Journal of Neuroendocrinology, 2002, Vol. 14, 450–457

Maternal Regulation of the Hypothalamic-Pituitary-Adrenal Axis in the 20-Day-Old Rat: Consequences of Laboratory Weaning

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Key words: weaning, maternal deprivation, postnatal development, fasting, rat.

Abstract

There is a large body of evidence that the development of the hypothalamic-pituitary-adrenal (HPA) system in the rat is under maternal regulation. One method used to study the influence of the dam–pup interaction in neonates and weanlings is the separation of mother and litter for 24 h. Previous studies showed that, even at the time of weaning, maternal deprivation results in a dysregulation of the HPA axis at multiple levels. However, the maternal deprivation paradigm usually includes deprivation of food and water, and it was not clear to which extent the observed effects are due to either maternal cues or dehydration and fasting. The primary purpose of the present study was to determine the role of fasting and/or maternal separation on the HPA axis at the time of weaning. Pups at 20 days after parturition are capable of self-feeding and no longer require tactile stimulation to induce eliminative functions. The results indicated that 24 h of fasting led to increased basal levels and further increases in stress induced corticosterone secretion. Fasting also appeared to contribute to the down regulation of basal glucocorticoid receptor mRNA in the hippocampus. In contrast, abrupt weaning irrespective of fasting or dehydration resulted in a suppressed adrenocorticotropin hormone response to an injection of isotonic saline. Although there was an effect of maternal separation on corticotropin-releasing factor mRNA in the paraventricular nucleus, this effect was further exacerbated by the absence of food. Finally, all rats that were separated from their dams showed more efficient negative-feedback. Thus, different aspects of the HPA system appear to respond differentially to either the absence of food or the absence of the mother or both.

The development of the hypothalamic-pituitary-adrenal (HPA) system in the rat is largely under maternal regulation (1–3). During the so called stress hyporesponsive period (SHRP), which lasts from approximately days 4–14, the dam suppresses the HPA axis so that mild stressors do not elicit a corticosterone response in the pup (4, 5). Twenty-four hours of maternal deprivation disinhibits the SHRP and the pups show elevated basal corticosterone levels and a robust adrenocorticotropin hormone (ACTH) and corticosterone response following a mild stress (6, 7). The major aspects of maternal behaviour that have been isolated as key suppressors of the HPA system during the SHRP are feeding and tactile stimulation (8–10). Other factors, such as maternal corticosterone in the milk (1), contact with siblings or with an adult rat (i.e. male) (11) and kinesthetic or vestibular stimulation (12), did not have an influence on maternal deprivation effects.

The published data suggest that tactile stimulation and nutrition affect different aspects of the HPA axis. Suchecki *et al*. (8) reported that stroking suppresses the stress-induced elevations of ACTH due to maternal deprivation, but not corticosterone. Only the combination of feeding and stroking suppresses the elevation of corticosterone after stress. Van Oers *et al*. (9) further demonstrated that the down regulation of corticotropin-releasing factor (CRF) mRNA in the paraventricular nucleus (PVN) of the hypothalamus and mineralocorticoid receptor (MR) mRNA in the hippocampus

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following maternal deprivation can be reversed by stroking. However, glucocorticoid receptor (GR) mRNA down regulation following maternal deprivation in the PVN and the hippocampus could only be reversed by stroking in combination with feeding. It was difficult to study the effects of nutrition alone as feeding at these ages needs to be combined with anogenital stroking to induce urination and defecation. However, these findings do suggest that nutrition primarily affects the adrenal and corticosterone while ACTH and central variables of the stress system are mainly affected by tactile stimulation.

Some of these effects have also been observed in pups outside the SHRP. Maternal deprivation at 18–20 days results in profound changes of the HPA system. Even though the post-SHRP pup shows a normal corticosterone response to mild stressors, 24 h of maternal deprivation greatly enhance this response (3, 7, 11, 13, 14). Interestingly, the ACTH response following an isotonic saline injection is suppressed in the older deprived rats (15). This effect is paradoxical to that which has been found during the SHRP. Furthermore, Smith *et al*. (14) reported a down regulation of basal CRF mRNA and c-*fos* mRNA stress levels in the PVN after deprivation in the older pup. The dam–pup interactions are different at this age. Anogenital licking to induce urination and defecation and a feeding apparatus are no longer required (16). It appears likely that the effects observed in older pups are largely due to nutritional deficits. However, tactile stimulation has still been shown to have a regulatory effect around the age of weaning in systems other than the HPA axis. Wang *et al*. (17) reported that the absence of tactile stimulation, but not the absence of food, suppresses basal ornithine decarboxylase (ODC) levels and tissue ODC response to trophic factors. These data suggest a regulatory role of the mother not only during the SHRP, but also at least until weaning.

The present experiment was designed to examine the effects of nutrition independent from those of tactile stimulation in rats at the time of weaning. Common laboratory practice is to separate the pup from the mother between 20 and 22 days of age. Laboratory weaning happens earlier and more abruptly than it would be in the absence of human management, with the immediate effects on the infant being mostly unknown. Since, at 20 days, the pups are capable of feeding, urinating and defecating without maternal help, it was possible to examine nutrition separately from maternal contact by removing the mother with or without food. A feeding apparatus and anal stroking, as used in younger rats, was not necessary (8, 9). Therefore, we were able to examine the effects of feeding and dehydration directly and test the hypothesis that the effects of maternal deprivation at this age are predominantly due to the lack of nutrition. Furthermore, the study addressed the question of neuroendocrine consequences of early weaning in the laboratory rat.

