The Journal of Neuroscience, March 1993, 13(3): 1097–1105

Increased Plasma ACTH Responses to Stress in Nonhandled Compared with Handled Rats Require Basal Levels of Corticosterone and Are Associated with Increased Levels of ACTH Secretagogues in the Median Eminence

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Postnatal handling of rat pups is known to alter permanently hypothalamic-pituitary-adrenal (HPA) responses to a wide variety of stressors. As adults, handled (H) and nonhandled (NH) animals also differ in sensitivity to the delayed negative-feedback effects of glucocorticoids on stress-induced HPA activity. However, it is not understood how handling alters neuroendocrine responses to stressful stimuli, and how differences in feedback sensitivity might account for changes in HPA activity both during and following stress. In the present studies, plasma ACTH responses to both restraint and ether stress were significantly greater in NH compared with H animals. Administration of 100 μg/kg corticosterone (B) immediately prior to restraint stress significantly decreased subsequent plasma ACTH responses to restraint to the same extent in H and NH animals, suggesting that H and NH animals do not differ in glucocorticoid fast feedback. H and NH animals adrenalectomized (ADX) 5 d prior to testing did not differ in plasma ACTH responses to restraint stress, demonstrating that the differences between the groups are dependent upon the presence of circulating B. However, the handling effect was apparent in ADX animals provided with a low level of B replacement (~5–6 μg/dl). B replacement significantly decreased plasma ACTH levels under both basal conditions and in response to restraint stress in H/ADX rats. In contrast, although B replacement also decreased basal ACTH levels in ADX/NH rats, there were no differences in plasma ACTH responses to restraint between NH/ADX and NH/ADX+B animals. Thus, plasma ACTH responses to restraint were significantly greater in ADX+B/NH compared with ADX+B/H rats. Thus, a low level of B replacement was able to restore the difference between H and NH animals in plasma ACTH responses to stress. This finding shows that the differences between H and NH animals in HPA responses to stress can occur independently of stress-induced elevations in plasma B levels. Finally, we found that resting-state levels of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the median eminence were significantly higher in NH animals compared with H animals. Taken together, these findings suggest that H and NH animals differ in delayed negative feedback, and that this difference occurs in response to low levels of B and is reflected in differential rates of CRH and AVP synthesis in hypothalamic neurons. Assuming that the difference in ACTH secretagogues exists in readily releasable storage pools in the median eminence, this would mean that any stress-induced neural signal that activates hypothalamic CRH/AVP neurons would result in a greater release of CRH and/or AVP in NH animals, leading to a larger ACTH response, and a larger and more prolonged increase in plasma B. Thus, differences between H and NH animals in HPA activity both during and following stress can occur under a variety of stressful conditions and independently of the stress-induced increase in plasma B. These findings are also consistent with earlier findings of increased hippocampal glucocorticoid receptor density in the H animals.

[Key words: neonatal handling, ACTH, glucocorticoids, glucocorticoid receptors, corticotropin-releasing hormone (CRH), arginine vasopressin (AVP)]

Postnatal handling of rat pups permanently alters hypothalamic-pituitary-adrenal (HPA) responses to a variety of stressful stimuli (Levine, 1957, 1962, 1970; Levine et al., 1967; Ader and Grota, 1969; Hess et al., 1969; Meaney et al., 1988a, 1989). These studies have shown that the development of rudimentary endocrine responses to stress is subject to environmental regulation. Moreover, these studies also provide a model for the study of individual differences in neuroendocrine systems, and how such differences might predict vulnerability to stress-induced pathology (see Levine, 1970; Meaney et al., 1988). Nevertheless, despite the long history of the neonatal handling paradigm, we know rather little about the mechanisms that underlie the differences in HPA activity between handled (H) and nonhandled (NH) animals.

In general, adult rats handled daily for the first 3 postnatal weeks exhibit markedly reduced HPA responses to a wide variety of stressors. This difference has been most commonly reported as a smaller increase in plasma corticosterone (B) levels during stress (see references cited above) and a faster return to basal B levels following the termination of the stress (Hess et al., 1969; Meaney et al., 1985a, 1988, 1989). The faster rate of

Received May 26, 1992; revised Sept. 8, 1992; accepted Sept. 17, 1992.

This research was supported by grants from the Medical Research Council (MRC) to M.J.M. and by NIMH Grant MH45216 to P.M.P. M.J.M. is an MRC scientist.

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decline in plasma B levels following stress in the H animals is not due to differences in the plasma clearance rate for B, nor is it associated with differences in plasma corticosteroid-binding globulin (CBG) levels (Grota, 1975; Meaney et al., 1989, 1992). Although less well characterized, there is also evidence for a decrease in stress-induced levels of plasma ACTH (Meaney et al., 1989) and bioactive corticotropin-releasing factor in H animals (Zarrow et al., 1972).

