

Stress-Induced Sensitization of the Hypothalamic-Pituitary Adrenal Axis Is Associated with Alterations of Hypothalamic and Pituitary Gene Expression

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Key Words

Corticotropin · Corticotropin-releasing hormone · Paraventricular nucleus · Proopiomelanocortin · Adrenal steroids · cFos · Stress

Abstract

We have previously reported that inescapable tail shock (IS) produces persistent changes in hypothalamic-pituitary-adrenal (HPA) axis function. These changes are manifest as an elevation in basal corticosterone (CORT) levels, a sensitization of adrenocorticotropin hormone (ACTH) and CORT responses to subsequent challenge, and a failure of dexamethasone to suppress both the ACTH and CORT responses to a subsequent challenge. The experiments presented here examine IS-induced alterations in the responsiveness of the HPA axis, particularly at the level of the anterior pituitary. The data presented show that adrenalectomy does not abolish the IS-induced sensitization of the HPA axis, suggesting that the sensitization is not solely caused by a defect in glucocorticoid negative feedback. Analysis of gene expression in the anterior pituitary revealed that IS exposure persistently elevated basal levels of proopiomelanocortin (POMC; the precursor to ACTH) mRNA and sensitized the

POMC hnRNA and c-fos mRNA response to a subsequent challenge. Analysis of gene expression in the parvocellular division of the paraventricular nucleus of the hypothalamus (pPVN) after IS exposure revealed that basal levels of corticotropin-releasing hormone (CRH) mature mRNA are elevated and the c-fos mRNA response to a subsequent challenge is enhanced. Finally, a blunted in vitro ACTH response to CRH challenge is observed after IS exposure. These data suggest that the ultimate source of the IS-induced sensitization is not the anterior pituitary and implicate an increased drive on the anterior pituitary from the pPVN.

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Introduction

A variety of stressors produce persistent alterations in basal corticosterone (CORT) levels and alter the responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to subsequent challenge [1–3]. Increased or maintained HPA responses to a novel stress are often observed in repeatedly stressed animals [4–7]. Inescapable tail shock (IS) is unusual in being an acute stressor that produces persistent changes in the function of the HPA axis. After

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exposure to a single session of IS, elevated basal levels of CORT can be observed at the diurnal trough of the circadian rhythm [8]. Furthermore, IS exposure sensitizes the HPA axis, such that ACTH and CORT responses to a subsequent challenge are exaggerated, especially during the rising phase of the response [9]. These persistent changes in HPA function are pronounced 24–48 h after a single session of IS and no longer present 5–7 days later. Since the ACTH response to challenge is exaggerated in IS rats, there must be an alteration in the pituitary and/or brain that contributes to the sensitization of the HPA axis response after IS. However, the proximate cause of the exaggerated ACTH response in IS rats is unknown.

A variety of signals impinge upon the anterior pituitary to regulate the secretion of ACTH. The hypothalamic neuropeptides, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), exert a stimulatory influence on ACTH secretion. CRH neurosecretory cells are located in the dorsomedial parvocellular subdivision of the paraventricular nucleus of the hypothalamus (pPVN) and about 25–50% of these cells also contain detectable levels of AVP [10, 11]. Axons of these cells terminate in the external zone of the median eminence and release their contents into the pituitary portal capillaries upon neuronal activation. ACTH, in turn, acts at the adrenal gland to induce the release of glucocorticoids (GCs), namely CORT, in the rat. In a classic neuroendocrine negative feedback loop, GCs act via the GC receptor (GR) at the anterior pituitary (and elsewhere) to inhibit ACTH release. Integration of both positive and negative secretory signals at the anterior pituitary is a major determinant in the final output of the HPA axis.

Thus, over-activity of the HPA axis may be caused by a diminution of GC negative feedback inhibition and/or augmentation of the effectiveness of ACTH secretagogues at the anterior pituitary. Intriguingly, some stressors reduce GR expression and/or impair GC negative feedback [12–14]. IS does not persistently alter GR expression in the pituitary [15]. However, rats previously exposed to IS are resistant to the negative feedback effects of peripherally acting doses of dexamethasone (DEX; a GR agonist), suggesting either impairment in the functioning of the GR at the anterior pituitary or increased stimulatory input to the anterior pituitary that overcomes the suppressive effects of DEX [16].

