

Long-term effects of maternal deprivation on the corticosterone response to stress in rats

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Sucheck, Deborah, and Sergio Tufik. Long-term effects of maternal deprivation on the corticosterone response to stress in rats. *Am. J. Physiol.* 273 (*Regulatory Integrative Comp. Physiol.* 42): R1332–R1338, 1997.—Twenty-four hours of maternal deprivation result in activation of the infant rat's adrenocortical axis. In the present study we examined the long-term effects of maternal deprivation on the corticosterone (Cort) response to stress. Pups were maternally deprived (Dep) on postnatal day (PND) 11 and tested immediately (PND 12) or returned to their mothers and tested at later ages. Testing consisted of a time course of the Cort response to a saline injection (5, 15, 30, and 60 min). At PND 12, the response of Dep pups was higher than that of nondeprived (non-Dep) pups. No group differences were observed at PND 16 and 22. On PND 30, Dep rats showed lower Cort levels than non-Dep pups at 0, 5, and 30 min after saline. At PND 60, non-Dep females showed higher Cort levels than males at 5, 15, and 30 min. This gender difference for Dep pups was observed only at 5 min. Male and female Dep animals presented lower Cort levels than non-Dep counterparts at 60 and 30 min after saline, respectively. These findings indicate that maternal deprivation effects on Cort secretion are long lasting. Dep rats showed a smaller adrenal response to stress at PND 30, whereas as adults the stress response was similar but the turnoff was different.

glucocorticoids; stress-hyporesponsive period; adult rats

THE DEVELOPING ORGANISM differs from the adult in many aspects. One such aspect is related to the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Whereas adults secrete glucocorticoids (GCs) in a circadian fashion and in response to stressful stimuli, the infant rat's HPA axis appears to lack such activities. Approximately on postnatal day (PND) 4, the infant rat enters the so-called stress-hyporesponsive period (SHRP). This period lasts until PND 14 and is characterized by reduction of adrenal sensitivity, evidenced by the fact that exogenous administration of adrenocorticotropic hormone (ACTH) does not result in a significant adrenal output (for review, see Refs. 26 and 28). It appears that low levels of GCs during this critical period are essential for a normal development (19), because exposure to high levels of GCs has widespread deleterious effects on the developing central nervous system (2) and on some functional parameters of the adrenocortical axis (29).

The underlying mechanism of the adrenal insensitivity to ACTH was investigated recently. Both in vivo (41) and in vitro (1) data point to a nadir of adrenal sensitivity around PND 10. Examination of steroidogenesis during development shows that immunoreactive levels of cytochrome P-450 enzymes do not change during neonatal development. Immunoreactive levels of 3- β -hydroxysteroid dehydrogenase, however, are

lower in PND 1 and 10 rat pups compared with adults, although the enzyme activity does not parallel changes in immunoreactivity; this parameter is similar in PND 10 and adult rats, and both are higher than that of PND 1 pups. The authors suggest that enzymes involved in steroidogenesis are differentially regulated during development, with no apparent correlation between enzyme activity and immunoreactivity levels of the proteins, probably due to the presence of different isoforms of these enzymes during this period in life (21).

Development of circadian rhythmicity (15) and negative feedback capacity occurs much later in development (8). The first appearance of circadian rhythmicity of plasma corticosterone (Cort) can be detected at PND 18 in overfed and standard-fed rats, with a slight delay in underfed animals, i.e., the rhythm is present at PND 21. In young animals, the peak of plasma Cort levels is observed during the dark period, whereas in adult rats it shifts to the end of the dark period (15). Both Goldman and co-workers (8) and Vázquez and Akil (37) showed that the negative feedback mechanism in the rat is active from PND 25 on. Although return to basal levels is somehow delayed at this age, it more closely resembles that of the adult rat (8, 37).

The theories formulated to explain the SHRP include factors of intrinsic origin, such as an enhanced negative feedback at the brain and pituitary levels (39), and immaturity of neural pathways that project to the hypothalamus (5). There is a growing body of evidence, however, indicating that inhibition of the infant's HPA axis also occurs as a result of maternal care (3, 14, 25, 32).

