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Persistent Hormonal Effects of Stress Are Not Due to Reduced Food Intake or Exposure to Stressed Rats

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Exposure to inescapable stress elicits persistent effects on the physiology and behavior of rats. Elevated basal plasma corticosterone concentrations have been observed for several days after cessation of stress. In this study, we measured hormonal concentrations in multiple axes at multiple levels, 24 h after one or three consecutive exposures to the same stress paradigm. The data indicated persistent activation of plasma corticosterone and prolactin concentrations, whereas plasma triiodothyronine, thyroxine, luteinizing hormone, and growth hormone concentrations were inhibited after either one or three stress sessions. In addition, we isolated the effects of restraint/tail shock *per se* from the effects of being moved and exposed to other stressed rats, and from the effects of reduced feeding produced by our stress protocol. The data clearly indicated that the stress paradigm, rather than exposure to stressed rats or decreased nutrient intake, is necessary to induce the persistent physiologic changes we observe after stressor exposures.

Key Words: Stress; pituitary; adrenal axis; growth hormone; prolactin.

Introduction

Organisms exposed to stressors undergo an enormous number of changes in physiology and behavior *(1)*. These changes appear to be adaptive in the short term but may be harmful if they persist temporally beyond the threat *(2)*. Our research has, for a number of years, focused on those persistent changes after inescapable stressor exposure, under the assumption that these are the most interesting and relevant for health *(3)*. Exposure to our standard stress procedure (40, 2-mA tail shocks delivered over a 2-h period) causes persistent changes in plasma corticosterone *(4)*.

While the hypothalamic pituitary adrenal (HPA) axis is considered one of the primary stress-responsive systems, exposure to stress also alters hormone levels within the thyroid, reproductive, and growth hormone (GH) axes. The literature has described how acute stressor exposures affect each of these axes. However, little is known of how exposure to repeated stressors might persistently alter the state of these axes. One of our recent reports *(5)* addressed this area of question. Rats were subjected to either one or three sessions of our restraint/tail shock stress procedure or served as unhandled controls. We measured plasma concentrations of corticosterone, adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), thyroxine (T_4), triiodothyronine (T_3), prolactin (PRL), and GH, as well as the reproductive hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone. In addition, we measured the pituitary content of the anterior pituitary hormones and hypothalamic corticotropin-hormone like (CRH-LI) concentrations. The data indicated that three stress sessions produced persistent activation of all aspects of the HPA axis and persistently inhibited the reproductive and thyroid axes *(5)*. However, the plasma ACTH and PRL concentrations were higher than expected, indicating that the controls were actually stressed prior to or during the sacrifice.

Features of the stressor and the stress response may account for persistent neuroendocrine abnormalities. First, rats exposed to stress vocalize and emit odors; these odors are not only stress responses but may be considered stressors in their own right. For example, exposure of conspecifics to such odors can in turn induce corticosterone responses *(6)*. Thus, the presence of these stress odors may contribute to persistent activation after inescapable stressor. Second, it is well known that exposure to intense inescapable stressors produces anhedonia *(7)*. Rats transiently decrease nutrient intake after exposure to inescapable stressors and there is an intendent decrease in body weight. The endocrine milieu 24 h after exposure to inescapable stressors may reflect the adaptive processes set in motion by decreased nutrient intake. There is considerable overlap between endocrine

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Table 1
Body and Raw Organ Weights ± SEM

Group	Body weight (g)	Adrenal weight (mg)	Pituitary weight (mg)	Hyppothalamic weight (mg)
HCC	427.9 ± 5.6	59.9 ± 2.0	12.4 ± 0.4	35.4 ± 2.0
WIT	428.8 ± 9.9	61.8 ± 2.4	11.5 ± 0.7	33.2 ± 2.3
RFC	403.2 ± 6.6[a]	60.3 ± 2.7	12.1 ± 0.6	36.1 ± 2.1
1DS	402.5 ± 9.0[a]	63.1 ± 2.9	12.1 ± 0.3	31.7 ± 2.3
3DS	379.5 ± 2.7[b]	60.8 ± 2.5	10.6 ± 0.5	36.0 ± 1.7

[a] $p < 0.05$ vs HCC.
[b] $p < 0.05$ vs HCC, WIT, RFC, and 1DS.

changes in response to restricted feeding and inescapable stressor (8). Restricted feeding is associated with elevated corticosterone *(8)* and reduced GH *(9)* and thyroid hormones *(10)*.

