

Research report

Long-term changes in mineralocorticoid and glucocorticoid receptor occupancy following exposure to an acute stressor

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Accepted 24 August 1999

Abstract

Stressors produce rapid activation of the hypothalamic–pituitary–adrenal axis, which typically resolves within 60–90 min following termination of the stressor. In addition, some stressors such as inescapable tailshock (IS) also produce elevated basal levels of corticosterone (CORT), and reduced serum levels of corticosteroid binding globulin (CBG). The elevated basal levels of CORT produced by IS are only observed at the trough of the circadian rhythm of CORT secretion, and are sustained for 2–3 days following stressor termination. The goal of the following experiments was to determine the extent to which the elevated basal levels of CORT observed following IS exposure produced greater corticosteroid receptor occupancy in the brain and pituitary. To do so, rats ($n = 8–10$ per group) received either sham or bilateral adrenalectomy (with CORT replacement in their drinking water; 25 $\mu\text{g}/\text{ml}$) and were given 3 days to recover. Rats were then exposed to 100 ISs (1.6 mA, 5 s each) administered on a 60 s variable intertrial interval, or remained in their home cages. As seen previously, IS produced an increase in basal CORT (5 $\mu\text{g}/\text{dl}$) and a decrease in CBG (30% decrease). Rats were sacrificed 24 h following IS for trunk blood samples and brain dissections. IS exposure had very little effect on corticosteroid receptor protein expression as determined by mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) binding levels in ADX rats. In addition, no changes in whole cell GR levels (as detected by Western blot) were observed in sham rats exposed to IS. On the other hand, IS exposure led to greater occupancy of MR (ranging from 25%–50%) in hippocampus, hypothalamus, pituitary, and posterior cortex. IS also produced greater occupancy of GR (approximately 20%) in hypothalamus and posterior cortex. These long-term changes in corticosteroid receptor activation, evident 24 h after IS exposure, may be responsible for some of the long-term neural, behavioral and immune changes observed following this acute stress procedure. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Corticosterone; Corticosteroid receptor; Western blotting; Adrenalectomy; Rat

1. Introduction

The acute hypothalamic–pituitary–adrenal (HPA) axis response to stressors usually dissipates within 60–90 min following the termination of the stressor. However, some stressors also produce long-term elevations in basal levels of corticosterone (CORT) [8,20,21,30,39]. These elevated basal levels of CORT are generally only observed at the trough of CORT's circadian rhythm, typically constitute a 5–15 $\mu\text{g}/\text{dl}$ change from normal baseline values, and can persist for several days following stressor termination. Moreover, some of these same stressors also reduce plasma levels of corticosteroid binding globulin (CBG; also re-

ferred to as transcortin) [2,41], the carrier protein for CORT [47]. Since 90% of circulating CORT is bound to CBG under basal conditions [47], and CORT cannot bind to its intracellular receptors while bound to CBG [31], the bioactivity of CORT is at least in part regulated by circulating levels of CBG. Thus, increased basal levels of CORT would be expected to have a much larger impact on target cells when CBG levels are diminished than when CBG levels are normal [28].

The effects of CORT are mediated by two high affinity intracellular receptors. These two receptors are referred to as mineralocorticoid receptors (MR; or Type I receptors) and glucocorticoid receptors (GR; or Type II receptors). The affinity of MR ($K_d = 0.5–1$ nM) for CORT is greater than that of GR ($K_d = 5–10$ nM), which leads to greater occupancy of MR under basal CORT conditions [33,42].

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Although unoccupied MR and GR receptors are predominantly located in the cytoplasm [6,35] until they become activated by CORT, there is also some evidence for unoccupied MR and GR in the nucleus [4,45]. Nevertheless, activation of cytoplasmic receptors leads to rapid receptor translocation to the nucleus where the receptor acts as a hormone-activated transcription factor [13]. Thus, the number of cytoplasmic CORT receptors reflects the number of unoccupied CORT receptors, while the number of nuclear CORT receptors reflects the number of occupied/activated CORT receptors. The relative proportion of occupied receptors in the rat given different circulating levels of CORT has been well characterized [33,42,44].

We have previously reported that inescapable tailshock (IS) is one such stressor that produces both elevated basal levels of CORT and reduced plasma levels of CBG [9,15]. Furthermore, the increase in basal CORT and the concomitant decrease in plasma CBG led to a fourfold increase in the amount of “free” CORT observed in plasma [15]. Although it is expected that elevated basal levels of CORT, especially in the face of reduced CBG, would occupy more receptors in the brain, it is impossible to predict the relative proportion of MR and GR occupancy under such conditions. The majority of MR in the brain are occupied by CORT at all times because of the high affinity of this receptor for CORT. Nevertheless, in the morning, at the trough of the circadian pattern of CORT secretion, a significant proportion of MR in the brain are unoccupied (10%–30%) [33,42]. In the pituitary an even higher proportion of MR are unoccupied at this time of day [42], probably as a result of the buffering effects of a high local concentration of CBG [11,12]. Due to the lower affinity of GR for CORT, in the morning virtually all of the GR in the pituitary and the brain are unoccupied by CORT [33,42]. Thus, one primary aim of this study was to examine the impact of the IS-induced elevation of basal levels of CORT and reduced CBG levels on MR and GR occupancy in brain and pituitary.

