Prenatal exposure to dexamethasone alters hippocampal drive on hypothalamic-pituitary-adrenal axis activity in adult male rats

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Hippocampal MR exhibit a 10-fold higher affinity for endogenous corticosterone (CORT) compared with GR (10). Even during periods of low CORT circulation, such as the circadian trough, hippocampal MR are substantially occupied and exert an inhibitory tone on the HPA axis (reviewed in Refs. 10, 15, 39). As the cycle progresses toward its peak, CORT circulation increases and progressively occupies hippocampal GR (40). Moreover, it has been shown that in the rat MR number and capacity increase significantly in the evening, whereas GR capacity at the two time points remains constant (42). Because MR are extensively occupied during the entire cycle, circadian changes in MR number affect the magnitude of CORT signaling and support the hypothesis that MR expression is crucial to the tonic activation and coordination of circadian-driven processes (9, 12, 48). However, MR do not act independently. In fact, numerous studies suggest that the relationship between hippocampal MR and GR is a more critical determinant of circadian-driven HPA axis activity compared with the actions of either receptor alone (6, 22, 33, 34). The relationship between MR and GR is also critical to negative feedback as the two receptors act coordinately to reduce CORT secretion following exposure to stress.

Because intracellular CORT concentration is the major determinant of hippocampal MR:GR action, it is important to consider the activity of intraneuronal 11β-hydroxysteroid dehydrogenases (11β-HSDs), which are critical to this process (13, 47). In normal rodents, CORT and its inactive form deoxycorticosterone (DOC) circulate in nearly equal concentrations; however, active CORT is substantially complexed with binding proteins in the blood and only a small fraction is capable of diffusing into neurons (10). In contrast, deoxycorticosterone circulates freely and readily enters cells, where it may be reactivated. 11β-HSDs catalyze the tissue-specific interconversion of endogenous glucocorticoids (cortisol and corticosterone) and inert 11-keto metabolites (cortisone and 11-dehydrocorticosterone) (2, 23). Two isoforms, denoted type I and type II, have been characterized for 11β-HSD. These isoforms are encoded by two different genes and display distinct directionality and ontogeny (reviewed in Ref. 16). 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD-1) is an NADPH-dependent reversible oxidoreductase present in a variety of tissues and is highly expressed in the hippocampus (24, 49). Conversely, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) is an exclusive NAD-dependent 11β-dehydrogenase and is not significantly expressed in the adult rat brain (11, 47).

Although physiological levels of CORT are a prerequisite for normal fetal brain development, exposure to excess endoge-
enal or synthetic glucocorticoid, particularly during the last third of gestation, has been shown to adversely affect both the neuroendocrine axis and behavior of the offspring (50, 57). During the later stages of fetal development, the basal set points for expression of genes regulating the HPA axis are programmed according to genotype. If the fetal endocrine environment is abnormal, for example if glucocorticoid (GC) is in excess, genetic programming may be “reset” toward HPA axis hyperactivity (10). This outcome has been associated with low birth weight, as well as stress-related cardiovascular, metabolic, and neuroendocrine disorders in adulthood (4, 27, 37).

Dexamethasone (DEX), a synthetic GC (sGC), is administered in ~10% of human pregnancies (32) due to its effectiveness in suppressing uterine contractions and promoting lung maturation (21, 35). However, studies in rat models have shown that DEX exposure in utero may give rise to adverse effects in adulthood. In rats, active CORT is substantially lowered during the last third of gestation. In particular, we explore the potential for the interaction among hippocampal GR, MR, and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2). However, CBG cannot bind DEX, and 11β-HSD-2 cannot inactivate DEX (35). Moreover, in adult animals the mdr1a P-glycoprotein in the blood-brain barrier protects neurons from DEX via a protein pump (10, 30), but this regulatory mechanism is underdeveloped in the fetus (35). These factors suggest that maternal DEX administration is capable of elevating levels of sGC in fetal neurons.