Mammalian development is characterized by a prolonged period of dependence on maternal care. The dam provides the infant with nutrients, warmth, tactile stimulation, and protection, to name only a few of the more obvious maternal behaviors. Less obvious, however, is the regulatory role the mother exerts on the pup's physiology. For instance, separation from the mother results in a decrease in heart rate, respiratory rate, and growth hormone secretion (9–12). Specifically in the case of HPA axis activity, 24 h of maternal separation induces an immediate increase of basal and stress- and ACTH-induced levels of Cort, in addition to increased stress-induced ACTH plasma levels (14, 32). Release of Cort is mainly, but not entirely, regulated by feeding (24), whereas ACTH secretion is completely inhibited by anogenital stroking (34).

The prolonged effects of maternal deprivation on Cort secretion have not been fully examined. Reunion of 12-day-old pups, previously maternally deprived (Dep) for 24 h, with their mothers for 4 days is capable of resetting Cort basal levels, but not the response to novelty nor to a saline or hypertonic saline injection.

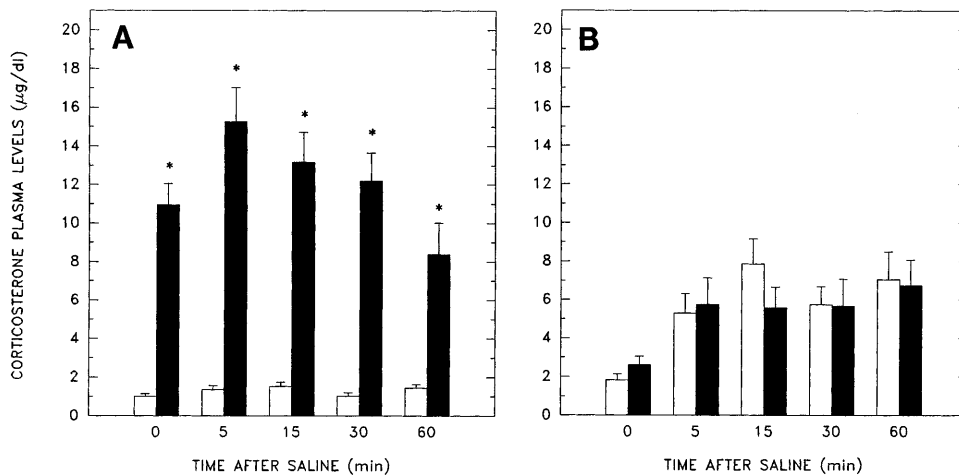


Fig. 1. A: corticosterone plasma levels (means \pm SE) of 12-day-old rat pups, either nondeprived (open bars) or deprived of their mothers for 24 h (filled bars). Pups were sampled before (0 min) or at different time intervals (5, 15, 30, 60 min) after a saline injection. Number of animals was 9–12/group. *Differs from nondeprived pups, $P < 0.05$. B: corticosterone plasma levels (means \pm SE) of 16-day-old rat pups, left undisturbed (open bars) or deprived of their mothers for 24 h (filled bars), on postnatal day 11. Pups were sampled before (0 min) or after a saline injection at different time intervals (5, 15, 30, 60 min). Number of animals was 6–11/group.

This study, however, investigated only one age after maternal deprivation (27). Therefore, it remains to be determined whether or not these effects are long lasting. The present study sought to examine the prolonged effects of maternal deprivation on Cort secretion in response to a mild stress.

METHODS

Subjects

The animals were Wistar rat pups bred in the animal colony of the Department of Psychobiology from Universidade Federal de São Paulo. The date of birth was designated *day 0*. On *PND 1*, litters were culled to 10 pups (5 males and 5 females) and housed with their mothers in plastic cages and provided with rat chow and tap water in the animal room, with controlled temperature ($25 \pm 2^\circ\text{C}$) and a 12:12-h light-dark cycle (lights on at 7:00 AM). Cages were cleaned every other day as part of the animal room routine. Cleaning consisted of placing mother and infants in a separate cage and returning them to the home cage after changing the sawdust shaving.