The current study was conducted to reexamine our prior study *(5)* with nonstress control levels, and to examine the influence of the secondary features of inescapable stressors such as exposure to stress odors and restricted feeding on the endocrine status of rats. To that end, two additional control groups were included: rats exposed to the odors/vocalizations of inescapably stressed rats, and rats whose available food was restricted to the amount eaten by inescapable stressor rats.

Results

Weights

All weight data are presented in Table 1. For body weight, analysis of variance (ANOVA) revealed a significant main effect of treatment: ($F[4, 53] = 17.0$; $p < 0.00001$). With respect to the a priori comparisons, the 3 d of stress (3DS) group weighed less than the other four groups (home cage controls [HCC], witness controls [WIT], restricted feeding controls [RFC], and 1 d of stress [1DS]; $p < 0.05$). The RFC and 1DS rats weighed less than the HCC rats ($p < 0.05$). Stressor exposures did not cause changes in adrenal weight, by using values unadjusted for body weight ($F[4, 45] < 1$). There were also no differences in either pituitary or hypothalamic weight (F's < 1).

Adrenocortical Axis

The HPA axis data are presented in Fig. 1. All pituitary hormone contents, except for ACTH, are presented in Table 2. The one-way ANOVAs showed significant effects of chronic stress on plasma corticosterone and ACTH concentrations, pituitary ACTH content, and hypothalamic CRH-LI concentrations. For plasma corticosterone concentrations, the ANOVA revealed a significant main effect of treatment ($F[4, 50] = 7.6$; $p < 0.0001$). The 3DS rats had higher corticosterone concentrations than the HCC, RFC, and WIT rats ($p < 0.05$). Also, the 1DS rats had higher corticosterone concentrations than the HCC rats ($p < 0.05$). For plasma ACTH concentrations, ANOVA also showed a significant main effect of treatment ($F[4, 49] = 8.6$; $p < 0.001$). The 3DS rats had higher plasma ACTH concentrations than the RFC and WIT rats ($p < 0.05$). For pituitary ACTH content, ANOVA revealed a significant main effect of treatment ($F[4, 20] = 3.0$; $p < 0.05$). The 3DS rats had lower levels of pituitary ACTH than the RFC rats ($p < 0.05$). For hypothalamic CRH-LI concentrations, ANOVA revealed a significant main effect of treatment ($F[4, 17] = 6.0$; $p < 0.01$). Both the 1DS and 3DS rats had higher CRH-LI concentrations than the HCC rats ($p < 0.05$).

Thyroid Axis

The thyroid axis data are presented in Fig. 2. For plasma TSH concentrations, ANOVA showed a significant main effect of treatment ($F[4, 53] = 3.1$; $p < 0.05$). Here, the RFC rats had lower concentrations than the HCC rats ($p < 0.05$). For plasma T_3, ANOVA revealed a significant main effect of treatment ($F[4, 50] = 6.7$; $p < 0.001$). The 3DS rats had lower plasma T_3 concentrations than the HCC, RFC, and WIT rats ($p < 0.05$). The 1DS rats also had lower plasma T_3 concentrations than the HCC rats ($p < 0.05$). For plasma T_4 concentrations, ANOVA revealed a significant main effect of treatment ($F[4, 50] = 14.9$; $p < 0.00001$). The 3DS group had lower T_4 concentrations than the other four groups (HCC, RFC, WIT, and 1DS; $p < 0.05$). The 1DS rats also had lower T_4 concentrations than the HCC rats ($p < 0.05$).