The difference in poststress plasma B levels has been explained on the basis of the enhanced sensitivity to the negative-feedback effects of elevated glucocorticoid levels in the H animals (Meaney et al., 1991). Indeed, H animals show a greater sensitivity to the negative-feedback effects of exogenously administered glucocorticoids (Meaney et al., 1989). The differences in negative-feedback sensitivity to elevated glucocorticoid levels and in HPA responses to stress between H and NH animals have in turn been associated with the increased density of hippocampal glucocorticoid (type II corticosteroid) receptors in the H animals (Meaney et al., 1985b, 1987, 1988a, 1989; Sarrieau et al., 1988; Mitchell et al., 1990). Thus, a chronic B treatment regimen that reversed the handling-induced increase in hippocampal glucocorticoid receptor binding capacity, by downregulating receptor levels to values comparable to those of NH animals, also eliminated the difference in poststress B levels (Meaney et al., 1989). The results of these and other studies have suggested that the increase in glucocorticoid receptor sites in the hippocampus is a critical feature for the handling effect on HPA function. The increase in receptor density appears to increase the sensitivity of the hippocampus to elevated levels of circulating glucocorticoids, enhancing the efficacy of negative-feedback inhibition on HPA activity, and serving to reduce poststress secretion of ACTH and B in H animals. This idea is consistent with our emerging view of the hippocampus as a major glucocorticoid target site in HPA regulation (see McEwen et al., 1986; Sapolsky et al., 1986; Dallman et al., 1987; Jacobson and Sapolsky, 1991).

Our current understanding is that the difference between the H and NH animals emerges under conditions of elevated glucocorticoid levels, such as those seen during and immediately following stress. Interestingly, young adult H and NH animals do not differ in basal levels of either ACTH or B (Meaney et al., 1989, 1992). However, this hypothesis is based largely on measures of plasma B, with little information concerning plasma ACTH levels during stress. Dallman's laboratory has shown that in adrenalectomized (ADX) animals provided B in their drinking water, the termination of ACTH responses following stress was as efficient as in intact animals (Jacobson et al., 1988). Moreover, under certain conditions, the termination of plasma ACTH responses to stress can occur independently of circulating B (Bradbury et al., 1991). These data clearly challenge the notion that the poststress inhibition of ACTH release is dependent upon the presence of elevated glucocorticoid levels.

In the present study, we have focused on plasma ACTH responses to restraint stress in H and NH animals, and provided measures of ACTH secretagogue activity in these animals. In a second study, we compared ACTH responses to stress in H and NH (1) ADX animals, which lack any glucocorticoid negative-feedback signal; (2) ADX animals provided the equivalent of a basal B signal (ADX+B), but lacking the negative-feedback signal associated with stress-induced increases in B; and (3) intact, sham-operated animals (SHAM), which possess both basal and stress-induced glucocorticoid signals. These animals were studied 5 d following ADX. At this time the HPA axis has generally stabilized in ADX+B animals (see Dallman et al., 1987; Bradbury et al., 1991). The results of these studies suggest that differences between H and NH animals in poststress HPA activity occur independently of stress-induced changes in circulating B levels and that the differences in negative-feedback sensitivity between H and NH animals are reflected in median eminence levels of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) even under basal conditions.

Materials and Methods

Animals. The animals used in these studies were male Long–Evans, hooded rats (Charles River Canada, St. Constant, Quebec, Canada), the offspring of dams mated in our animal colony. Handling consisted of removing the mother and then the pups from the cage, and placing the pups into a plastic container lined with bedding material for 15 min. The pups, followed by the mother, were then returned to their cage. Handling occurred once per day for the first 3 postnatal weeks. The NH animals were left completely undisturbed throughout this period. On day 22, the animals were weaned and housed in same-sex, same-treatment groups of three animals per cage. The animals were maintained on a 12 hr:12 hr light : dark schedule (lights on at 08:00) 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 and were randomly selected from approximately 10 litters. All testing was performed during the light phase of the cycle between 12:00 and 15:00.

Adrenalectomy was performed under Metophane (methoxyflurane; Pitman-Moore, Inc., Washington Crossing, NJ) anesthesia, and all ADX animals were provided with 0.9% saline as drinking water. For studies of median eminence content of CRH and AVP, animals were rapidly (i.e., <10 sec) killed by decapitation following removal from the home cage between 11:00 and 13:00. 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. 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 (~150 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 100 μg/kg B (in EtOH:saline, 1:9) 3 min prior to restraint testing in order to examine fast-feedback effects. In this experiment, blood samples were taken immediately following a 10 min period of restraint. Ether stress was performed by placing animals into a large, covered beaker containing ether-soaked cotton balls. The animals were removed from the beaker after 2.5 min and a blood sample was taken immediately, and at various times afterward. Blood samples for corticosterone or CBG measurement were taken into 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, centrifuged, and stored at −80°C.

In a separate study, animals were ADX 5 d prior to testing. Immediately following adrenalectomy, one-half of the animals were implanted with fused 150 mg steroid pellets comprised of 50% B and 50% cholesterol (see Meyer et al., 1979). SHAM animals received the anesthetic and bilateral incisions above the adrenals. Two days later all animals were implanted with jugular catheters. Stress testing was performed on day 5, 3 d following catheter implants.

Radioimmunoassays. Plasma B was measured by the radioimmunoassay of Krey et al. (1975) with a highly specific B antiserum (B3-163, Endocrine Sciences, Tarzana, CA), ^{3}H-B (101 Ci/mmol; New England Nuclear, Boston, MA) as tracer, and 1 μl of plasma. The minimum level of detection with the assay was 10 pg. The antiserum cross-reacts slightly with desoxycorticosterone (~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 de-

scribed 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 cross-reacts 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-melanocyte-stimulating hormone (β-MSH), and α- and β-lipotropin. The intra- and interassay coefficients of variation were 18% and 8%, respectively. The minimal detectable level was 1 pg.