Deprivation Procedure

On *PND 11*, mothers were removed from and pups remained in the home cage. Home cages were placed in a separate room, on top of electric heating pads (General Electric), set at $30\text{--}33^\circ\text{C}$, under the same conditions as the animal room. Maternal deprivation lasted 24 h. At the end of the deprivation period, pups were reunited with their mothers until time of testing or weaning or were tested immediately (*PND 12*). Nondeprived (non-Dep) pups remained with their mothers in the home cages in the animal room. For both conditions, cage bedding was changed every other day.

Testing Procedure

Pups were tested on *PND 12* (immediately after the end of the deprivation period) or on *PND 16, 22, 30, and 60*. In each litter, two pups (1 male and 1 female) were decapitated immediately for determination of Cort basal levels (0 min). The remainder of the pups was injected intraperitoneally with 0.9% saline (volume of 0.1 ml/100 g body wt) and returned to their home cages for 5, 15, 30, or 60 min, in the same room as they were injected. After the allotted period of time had elapsed, pups or adult animals were brought to an adjacent room, the decapitation room, and sampled. Trans-

port of animals and sampling did not last more than 2 min. Sampling was carried out between 10:00 AM and 12:00 PM. Forty-eight litters were used and the experimental design was two (conditions: non-Dep, Dep) by five (times: 0, 5, 15, 30, and 60 min) by two (sex: male, female). Time and sex were the intralitter variables.

Blood Sample Procedure

Trunk blood of each pre- or postweaning animal was collected in pre-cooled vials containing EDTA (60 mg/ml). Blood was centrifuged for 20 min at 2,300 revolutions/min at 2°C . Plasma was collected in plastic tubes and kept at -20°C until radioimmunoassay for Cort (ICN Biomedicals) was performed. The sensitivity of the assay was $0.125 \mu\text{g/dl}$. The intra- and interassay coefficients of variation were 7.3 and 6.9%, respectively.

Data Analysis

For each age, Cort data were analyzed by means of a three-way analysis of variance (ANOVA) procedure, with condition, time, and sex as the main factors. When sex was determined not to be a significant factor, data were collapsed across this variable. Post hoc analysis was performed by the Newman-Keuls procedure. Weight data were analyzed, at each age and each sex, by the Student's *t*-test for independent variables. The level of significance was set at $P < 0.05$.

RESULTS

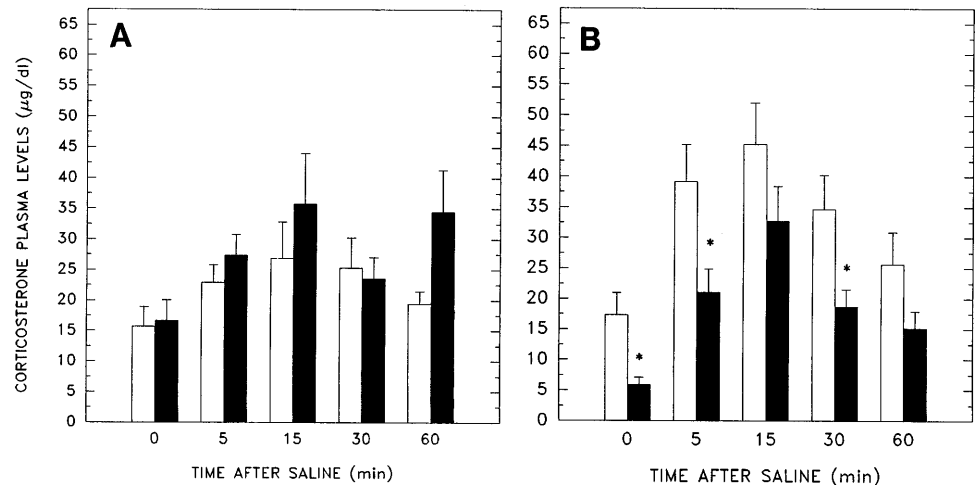
PND 12

ANOVA revealed an interaction between time and group [$F(1,91) = 3.112$; $P < 0.02$]. Analysis of this interaction showed that Cort levels of Dep pups were higher than those of their non-Dep counterparts at all time points after saline injection. Moreover, non-Dep animals did not show a Cort response to stress, whereas Cort levels of Dep pups were higher at 5 min than basal levels, and at 5, 15, and 30 min than 60 min (Fig. 1A).