Reproductive Axis

The reproductive axis data are presented in Fig. 3. For plasma LH concentrations, ANOVA revealed a significant main effect of treatment ($F[4, 47] = 5.4$; $p < 0.01$). The 3DS rats had lower concentrations of plasma LH than the HCC, RFC, and WIT rats ($p < 0.05$). No significant differences were seen in plasma FSH concentrations among any of the groups. For plasma testosterone concentrations, ANOVA revealed a significant main effect of treatment ($F[4, 48] = 3.2$; $p < 0.05$). The RFC rats had lower testosterone concentrations than the 3DS rats ($p < 0.05$).

Other Hormones

The PRL and GH data are presented in Fig. 4. For plasma GH concentrations, ANOVA revealed a significant main effect of treatment ($F[4, 53] = 44.4$; $p < 0.00001$). The 3DS rats had lower concentrations of GH than the HCC rats ($p < 0.05$). In fact, all the other groups had lower plasma GH concentrations than the HCC rats (RFC, WIT, and 1DS; $p < 0.05$). For plasma PRL concentrations, ANOVA revealed a significant main effect of treatment ($F[4, 52] = 7.6$; $p < 0.0001$). The 3DS rats had higher plasma PRL concentrations than the HCC, RFC, and WIT rats ($p < 0.05$), and the 1DS rats also had higher plasma PRL concentrations than the HCC rats ($p < 0.05$).

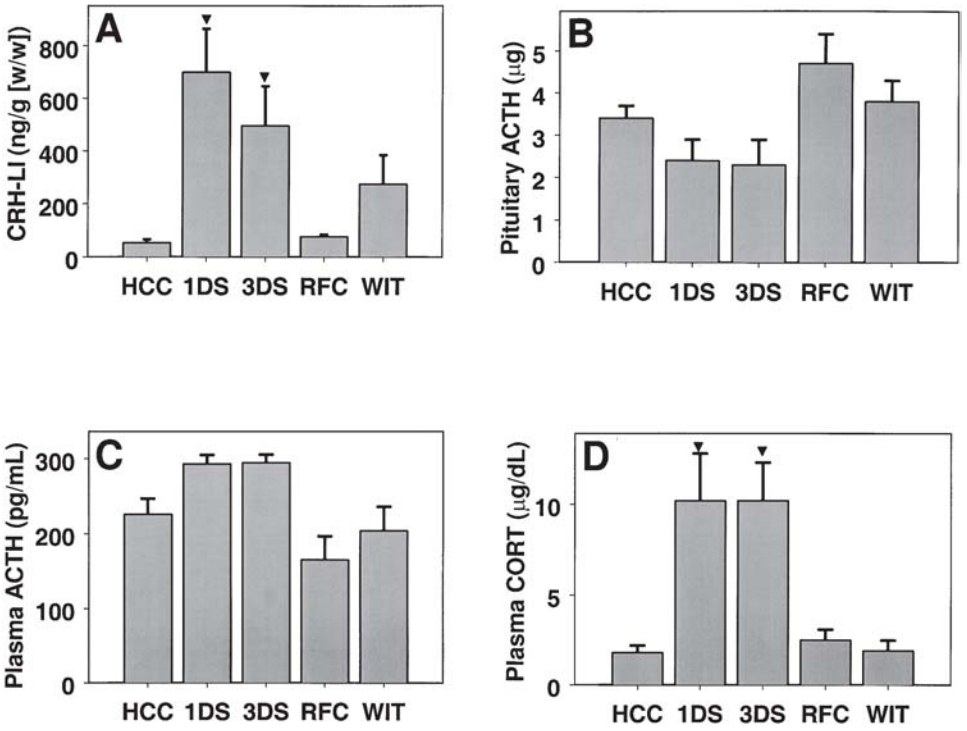

Fig. 1. Effect of stress, witnessing stress, and restricted feeding on HPA axis hormones. (**A**) Hypothalamic CRH; (**B**) pituitary ACTH; (**C**) plasma ACTH; (**D**) plasma corticosterone (CORT). ▼, Significant difference ($p < 0.05$) between the stress group indicated and the HCC group.