Many stressors produce persistent elevations in expression of CRH and/or AVP and, thus, may produce conditions of increased stimulatory input to the anterior pituitary. Exposure to chronic variable stress, immobilization, repeated hypertonic saline injections, or a single injection

of the proinflammatory cytokine tumor necrosis factor- α increases basal expression of CRH mRNA in the parvocellular PVN [12, 17–19]. Additionally, foot shock enhances the CRH immunoreactivity induced by subsequent exposure to an electrified prod [20]. Foot shock also persistently increases the percentage of CRH neurons in the pPVN that co-store the AVP peptide [21]. Similarly, repeated restraint increases the number of cells containing AVP mRNA in the pPVN and sensitizes the AVP hnRNA response to a subsequent injection of hypertonic saline [17, 22]. Aged rats have a greater percentage of CRH neurons that co-express AVP mRNA and peptide, and rats selectively bred for high anxiety-related behavior (HAB) also exhibit elevated basal levels of AVP peptide within the pPVN [23–26]. Furthermore, pretreatment with a selective V1 receptor antagonist abolished the DEX nonsuppression in both aged and HAB rats [23–26].

In sum, IS produces profound and persistent changes in the HPA axis that have been observed at the level of circulating ACTH and CORT. The proximate cause of these changes is unknown. Thus, the primary goals of these studies were to determine (1) whether IS-induced sensitization of the HPA axis occurs in the absence of GC negative feedback and (2) whether IS sensitizes subsequent responses in the anterior pituitary and/or PVN.

Material and Methods

Animals

Adult male Sprague-Dawley rats (250 g at purchase; Harlan Sprague Dawley, Indianapolis, Ind., USA) were used in all studies. Animals were individually housed in hanging metal cages at $25 \pm 1^\circ\text{C}$ with a 12:12-hour light:dark cycle (lights on at 06.00 h). Standard rat chow and water were freely available. Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Stressor Protocol

Animals either remained undisturbed in their home cages as controls (HCC) or were exposed to a single session of inescapable tail shock (IS). All IS treatments occurred between 07.00 and 10.00 h in a separate room from the HCC. The stressor protocol involved placing the animal in a Plexiglas restraining tube (23.4 cm long and 7 cm in diameter) with its tail protruding from the end. Copper strips coated with electrode paste were then securely fastened to the tail of each rat and taped in place. A computer (IBM PC, model No. 5150) delivered 100 1.6-mA 5-s tail shocks, with a variable inter-trial interval ranging from 30 to 90 s (average of 60 s). The number of animals shocked simultaneously ranged from 8 to 12. Thus, animals were in close proximity and were not isolated from each other during the IS session.

In some experiments, rats were also exposed to pedestal stress 24 h following IS or HCC treatment. Pedestal stress consisted of placing a single rat on an elevated, uncovered square pedestal (33 cm²; raised 61 cm above the ground). The pedestals were located in a separate room from the HCCs. Rats were removed from the pedestal without being returned to their home cages and quickly decapitated.

Adrenalectomy Surgery

Bilateral adrenalectomies (ADXs) were aseptically performed under isoflurane anesthesia. All tissues removed from the animal were carefully examined to ensure complete removal of the adrenal glands. Sham-operated animals received the identical procedure except that the adrenal glands were gently manipulated with forceps, but not removed. ADX animals received CORT replacement in their drinking water since this method has been shown to mimic the normal circadian pattern of CORT secretion [27]. CORT (Sigma-Aldrich Incorporated) was initially dissolved in ethyl alcohol (EtOH) and diluted to a final concentration of 25 µg/ml in 0.2% EtOH, 0.9% saline. Sham animals received drinking water containing 0.2% EtOH.

Corticotropin-Releasing Hormone

Rat/human CRH (Sigma) dissolved in sterile saline was injected intraperitoneally (i.p.) at doses of 1 or 3 µg/kg. Control injections were equivalent (1 ml/kg) vehicle. Rat/human CRH was also dissolved in Earle's Balanced Salt Solution (Sigma) for use *in vitro*.

In vitro Assessment of Anterior Pituitary Function

The entire pituitary gland was removed from the skull after rapid decapitation. The neurointermediate lobe was dissected away and the anterior lobe of the pituitary was halved using a scalpel blade. Anterior pituitary halves were placed in tissue culture wells with inserts (Corning Incorporated) submerged in 2.5 ml Earle's Balanced Salt Solution. Tissue segments were incubated for 2.5 h at 37 °C in 5% CO₂/95% air and incubation media was replaced at 0.5 and 1.5 h. After 2.5 h, tissue segments were placed in a dish containing CRH or vehicle and incubated at the above conditions for 1 h. Aliquots of media were collected in plastic tubes and stored at -80 °C until assayed for ACTH. Upon completion of the experiment, wet weights of tissue segments were obtained and ACTH levels are ultimately expressed per milligram of tissue.