Materials and methods

Animals

Hybrid offspring of Sprague-Dawley females and Long-Evans males were used in this study. Offspring were bred in the rat colony at the University of Delaware. A total of 32 litters were used. Pregnant females were checked for litters daily at 09.00 h. If litters were found, the day of birth was defined as day 0 for that litter. On the day after parturition, day 1, each litter was culled to eight healthy pups (four males and four females), and each mother and her offspring was transferred to a clean polycarbonate cage. Litters remained undisturbed until used in the study. All rats were housed under a 12 : 12 light/dark cycle (lights on at 07.00 h EST) and constant temperature (25 ± 2 °C) conditions. Food and tap water was provided *ad libitum*. The experiment was carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Deprivation procedure

Male and female pups were examined at postnatal day 20. Litters were randomly assigned to any of the four different conditions during the last 24 h before testing, three maternally deprived (DEP) conditions and one non-deprived (NDEP) condition. For the deprived conditions, litters were separated from their mothers for 24 h and kept without any food or water available (DEP − F − W), or provided either with food and water (DEP + F + W) or only with water (DEP − F + W) *ad libitum*. Deprivation was carried out in a separate room in the animal facility under similar lighting and temperature conditions as mentioned previously. Mothers nursing litters selected for maternal deprivation were removed from their home cages 24 h prior to the day of experimentation. All of the pups remained in the home cage, which was placed on heating pads maintained at 30–33 °C for 24 h. Non-deprived litters were left undisturbed with their mothers in the rearing room until the day of testing. All pups were colour-marked on their tails for identification purposes and weighed before and after the deprivation procedure.

Testing procedure

At the initial time of treatment (t_0), one male and one female pup from the litter were weighed and sacrificed by decapitation, serving as noninjected controls. If the tested litter was a nondeprived control the mother was removed immediately before t_0. The remaining three male and three female pups from the litter were weighed and received a single injection of isotonic sterile saline (0.9%; volume 0.1 ml/10 g bodyweight, i.p.; 27-gauge needle), which served as the mild stressor. Sodium chloride solution was nonpyrogenic and endotoxin free (Sigma, St Louis, MO, USA). Injected pups were returned to their home cage (without the mother) and each cage was placed on a heating pad maintained at 30–33 °C for the testing duration. At time points 15, 30 and 60 min, one male and one female pup were sacrificed from each home cage.

Sampling and hormone assays

Trunk blood was collected individually in labelled 1.5 ml ethylenediametetraacetic acid (EDTA)-coated microcentrifuge tubes at the time of decapitation. The samples were kept on ice and later centrifuged for 20 min at 2000 r.p.m. at 5 °C. The plasma was then transferred to clean 1.5-ml microcentrifuge tubes and stored at −20 °C. Commercially available kits were used for the determination of plasma ACTH and corticosterone [INCSTAR Corp., Stillwater, MN, USA (sensitivity 15 pg/ml) and ICN Biomedicals, Inc., Cleveland, OH, USA (sensitivity 0.125 μg/dl)]. At the designated time after the saline injection (15, 30 and 60 min), brains were rapidly removed and frozen in 2-methylbutane at −50 °C, then stored at −70 °C until processing for *in situ* hybridization.

In situ *hybridization*

Frozen brains were sectioned at −20 °C in a cryostat microtome at 16 μm in the coronal plane through the level of the hypothalamic PVN and the dorsal hypothalamus. The sections were thaw-mounted on Superfrost slides (VWR Scientific, West Chester, PA, USA), dried at 35 °C on a slide warmer and kept at −70 °C. Using ^{35}S-UTP labelled riboprobe, hybridization histochemical localization of CRF mRNA or the GR mRNA transcript were performed as described previously (18). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol, and delipidated in chloroform. The cRNA probes for CRF and GR contained the full length coding regions of rat CRF and GR (respectively). The antisense cRNA probes were transcribed from a linearized plasmid using SP6 polymerase (CRF mRNA) or T7 polymerase (GR mRNA) according to the instructions supplied with the kit (Ambion, Inc., Austin, TX, USA). Tissue sections (two brain sections per slide) were saturated with 100 μl of hybridization buffer (20 mM Tris-HCL (pH 7.4), 50% formamide, 300 mM NaCl, 1 mM EDTA (pH 8), 1 × Denhardt's, 250 μg/ml yeast transfer RNA, 250 μl/ml

total RNA, 10 mg/ml salmon sperm DNA, 10% dextran sulphate, 100 mM dithiothreitol, 0.1% SDS and 0.1% sodium thiosulphate) containing 1.5×10^6 c.p.m. ^{35}S-labelled riboprobe. Brain sections were coverslipped and incubated overnight at 54 °C. The following day the sections were rinsed in 4 × standard saline citrate (SSC), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 × SSC for 1 h at 65 °C and dehydrated through traded concentrations of alcohol. The slides were apposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY, USA) for 5 days for CRF mRNA and 14 days for GR mRNA.

Statistical analysis

The experiment had the following experimental design: 4 conditions (NDEP, DEP−F−W, DEP−F+W, DEP+F+W) × 4 treatments [basal (0 min) and at 15 min, 30 min, 60 min after saline injections] × 2 sex (male, female). Eight litters were used in each condition.

The results were analysed by means of a 4 (conditions) × 4 (treatment) × 2 (sex) analysis of variance procedures (ANOVA) with $P < 0.05$ considered statistically significant. Where appropriate, tests of simple main and interaction effects and subsequent posthoc comparisons were made by Newman–Keuls procedures. The initial analysis included sex as a factor; once it was determined that sex was not a significant factor, the data were collapsed across this variable and analysed by means of 4 (conditions) × 4 (treatments).

Autoradiographs were digitized, and relative levels of mRNA were determined by computer-assisted optical densitometry (NIH Image, Bethesda, MD, USA). The mean of 4–8 measurements was taken from each rat.

Results

Comparison of body weight

ANOVA revealed an interaction between the different conditions [$F(3,252) = 121.7$, $P < 0.0001$]. When compared to the average weight at postnatal day 19 (48.4 ± 0.27 g), NDEP and DEP+F+W pups gained weight, DEP−F−W and DEP−F+W lost weight. The weight of DEP−F+W was significantly higher compared to DEP−F−W. Pups in the NDEP condition showed the significantly highest weight of all groups at postnatal day 20 (Fig. 1).