For peptide extraction, median eminence fragments were placed in 2 ml of 45°C 1.0 *N* acetic acid:0.5 *N* HCl (1:1 v/v) containing pepstatin (4.5 μg/ml) and, for CRH extraction, 0.5% β-mercaptoethanol. After 5 min, solutions were cooled, sonicated, and centrifuged (5000 × *g*, 30 min, 4°C). Supernatants were then lyophilized and frozen at −70°C. Immediately prior to assay, samples were reconstituted in 0.5 ml assay buffer neutralized.

CRH levels were determined by radioimmunoassay (see Plotsky et al., 1985) using antiserum rC-70 (Dr. W. Vale, The Salk Institute, La Jolla, CA) at a final dilution of 1:150,000 with synthetic rat CRH-41 as standard (Dr. J. Rivier, The Salk Institute, La Jolla, CA) and iodinated by the chloramine-T method for tracer. The minimal detectable level was 2 pg/ml. The intra- and interassay coefficients of variation were 3.6% and 10.6%, respectively.

AVP and oxytocin radioimmunoassays (see Plotsky et al., 1985) were performed using antisera R-71 to AVP and R-421 to oxytocin (Dr. D. A. Fisher, Harbor–UCLA Medical Center, Torrance, CA) in final dilutions of 1:112,000 and 1:12,800, respectively. Synthetic peptides (Dr. J. Rivier) were used for tracer preparation (see above) and standards. The minimal detectable level for each peptide was 1 pg/ml. The intra- and interassay coefficients of variation were 6.2% and 12.1%. The cross-reactivity of synthetic oxytocin with the AVP antiserum was <0.2%, while that of synthetic AVP for the oxytocin antiserum was <0.02%. The results of the CRH, AVP, and oxytocin radioimmunoassays are expressed as μg/mg protein.

Plasma CBG assay. Plasma CBG levels were measured by a method described by Martin et al. (1975) in plasma samples taken 2 hr into the light cycle (10:00). For the measurement of CBG, endogenous steroids were removed from plasma samples by passing the sample through 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 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-B 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 B. 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 TEDGM into minivials, which were then filled with 5 ml of Liquiscent (National Diagnostics, Somerville, NJ) and counted in a Packard scintillation counter at 40% efficiency. Protein content was determined by the method of Bradford (1976), and the results were expressed as picomoles of binding/mg protein.

Glucocorticoid receptor occupancy. Glucocorticoid receptor occupancy in hippocampal tissue from H and NH animals was estimated by comparing receptor binding in ADX+B animals, prepared as described above, with animals adrenalectomized (ADX) 16 hr prior to death using a previously described procedure (see Reul and De Kloet, 1985; Meaney et al., 1988b: Sapolsky et al., 1990). Following decapitation, the brain was quickly removed and placed on ice, and the hippocampus was dissected, frozen on dry ice, and stored at −80°C. Tissue was homogenized in TEDGM (pH adjusted to 7.4) and the homogenate was centrifuged at 2°C for 60 min at 105,000 × *g* in a Beckman L8-80 ultracentrifuge. Aliquots (150 μl) of the soluble fraction prepared from the hippocampal tissue were incubated with 100 μl buffer containing a saturating 20 nM concentration of ^{3}H-dexamethasone (87.0 Ci/mmol; New England Nuclear, Boston, MA) at 0–2°C for 4 hr. Nonspecific binding was determined in parallel incubations containing a 200-fold excess of unlabeled synthetic glucocorticoid, RU 28362. RU 28362 has been shown to bind to the glucocorticoid receptor selectively, showing very little affinity for the mineralocorticoid receptor (see Reul and De Kloet, 1985; McEwen et al., 1986). Sephadex LH-20 columns (4 × 1 cm), equilibrated with TEDGM, made from disposable pipette tips, were used to separate bound from unbound steroid. 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 TEDGM into minivials, which were then filled with 5 ml of Liquiscent (National Diagnostics, Somerville, NJ) and counted in a Packard scintillation counter at 40% efficiency. Protein content was determined by the method of Bradford (1976) and the results were expressed as femtomoles of binding/mg protein. Protein concentrations ranged from 300–500 μg/ml.

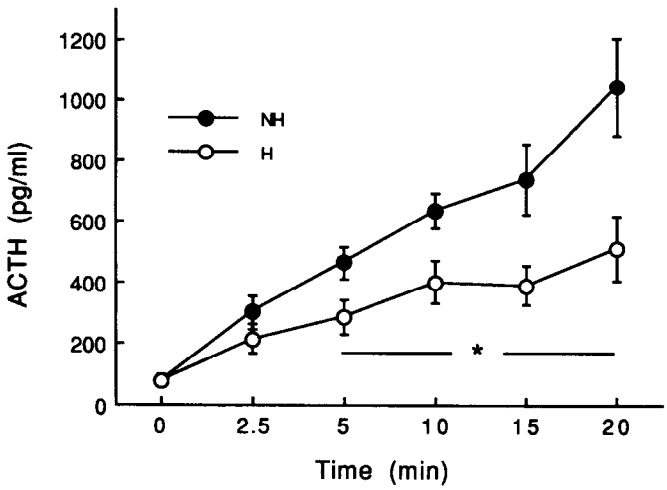

Figure 1. Plasma ACTH levels in H and NH rats (n = 8 per group) sampled prior to and at various time points during restraint stress. Values above the *line with the asterisks* (*) differ at $p < 0.05$.

