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Sex differences in rats: Effects of chronic stress on sympathetic system and anxiety

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Abstract

In this study we tested whether periodic maternal deprivation (MD) (4.5 h daily during the first 3 weeks of life) caused chronic changes in anxiety and medullo-adrenal responses to chronic stress in either male or female adult (2.5 months of age) rats, or both. Repeated maternal deprivation had a sex-specific effect on epinephrine (E) and norepinephrine (NE) levels: an increase in both measures was observed only in females. Unpredictable stress did not produce changes on plasma catecholamine levels either in males or females. However, when the females were maternally deprived as well as stressed they showed an increase in plasma NE $p<0.05$. On the other hand, non-maternally deprived (NMD), maternally-deprived and stressed males showed high levels of catecholamines compared to females $p<0.001$. In the elevated plus maze test, MD-treated males displayed a slight increase in anxiety-related behavior compared with NMD rats. This was indicated by a reduction in the time spent on the open arms, whereas females showed less anxiety, indicated by an increase in the number of entries, and in the time spent on the open arms. After exposure to chronic stress only the females displayed decreased anxiety-related behavior. These results suggest that there are sex-induced effects in emotional reactivity, perception of the stressor and in the evaluation of novel situations. Thus, maternal deprivation and chronic variable stress caused both long-term alterations in sympathetic response and gender-dependent changes in the anxiety index of adult rats.

Keywords: Functional sex differences; Stress; Maternal deprivation; Epinephrine; Norepinephrine; Anxiety

1. Introduction

Exposure of laboratory animals to a variety of stressors activates the sympathoadrenal system (SAS) and the hypothalamic–pituitary–adrenocortical (HPA) system, resulting in an increase in levels of catecholamines (CA) released from the SAS. Simultaneously, corticotropin-releasing hormone (CRH) produced in the hypothalamus increases the release of ACTH and adrenal corticosteroids from the HPA system [1,2].

A substantial part of brain development happens after birth, and consequently is subject to environmental influences such as maternal separation, hypothermia, and food deprivation, some of which may negatively affect brain maturation [3,4].

Acute or repeated long-term separation from the dam is considered to be one of the most potent natural stressors during development. Neonatal maternal deprivation induces long-term alterations on HPA axis sensitivity and medullo-adrenal secretion [5,6]. Male rats that undergo repeated separation show greater hypothalamic–pituitary–adrenal activity, both basally and in response to an acute stressor upon reaching adulthood [7,8]. In previous works, we demonstrated that plasma epinephrine (E) and norepinephrine (NE) concentrations were significantly increased in adult female rats that were maternally deprived [6]. Furthermore,

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postnatal treatments, like repeated maternal separation, induce long-term changes, including stress hyperresponsiveness, anxiety-like behavior and cognitive deficits, which can persist throughout adulthood [9,4].

There is great evidence of sexual differences in basic neural processes and behaviors.

One of the fundamental differences between the mammalian sexes is the expression of genes on the Y chromosome, the protein products of which promote differentiation of the primordial gonads in the testes in the fetal male [10]. In early studies, investigators found that during species-specific critical periods, male hormones, such as testosterone, or metabolites of testosterone, masculinized the brain, the hypothalamic–pituitary–gonadal axis, and behavior, which laid the foundation for species-typical sexual responses as adults [11,12].

Sexually dimorphic behaviors in mammals can be considered the final result of reciprocal influences among genes, gonadal sex, hormonal sex, organizational and activational effects of hormones on the brain, learning, social and other environmental influences [11].

It appears that maternal separation during early development does not adversely affect females as much as males. Male rats that undergo maternal separation for 3–4.5 h a day for the first 2 or 3 weeks of life exhibit an increase in fearfulness in adulthood [13]. For instance, female rats that were maternally separated did not show an increase in their fear-related behaviors in adulthood compared to females that were not maternally deprived [14]. Another study showed that when males and females are maternally separated, males show more fear and anxiety-related behavior in adulthood compared to maternally separated females [5,15].

The aim of the present study was to investigate whether neonatal maternal deprivation produces long-term changes in medullo-adrenal activity and anxiety response to stress in male and female rats. We hypothesized that repeated separation from the mother would result in a significant increase in the offspring's sympathetic reactivity to stress and consequently an increase in emotional reactivity, which would be expressed in a gender-dependent way. Thus, in adult animals, previously exposed to periodic maternal deprivation, we monitored not only the behavioral response in the elevated plus maze (a relevant non-conditioned test for anxiety-related behavior), but also assessed medullo-adrenal activity after exposure to chronic stress.