ACTH Assay

Plasma and media levels of ACTH were determined by radioimmunoassay. Plasma samples (50 µl), media samples (20 µl), and ACTH standards (10–1,000 pg/ml) were incubated overnight at 4 °C with antiserum (rabbit antibody Rb7; courtesy of Dr. William England, University of Minnesota) and 100 µl of [¹²⁵I]-ACTH. Goat anti-rabbit IgG (Calbiochem) and normal rabbit serum were added and allowed to incubate for 30 min before the antibody-bound ACTH was separated from free ACTH with a PBS buffer containing 5% PEG. The bound fraction was counted with a gamma counter (Wallac, 1470 Wizard).

RNA Extraction and DNase Treatment

Total RNA was isolated after homogenization of the anterior pituitary in Trizol reagent according to the manufacturer's instructions. Chloroform (200 µl) was added to each tissue homogenate. Samples were centrifuged (12,200 g, 15 min, 4 °C) and the aqueous phase was transferred to a clean tube. Equivolume isopropanol was

added to the sample. Samples were centrifuged (12,200 g, 10 min, 4 °C) and supernatants discarded. The pellet was washed with 75% ethanol and centrifuged (7,500 g, 10 min, 4 °C). Supernatants were discarded and the pellet was allowed to air dry before reconstitution in sterile, RNase free H₂O (Baxter).

DNA-freeTM kits (Ambion) were used according to the manufacturer's instructions to remove contaminating genomic DNA from samples. Equal amounts of DNase I (4 units) and 10 × DNase buffer were added to each sample. Samples were incubated at 37 °C for 30 min. DNase inactivation reagent was added to each sample and samples were incubated at room temperature for 2 min. Samples were centrifuged (12,200 g, 2 min) and the supernatant was transferred to a clean tube.

The integrity of the RNA was verified by ethidium bromide visualization of the 28S and 18S bands by denaturing agarose gel electrophoresis.

Reverse Transcription-Polymerase Chain Reaction

Concentration of total RNA was determined by spectrophotometry at 260 nm. Total RNA was diluted to 0.25 µg/µl. First strand cDNA was synthesized by random-priming using 2.5 µg of total RNA, 50 ng of DNA random hexanucleotides, and 200 U of Superscript II RNase H⁻ reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Reverse transcription was carried out at 42 °C for 70 min and terminated by heating to 95 °C for 10 min.

PCRs were performed in duplicate. Aliquots of cDNA (2 µl) were amplified using TaqBeadTM Hot Start Polymerase wax beads (1.25 U/bead; Promega Corporation) in a reaction volume of 50 µl that contained 5 × Green GoTaqTM Reaction Buffer (Promega Corporation), dNTPs (0.2 mM each), and 0.4 µM gene specific primers. The sense primer for POMC hnRNA was 5'-GGCTCACACATTGGGCCTCC-3' and was complementary to a region within the second intron of the POMC gene. The sense primer for POMC mature mRNA was: 5'-CAGGACCTCACCACGGAAAG-3'. The common anti-sense primer used for both POMC hnRNA and mature mRNA was: 5'-CTTCCAGCTCCCTCTTGAAC-3', which results in the amplification of a 395-bp product for POMC hnRNA and a 399-bp product for POMC mRNA. The primers for c-fos were: 5'-GGAA-TAAGATGGCTGCAGCC-3' (sense) and 5'-GTCACAGACAT-CTCCTCTGG-3' (anti-sense), which amplify a 219-bp product. The primers for cyclophilin were: 5'-GAGAAAGTTCCAAGACAGCA-GAAA-3' (sense) and 5'-CTGAGCTACAGAAGGAATGGTTTGA-3' (anti-sense), which amplify a 470-bp product. Denaturation was performed in each PCR at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min (for the final step, extension was for 7 min). cDNA was amplified 31 (POMC hnRNA), 29 (c-fos mRNA), 25 (cyclophilin mRNA), or 23 (POMC mRNA) cycles. After amplification, aliquots of PCR products (10 µl) were electrophoresed on 2% (w/v) agarose gels. Gels were run at 100 V for 1 h, stained with ethidium bromide (0.5 µg/ml) for 7 min, and washed in water for 1 h. Gels were then photographed under ultraviolet light using a Gel Doc 2000 System (BioRad Laboratories). Band densities were obtained by densitometric measurements of the PCR products using public domain software Scion Image Beta 4.02 Win (Scion Image Corporation). The software can be downloaded from http://www.scioncorp.com/frames/fr_scion_products.htm. The amount of mRNA was expressed as a ratio of densitometric measurements derived from the gene-specific mRNA and cyclophilin mRNA.

