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Maternal Adrenalectomy and Adult Offspring in a Conflict Situation in the Rat

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CATALANI, A., E. TOTH, B. GAMBINI, A. GIULIANI, G. LORENTZ AND L. ANGELUCCI. *Maternal adrenalectomy and adult offspring in a conflict situation in the rat.* PHARMACOL BIOCHEM BEHAV 32(1) 323–329, 1989.—The effects of the absence of maternal adrenals during pregnancy (P), during lactation (L), during pregnancy and lactation (PL) were studied on pain suppressed behavior (punished drinking test) of the adult offspring in comparison with controls (C). The female L offspring showed a lower responsiveness to the anxiogenic stimulus, as demonstrated by increased water intake, decreased percentage of ineffective licks, and decreased time to perform 300 licks compared to C. The male L behavior was not affected. Reduced growth was not responsible for the reduced anxiogenic reactivity because also both male and female PL offspring had lower weight than C, but did not show any significant effect. Pain threshold in the tail flick test was the same in all types of offspring. Thus, absence of maternal adrenals, specifically during lactation, significantly affects behavior of female offspring. It is discussed whether this is due to the lack of a physiological influence of maternal adrenal hormones on brain ontogenesis (hippocampal glucocorticoid receptors), or on the development of the brain-pituitary-adrenal system during neonatal life of the offspring.

Hypothalamo-pituitary-adrenal axis   Adult offspring behavior   Punished drinking test
Neonatal brain endocrine ontogenesis

EXPERIMENTAL evidence exists that corticosterone treatment in rats during the first postnatal week produces hyperresponsiveness and hyperemotionality (27); it has also been reported that administration of corticosterone to lactating rats (3), or of adrenocorticotropin to the mother during pregnancy (31), has striking effects on the behavior of adult offspring. Similarly, when pregnant rats are exposed to stressors, activating the hypothalamo-pituitary-adrenal axis, offspring behavior is affected (11,32). Furthermore, according to some authors, prenatally stressed offspring are more emotional and vulnerable to subsequent stress in adult life (10), while others suggest that in the same situation a decrease in emotionality occurs (1, 15, 22).

Effects of maternal deficiency of adrenal hormones on offspring behavior have also been reported: females born to mothers adrenalectomized prior to mating had significantly higher avoidance scores in the shuttle box, and an increased activity in the open field (16). In contrast, other studies failed to demonstrate that altered pituitary-adrenal function in the mother induces behavioral changes in the offspring: rats born to mothers adrenalectomized on day 10 or 16 of pregnancy (13), or hypophysectomized on day 13 of gestation (26), or treated with dexamethasone from day 2 to 17 of gestation (12), were found to be no different from controls in some behavioral tests.

The present study is aimed to extend the previous investigations on the long term effects of manipulation of the maternal hypothalamo-pituitary-adrenal function (3,4), with regard to emotionality in the adult offspring. Rats born to and/or lactated by adrenalectomized mothers were tested in a conflict situation, since there is evidence that this procedure produces "fear and stress" anxiety of the corticosterone releasing type (18).