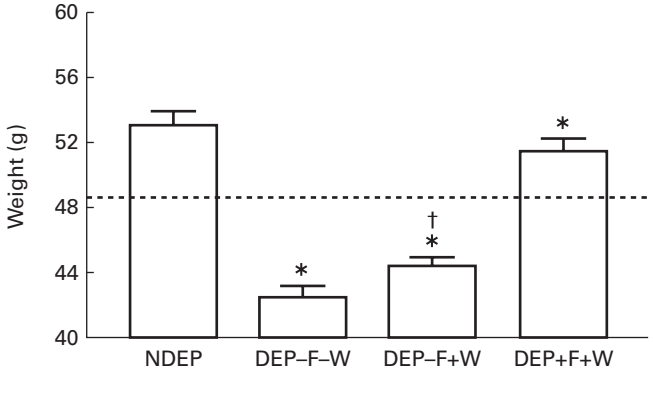

FIG. 1. Body weight in 20-day-old deprived and nondeprived rats. The dotted line marks the average weight of all pups at postnatal day 19 (48.4 ± 0.27 g). Note that both NDEP and DEP+F+W pups gain weight, while pups deprived without food (DEP−F+W and DEP−F−W) loose weight during the deprivation period. Data represent mean ± SEM. *Significant from NDEP, †significant from DEP−F–W and DEP+F+W, $P < 0.05$.

Plasma corticosterone levels

ANOVA revealed an interaction between condition and treatment [$F(3,252) = 6.49$, $P < 0.0001$]. All groups showed a significant corticosterone response following 15 min after saline injection. Plasma corticosterone levels in the NDEP group remained elevated over the time of testing, while in all the deprived groups plasma corticosterone levels returned to baseline within 60 min. We detected significant elevation of basal and stress-induced corticosterone in the DEP−F−W and the DEP−F+W group compared to the NDEP controls. Pups from the DEP+F+W condition did not differ significantly in their basal corticosterone levels and their 30 min response compared to their NDEP counterparts. However, they did show a faster response (significantly higher corticosterone levels at 15 min compared to NDEP) and 60 min following saline stress corticosterone levels returned to baseline (significantly lower compared to NDEP) (Fig. 2).

Plasma ACTH levels

ANOVA revealed an interaction between condition and treatment [$F(3,252) = 7.77$, $P < 0.0001$]. All groups showed a significant ACTH response 15 min after saline injection. Plasma ACTH levels in the NDEP group remained elevated over the time of testing, while in all the deprived groups ACTH levels returned to baseline within 30 min. Stress-induced ACTH elevation in all the deprived groups was significantly lower compared to the NDEP controls. No significant difference could be detected between the deprived conditions (Fig. 3).

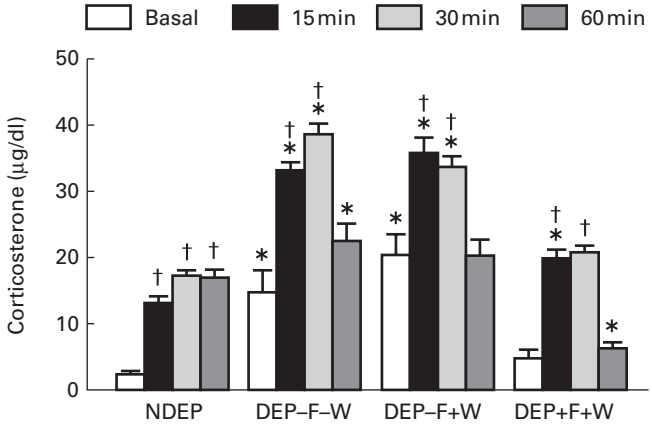

FIG. 2. Basal and stress-induced (saline injection) plasma corticosterone values in 20-day-old deprived and nondeprived rats. Basal and stress values of corticosterone in the DEP−F−W and DEP−F+W group were significantly elevated compared to the NDEP group. With the availability of food and water during the deprivation period (DEP+F+W), no significant differences were detected between basal and 30 min stress-induced corticosterone compared to the NDEP controls. In all the deprived groups, corticosterone values returned to baseline levels within 60 min. In the NDEP control group, corticosterone levels remained elevated during the time of testing. Data represent mean ± SEM. *Significant from NDEP counterparts, †significant from basal levels, $P < 0.05$.

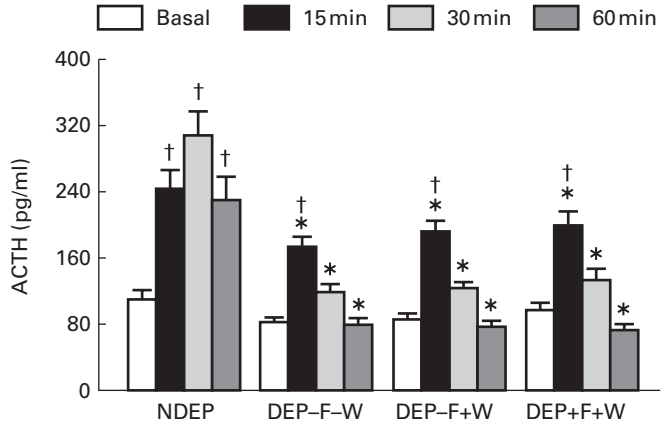

FIG. 3. Basal and stress-induced plasma adrenocorticotropin hormone (ACTH) values in 20-day-old deprived and nondeprived rats. No significant differences between the groups were detected in ACTH basal values. Following saline stress all deprived groups showed a significantly lower response compared to the NDEP controls. No significant differences were detected between the deprived conditions. After 30 min ACTH levels in all deprived conditions returned to baseline levels (no significant differences could be detected). In the NDEP controls, ACTH levels remained elevated during the time of testing. Data represent mean ± SEM. *Significant from NDEP counterparts, †significant from basal levels, $P < 0.05$.