In situ Hybridization

After decapitation, whole brains were removed, flash frozen in isopentane chilled to -30°C , and stored at -80°C until assay. Brains were sectioned ($10\ \mu\text{m}$) at the level of the PVN on a cryostat, thaw mounted onto poly-*L*-lysine-coated slides, and stored at -80°C . Prior to hybridization, tissue was fixed in a 4% paraformaldehyde solution for 1 h. Slides were then washed in $2\times$ standard sodium citrate (SSC) and acetylated in 0.1 *M* triethanolamine containing 0.25% acetic anhydride. Slides were washed again and dehydrated in a series of graded, diminishing ethanol concentrations, and allowed to dry.

^{35}S -UTP or ^{35}S -UTP/ ^{35}S -CTP-labeled cRNA probes were generated from cDNA subclones in transcription vectors for CRH and AVP mRNA. Reaction mixture for labeling of riboprobes consisted of sterile filtered water, T7 transcription buffer, linearized plasmids (courtesy of Drs. Serge Campeau and Heidi Day, University of Colorado), ^{35}S -UTP (mRNA probes), dithiothreitol, RNase inhibitor, T7 polymerase, and remaining nucleotides. The transcription reaction occurred at 37°C over 2 h. The radiolabeled probe will be separated from the mixture using a Sephadex G50–50 column. Fractions containing the most radioactivity were used as probe.

Riboprobes were diluted in hybridization buffer (formamide, sterile water, SSC, Denhart's solution, yeast RNA, sodium phosphate buffer, dextran sulfate) to a concentration of 1–2 million counts per slide ($65\ \mu\text{l}/\text{slide}$). The diluted probe was applied to each slide underneath a coverslip. Slides were placed in sealed plastic boxes lined with chromatography paper moistened with 50% formamide in dH_2O and incubated overnight at 55°C . The following day, coverslips were floated off in $2\times$ SSC and rinsed several times. Slides were then incubated in RNase A at 37°C for 1 h, washed in successive decreasing concentrations of SSC, and incubated in $0.1\times$ SSC at 70°C for 1 h. Finally, slides were dehydrated in successive increasing concentrations of ethanol and exposed to X-ray film. Semiquantitative analyses were performed on digitized images from X-ray film (NIH image).

Slides for AVP mRNA were then dipped in NTB2 emulsion (diluted 1:1; Eastman Kodak, Rochester, N.Y., USA) and stored at 4°C for 28 days. Emulsion-dipped slides were developed in D-19 developer (Eastman Kodak), counterstained with Cresyl Violet, dehydrated in graded series of alcohol, placed in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, Pa., USA). To allow reliable comparisons between groups, sections from each group were processed together.

Results

Experiment 1 – Effects of ADX on Sensitization of the HPA Axis

Methods and Rationale

Previous data have suggested that IS-induced sensitization of the HPA axis may be due, in part, to impaired GC negative feedback in animals previously exposed to IS [16]. If differences in HPA axis responsiveness between animals previously exposed to IS and non-stressed controls are solely due to differences in the efficiency of GC

negative feedback, then complete elimination of adrenal GCs should eliminate differences in HPA axis responsiveness. To test this hypothesis, rats ($n = 46$; 5–7/group) underwent bilateral ADX or sham surgery. ADX rats were given basal CORT replacement for 2 days after surgery. Since negative feedback effects of GCs may operate in a time domain of days, basal CORT replacement was stopped (but saline replacement continued) in ADX rats 24 h before rats were exposed to IS or remained in their home cages (day 4 after surgery). On day 5 after surgery, rats were placed on an elevated, uncovered square pedestal ($33\ \text{cm}^2$; raised 61 cm above the ground) for 0 or 15 min. Pedestal exposure has previously been shown to induce a larger ACTH response in animals previously exposed to IS [9]. Following this treatment, unanesthetized rats were rapidly decapitated and trunk blood was collected for measurement of ACTH levels.

