
MATERNAL SEPARATION AND LESION OF ADTN ALTERS ANXIETY AND ADRENAL ACTIVITY IN MALE RATS

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The present study investigated the effect of early maternal separation on anxiety and hypophyso-adrenal system activity to anterodorsal thalamic nuclei (ADTN) lesion in male rats as adults in order to compare this with previous results with

Received 14 May 2008; accepted 7 August 2008.

The Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Grant 02593) and the Secretaría de Ciencia y Técnica (SECyT, Grant 16206) supported this work.

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female rats. During the first 3 weeks of life, male rats were isolated 4.5 hr daily and tested as adults. Thirty days after ADTN lesion we found that adrenocorticotrophic hormone (ACTH) plasma levels were affected neither by maternal separation nor by ADTN lesion. Plasma corticosterone (CORT) concentration was increased with lesion of the ADTN in maternally separated rats. A significant increase in plasma catecholamine concentration was induced by early maternal separation. In ADTN-lesioned rats, plasma norepinephrine (NE) concentration was significantly lower than in the respective sham-lesioned groups. In terms of anxiety, there were no significant effects of early experience. However, the ADTN lesion tended to decrease anxiety-related behavior.

Keywords ACTH, ADTN, anxiety, catecholamines, corticosterone, maternal separation

INTRODUCTION

For the last two decades, we have been studying the influence of different neural centers of the limbic system on the hypothalamic–pituitary–adrenal (HPA) system and the sympathetic–adrenal–medullary (SAM) system in response to stress.

Our previous studies led us to place the anterodorsal thalamic nuclei (ADTN) among the extrahypothalamic limbic centers, which are involved in regulating corticosterone (CORT) and adrenal catecholamine secretion or release in rats. We demonstrated an inhibitory influence of the ADTN on adrenal function (Suárez & Perassi, 1988, 1990, 1993; Suárez, Perassi, & Loyber, 1987).

Then, we focused on characterizing the long-term consequences of neonatal maternal separation on behavioral and neuroendocrine response to chronic stress in adult life. That study investigated especially the effects of maternal-infant separation on the regulation exerted by the ADTN on SAM, adrenocorticotrophic hormone (ACTH), and CORT secretion in female rats.

Lesions of the ADTN provoked an increase in plasma ACTH, norepinephrine (NE), and epinephrine (E) concentration in maternally separated (MS) animals (Suárez et al., 2001). All that work was realized in female rats so that the effect of maternal separation on female rats has already been reported. However, it appears that maternal separation during early development does not adversely affect females as much as males (Russell et al., 2003) For instance, female rats that were MS did not show an increase in their fear-related behaviors in adulthood compared to females that were not MS (Boccia & Pedersen, 2001). Our study showed that, when males and females are MS, males show more

anxiety behavior and higher catecholamine levels in adulthood compared to MS females (Renard, Suárez, Levin, & Rivarola, 2005).

Another study showed that, unlike males, female rats that have experienced maternal separation do not show a heightened endocrine stress response in reaction to a stressor (Wigger & Neumann, 1999). Although in rats there appears to be a sex difference in their response to maternal separation (Slotten, Kalinichev, Hagan, Marsden, & Fone, 2006), it should be noted that increased anxiety and fearfulness have been reported in both male and female nonhuman primates that have undergone severe maternal separation (Suomi, Collins, Harlow, & Ruppenthal, 1976).

In addition to these behavioral and endocrine effects, males that undergo repeated separation show greater HPA activity. This is both basal and in response to an acute stressor upon reaching adulthood (Ladd et al., 2000; Liu, Caldji, Sharma, Plotsky, & Meaney, 2000; Plotsky & Meaney, 1993). Thus, it appears that early life stressors in the form of maternal separation can have a lasting influence on the physiology and behavior of offspring in rats.

On the basis of the above considerations, the aim of the present study was to investigate the long-term consequences of neonatal maternal separation on anxiety and neuroendocrine response in adult life.

This study focused especially on the effects of maternal-infant separation on the regulation exerted by the ADTN on SAM secretion and the HPA system and through these on anxiety indices. With this purpose in mind, we analyzed plasma concentration of NE, E, CORT, ACTH, and anxiety indices in adult male rats that were subjected to periodic maternal separation as neonates. The findings are compared with previous data from female rats.

