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Neonatal Endotoxin Exposure Alters the Development of the Hypothalamic-Pituitary-Adrenal Axis: Early Illness and Later Responsivity to Stress

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The long-term consequences of neonatal endotoxin exposure on hypothalamic-pituitary-adrenal axis (HPA) function were assessed in adult female and male Long–Evans rats. At 3 and 5 d of age, pups were administered endotoxin (*Salmonella enteritidis*, 0.05 mg/kg, i.p.) at a dose that provokes a rapid and sustained physiological response, but with no mortality. As adults, neonatally endotoxin-treated animals exhibited significantly greater adrenocorticotrophic hormone (ACTH) and corticosterone responses to restraint stress than controls. In addition, dexamethasone pretreatment was less effective in suppressing ACTH responses to restraint stress in endotoxin-treated animals than in controls, suggesting decreased negative-feedback sensitivity to glucocorticoids. Neonatal endotoxin treatment elevated resting-state median eminence levels of corticotropin-releasing hormone (CRH) and arginine vasopressin in adult male animals, and arginine vasopressin in both adult males and females. Neonatal exposure to endotoxin also increased CRH mRNA expression in the paraventricular nucleus of the hypothalamus of adult males, with no difference in females. Finally, glucocorticoid receptor density was reduced across a wide range of brain regions in the neonatal endotoxin-treated, adult animals. These data illustrate the interactive nature of immune and endocrine systems during development. It appears that endotoxin exposure during critical stages of development decreases glucocorticoid negative-feedback inhibition of ACTH secretagogue synthesis, thus increasing HPA responsiveness to stress. The implication of these findings is that exposure to gram-negative LPS in early life can alter the development of neural systems which govern endocrine responses to stress and may thereby predispose individuals to stress-related pathology.

[Key words: hypothalamic-pituitary-adrenal axis, development, endotoxin, adrenocorticotropin, corticotropin-releasing factor (hormone), vasopressin]

There are considerable individual differences in the response of the hypothalamic-pituitary-adrenal (HPA) axis to stress and these differences appear to be related to vulnerability to several forms of pathology (see Chrousos and Gold, 1992; McEwen and Steller, 1993). The variability in HPA responses to stress derives from organismic variables such as sex, reproductive state, and age, as well as genetic background (e.g., Le Mevel et al., 1979; Sapolsky et al., 1984; Shanks et al., 1990; Walker et al., 1990, 1992; Viau and Meaney, 1991; Meaney et al., 1993). In addition, postnatal environmental events are known to alter the development of the HPA response to stress (Levine, 1957, 1962; Levine et al., 1967; Ader and Grota, 1969; Meaney et al., 1988, 1989; Plotsky and Meaney, 1993; Viau et al., 1993). For example, as adults, neonatally handled animals exhibit dampened HPA responses to stress compared with nonhandled animals (Levine, 1957, 1962; Hess et al., 1969; Meaney et al., 1988, 1989; Viau et al., 1993). In contrast, adult animals exposed to repeated periods of prolonged maternal separation as neonates display increased HPA responses to stress (Plotsky and Meaney, 1993). These effects persist throughout the life of the animal and the resulting differences in HPA activity are associated with altered T-dependent antibody responses (Bhatnagar et al., 1992) and the incidence of age-related neuropathology (Meaney et al., 1988).

Immune activation elicits both HPA and central catecholamine alterations similar to those seen following stress (Blalock, 1984; Dunn et al., 1987, 1989; Zalcman et al., 1991; Anisman et al., 1993; Rivier et al., 1993; Stenzel-Poore et al., 1993). Furthermore, the immune and HPA systems are mutually regulatory, and these interactions partly determine the impact of stress on immune functioning (Munck et al., 1984; Irwin et al., 1990; Berkenbosch et al., 1991; Anisman et al., 1993; Rivier et al., 1993). Given the close, functional association between the HPA axis and immune system, we wondered whether endotoxin exposure and subsequent exposure to immune factors associated with acute phase responses during early life might alter the development of the HPA axis. Endotoxin administration reliably activates HPA responding in the neonate, and this effect appears to be mediated by corticotropin-releasing hormone (CRH) release (Witek-Janusek, 1988; Shanks and Meaney, in press a,b). Considering the immune–HPA interactions early in postnatal life, when HPA development is susceptible to envi-

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ronmental influences, it seems interesting to ask whether such interactions might permanently alter HPA development. This question is potentially of considerable importance since early postnatal life in mammals represents a period of considerable bacterial colonization (largely in the nasopharynx, intestine, and vagina), and the newborn is very susceptible to infection (see Williams, 1981).

There are limited data indicating long-term consequences following endocrine–immune interactions during the first week of life. In particular, it has been demonstrated that early endocrine events have considerable impact on immune development of tolerance (Pierpaoli, 1981). Conversely, immune challenge during the first week of life with Newcastle Disease virus is associated with alterations in both gonadal and adrenal weights (O'Grady and Hall, 1991). These data indicate that endocrine and immune processes probably develop dependently; however, to the best of our knowledge little or nothing is known about early exposure to endotoxin and its long-term effects on neural control of endocrine function.

In the present studies we examined the effects of postnatal endotoxin exposure on the development of the HPA axis in male and female rats. We have used a dose of *Salmonella enteritidis* sufficiently low so as to not produce any mortality (see Shanks et al., in press a). Nevertheless, this endotoxin dose provokes an HPA response, increased blood glucose levels, and results in greatly reduced behavioral activity (see Witek-Janusek, 1988; Shanks et al., in press a,b). Animals were treated during the first week of life, a period during which environmental events such as handling or maternal separation have been found to permanently alter HPA development (references cited above). Animals were then left untreated and examined at 4–5 months of age as sexually mature, adult animals. The results of these studies show that exposure to gram-negative LPS in early life can permanently alter the development of neural systems which govern endocrine responses to stress.