A second aim of this study was to determine whether IS exposure would produce a change in MR or GR protein expression in brain or pituitary. Several chronic stress paradigms have been shown to reduce MR and GR mRNA [18,25] or receptor binding levels in the rat brain [14,36]. Many of these effects are dependent upon the sustained high levels of CORT that are produced during the stressor. Importantly, IS exposure produces a peak plasma CORT response (typically 40–50 $\mu\text{g}/\text{dl}$) that is comparable in magnitude to other stressors which are known to downregulate MR and GR expression (see Ref. [10] for a recent characterization). Thus, we sought to determine whether an intense acute stressor, such as IS, is able to alter MR or GR levels. Theoretically, a downregulation of MR or GR could account for the elevated basal levels of CORT observed 24–48 h after IS exposure.

In order to assess whether IS produced changes in either corticosteroid receptor expression or occupancy we have

utilized a combination of corticosteroid receptor measurement strategies. By using a cytosolic corticosteroid receptor binding assay we can measure the number of available (unoccupied) MR and GR present in brain and pituitary at the time of animal sacrifice [33,44]. By comparing the number of available receptors in tissue of adrenal-intact rats to those of adrenalectomized rats we can make inferences about whether differences in available receptor levels reflect differences in receptor expression or receptor occupation. To further assess the possibility of IS induced changes in GR protein expression we have used a whole cell Western blot procedure. This Western blot procedure has the advantage over the receptor binding assay of being able to determine corticosteroid receptor protein expression levels in the adrenal-intact rat. We were not able to use this procedure to measure MR protein expression because the low levels of MR present in adrenal-intact rats are below the detection limits of our Western blot procedure.

2. Materials and methods

2.1. Animals

Adult male viral-free Sprague–Dawley rats (350–400 g; Harlan Labs.) were individually housed in suspended wire cages (24.5 cm \times 19 cm \times 17.5 cm) with food and water available ad libitum. Colony conditions were maintained at 22°C on a 12:12 h light:dark cycle (lights on 0700–1900 h). Rats were given at least two weeks to habituate to the colonies prior to experimentation. Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

2.2. Experiment 1 — IS effects on available corticosteroid receptors

Thirty eight rats ($n = 8$ –10 per group) were randomly assigned to either adrenalectomy-home cage control (ADX-HCC), adrenalectomy-inescapable shock (ADX-IS), Sham-IS, or Sham-HCC groups. Bilateral ADXs were aseptically performed under halothane anesthesia (Halocarbon Laboratories). Sham-operated animals followed the identical procedure except that the adrenal glands were gently manipulated with forceps but not removed. Steroid replacement began for ADX animals immediately after surgery. The ADX rats served as a means to determine whether there was a change in MR or GR protein expression 24 h after IS. By insuring that no CORT was present in the ADX rats at the time of sacrifice, the available corticosteroid receptor binding levels represent total cellular receptor levels [34,44]. It was important, however, to maintain in these ADX rats a normal circadian pattern of basal CORT exposure to prevent the upregulation of GR

that is observed with long-term ADX [43]. Thus, ADX rats were given CORT replacement in their drinking water until the night before sacrifice. This replacement method has been shown to mimic the normal circadian pattern of CORT secretion [19], thereby eliminating potential neurochemical alterations that occur in the absence of basal CORT replacement (e.g., Refs. [5,24]). CORT was initially dissolved in ethyl alcohol (EtOH) and diluted to a final concentration of 25 $\mu\text{g}/\text{ml}$ in 0.2% EtOH, 0.5% saline. Sham animals received drinking water containing 0.2% EtOH.

After 3 days of recovery, IS rats were transported to an adjacent room and placed in a Plexiglas restraining tube (15×7 cm) where 100 tailshocks (1.6 mA, 5 s each, variable inter-trial interval 60 s, range 30–90 s) were administered. The duration of the IS procedure was approximately 2 h, and IS was administered during the AM (1000–1200 h). HCC rats remained undisturbed in their home cages during this time. Since IS rats drink a large bolus of water in the first few hours following termination of the IS session (unpublished observations), all ADX rats were given 0.5% saline/0.2% EtOH water until lights out. ADX rats were then given CORT water (described above) for 2 h, then were given saline until lights on the next day. CORT water was available to rats for only 2 h during the evening before sacrifice in order to prevent neurochemical alterations which are typically observed following ADX, and to ensure that any exogenous CORT would be cleared prior to decapitation.

Twenty-four hours after IS, rats were killed by rapid decapitation and trunk blood was gathered in heparinized tubes. Brain tissues were dissected and frozen immediately at -70°C until the time of assay. These procedures were conducted rapidly and quietly in an adjacent room in order to minimize stress to the animals.

2.3. Experiment 2 — IS effects on whole cell GR

In the first experiment, the effect of IS on available cortico-steroid receptor binding was measured. By comparing available receptor levels in ADX rats (total receptor levels) to those of adrenal-intact rats (unoccupied receptor levels) inferences can be made about the effect of IS-induced elevated basal CORT levels on relative MR and GR occupation. However, those inferences are dependent on the assumption that any effect that IS may have on corticosteroid receptor protein expression would be equivalent between ADX and adrenal-intact rats. In this second experiment, we used a whole-cell Western blot procedure to directly measure GR protein expression in adrenal-intact rats 24 h after IS. The experimental procedure was identical to the first experiment with the exception that only adrenal-intact rats were used.