MATERIAL AND METHODS

Animals and Housing

Male Wistar rats were used in this study. All rats were subjected to the same conditions. They were housed in a temperature-controlled room ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) under artificial illumination (12:12 hr light–dark; lights on at 07:00 hr), with water and food available ad libitum. All rats were handled daily by the same investigator from weaning. This consisted of picking up each rat from its home cage by placing the hand over its back, with the thumb and forefinger pressing its forelegs toward its head. Each rat was then placed briefly in another cage and finally returned to its home cage. On the day of sacrifice, the rat was picked up in the same way but instead of being placed in a cage it was

immediately decapitated (Loyber, Perassi, Leucona, & Peralta, 1977). Rats were decapitated between 09:00 and 12:00 hr. Experiments were performed in accordance with protocols approved by the Animal Care Committee of the University of Córdoba, Argentina.

Separation Procedure

On postnatal day 1, litters were culled to eight pups (four females and four males when possible). Pups were separated from their mother daily for 4.5 hr during the first 3 weeks of life (Ogawa et al., 1994). Each separation consisted of removing the mother from the home cage, placing her alone in a cage in the same room and, after 4.5 hr, returning her to the home cage. Separations were carried out between 08:00 and 12:30 hr. Nondeprived control litters remained with the mother until weaning on postnatal day 21. After weaning, the male rats were selected and housed together in standard cages until 8 weeks of age, at which time the ADTN lesions were performed. Both nonmaternally separated (NMS) and MS rats were randomly assigned to two groups: (a) ADTN-lesioned and (b) sham-lesioned.

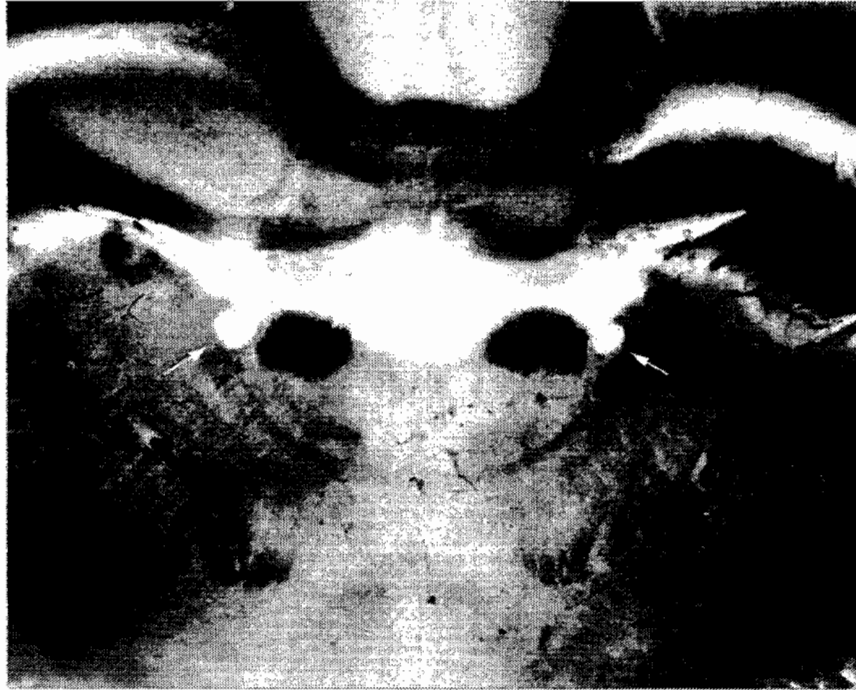
Surgery

At the time of operation, the animals were about 8 weeks old and weighed 230–270 g. The rats were anaesthetized with 2,2,2-tribromoethanol (200 mg/kg intraperitoneal (i.p.)) and then placed in a stereotaxic instrument. The skull vault was surgically exposed and holes were drilled bilaterally, permitting an electrode to be stereotaxically guided into the ADTN. The stereotaxic coordinates (König & Klippel, 1963) for the ADTN were 5.3 mm anterior to the lambda, 1.2 mm lateral to the sagittal suture, 1.0 mm above the horizontal zero plane, and 3.8 mm below the surface of the skull. Bilateral electrolytic lesions were made by passing 1 mA of cathodic current through the insulated tips (0.25 mm diameter) of a stainless steel electrode for 30 s. The same procedure was performed on sham-lesioned animals, but no current was passed through the electrodes. A schematic representation of the location of the lesion points is presented in Figure 1.