Previous investigations of rats exposed to excess levels of maternal or sGC in utero have focused on a few select components, such as hippocampal MR, GR, or hypothalamic CRH, in an attempt to deduce the source of HPA axis destabilization (3, 14, 25). However, we hypothesize that a spectrum of HPA hormones and stress-related factors act in coordination to produce the observed effects of excess glucocorticoid exposure during late gestation. In particular, we explore the potential for interaction among hippocampal GR, MR, and 11β-HSD-1. In this study, we measured the expression of hippocampal GR, MR, and 11β-HSD-1 mRNAs, hypothalamic GR, CRH, and AVP mRNAs, pituitary GR mRNA, and the hormones ACTH and CORT in adult male rats exposed to DEX or vehicle during the last third of gestation. We also measured gene expression and hormone levels in control and DEX-exposed male offspring in response to a 2-h restraint stress challenge followed by a 1-h recovery period. Our results suggest that persistent changes occur in regulatory components of the HPA axis of adult male offspring following DEX exposure in utero.

EXPERIMENTAL PROCEDURES

Animals, DEX treatment, and tissue collection. Twenty four time-mated Sprague-Dawley dams (250–350 g) were obtained from Hilltop Laboratories on gestation day (GD) 4. It is not likely that maternal stress resulting from transportation affected fetal HPA axis programming, as this axis does not begin to develop until GD 12 or 13 (31, 55). In addition, pregnant dams had more than 1 wk of acclimatization before DEX treatment. During the third week of gestation, dams were randomly assigned to receive daily injections (sc) of DEX (125 μg·kg⁻¹·day⁻¹; n = 16; Sigma, St. Louis, MO) or vehicle (saline + 0.4% ethanol, n = 16) during days 14–19 of gestation, according to previous studies (17, 25). We observed that administering maternal DEX at doses of 200 μg·kg⁻¹·day⁻¹ or greater resulted in significant growth retardation and lethality in the offspring. Day 0 is defined as the morning of appearance of the vaginal plug, and rat gestation lasts 21–22 days. All animals were maintained under conditions of controlled lighting (0600 to 1800) and temperature (23°C) and given food and water ad libitum. The male offspring (n = 124) were weaned on postnatal day 21 and housed three per group according to litter. Adult males were separated into four groups: control unrestrained, DEX-exposed unrestrained, control restraint-stressed, and DEX-exposed restraint-stressed. The unrestrained control and DEX-exposed 90-day-old males were killed during the rising phase of the circadian peak (1500 to 1700) and prepared for study. The remaining males were exposed to restraint stress for 2 h within a time period of 1300 to 1600 in a wire mesh tube. Following a 1-h recovery from stress, rats were brieﬂy exposed to CO2 in a precharged chamber and immediately decapitated by guillotine. Animals were killed in a staggered fashion of two control followed by two DEX-exposed rats during the hours of 1600 to 1700. We chose to stress and kill both control and DEX-exposed rats during the rising phase of the circadian cycle rather than during the circadian trough, as previously done by others, to determine whether significant differences persisted in the expression of HPA axis regulatory components during a period of heightened circulating CORT. Immediately upon death, trunk blood was collected, and whole pituitary, hypothalamus, and hippocampus tissues were rapidly excised and stored in RNAlater (Ambion, Austin, TX). These studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all animal procedures have been reviewed and approved by the Animal Care and Use Committee of Bucknell University.