Results

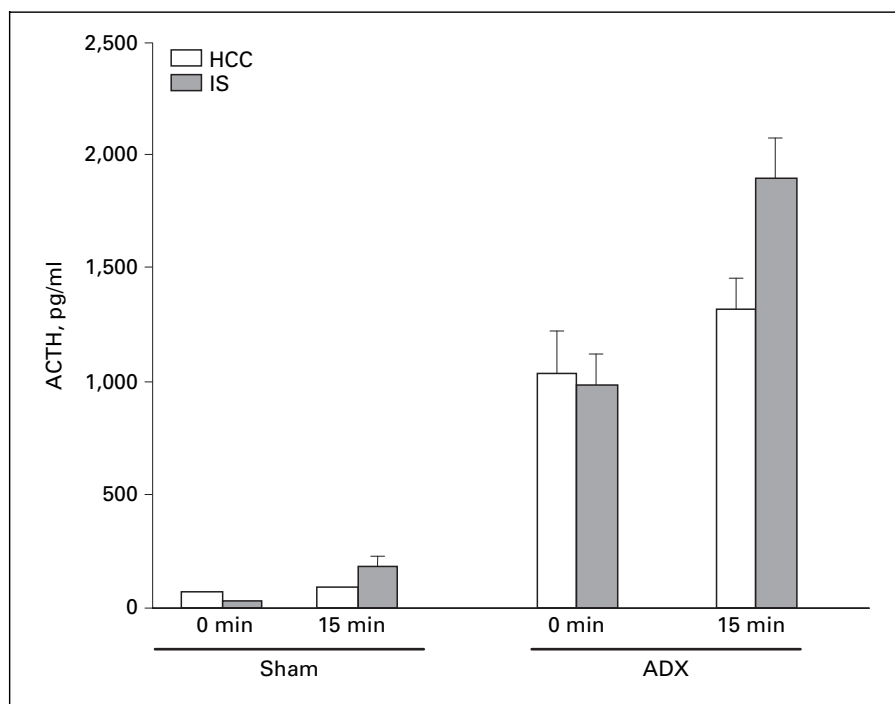
As expected, ADX rats had dramatically elevated ACTH levels as compared to sham rats. Moreover, the ACTH response to placement on an elevated pedestal was enhanced in rats previously exposed to IS, regardless of their adrenal status (fig. 1). These observations were confirmed by statistical analyses. ANOVA revealed a significant surgery \times stress (IS vs. HCC) \times challenge (no stress vs. pedestal) interaction ($F(1,38) = 5.471$, $p < 0.05$). Post hoc tests confirmed that ADX rats had significantly higher levels of circulating ACTH ($p < 0.0001$). Furthermore, prior IS exposure by itself did not alter ACTH levels 24 h later ($p > 0.05$). However, as compared to HCC rats, rats previously exposed to IS had significantly increased ACTH levels after placement on an elevated pedestal regardless of whether they were sham ($p < 0.05$) or ADX ($p < 0.005$).

Experiment 2 – Effects of IS Exposure on Expression of CRH and AVP mRNA in the pPVN and POMC mRNA in the Anterior Pituitary

Methods and Rationale

The results of experiment 1 indicate that impaired GC feedback alone cannot account for the sensitized ACTH response observed in rats previously exposed to IS. Thus, there must be increased drive on the anterior pituitary that leads to enhanced ACTH levels in rats previously exposed to IS. Persistent increased drive on the anterior pituitary may result in elevated POMC mRNA levels.

Fig. 1. Inescapable tail shock (IS)-induced sensitization of the ACTH response to pedestal challenge in adrenalectomized (ADX) rats. ADX or sham-operated rats were exposed to IS or remained in their home cages as controls (HCC). One day later rats remained in their home cages or were placed on an elevated pedestal. Unanesthetized rats were rapidly decapitated 15 min following placement on the pedestal. ADX increased circulating ACTH levels, but did not alter IS-induced sensitization of the ACTH response to pedestal. Values are means \pm SE.



Therefore, to examine the effects of IS on CRH and AVP mRNA expression in the pPVN and POMC mRNA expression in the anterior pituitary, rats ($n = 14$; 7/group) were exposed to IS or remained in their home cages. 24 h later unanesthetized rats were rapidly decapitated. Brains were removed and processed for in situ hybridization as detailed in the Materials and Methods section. Anterior pituitary glands were also removed, flash frozen in liquid nitrogen, and stored at -80°C until RNA extraction and subsequent analysis by RT-PCR.

Results

POMC mRNA. Exposure to IS produced a persistent elevation in the expression of POMC mRNA in the anterior pituitary (fig. 2a). This observation was supported by statistical analysis. ANOVA revealed a main effect of stress ($F(1,8) = 5.793$, $p < 0.05$).

CRH mRNA. Exposure to IS produced a persistent elevation in the expression of CRH mRNA in the pPVN (fig. 2b). This observation was supported by statistical analysis. ANOVA revealed a main effect of stress ($F(1,13) = 12.141$, $p < 0.001$).

AVP mRNA. Exposure to IS did not alter the expression of AVP mRNA in the pPVN (fig. 2c).