METHOD

A total of 104 COBS Wistar virgin female rats (Charles River, Calco Italy) at about seventy days of age and weighing 160–180 g were housed, four per cage, with one male for one week and pregnancies were daily determined (presence of spermatozoa in the genital tract). The positive animals were immediately housed singly. Forty-seven out of 77 pregnant rats were adrenalectomized (dorsal approach under light ether anesthesia) on 14th day of pregnancy, and then maintained on 0.9% saline in place of tap water. The remaining 30 rats were sham operated. Mothers which failed to deliver or did not survive after adrenalectomy or sham operation during or before delivery were 16/47 (34%) for the adrenalectomized and 5/30 (16%) for the sham operated animals. As soon as possible (in no case more than 8 hours) after delivery, litters were culled to six pups (3 females and 3 males) to prevent malnutrition, and moved from the biological mother to a foster mother. Litters of 7 adrenalectomized mothers were crossed with those of 7 sham operated mothers; cross-fostering was also accomplished within litters of 14 sham operated mothers and within litters of 20 adrenalectomized mothers, so that no pup was nursed by its biological mother; in the latter two instances not all litters were used. Weaning was carried out on the 21st day. Four female and four male groups were obtained, each of 10–20 animals randomly chosen out from 5 to 7 litters: consequently each litter contributed to each group with 2–3 animals. This procedure was similar to that adopted in studies on behavioral consequences of maternal manipulation on the offspring (19,23). The groups were: rats born to and lactated by sham operated mothers (controls, C groups, 12 females and 18 males, respectively); rats born to adrenalectomized mothers and lactated by sham operated foster mothers (absence of adrenals during pregnancy, P groups, 16 females and 19 males, respectively); rats born to sham operated mothers and lactated by adrenalectomized foster mothers (absence of adrenals during lactation, L groups, 10 females and 11 males, respectively); rats born to adrenalectomized mothers and lactated by adrenalectomized foster mothers (absence of adrenals during pregnancy and lactation, PL group, 16 females and 20 males, respectively). When even one member of the litter died, the whole litter was discarded; this occurrence was 0/6 for the C group, 0/7 for the P group, 2/7 for the L group, 3/10 for the PL group. Rats were housed 3 per cage and maintained at 24±2°C with a 7 a.m.–7 p.m. light period. All rats underwent the conflict test at the age of five months; during the 2 weeks before testing all rats were gentled by daily handling.

Body Weight

Body weights were taken at weaning and at 12, 18, 24 and 28 weeks.

Milk Composition Determination

Four adrenalectomized and four sham operated mothers on 8th day of lactation were instrumentally milked on light ether anesthesia and oxitocin stimulation (5 I.U. SC). Milk was analyzed for protein, sugar and fat content according to standard procedures (29). Litters of these mothers were discarded.

Tail Flick Test

The eight groups of offsprings were tested for algesic threshold with the tail flick procedure (7), two weeks before the conflict procedure, in order to ascertain differences among groups in pain sensitivity which could influence their performance.

Basal Water Intake

Daily water intake in each cage was recorded for 5 days before the conflict procedure, in order to ascertain differences among groups which could influence their performance.

Conflict Test Apparatus and Procedure

A modified Vogel *et al.* (41) conflict test procedure was used. The testing apparatus consisted of a Plexiglas chamber 37×26×13 cm with a stainless steel floor, equipped with a graduated all glass drinking tube, the nozzle of which contained a wired stainless steel coil. Animals in their home cage were water deprived 48 hours prior to either the training or the testing session, with food constantly available. Training consisted in moving the rat to the testing chamber, allowing it to freely drink tap water for 10 minutes, and then immediately returning it to the home cage. One hour later, the rat was allowed to drink for 24 hours. After a second 48 hour period of water deprivation, the rat was placed again in the test chamber, the drinking tube was now connected to a shocker (the other pole was the stainless steel floor) so that the animal received a constant intensity current (210 μA) every time it attempted to drink. The apparatus could automatically measure the parameters of consummatory behavior. The test was always carried out between 7 a.m. and 12 a.m.

The following measurements were made: 1) latency to the first lick, 2) time to complete 300 licks, 3) water intake in 10 minutes and 4) percentage of ineffective licks in 10 minutes expressed as:

$$\frac{\text{total licks} - \text{effective licks}}{\text{total licks}} \times 100$$

Effective licks were calculated by multiplying the number of licks necessary to drink 1 ml in the training session (without the shock) by the number of ml assumed in the test session. The number of ineffective licks was considered a more precise measure of anxiety than the water intake. In fact, animals could have consumed a similar amount of water either by a greater (more anxiety) or smaller (less anxiety) number of licks. Measurements 1 and 2 evaluate the drive of the animal in the first minutes of the test, when the rat has to cope both with the environmental change and the shock for the first time. Measurements 3 and 4 evaluate the overall performance of the animal.