Elevated Plus Maze

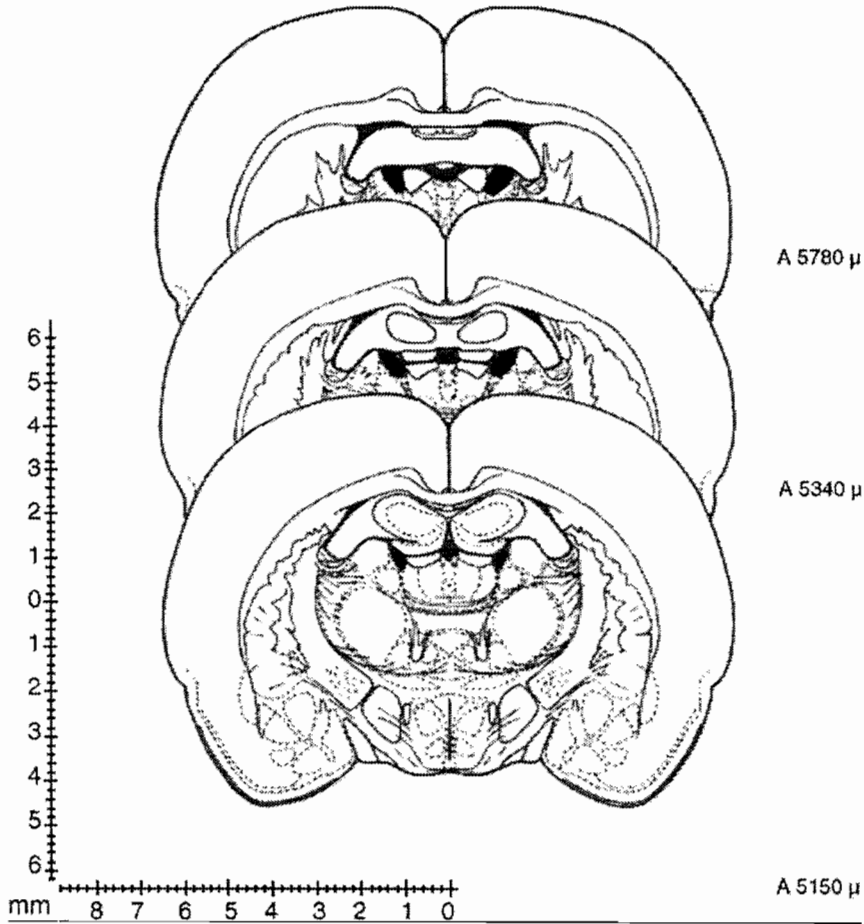
Twenty-nine days after surgery, rats were tested in the elevated plus-maze apparatus. At the same age, the unstressed group was also tested. The elevated

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Figure 1. Photograph and diagram of damage following lesion of anterodorsal thalamic nuclei (plane A: 5340 from the atlas of König & Klippel, 1963).

plus-maze test is based on creating a conflict between the rat's exploratory drive and its innate fear of open and exposed areas. Thus, decreased open arm exploration was taken to indicate enhanced anxiety-related behavior. As described in detail by Liebsch et al. (1995), the apparatus consisted of a plus-shaped platform elevated 50 cm from the floor. Two of the opposing arms (50 × 10 cm) were closed by 40 cm high side and end walls (closed arms), while the other two arms had no walls (open arms). At the beginning of the test, the rat was placed onto the central area (10 × 10 cm) of the maze. During the 5-min exposure the following parameters were recorded: number of entries into open arms, number of entries into closed arms, and time spent on the open arms. Two indices of anxiety were obtained: the number of entries into open arms expressed as a percentage of the total number of entries, and the amount of time spent in the open arms expressed as a percentage of total time. Between each session, the maze was wiped clean. Behavioral testing was conducted in a quiet room. Rats were transported to the experimental room 2 hr before behavioral testing to habituate them to the new environment.

Assays of Hormones

Thirty days after the lesion, the rats were decapitated with a guillotine for small animals within 5–7 s after being taken from their home cage. Immediately after decapitation, trunk blood was collected into heparinized plastic tubes kept on ice and centrifuged. Individual plasma samples were frozen and stored for subsequent determination of E, NE, ACTH, and CORT concentration.