Materials and Methods

Animals. Pregnant Long–Evans dams were received from Charles River, St. Constant, Québec at 18 d gestation. Litters were randomly assigned to the different experimental groups (four litters per group), and litter size varied between 12–15 pups. Rat pups were injected intraperitoneally on days 3 and 5 of life with either 0.05 mg/kg *Salmonella enteritidis* endotoxin (Sigma, St. Louis, MO) in sterile saline (injection volume not exceeding 0.1 cc) or an equivalent volume of sterile saline vehicle, or were left untreated. We previously determined that HPA responding in the neonate following a single endotoxin injection was elevated for slightly less than 48 hr (Shanks and Meaney, in press a); therefore, we injected the animals twice during the first week (days 3 and 5 of life) to provoke immune activation for days 3–7 of life.

On day 22 of life the animals were weaned and housed in same-sex, same-treatment groups of three animals per cage. Animals were maintained on a 12:12 light : dark schedule (lights on at 0800 hr) with free access to food (Purina Lab Chow) and water. The animals used in these experiments were 4--5 months of age (350–400 gm) at the time of testing. All testing was performed during the light phase of the cycle between 12:00 and 15:00 hr.

Adrenalectomy was performed under metophane (Methoxyflurane; Pitman-Moore, Inc., Washington Crossing, NJ) anesthesia and all adrenalectomized animals were provided with 0.9% saline as drinking water. For studies of resting-state median eminence peptide content, animals were rapidly (i.e., <10 sec) killed by decapitation following removal from the home cage between 11:00 and 13:00 hr. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the McGill University Animal Care Committee.

Stress testing and blood sampling. Three days prior to testing, animals were implanted with indwelling jugular catheters under Metophane anesthesia and single housed for the remainder of the study (see Viau et al., 1993). Restraint stress was performed using tubular, plastic restrainers lined with foam rubber. The animals were placed into the restrainers for a 20 min period. A blood sample (~ 300 μl) was taken immediately before the animal was placed into the restrainer and less than 10 sec following removal from the home cage. This sample was used to obtain estimates of basal ACTH and B levels prior to stress. Blood samples (~ 150 μl) were then taken from the same animals via a jugular catheter at various times during restraint.

In one experiment animals were injected subcutaneously with 10 μg/kg dexamethasone (in ETOH : saline/1:9) or vehicle 3 hr prior to restraint testing in order to examine glucocorticoid negative-feedback effects. In this experiment, blood samples were taken prior to and at 10 and 20 min of restraint. This dose of dexamethasone was chosen on the basis of previous dose–response studies using this same procedure (Meaney et al., 1989) to define differences in glucocorticoid negative-feedback sensitivity between handled and nonhandled rats.

Blood samples for corticosterone or corticosteroid-binding globulin (CBG) measurements were taken in heparinized tubes, placed on ice, centrifuged, and stored at $-30°C$ until assayed. Blood samples for ACTH assays were taken in tubes containing EDTA and Trasylol. Samples were centrifuged and stored at $-80°C$.

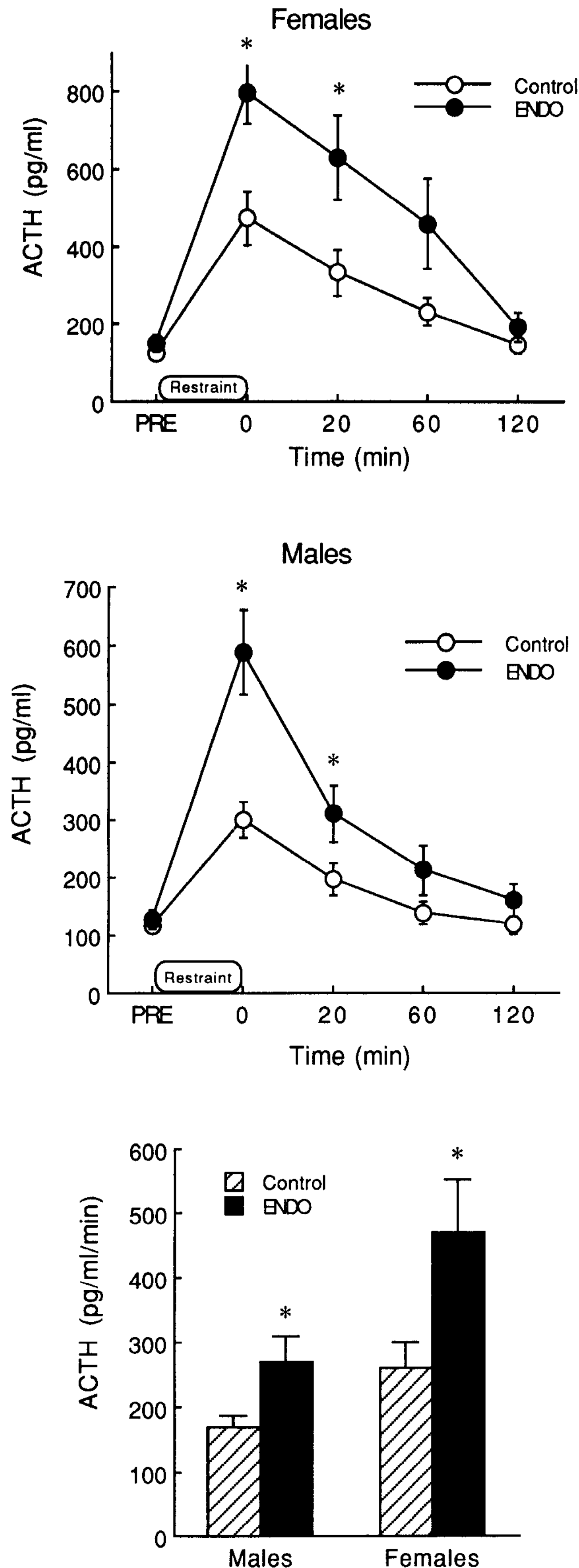
Radioimmunoassays. Plasma corticosterone was measured by the radioimmunoassay of Krey et al. (1975) with a highly specific corticosterone antiserum (B3-163, Endocrine Sciences, Tarzana CA), ^{3}H-corticosterone (101 Ci/mmol, New England Nuclear, Boston, MA), as tracer, and 1 μl of plasma. The minimum level of detection with the assay was 5 pg/ml. The antiserum crossreacts slightly with desoxycorticosterone ($\sim 4\%$), but not with cortisol ($<1\%$). The intra- and interassay coefficients of variation were 8.9 and 11.2%, respectively.