A high latency to the first lick, a long time to complete 300 licks, a high percentage of ineffective licks and a small water intake were assumed to indicate a high level of anxiety.

Statistical Analysis

Data are presented as the mean±standard error of the mean (S.E.M.). Animals' weights were analyzed by means of a linear trend analysis with a two-factor mixed design with repeated measure on one factor (5,43). Behavioral data were analyzed (ANOVA) as a 2×2 factorial (two levels: absence or presence of adrenals during pregnancy or lactation) design using treatment means as single observations because of an unequal number of animals per group (8). However as the levels of the factors were simply presence or absence, the results of the factorial design may be misleading (8), thus, Newman-Keuls' test for the significance of the differences between pairs of means was subsequently used. Moreover, the behavioral variables were analyzed with a noninferential approach [Cluster analysis procedure (K means)] (9); prior to this a factor analysis was used for the selection of variables (28). Time latencies in the tail flick test were analyzed by the Mann-Whitney U-test.

RESULTS

Milk Composition

No clearcut differences were observed in the content of fat, protein and sugar in milk between adrenalectomized (16.1, 5.21, 1.03 respectively) and sham operated (14.9, 4.7, 0.93 respectively) animals.

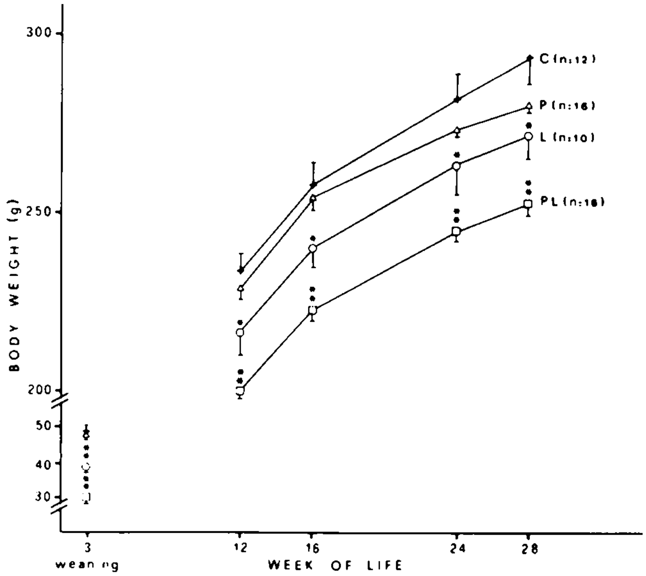

FIG. 1. Mean body weights (±S.E.M.) of female offspring at different ages. C=control, P=absence of maternal adrenals during pregnancy, L=during lactation, PL=during both pregnancy and lactation. Number of animals in parentheses. *$p<0.05$ and **$p<0.01$ with Newman-Keuls' test versus C.