Rats ($n = 9$ – 10 per group) were randomly assigned to either HCC or IS treatment. IS was administered as described above. Twenty-four hours following IS termina-

tion, all rats were killed by rapid decapitation and brains were dissected and frozen. Trunk blood samples were collected for the measurement of CORT.

2.4. Assays

2.4.1. CORT

Total plasma CORT levels were measured by radioimmunoassay using rabbit antiserum (antibody B21–42; Endocrine Sciences, Tarzana, CA). This antiserum has very low cross reactivity with other glucocorticoids and their metabolites. The assay sensitivity was 0.5 $\mu\text{g}/\text{dl}$ (assay volume = 20 μl plasma). Both the intraassay and interassay coefficients of variation were less than 9%.

2.4.2. CBG

Plasma CBG levels were assessed using a competitive binding assay adapted from Ref. [47]. The samples were initially diluted 1:200 in buffer (pH = 8.0) consisting of 10 mM Trizma base, 1.0 mM EDTA, 10% Glycerol (v/v), and 1.0 mM dithiothreitol. The diluted sample was then mixed with a saturating concentration of ^3H -CORT (15 nM) \pm unlabelled CORT (10 μM) at a final dilution of 1:600 and allowed to incubate over night at 4°C . Bound and unbound steroids were separated using activated charcoal (executed in duplicate). The bound fraction was mixed with scintillation cocktail and counted with a liquid scintillation counter (TriCarb 1600TR, Packard, Meriden, CT). Data were expressed as nmol specific ^3H -CORT binding/ μl serum. Since one CORT molecule binds per CBG molecule, this is equivalent to nmol CBG/ μl serum.

2.4.3. Cytosolic corticosteroid receptors

Corticosteroid receptors were measured in the cytosolic fraction of brain homogenates as previously described by Spencer et al. [44]. Homogenization and incubation buffer consisted of 10 mM Tris, 20 mM molybdic acid, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerin (v/v) in distilled water (pH = 7.4 at 4°C). Brain structures were homogenized in 500 μl of buffer with a motor driven Teflon pestle and centrifuged for 30 min at $100,000 \times g$ to generate cytosol. The supernatant containing the soluble tissue fraction (cytosol) was used for subsequent receptor binding measurements. Cytosol from individual tissue samples were incubated overnight at 4°C in the presence of a saturating (20 times K_d) concentration of ^3H -dexamethasone (15 nM) \pm competitor. Specific binding of ^3H -dexamethasone to GR was determined by the amount of total binding displaced by the selective GR ligand, RU28362 (0.5 μM) [7]. Specific binding of ^3H -dexamethasone to MR was determined by subtracting the amount of binding displaced by unlabelled dexamethasone (10 μM) from the amount that was not displaced by RU28362.

The macromolecular bound fraction of ^3H -dexamethasone was collected by gel filtration over mini LH-20 Sephadex columns (in triplicate). The bound fraction was

mixed with scintillation cocktail and counted with a liquid scintillation counter. Final specific binding was expressed as fmol/mg cytosolic protein. Protein determinations were made with the Bradford method [3], using bovine serum albumin as the protein reference.

2.4.4. Western blotting for whole cell GR

Frozen brain parts were individually sonicated in a 50 mM Tris buffer (pH 7.2, 4°C) containing 6 mM MgCl₂, 1 mM EDTA, 10% (wt/vol) sucrose, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamide, 1 mM leupeptin, 1 μg/ml of pepstatin, 1 μg/ml antipain, 1 μg/ml aprotinin, and 1 μg/ml of soybean trypsin inhibitor. SDS was not included in the sonication buffer because pilot studies in our laboratory have demonstrated that sonication alone is sufficient to release both cytoplasmic and nuclear fractions containing GR (unpublished observations). Homogenates were ultracentrifuged (105,000 × *g*) and the resulting supernatants from each sample were adjusted to a final protein concentration of 4 μg/μl (DC protein assay, Bio-Rad). Supernatants were mixed with Laemmli's sample buffer and boiled for 5 min. Samples (50 μg) were loaded onto 8% Tris–glycine acrylamide gels (Novex) and separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were electrophoretically transferred from gels to PVDF membrane. GR protein was detected on PVDF blots by the monoclonal antibody, BUGR2 (Affinity BioReagents, Golden, CO). Immunopositive bands were visualized by a chemiluminescent method

(ECL, Amersham). The optical density of GR reactive bands (approximately 97 kDa) visible on X-ray film were measured with an image analysis system using NIH Image-ware. Data are expressed as mean optical density.

2.5. Statistics

In Experiment 1, water consumption data, plasma levels of CORT and CBG, and receptor binding data for each brain region and receptor type were analyzed using a 2 × 2 (Sham and ADX vs. HCC and IS) analysis of variance (ANOVA). Individual *t*-tests were utilized to examine post-hoc differences between HCC and IS groups only where reliable interactions were previously established by ANOVA. In Experiment 2, all data were analyzed using a single factor (HCC vs. IS) ANOVA design.