Plasma (25 μl) ACTH was measured by the radioimmunoassay described by Walker et al. (1990) with an ACTH antiserum (IgG Corp., Nashville, TN) and ^{125}I-ACTH (Incstar, Stillwater, MN) as tracer. The ACTH antibody crossreacts 100% with $ACTH_{1-39}$, $ACTH_{1-18}$, and $ACTH_{1-24}$, but less than 1% with $ACTH_{1-16}$, β- endorphin, α-/and β-MSH, and α-/and β-lipotropin. The intra- and interassay coefficients of variation were 11 and 8%, respectively. The minimal detectable level was 10 pg/ml.

Determination of median eminence content for CRH and AVP was performed using an acid extraction procedure (see Suda et al., 1983). Median eminence samples were homogenized by sonication in 0.1 N HCl with 0.001% β- mercaptoethanol, 0.5% ascorbic acid, 0.01% bacitracin, and 0.001% phenylmethylsulfonylfluoride. Extracts were then centrifuged at 12,000 *g* for 30 min and aliquots of the extract were evaporated. Extracts were reconstituted with assay buffer and peptide levels were determined using radioimmunoassays (see Plotsky, 1985) with CRH and AVP antibodies (Peninsula Laboratories Inc., Belmont, CA) and ^{125}-CRH and ^{125}-AVP as tracers. The CRH antibody crossreacts 100% with rat and human CRH, while the AVP antibody crossreacts 100% with [ARG^{8}]-vasopressin. Sensitivity of the CRH and AVP assays was 15 pg/tube and 4 pg/tube, respectively. Intra- and interassay variability was 2.4 and 4.0% for CRH and 3.1 and 6.2% for AVP. Protein levels were determined using an aliquot of the extract that was taken prior to centrifugation with the method of Bradford (1976). Values are expressed as pg/μg protein.

Plasma CBG. Plasma CBG levels were determined by single-point assays using the method of Martin et al. (1975), with slight modifications (see Meaney et al., 1992). Plasma samples (20 μl) were stripped of endogenous corticosterone using a 10×1 cm Sephadex LH-20 column and the plasma was diluted 1:50 with TEDGM (30 mM Tris, 1 mM EDTA, 10 mM sodium molybdate, 10% v/v glycerol, and 1 mM dithiothreitol; pH at 7.4). Aliquots (225 μl) of the diluted plasma were then incubated in buffer (150 μl) containing a saturating 80 nM concentration of ^{3}H-corticosterone for 90 min at 2–4°C (see Martin et al., 1975). Nonspecific binding was defined in parallel incubations by a 200-fold excess of cold corticosterone. Separation of bound from free was achieved using Sephadex LH-20 columns (4×1 cm), equilibrated with TEDGM, made from disposable pipette tips. Following the incubation, 100 μl of the incubates were washed into the columns with 100 μl of TEDGM. The columns were eluted 30 min later with 500 μl of TEGM into minivials, which were then filled with 5 ml of Liquiscent (National Diagnostics, Somerville, NJ), and counted in a Packard scintillation counter at 60% efficiency. Protein content was determined by the method of Bradford (1976) and the results were expressed as picomols (pmols) ^{3}H-corticosterone binding/mg protein.

Glucocorticoid receptor binding. Adrenalectomy was performed 16 hr



prior to death to ensure clearance of endogenous steroid (see McEwen et al., 1986). Animals were killed by rapid decapitation and tissues collected for subsequent glucocorticoid receptor-binding determinations. Brains were removed quickly, then placed on ice, and the frontal cortex, hippocampus, and hypothalamus dissected, then frozen at −80°C until assayed.

The tissue was homogenized in TEDGM (pH adjusted to 7.4). The homogenate was then centrifuged at 2°C for 60 min at 105,000 × *g*. Aliquots of the soluble fraction were incubated with a saturating 10 nM concentration of ^{3}H-dexamethasone and nonspecific binding was determined in a parallel set of incubates containing a 500-fold excess of unlabeled RU 28362. RU 28362 has been shown to selectively bind to the glucocorticoid receptor, showing very little affinity for the mineralocorticoid receptor (see Reul and DeKloet, 1985; McEwen et al., 1986). Thus, these binding conditions define glucocorticoid receptor density.

Separation of bound from free radioligand was achieved using Sephadex LH-20 columns (4 × 1 cm) made from disposable pipette tips and equilibrated with TEDGM. Following incubation, 100 μl of the incubate was washed into the columns with 100 μl of TEDGM. The columns were eluted 30 min later with 500 μl of TEGM into minivials, which were then filled with scintillation cocktail and counted in a Packard scintillation β counter at an efficiency of 40%. Protein content of the supernatants was determined using the method of Bradford (1976) and results expressed as fmols bound ^{3}H-dexamethasone/mg protein specific binding.