PND 16

A main effect of time was detected [$F(4,71) = 5.934$; $P < 0.0004$]. Both groups showed an enhanced Cort response to the injection at all time points over basal values (Fig. 1B).

Fig. 2. *A*: corticosterone plasma levels (means \pm SE) of 22-day-old rat pups, left undisturbed (open bars) or deprived (filled bars) of their mothers for 24 h, on postnatal *day 11*. Pups were sampled before (0 min) or after a saline injection at different time intervals (5, 15, 30, 60 min). Number of animals was 6–14/group. *B*: corticosterone plasma levels (means \pm SE) of 30-day-old rats, left undisturbed (open bars) or deprived (filled bars) of their mothers for 24 h, on postnatal *day 11*. Animals were sampled before (0 min) or at different time intervals (5, 15, 30, 60 min) after a saline injection. Number of animals was 8–13/group. *Differs from nondeprived pups, $P < 0.05$.



PND 22

Fig. 2*A* shows the results observed on animals tested at PND 22. No differences in non-Dep and Dep stress response were observed at this age. Likewise, groups did not differ from each other.

PND 30

Main effects of time [$F(4,84) = 7.92$; $P < 0.0001$] and group [$F(1,84) = 18.78$; $P < 0.0001$] were revealed. Post hoc analysis showed that Cort levels of non-Dep rats were increased 15 min after stress over basal levels. For Dep animals, Cort response was higher at 5 and 30 min than basal levels. A further elevation was observed at 15 min, when levels were higher than all other time points. Comparison between non-Dep and Dep animals at each time point revealed that non-Dep Cort levels were higher than those of Dep at 0, 5, and 30 min (Fig. 2*B*).

PND 60

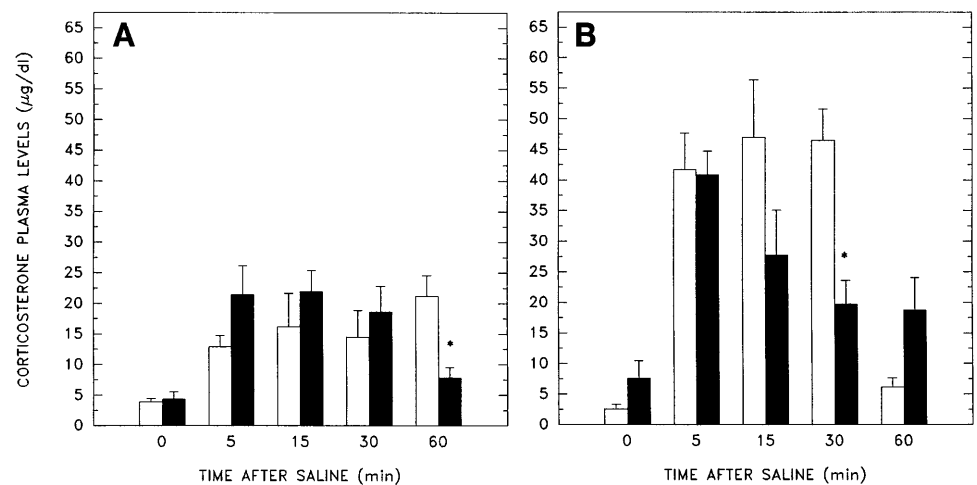
ANOVA revealed a three-way interaction [$F(4,117) = 7.51$; $P < 0.0001$]. Analysis of the sex difference indi-

cated that non-Dep females showed higher Cort levels than males at 5, 15, and 30 min after saline administration, whereas this difference between Dep females and males was present only at the 5 min time point. Because Cort response of female animals was more intense than that of males, a separate ANOVA was performed for each gender, with time and group as the factors.

Males. An interaction between time and group was observed [$F(4,61) = 3.57$; $P < 0.02$]. Post hoc analysis of this interaction revealed that Cort levels of non-Dep males were higher at 60 min than basal. Dep male rats showed augmented hormone levels at 5, 15, and 30 min over basal. Non-Dep values were higher than Dep levels 60 min after saline injection (Fig. 3*A*).