3. Results

3.1. Experiment 1 — IS effects on available corticosteroid receptors

3.1.1. IS effects on water consumption

Since rats frequently drink a large quantity of water in the first few hours after IS exposure (unpublished observations), all ADX rats were given 0.5% saline/EtOH water during this period. As expected, rats exposed to IS drank significantly more water in the afternoon following IS

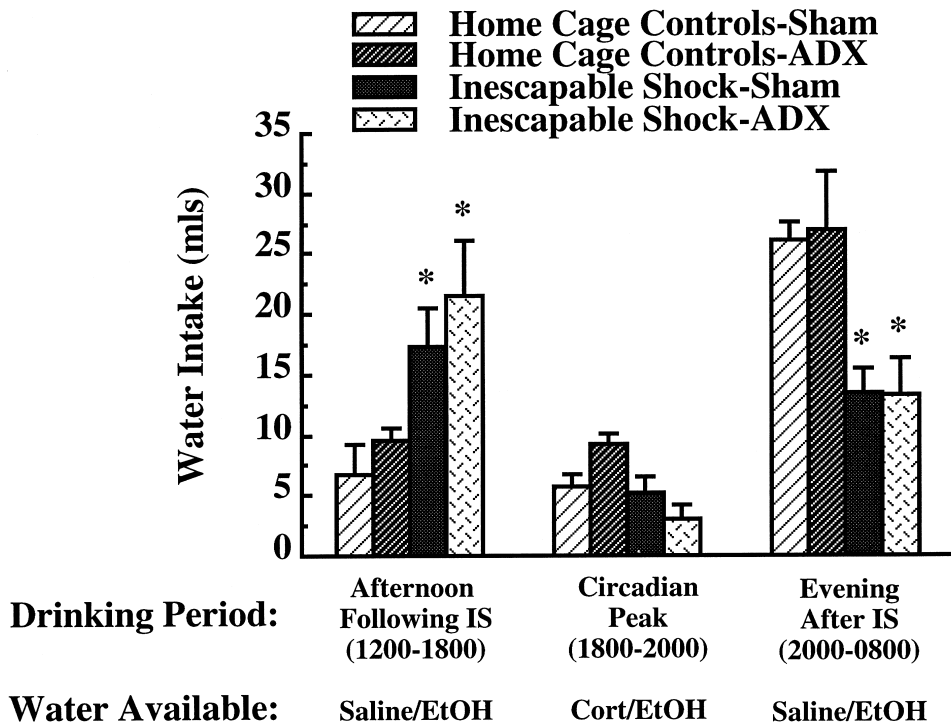


Fig. 1. IS alters drinking patterns. Rats exposed to IS drink significantly more than HCC rats for the first few hours immediately following the shock session. However, during the dark cycle on the night following IS, IS rats drink significantly less than HCC rats.

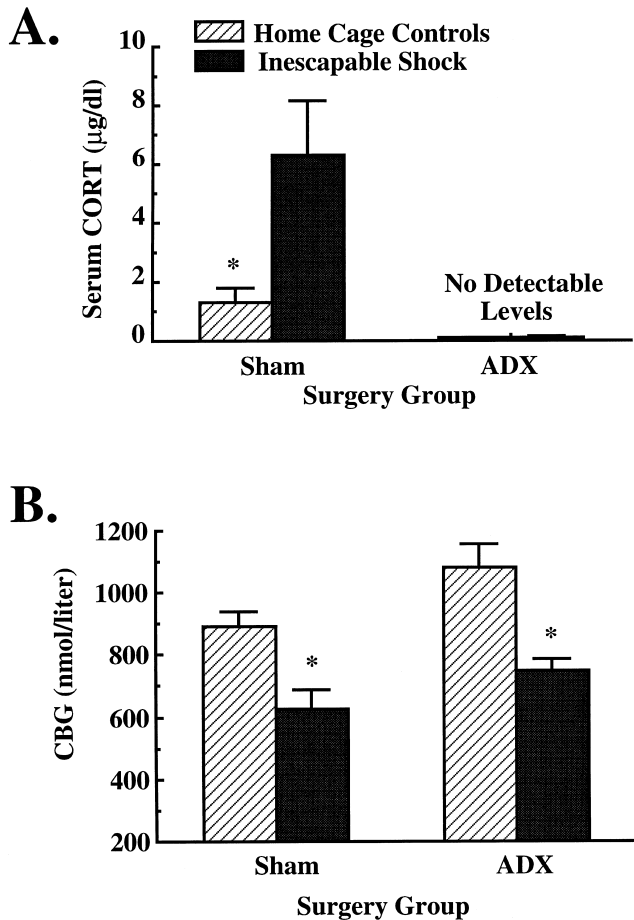


Fig. 2. Basal CORT levels 24 h after IS. (A) IS exposure led to elevated basal levels of CORT during the circadian trough of CORT secretion. This effect typically persists for 2–3 days (see Ref. [15] for a complete time-course). No CORT was detectable in the serum of adrenalectomized rats. (B) IS also significantly decreased serum levels of CBG. The effects of IS on serum CBG were not affected by adrenalectomy.

treatment [$F(1,34) = 12.7$, $p < 0.01$]. However, no reliable differences were observed in water consumption during the first 2 h after lights out. Furthermore, IS treated rats drank significantly less overnight compared to HCC rats [$F(1,34) = 17.9$, $p < 0.0001$]. No effects of adrenalectomy were observed in the overnight water consumption. We collected and presented these data as a rationale for why ADX rats were given saline/EtOH water to drink in the first few hours after IS. For ease of presentation, all water consumption data are presented in a single graph (Fig. 1).