2. Material and methods

2.1. Animals and both male and female Wistar rats were used

All animals were subjected to the same conditions: they were housed in a temperature controlled room (22±2 °C) under artificial illumination (12:12 h light/dark; lit on at 07:00 a.m.). Water and food were given ad libitum. All the subjects were handled daily by the same researcher from weaning until they were decapitated, to minimize stress reactions to manipulation. Handling consisted of picking up each animal from its home cage by placing the hand over the animal's back, with the thumb and forefinger pressing its forelegs towards its head, then the animal was placed in another cage for a while and finally, put back in its home cage. The day of sacrifice the animal was picked up in the same way but instead of being placed into a cage, it was immediately decapitated.

At the time of decapitation all the animals were 2.5 month old and weighed 200–300 g.

Rats were decapitated between 09:00 and 12:00 a.m., on diestrus in the case of the female rats, in order to avoid unwanted variability in circulating hormone levels, linked to diurnal fluctuations and the estrus cycle. Diestrus was determined by examination of vaginal smears.

Experiments were performed in full accordance with protocols approved by the Animal Care and Use Committee of Córdoba University, Argentina.

2.2. Separation procedures

On postnatal day 1, litters were culled to eight pups (four females and four males when possible). The rat pups were daily deprived of their mother for 4.5 h. during the first 3 weeks of life [16]. Each separation consisted of removing the mother from the home cage. She was placed alone in a cage in the same room. After 4.5 h, the mother was returned to the home cage. Separations were carried out between 08:00 am and 12:30 pm. Non-deprived rats remained undisturbed in the maternal cage, except for a bedding change twice a week, until the weaning age at postnatal day 30.

After weaning, male and female rats were selected and housed in standard cages. Both NMD and MD rats were randomly divided into two groups: the first was subjected to the plus maze test, while the second was subjected to unpredictable stress and the plus maze test. Unrelated subjects were used to avoid confounding litter effects (each experimental group was made up of subjects from at least 3 litters).

2.3. Variable chronic stress

At 48 days of age, the rats were exposed to a 24-day variable stressor paradigm (modified Katz's stress model [17]). Individual stressors are listed in Table 1.

The type of stressor and the day on which it was applied were chosen by using a random number table, except for that on day 24. In this case, noise was used as a stressor on the day preceding the plus maze test, to avoid the unpredictability associated with this chronic stress model [18]. The following stressors were used: a) 4 h of noise produced by an alarm bell (85 dB); b) ether anesthesia until loss of reflex; c) two intraperitoneal (i.p.) injections of isotonic saline; d) 24 h of food deprivation, e) 1 h of restraint inside a 6 cm diameter metal cylinder.

Table 1
Variable chronic stress (Katz's modified model)

Day	Stressor	Hour
1	Ether anesthesia	12:00 a.m.
2	Immobilisation	10:00 a.m.–11:00 a.m.
3	Noise	09:00 a.m.–01:00 p.m.
4	Two saline injections	11:00 a.m. and 03:00 p.m.
5	Ether anesthesia	10:00 a.m.
6	Fasting	For 24 h
7	Rest day	
8	Ether anesthesia	09:30 a.m.
9	Noise	09:30 a.m.–01:30 p.m.
10	Two saline injections	11:30 a.m. and 03:30 p.m.
11	Immobilisation	03:00 p.m.–04:00 p.m.
12	Noise	10:00 a.m.–02:00 p.m.
13	Fasting	For 24 h
14	Rest day	
15	Ether anesthesia	01:00 p.m.
16	Immobilisation	03:00–04:00 p.m.
17	Noise	09:30 a.m.–01:30 p.m.
18	Two saline injections	12:00 a.m. and 04:00 p.m.
19	Noise	09:30 a.m.–01:30 p.m.
20	Fasting	For 24 h
21	Rest day	
22	Ether anesthesia	11:45 a.m.
23	Immobilisation	10:00–11:00 a.m.
24	Noise	09:00 a.m.–01:00 p.m.

Animals were subjected to various stressors for 24 days.