Table 2
Pituitary Levels of Hormones (μg) ± SEM with (*n*)

Group	LH	FSH	TSH	GH	PRL
HCC	46.7 ± 1.9 (6)	28.9 ± 2.2 (6)	3.7 ± 0.1 (6)	209.1 ± 20.5 (6)	2.1 ± 0.3 (6)
WIT	55.6 ± 4.5 (6)	27.7 ± 2.2 (6)	4.8 ± 0.4 (5)	208.4 ± 10.6 (5)	2.7 ± 0.3 (5)
RFC	55.6 ± 3.9 (6)	40.9 ± 4.4 (6)	6.4 ± 0.9 (6)[a]	275.8 ± 33.5 (6)	3.1 ± 0.5 (6)
1DS	47.1 ± 3.5 (6)	33.1 ± 1.0 (6)	4.6 ± 0.2 (6)	320.4 ± 32.7 (6)	2.9 ± 0.5 (6)
3DS	61.6 ± 13.9 (3)	51.5 ± 21.7 (3)	5.4 ± 0.8 (3)	271.4 ± 60.6 (3)	6.9 ± 2.4 (3)[b]

[a] $p < 0.05$ vs HCC.
[b] $p < 0.05$ vs HCC, RFC, WIT, and 1DS.

Discussion

A primary goal of the present study was to examine the persistent endocrine changes observed 24 h after cessation of an inescapable stressor. Consistent with our earlier work *(5)*, exposure to a single session of inescapable stress resulted in elevated plasma corticosterone, ACTH, and PRL concentrations and suppressed thyroid hormone concentrations. However, the control plasma ACTH and PRL concentrations given herein are consistent with nonstress levels in contrast to the stress levels of our earlier study *(5)*. As in the earlier study, the endocrine status of rats exposed to three sessions of inescapable stressor was similar to that for rats given a single session; adaptation to inescapable stressor was not apparent.

A major strength of the present study is that we simultaneously measured hormone concentrations in multiple axes at multiple levels within each axis in the same rats. However, there are limitations to interpreting hormone results from only one blood collection. These animals were decapitated to obtain enough blood to measure all the hormonal axes at the same time as well hypothalami and pituitaries. Sequential bleeding from these animals may have revealed differences in episodic or diurnal hormone secretion.

In contrast to our earlier study *(5)*, persistent changes within the reproductive axis were not as pervasive. In that study *(5)*, stressed rats had lower plasma concentrations of testosterone, LH, and FSH. In the present study, only plasma LH concentrations were suppressed in 3DS rats. Failure to observe suppressed plasma testosterone concentrations after exposure to stress may have been owing to the lower concentrations observed in the HCC group. Over all groups, testosterone concentrations were approx 50% lower than in our earlier study *(5)*. However, this unexpected lack of