The catecholamines in 500 μ L aliquots of plasma were partially purified by batch alumina extraction, separated by reverse-phase high-pressure liquid chromatography (RF-HPLC) using a 4.6 × 250 mm Zorbax R × C18 column (New England Nuclear, Du Pont, Boston, MA, USA). The quantification was made by current produced upon exposure of the column effluent to oxidation and then reducing potentials in series using a triple-electrode system (Coulchem II, ESA, Bedford, MA, USA) (Eisenhofer et al., 1986). Recovery through the alumina extraction step averaged 70%–80% for catecholamines. Catechol concentrations in each sample were corrected for recovery of an internal standard dihydroxybenzylamine. The detection limit of the assay was about 15 pg per volume assayed for each catechol. The electrochemical responses were linear ($r = 0.99$) for amounts of NE or E from 50 to 2000 pg. The interassay variations were 14% and 15% and the intraassay variation coefficient was 10% for E and NE.

Plasma ACTH was analyzed using a commercial radioimmunoassay kit (Incstar Corporation). The sample, first antibody, and tracer were combined and incubated for 16–24 hr at 2°C–8°C. A preprecipitated second antibody complex was added to separate the bound tracer from the free tracer. The assay was then centrifuged and decanted after a 15-min incubation at 20°C–25°C. The normal range in plasma was found to be 0–71 pg/mL (Incstar Laboratory). Concentrations of plasma CORT were determined by radioimmunoassay using an antiserum raised in sheep against CORT-21-hemisuccinate bovine serum albumin, using a 1:10 ether extraction to avoid interference of other plasma components. Detection limits were 0.1 mg/dl; intraassay variation was 8%; and interassay variation was 10%. The radioimmunoassay of CORT was done in triplicate (Ratka, Sutanto, Bloemers, & De Kloet, 1989).

Histology

The brains of lesioned animals were fixed in 10% formaldehyde and subsequently sliced in a coronal plane on a cryostat-mounted microtome and examined histologically to verify the location of lesions. Data from animals with nonsatisfactory lesions (<80% of damage) were not included in the statistical evaluation.

Statistical Method

Statistical significance of the data was determined by two-way analysis of variance (ANOVA) (factors: maternal separation \times ADTN lesion), and individual group means were compared by Tukey's test. Significance was set at $p < .05$.

RESULTS

Endocrine Responses

Early maternal separation induced a decrease in plasma ACTH in both sham and lesioned rats compared to NMS but this did not reach statistical significance (Figure 2).

In NMS and MS animals, there was no effect of the ADTN lesion on plasma ACTH concentration (Figure 2).

In MS animals, the lesion of the ADTN produced an increase in plasma CORT concentration compared to sham-lesioned ($F(1-18) = 7.39, p < .01$). On

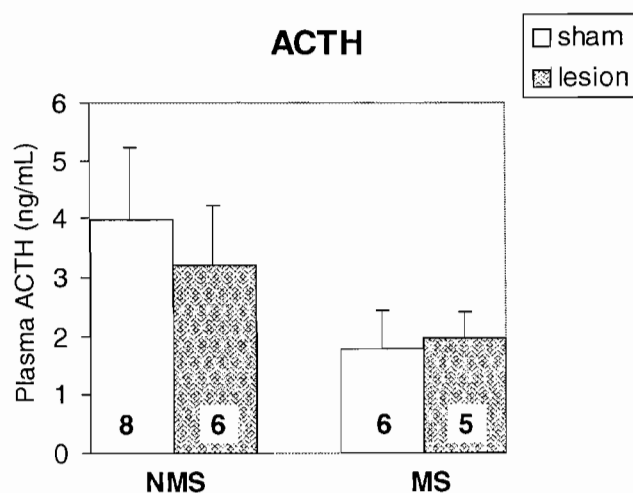


Figure 2. Plasma ACTH response to ADTN lesion in nonmaternally separated and maternally separated rats. Mean values \pm SE are presented. The number of rats per group is given beneath each bar (two-way ANOVA followed by Tukey's post hoc test).

the other hand, MS ADTN-lesioned animals showed an increase in CORT levels compared to NMS-lesioned animals ($F(1-18) = 22.21, p < .001$). However, there was no effect of the ADTN lesion on plasma CORT concentration in the NMS groups (Figure 3).