CRH mRNA in situ *hybridization.* Following rapid decapitation, brains were removed and frozen quickly in isopentane. Brains were blocked and 15 μm cryostat slices were mounted on polylysine-coated slides, dessicated under a vacuum at room temperature, then stored at −80°C. Preparation of the CRH mRNA riboprobe as well as the *in situ* hybridization procedure have been previously described (see Watts and Swanson, 1989). CRH mRNA *in situ* hybridization was performed using a probe generously provided by Dr. Kelly Mayo (Northwestern University). The probe was constructed from a 380 bp PvuII to SphI fragment ligated into pGEM-4. The insert corresponds to the C-terminus of the preproCRH molecule that includes the entire CRH peptide. Slides were warmed to room temperature, then postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 10 min at room temperature, and washed in three changes of 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) in sterile water containing 0.02% diethylpyrocarbonate (Depc H_2O). Hybridization was performed at 45°C for 16–20 hr in buffer containing 50% formamide, 600 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% bovine serum albumin, 20 μg/ml denatured salmon sperm DNA, 25 μg/ml yeast tRNA, 10% dextran sulphate, 10 mM dithiothreitol, and 2 × 10^7 CPM/ml of ^{35}S-UTP-labeled cRNA antisense probe. Slides were rinsed in 2 × SSC, treated with RNAse I (30 μg/ml; Pharmacia) for 30 min at room temperature, then rinsed again in 2 × SSC, and washed in a final stringency of 0.1 SSC (containing 14 mM β-mercaptoethanol) for 60 min at 60°C. Sections were then dehydrated with increasing concentrations of ethanol (0.3 M sodium acetate), dried, and exposed to β-max Hyperfilm (Amersham) for 10 d.

CRH mRNA expression was determined using integrated optical density measurements from the β-max Hyperfilm (see Watts and Swanson, 1989). Measures were taken from slides bearing the maximal extent of the parvocellular region of the PVN (ppPVN), as determined by adjacent Nissl-stained sections. The optical density of CRH mRNA hybridization in left and right ppPVN for each animal was measured using an image analysis system and the average was calculated after subtracting background.

Statistical analysis. The hormone data were analyzed using an analysis of variance model with one repeated measure (time) and Scheffe post hoc analysis. Integrated hormone levels (area under the curve) were calculated using the Trapezoidal rule. Analysis of between groups differences was performed using *t* tests.

We performed a preliminary analysis on the data from saline and untreated animals and found that these animals did not differ on any measure ($p > 0.5$ for all comparisons). This finding is consistent with

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Figure 1. Mean (±SEM) plasma ACTH levels in control and neonatal endotoxin-treated (*ENDO*) animals prior to, during, and following a 20 min period of restraint stress. The *bottom frame* shows integrated plasma ACTH responses to stress in these groups (n = 8–10/group). *Indicates significant group difference, $p < 0.05$.

our previous studies showing that animals receiving one or two injections as neonates do not differ from untreated controls on measures of adult HPA function (see Meaney et al., 1987). Hence, the data from these groups was pooled and is presented as a single control group.

Results

Stress responding

Plasma ACTH and corticosterone responses to 20 min of restraint stress were significantly higher in neonatally endotoxin-treated animals compared with controls (Figs. 1 and 2). There were no differences in prestress measures of either ACTH or corticosterone (Figs. 1 and 2). Thus, differences in HPA responses to stress were not associated with differences in prestress, basal glucocorticoid levels. For both females and males, endotoxin-treated animals showed significantly higher peak plasma ACTH levels during restraint and at 20 min following the termination of restraint (Fig. 1). Plasma ACTH levels remained elevated at 60 min in the endotoxin-treated animals; however, this difference was not significant. As expected on the basis of these differences, the integrated plasma ACTH response to restraint was substantially (almost twofold) higher in the male and female endotoxin-treated animals (Fig. 1).

Peak responses of plasma corticosterone to restraint were significantly higher in male, but not female, endotoxin-treated treated animals (Fig. 2). However, plasma corticosterone responses in the poststress period were higher in both male and female endotoxin-treated animals, reflecting the differences in plasma ACTH levels. Plasma corticosterone levels remained elevated to a greater extent in females than in males. Analysis of the plasma corticosterone responses showed that integrated corticosterone response to restraint stress was significantly higher in the both male and female neonatal endotoxin-treated animals (Fig. 2). Thus, the corticosterone data paralleled that for plasma ACTH. In addition, we calculated ACTH : corticosterone ratios and found no differences across the groups (data not shown). This suggests that there were no effects on adrenal sensitivity to ACTH.

Plasma CBG levels

Mean plasma CBG levels (pmols bound ^{3}H-corticosterone/mg protein) did not differ as a function of neonatal endotoxin treatment for either males (controls = 7.1 ± 0.5, endotoxin treated = 7.6 ± 0.7 pmol/mg protein) or females (controls = 17.2 ± 1.0, endotoxin treated = 16.4 ± 0.8 pmol/mg protein). However, as previously reported (e.g., Gala and Westphal, 1965), females had significantly ($p < 0.0001$) higher levels of plasma CBG than did males. The free corticosterone signal is proportional to the non-CBG bound fraction of the circulating hormone (Pardridge et al., 1983). Thus, the absence of any difference in plasma CBG between the endotoxin-treated and control groups suggests that the total corticosterone levels accurately reflected the differences in free hormone levels.

Median eminence CRH and AVP levels, and PVN levels of CRH mRNA expression

Neonatal endotoxin treatment resulted in long-term changes in resting-state levels of ACTH secretagogues in the median eminence. However, the pattern of these effects depended upon gender. Among males, resting levels of median eminence CRH content were significantly elevated in endotoxin-treated animals (Fig. 3). This effect was not observed in females. In contrast, median eminence AVP levels were significantly higher in both male and female neonatal endotoxin-treated animals compared with controls (Fig. 3).

