SuperFrost®Plus slides and then processed for in situ hybridization of CRF mRNA. Slides were fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer for 10 min. After dehydration through graded ethanol, sections were immersed in chloroform for 10 min, rehydrated with ethanol and air dried. The oligonucleotide used was 5′-AGGGCAGAGCAGTTAGCTCAGCAAGCTCAC-3′ (Sigma Genosis, UK). The probe was 3′-tail labelled with αS [^{35}S] dATP (specific activity >1000 Ci/mmol, Amersham Biosciences, UK). Negative controls including sense oligonucleotide showed minimal background signals. Relative abundance of CRF mRNA was determined by densitometry quantification of autoradiograms using the Microcomputer Imaging Device (Imaging Research, St Catherines, Ontario, Canada) corrected for non-specific signals. Optical density values were calibrated to ^{35}S tissue equivalents using ^{14}C microscales (Amersham, UK). Densitometric values from three sections of each animal were averaged and expressed as nCi/g tissue. CRF mRNA expression in MS groups was expressed as a percentage of their respective controls.

2.15. Western blotting

Cytosolic and nuclear extract preparations from the hippocampus of acutely stressed rats were homogenized in a 50 mM Tris buffer (pH 7.2, 4 °C), as previously described (Spencer et al., 2000). Each sample was adjusted to a final protein concentration of 4 mg/ml (DC protein assay; Bio-Rad, Hercules, CA). Extracts were mixed with Laemmeli's sample buffer boiled for 5 min. Samples (40 μg) were loaded onto 8% bisacrylamide gels and separated by SDS-PAGE. Separated proteins were electrophoretically transferred from gels to PVDF membranes. Glucocorticoid receptor protein was detected with the monoclonal antibody, GR M-20 (1:2000 TBST; Santa Cruz Biotechnology Inc., CA). Immunopositive bands were visualized by a chemiluminescent method (ECL; Amersham, Arlington Heights, IL). The optical density of glucocorticoid receptor-reactive bands (~97 kDa) visible on X-ray film was determined densitometrically (Murphy et al., 2002).

2.16. Data analysis

Data were analysed by SPSS for Windows, release 11.0. Normality was checked by Shapiro-Wilks's test ($p>0.05$). Behavioural data were analysed by unpaired *t*-tests (when only MS vs AFR groups are compared), or one-way or two-way analysis of variance ANOVA. Neuro- and bio-chemical data was analysed by unpaired *t*-tests (AFR vs MS groups). For all analyses, post hoc comparisons were conducted if appropriate, using Tukey protected least significance test. Data are presented as mean ± SEM and the level of significance for ANOVA and post hoc testing was set at $p<0.05$.

3. Results

3.1. Behavioural characterization

Locomotor activity was not modified by rearing (Student's *t*-test), and total path length travelled was 6910.01 ± 300.92 for AFR ($n=10$) and 7704.13 ± 378.52 ($n=10$) for MS rats.

In the Porsolt forced swimming test (Fig. 1A), MS ($n=10$) produced a significant increase in immobility compared with AFR ($n=10$) rats (Student's *t*-test, $p<0.05$).

In the sucrose intake test, there was an interaction [$F_{1,34}=3.66$, $p<0.05$] between rearing condition (AFR vs MS) and water/sucrose intake. Even though all animals tested showed preference for the sucrose solution compared with water, consumption of sucrose intake (sucrose/weight of the rat) was reduced in MS ($n=12$) rats compared with the AFR group ($n=12$) (Student's *t*-test, $p<0.01$), a finding that can be interpreted as a sign of anhedonia (Fig. 1B). There were no differences (Student's *t*-test) in water intake between AFR rats (0.018 ± 0.003 ml/g) and MS rats (0.012 ± 0.001 ml/g).

MS rats ($n=10$) showed increased anxiety-like behaviour (Fig. 1C), as statistical analysis revealed that MS rats spent a significantly smaller time in the open arms of the elevated plus maze than controls ($n=10$) (Student's *t*-test, $p<0.01$). No differences were found in the number of entries into the different arms of the elevated plus maze.