RNA isolation and RT-PCR. Total RNA was isolated from individual pituitary, hypothalamus, and hippocampus tissues using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Purified total RNA was reverse transcribed using RETROscript (Ambion) in accordance with the manufacturer’s recommended procedure. Real-time PCR was performed on an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Green Supermix (Bio-Rad). Primers were constructed (Table 1) using the NCBI online database (www.ncbi.nlm.nih.gov) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequence specificity of each primer pair was confirmed using BLAST, and all primers were designed using MWG Oligo Synthesis (High Point, NC). Reactions were run in 96-well optical plates, with each well containing 12.5 μl Supermix, cDNA corresponding to 2 μg RNA input (diluted 1:10), and 0.05 μM each of forward and reverse primers in water. Target genes were amplified through the following thermocycling program: 95°C for 3’, 40 × 15” PCR cycles at 95°C, 60°C for 1’, and 55°C for 1’. At the end of the program, 80 repeats of 15” each accompanied by a temperature ramp of 0.5°C/minute were performed, during which dissociation curve data were collected to verify that only target sequences were amplified. CRH and AVP genes were amplified from hypothalamic-derived cDNA, GR was amplified from pituitary, hypothalamic, and hippocampal-derived cDNA, and MR and 11β-HSD-1 genes were amplified from hippocampal-derived cDNA. In each sample, the gene of interest was coamplified with the standard housekeeping gene, 18S ribosomal RNA, to control for differences in pipette volume. A real-time PCR was conducted for each primer pair in which cDNA samples were substituted with dH2O to verify that exogenous DNA was not present. Additionally, 2 μg of RNA isolated by the procedure described above were substituted for cDNA in a real-time PCR reaction to confirm that there were no genomic DNA contaminants in the RNA samples. Both negative controls showed no amplification after 35 cycles.

Data analysis. The cycle numbers at which amplified DNA samples exceeded a computer-generated fluorescence threshold level were normalized and compared to determine relative gene expression.
Higher cycle number values indicated lower initial concentrations of cDNA, and thus lower levels of mRNA expression. Each sample was run in triplicate, and averaged triplicates were used to assign cycle threshold (CT) values. dCT values were generated by subtracting experimental CT values from the CT values for 18S targets coamplified with each sample (see Fig. 1 for an annotated real-time PCR graph). The group (control unrestrained, DEX-exposed unrestrained, control restraint-stressed, or DEX-exposed restraint-stressed) with the highest mean dCT value (lowest gene expression) per amplified gene target was set to zero and the mean dCT values of the other three groups were set relative to this calibrator (ddCT). The ddCT values were calculated as powers of 2 (2^ddCT), to account for the exponential doubling of the PCR.

ACTH and corticosterone radioimmunoassay. Trunk blood samples were centrifuged at 1,200 g for 10 min at 4°C. Serum aliquots were stored at −20°C for analysis by radioimmunoassay (ICN Biomedicals, Costa Mesa, CA). Corticosterone and ACTH were measured according to manufacturer’s protocols.

Statistical analysis. Hormonal output and gene expression in control offspring and animals exposed to DEX in utero (drug effect) under unrestrained or restraint-stressed conditions (stress effect) were compared using two-way ANOVA, followed by a one-way ANOVA when appropriate (SPSS statistical analysis software, Chicago, IL). Statistical significance was confirmed if P < 0.05.

RESULTS

Effects on weight and serum hormones. As shown in Table 2, DEX-treated dams gave birth to offspring with significantly lower birth weights compared with controls [F(1,123) = 13.57, P < 0.001]. This is in agreement with previous studies using a similar dose of DEX during the same gestational period (17, 25). As adults, DEX-exposed offspring continued to exhibit significantly reduced weights compared with controls [F(1,121) = 5.69, P < 0.02].

All adult offspring exhibited a significant increase in ACTH following restraint stress [stress effect, F(1,121) = 5.185, P = 0.025]. Moreover, DEX-exposed animals had significantly higher levels of serum ACTH compared with the control group in the unrestrained or restraint-stressed condition [drug effect, F(1,121) = 9.487, P = 0.003]. However, the ACTH response to stress did not differ between control and DEX-exposed

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Table 1. Primer sequences (GenBank/NCBI) used in real-time PCR