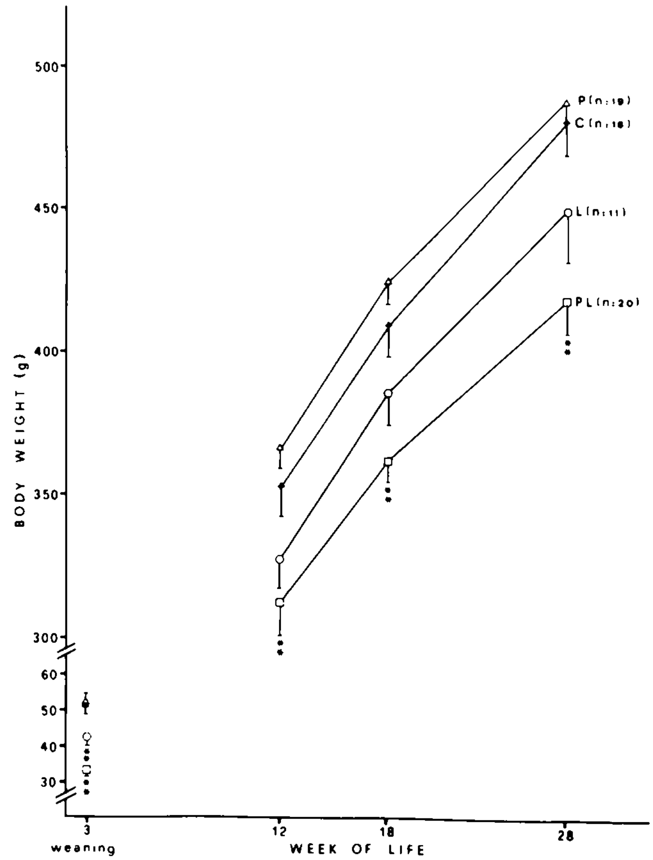

FIG. 2. Mean body weights (−S.E.M.) of male offspring at different ages. C=Control, P=absence of maternal adrenals during Pregnancy, L=during Lactation, PL=during both Pregnancy and Lactation. Number of animals in parentheses. *$p<0.05$ and **$p<0.01$ with Newman-Keuls' test versus C.

Body Weight

Figure 1 shows mean body weights of female offspring at different ages. The linear trend analysis revealed a significant effect of the absence of maternal adrenals during lactation on offspring body weights [$F(1,26)=7.16$, $p<0.05$ for L animals, and $F(1,26)=38.93$, $p<0.001$ for PL animals versus C]. A variance analysis for the single days showed that the differences between L, or PL animals and C were present both at weaning and in adulthood (see figure for levels of significance). A significant groups × days interaction was present for PL offspring [$F(4,104)=4.17$, $p<0.01$ versus C]; this group was the only one in which the difference in body weight from controls went together with a difference in growth rate. The analysis also showed a significant difference in body weight between L and PL animals, $F(1,24)=9.08$, $p<0.01$. No significant differences in body weight and growth rate were observed between P and C.

Weights of male offsprings are reported in Fig. 2. The PL offspring weight was significantly lower than C, $F(1,36)=24.32$, $p<0.001$. A day by day analysis revealed that PL rats' body weight was lower than that of C at weaning and in adulthood, while for L animals the difference was present only at weaning (see figure for levels of signfiicance). As for female offspring, a significant groups × days interaction growth rate was present only for the PL offspring, $F(3,108)=4.77$, $p<0.01$. Body weight of PL animals was significantly lower than that of the L offspring, $F(1,29)=12.7$, $p<0.01$. The analysis did not reveal any difference in weight between L, or P animals and C.

In spite of the significant differences in body weight, no signs of poor health were observed (fur appearance, resistance to grips, muscle tone and mortality rate) both at weaning and in adulthood.

Tail Flick

With this test no differences were observed in pain sensitivity within the four experimental groups of female offspring or within the four experimental groups of male offspring [medians (sec): female C 4.3, P 3.7, L 5.1, PL 4.5; male C 4.1, P 3.8, L 3.4, PL 3.8; Mann-Whitney U-test].

Basal Water Intake

No significant differences were observed in daily water intake among the four groups of offspring [$F(3,53)=1.64$, ns for male, and $F(3,38)=0.61$, ns for female].

Conflict Test

In this test the behavior of C animals was no different from that routinely observed in unfostered animals in our laboratory: for instance values of latency time to the first lick, time to make 300 licks, water intake and percentage of uneffective licks were in the former 21±8, 231±43, 3.6±0.5 and 40±5, respectively; in the latter 16±3, 234±44, 3.9±0.9 and 36±4.3, respectively.