3.2. HPA responsiveness in MS animals

Fig. 2A shows an autoradiogram representative of sections hybridized for CRF mRNA in the PVN. Quantification of the signal (Fig. 2B, $n=5$ for both the AFR and MS groups)

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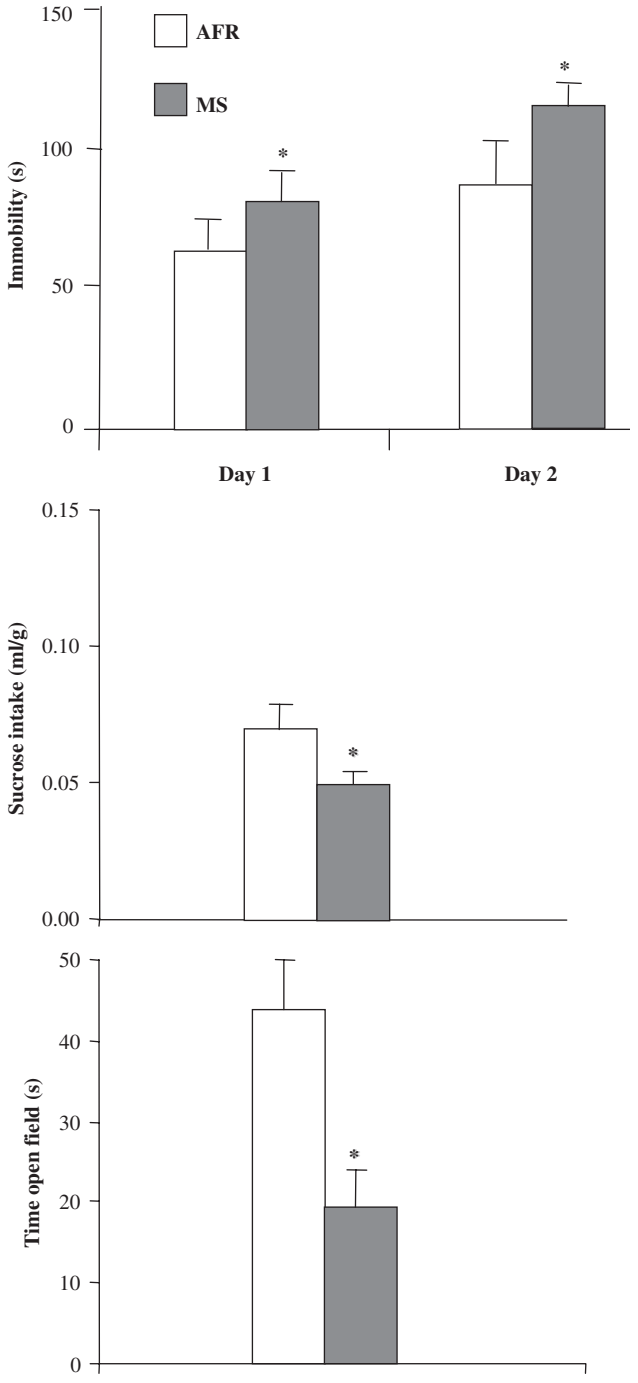

Fig. 1 Behavioural characterization of maternal separation (MS) rats (A) Effects of MS in the Porsolt forced swimming test. Results are expressed as immobility time. (B) Effects of MS in sucrose intake. Results are expressed as consumption of sucrose/weight of the rat. (C) Effects of MS in the elevated plus maze. Results are expressed as time spent in the open arms of the maze. $^{*}p<0.05$ vs control (AFR) rats, Student's *t*-test.

revealed an increase in CRF mRNA density in MS animals in the PVN (Student's *t*-test; $p<0.01$).