2.4. Elevated plus maze

Twenty-four hours after the last stressor, rats were tested in the elevated plus maze apparatus. At the same age, the unstressed group was also tested. The elevated 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. [19], the apparatus consisted of a plus-shaped platform elevated 50 cm from the floor. Two of the opposing arms (50×10 cm) were enclosed by 40 cm-high side and end walls (closed arms), whereas the other two had no walls (open arms). At the beginning of the test, each rat was placed onto the central area (10×10 cm) of the maze facing a closed arm, and was allowed to explore the plus maze freely. During the 5-min exposure the following parameters were recorded: number of entries into open arms, number of entries into closed arms, 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 the total time. Between each session the maze was wiped clean. Behavioral testing was conducted in a quiet room. Animals were transported to the experimental room 2 h before the behavioral test to eliminate the stressor effects of the new environment.

2.5. Assays of hormones

Twenty-four hours after the test, the rats were decapitated with a small animal guillotine within 5–7 s after being taken from their home cage. Immediately after decapitation, trunk blood was collected and centrifuged. Individual plasma samples were frozen and stored for subsequent determination of epinephrine (E) and norepinephrine (NE) concentration.

Blood for a plasma catecholamine assay was poured into plastic tubes containing heparin, kept on ice. The catechols in 1000 μl 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 $R \times C18$ column (New England Nuclear, Du Pont, Boston, MA, USA). The quantification was made by current produced upon exposure of the column effluent to oxidizing and then, by reducing potentials in series using a triple-electrode system (Coulochem II, ESA, Bedford, MA, USA) [20]. Recovery through the alumina extraction step averaged 70–80% for catecholamines. Catechol concentrations in each sample were corrected for the recovery of an internal standard dihydroxybenzylamine.

2.6. Statistical method

Statistical significance of the data was determined by three-way analysis of variance (ANOVA) (factors: sex × maternal separation × stress) and individual group means were compared by the Tukey test. Significance was set at $p < 0.05$.

3. Results

The effect of maternal deprivation and stress on plasma norepinephrine (NE) of male and female rats is displayed in Fig. 1.

Unstressed plasma levels of NE did not differ in maternally deprived (MD) and non-maternally deprived (NMD) males. Mean basal levels of plasma NE were not

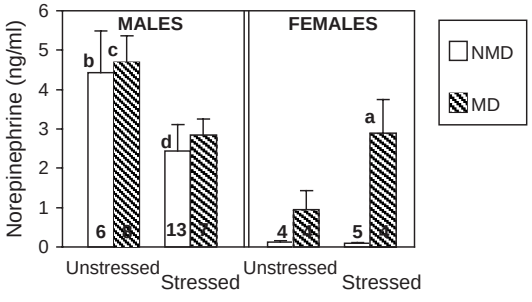

Fig. 1. Plasma norepinephrine concentration in non-maternally-deprived (NMD) and maternally-deprived (MD) males and females subjected to unpredictable stress. Mean±SE. The number of animals per group is included inside each bar. (a) Significant differences $p < 0.05$ vs. respective NMD. (b), (c) and (d) Significant differences $p < 0.05$ vs. respective females.

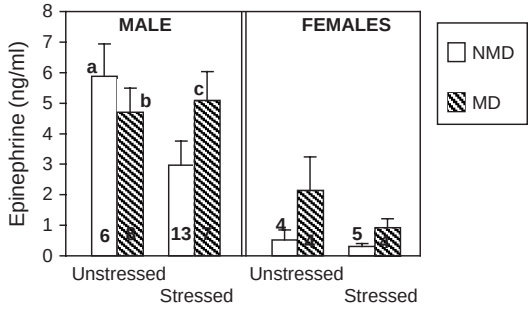

Fig. 2. Plasma epinephrine concentration in non-maternally-deprived (NMD) and maternally-deprived (MD) males and females subjected to unpredictable stress. Mean±SE. The number of animals per group is included inside each bar. (a), (b) and (c) Significant differences $p<0.05$ vs. respective females.

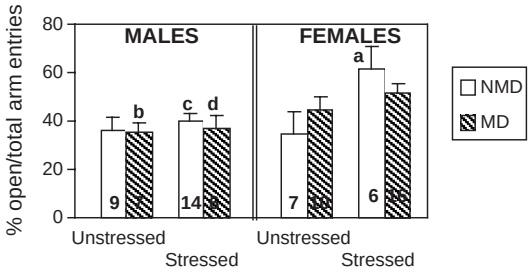

Fig. 4. Number of entries on the open arms (expressed as percentage) during a 5 min exposure to the elevated plus maze test of non-maternally-deprived (NMD) and maternally-deprived (MD) male and female rats, subjected to unpredictable stress. Mean±SE. The number of animals per group is included inside each bar. (a) Significant difference $p<0.05$ vs. respective unstressed. (b), (c) and (d) Significant difference $p<0.05$ vs. respective females.

significantly altered by stress either in NMD or MD male rats.