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Primer Sequence 5’–3’</th>
<th>Accession No.</th>
<th>Length, nt</th>
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<tr>
<td>CRH</td>
<td>Fwd: TCACCTTCGAGCTGTCCAGG</td>
<td>NM 031019</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Rev: GGAAATGAAATGGCCCTTTT</td>
<td>NM 016992</td>
<td>98</td>
</tr>
<tr>
<td>AVP</td>
<td>Fwd: CCGATGTGGACAGGCTTTTTT</td>
<td>NM 017081</td>
<td>88</td>
</tr>
<tr>
<td>11β-HSD-1</td>
<td>Fwd: TGCTCTGATGGTTTCCTTTT</td>
<td>M14053</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Rev: GAGGCGGAGGACACAGAGAG</td>
<td>X74498</td>
<td>137</td>
</tr>
<tr>
<td>GR</td>
<td>Fwd: ACCTCGATGGCCAAATGACC</td>
<td>M11188</td>
<td>76</td>
</tr>
<tr>
<td>MR</td>
<td>Fwd: AAAGGCTGGATGGAGGAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>Fwd: AAAGGCTCACCACATCCAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: GGCCTGGAAGAGCTGCTGTA</td>
<td></td>
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</tr>
</tbody>
</table>

Final concentration of each primer was 1 μM. CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; 11β-HSD-1, 11-β-hydroxysteroid dehydrogenase-1; GR, glucocorticoid receptor; MR, mineralcorticoid receptor.
animals since the interaction, drug × stress, was not significant. In addition, all adult offspring exhibited a significant increase in CORT following restraint stress \(F(1,121) = 65.769, P < 0.0005\), and the CORT levels in DEX-exposed offspring were significantly higher than the control group under both unrestrained and restraint-stressed conditions \(F(1,121) = 5.743, P = 0.018\). Again, the drug × stress interaction was not significant and indicated that the CORT response to stress did not differ between control and DEX-exposed offspring. Taken together (Table 2), these results suggest a long-lasting metabolic disturbance following fetal exposure to DEX.

**Changes in gene expression.** To assess potential perturbations in the HPA axis at the level of mRNA expression, we examined multiple gene targets in the pituitary, hypothalamus, and hippocampus of control and DEX-exposed animals that were unrestrained or subjected to a 2-h restraint period followed by a 1-h recovery. Our results show that GR mRNA expression in the pituitary was significantly upregulated in response to restraint stress in all animals [Fig. 2; \(F(1,121) = 29.625, P < 0.0005\)]; however, the magnitude of this upregulation in response to restraint stress was significantly less in the pituitaries of the DEX-exposed compared with control animals [Fig. 2; \(F(1,71) = 4.460, P = 0.038\)].

We found no significant differences in hypothalamic GR mRNA expression in the control × DEX-exposed group under either treatment condition (Fig. 3A). In contrast, hypothalamic AVP mRNA was not perturbed (Fig. 3B). As expected, all animals subjected to restraint stress significantly increased hypothalamic CRH mRNA expression [Fig. 3C; \(F(1,92) = 8.876, P < 0.004\)]; moreover, CRH mRNA expression level in the DEX-exposed group was significantly higher compared with the control group under the unrestrained and restraint-stressed condition [Fig. 3C; \(F(1,92) = 10.443, P = 0.002\)]. No significant difference was detected in the drug × stress interaction indicating that the expression of CRH mRNA in response to stress was similar in both the control and DEX-exposed animals.

In the hippocampus, all animals significantly reduced GR mRNA expression in response to stress [Fig. 4A; \(F(1,105) = 61.214, P < 0.0005\]), and no significant difference was observed in the stress response between control and DEX-exposed animals. In contrast, hippocampal MR expression levels in control and DEX-exposed animals were significantly different [\(F(1,105) = 5.295, P = 0.023\)]. Further analysis using a one-way ANOVA demonstrated that MR expression was significantly different between the control and DEX-exposed group only under the unrestrained condition [Fig. 4B; \(F(1,71) = 7.795, P = 0.018\)]. In addition, we measured the expression of 11β-HSD-1 in the hippocampus. This enzyme has been shown to regulate local reactivation of inactive corticosterone in hippocampal neurons, thus amplifying tissue exposure to corticosterone and potentially contributing to the modulation of circadian-driven HPA activity. Interestingly, all animals exhibited a significant downregulation of this gene in response to restraint stress [Fig. 4C; \(F(1,85) = 21.511, P < 0.0005\]); however, 11β-HSD-1 mRNA expression level was significantly higher in DEX-exposed male offspring compared with controls in both the unrestrained and restraint-stressed condition [Fig. 4C; \(F(1,85) = 8.678, P = 0.004\)]. The magnitude of the stress response did not differ between the two groups.