As shown in Fig. 3, increases in corticosterone ($n=12$ for both the AFR and MS groups) and ACTH levels ($n=12$ for both the AFR and MS groups) in response to an acute swimming stress were significantly higher in MS rats (Student's *t*-test; $p<0.005$). During the MS period, corticosterone and ACTH levels in pups ($n=10$) were significantly higher than those of AFR pups (Student's *t*-test; $p<0.005$): corticosterone levels were 165.12 ± 27.95 and 699.62 ± 84.12 ng/ml, and ACTH values were 60.35 ± 6.32 and 217.16 ± 94.44 pg/ml for AFR and MS pups, respectively.

In the hippocampus of MS rats (Fig. 4), there was a lower glucocorticoid receptor density ($n=6$), measured both in cytosolic and nuclear extracts, compared to AFR rats ($n=8$) (Student's *t*-test, $p<0.01$).

3.3. Effect of maternal separation on cognition

In the acquisition phase of Morris water maze task (Fig. 5A), overall analysis (repeated measures ANOVA) showed no significant effect of rearing in distance swam to find the platform ($n=10$ for both the AFR and MS groups). However, rearing produced a statistically significant impairment in the retention phase (Fig. 5B), and distance swam by animals searching for the platform was significant lower in the MS group compared to AFR rats (Student's *t*-test, $p<0.05$, $n=10$ for both the AFR and MS groups). Swim speed was not affected by rearing in neither the acquisition phase nor the retention phase, indicating absence of effects of MS on locomotor activity.

In the novel object recognition test, there was no difference in the total amount of time spent exploring two identical objects between AFR and MS groups. However, MS rats showed a learning impairment (Student's *t*-test, $p<0.01$, $n=12$ for both the AFR and MS groups) as the discrimination index was significantly lower (72.00 ± 4.28 vs 54.54 ± 3.69, respectively).

3.4. Effects of mifepristone treatment on MS animals

In the forced swimming test (Fig. 6A), there was a main effect of treatment in the immobility time measured, and mifepristone was able to reduced the enhanced immobility time associated to MS [$F_{1,40}=4.052$, $p<0.05$], ($n=8$–12 per group).

When animals were treated with mifepristone before the first exposure to the objects in the novel object recognition test, statistical analysis indicates a significant interaction between rearing and mifepristone treatment on the measure of discrimination between new and familiar objects [$F_{1,43}=4.83$, $p<0.05$], ($n=8-12$ per group). Further analysis (Student's *t*-test) revealed that MS animals treated with mifepristone did not show any memory impairment (Fig. 6B).

3.5. Involvement of the noradrenergic system in the effects of maternal separation

Increases (%) in noradrenaline content in response to an acute swimming stress were significantly higher in MS rats compared to AFR rats both in the hippocampus (1474.41 ± 62.50 vs 1726.05 ± 80.91, Student's *t*-test, $p<0.05$, $n=10$ for both the AFR and MS groups) and frontal cortex

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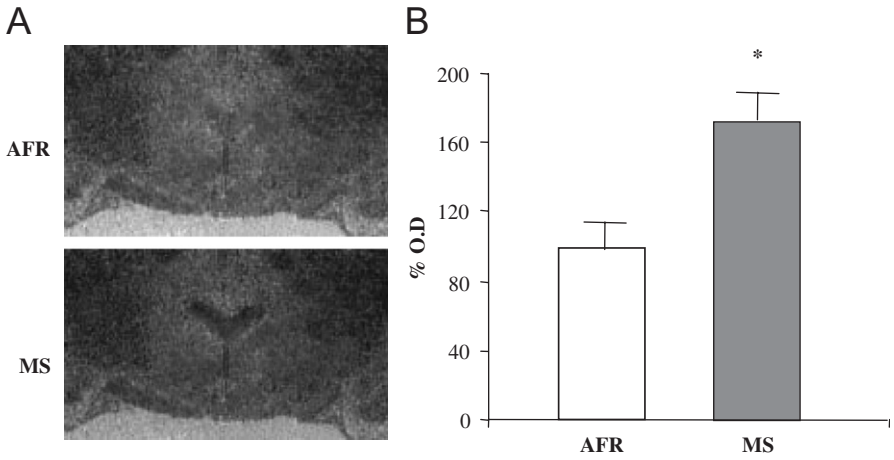

Fig. 2 (A) Autoradiograms of sections hybridized with CRF antisense cRNA probes in the paraventricular hypothalamus nucleus (PVN) in control (AFR) and MS rats. (B) Densitometry analysis of data ($n = 5$). Data are expressed as percentage of optical density (OD) values of control rats (AFR); $^*p < 0.01$ vs AFR rats. MS: maternal separation rats. Data (in nCi/g tissue) are as follows: AFR: 335.09 ± 15.67 and MS: $615.08 \pm 28.97^*$.