Females. Similarly, an interaction between time and group was observed [$F(4,56) = 5.67$; $P < 0.001$]. Non-Dep females showed increased Cort levels at 5, 15, and 30 min, compared with 0 and 60 min. Cort response of Dep females was higher at 5 and 15 min than levels at 0 min. In addition, Cort values at 5 min were higher than those at 30 and 60 min after saline. Comparison between groups showed higher levels of Cort in non-

Fig. 3. *A*: corticosterone plasma levels (means \pm SE) of 60-day-old male rats, left undisturbed (open bars) or deprived (filled bars) of their mothers for 24 h, on postnatal *day 11*. Animals were sampled before (0 min) or after a saline injection at different time intervals (5, 15, 30, 60 min). Number of animals was 5–8/group. *Differs from nondeprived pups, $P < 0.05$. *B*: corticosterone plasma levels (means \pm SE) of 60-day-old female rats, left undisturbed (open bars) or deprived (filled bars) of their mothers for 24 h, on postnatal *day 11*. Rats were sampled before (0 min) or at different time intervals (5, 15, 30, 60 min) after a saline injection. Number of animals was 5–10/group. *Differs from nondeprived pups, $P < 0.05$. For further differences between time points or genders, refer to RESULTS.



Dep females than their Dep counterparts at 30 min (Fig. 3B).

Body Weight

At all ages, except 60 days, both Dep male and female rats exhibited smaller body weight than their non-Dep counterparts ($P < 0.03$). Sixty-day-old Dep males were heavier than their non-Dep counterparts ($P < 0.03$), whereas non-Dep and Dep 60-day-old females did not differ from each other (Table 1).

DISCUSSION

The results of the present study indicate the effects of maternal deprivation are long lasting. Contrary to its immediate effects, however, maternal deprivation renders the adult offspring less responsive to a mild stress. This effect is more clearly observed at 30 days of age. At *PND 60*, hormone levels of non-Dep males and females were higher than those of their Dep counterparts at some time points after saline. Moreover, a closer inspection of the characteristics of the Cort response revealed that 30 day-old Dep animals responded faster to the stimulus: the onset of response of Dep animals occurred 5 min after the stimulus, whereas this increased response was only detected at 15 min in non-Dep animals. Similarly, Dep 60-day-old male rats responded to the stimulus with augmented Cort levels at 5 min, and this response remained so elevated until 60 min after the stimulus, when it became undistinguishable from nontreated levels. Differences in Cort secretion in non-Dep male rats were only observed at the 60 min time point. This persistent elevation may not reflect solely the response to the saline injection; rather, it may be a summation of this stimulus and the transport of animals to the decapitation room. The time course of Cort secretion of 60-day-old females was not so distinct between non-Dep and Dep animals. The turnoff of the Cort response in 60-day-old female Dep rats, however, did not appear to be as efficient as that of non-Dep females. The reason for this isolated difference is not evident. Whether this is a result of maternal depriva-

tion-induced changes in female hormones leading to alteration on Cort clearance requires additional investigation.

The results of 12-day-old pups, maternally deprived for 24 h, are in agreement with previous findings (14, 25, 33, 34), although rat strain and conditions of cage cleaning were different between those and our study. At *PND 16*, both non-Dep and Dep animals exhibited increased Cort response to the stimulus, in accordance with the well-known fact that, at this age, pups are emerging from the SHRP (26, 28). Recently, van Oers et al. (36) reported that 20-day-old rat pups, separated from their mothers for 24 h on *PND 3*, exhibit higher ACTH response to stress, whereas this manipulation on 7-day-old pups does not result in altered ACTH release, and on *PND 11*, ACTH response of 20-day-old pups to stress is lower than that presented by normally reared pups. These groups, however, showed no differences of Cort levels compared with non-Dep pups. These findings evidence that maternal deprivation persistently alters the activation of the HPA axis. Moreover, they indicate that depending on the age when maternal deprivation is imposed, adrenal sensitivity is altered in different ways. If the reduced ACTH response to saline injection observed in animals deprived on *PND 11* is long lasting, it would be legitimate to speculate that our results on 30-day-old rats, i.e., reduced Cort response to the saline injection, were a consequence of this effect.