Early maternal separation induced a significant increase in plasma NE concentration in both sham and lesioned rats, compared with the NMS

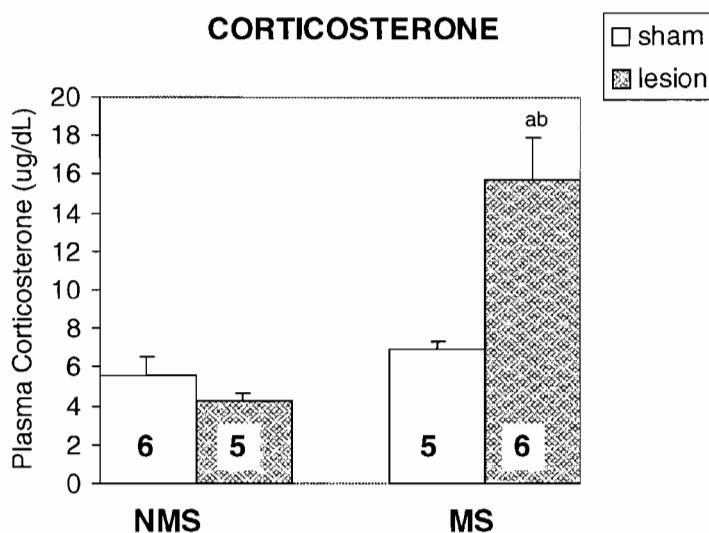


Figure 3. Plasma Corticosterone response to ADTN lesion in nonmaternally separated and maternally separated rats. Mean values \pm SE are presented. The number of rats per group is given beneath each bar. (a) Significant difference $p < .005$ vs. sham lesioned. (b) Significant difference $p < .05$ vs. nonseparated group (two-way ANOVA followed by Tukey's post hoc test).

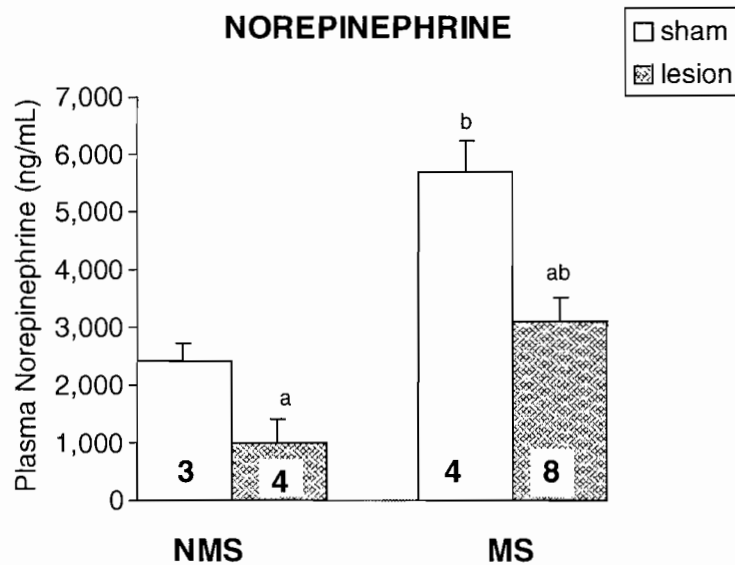


Figure 4. Plasma norepinephrine response to ADTN lesion in nonmaternally separated and maternally separated rats. Mean values \pm SE are presented. The number of rats per group is given beneath each bar: (a) significant difference $p < .005$ vs. sham lesioned; (b) significant difference $p < .05$ vs. nonseparated group (two-way ANOVA followed by Tukey's post hoc test).

($F(1-15) = 30.55, p < .001$). In ADTN-lesioned NMS and MS rats, plasma NE concentration was significantly lower than in the respective sham-lesioned groups ($F(1-15) = 1.93, p < .001$) (Figure 4).

Following ADTN lesion, there was no significant difference in plasma E between sham and lesioned NMS and MS animals. On the other hand, maternal separation induced a significant increase in values of plasma E in both sham and lesioned rats, compared with NMS ($F(1-16) = 12.27, p < .01$) (Figure 5).

Behavioral Responses

In the elevated plus-maze test, the open arm entries were similar in MS and NMS rats. There was no effect of the ADTN lesion on the open arm entries in either MS or NMS groups. The ADTN lesion tended to increase the percentage of time spent on the plus maze in MS rats, but this was not statistically significant. This could reflect decreased anxiety-related behavior after repeated maternal separation combined with the ADTN lesion (Figure 6).

DISCUSSION

Early traumatic experiences are believed to play a major role in the development of adult psychological disorders, including anxiety (Heim & Nemeroff, 2001; Pryce & Feldon, 2003).