**DISCUSSION.** The present study shows that select changes in the expression of key HPA axis regulatory components occur in adult male rats following exposure to DEX in utero. In agreement with earlier work, we found a significant increase in hypothalamic CRH mRNA in the DEX-exposed group compared with controls in both the unrestrained and restraint-stressed animals (57). Although altered mRNA expression does not consistently predict the magnitude or functionality of corresponding protein products, in this case, the increase in CRH mRNA was asso-

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**Table 2. Effects on birth weight, adult weight, and serum hormones following prenatal exposure to DEX**

<table>
<thead>
<tr>
<th>Birth wt, g</th>
<th>Adult wt, g</th>
<th>ACTH, pg/ml</th>
<th>CORT, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unrestrained</strong></td>
<td><strong>Restraint Stressed</strong></td>
<td><strong>Unrestrained</strong></td>
<td><strong>Restraint Stressed</strong></td>
</tr>
<tr>
<td>Control</td>
<td>6.63±0.08</td>
<td>458.0±6.9</td>
<td>257±23</td>
</tr>
<tr>
<td>DEX-exposed</td>
<td>5.22±0.09</td>
<td>434.0±5.9*</td>
<td>363±36*</td>
</tr>
</tbody>
</table>

Values are means ± SE. \(\dagger P < 0.0005, \star P < 0.005, * P < 0.05\) indicate significant difference between dexamethasone (DEX)-exposed animals compared with controls using 1-way ANOVA for weights and a 2-way ANOVA for serum hormone analysis. ACTH, adrenal corticotropin hormone; CORT, corticosterone.
associated with a significantly higher level of serum ACTH and CORT in DEX-exposed rats. In contrast, we measured no change in hypothalamic AVP mRNA expression in the experimental animals. Hypothalamic and pituitary GR mRNA levels were not significantly different in unrestrained, DEX-exposed adult male rats compared with controls. The lack of a disturbance at these sites under this condition supports the hypothesis that GR at the level of the hypothalamus and pituitary may be more involved in negative feedback following a CORT surge rather than in regulating diurnal fluctuations of CORT during baseline activity (9, 10, 52). This is further supported by our data showing that pituitary GR mRNA significantly increases in both the control and DEX-exposed animals in response to restraint stress; however, the increased expression was significantly less in the DEX-exposed offspring compared with controls. It is possible that the observed spike in pituitary GR mRNA expression transiently increases GR to provide a short-term increase in the capacity for GR-mediated negative feedback before receptor downregulation. The significantly lower level of pituitary GR mRNA expression in the DEX-exposed males compared with controls during this immediate response to stress may represent a blunting of the negative feedback in the experimental animals as the drug × stress interaction was not significantly different. All values are means ± SE.

As expected, all of the animals downregulated GR mRNA in response to restraint; however, the expression of hippocampal GR mRNA was essentially unchanged when comparing DEX-exposed and control animals during unrestrained or restraint-stressed conditions. In contrast, we found that MR mRNA expression did not change significantly in response to restraint stress but was significantly lower in the unrestrained DEX-exposed rats compared with controls. Our mRNA measurements agree with receptor functionality studies that demonstrate a significant reduction in binding by hippocampal MR, but not GR, in adult male offspring exposed to excessive amounts of maternal glucocorticoid during gestation (3, 26, 56).