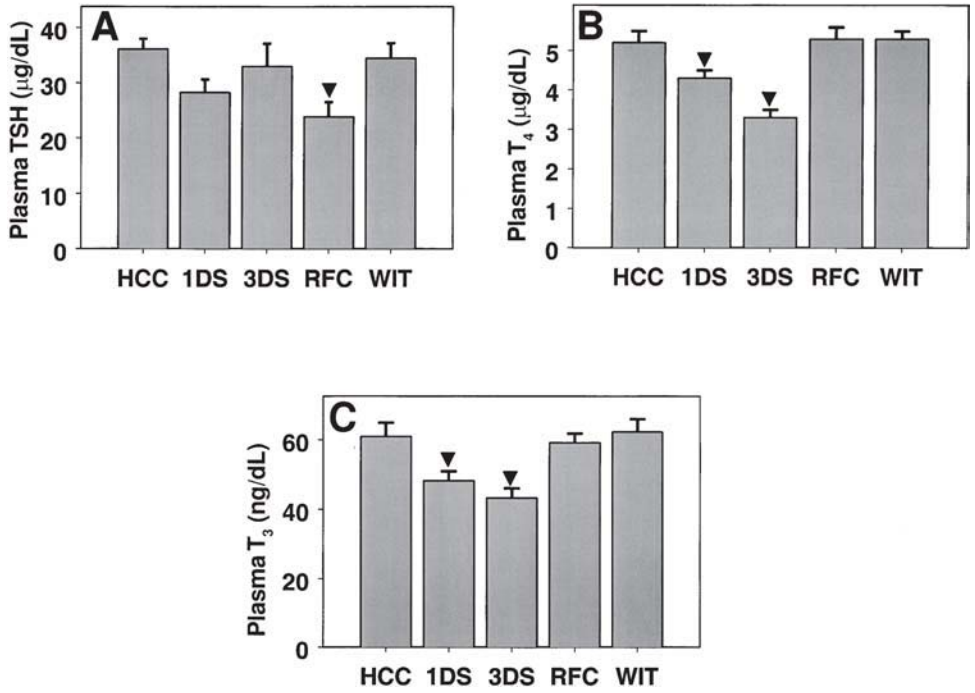

Fig. 2. Effect of stress, witnessing stress, and restricted feeding on thyroid axis hormones. (**A**) plasma TSH; (**B**) plasma T_3; (**C**) plasma T_4. ▼, Significant difference ($p < 0.05$) between the stress group indicated and the HCC group.

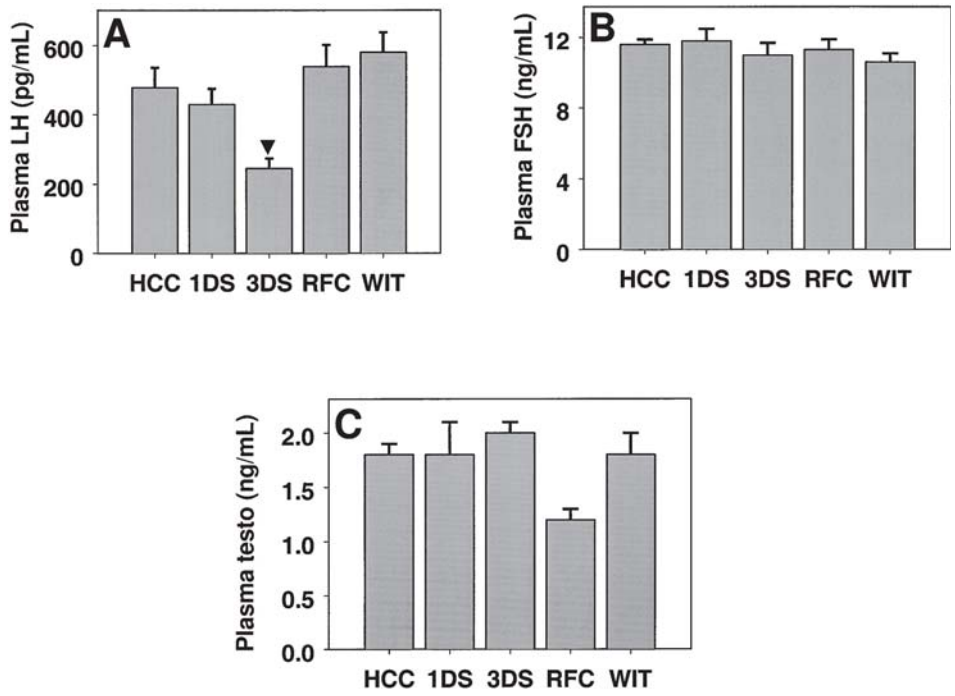

Fig. 3. Effect of stress, witnessing stress, and restricted feeding on reproductive hormones. (**A**) plasma LH; (**B**) plasma FSH; (**C**) plasma testosterone (testo). ▼, Significant difference ($p < 0.05$) between the stress group indicated and the HCC group.

suppression of testosterone is inconsistent with the findings of other studies *(5,11,12)* and currently lacks a solid explanation. The pattern for LH and FSH fit the literature *(13)*. In general, exposure to stress results in suppressed plasma LH; plasma FSH may also be suppressed, but the magnitude of such changes were smaller than that observed for LH *(13)*. Taken together, the data indicate that persistently suppressed plasma LH concentrations is the most consistent finding among the peripheral gonadotropic hormones.