3.1.2. Plasma CORT and CBG

Plasma levels of CORT were measured in blood samples collected 24 h after IS. A significant interaction between stress condition and surgical manipulation was observed in plasma CORT [$F(1,32) = 6.8$, $p < 0.05$]. The completeness of the ADX surgery was verified by levels of CORT that were undetectable or at the floor of assay sensitivity. In contrast, basal levels of CORT were detected in Sham-HCC rats, and exposure to IS significantly

increased basal levels of CORT 24 h following stressor termination (Fig. 2A). Exposure to IS also significantly reduced plasma CBG levels [$F(1,32) = 16.9$, $p < 0.001$]. However, no reliable interaction between stress condition and surgical manipulation was observed. Thus, ADX had no effect on the IS-induced reduction in serum CBG (Fig. 2B).

3.1.3. ADX effects on MR and GR binding levels

The number of unoccupied corticosteroid receptors was assessed by measuring cytosolic MR and GR binding levels. Significant main effects of surgical condition were observed in MR binding levels in all brain regions examined. That is, higher levels of cytosolic MR binding were observed in ADX rats than sham-operated controls in hippocampus [$F(1,34) = 202.2$, $P < 0.001$], hypothalamus [$F(1,34) = 59.8$, $P < 0.001$], pituitary [$F(1,34) = 41.9$, $P < 0.001$] and posterior cortex [$F(1,34) = 157.2$, $P < 0.001$].

Similar effects were observed in GR binding levels of ADX rats. Higher cytosolic GR levels were observed in the hippocampus [$F(1,34) = 28.0$, $P < 0.001$], hypothalamus [$F(1,34) = 58.9$, $P < 0.001$], and posterior cortex [$F(1,34) = 20.8$, $P < 0.001$] of ADX rats. However, no main effect of ADX was observed in pituitary GR binding.

SHAM

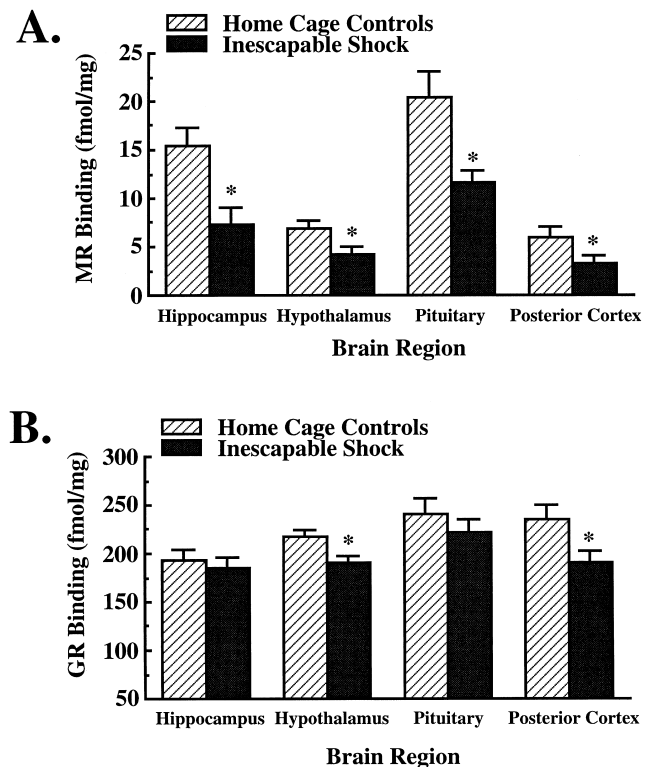


Fig. 3. Number of available CORT receptors in sham rats 24 h after IS. The elevated basal levels of CORT produced by IS exposure led to fewer available MRs in all brain regions examined. Similar effects were also observed in GRs in the hypothalamus and posterior cortex.