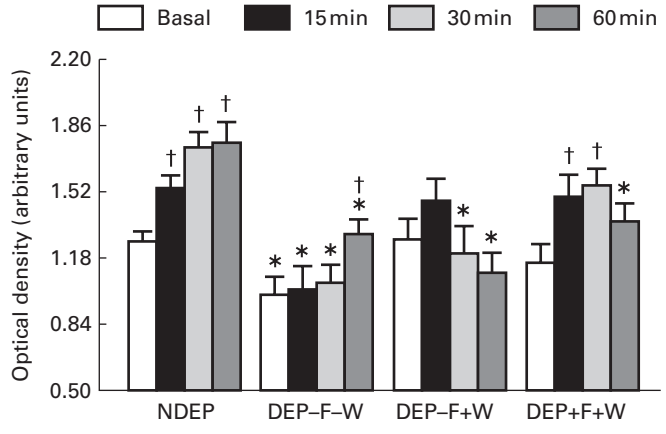

FIG. 4. Basal and stress induced expression levels of corticotropin-releasing factor (CRF) mRNA in the hypothalamic paraventricular nucleus (PVN) of 20-day-old deprived and nondeprived rats. NDEP controls show a significant increase of CRF mRNA 15 min following saline stress. Deprivation without food and water (DEP − F − W) significantly decreases CRF mRNA basal levels in the PVN and a significant increase after stress was only detected after 60 min. Deprivation with water (DEP − F + W) reinstates CRF mRNA basal levels, but the stress response is diminished. DEP + F + W pups display basal CRF mRNA levels not significantly different from NDEP. At 60 min, however, CRF mRNA levels are significantly lower compared to NDEP. Data represent mean ± SEM. *Significant from NDEP counterparts, †significant from basal levels, $P < 0.05$.

CRF mRNA in the PVN

ANOVA revealed an interaction between condition and treatment [$F(3,252) = 3.29$, $P < 0.0009$]. NDEP rats showed a significant increase in CRF mRNA 15 min after the saline injection (Fig. 4). This elevation peaked at 30 min and remained elevated until 60 min. Maternal separation in all the deprived conditions resulted in a significant suppression of CRF mRNA expression (area under the curve, Fig. 5). However, there were also significant differences between the three deprived conditions. DEP − F − W pups showed significantly lower basal CRF mRNA expression and a significant elevation of CRF mRNA could only be detected 60 min following saline injection. Pups in the DEP + F + W group showed a significant increase in CRF transcript 15 min after the stressor. However, 60 min following saline stress, CRF mRNA levels declined to a significantly lower level compared to the NDEP controls. CRF mRNA levels of the DEP − F + W rats did not show a significant increase following saline stress and their CRF mRNA levels at 30 and 60 min were significantly lower compared to NDEP.

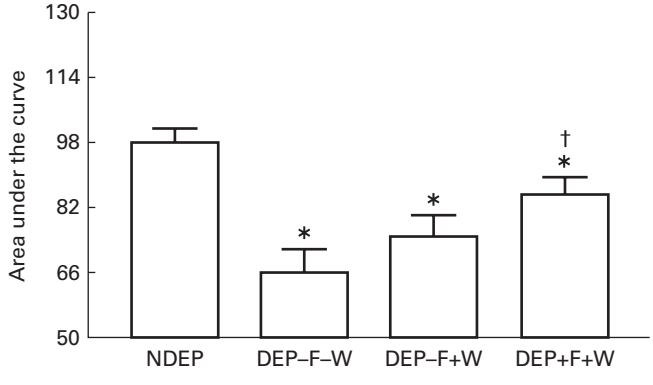

FIG. 5. Area under the curve of corticotropin-releasing factor (CRF) mRNA expression in the hypothalamic paraventricular nucleus (PVN) of 20-day-old deprived and nondeprived rats. In all deprived groups, we detected a significant reduction of the overall CRF mRNA levels in the PVN. Within the deprived conditions, DEP + F + W pups showed significantly higher CRF mRNA levels (area under the curve) compared to DEP − F − W. Data represent mean ± SEM. *Significant from NDEP, †significant from DEP − F − W, $P < 0.05$.

GR mRNA in the hippocampus

The strongest signal of GR mRNA levels could be observed in the CA1 area of the hippocampus, while this was intermediate in the dentate gyrus (DG) and low in the CA3 area. In the CA1 area, ANOVA revealed an interaction between condition and treatment [$F(3,252) = 14.34$, $P < 0.0001$]. DEP − F − W and DEP − F + W resulted in a significant down regulation of GR mRNA basal levels in the CA1 area. There were no changes of GR mRNA in NDEP, DEP − F − W and DEP − F + W rats over the time of testing. In DEP + F + W pups, however, we observed a significant down regulation of GR transcript 60 min after the onset of the stressor. No significant effect of treatment or time could be observed in any of the other measured areas of the hippocampus (CA3, DG) (Fig. 6).

Discussion

Previous studies have shown that maternal separation around the time of weaning (18 days after parturition) had significant

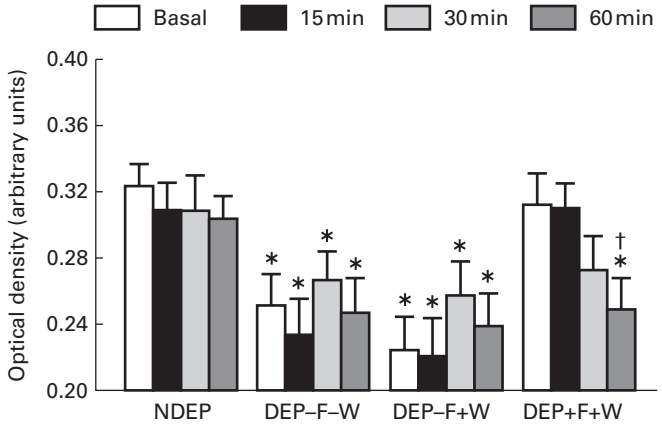

FIG. 6. Basal and stress-induced expression levels of glucocorticoid receptor (GR) mRNA in the CA1 area of the dorsal hippocampus of 20-day-old deprived and nondeprived rats. Basal expression levels of GR mRNA in DEP − F − W and DEP − F + W pups are significantly reduced compared to NDEP controls. In DEP + F + W pups, saline stress resulted in a significant down regulation of GR mRNA after 60 min. Data represent mean ± SEM. *Significant from NDEP, †significant from basal, $P < 0.05$.

effects on the HPA axis. Both basal and stress induced corticosterone were elevated. Paradoxically ACTH, PVN c-*fos* and CRF gene expression were down regulated (19). In these studies, maternal separation also included food and water deprivation (14, 15). The primary aim of the current study was to determine the differential role of fasting and/or maternal separation on the HPA axis at the time of weaning. The results indicate that maternal deprivation around the time of weaning altered the HPA axis at multiple levels. However, different aspects of the HPA system appear to respond differentially to either the absence of food or the absence of the dam or both. The results also indicated that 24 h of fasting led to increased basal levels and further increases in stress-induced corticosterone secretion. Fasting also appeared to contribute to the down regulation of basal GR mRNA in the CA1 region of the hippocampus. In contrast, abrupt weaning was responsible for the suppressed ACTH response to an injection of isotonic saline. Although there was an effect of maternal separation on CRF mRNA, this effect was further exacerbated by the absence of food. Finally, rats that were separated from their dams showed more efficient negative-feedback.