In contrast to our results, other studies have measured a downregulation of both MR and GR in response to late-gestational exposure to DEX (14, 25, 57). These studies claim that reduction in both receptors was associated with a blunted negative feedback system and a constitutively hyperactive HPA axis. However, recent data emphasize alternate roles for MR and GR during the rising phase of the circadian cycle,
when MR-mediated tonic inhibition is overcome by GR-mediated facilitation of the HPA axis (reviewed in Refs. 8, 10, 52). It is probable that hippocampal MR is more involved in homeostatic maintenance (15), whereas hippocampal GR may be more influential in establishing drive and facilitation of the HPA axis during the circadian rising phase and in mediating HPA feedback inhibition in response to stress (reviewed in Refs. 10, 52). This hypothesis is supported by evidence showing that intrahippocampal GR antagonists suppress ACTH and inhibit HPA activity in the rat, whereas intrahippocampal MR antagonists have the opposite effect of enhancing circadian-driven HPA activity by increasing basal afternoon levels of both ACTH and CORT (52). Our data agree with this evidence by demonstrating that a significant reduction in hippocampal MR mRNA expression during the afternoon period is associated with a significant increase in both ACTH and CORT output. These findings support the hypothesis that reductions in MR, without a change in GR, are associated with HPA hyperactivity.

The most interesting finding with regard to the effects of prenatal DEX exposure on the HPA axis is the perturbation we detected in the expression of hippocampal 11β-HSD-1 mRNA. The expression of this gene, in conjunction with that of MR and GR, is critical to glucocorticoid action at the level of the hippocampus. Although 11β-HSD-1 is bidirectional in hippocampal homogenates (24), it has recently been purported that under conditions of low circulating CORT, hippocampal 11β-HSD-1 is more highly expressed and acts almost exclusively as a reductase by regenerating active CORT from deoxyCORT in hippocampal neurons both in vitro (19, 38) and in vivo (1). Studies of this enzyme’s expression and directionality elsewhere in the brain are rare and inconclusive (reviewed in Refs. 46, 47). Moreover, localization of 11β-HSD-1 to hippocampal neurons presents the possibility that this enzyme modulates the intraneuronal CORT concentration and thus the relationship of MR and GR. Because recent findings have shown that 11β-HSD-1 activity parallels mRNA expression (20), the overall downregulation of 11β-HSD-1 mRNA observed in both our DEX-exposed and control animals compared with controls under the unrestrained or restraint-stressed condition (*P < 0.0045). However, 11β-HSD-1 mRNA expression was significantly higher in the DEX-exposed animals compared with controls under the unrestrained or restraint-stressed condition (**P < 0.0005). No significant difference was observed in the response to stress between control and DEX-exposed animals. All values are means ± SE.
high levels of DEX, a glucocorticoid that has the potential to traverse the fetoplacental barrier and penetrate fetal neurons. Because DEX exposure in our animals was conducted at a time when critical set points for basal HPA activity are established, we propose that an adaptive response in the regulatory signals controlling 11β-HSD-1 promoted a heightened expression of this enzyme to compensate for the low endogenous CORT present in the fetal milieu.

In summary, our data suggest that adult male offspring exposed to DEX in utero exhibit 1) an increased hypothalamic CRH mRNA expression, 2) a significantly reduced hippocampal MR mRNA expression and shift in the MR:GR balance during baseline activity, as well as a 3) persistent increase in 11β-HSD-1 mRNA expression in hippocampal neurons. The cumulative effect of these changes would be to induce chronically elevated levels of intraneuronal CORT, a hyperactive HPA axis, and persistently elevated levels of circulating CORT. Our data support this interpretation. In addition, we found that the initial response to stress was altered in DEX-exposed rats at the level of the pituitary. More specifically, we found that pituitary GR expression appears to be less tightly regulated during the initial rise in GR mRNA before receptor downregulation.

Recent studies have shown that if prenatally DEX-exposed offspring are adopted by a healthy control mother, her postnatal care is capable of reversing the adverse physiological and brain receptor systems. The surgical neonate.

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GRANTS

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