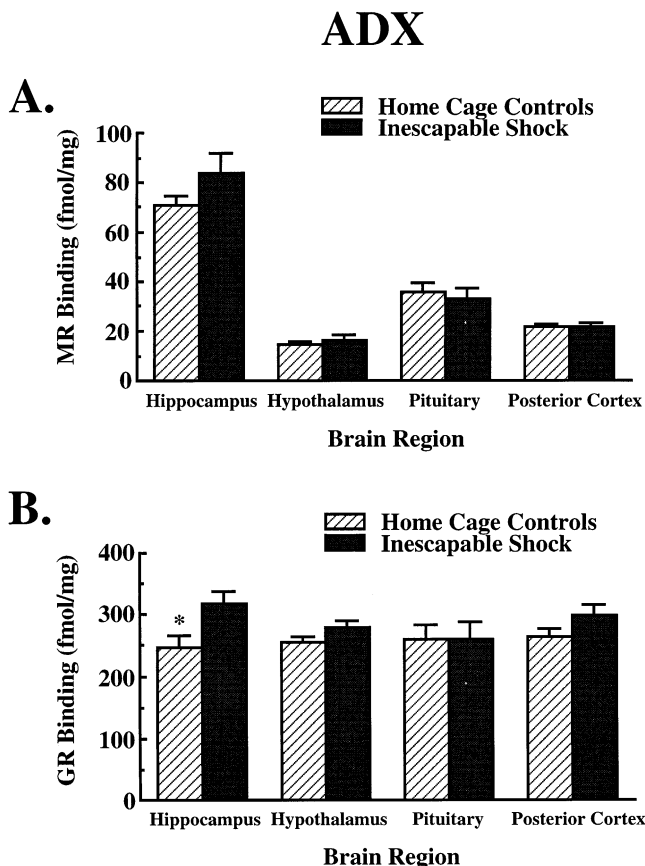


Fig. 4. Number of available CORT receptors in adrenalectomized rats 24 h after IS. As a measure of total receptor levels, the number of available receptors in adrenalectomized rats was also examined. No reliable differences were observed in MR levels between IS and HCC rats. However, more available GRs were observed in the hippocampus of IS treated rats than HCCs.

3.1.4. IS effects on MR and GR binding levels

No main effects of stress condition were observed in MR or GR binding in any of the four regions examined. However, reliable interactions between stress condition and surgical manipulation were observed in hippocampus [$F(1,34) = 5.0$, $P < 0.05$], hypothalamus [$F(1,34) = 3.6$, $P < 0.05$], pituitary [$F(1,34) = 9.3$, $P < 0.05$], and posterior cortex [$F(1,34) = 6.8$, $P < 0.05$]. Post-hoc analyses revealed reliable decreases in unoccupied MR receptors in Sham-IS rats when compared to Sham-HCC rats in each of these regions. No reliable differences were observed in MR binding of ADX-IS rats when compared to ADX-HCC rats (Fig. 3A and Fig. 4A) in any of the regions examined.

The effects of IS on GR binding were similar to that observed in MR binding, but were restricted to specific regions. Reliable interactions between stress condition and surgical manipulation in GR binding levels were observed only in hippocampus [$F(1,34) = 5.7$, $P < 0.05$], hypothalamus [$F(1,34) = 11.6$, $P < 0.01$], and posterior cortex [$F(1,34) = 9.7$, $P < 0.01$]. These interactions reflected reliable decreases in hypothalamus and posterior cortex GR binding. Interestingly, a reliable increase in hippocampal

GR binding was observed in ADX-IS rats when compared to ADX-HCC rats (Fig. 3B/Fig. 4B).

3.2. Experiment 2 — IS effects on whole cell GR

Prior to processing of brain tissues to measure whole cell GR levels, serum CORT levels were examined. As in the previous experiment, IS once again produced elevated basal levels of CORT 24 h later (HCC = 1.4 ± 0.6 $\mu\text{g}/\text{dl}$; IS = 5.4 ± 1.0 $\mu\text{g}/\text{dl}$) [$F(1,16) = 11.2$, $P < 0.01$], as well as reduced serum CBG (HCC = 589.1 ± 21.4 ; IS = 419.6 ± 21.4) [$F(1,16) = 31.3$, $p < 0.0001$]. Western blot analysis demonstrated that no differences in whole cell GR content occurred following exposure to IS in the hippocampus [$F(1,16) = 0.3$, $p > 0.05$], hypothalamus [$F(1,16) = 1.9$, $p > 0.05$], pituitary [$F(1,16) = 0.5$, $p > 0.05$], or posterior cortex [$F(1,16) = 1.1$, $p > 0.05$]. It is important to note here that each brain region was analyzed in a separate run of the Western blot procedure. Thus,

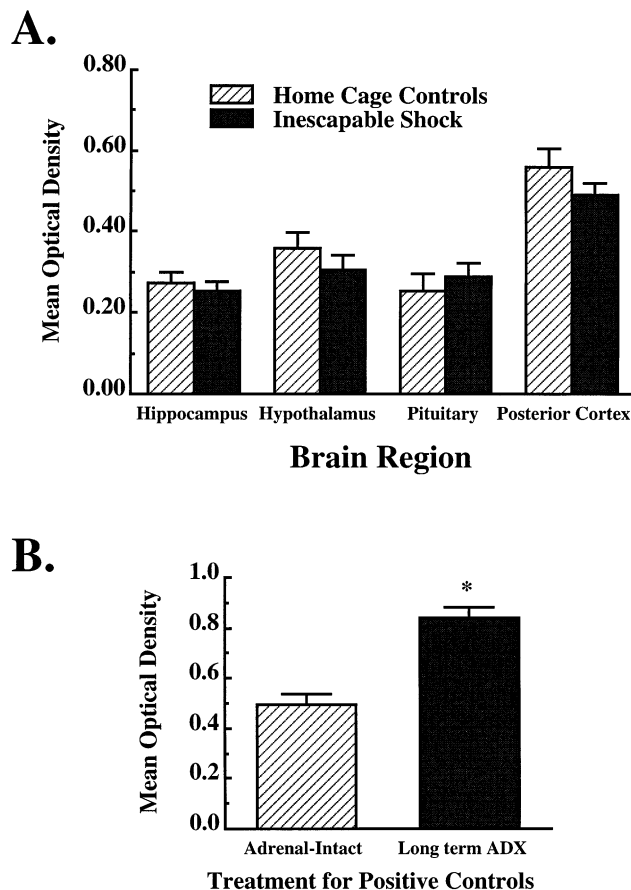


Fig. 5. Whole cell GR levels 24 h after IS. IS had no effect on whole cell GR levels in any of the brain regions examined (Panel A). In order to ensure that our Western blotting procedure was sensitive to treatment effects, positive control samples from pooled hippocampi of adrenal intact and long-term ADX were incorporated into each procedure and are plotted in Panel B. Since each brain region was examined in a different assay, relative differences in whole cell GR levels between regions cannot be inferred from these data.

relative differences in total GR levels across brain regions should not be inferred. Optical density values for all brain regions are presented on a single graph for ease of viewing (Fig. 5A).