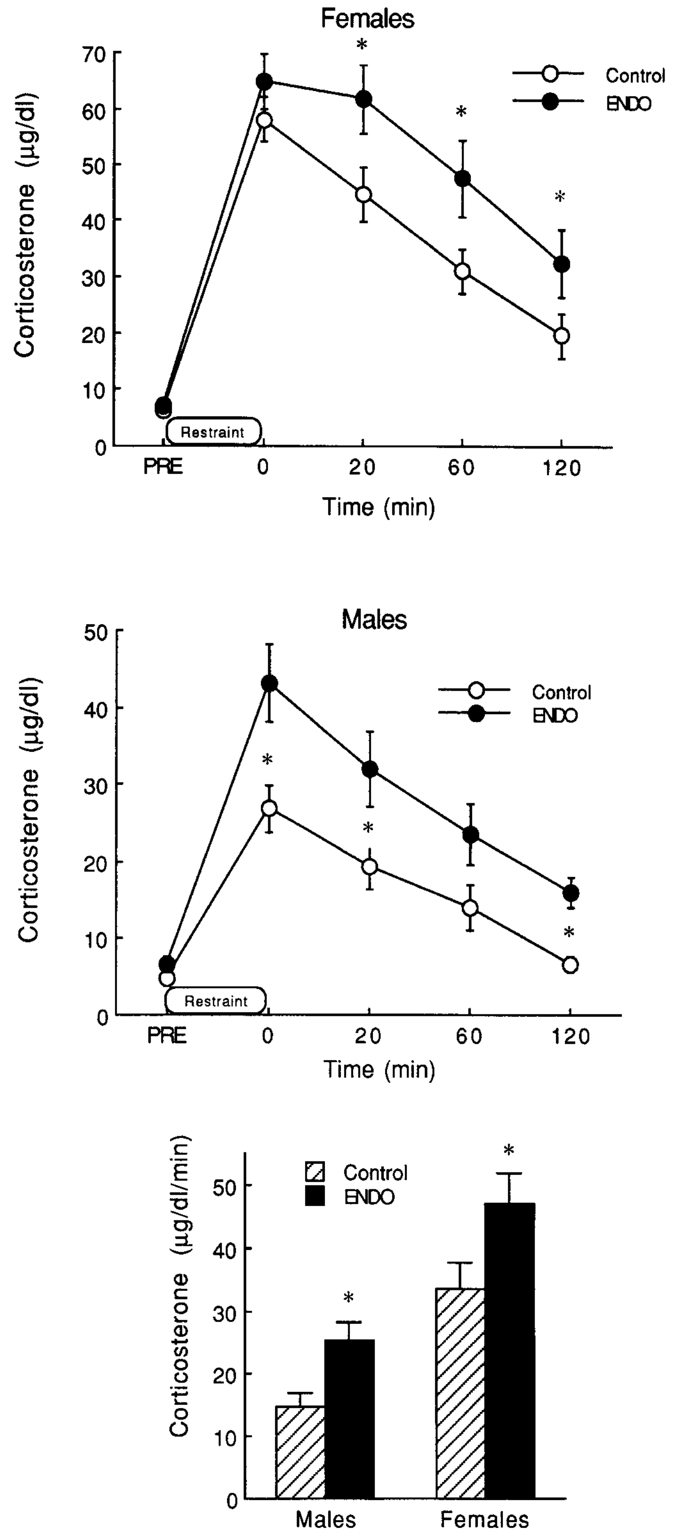

Figure 2. Mean (±SEM) plasma corticosterone levels in control and neonatal endotoxin-treated (*ENDO*) animals prior to, during, and following a 20 min period of restraint stress. The *bottom frame* shows integrated plasma corticosterone responses to stress in these groups (n = 8–10/group). *Indicates significant group difference, $p < 0.05$.

The analysis of the autoradiograms revealed significantly increased CRH mRNA levels in male endotoxin-treated animals

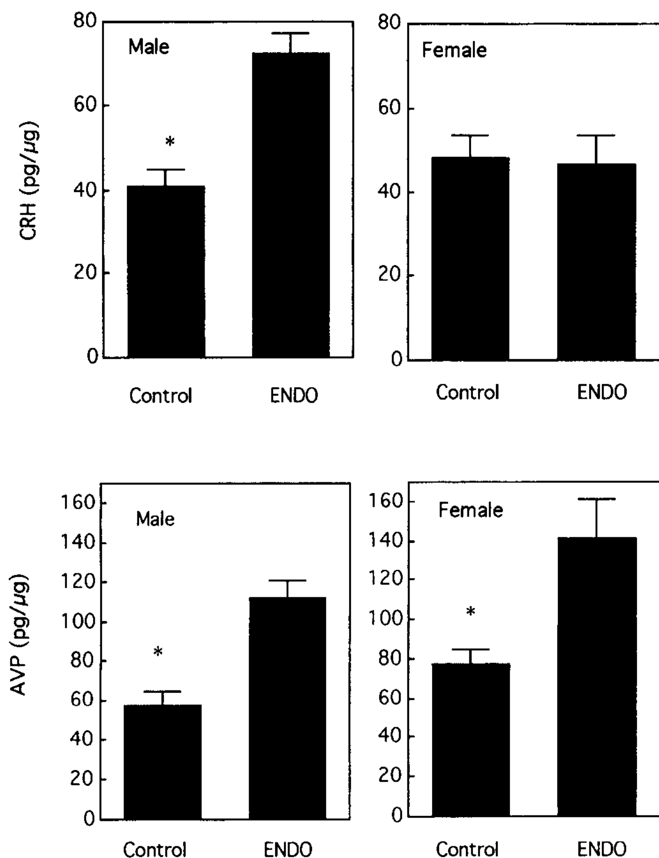

Figure 3. Mean (±SEM) levels (μg/mg protein) of median eminence CRH (*top*) and AVP (*bottom*) in control and neonatal endotoxin-treated (*ENDO*) animals (n = 7–8/group). *Indicates significant group difference, $p < 0.001$.