In the ADX+B animals, a 4 hr incubation period reflects binding only to the unoccupied receptor sites; hormone-bound receptor sites, whether soluble or DNA bound, are masked by the endogenous ligand. In ADX animals the absence of steroid renders receptor sites available for radioligand binding in the soluble fraction and decapitation occurs at a time that precedes the upregulation of receptor sites (see McEwen et al., 1986). Thus, receptor binding in 16 hr ADX animals serves as an estimate of the total receptor binding capacity. It is then possible to estimate the receptor occupancy by subtraction (ADX values − binding at 4 hr). These data were expressed as the percentage of receptor occupancy [(ADX binding − ADX+B binding)/ADX binding] for both H and NH animals.

Statistical analysis. Integrated plasma ACTH levels (area under the curve) were calculated using the trapezoidal method. Data derived from group × time designs were analyzed using analysis of variance with one repeated measure (time of sample), and Scheffé post hoc tests were used, where appropriate, to explore the source of significant effects. Group data were analyzed using unpaired *t* tests.

Results

Plasma ACTH during stress

Integrated plasma ACTH levels were significantly ($p < 0.01$) higher during the 20 min restraint stress in the NH animals (409.8 ± 60 vs. 243.8 ± 40 pg/ml/min). Differences in plasma ACTH levels were apparent at 5, 10, 15, and 20 min following the onset of restraint (Fig. 1). Plasma ACTH levels in NH animals were significantly ($p < 0.05$) higher at 20 min into restraint than at any other time point. In contrast, plasma ACTH levels in the H animals peaked by 10 min (i.e., there was no statistically reliable difference between the 10 and 20 min samples in the H animals). The difference in plasma ACTH levels was reflected in significantly higher plasma levels of B in the NH animals. Thus, integrated B levels over the same time period were significantly ($p < 0.01$) higher in the NH animals (25.6 ± 1.3 vs. 16.4 ± 1.3 μg/dl/min).

The results for ether stress were comparable to those for restraint (data not shown). Integrated plasma ACTH levels over the 20 min period following exposure to ether were significantly

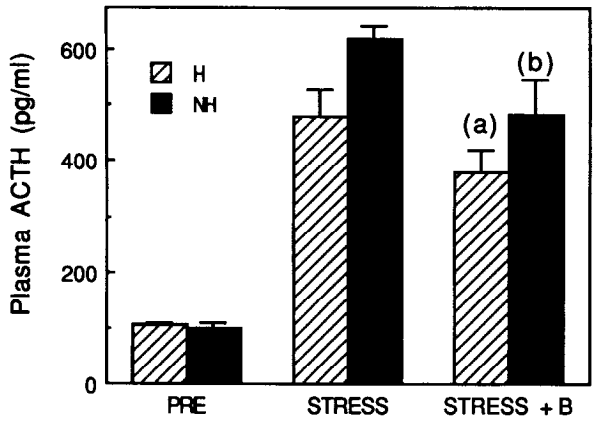

Figure 2. Plasma ACTH levels during restraint stress in H or NH animals treated with 100 μg/kg B or saline/EtOH vehicle. Samples were taken 10 min following the onset of restraint. The prestress samples (*PRE*) were taken from untreated animals. *a*, differs at $p < 0.05$ from vehicle-treated (*STRESS*) H animals; *b*, differs at $p < 0.05$ from vehicle-treated (*STRESS*) H animals; $n = 5$ or 6/group.

($p < 0.01$) higher in the NH animals (1453.6 ± 177 vs. 902.0 ± 148 pg/ml/min).

Glucocorticoid fast feedback

Administration of exogenous 100 μg/kg B 3 min prior to restraint stress significantly ($p < 0.05$) reduced plasma ACTH levels in both H and NH animals (Fig. 2). As expected, NH animals showed significantly ($p < 0.05$) higher plasma ACTH levels and therefore the suppression data were analyzed as the percentage change following B treatment. There was no difference in the magnitude of the suppression of ACTH in H (29 ± 2%) vs. NH (28 ± 3%) following pretreatment with B. The magnitude of the suppression of restraint-induced increases in plasma ACTH was not great, and this is consistent with the recent finding using a very similar paradigm (Young et al., 1990).

Adrenalectomy with B replacement

The 50% B pellets provided a plasma B signal that was in the low physiological range (ADX+B/H, 5.6 ± 0.9 μg/dl; ADX+B/NH, 6.2 ± 1.2 μg/dl) and comparable to the integrated B levels achieved over the diurnal cycle (see Dallman et al., 1987). These animals did not differ in plasma CBG levels (Table 1). The same plasma samples were then used to calculate the percentage of free B using the procedure described by Martin et al. (1975). Based on these estimates, the concentration of free B was 10.4 ± 3.2 nM in ADX+B/N rats and 11.7 ± 2.2 nM in ADX+B/NH rats. These B levels resulted in comparable levels of glucocorticoid receptor occupancy in hippocampal tissue (ADX+B/H, 37.3 ± 10%; ADX+B/NH, 41.2 ± 8%; $n = 6$ per group). This level of receptor occupancy corresponds closely to the study of Sapolsky et al. (1990), where glucocorticoid receptor occupany was examined over a wide range of plasma B levels. Note that these data are presented as the percentage occupancy of the total receptor population for each group. As reported in several previous studies, receptor binding in animals ADX 16 hr prior to death differed significantly (H, 151 ± 9 fmol/mg protein; NH, 103 ± 10 fmol/mg protein; $p < 0.01$; $n = 6$ per group), reflecting the increased hippocampal glucocorticoid receptor density in the H animals.