Interestingly, high endogenous Cort levels induced by maternal deprivation did not appear to be detrimental for the adrenocortical response of adult rats to stress, contrary to the effects of exogenous administration of GCs on some adrenal parameters, such as adrenal relative weight (29). Several studies that examined the effects of neonatal administration of GCs show that 20- to 25-day-old pups present lower plasma ACTH levels in response to ether and increased feedback sensitivity in response to dexamethasone, without change of Cort-binding globulin binding capacity (7). When tested at 45–48 days of age, hydrocortisone-treated female rats show decreased plasma Cort response to novelty (mild stress) and to ether (intense stress), whereas reduced Cort levels in males is seen in response to novelty, but not ether. The authors suggest an impairment of the adrenocortical response to stress at this age (6). The adrenocortical response to ACTH and to immobilization is also preserved in 30-day-old rats, neonatally treated with hydrocortisone, although the circadian rhythm of plasma Cort levels is obliterated at this age, but not in 80-day-old male rats (13). It is important to bear in mind, however, that these steroids are administered at different ages and in pharmacological doses, and they probably do not mimic accurately a more physiological situation, such as maternal deprivation.

The possibility that maternal reunion is capable of reverting or attenuating possible harmful effects of increased Cort levels during infancy is a matter of speculation and requires further investigation. The ability of a lactating anesthetized dam to suppress the adrenocortical response of Dep pups to stress was

Table 1. Weight of normally reared and maternally deprived pups at different ages

Age	Non-Dep, g		Dep, g	
	Males	Females	Males	Females
<i>PND 12</i>	20.42 ± 0.63 (26)	20.17 ± 0.57 (24)	18.60 ± 0.28* (24)	17.06 ± 0.26* (23)
<i>PND 16</i>	24.40 ± 0.73 (21)	24.21 ± 0.60 (26)	19.59 ± 0.66* (22)	18.52 ± 0.56* (26)
<i>PND 22</i>	40.95 ± 1.55 (10)	39.81 ± 1.05 (13)	30.12 ± 0.84* (26)	28.77 ± 0.89* (22)
<i>PND 30</i>	77.81 ± 2.18 (26)	74.72 ± 1.88 (30)	68.67 ± 2.00* (26)	64.67 ± 2.00* (30)
<i>PND 60</i>	214.06 ± 5.75 (36)	149.5 ± 3.09 (38)	228.62 ± 3.41* (39)	155.85 ± 3.36 (39)

Values are means ± SE, with no. of animals/group in parentheses. Maternal deprivation occurred on postnatal day (*PND*) 11 and lasted for 24 h. Non-Dep, nondeprived; Dep, maternally deprived. Comparisons were made within the same gender, between non-Dep and Dep pups. * $P < 0.03$, Student's *t*-test for independent variables.

previously demonstrated. Thus 12-, 16-, and 20-day-old Dep pups exposed to novelty in the presence of a lactating dam showed a suppressed Cort response, with or without a milk infusion. This effect was not obtained when pups were tested in the presence of a male or kept alone (30, 31). However, these studies only examined the effects of the presence of a dam during the testing procedure and not the consequences of the mother-infant reunion. Rosenfeld and co-workers (27) recently showed that only pups deprived of their mother for 24 h remain hyperresponsive to several stressors, after 4 days of reunion with their mothers, although basal levels are undistinguishable from those of non-Dep pups. The same results were not observed after 1 or 3 periods of 8 h of maternal deprivation, suggesting that there is a critical period, between 8 and 24 h, in which maternal deprivation effects appear to be long lasting. Therefore, the length, in addition to the regimen of exposure to maternal separation appears to be of critical importance. Although our results do not completely replicate their *PND 16* data, a direct comparison is not possible because they used a different strain of rats and we examined different ages.