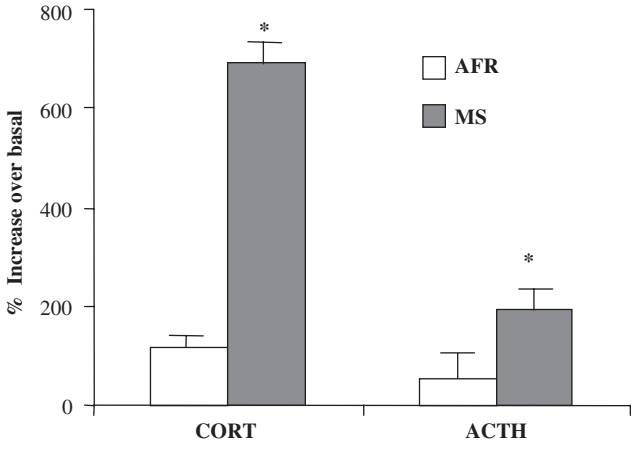

Fig. 3 Effect of maternal separation (MS) on plasma corticosterone and ACTH responses to an acute stressor (15 min swimming). Data are presented as percentage increase over basal values; $^*p < 0.001$ vs control (AFR) rats, Student's t-test. Basal levels were 68.45 ± 6.30 and 70.19 ± 5.59 ng/ml (corticosterone) and 178.05 ± 28.08 and 165.52 ± 48.28 pg/ml (ACTH) for AFR and MS groups, respectively.

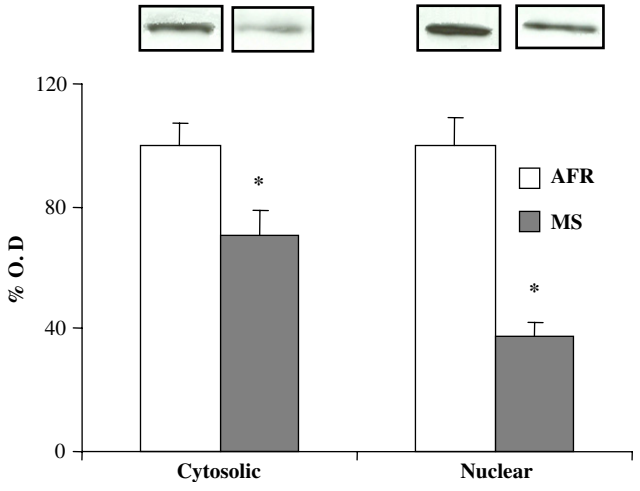

Fig. 4 Distribution of glucocorticoid receptor protein, in nuclear and cytosolic extracts from hippocampus of stressed AFR and MS rats. Data are expressed as percentage of optical density (OD) values of control rats (AFR). MS: maternal separation rats. $^*p < 0.01$ vs AFR rats, Student t-test.

(1423.40 ± 60.62 vs 1885.66 ± 153.84, Student's t-test, $p < 0.01$, $n = 10$ for both the AFR and MS groups).

In the forced swimming test, statistical analysis indicates a significant interaction between rearing and treatment with the β-adrenoceptor antagonist propranolol on the immobility time [$F_{1,36} = 5.91$, $p < 0.01$], ($n = 8-12$ per group). Further analysis revealed that MS animals treated with propranolol had immobility times similar to those of AFR rats (Fig. 7A).

In the novel object recognition test, propranolol reversed memory impairments associated to MS in rats (Fig. 7B). Statistical analysis revealed a significant interaction between propranolol treatment $\times$ rearing [$F_{1,40} = 4.070$, $p < 0.05$], ($n = 10$ per group).