Experiment 3 – Effects of IS Exposure on Transcriptional Responses to Elevated Pedestal Exposure

Methods and Rationale

The results of experiment 2 indicated that IS exposure persistently alters basal expression of POMC and CRH mRNA in the anterior pituitary and pPVN, respectively. To determine whether IS exposure also alters the response of these regions to subsequent challenge, the transcriptional responses within these regions were examined. Examination of intronic sequences allows the assessment of newly transcribed RNA, or hnRNA, which reflects changes in gene transcription and is a much more sensitive index of rapid cellular responses than measurement of steady-state mRNA levels [28]. Immediate early genes (IEG), such as *c-fos*, are reliable indicators of cellular activation as a result of their inducibility by a variety of different stimuli and have been widely used to characterize cellular activation. Thus, examination of POMC hnRNA and *c-fos* mRNA levels in response to pedestal stress provide a direct view of rapid stimulus-induced transcriptional activation and whether prior IS exposure alters the magnitude and timing of these responses. To test whether prior IS exposure sensitizes the transcriptional response of the anterior pituitary and pPVN to placement on an

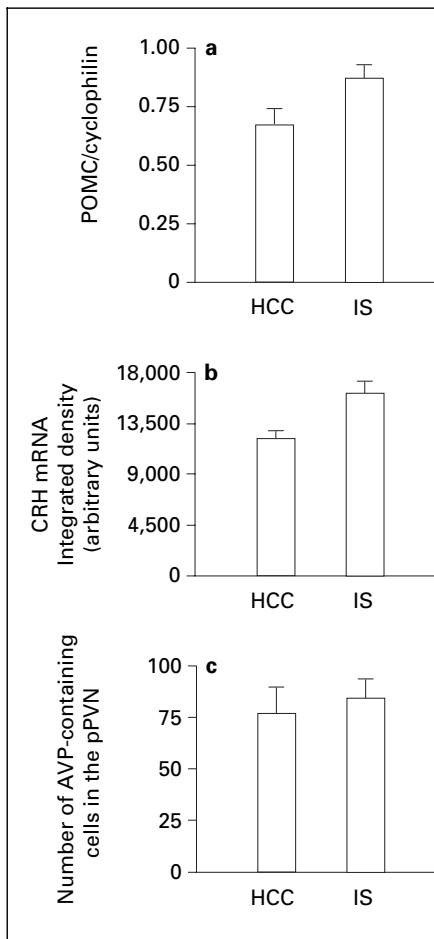


Fig. 2. Effects of inescapable tail shock (IS) on basal gene expression in the pPVN and anterior pituitary. Rats were exposed to IS or remained in their home cages as controls (HCC). 24 h later, unanesthetized rats were rapidly decapitated. Prior IS exposure elevated POMC mRNA expression in the anterior pituitary gland as assessed by RT-PCR and CRH mRNA expression within the pPVN as assessed by in situ hybridization. **a** POMC mRNA in the anterior pituitary. **b** CRH mRNA in the pPVN. **c** AVP mRNA in the pPVN. Values are means \pm SE.

elevated pedestal, rats ($n = 47$; 7 or 8/group) were exposed to IS or remained in their home cages. 24 h after this treatment, rats were placed on an elevated pedestal for 0, 7.5, or 15 min. Following this treatment, unanesthetized rats were rapidly decapitated and trunk blood, anterior pituitaries, and whole brains were collected. Trunk blood was processed as described in the Materials and Methods section and ACTH levels were assessed by radioimmunoassay. Anterior pituitaries were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. *c-fos* mRNA and POMC hnRNA levels were assessed by RT-

PCR. Brains were sectioned at the level of the PVN on a cryostat and analyzed for *c-fos* mRNA by in situ hybridization.

Results

Circulating ACTH. Consistent with prior results, placement on an elevated pedestal increased circulating ACTH levels in rats previously exposed to IS, but not in HCC rats (fig. 3a) [9]. This observation was supported by statistical analysis. ANOVA revealed a significant stress (HCC vs. IS) \times challenge (no stress vs. 7.5 min pedestal vs. 15 min pedestal) interaction ($F(2,40) = 3.267$, $p < 0.05$). Post hoc tests revealed that prior exposure to IS did not, by itself, increase ACTH levels 24 h later ($p > 0.05$). However, as compared to HCC rats, rats previously exposed to IS had significantly increased ACTH levels after placement on an elevated pedestal for 7.5 ($p < 0.01$) or 15 ($p < 0.05$) min.

Anterior Pituitary *c-fos* mRNA. Placement on an elevated pedestal increased *c-fos* mRNA expression in the anterior pituitary to a greater extent in rats previously exposed to IS (fig. 3b). These observations were supported by statistical analysis. ANOVA revealed a significant stress \times challenge interaction ($F(2,38) = 3.581$, $p < 0.05$). Post hoc tests revealed that prior exposure to IS did not, by itself, increase *c-fos* mRNA expression in the anterior pituitary 24 h later ($p > 0.05$). However, as compared to HCC rats, rats previously exposed to IS had significantly increased *c-fos* mRNA expression after placement on an elevated pedestal for 7.5 ($p < 0.01$) or 15 ($p < 0.05$) min.