On the other hand, in female rats, basal plasma NE levels were higher in MD compared with NMD, but did not reach statistical significance. In maternally deprived females, exposure to variable chronic stress produced an increase in plasma NE compared to NMD-stressed female rats $F(7,50)=3.64$, $p<0.05$.

Regarding sex differences, a comparison of male and female rats revealed that both NMD and MD males showed higher basal NE levels $F(7,50)=21.13$, $p<0.001$. In response to chronic stress, plasma NE concentration was higher in males $F(7,50)=21.13$, $p<0.001$, compared to females with the same treatment. However, when comparing male and female MD rats, exposure to stress did not yield any differences in NE plasma levels.

Fig. 2 shows plasma epinephrine (E) concentration in maternally-deprived and stressed male and female rats.

As indicated, neither maternal deprivation nor exposure to chronic stress induced any changes in plasma E levels in males. No significant differences were evident in plasma E in MD-stressed male rats. Similarly, in female rats, plasma E remained practically unchanged compared with control females.

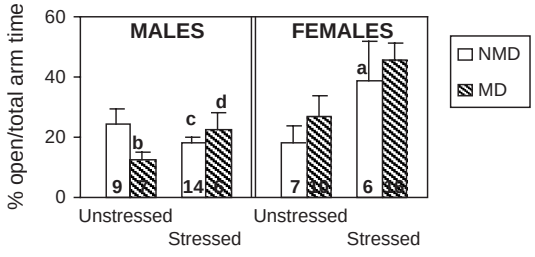

Fig. 3. Time spent on the open arms (expressed as percentage) during a 5 min exposure to the elevated plus maze test of non-maternally-deprived (NMD) and maternally-deprived (MD) male and female rats, subjected to unpredictable stress. Mean±SE. The number of animals per group is included inside each bar. (a) Significant difference $p<0.05$ vs. respective unstressed. (b), (c) and (d) Significant difference $p<0.05$ vs. respective females.

In relation to sex differences, both NMD and MD males showed higher basal E levels $F(7,50)=29.55$, $p<0.001$.

In MD-stressed animals, plasma E concentration in males was also significantly higher $F(7,50)=29.55$, $p<0.001$, compared to females with the same treatment.

Figs. 3 and 4 show behavioral parameters during a 5-min exposure to the elevated plus maze of male and female adult postnatally-stressed rats, and their respective controls.

Statistical analysis revealed that neither MD nor stress, induced changes in males in the time spent on, and the number of entries into the open arms. Maternal deprivation did not produce alterations in anxiety-related behavior in females. Nevertheless, after stress, females showed a significant increase in time spent on, and the number of entries into the open arms of the plus maze, compared to unstressed animals $F(7,74)=6.85$, $p<0.01$.

The comparison of male and female animals revealed significant differences in anxiety-related behavior during the elevated plus maze test. This was indicated by a reduction in the time spent on $F(7,74)=8.06$, $p<0.01$, and the number of entries $F(7,74)=8.38$, $p<0.01$ into open arms in maternally-deprived males compared to females subjected to maternal deprivation.

Both maternally deprived and NMD stressed males, showed a decrease in time spent on $F(7,74)=8.06$, $p<0.01$, and the number of entries $F(7,74)=8.38$, $p<0.01$ into open arms, compared to females subjected to the same treatments.

Table 2 shows a summing-up of the findings with a direct gender comparison of NE, E and anxiety.

Table 2
Direct male and female comparison of norepinephrine, epinephrine and anxiety in non-maternally deprived (NMD) and maternally deprived (MD) rats, subjected to plus maze test, and to unpredictable stress and plus maze test

		NE		E		Anxiety	
		♂	♀	♂	♀	♂	♀
NMD	Plus maze	>		>		–	
	Stress+plus maze	>		–		>	
MD	Plus maze	>		>		>	
	Stress+plus maze	–		>		>	

4. Discussion

In the present study we have investigated gender-dependent effects on sympatho-medullo-adrenal (SMA) activity and the anxiety index of maternally-deprived (MD) and stressed animals.

We demonstrated that maternal separation, repeated daily for 4.5 h. during the first 3 weeks of life had a sex-specific effect on E and NE levels: an increase in both measures was observed only in females, although this fact did not reach statistical significance.