4. Discussion

In humans, the experience of adverse events early in life is associated with an increased risk of development of psychiatric disorders in adulthood. This association has led to the belief that stress of early adverse experiences programs changes in the brain, which persist throughout lifetime and predispose an individual to the development of depression (Heim and Nemeroff, 2001). Rat models of early life adversity include those in which the neonatal animals are periodically deprived of contact with the dam, usually known as maternal separation (MS). MS alters the HPA function and the ability of the organism to respond to, cope with and adapt to stressful stimuli. Supporting this idea and

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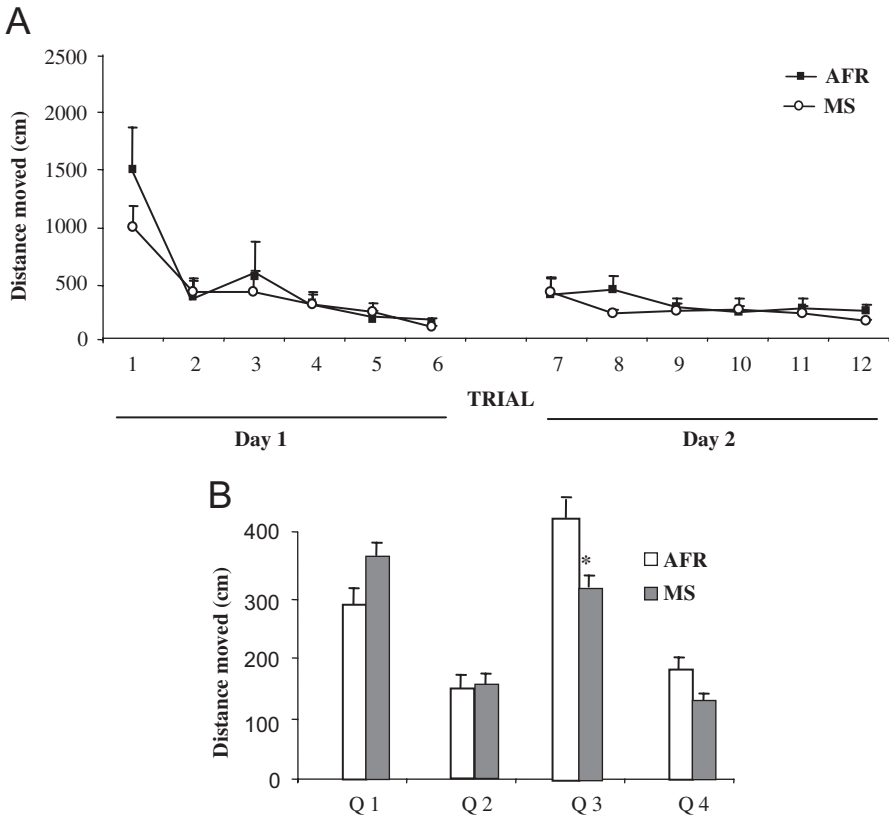

Fig. 5 Effect of maternal separation (MS) in the Morris water maze test, acquisition (A) and retention phase (B). In (A), data represent distance swam to find the platform, in (B) data are presented as distance swam in each of the quadrants. Platform used to be located on quadrant (Q) 3. $^{*}p<0.05$ vs control (AFR) rats, Student's *t*-test.