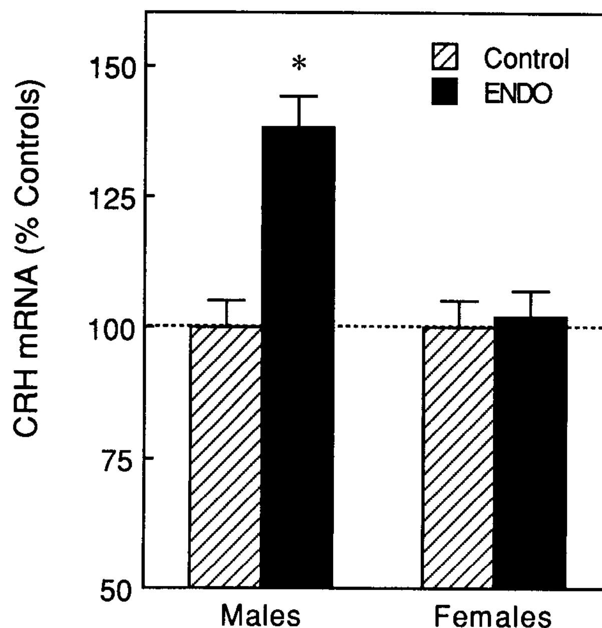

Figure 4. Mean (±SEM) levels of CRH mRNA expression in control and neonatal endotoxin-treated animals as a percentage of the control values (mean for control group = 100%). The raw data were derived from optical density measurements of autoradiograms following *in situ* hybridization (n = 5/group). *Indicates significant group difference, $p < 0.001$.

compared with controls (Fig. 4). However, consistent with the measures of median eminence peptide content, there was no difference in CRH mRNA expression between endotoxin-treated and control females.

Dexamethasone suppression of plasma ACTH responses to restraint stress

As expected, plasma ACTH responses to restraint stress were significantly greater in the saline-pretreated, endotoxin-treated animals compared with saline-pretreated controls (Fig. 5, and note the differences in the range of values for the ordinates). Moreover, there were substantial differences in the response of the neonatal endotoxin-treated and control animals to dexamethasone pretreatment. Restraint-induced elevations in plasma ACTH were effectively suppressed by dexamethasone pretreatment in both male and female control animals. Thus, plasma ACTH levels in dexamethasone-pretreated control animals did not differ from basal values at either 10 or 20 min of restraint. At both time points, plasma ACTH levels were significantly lower in the dexamethasone-pretreated controls compared with saline-pretreated controls.

In contrast, dexamethasone pretreatment reduced, but did not eliminate, restraint-induced increases in plasma ACTH in neonatal endotoxin-treated animals. In the endotoxin-treated animals, both dexamethasone- and saline-pretreated animals showed significant plasma ACTH responses to restraint (i.e., significantly greater than basal ACTH level; Fig. 5). Overall, the plasma ACTH responses were somewhat decreased following dexamethasone pretreatment in endotoxin-treated rats; however, plasma ACTH levels were significantly different from saline-pretreated animals only at 20 min into restraint in the female animals. While dexamethasone pretreatment reduced plasma ACTH responses to restraint stress in both groups, this effect was more marked in the control animals. These data indicate that glucocorticoid negative feedback is less effective in the neonatal endotoxin-treated animals.

Glucocorticoid receptor binding

As indicated in Table 1, neonatal endotoxin treatment decreased glucocorticoid receptor density in the hypothalamus, hippocampus, and frontal cortex of adult animals, regions known to regulate HPA responses to stress. These differences were in the range of 20–30% decreases in the endotoxin-treated rats. Overall the effect was significantly decreased glucocorticoid receptor sites in the forebrain of endotoxin-treated animals compared with controls.

Discussion

Endotoxin exposure during the first week of life altered the development of the HPA axis. In comparison to controls, neonatally endotoxin-treated animals secreted higher levels of ACTH and corticosterone in response to stress and increased resting-state median eminence levels of CRH (in males) and of AVP (in both males and females). In addition, CRH mRNA expression in the PVN was increased in endotoxin-treated males compared with controls. The effects on CRH and AVP levels most likely reflect differences in readily releasable storage pools of these peptides in axon terminals located in the median eminence. Thus, a stress-related excitatory signal at the level of the