Plasma ACTH levels were significantly ($p < 0.0001$) elevated following ADX in both H and NH rats (ADX/H, 655 ± 63 pg/ml; SHAM/H, 65 ± 6 pg/ml; ADX/NH, 727 ± 85 pg/ml; SHAM/NH, 60 ± 7 pg/ml). B replacement was sufficient to reverse at least partially the ADX-induced increase in basal plasma ACTH in both H (ADX+B/H, 139 ± 46 pg/ml) and NH animals (ADX+B/NH, 145 ± 76 pg/ml; also see Fig. 3). Moreover, the ADX-induced increase in plasma CBG levels was reversed with B replacement in both H and NH animals (see Table 1).

Table 1. Mean ± SEM plasma CBG levels (pmol/mg protein) in H and NH animals 5 d following ADX, ADX+B, or SHAM treatment ($n = 8$ per group)

	SHAM	ADX	ADX+B
H	11.4 ± 0.5	20.5 ± 1.9*	14.1 ± 0.7
NH	13.2 ± 0.8	25.2 ± 1.6*	14.8 ± 1.1

* Significantly different from SHAM animals ($p < 0.01$).

As in previous experiments, plasma ACTH levels in response to restraint stress were significantly ($p < 0.05$) higher in the intact (SHAM) NH animals compared with intact (SHAM) H animals both during and following stress (Fig. 3, Table 2). The integrated plasma B response to restraint was also significantly higher in the SHAM/NH compared with SHAM/H rats (30.3 ± 4.1 vs. 20.9 ± 2.2 μg/dl/min; $t = 1.91$, $p < 0.05$). H/ADX and NH/ADX animals showed a significant ($p < 0.0001$) increase in plasma ACTH levels in response to restraint stress. At 40 min following the termination of stress, plasma ACTH levels were subsequently reduced to values that were comparable to those observed prior to stress. There were no significant differences in plasma ACTH levels at any time between ADX/H and ADX/NH animals. Among the NH animals, plasma ACTH levels did not differ between ADX and ADX+B animals at any time point, except the basal sample (see Fig. 3, PRE). Moreover, the integrated plasma ACTH response was virtually indentical in these animals (see Table 2). In contrast, among H animals plasma ACTH levels were significantly ($p < 0.05$) lower in the ADX+B animals than in the ADX animals at 20 and 40 min poststress (see Fig. 3). Plasma ACTH levels in ADX+B/H animals were lower than in ADX/H during stress, but these differences during restraint were partially masked by the variability among the ADX+B animals. Nevertheless, these differences approached significance at 10 min ($p = 0.05$) and 20 min of restraint stress ($p > 0.05 < 0.10$), and were significant at 20 min ($p < 0.05$) and 40 min ($p < 0.01$) following stress. Note as well that the integrated plasma ACTH level in the ADX+B/H animals was significantly ($p < 0.01$) lower than that for the ADX/H animals (see Table 2). In contrast, the integrated plasma ACTH response to restraint in ADX+B/NH animals did not differ from that of ADX/NH animals. Most importantly, there were highly significant differences in ACTH responses to restraint stress between ADX+B/H and ADX+B/NH animals (see Fig. 3). ADX+B/NH animals had significantly higher plasma ACTH levels at 10 min into restraint, and at both 20 and 40 min following restraint. Overall, the integrated plasma ACTH response to restraint was almost twofold higher ($p < 0.01$) in the ADX+B/NH compared with ADX+B/H animals (see Table 2). Finally, a striking difference between these groups was the magnitude of the increase in plasma ACTH during stress. We estimated the peak ACTH to stress by averaging the two samples taken during stress (10 and 20 min) and subtracting the