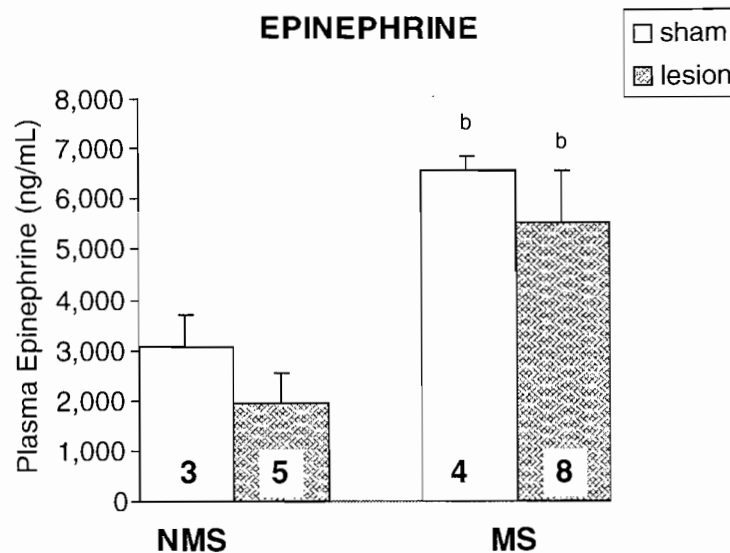


Figure 5. Plasma epinephrine response to ADTN lesion in nonmaternally separated and maternally separated rats. Mean values \pm SE are presented. The number of rats per group is given beneath each bar: (b) significant difference $p < .05$ vs. nonseparated group (two-way ANOVA followed by Tukey's post hoc test).

In the present study, we have investigated the effect of early maternal separation on HPA activity response to ADTN lesion in male rats as adults in order to compare this with previous results with female rats.

We have previously demonstrated that the ADTN exerts an inhibitory influence on HPA axis activity, since the lesion of this limbic structure provoked an increase in ACTH and CORT plasma levels (Suárez & Perassi, 1997).

In previous works on maternal separation, we demonstrated that repeated maternal separation for 4.5 hr daily during the first 3 weeks of life produced long-term changes in HPA axis function in female rats (Suárez et al., 2001). In the present work, in males no differences were found in plasma ACTH. However, in maternally separated rats, an ADTN lesion produced a significant increase in the plasma concentration of CORT, and their overall plasma CORT concentration was elevated compared with nonseparated rats. These results regarding the effect of ADTN lesion in separated male rats are different from our previous findings in which the lesion of the ADTN in females rats evoked a decrease in CORT (Suárez et al., 2001), suggesting a sex-specific effect on the HPA response.

Bibliography indicates that testosterone can inhibit HPA axis function, and estrogen can enhance it (Handa, Burgess, Kerr, & O'Keefe, 1994). Gonadal steroids can act at multiple levels within the HPA axis: at the hypothalamus, affecting corticotrophin releasing hormone and arginine-vasopressin synthesis;