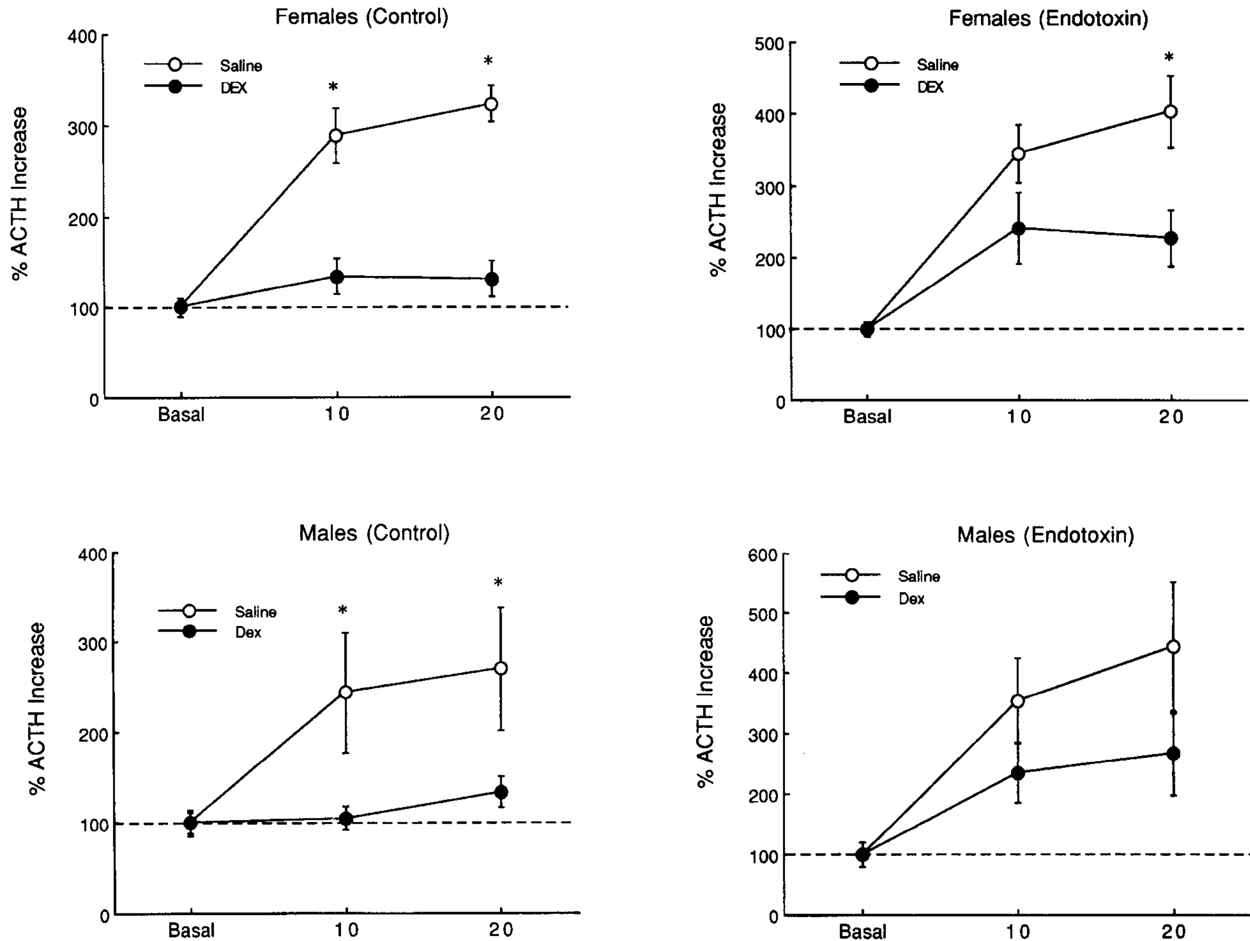

Figure 5. Mean (±SEM) plasma ACTH levels at 10 or 20 min following the onset of restraint stress in control and neonatal endotoxin-treated (*ENDO*) animals. The data are expressed as the percentage of basal ACTH levels for each group, thus showing the magnitude of the stress-induced increase in plasma ACTH (n = 6–8/group). Animals were pretreated 3 hr prior to testing with either saline or dexamethasone (10 μg/kg; DEX). *Indicates significant group difference, $p < 0.001$.

hypothalamus likely results in greater CRH and AVP release in the endotoxin-treated animals and, in turn, a greater plasma ACTH signal. Although the increased stress-induced release of CRH/AVP from PVN neurons of neonatal endotoxin-treated animals remains to be confirmed, this idea is consistent with the findings that median eminence CRH (e.g., Murakami et al., 1989) and AVP (Viau et al., submitted) levels are strongly correlated with ACTH responses to restraint stress. Indeed, the differences in the terminal pools of CRH and AVP suggest that endotoxin-treated and control animals would differ in stressors mediated by either secretagogue. Note, pituitary ACTH responses to restraint stress are mediated by dynamic variations in both CRH and AVP (Linton et al., 1985; Nakane et al., 1985).

Although median eminence levels of AVP were elevated in both male and female endotoxin-treated animals, the effect on CRH levels was observed only in males. This was true both for median eminence peptide content and for mRNA levels. It is not clear why the effect of early endotoxin treatment on measures of CRH synthesis was not observed in females. However, hypothalamic CRH mRNA and median eminence CRH levels in female rats have been shown to vary considerably over the estrus cycle (Hiroshige and Wada-Okada, 1973). In the present study, the estrus state of the females was not controlled, and it may be that the effects of the early endotoxin treatment were shrouded by those associated with the changes in the estrus cycle. Otherwise, we really have no explanation for this finding. In every other respect, the effects of early endotoxin treatment are essentially the same for both males and females.

The changes in ACTH secretagogue content were also associated with differences in negative-feedback sensitivity to circulating glucocorticoids. Glucocorticoid pretreatment inhibited plasma ACTH responses to stress to a significantly greater extent

Table 1. Mean (±SEM) glucocorticoid receptor binding levels (fmol ^{3}H-dexamethasone/mg protein specific binding) in various brain regions in a control and neonatal endotoxin-treated (ENDO) animals (n = 6-8/group)

	ENDO	Control
Males		
Hypothalamus	91.6 ± 8*	117.0 ± 8
Hippocampus	138.4 ± 6*	178.9 ± 14
Frontal cortex	142.8 ± 14*	170.1 ± 9
Females		
Hypothalamus	129.6 ± 12†	168.7 ± 14
Hippocampus	150.5 ± 11*	192.8 ± 9
Frontal cortex	164.3 ± 9*	195.9 ± 10

* Significant group differences at $p < 0.05$.

† Significant group difference at $0.10 > p > 0.05$.

in control than in endotoxin-treated animals. Glucocorticoids are known to regulate the synthesis and release of hypothalamic CRH and AVP (e.g., Hillhouse and Jones, 1976; Plotsky and Vale, 1984; Plotsky et al., 1986; Plotsky and Sawchenko, 1987; Sawchenko, 1987a,b; Owens et al., 1990; Imaki et al., 1991). Indeed, this process is considered as a basis for delayed glucocorticoid negative-feedback effects (see Dallman et al., 1992). Thus, it is possible that the increased synthesis of ACTH secretagogues in the endotoxin-treated animals occurs in response to a decreased tonic, glucocorticoid negative-feedback signal.