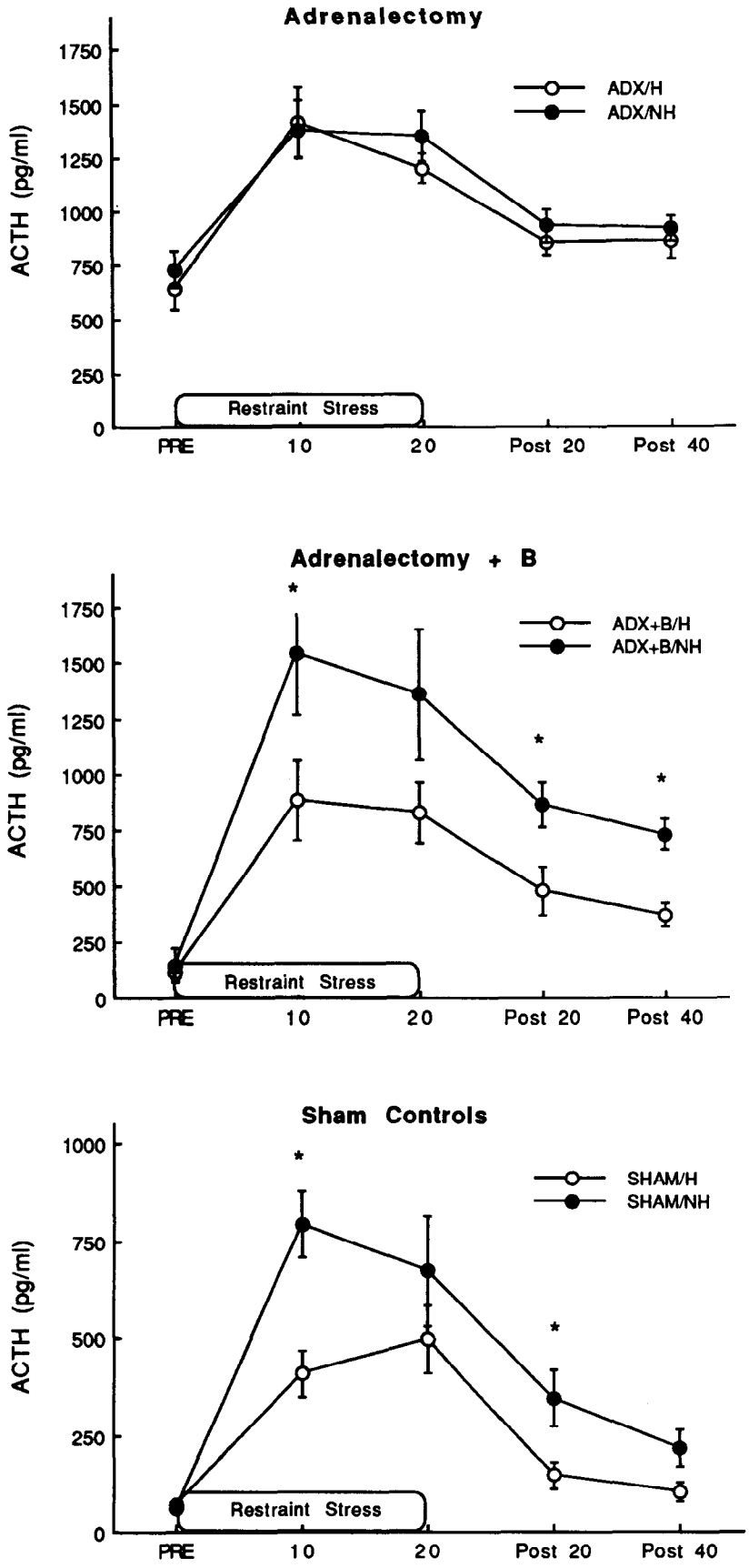

Figure 3. Plasma ACTH levels prior to (*PRE*), during, and at 20 and 40 min following a 20 min restraint stress in ADX, ADX+B, and SHAM H and NH animals. The *asterisks* indicate values that differ at $p < 0.05$.

Table 2. Mean ± SEM integrated plasma ACTH levels (pg/ml/min) with restraint stress in H and NH animals 5 d following ADX, ADX+B, or SHAM treatment (n = 8 per group)

	SHAM	ADX	ADX+B
H	326.7 ± 68*	1031.5 ± 61	608.9 ± 142**
NH	448.2 ± 83	1070.1 ± 111	1091.1 ± 111

* Significantly different from same-treatment NH animals ($p < 0.05$).
** Significantly different from same-treatment NH animals ($p < 0.01$).

basal (Fig. 3, PRE) ACTH value for each animal. This analysis revealed that the ACTH response to restraint was significantly higher in ADX+B/NH rats compared with all other groups, including ADX animals (Table 3). These data further underscore the differences between ADX+B/H and ADX+B/NH animals.

ACTH secretagogues

CRH and AVP content in median eminence extracts from intact H and NH animals differed significantly ($p < 0.01$; Fig. 4). Under resting-state conditions, CRH content was 85% higher and AVP content 45% higher in the NH animals. There were no differences in oxytocin levels (H, 301.6 ± 53 μg/mg protein; NH, 313.9 ± 58 μg/mg protein).

Discussion

NH animals showed higher plasma ACTH levels during both restraint and ether stress than H animals. However, these differences in ACTH secretion between H and NH animals were apparently not due to differences in glucocorticoid fast feedback. Glucocorticoid administration immediately prior to restraint stress moderately dampened plasma ACTH responses, and the magnitude of the supression was similar in H and NH animals (see Fig. 2).