Regarding plasma GH concentrations, the data presented herein are consistent with data found in the literature *(13, 14)* where stress has reduced plasma GH levels. However, in

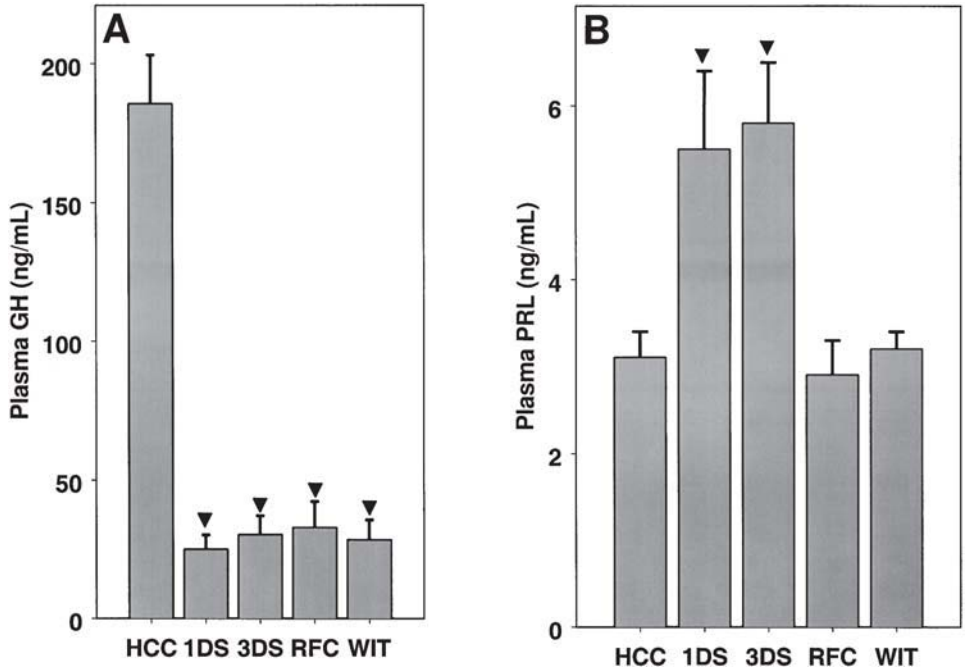

Fig. 4. Effect of stress, witnessing stress, and restricted feeding on plasma GH and PRL. (**A**) plasma GH; (**B**) plasma PRL.▼, Significant difference ($p < 0.05$) between the stress group indicated and the HCC group.

our earlier study *(5)*, we failed to show this inhibition, perhaps because the control concentrations were low.

The present study was designed to evaluate two features of the stress response and their possible role in producing persistent endocrine abnormalities. Of the plasma hormones measured, exposure of conspecifics to stressed rats resulted only in suppressed plasma GH. Previous work found persistent adrenocortical activation in conspecifics, but exposure was more frequent and of longer duration *(15)*.

We also included a control group for the self-restricted feeding of stressed rats. Restriction of feeding for 3 d, to a level comparable to that for stressed rats, did result in lower body weights. However, restriction did not result in reductions in body weight comparable with that of rats stressed for 3 d. This finding supports our contention that a reduction in food intake is not the sole reason that rats exposed to multiple stress sessions lose weight *(15)*. Regarding organ weight, stress did not cause any change in adrenal, pituitary. or hypothalamic weight. This reinforces the caveat that using adjusted organ weight values in rats that lose weight has inherent interpretational flaws. Although other studies have reported changes in adrenal weight after stress *(16)*, those studies have typically analyzed values adjusted for body weight. Our data seem to suggest that stress, at least relatively short-term stress, does not alter the weight of the adrenal, pituitary, and hypothalamus.