The behavior in the conflict test of the four experimental groups of female and male offspring is shown in Figs. 3 and 4, respectively. The 2×2 factorial design showed that the

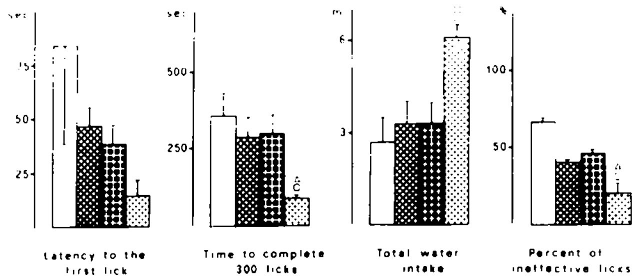

FIG. 3. Behavior in the conflict test of female offspring: C (open columns), P (cross hatched columns), PL (columns with squares) and L (dotted columns). Each column represents the mean±S.E.M.; ○, □, △=$p<0.05$ with Newman-Keuls' test versus C, P and PL respectively. Two × two factorial analysis in the test.

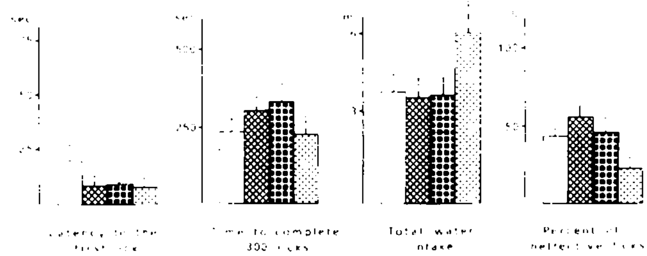

FIG. 4. Behavior in the conflict test of male offspring: C (open columns), P (cross hatched columns), PL (columns with squares) and L (dotted columns). Each column represents the mean+S.E.M.; ○, □, △=$p<0.05$ with Newman-Keuls' test versus C, P and PL respectively. Two × two factorial analysis in the test.

TABLE 1

FACTORIAL ANALYSIS: MATRIX OF FACTOR LOADINGS OF DRINKING BEHAVIOR VARIABLES

Variables	Factors 1	2	3	4
Latency to the first lick	0.46	0.88	−0.05	−0.01
Time to make 300 licks	0.81	−0.20	−0.49	0.22
Water intake	−0.87	0.18	−0.02	0.44
% uneffective licks	0.83	−0.09	0.48	0.25
Total variance percentage	58.6	21.7	11.9	7.7

behavior of female offspring was not affected by the absence of maternal adrenals during pregnancy; on the contrary, the absence of maternal adrenals during lactation significantly affected the time to perform 300 licks, $F(1,47)=4.57$, $p<0.05$, the percentage of ineffective licks, $F(1,47)=4.35$, $p<0.05$, and the water intake, $F(1,47)=4.69$, $p<0.05$. Latency time to the first lick was unaffected. The analysis also revealed a significant interaction of the absence of adrenals during P with the absence during L in the time to perform 300 licks, $F(1,47)=4.89$, $p<0.05$, the water intake, $F(1,47)=4.69$, $p<0.05$, and the percentage of ineffective licks, $F(1,47)=6.45$, $p<0.05$. So it appeared that the behavior of offspring whose biological and foster mothers were both adrenalectomized was not simply the result of the algebraic sum of the effect of the absence of adrenals in the two periods of perinatal life. The Newman Keuls' test for the significance of the difference between pairs of means indicated that in L offspring the time to perform 300 licks and the percentage of ineffective licks were decreased and the water intake increased in comparison with C (see Fig. 3 for significance). The L offspring was also significantly different from the PL one in the time to perform 300 licks, water intake and percentage of ineffective licks. No significant differences were observed in groups P and PL versus C.