Among the earlier handling studies, perhaps the most reliable and pronounced difference in the HPA response to stress between H and NH animals was the elevated poststress levels of B in the NH animals. Even when the groups are statistically equated for differences in B achieved during stress, the rate of decline in plasma B is faster in the H animals (Meaney et al., 1989). In previous reports, we speculated that these differences occurred in response to the poststress negative-feedback signal associated with elevated glucocorticoid levels. H and NH animals differ in delayed negative-feedback sensitivity to elevated glucocorticoid levels (Meaney et al., 1989). Exogenous administration of either dexamethasone or B 3 hr prior to testing produces a greater suppression of stress-induced HPA activity in H animals than in NH animals; the ID_{50} values for both steroids are about 5–10 times lower in the H animals. Thus, we

Table 3. Mean ± SEM stress-induced elevation in plasma ACTH in H and NH animals 5 d following ADX, ADX+B, or SHAM treatment

	SHAM	ADX	ADX+B
H	385.0 ± 63*	715.2 ± 81	740.8 ± 126
NH	672.8 ± 91†	639.1 ± 101	1457.7 ± 240†,**

Values are (St10 + St20)/2 − prestress levels), and are the same as in Figure 3.
* Significantly different from remaining H animals ($p < 0.01$).
† Significantly different from same-treatment H animals ($p < 0.01$).
** Significantly different from remaining NH animals ($p < 0.01$).

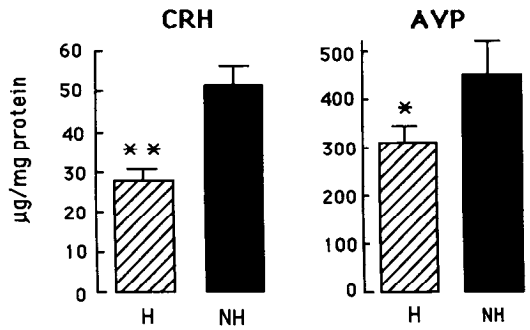

Figure 4. Median eminence levels of CRH and AVP in H and NH animals (n = 8 per group). *, **, values that differ at $p < 0.05$, $p < 0.01$, respectively.

believed that the difference in poststress HPA activity between H and NH animals was associated with a differential response to the negative-feedback signal associated with elevated glucocorticoid levels achieved during stress. However, the differences in ACTH secretion revealed in this study, together with the previous findings from Dallman's group, clearly raise an alternative hypothesis.

The integrated ACTH response to both restraint and ether was substantially higher in the NH animals than in the H animals. Maximal levels of B are achieved in response to levels of ACTH that are lower than normally occur in response to stress. However, the adrenal is known to respond not only to the magnitude of the elevation in ACTH, but also to the integrated ACTH signal (i.e., ACTH levels over time). A higher integrated level of ACTH results in a prolonged period of elevated B secretion. Thus, the differences in poststress B levels in H and NH animals might be associated with the differences in the integrated ACTH levels and not with a poststress, negative-feedback signal.

In order to test this idea, we examined plasma ACTH responses to stress in ADX, ADX+B, SHAM, H, and NH animals. First, it is important to note that there were no differences in plasma ACTH levels at any time between ADX/H and ADX/NH animals at 5 d following ADX. Thus, the removal of circulating B completely eliminated the differences in ACTH responses to stress between H and NH animals. There remains the possibility that differences between ADX/NH and ADX/H animals may have emerged had the animals been examined at a longer time following ADX. Nevertheless, the comparable ACTH responses in these animals is consistent with the idea that differences in HPA activity between H and NH animals occur in response to differences in glucocorticoid negative-feedback sensitivity. The question, then, is whether this difference in negative-feedback inhibition occurs in response to basal or stress-induced levels of B. The critical comparison here involves the ADX+B groups, where the basal B signal has been maintained, but where the negative-feedback signal associated with stress-induced increases in B has been eliminated. If the differences in poststress HPA activity between H and NH animals are dependent upon negative-feedback inhibition associated with elevated glucocorticoid levels then ADX+B/H and ADX+B/NH animals should not differ in plasma ACTH levels. This was clearly not the case. H and NH animals provided with only basal levels of B replacement, like intact animals, showed significant differences in plasma ACTH levels both during and following stress (see Fig. 3, Table 2). Thus, the difference in

poststress HPA activity between H and NH animals is not uniquely dependent upon a stress-induced elevation in plasma B levels. These results are consistent with recent studies showing that plasma ACTH levels during and following stress are tightly regulated by basal glucocorticoid levels (see Akana et al., 1992).

It is also important to note the specificity of this effect. The B replacement regimen used in this study was sufficient to attenuate greatly the ADX-induced increase in basal ACTH and plasma CBG in both H and NH animals, but altered stress-induced ACTH secretion only in the H animals. A number of previous studies have shown that in laboratory rats, B pellet replacement of this order of magnitude is sufficient to correct basal ACTH secretion (Akana et al., 1988; Jacobson et al., 1988) and CBG production (Levin et al., 1987), but not stress-induced increases in plasma ACTH; these findings are comparable to our data with ADX+B/NH rats. Indeed, the largest plasma ACTH response to restraint stress was observed in the ADX+B/NH animals (see Table 3). By contrast, basal B replacement did at least partially attenuate the ACTH hypersecretion with stress in ADX+B/H rats. The reduced ACTH secretion in response to stress in the ADX+B/H animals is consistent with previous data showing increased glucocorticoid negative-feedback sensitivity in H animals. Taken together, these data indicate that differences in HPA response to stress between H and NH animals are dependent upon the presence of glucocorticoids, but are not dependent upon stress-induced elevations in glucocorticoid levels.