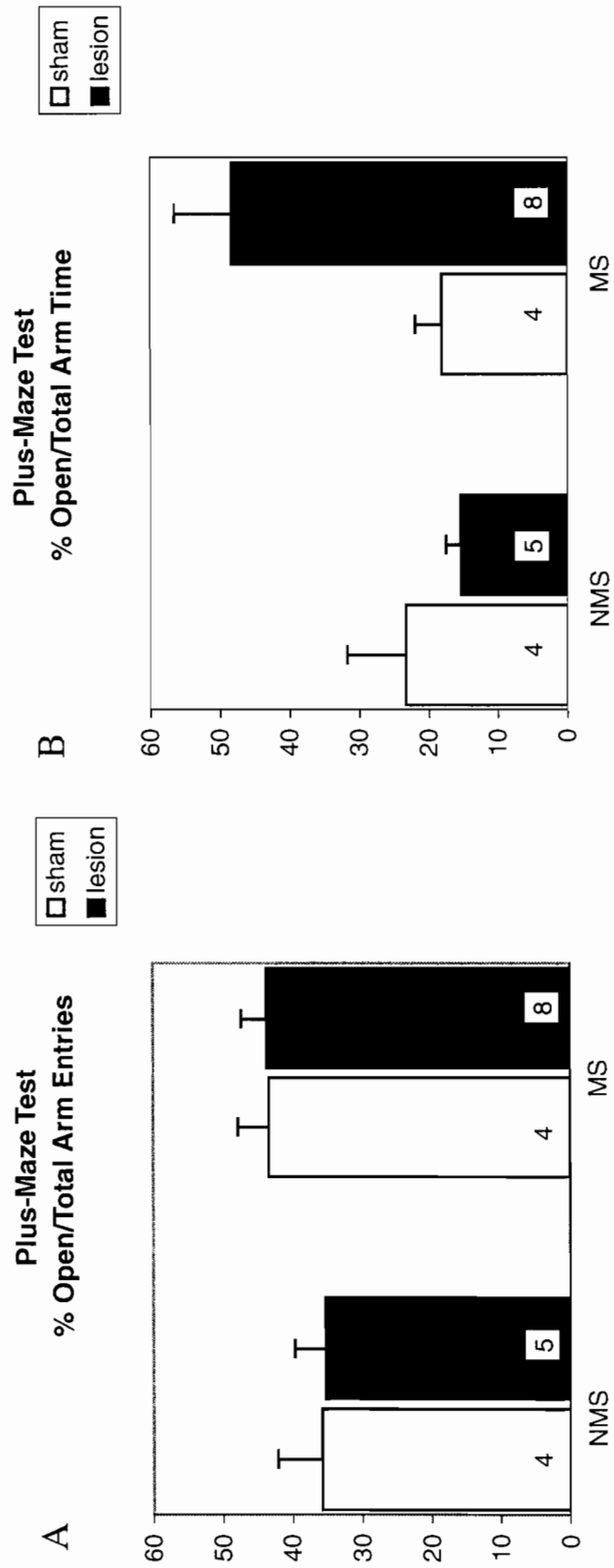


Figure 6. Behavioral parameters during a 5-min exposure to elevated plus-maze test of nonmaternally separated and maternally separated rats in sham- and ADTN-lesioned rats. Each column represents the mean \pm SE of number of entries (panel A) and time spent on the open arms (panel B) (expressed as percentages).

at the anterior pituitary, affecting adrenocorticotropin hormone release; and/or at the adrenal gland, affecting glucocorticoid synthesis (Pfaff, 2002; Viau & Meaney, 1996; Williamson, Bingham, & Viau, 2005).

One mechanism by which androgens and estrogens modulate stress responses is through binding to their respective genomic receptors in the central nervous system (CNS). The distribution and regulation of androgen and estrogen receptors suggest areas of the brain where gonadal steroids can influence the HPA axis stress response. In rats, the ADTN are connected to the cingulate cortex, especially to the retrosplenial area and, via this and the entorhinal area, the ADTN are connected to the hippocampus, septal, amygdaloid, and mammillary nuclei (Zeman & Innes, 1963). Therefore, the influence of the ADTN on ACTH and CORT secretion may be via more or less direct routes to the hypothalamus through the extrahypothalamic structures that regulate the HPA system. When considering Ladd results (Ladd, Owens, & Nemeroff, 1996), the possibility emerges that the lowered plasma ACTH values in our MS rats could be caused by reduced corticotropin-releasing hormone (CRH) secretion or CRH binding in the pituitary. In addition, it is possible that chronic stress induced by periodic maternal deprivation might reduce CRH receptors concentration in anterior pituitary (Aguilera, Hauger, Pharwood, Millan, & Catt, 1989).

In the current study, diminution of circulating ACTH was induced by neonatal deprivation; however, plasma CORT was differentially affected by deprivation. In MS-lesioned rats, plasma CORT increased compared to nondeprived. The dissociation between plasma ACTH and plasma CORT concentration could be a result of direct sympathetic activation of the adrenal cortex or another mechanism. There is evidence suggesting that elevation in plasma corticosteroids levels is not always accompanied by increased ACTH levels. A number of non-ACTH-dependent mechanisms may be involved in the control of adrenocortical secretion (Labrie et al., 1987), including direct autonomous innervation (Holzwarth, Cunningham, & Kleitman, 1987). Taking into consideration the results obtained in the present work that MS rats exhibited elevated plasma E values, the higher values of plasma CORT might be the result of hyperactivity of sympathetic nervous system.

In summary, these data indicate that postnatal maternal separation in male rats seems to enhance sensitivity to the inhibitory influence of ADTN on CORT secretion.