Consistent with our previous studies (13), 24 h of maternal deprivation resulted in exaggerated basal and stress-induced levels of corticosterone. The increases in circulating levels of corticosterone were completely reversed when the rats were provided with food/water during the period of separation from the mother. The availability of water during the deprivation period had little influence on corticosterone. Thus, the suppression of both basal and stress-induced corticosterone appears to be attributable to feeding. We hypothesized that, during SHRP, feeding suppresses the adrenal response to stress by diminishing the sensitivity of the adrenal to its trophic hormone ACTH (9). The data from the current experiment indicate that this phenomenon is not restricted to the period in development when the adrenal appears to be quiescent (days 4–14). Thus, at the time of weaning, when the pups are capable of eliciting a significant elevation of corticosterone following stress, feeding still appears to be a rate-limiting factor on the magnitude of the corticosterone response.

There are several potential mechanisms that appear to influence adrenal sensitivity. Walker (20) reported that a chemical sympathectomy abolished the enhanced adrenal sensitivity to ACTH induced by maternal separation in 10-day-old pups, thus suggesting a role for the adrenal medulla in stimulating steroid secretion from the adrenal cortex following maternal deprivation. There is evidence from both in-vitro and in-vivo experiments that catecholamines can stimulate steroid secretion in the adrenal cortex via β-adrenergic receptors (21–25). It is possible that there are inhibitory influences exerted by the dam on the autonomic nervous system of the adrenal. As a consequence, an increased release of nor-adrenaline and adrenaline from the adrenal medulla in maternally deprived pups would amplify the paracrine action of these amines on the adrenal cortex (26–28), which would be manifested in an increased adrenal sensitivity.

There are at least two additional mechanisms that could account for the decreased adrenal sensitivity during development. Zilz *et al*. proposed a role for peripheral benzodiazepine receptors (PBR) in regulating adrenal sensitivity (29). This intracellular molecule is believed to assist in the transport of cholesterol from intracellular stores to inner mitochondrial membranes for steroidogenesis. Cholesterol transport is initiated in adrenocortical cells in response to cAMP formation that results from activation of the ACTH receptor by its ligand. In the *in situ* hybridization study by Zilz *et al*., PBR binding capacity and immunoreactive PBR content directly paralleled that of ACTH-induced steroidogenesis. During the period of development when the adrenal is insensitive to ACTH, PBR levels were considerably reduced compared to the adult. It is therefore possible that PBR may be increased during maternal deprivation. Insofar as it has been demonstrated that fasting is responsible for the increased sensitivity of the adrenal gland in DEP pups, this would be a nutritionally mediated process. Recently, there have been several reports suggesting a role for leptin in the regulation of the HPA axis (30–32). Leptin, a product of the ob gene in adipocytes (33), appears to affect the HPA axis at multiple levels. In the neonate, increased leptin levels have been shown to reduce ACTH secretion and stress-induced expression of CRF mRNA. Relevant to this discussion is the fact that leptin appears to directly inhibit secretion of glucocorticoids *in vitro* from primary cell lines derived from dispersed human and rat adrenal glands via binding to leptin receptors (OB-R) on these adrenocortical cells (34). In addition, neonates are exposed to high levels of leptin postnatally (35). The surge of leptin during this period of development corresponds with the down regulation of corticosterone from the rat adrenal. Presumably, one source of the increased leptin would be derived from the maternal milk (31). Thus the 24-h fast which accompanies maternal deprivation would be expected to result in a marked decrease in circulating leptin, and this would remove the inhibitory influence of leptin on the neonatal adrenal. Fasting might also induce down regulation of the leptin receptor in the DEP pups. Although we have entertained three different

possible mechanisms that may be responsible for the regulation of the adrenal sensitivity, the only one of these that is directly related to feeding at this time would involve the regulation of the adrenal by leptin.

The phenomenon of enhanced adrenal sensitivity is even more striking in light of the data obtained on the ACTH response to stress. In contrast to the effects of maternal deprivation during the SHRP when the ACTH response is enhanced (9), deprived pups around the time of weaning show a marked diminution of the ACTH response. Thus, although maternally deprived 20-day-old rats have reduced circulating levels of ACTH, those pups that were fasted responded to these reduced ACTH levels with robust corticosterone secretion. Non-deprived pups exhibit the lowest adrenal sensitivity to ACTH compared to their deprived counterparts (with or without feeding). Whereas there are numerous reports that feeding is involved in the regulation of the adrenal, the maternal deprivation effects on ACTH in weanling pups are not reversed by feeding. Regardless of the pups' nutritional status, removal of the dam results in a down regulation of ACTH secretion. Thus, in one way, the weanling pups resemble their younger counterparts both in respect to the regulation of the adrenal and the regulation of ACTH. Feeding directly influences the adrenal, whereas the absence of some dimension of maternal behaviour or dam–pup interaction is responsible for regulating pituitary ACTH. In the neonate, a component of maternal behaviour, stroking, reverses the increased ACTH following stress (9). However, Stanton *et al.* have previously demonstrated that maternal contact suppressed the corticosterone response to novelty in weanling pups (13). In these studies, contact with an anaesthetized dam was sufficient. Thus, the activity of the dam did not appear to be relevant. In addition, Wang *et al.* showed that maternal touch can influence ornithine decarboxylase expression in 20-day-old rats, demonstrating the importance of maternal contact in a different system (17). The 'normal' weaning process is much more gradual than the abrupt separation from the dam that was used for these experiments, and which is the more usual pattern of weaning employed in many animal facilities. Cook *et al.* reported that different patterns of weaning have long-term consequences on the response of the HPA axis (36). Differences in the corticosterone response to restraint in the adult rat have been attributed to changes in maternal contact at the time of weaning. Animals that were weaned gradually showed a reduced response to stress compared to rats that were weaned abruptly at day 21. These studies support our hypothesis that maternal contact regulates pituitary ACTH even around the time of weaning. However, which part of the maternal behaviour or contact is responsible for the observed short and long-term changes in HPA activity remains unclear.