Restricted feeding altered the basal hormone concentrations of rats. Consistent with the findings of Marti et al. *(14)*, restriction of feeding resulted in suppressed plasma TSH; however, it did not affect plasma T_3 and T_4 concentrations. Contrary to expectation *(8)*, feeding restriction did not affect plasma corticosterone concentrations. Plasma PRL concentrations were also unaffected by food restriction, although suppressed plasma GH concentrations were evident. Together, these hormonal patterns suggest that the food restriction regimen, although not a chronic stressor, has metabolic consequenecs evident in the thyroid and GH axes.

Overall, exposure to repeated stressors produced persistent hormonal abnormalities similar in degree and breadth as those apparent after a single stress session. Persistent activation of the HPA axis, elevated plasma PRL concentrations; and inhibition of plasma T_3, T_4, and LH concentrations were not secondary to reduced feeding or chronic exposure to stress-related odors/vocalizations. Plasma GH concentrations appear to he sensitive to even mild stressors, an assertion that agrees with other data *(12)*. The present study provides the basic information for the effect of stress on persistent changes in multiple hormonal axes at multiple levels in these axes. Future work will determine the nature of interaxes interactions in the development and maintenance of a chronic stress state.

Materials and Methods

Subjects

Sixty male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA) and housed individually in shoebox cages. The rats were 80 d old and weighed about 375 g. They were maintained on a 12-h light:12-h dark cycle with lights on at 7:00 AM. Purina rat chow and water were

available ad libitum except for the RFC. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Medical Center. Body weight changes and plasma cholesterol levels from this experiment have been reported previously *(17)*.

Design and Procedure

After 2 wk under these conditions, rats were divided into three cohorts run on three successive weeks. Rats were stratified by body weight and then randomly assigned to one of five groups. As in our prior report *(5)*, rats were exposed to either 3 d of the stress regimen (3DS group), exposed to 1 d of the stress regimen (1DS group) on the last day of stress for the first group, or remained home cage controls (HCC group). We added two additional groups to the present study. One group of rats was transferred to the stress room for the 3 d of stressor exposure for the 3DS group (witness controls or WIT group). Another group of rats had their feeding restricted (RFC group) because exposure to the stress regimen results in a loss of both food intake and body weight *(5)*. The RFC group was given the average amount of food that 3DS rats ate in previous experiments *(15)*. Food restriction was accomplished in this manner, as opposed to a direct yoking procedure, to ensure that the timing of sacrifice was the same across groups. Food intake in the RFC and 3DS groups was not different and averaged approx 20 g/d over the 3 d of stress *(17)*.

The stress regimen has been previously described in detail elsewhere *(3)*. Briefly, stress sessions began at 9:00 AM when rats were moved into the laboratory from their colony room. The 1DS animals were exposed to one 2-h session and the 3DS animals were exposed to one 2-h session each day for 3 d. Each 2-h stress session consisted of loose restraint in hardware cloth tubes and the delivery of 40, 2-mA tail shocks (on for 166 of 200 ms during each 3-s shock). The amperage of the shocks was verified throughout the stress session, and the shocks were delivered on a variable interval schedule.

Trunk blood was obtained via decapitation 22 h after the end of the last stressor exposure (9:00 AM). Five milliliters of blood was collected into polypropylene tubes on ice containing Na_2-EDTA and Trasylol through heparinized funnels, and then centrifuged at 4°C, and the plasma was stored frozen at −80°C until assayed. The medial basal hypothalami and pituitaries were rapidly dissected, weighed, and frozen at −80°C. The hypothalami were extracted with 10 vol of boiling water, whereas pituitary samples were extracted with 100 vol of 0.9% NaCl at 40°C for all pituitary hormones except ACTH in which the extract was boiled *(18)*. These samples were chilled to 4°C and then centrifuged at 2000*g* for 30 min at 4°C. The hypothalamic supernatants were removed and stored frozen at –80°C until assayed for CRH-LI, whereas the pituitary extracts were assayed for ACTH, TSH, PRL, GH, FSH, and LH.