Anterior Pituitary POMC hnRNA. Placement on an elevated pedestal increased POMC hnRNA expression in the anterior pituitary and prior exposure to IS significantly enhanced this effect (fig. 3c). This observation was supported by statistical analysis.

ANOVA revealed a significant stress \times challenge interaction ($F(2,39) = 4.921$, $p < 0.05$). Post hoc tests revealed that prior exposure to IS did not, by itself, increase POMC hnRNA expression in the anterior pituitary 24 h later ($p > 0.05$). However, as compared to HCC rats, rats previously exposed to IS had significantly increased POMC hnRNA expression after placement on an elevated pedestal for 7.5 ($p < 0.0001$) or 15 ($p < 0.05$) min.

PVN *c-fos* mRNA. Prior exposure to IS enhanced the increase in PVN *c-fos* mRNA expression induced by placement on an elevated pedestal (fig. 3d). ANOVA yielded a significant stress \times time (on pedestal) interaction ($F(2,41) = 6.81$, $p < 0.005$). Post hoc tests revealed that rats previously exposed to IS or HCC treatment did

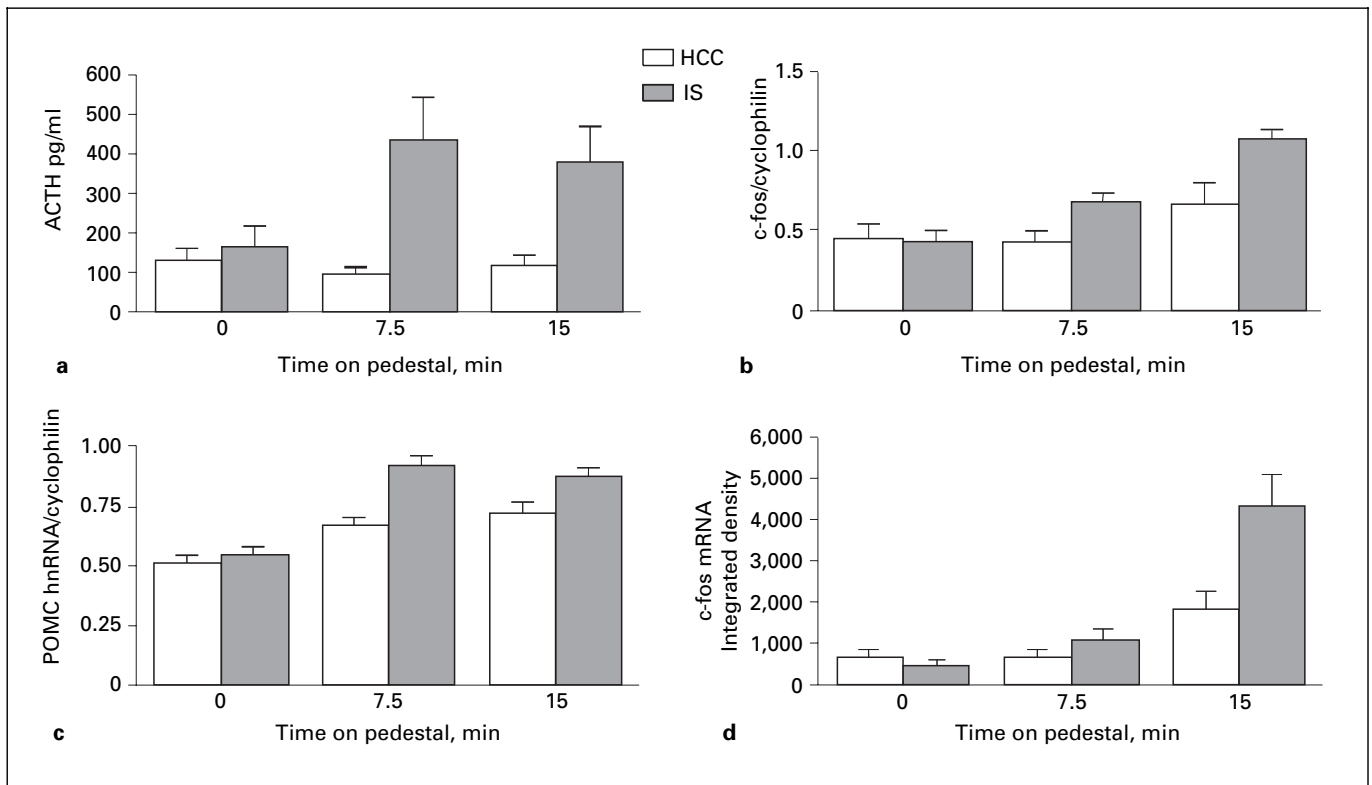


Fig. 3. Effects of inescapable tail shock (IS) on the response of the anterior pituitary and pPVN to subsequent challenge. Rats were exposed to IS or remained in their home cages as controls (HCC). 24 h later, rats were placed on an elevated pedestal. Unanesthetized rats were rapidly decapitated 0, 7.5, or 15 min following placement on the pedestal. RT-PCR was used to assess gene expression in the anterior pituitary and in situ hybridization was used to assess gene expression in the pPVN. Prior exposure to IS sensitized the circulating ACTH (a), anterior pituitary c-fos mRNA (b), anterior pituitary POMC hnRNA (c), and pPVN c-fos mRNA (d) response to subsequent pedestal exposure. Values are means \pm SE.

not differ in the expression of PVN c-fos mRNA 0 and 7.5 min after placement on an elevated pedestal (both $p > 0.05$). However, when placed on an elevated pedestal for 15 min, rats previously exposed to IS exhibited a larger increase in PVN c-fos mRNA expression as compared to HCC rats ($p < 0.05$).