The results of the receptor-binding studies provide a potential mechanism for the difference in glucocorticoid feedback sensitivity between control and endotoxin-treated animals. Glucocorticoid receptor density was significantly reduced in the hypothalamus, hippocampus, and frontal cortex of endotoxin-treated animals. These structures have been shown to mediate the inhibitory effects of glucocorticoids on (1) CRH and AVP synthesis in PVN neurons and (2) ACTH release in response to stress (see Feldman and Conforti, 1980; Kovacs and Makara, 1986; McEwen et al., 1986; Sapolsky et al., 1986; Sawchenko, 1987a; Jacobson and Sapolsky, 1991; Dallman et al., 1992; Diorio et al., 1993). Thus, neonatal endotoxin exposure decreases glucocorticoid receptor binding in structures known to mediate glucocorticoid inhibition of HPA activity. These changes in glucocorticoid receptor expression could then represent one way in which early cytokine signals "program" the responsivity of the HPA axis to stress. The decrease in glucocorticoid receptor sites, and thus in glucocorticoid sensitivity, in regions known to govern HPA activity could reduce tonic inhibition of CRH and AVP synthesis, resulting in increased release of ACTH secretagogues in response to stress. This hypothesis suggests that neonatal endotoxin exposure alters HPA responses to stress by influencing the development of glucocorticoid receptor expression in neuronal populations that regulate HPA activity, rather than directly on hypothalamic CRH/AVP neurons.

The mechanisms underlying these effects are not clear; however, it is possible that cytokine release following endotoxin exposure may alter glucocorticoid receptor development. Numerous cytokines (e.g., IL-1, IL-6) are released from various tissues following endotoxin exposure and can cross the blood–brain barrier (Banks et al., 1993; Muramani et al., 1993; Schobitz et al., 1993). It is interesting that central administration of IL-1 can alter hippocampal glucocorticoid receptor binding in adult animals (Weidenfeld et al., 1988).

In addition, it is interesting that the effects of early endotoxin exposure on HPA development parallel those of maternal separation. Maternal separation during early postnatal life results in increased hypothalamic CRH mRNA expression and enhanced HPA responses to stress in adulthood (Plotsky and Meaney, 1993). Although not directly measured, we observed reliable changes in mother–pup interactions following endotoxin administration to neonates. At 3–4 hr postinjection, the dam was consistently off the nest, and this persisted until about 8 hr following treatment. Alterations in maternal behavior likely reflect attempts by the dams to regulate body temperature and avoid tactile contact with pups. Both nest temperature and tactile contact are important factors determining HPA function in neonates (Sucheki et al., 1993) and may have long-term consequences for endocrine function (Stanton et al., 1988). Interestingly, postnatal handling, which seems to increase contact between mother and pups (Jans, Woodside, and Meaney, unpublished observations), results in decreased hypothalamic CRH mRNA expression and reduced HPA responses to stress (Meaney et al., 1989; Plotsky and Meaney, 1993; Viau et al., 1993). These effects appear to be due to increased glucocorticoid receptor expression in the hippocampus and the frontal cortex (Meaney et al., 1985, 1989; Sarrieau et al., 1988; O'Donnell et al., in press), a pattern exactly opposite to that of early endotoxin exposure. Consequently, factors secondary to the physiological responses to endotoxin exposure may also contribute to the altered HPA development.

These findings suggest that the differentiation of glucocorticoid receptor systems within neural structures that regulate HPA function is sensitive to a variety of environmental signals during the postnatal period. These signals can act to permanently increase or decrease glucocorticoid receptor expression within specific neural structures, and in so doing determine the responsiveness of the HPA axis to stress throughout the lifetime of the animal. We believe that these effects reflect a naturally occurring plasticity whereby the early environment is able to "program" rudimentary, biological responses to threatening stimuli. The activation of the adrenocortical/sympathoadrenal responses to stress is both essential for survival and metabolically very costly. Increased glucocorticoid responses to stress are associated with enhanced immunosuppression (see Munck et al., 1984) and increased risk for neuropathology in later life (Sapolsky et al., 1986; Meaney et al., 1988; Issa et al., 1990). While it is clearly in the animal's best interest to activate these neuroendocrine systems in response to threat, exaggerated or unnecessary activity is also damaging. Hence the importance of an appropriate level of response to the threat. The Norway rat inhabits a tremendous variety of ecological niches, each with varied sets of environmental demands. We think that the plasticity observed in these developmental studies allows the animal to adapt defensive systems to the unique demands of the environment. Since mammals usually spend their adult life in an environment that is either the same or quite similar to that in which they were born, developmental "programming" of CNS responses to stress in early life is likely to be of adaptive value to the adult.

The current data represent an interesting example of this process. Under normal conditions, early postnatal life represents a period of bacterial colonization, a time when a previously sterile milieu is inhabited by micro- organisms which are likely to remain as residents throughout the life of the animal. We can assume that there are considerable differences in the degree of colonization as well as in the species and the threat they represent (see Williams et al., 1981). It is also reasonable to assume that these processes as well as the level of pathogens in the environment represent a developmental signal that approximates the level of immunological challenge that is likely to confront the animal over the course of its life. This signal, in turn, appears to be capable of regulating the development of the HPA axis. The HPA axis represents an important regulator of immune function, and Munck et al. (1984) have argued that in the absence of adequate adrenocortical regulation, elevated immune activity can present a cytotoxic threat to the animal. Animals which show inadequate adrenocortical responses to immune challenge are highly vulnerable to autoimmune disorders (see Sternberg et al., 1989) or to the toxic effects of exaggerated immune responses (see Mason et al., 1991). These effects likely involve glucocorticoid production. Following adrenalectomy or treatment with the glucocorticoid receptor antagonist RU 38486, animals are more susceptible to endotoxic shock (Grundmann et al., 1992; Lazar et al., 1992; Ramchandra et al., 1992). In

this context, the enhanced HPA responsivity of the endotoxin-treated animal is probably of adaptive value. What is curious is why (and how) this enhanced level of HPA responsivity becomes generalized to other forms of stress, and this may represent the biological cost extracted from the animal in anticipating (and adapting to) a higher level of immunological challenge. Seen in this light, it is clear that the endotoxin-treated animals in the present study (or the nonhandled animals in earlier research) are not biologically "less fit" than control (or handled) animals. These animals are simply different, and the differences can be at least partially understood in terms of the early environmental events.

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