It has been reported that the dynamics of the expression of the CRF gene in the neonate differs markedly from that observed in the adult (15). In response to a saline injection, increased CRF mRNA is evident 15 min following injection. This is in sharp contrast to the adult, which requires from 2–4 h following stress to respond and, in many instances, a relatively severe challenge is required before these changes are observed (37, 38). These patterns of rapid gene expression are not limited to the SHRP but appear to persist in the preweaning rat at day 18. The rapid increases in CRF mRNA are once again observed in the 20-day-old rat that remains with the dam. Maternal deprivation markedly alters these patterns of CRF gene expression. Although, in general, separation from the dam results in a down regulation of CRF mRNA, each deprivation condition appears to produce a pattern of gene expression that is unique to the conditions of deprivation.

Control of the secretion of ACTH is assumed to be primarily a function of CRF released from the nerve terminals of the median eminence. Acting via the CRF-1 receptors in the pituitary, CRF stimulates the synthesis and secretion of ACTH from the pituitary corticotrophin cells. Therefore, it would be expected that if CRF were down regulated, then the activation of the pituitary corticotrophs would be reduced accordingly. Thus, we would predict that the reduced ACTH response observed in all of the deprived groups would be reflected by a reduction in CRF released, which would consequently be reflected in a concomitant reduction in the CRF gene expression in the PVN required to replace the released CRF. The data indicate that, in all of the deprived groups, there is an overall reduction in CRF mRNA in the PVN, which is consistent with the reduced ACTH response. However, unlike the down regulation of ACTH, which is similar in all deprived pups, there are significant differences in CRF gene expression between the deprived groups that appear to be related to the availability of food. Thus, the reduction of CRF mRNA in the DEP + F + W groups is not as pronounced as the DEP − F − W. It has been well established in the adult rat that elevated levels of circulating corticosterone suppress both ACTH and CRF gene expression. Although the data suggest that separation from the dam can influence the expression of the CRF gene, there appears to be an additional process that produces even further suppression. Given that both basal and stress induced corticosterone levels are significantly elevated in the DEP − F − W and the DEP − F + W groups, the marked suppression of CRF mRNA in these rats could be a function of the combination of the influence of maternal separation and a more intense negative feedback signal. Insofar as basal levels of corticosterone are elevated in these two groups, it can be inferred that there was a prolonged exposure of high levels of corticosterone that would act via the GRs in the PVN.

It has been proposed that the glucocorticoid control of the HPA system has two modes of operation: (i) a 'proactive' mode that acts to regulate basal levels of HPA activity and is involved in control of the sensitivity of the HPA system for stressors and (ii) a 'reactive' mode that facilitates the termination of the stress-induced levels of ACTH and corticosterone (39). Whereas the proactive mode can be observed early in development, reactive glucocorticoid control is a late-developing process. Vazquez and Akil demonstrated that in weanling rats the stress response is not terminated as efficiently as in the adult (40). The results obtained in the current experiment indicate the NDEP rats do not terminate their ACTH and corticosterone responses for at least 60 min following stress, which is in agreement with previously published reports. In contrast, all of the DEP pups show significant negative-feedback and by 60 min have either returned to, or were below their basal levels. Insofar as this

effect is observed in the DEP + F + W, it is not dependent on nutrition and reveals a further aspect of HPA regulation that is influenced by the presence of the dam. In the neonate during the SHRP, when corticosterone and ACTH levels are elevated, there is little evidence of reactive negative-feedback and these levels can remain elevated for as long as 4 h following stress (15). In the adult, reactive negative-feedback in response to an acute challenge is very rapid. ACTH levels can return to basal within 15 min and corticosterone is rarely elevated for longer than 60 min (41). Weaning appears to represent a transitional period where, unlike the neonate, the 20-day-old rat has the capacity to elicit a robust corticosterone and ACTH response to stress. However, unlike the adult, the capacity for rapid termination of the peripheral endocrine responses still appears to be deficient. A stimulating function of weaning on development has also been reported for the expression of different opioid receptor subtypes (42, 43). Thus, the postweaning period with the first prolonged absence of the mother appears to accelerate the maturation of at least some functional aspects of the brain following weaning.

There is an abundant literature that GRs play a crucial role in corticosterone mediated negative-feedback (44), in particular in reactive negative-feedback (39). GRs are widely distributed throughout the central nervous system and are present in the PVN, pituitary, cortical regions, hippocampus and ascending aminergic pathways (45). Special attention has been given to the GRs in the hippocampus. Several investigators have postulated that the number of GRs in the hippocampus is involved in the efficiency of negative-feedback (18, 46–49). To extend our understanding of the HPA axis in the weanling rat, we examined GR mRNA in the hippocampus in weanling nondeprived and deprived pups. The decision to examine the hippocampus was based upon other data that suggested that especially the CA1 region was highly sensitive to corticosterone (50). There were clear differences in GR mRNA between the groups. DEP − F − W and DEP − F + W pups showed a down regulation of basal GR mRNA compared to NDEP. The DEP + F + W had basal levels that were equal to the NDEP group. Thus, as in younger pups (9), feeding also influences GR expression in the hippocampus. The down regulation of basal GR mRNA levels in the hippocampus is likely a consequence of increased levels of circulating corticosterone in the DEP rats that were deprived of food. However, with the available data, no clear conclusion can be made on the question of negative feedback regulation by GRs. In our study, we only examined GRs in the hippocampus and therefore we need to exercise caution before concluding that there is no clear relationship between CRF message and negative-feedback. Without the information on the expression of GR gene in the PVN and pituitary, our conclusions are limited. However, the critical importance of hippocampal GR expression in both the activation of the HPA axis and negative-feedback has been proposed by several investigators (39, 48, 51). Thus, albeit the GR results are limited in scope, they do not support the proposed role of the hippocampal GR receptors on negative-feedback insofar as efficient reactive negative-feedback is observed in our experiments in weanling rats that exhibit a marked down regulation of GR expression in the hippocampus.