Radioimmunoassay of CRH-LI

Radioimmunassay (RIA) for CRH-LI was performed as previously described, with CRH as the standard *(19)*. The antibody to h(rat) CRH was purchased from IgG Corporation (Nashville, TN), and iodinated CRH was purchased from Amersham (Arlington, IL). The assay sensitivity was 1 pg/tube, and the intraassay variability was 8%.

RIA of ACTH

Plasma samples for the ACTH assays were first extracted using Quso G-32 and eluted with acidified acetone. Then the ACTH RIA was performed as previously described *(19)*. Briefly, the antibody to ACTH was purchased from IgG Corporation, and iodinated ACTH was purchased from Amersham. Synthetic ACTH 1-39 was used as the standard and for labeling. The assay sensitivity variability was 1 pg/tube, and the aliquot size for the assay was 50 µL, and the intraassay variability was 6%.

RIA of Corticosterone

Plasma corticosterone was assayed using a double-antibody RIA kit (RSL ^{125}I Corticosterone Kit no. 07-120102; ICN, Carson, CA). The assay was performed as previously described *(17)*. The minimum detectable concentration was 0.15 µg/dL, and the aliquot size was 5 µL, and the intraassay variability was 7.0%.

RIAs of Thyroid Axis Hormones, Reproductive Axis Hormones, GH, and PRL

Plasma TSH, LH, FSH, GH, and PRL levels were assayed directly using a double-antibody RIA with reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). NIDDK-rTSH-RP-2 was used as the standard for TSH, NIDDK-rFSH for FSH, LH-RP2 for LH, rGH for GH, and R-Prolactin-RP-2 for PRL. The assay sensitivities were 50 pg/tube for pituitary TSH, 2 µg/dL for plasma TSH, 10 pg/tube for LH, 20 pg/tube for FSH, 50 pg/tube for GH, and 10 pg/tube for PRL. The aliquot sizes for the assays were 25 µL for TSH, 100 µL for LH, 100 µL for FSH, 25 µL for GH, and 25 µL for PRL. The intraassay variability was 5.4% for TSH, 2.2% for LH, 4.2% for FSH, 10.5% for GH, and 8% for PRL. Plasma T_4 and T_3 were measured using coated-tube, double-antibody RIA kits for total T_3 and T_4 from ICN as described previously *(17,20)*. The minimum detectable levels were 0.1 µg/dL for the T_4 assay and 0.1 ng/dL for the T_3 assay. The aliquot sizes for the assays were 25 µL for T_4 and 100 µL for T_3, and the intraassay variability was 4.7% for T_4 and 3.2% for T_3. Plasma testosterone levels were assayed after ether extraction using antiserum supplied by ICN as described previously *(21)*. The minimum detectable level was 0.1 ng/mL. The aliquot size for the testosterone assay was 100 µL, and the intraassay variability was 5.0%.

RIA data reduction for all the assays was performed on an IBM PC using 4-parameter log-logit curvefit software

(M.L. Jaffe and Associates, Jupiter, FL). All samples for a hormone were run in the same assay and in duplicate.

Statistical Analyses

We performed separate ANOVAs for each of the dependent measures. Group and cohort were both factors in the analysis. Cohort was analyzed but not evaluated in any model. Consistent with the experimental design, we formulated *a priori* comparisons between groups. The seven *a priori* comparisons were HCC vs the other four groups, and 3DS vs the three other groups. Comparisons between the HCC and other groups were analyzed with Dunnet's multiple comparison tests for *a priori* comparisons involving a control mean. Comparisons between the 3DS group and the other groups were done with Dunn's tests. For all planned comparisons, 0.05 was used as the significance criterion.

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