These findings also suggest that negative-feedback differences between H and NH animals can occur in response to basal B levels. These differences appear to be reflected in median eminence levels of CRH and AVP (but not oxytocin). Thus, under resting-state conditions, CRH and AVP levels in the median eminence were significantly higher in the NH animals. We have recently found that hypothalamic CRH mRNA levels are about 2.5-fold higher in NH compared with H animals (P. M. Plotsky and M. J. Meaney, unpublished observations). Thus, under resting conditions, hypothalamic CRH and AVP synthesis appears to be elevated in NH rats, a difference that occurs in the presence of basal glucocorticoid levels.

The differences in median eminence levels of CRH and AVP offer an important insight into understanding the nature of the differences in glucocorticoid negative-feedback between H and NH animals. First, it should noted that H and NH animals do not differ in basal levels of ACTH or B (Meaney et al., 1989, 1992; see also Figs. 1–3). It seems likely, therefore, that the differences in CRH and AVP represent differences in readily releasable storage pools of these peptides in axon terminals located in the median eminence. The excitatory signal at the level of the paraventricular nucleus of the hypothalamus associated with stress likely results in greater CRH and AVP release in the NH animals. This, in turn, would result in a greater plasma ACTH signal. This idea is consistent with the finding that H and NH animals differ in plasma ACTH responses to a wide variety of stressors. Indeed, the differences in the terminal pools of CRH and AVP suggest that H and NH animals would differ in stressors mediated by either secretagogue. Note that pituitary ACTH responses to both restraint and ether stress appear to be mediated by dynamic variations in both CRH and AVP (Linton et al., 1985; Nakane et al., 1985; Antoni, 1986; Rivier and Plotsky, 1986; Plotsky, 1987). This hypothesis has been at least partially confirmed in one recent study. Plotsky and Meaney (unpublished observations) found that CRH release from the median eminence in response to restraint stress was significantly greater in NH compared with H rats.

It is also important to note that ADX+B animals do not differ in corticotrope sensitivity to CRH (Akana et al., 1986), and that differences in ACTH release in response to stress most likely reflect differences in neural regulatory components of ACTH secretion (Plotsky et al., 1986; also see Akana et al., 1988). This idea is also consistent with available information on the role of the hippocampus in mediating glucocorticoid inhibition of HPA activity. Hippocampal lesions result in a prolonged elevation of B following stress (Sapolsky et al., 1984). Herman et al. (1989) found that hippocampal lesions resulted in increased CRH and AVP mRNA levels in the hypothalamus under basal B conditions. Cortical lesions produced a similar, albeit less pronounced, effect. Moreover, Sapolsky et al. (1990) found that portal concentrations of CRH and AVP were negatively correlated with hippocampal glucocorticoid receptor occupancy. Interestingly, hippocampal glucocorticoid receptor occupancy was significantly correlated with resting (prestress) portal concentrations of both CRH and AVP. These findings suggest that an increased glucocorticoid receptor signal at the level of the hippocampus is associated with decreased levels of hypophysial CRH and AVP.

H and NH animals have been found to differ in glucocorticoid receptor density and this difference is observed only in the hippocampus and frontal cortex (reviewed in Meaney et al., 1991; see also earlier references). There are no differences in glucocorticoid receptor density between H and NH animals in hypothalamus, pituitary, amygdala, or septum (Meaney et al., 1985b) or in hippocampal mineralocorticoid (type I corticosteroid) receptors (Sarrieau et al., 1988; Meaney et al., 1989, 1992). The differences in hippocampal glucocorticoid receptor density are consistent with the decreased CRH and AVP content in the median eminence of the H rats (and analogous to the findings of Sapolsky et al., 1990). Note that we found substantial glucocorticoid receptor occupancy (~35–40%) in response to the B-replacement dose used in this study in both H and NH animals. Although the percentage of receptor occupancy was comparable between the two groups, the significantly increased glucocorticoid receptor density in the H rats suggests that the actual magnitude of the hippocampal glucocorticoid receptor signal was substantially higher in H animals.

On the basis of these data, it seems reasonable to propose that (1) H and NH animals differ in delayed negative feedback (Meaney et al., 1989), and that this difference is reflected in differential rates of CRH and AVP synthesis in the paraventricular nucleus of the hypothalamus; (2) that differences in negative-feedback regulation are apparent even in response to basal B signals; and (3) in response to stress there is a greater release of CRH and/or AVP in the NH animals, (4) giving rise to a greater increase in plasma ACTH levels and (5) a greater increase in plasma B levels, which persists for a longer period of time (i.e., higher poststress plasma B levels in the NH animals). Thus, differences between H and NH animals in HPA activity both during and following stress can occur independently of the stress-induced increase in plasma B.

In summary, postnatal handling during the early postnatal period leads to increased glucocorticoid receptor density in the hippocampus and is associated with enhanced negative-feedback control over HPA function. It is likely that this plasticity reflects a basic process, whereby the early environment is able to "fine tune" the sensitivity and efficiency of certain neuroen-

docrine systems that mediate the animal's response to stimuli that threaten homeostasis. Such plasticity is likely to be of considerable importance to a species like the rat, which prospers in a tremendous range of ecological niches. The differences in negative-feedback sensitivity appear to be associated with differences in CRH and AVP release during stress, and thus determine, in part, individual differences in the sensitivity of the HPA axis to stress. Under the appropriate conditions, these differences in HPA function appear to predict vulnerability to neuropathology in later life (see Meaney et al., 1988a).

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