In male offspring, the factorial design revealed that the percentage of ineffective licks was affected by the absence of adrenals during pregnancy, $F(1,58)=5.66$, $p<0.05$. The absence of adrenals during pregnancy or lactation had no effect

TABLE 2

CLUSTER ANALYSIS: NATURAL CLASSES PROFILES

	Time to Complete 300 Licks x	DS	Total Water Intake x	DS	% Ineffective Licks x	DS
Female						
I cluster	95.4	38.5	6.3	1.5	18.3	18.2
II cluster	253.2	160.1	2.9	1.2	36.0	15.0
III cluster	600.0	0	0	0.1	97.1	10.1
Entire set	271.1	225.3	3.8	2.9	43.9	36.3
Male						
I cluster	167.1	94.6	6.5	1.8	16.8	25.6
II cluster	216.3	93.8	3.0	1.5	52.5	18.8
III cluster	600.0	0	1.0	2.2	65.7	33.0
Entire set	273.9	182.1	3.7	2.7	43.5	30.9

on latency to the first lick, time to make 300 licks and water intake. No interaction of the absence of adrenals during P with the absence during L was observed. Multiple comparisons with the Newman Keuls' test did not demonstrate an increase in percentage of ineffective licks of the offspring born to adrenalectomized mothers, whether or not the foster mother was adrenalectomized. The factor analysis, previous to the cluster analysis, put in evidence two major factors (Table 1): the first includes three variables: time to make 300 licks, percentage of uneffective licks and water intake; the second one includes the latency to the first lick. The cluster analysis, separately performed on females and males using the first three variables, revealed the following remarkable features: a) the presence of three natural classes characterized as follows: first class: short time to make 300 licks, low percentage of uneffective licks and high water intake (low responsiveness to anxiogenic stimuli); second class: long time to make 300 licks, high percentage of uneffective licks and small water intake (high responsiveness to anxiogenic stimuli); third class: intermediate values of the three variables (medium responsiveness to anxiogenic stimuli) (Table 2); b) Table 3 shows an asymmetrical clustering of

TABLE 3

DISTRIBUTION OF EXPERIMENTAL GROUPS IN THE NATURAL CLASSES

Experimental Groups	Clusters 1	2	3
Female			
C	4	2	6
P	5	6	3
L	9	1	0
PL	5	4	4
Male			
C	5	8	2
P	5	8	5
L	6	3	1
PL	5	10	4

Clusters indicate the degree of responsiveness to anxiogenic stimuli: cluster 1: low, 2: intermediate, 3: high anxiety.

female L offspring while the animals of the other groups are spread over the three clusters. The exact Fisher test on the extreme classes (1 and 3) confirmed the different distribution of L females compared to C ones ($p<0.01$).

DISCUSSION

The present findings indicate that the behavior of the offspring lactated by an adrenalectomized mother can be significantly affected in an anxiety-provoking situation.

In the punished drinking test, female offspring of this type (L) showed lower conflictuality compared to controls, as demonstrated by: 1) the increased water intake despite the presence of an aversive stimulus, 2) the decreased percentage of ineffective licks, and 3) the shorter time to perform 300 licks. Since no differences in basal condition daily water intake and algesic threshold were observed among the various types of offspring, the above results strongly suggest an effect on anxiety, although such an effect should be confirmed in other anxiogenic situations. However in studies to be published a reduction in the grooming activity in the open field, and an increase in holeboard exploration have been observed in this type of offspring.

Interestingly, a significant interaction of absence of adrenals during P with absence of adrenals during L in the parameters of the conflict test was present in the female offspring, i.e., the absence of maternal adrenals during pregnancy did not affect the female offspring behavior, although prevented the effect of the same absence during lactation. This interaction may be explained by considering the fetal adrenal hypertrophy secondary to maternal deficiency (6) in the PL offspring (not in L offspring), which produces in the fetal and neonatal life, plasma corticosterone levels higher than normal (37).

Although no hierarchal design analysis for "litter effect" was applied (2), such an effect is unlikely to have occurred, considering the relatively high number of litters used, compared to the number of animals from each litter in all experimental groups. Moreover, the consistence of the evaluations obtained with two completely different approaches, cluster analysis and classic inferential statistics, demonstrates the robust structure of the data. It is to be noted that the female L offspring were so homogeneous in their behavior in the punished drinking test that they were almost totally concentrated in one natural class, further underlining the absence of a "litter effect."