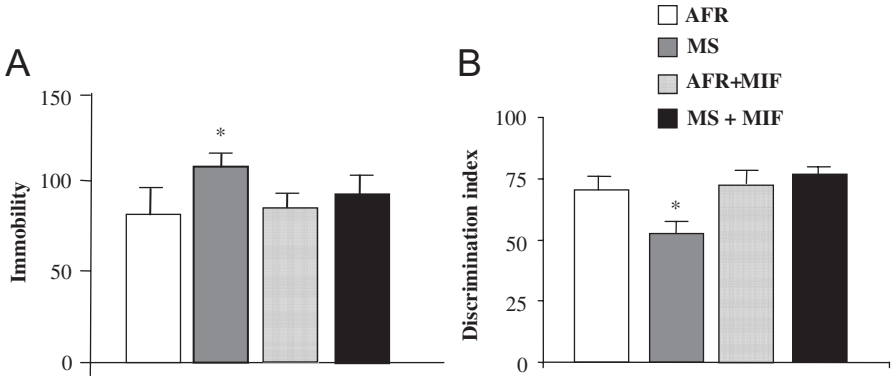

Fig. 6 Effects of mifepristone treatment on maternal separation (MS) rats. (A) Forced swimming test (immobility time). (B) Novel object recognition test. Maternal separation (MS) and mifepristone treatment on the novel object recognition test (discrimination index:time exploring the new object/total exploration time $\times$ 100). $^{*}p<0.01$ vs rest of the groups, two-way ANOVA (treatment $\times$ rearing). MIF: mifepristone treatment, 10 mg/kg SC.

accordingly to previous reported works, we have found that, compared to normally reared animals, MS rats show in adulthood depressive-like behaviour in the forced swimming test (Willner, 1990; Plotsky et al., 1998; Hall, 1998; Ladd et al., 2000), anhedonic behaviour (Willner et al., 1987; Zurita and Molina, 1999; Huot et al., 2001) and anxiety behaviour (Wigger and Neumann, 1999; Huot et al., 2000), increased HPA axis responsiveness to stressors (Rosenfeld et al., 1992; Plotsky and Meany, 1993; Ladd et al., 1996; Wigger and Neumann, 1999) and elevated CRF mRNA in the PVN (Plotsky and Meany, 1993; Ladd et al., 1996). Therefore, neonatal MS in the rat can be considered as an animal model of vulnerability to development of depression-like syndrome and an enhanced stress responsiveness (Sanchez et al., 2001; Ladd et al., 2005). It can be suggested that alterations in the behavioural phenotype associated to stress are related to the increase HPA axis responsiveness to stressors as, in our hands, the depressive-like behaviour

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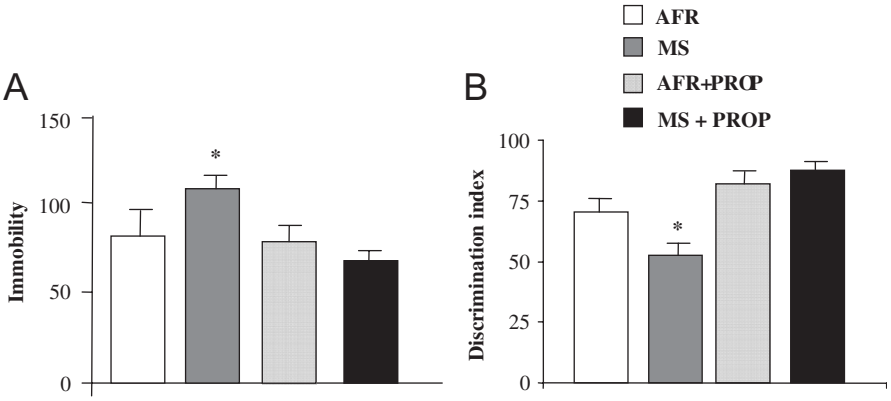

Fig. 7 Effects of propranolol treatment on maternal separation (MS) rats. (A) Forced swimming test (immobility time). (B) Novel object recognition test. Maternal separation (MS) and propranolol treatment on the novel object recognition test (discrimination index:time exploring the new object/total exploration time × 100). $^{*}p<0.01$ vs rest of the groups, two-way ANOVA (treatment × rearing). PRO: propranolol treatment, 2 mg/kg SC.

was reversed by administering the glucocorticoid receptor antagonist mifepristone. In this regard, in clinical studies, it has been recently shown that by regulating the HPA axis, mifepristone may be effective in the treatment of psychotic major depression (Flores et al., 2006) and bipolar disorder (Young et al., 2004).