Since long-term ADX in the absence of CORT replacement reliably increases the expression of GR in the hippocampus, we used pooled hippocampi from long-term (3–5 day) ADX and adrenal intact animals as positive controls that are included on each Western blot. Thus, these positive controls were also quantified and analyzed (one adrenal intact and one long-term ADX sample per gel \times 2 gels per brain region \times 4 brain regions = 16 standards) in the present assays. Pooled hippocampi from long-term ADX (no CORT replacement) showed a reliable increase in whole cell GR levels when compared to hippocampi from adrenal intact rats [$F(1,14) = 32.3$, $p < 0.0001$] (Fig. 5B).

4. Discussion

These data replicate our previous finding that IS exposure produces long-term elevations in basal levels of CORT and a decrease in plasma CBG [9,15]. In addition, we have demonstrated that as a result of these changes, there are fewer unoccupied MR detected by the receptor binding assay in the hippocampus, hypothalamus, pituitary, and posterior cortex. Similarly, we observed a significant decrease in unoccupied GR in hypothalamus and posterior cortex of IS treated rats. IS did not produce a decrease in corticosteroid receptor binding levels of ADX rats or a decrease in whole-cell GR immunoblot levels of adrenal-intact rats. Therefore, the decreases in available MR and GR levels most likely reflect an increased occupancy of corticosteroid receptors by the IS-induced elevation in basal CORT levels. It is important to note that the changes in receptor occupation (approximately 25%–50% increased occupancy of MR, and 20% increased occupancy of GR) is the pattern of results that one would expect based on the affinities of the two receptors for CORT [28,33]. That is, the largest changes in receptor occupancy were observed in MR, which have a higher affinity for CORT than GR.

One possible alternate explanation for the decrease in available corticosteroid receptor binding observed after IS in adrenal-intact rats is that the CORT receptors occupied during IS have not been recycled or replenished within 24 h. However, Meaney et al. [26] have previously shown that cytoplasmic receptor levels return to approximately normal levels by 4 h following termination of immobilization stress. Currently there are no published examples of stressors that selectively interfere with the normal recycling and replenishment of CORT receptors. Thus, it is unlikely that the greater occupancy of CORT receptors observed 24 h after IS reflects residual changes in occupancy that occurred during the IS session.

Although it is expected that elevated basal levels of CORT, especially in conjunction with a decrease in CBG, would occupy more receptors, it was impossible to predict the magnitude of the changes in receptor occupation that would be observed following IS exposure. These data demonstrate that at the trough of the circadian rhythm of CORT secretion, a time when the majority of GR are normally unoccupied [33,42], IS treated rats had significantly more GR occupation in the hypothalamus and cortex. Although receptor occupancy was only measured at one time point (24 h) after IS, the elevated basal CORT and reduced CBG typically persist for 48–72 h following termination of the stressor. Thus, target cells may be exposed to unusually high activation of CORT receptors for several days following the stressor, rather than just during the stressor. Furthermore, one might have predicted that such small increases in basal CORT would only occupy more MR and that the increased MR occupation might be restricted to the CNS due to the buffering capacity of CBG in the periphery. However, the present data clearly demonstrate that relatively subtle increases in basal CORT impact both MR and GR in the brain and MR in the pituitary.

In contrast, greater occupancy of GR was not observed in the pituitary. deKloet et al. [11,12] have previously demonstrated that the pituitary contains high levels of CBG that may serve to buffer the cells from glucocorticoid negative feedback. However, it is important to note that we also failed to see increased occupancy of GR in the hippocampus. This is much less likely to be a result of a local buffering effect of CBG since this large protein is not able to cross the blood brain barrier. Interestingly, we did observe an increase in hippocampal GR binding in ADX rats after IS, suggesting that there may have been an IS-induced upregulation of this receptor protein expression. However, in the second experiment, we did not observe an increase in hippocampal GR protein level after IS as assessed by immunoblot.

It is perhaps not surprising that MR and GR protein levels were unaffected by IS exposure, since decreased GR is typically only observed following stressors that are chronic [14,36]. This holds true for changes in GR produced by exogenous CORT; only prolonged administration of CORT or supraphysiological regimens of CORT are sufficient to downregulate GR [17,43].