Maternal separation also modifies adrenal medullary secretion in adulthood. Neonatal maternal separation was found to result in significantly greater plasma E concentration than nonseparated controls. In the same way, maternal

separation in female rats produced an increase in NE concentration (Suárez et al., 2001). These results suggest that maternal separation enhances sympathetic nervous system activity both in males and females.

Concerning the role of the ADTN, nonseparated male rats showed a decrease in plasma NE concentration after lesion of the ADTN, but did not show changes in plasma E concentration. On the other hand, when these rats were maternally separated, this produced a decrease in plasma NE and E, but this did not reach statistical significance. In previous work we found that, in separated female rats, ADTN lesion produced a significant increase in plasma concentration of both E and NE. In summary, our results indicate that ADTN activity is sex-dependent, because males showed decreased catecholamine concentration compared to females.

Numerous brain structures have been described as developing and/or functioning in a sexually dimorphic manner, including regions important in high-level cognitive processing such as the hippocampus, amygdala, and prefrontal cortex (Cahill, 2006). In some cases, sexually dimorphic brain activation does not seem to correlate with sex-specific behavioral differences, perhaps indicating that in some cases sex differences in brain function may compensate for structural differences.

Therefore, we can assume that in this case the ADTN functions as an activator limbic structure of the adrenal medullary system in males, while it acts as inhibitor in females.

In addition to the effects on HPA axis hormone secretion and adrenal medullary responses, the maternally separated rats also differed in behavioral and emotional responses (Suárez et al., 2001).

Recently, interest in the investigation of long-term behavioral consequences of maternal separation has been stimulated by the hypothesis that it might constitute an animal model for the origins of human psychological and physical diseases (Cirulli, Santucci, Laviola, Alleva, & Levine, 1994; Ellenbroek & Riva, 2003; Faure, Uys, Marais, Stein, & Daniels, 2007; Francis, Caldji, Champagne, Plotsky, & Meaney, 1999; Lehmann, Stöhr, & Feldon, 2000; Wigger & Neumann, 1999). There is some evidence of behavioral alterations in MS animals that resemble those observed in patients with affective disorders (Caldji, Francis, Sharma, Plotsky, & Meaney, 2000; Huot, Thirivikraman, Meaney, & Plotsky, 2001; Ladd et al., 2000; Rhees, Lephart, & Eliason, 2001; Wigger & Neumann, 1999).

Elevated plus-maze testing has been validated as a measure of fear or emotional behavior, i.e. anxiety, in the rat (Pellow, Chopin, File, & Briley, 1985; Pellow & File, 1986).

While the majority of investigators examined the effects of maternal separation only in male offspring, those that studied both males and females found sex-dependent effects (Kalinichev, Easterling, & Holtzman, 2001; Kalinichev, Easterling, Plotsky, & Holtzman, 2002; McIntosh, Anisman, & Merali, 1999; Wigger & Neumann, 1999). In this study, in terms of behavior, there were no significant effects of early experience. However, maternally separated rats with ADTN lesion tended to spend more time in the open arms than the nondeprived rats, indicating that maternally separated male rats may be less anxious than nonseparated rats. This is in accordance with other studies where maternal separation does not affect anxiety-like behavior in male rats (Huot, Gonzalez, Ladd, Thirivikraman, & Plotsky, 2004; Marmendal, Roman, Eriksson, Nylander, Fahlke, 2004; Pellow & File, 1986; Shalev & Kafkafi 2002; Zimmernberg & Kajunski, 2004), or even leads to a decrease in such behaviors (Ploj, Roman, & Nylander, 2002). In female rats we found that maternal separation as well as ADTN lesion produces a decrease in anxiety-related behaviors (Suárez et al., 2004). GABA plays a major role in central regulation of HPA stress responses and is also involved in anxiety. It has been reported that in maternally separated males there is significant reduction of GABA_A receptor levels in the medial prefrontal cortex, as well as in locus coeruleus and nucleus tractus solitarius (Caldji et al., 2000). This decrease in GABA-binding sites might result in potentiated noradrenergic stimulation of CRH synthesis and release (Plotsky, Cunningham, & Widmaier, 1989) and contribute to increased CORT and ACTH responses to stress observed in maternally separated animals (Huot et al., 2001; Liu et al., 2000). This argument could explain the results observed in this work in MS and ADTN-lesioned males.

In summary, these data indicate that anxiety and neuroendocrine responses of adult male and female rats may be modulated by maternal separation during early development and that the ADTN participate in a sex-dependent manner on HPA axis hormone secretion and adrenal medullary responses.

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