The magnitude of the HPA response to acute stress is a function of CRF release, which activates the pituitary–adrenal system. There are also modulatory influences, mainly glucocorticoid negative feedback that inhibits CRF synthesis and release, thus dampening HPA responses to stress (De Kloet et al., 1998). The hippocampus, which exhibits a high density of corticosteroid receptors, plays an important role in the negative regulation of the HPA axis (Jacobson and Sapolsky, 1991). It has been suggested that there is a glucocorticoid cascade hypothesis (Sapolsky et al., 1986), which proposes that elevated glucocorticoids leads to hippocampal neuronal loss and therefore glucocorticoid receptor loss. Given that hippocampal glucocorticoid receptors mediates glucocorticoid negative feedback, their loss promotes further increase in glucocorticoid levels. Accordingly, a decreased inhibitory signal to the PVN CRF neurons might be expected to follow from the down-regulation of hippocampal glucocorticoid receptors reported here, and previously observed in maternally separated rats (Sutanto et al., 1996, Ladd et al., 2004). Therefore, the stress hyperresponsiveness observed in MS rats has been attributed, at least in part, to impaired glucocorticoid-mediated feedback sensitivity mediated by hippocampal glucocorticoid receptors (Plotsky et al., 1986; Vazquez, 1998; Ladd et al., 2004). Changes in glucocorticoid receptor expression may be secondary to an increased glucocorticoid tone. However, there is also evidence that altered glucocorticoid receptor transcription may be a primary event in the development of individual stress responsiveness, at least in Long Evans rats (Francis et al., 2002; Weaver et al., 2004).

MS rats exhibited a significant cognitive impairment in two different tests: the Morris water maze task and the novel object recognition test. In addition, the reduced sucrose intake it can also be explained as impaired recognition memory. It is well established that animals initially show a neophobic response to novel tastes (e.g. Bermudez-Rattoni, 2004). Therefore, it seems possible that reduced sucrose consumption can be explained by impaired recognition of sucrose as a purported familiar taste after the training. Stress and exposure to glucocorticoids early in life have been associated with impairments in learning and memory (Huot et al., 2002) and hippocampal atrophy. Plasticity of hippocampal circuitry, essential for its function in learning and memory, may increase its vulnerability to various insults including stress. The majority of hippocampal granule neurons develops and extends their axons between PNDs days 1 and 21 (Amaral and Dent, 1981). This peak period of neurogenesis overlaps the stress hyporesponsive period (PNDs 4–14). Exposure to elevated levels of corticosterone during the neonatal period (as shown in pups by significant higher levels of corticosterone compared to controls during the MS period), may affect hippocampal development (Huot et al., 2002). In this sense, MS rats have been described to exhibit decreased mossy fibre density in the stratum oriens region of the hippocampus (Huot et al., 2002). Therefore, MS during critical periods of hippocampal development can disrupt hippocampal cytoarchitecture in a stable manner, which may contribute to the learning deficits observed in these animals. Long-term adaptations in corticosteroid receptor density, as seen in the present work, may also subserve cognitive deficits associated with early adverse experience. In rodents, impaired glucocorticoid receptor function compromises cognitive and spatial capacity (De Kloet et al., 1999). It has been suggested that glucocorticoid effects on memory retrieval depend, at least in part, on activation of glucocorticoid receptors in the hippocampus, as the administration of a glucocorticoid receptor agonist infused into the hippocampus before retention induces memory retrieval impairment (Roozendaal et al., 2003).

In addition, MS rats exhibited a stress hyperresponsive HPA in adulthood that could be contributing to the cognitive deficits associated to early life adverse experiences. Elevation of circulating corticosterone concentrations by systemic administration of corticosterone or glucocorticoid receptor agonists exerts an inhibitory influence on learning