Apparently, the length of maternal separation is of vital importance in determining the long-term effects of this manipulation. Thus pups subjected to a 2-h period of maternal separation for 28 days show hyperactivity and increased grooming in the open field, growth retardation, and immune suppression, suggesting an alteration of the endogenous opioid system (38). Maternal separation for 4.5 h during the first 3 wk of life, on the contrary, induces a reduction of Cort response to a 2-h restraint stress and increased inhibition of Cort response by dexamethasone, indicating increased negative feedback efficiency in separated pups. No difference in hippocampal GC and in cerebral cortex serotonin and adrenaline receptor densities is observed between separated and control animals. According to the authors, these results suggest that periodic maternal separation for 4.5 h renders the animals less sensitive to environmental stimuli in adulthood (22). These effects were shown to persist until *week 92* of life (20). A comparison between the effects of early handling and 180 min of maternal separation on some variables related to corticotropin-releasing factor (CRF) showed that handled rats presented lower basal hypothalamic CRF mRNA and content than maternally separated and control animals. Conversely, the latter groups showed higher Cort response to a restraint stress than handled rats (23).

Another interesting finding from our study is that gender differences in Cort response to stress were attenuated in Dep pups. Whereas non-Dep females showed a higher magnitude of Cort response than males at all time points except basal and 60 min, Cort levels of Dep females were higher than those of males only at the 5 min time point. We are unable, at this moment, to explain such attenuation in sex difference of Cort response in our Dep pups. Because we did not anticipate such an effect, possible alterations in estrous cycle were not followed. Therefore, any alteration in

sexual hormones that might have been induced by maternal deprivation requires a deeper investigation. An alternative explanation may come from a recent report in which 24 h of maternal deprivation on *PND 3* was shown to differentially alter the population of Cort receptors in the hippocampus of 48-day-old males and females. Whereas males show a downregulation of GC and mineralocorticoid receptors (GRs and MRs, respectively), females exhibit an upregulation of GRs at *PND 48*. These alterations were intensified when Dep pups received an ACTH injection at the end of the deprivation period, suggesting that increased circulating levels of either ACTH (from the injection) and/or Cort (as a consequence of the injection) appear to have a long-lasting influence on GRs and MRs that is dependent on the gender (35).

The fact that reductions of the Cort response in Dep pups were more consistent at *PND 30* indicates that this effect of maternal deprivation, although long lasting, may not be permanent. Similar results were observed with the pups' weight gain, in which Dep pups presented a smaller weight than their non-Dep counterparts until *PND 30*. It is not possible to affirm that reduced body weight of Dep pups is a function of reduced food consumption. On the basis of data showing the influence of feeding on the HPA axis activity, i.e., increased activation of the HPA axis as a consequence of starvation (4), and the dependence of diurnal Cort response to ACTH on time of feeding (40), it appears unlikely these animals were in any way underfed compared with non-Dep rats. Our results are, to a certain extent, in agreement with previous studies reporting that neonatal cortisol treatment results in depressed body weight gain even at 60- or 80-day-old rats (16, 29).

Contrary to the early handling paradigm, in which old handled rats present decreased Cort response to stress in addition to a more efficient negative feedback and diminished GR loss than nonhandled animals (17, 18), the effects of maternal deprivation at much older ages is unknown. At present, our results appear to indicate that maternal deprivation results in long-lasting effects on Cort response to a mild stress.

Perspectives

Our results showed the Cort response to a mild stimulus is reduced in prepubertal and, to a certain degree, in adult rats previously deprived of their mothers for 24 h, at *PND 11*. These results do not, however, imply that the same pattern of response can be expected for most stressors. Whether most intense stimuli or stressors applied for longer periods (for instance, restraint or novelty for 1 or 2 h) result in similar responses is unknown.

It would be interesting to examine both ACTH and Cort responses of Dep rats to different kinds of stress. In addition, the behavioral response of Dep rats to adverse situations, such as the open-field and the elevated plus-maze could shed some light on possible maternal deprivation-induced alterations on brain mechanisms, other than the HPA axis itself.

Finally, on the basis of the early handling literature, it appears worth verifying the consequences of 24-h maternal deprivation on the hippocampal function of aged rats, because this brain region contains both GRs and, therefore, is a target area of Cort action.

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