In summary, our study demonstrates that abrupt weaning at 20 days of age results in pronounced changes in HPA activity of the rat. Although some of the effects of abrupt weaning can be attributed to food deprivation, others appear to be a consequence of the loss of the dam. Pups at this age appear to be in a transitional process and still dependent upon the dam for the regulation of the HPA axis. Food deprivation increases adrenal sensitivity and the resulting high levels of corticosterone appear to down regulate basal levels of GR mRNA. However, some, as yet unspecified, maternal signal(s) or a disruption of the dam–pup interaction regulates the hypothalamic-pituitary components of the HPA axis. In pups during the SHRP, it has been shown that anogenital stimulation can restore the changes seen with maternal deprivation (9). In the weanling rat, there is little evidence that anogenital stimulation is provided by the dam since, at this age, the pup is capable of controlling its own eliminative processes. Although the weanling pup can survive without the presence of the dam at this age, the HPA system still requires the dam's presence for normal development and abrupt disruption of the dam–pup interaction markedly alters the HPA axis at every level that we have examined.

Acknowledgements

We thank Dr Mark A. Smith for his support with the *in situ* hybridizations. This research was supported by grant MH-45006 from the National Institutes of Mental Health to Seymour Levine.

Accepted 27 February 2002

References

1 Cirulli F, Gottlieb SL, Rosenfeld P, Levine S. Maternal factors regulate stress responsiveness in the neonatal rat. *Psychobiology* 1992; **20:** 143–152.
2 Levine S. The ontogeny of the hypothalamic-pituitary-adrenal axis. The influence of maternal factors. *Ann NY Acad Sci* 1994; **746:** 275–288.
3 Rosenfeld P, Gutierrez YA, Martin AM, Mallett HA, Alleva E, Levine S. Maternal regulation of the adrenocortical response in preweanling rats. *Physiol Behav* 1991; **50:** 661–671.
4 Vazquez DM. Stress and the developing limbic-hypothalamic-pituitary-adrenal axis. *Psychoneuroendocrinology* 1998; **23:** 663–700.
5 Sapolsky RM, Meaney MJ. Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Res* 1986; **396:** 64–76.
6 Levine S, Huchton DM, Wiener SG, Rosenfeld P. Time course of the effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. *Dev Psychobiol* 1991; **24:** 547–558.
7 Stanton ME, Gutierrez YR, Levine S. Maternal deprivation potentiates pituitary-adrenal stress responses in infant rats. *Behav Neurosci* 1988; **102:** 692–700.
8 Suchecki D, Rosenfeld P, Levine S. Maternal regulation of the hypothalamic-pituitary-adrenal axis in the infant rat. The roles of feeding and stroking. *Brain Res Dev Brain Res* 1993; **75:** 185–192.
9 van Oers HJ, De Kloet ER, Whelan T, Levine S. Maternal deprivation effect on the infant's neural stress markers is reversed by tactile stimulation and feeding but not by suppressing corticosterone. *J Neurosci* 1998; **18:** 10171–10179.
10 van Oers HJ, De Kloet ER, Li C, Levine S. The ontogeny of glucocorticoid negative feedback: influence of maternal deprivation. *Endocrinology* 1998; **139:** 2838–2846.
11 Stanton ME, Levine S. Inhibition of infant glucocorticoid stress response: specific role of maternal cues. *Dev Psychobiol* 1990; **23:** 411–426.