Subsequent exposure to the unpredictable stress paradigm did not produce an effect on plasma catecholamine concentration either in males or in females. However, it showed a downward trend in these hormones in males subjected to unpredictable stress. On the other hand, when the females were maternally deprived and subjected to unpredictable stress, it produced an increase on plasma NE.

Bibliography indicates that testosterone can inhibit HPA axis function, and estrogen can enhance it [21]. 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 a similar way they could be acting on the sympathetic system, which is revealed by an increase in catecholamines in female rats subjected to maternal deprivation and unpredictable stress.

Despite many published works related to SMA response during unpredictable stress, the results are controversial. The characteristics of the response may change considerably depending on different variables such as the animals' strains [22], age [23], sex [24], stimulus type, intensity and duration [25,26]. Therefore, a modest increase, decrease and no alterations in plasma E and NE response in rats were reported [26].

Our results indicate that SMA activity is dependent on the animals' sex, because males showed higher NE and E concentration compared to females.

Non-maternally deprived, maternally-deprived and stressed animals showed high levels of these hormones compared to the respective females. These data indicate a differential concentration of catecholamines and higher medullo-adrenal activity in males that is consistent with previous results obtained in our laboratory.

Others investigators have found opposite results, with females showing higher catecholamine levels compared with males. However, the stress procedure was different as they used an acute stressor, and so these are not comparable with our results [24,27].

This differential activation of the adrenal medulla may depend on the gonadal steroids and the hypothalamo–pituitary–gonadal activity that can condition the sympathetic response.

Charmian [28] and Zimmerberg and Brown [29] held that the differences between sexes are related to the production of sexual hormone metabolites, for example D-hidrostenedione, or to neurosteroids that mediate the HPA response in extrahypothalamic areas.

In relation to anxiety, it is important to distinguish between the impact of brief periods of separation (handling), from those elicited by more protracted periods of maternal separation. Rat pups exposed to brief periods of handling during the first weeks of life exhibit less fear and/or anxiety later in life [30,31]. This has been interpreted to reflect an enhanced ability to cope with stressful events, and, thus, reduced vulnerability to stressor-induced behavioral impairments or pathologies.

Although it had been hypothesized that maternally-separated animals would be more anxious, this was found not to be the case. In fact we did not find significant effects of maternal separation compared with sex-matched NMD animals. Other authors [32] have reported similar results.

Interestingly the effects of maternal deprivation were gender-dependent in that this resulted in an increase of anxiety-related behavior in males compared to MD-treated females.

Unpredictable stress did not influence anxiety in males. In contrast, females displayed significantly less anxious behavior after being subjected to unpredictable stress.

The plus maze test results showed that males exhibited more anxiety in novel environments, after being subjected to maternal deprivation and/or unpredictable stress, compared with females. These data are consistent with other authors [5,9,15] who observed marked gender-dependent effects of periodic maternal-deprivation treatment (3 h/day) in the elevated plus maze. The effect of MD on emotional behavior was higher in males than in females, and it was found together with an increase in ACTH secretion in response to plus maze test exposition, only in males.

Kelly et al. [11] and Zimmerberg and Brown [29] stated that the diminution of anxiety in females is a consequence of the regulatory effect of estrogens and allopregnenolone. In females, higher circulating levels of progesterone are available to the brain to be converted into allopregnenolone. Higher levels of allopregnenolone in stressful situations would predict lower levels of anxiety in female rats compared to male rats. This sex difference has been reported both in an open-field activity test and in the plus maze test. When allopregnenolone is given to female rats, they show an increase in open arm entries on an elevated plus maze, indicating a decrease in anxiety [29]. These results suggest that there are sex-induced effects in emotional reactivity, perception of the stressor and in the evaluation of novel situations.

Numerous studies conducted by Levine and colleagues have demonstrated that separating a rat pup from the mother for 24 h, i.e., maternal deprivation, has a profound effect on the neuronal and endocrine responses to stress [33]. Thus, maternal deprivation and chronic variable stress caused

long-term alterations in sympathetic response and also gender-dependent changes in the anxiety index of adult rats.

Our data suggest that males are more sensitive to the effects of maternal deprivation and chronic stress.

Dimorphism often depends on the presence of sex hormones during a restricted period of development. However, sexual differences of the CNS and behavior may have an underlying genetic component, independent of any sex steroid-dependent mechanism. In addition, the environment can have a significant impact on the dimorphism and sexual differentiation of the CNS. Future studies of dimorphism should further our understanding of the neurochemical base of behavior.

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