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and memory (see rev. by Douma et al., 1998), and stress exposure or glucocorticoid administration profoundly impairs working memory (Arnsten and Goldman-Rakic, 1998; Wolf, 2003) and long-term glucocorticoid exposure resulted in an impaired maze learning performance (Endo et al., 1996). Okuda et al. (2004) found that glucocorticoids given immediately after object recognition training induced retention impairment at a 1 h interval, i.e., the same interval as used in the present study. Therefore, in order to prevent corticosterone from binding to glucocorticoid receptors, the glucocorticoid receptor antagonist mifepristone had to be administered shortly pretraining or immediately post-training, in order to achieve blockade of the action of corticosterone in the context of learning which then resulted in impaired storage of information. In our hands, mifepristone, 10 mg/kg (Pugh et al., 1997; Oitzl et al., 1998), was able to completely reversed memory deficits observed in MS rats in the novel object recognition test, supporting the hypothesis that elevated secretion of glucocorticoids may be associated to the cognitive deficits in the MS model.

As to the mechanisms responsible for the involvement of glucocorticoids in memory deficits, in animal studies, hyperglucocortisolaemia can potentiate excitotoxicity of hippocampal pyramidal neurons and chronic administration of high doses of corticosterone leads to hippocampal neuronal loss (Sapolsky et al., 1985). However, anatomical studies using stereology-based methodology have failed to confirm that chronically high glucocorticoid exposure results in evidence for increased hippocampal neuronal loss in rats (Leverenz et al., 1999). In addition, several studies indicate that the hippocampus is only minimally involved in memory for objects (Brown and Aggleton, 2001). Altogether, it seems glucocorticoid-induced neurotoxicity of the hippocampus may not be directly responsible for the memory deficits found in the present work.

A major source of input to the PVN arises form the brainstem noradrenergic neurons (Liu et al., 2000). CRF neurons in the PVN are potently stimulated by noradrenaline and there is a strong positive correlation of PVN noradrenaline activation and activity of the HPA axis (Pacak et al., 1995). In fact, it has been described that glucocorticoid feedback to inhibit CRF release in the PVN is via attenuation of noradrenergic activation (Pacak et al., 1995). It has also been shown that the impairing effects of glucocorticoids on memory retrieval and working memory depend on noradrenergic activation within the amygdala (Roozendaal et al., 2006b). Systemic administration of the β-adrenoceptor antagonist propranolol blocks the impairing effect of corticosterone on working memory, that is known to rely on the integrity of the medial prefrontal cortex (Fuster, 1991). Moreover, propranolol, infused into the hippocampus prevents the impairing effect of concurrent intra-hippocampal administration of a glucocorticoid receptor agonist on memory retrieval (Roozendaal et al., 2004a). These results indicate that glucocorticoids interact with noradrenergic mechanisms of the hippocampus and prefrontal cortex, areas reciprocally connected with the amygdala (Kim et al., 2001; Maroun and Richter-Levin, 2003), in regulating memory retrieval. In our hands, propranolol was able to reverse both the enhanced immobility time in the forced swimming test and the memory deficits associated to MS in rats, confirming the involvement of the noradrenergic system in behavioural changes and cognitive impairments mediated by glucocorticoids in the MS model. In addition to interacting with the noradrenergic signalling cascade at a postsynaptic level, glucocorticoids may influence noradrenergic function by altering the synthesis of noradrenaline (McEwen et al., 1987). In fact, we found that increases in noradrenaline levels in response to an acute swimming stress in the hippocampus and frontal cortex were significantly higher in MS animals.

In summary, we have shown that MS during the first weeks of life results in animals that exhibit, in addition to behavioural and neuroendocrine signs of elevated stress reactivity as adults, cognition impairments that seem to be mediated by elevated levels of glucocorticoids. These behavioural and cognitive deficits are reversed by administration of the glucocorticoid antagonist mifepristone and the β-adrenergic antagonist propranolol. Currently, mifepristone is in Phase III clinical trials for major depression and might be the first non-monoaminergic-based antidepressant on the market (Berton and Nestler, 2006). In addition, preliminary clinical evidence has been provided that glucocorticoid receptor antagonist may have useful cognitive-enhancing properties in bipolar disorder (Young et al., 2004). The present results may provide the proof-of-concept for the use of mifepristone in the treatment of depressive-like and memory disturbances in stress-related psychiatric disorders.

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