12 Pauk J, Kuhn CM, Field TM, Schanberg SM. Positive effects of tactile versus kinesthetic or vestibular stimulation on neuroendocrine and ODC activity in maternally-deprived rat pups. *Life Sci* 1986; **39:** 2081–2087.
13 Stanton ME, Wallstrom J, Levine S. Maternal contact inhibits pituitary-adrenal stress responses in preweanling rats. *Dev Psychobiol* 1987; **20:** 131–145.
14 Smith MA, Kim SY, van Oers HJ, Levine S. Maternal deprivation and stress induce immediate early genes in the infant rat brain. *Endocrinology* 1997; **138:** 4622–4628.
15 Dent GW, Smith MA, Levine S. Rapid induction of corticotropin-releasing hormone gene transcription in the paraventricular nucleus of the developing rat. *Endocrinology* 2000; **141:** 1593–1598.
16 Ader R, Grota LJ. Rhytmicity in the maternal behaviour of Rattus norvegicus. *Anim Behav* 1970; **18:** 144–150.
17 Wang S, Bartolome JV, Schanberg SM. Neonatal deprivation of maternal touch may suppress ornithine decarboxylase via down-regulation of the proto-oncogenes c-myc and max. *J Neurosci* 1996; **16:** 836–842.
18 Makino S, Smith MA, Gold PW. Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. *Endocrinology* 1995; **136:** 3299–3309.
19 Dent GW, Okimoto DK, Smith MA, Levine S. Stress-induced alterations in corticotropin-releasing hormone and vasopressin gene expression in the paraventricular nucleus during ontogeny. *Neuroendocrinology* 2000; **71:** 333–342.
20 Walker CD. Chemical sympathectomy and maternal separation affect neonatal stress responses and adrenal sensitivity to ACTH. *Am J Physiol* 1995; **268:** R1281–R1288.
21 Edwards AV, Jones CT. The effect of splanchnic nerve section on the sensitivity of the adrenal cortex to adrenocorticotrophin in the calf. *J Physiol (Lond)* 1987; **390:** 23–31.
22 Edwards AV, Jones CT. The effect of splanchnic nerve stimulation on adrenocortical activity in conscious calves. *J Physiol (Lond)* 1987; **382:** 385–396.
23 Jones CT, Edwards AV. The role of corticotrophin releasing factor in relation to the neural control of adrenal function in conscious calves. *J Physiol (Lond)* 1992; **447:** 489–500.
24 Engeland WC, Gann DS. Splanchnic nerve stimulation modulates steroid secretion in hypophysectomized dogs. *Neuroendocrinology* 1989; **50:** 124–131.
25 Hirata Y, Uchihashi M, Sueoka S, Matsukura S, Fujita T. Presence of ectopic beta-adrenergic receptors on human adrenocortical cortisol-producing adenomas. *J Clin Endocrinol Metab* 1981; **53:** 953–957.
26 Hinson JP. Paracrine control of adrenocortical function. A new role for the medulla? *J Endocrinol* 1990; **124:** 7–9.
27 Hinson JP, Puddefoot JR, Kapas S. Actions of vasoactive intestinal peptide on the rat adrenal zona glomerulosa. *J Endocrinol* 1999; **161:** 51–57.
28 Vizi ES, Toth IE, Szalay KS, Windisch K, Orso E, Szabo D, Vinson GP. Catecholamines released from local adrenergic axon terminals are possibly involved in fine tuning of steroid secretion from zona glomerulosa cells: functional and morphological evidence. *J Endocrinol* 1992; **135:** 551–561.
29 Zilz A, Li H, Castello R, Papadopoulos V, Widmaier EP. Developmental expression of the peripheral-type benzodiazepine receptor and the advent of steroidogenesis in rat adrenal glands. *Endocrinology* 1999; **140:** 859–864.
30 Heiman ML, Ahima RS, Craft LS, Schoner B, Stephens TW, Flier JS. Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. *Endocrinology* 1997; **138:** 3859–3863.
31 Trottier G, Koski KG, Brun T, Toufexis DJ, Richard D, Walker CD. Increased fat intake during lactation modifies hypothalamic-pituitary-adrenal responsiveness in developing rat pups: a possible role for leptin. *Endocrinology* 1998; **139:** 3704–3711.
32 Nye EJ, Bornstein SR, Grice JE, Tauchnitz R, Hockings GI, Strakosch CR, Jackson RV, Torpy DJ. Interactions between the stimulated hypothalamic-pituitary-adrenal axis and leptin in humans. *J Neuroendocrinol* 2000; **12:** 141–145.
33 Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nature Med* 1995; **1:** 1155–1161.
34 Pralong FP, Roduit R, Waeber G, Castillo E, Mosimann F, Thorens B, Gaillard RC. Leptin inhibits directly glucocorticoid secretion by normal human and rat adrenal gland. *Endocrinology* 1998; **139:** 4264–4268.
35 Ahima RS, Prabakaran D, Flier JS. Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. *J Clin Invest* 1998; **101:** 1020–1027.
36 Cook CJ. Patterns of weaning and adult response to stress. *Physiol Behav* 1999; **67:** 803–808.
37 Givalois L, Arancibia S, Tapia-Arancibia L. Concomitant changes in CRH mRNA levels in rat hippocampus and hypothalamus following immobilization stress. *Brain Res Mol Brain Res* 2000; **75:** 166–171.
38 Imaki T, Nahan JL, Rivier C, Sawchenko PE, Vale W. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. *J Neurosci* 1991; **11:** 585–599.
39 De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M. Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 1998; **19:** 269–301.
40 Vazquez DM, Akil H. Pituitary-adrenal response to ether vapor in the weanling animal: characterization of the inhibitory effect of glucocorticoids on adrenocorticotropin secretion. *Pediatr Res* 1993; **34:** 646–653.
41 Brett LP, Chong GS, Coyle S, Levine S. The pituitary-adrenal response to novel stimulation and ether stress in young adult and aged rats. *Neurobiol Aging* 1983; **4:** 133–138.
42 Kitchen I, Crook TJ, Muhammad BY, Hill RG. Evidence that weaning stimulates the developmental expression of a delta-opioid receptor subtype in the rat. *Brain Res Dev Brain Res* 1994; **78:** 147–150.
43 Kitchen I, Leslie FM, Kelly M, Barnes R, Crook TJ, Hill RG, Borsodi A, Toth G, Melchiorri P, Negri L. Development of delta-opioid receptor subtypes and the regulatory role of weaning. Radioligand binding, autoradiography and in situ hybridization studies. *J Pharmacol Exp Ther* 1995; **275:** 1597–1607.
44 Karanth S, Linthorst AC, Stalla GK, Barden N, Holsboer F, Reul JM. Hypothalamic-pituitary-adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function. *Endocrinology* 1997; **138:** 3476–3485.
45 Rosenfeld P, Van Eekelen JA, Levine S, De Kloet ER. Ontogeny of corticosteroid receptors in the brain. *Cell Mol Neurobiol* 1993; **13:** 295–319.
46 Dijkstra I, Tilders FJ, Aguilera G, Kiss A, Rabadan-Diehl C, Barden N, Karanth S, Holsboer F, Reul JM. Reduced activity of hypothalamic corticotropin-releasing hormone neurons in transgenic mice with impaired glucocorticoid receptor function. *J Neurosci* 1998; **18:** 3909–3918.
47 Kretz O, Reichardt HM, Schutz G, Bock R. Corticotropin-releasing hormone expression is the major target for glucocorticoid feedback-control at the hypothalamic level. *Brain Res* 1999; **818:** 488–491.
48 Meaney MJ, Aitken DH, Viau V, Sharma S, Sarrieau A. Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat. *Neuroendocrinology* 1989; **50:** 597–604.
49 Jacobson L, Sapolsky R. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr Rev* 1991; **12:** 118–134.
50 Vazquez DM, van Oers H, Levine S, Akil H. Regulation of glucocorticoid and mineralocorticoid receptor mRNAs in the hippocampus of the maternally deprived infant rat. *Brain Res* 1996; **731:** 79–90.
51 Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM, Meaney MJ. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* 1997; **277:** 1659–1662.