The effects of maternal adrenalectomy during lactation on adult offspring behavior could have occurred through different mechanisms: for instance, a maturation and growth deficit affecting behavior. Fat, protein and sugar contents of milk of adrenalectomized mothers were not substantially different from controls; however milk production could have been reduced, as a consequence of the lack of corticosterone which modulates the initiation and maintainance of lactation (30). The impairment of growth could also have been due to reduced corticosterone-operated transportation of fluid and salts through the gastrointestinal mucosa (17). Since we observed an effect on the behavior in the female L offspring only, while impairment of growth was present in other types of offspring (PL females, L and PL males), the reduced responsiveness to anxiogenic stimuli cannot be attributed to this impairment. The offspring growth and behavior could also have been affected by a deficit in maternal behavior (14, 35, 36, 42,); this could, for instance, influence the development of masculine sexual behavior (24). However, any possible deficit in maternal behavior could not have been the major factor responsible for female L behavior, since the same effect should have been observed even in PL females. Stimulation of the maternal pituitary-adrenal system as a "separation effect" could have influenced adult offspring behavior; however this effect could not have been present in the foster mothers of L group, because they were adrenalectomized. A similar "separation effect" was unlikely in the pups because in the early postnatal period their pituitary-adrenocortical responsiveness to stress was substantially suppressed (34).

Direct effects of changes in the maternal pituitary-adrenal system on neonatal brain in a period during which ontogenesis is accomplished could also have had an influence on behavior in adulthood (40). It is known that the hippocampus with its high concentration of glucocorticoid receptors is a principal neural target tissue for the steroids (20,33) and presence of corticosterone in milk has been reported by many laboratories (17). Some studies demonstrated that daily injection of the hormone on postnatal day 3–5 (25), neonatal handling (23), and maternal endocrine manipulation (4) result in a change in the glucocorticoid receptor system in the adult rat. The absence of the glucocorticoid hormone in the adrenalectomized lactating mother and, consequently, of corticosterone intake in pups through the milk could have had significant consequences by influencing brain development in general, and expression of the hippocampal glucocorticoid receptor in particular, inducing permanent effects on behavior. In fact, previous studies in this laboratory demonstrated that in the three-month-old male offspring, hippocampal specific binding of corticosterone, as well as basal and stress plasma level of the hormone, were affected by maternal adrenalectomy during lactation, both in the presence and the absence of adrenals during pregnancy (4). No data are available for female offspring which could help us to interpret our results. Turner and Weaver (39) described a marked sexual dimorphism in the adult rat hippocampal glucocorticoid binding, with females exhibiting a greater capacity than males. This could make females more sensitive to the effects of neonatal deficit of corticosterone. Moreover it has been shown that maternal estrogens penetrate the blood brain barrier in newborn males, but not in females (21); this could modify the brain sensitiveness to the lack of corticosterone.

We emphasize that the group most consistently different from any other group was that of animals born to sham operated mothers and lactated by foster adrenalectomized mothers. This is in accordance on the one hand with the finding of behavioral consequences of postnatal maternal stress on the offspring (19), and on the other hand with the findings of no effect of maternal pituitary-adrenal system manipulation during gestation on the offspring behavior if lactation was carried out by an intact foster mother (13,26). The findings in the present study of a reduced emotionality are in accordance with the increase in emotionality of the offspring of mothers stressed during pregnancy (38) and in rats treated with exogenous corticosterone in neonatal life (27).

Impaired development, modification of corticosterone hippocampal binding capacity and of hypothalamo-pituitary-adrenal axis activity in the offspring, as the main consequences of maternal adrenocortical insufficiency during lactation, may participate in the genesis of the behavioral effects in adulthood. However, further studies are needed to better clarify the lower responsiveness to anxiogenic stimuli as a consequence of the above occurrence.

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