The presence of elevated basal CORT for several days following termination of the IS session also requires discussion. One potential explanation for the elevated basal CORT is that IS treated rats have impaired negative feedback [22]. This hypothesis is especially warranted given the finding that elevated basal CORT occurs while at the same time there is greater occupancy of CORT receptors, which would be expected to provide more feedback inhibition. Our results indicate, however, that if there is an IS-induced decrease in CORT negative feedback, the decreased feedback is not a result of corticosteroid receptor

downregulation. At least there does not appear to be an IS-induced downregulation of corticosteroid receptors that is of sufficient magnitude and tissue generality to be detected by our present receptor measurement techniques. Another potential explanation for the elevated basal CORT is increased drive to the HPA axis which overcomes CORT negative feedback. IS could produce increased drive to the HPA axis as a result of neural sensitization, increased metabolic demand, or cognitive/perceptual changes, but these prospects remain to be determined.

Another important replication in the present data is the finding that ADX does not alter the IS-induced reduction in CBG. We [9] have previously demonstrated that IS-induced reductions in CBG occur in rats that have been pretreated with the GR antagonist RU38486, or received ADX prior to stressor exposure. Since CORT has the ability to dynamically alter CBG levels [47], and stressors produce a robust increase in serum CORT, many researchers have assumed that stress effects on CBG are unitarily mediated by CORT. Thus, it is noteworthy to mention that this is not always the case. Data by Fleshner et al. [15] support this conclusion, since an injection of CORT which mimics the IS-induced rise in CORT had no effect on serum levels of CBG, nor is it sufficient to produce elevated basal CORT. As a result of these findings, we have concluded that the IS-induced rise in CORT is neither necessary nor sufficient to produce the subsequent increase in basal CORT or the reduction in CBG.

Although we have not yet determined the mechanism(s) by which IS alters basal CORT and plasma CBG levels, our best indication at this point is that these changes occur as a result of acute phase activation. The acute phase response is a generalized systemic immune response that normally occurs following bacterial infection or antigen exposure. During the acute phase response, liver metabolism is altered such that the normal synthesis of carrier proteins such as albumin and CBG is inhibited, while synthesis of acute phase-positive proteins is initiated. In addition to changes in plasma proteins, acute phase activation also leads to behavioral alterations, fever production, and increases in basal CORT [48]. All of these changes have been observed following IS exposure [9], and can be blocked by central administration of alpha-melanocyte stimulating hormone (α -MSH; Ref. [29]) prior to IS. Importantly, central administration of α -MSH blocks most indices of acute phase activation when administered prior to infective agents. These findings suggest that the reduction in CBG is likely a result of acute phase activation. Indeed, many other researchers also consider reductions in CBG to be a highly sensitive measure of acute phase activation as well [32,37,38,46].

While the mechanism by which IS produces a relatively long-lasting increase in basal CORT and a reduction in CBG has yet to be determined, the consequences may be an important component of the long-term effects of IS. Determining the functional significance of this increased

amount of corticosteroid receptor activation following IS will require careful separation of the relative role of CORT elevations during IS from the subsequent basal CORT elevations. The use of selective corticosteroid receptor antagonists [23] to block CORT receptors during or after IS may prove to be a good strategy for these studies. The greater occupancy of MR and GR that we have observed in rats 24 h after IS represents not only a greater percent of receptor occupancy than is normally seen during the trough of CORT secretion, but in some cases represents occupancy of receptors (MR in the pituitary, GR in brain) that at this time of day appear to be typically completely unoccupied by CORT. The near maximal occupancy of MR observed in the brain during basal conditions 24 h after IS is similar to that observed in normal rats after acute stress or at the circadian peak of CORT basal secretion. On the other hand, the increased occupancy of GR in the brain 24 h after IS is still less than that observed under either acute stress or peak basal CORT conditions [42]. However, Akana et al. [1] have demonstrated that the daily mean level of CORT exposure in the rat is normally maintained within a narrow range. Thus, modest changes in daily MR and GR activation may have important consequences.

There is a wide range of neuronal processes that are regulated by the normal circulating levels of glucocorticoids. These processes include regulation of the levels of neurotransmitters and their receptors, regulation of intracellular signal transduction, and regulation of neuronal survival and morphology [16]. In light of these wide-ranging effects, it is perhaps not surprising that activity of the HPA axis is normally tightly regulated. Serotonin 5-HT_{1a} receptor expression is one example of a protein that is sensitive to moderate *in vivo* disruption in basal CORT levels. Meijer et al. [27] have recently demonstrated that elevated trough levels of CORT suppress serotonergic 5-HT_{1a} receptor mRNA and binding in the hippocampus. In these experiments, rats were implanted with CORT pellets that produced basal CORT levels virtually identical to those observed in rats exposed to IS. Furthermore, Short [40] have recently demonstrated similar reductions in 5-HT_{1a} receptor binding in the dorsal raphe nucleus of IS treated rats. Thus, the elevated basal CORT produced by IS exposure may be responsible for the downregulation of 5-HT_{1a} that is also observed following IS. Nevertheless, the physiological consequences of the increased receptor occupancy produced by IS and its generality to other stressors remain to be determined.

In summary, we found that the elevated basal CORT observed 24 h after IS exposure led to greater occupancy of MR and GR in the brain and MR in the pituitary. Interestingly, the increase in basal CORT cannot be accounted for by a downregulation of corticosteroid receptor expression. These findings suggest that target cells in the brain and pituitary may have greater corticosteroid receptor activation for up to several days following termination

of the stressor, and may account for some of the long-term neural, behavioral, and immunological consequences of acute stressor exposure.

Acknowledgements

Supported by NIH Grants MH45045 and DK49143.

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