Experiment 4 – Effects of IS on the ACTH Response to *in vitro* CRH Challenge

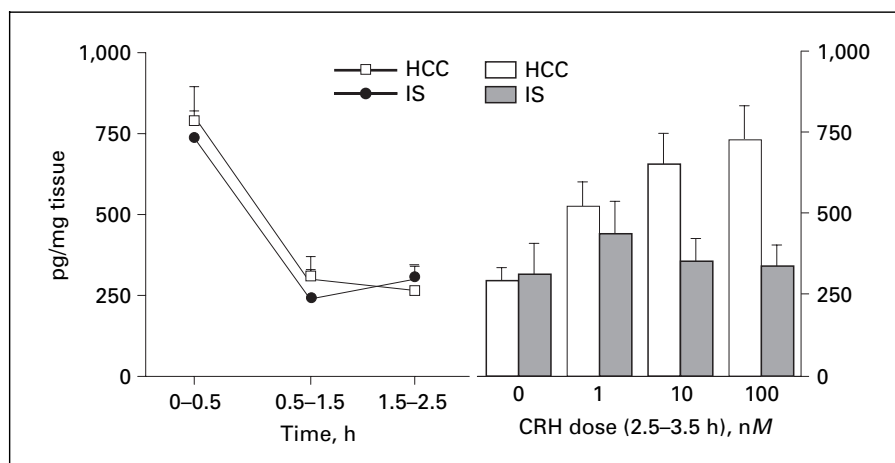
Methods and Rationale

The results of experiments 2 and 3 suggest that the activity, and possibly the output, of the PVN may be increased by IS. Repeated or prolonged stimulation with CRH reduces CRH receptor binding and can cause reduced ACTH responsiveness or desensitization [29–

31]. Indeed chronically activated corticotrophs generally show blunted ACTH response to CRH [32]. To test the hypothesis that IS exposure decreases the responsiveness of the anterior pituitary to CRH, quartered anterior pituitaries were assessed for their ability to release ACTH in response to *in vitro* CRH stimulation.

To test this hypothesis, rats ($n = 24$; 9–15/group) were exposed to IS or remained in their home cages. 24 h later unanesthetized rats were rapidly decapitated and anterior pituitary glands were removed, quartered, and maintained *in vitro* as described in the Materials and Methods section. One quarter anterior pituitary from each rat was stimulated with 0, 1, 10, or 100 nM CRH. Supernatant was collected each time the media was exchanged (i.e., 0.5, 1.5, and 2.5 h) and after stimulation for 1 h with CRH for measurement of ACTH levels.

Fig. 4. ACTH response to in vitro CRH challenge in inescapable tail shock (IS) rats. Rats were exposed to IS or remained in their home cages as controls (HCC). 24 h later, unanesthetized rats were rapidly decapitated and anterior pituitary glands were removed. Anterior pituitaries were quartered and subsequently stimulated with CRH in vitro. Prior IS exposure impaired the ACTH response to in vitro CRH challenge. Values are means \pm SE.



Results

ACTH levels from pre- and post-CRH-stimulated incubations were analyzed separately. Quartered anterior pituitaries secreted large amounts of ACTH during the first 0.5-hour incubation. The amount of ACTH released over the next 2 h was significantly reduced (fig. 4). Prior exposure to IS did not alter this pattern of ACTH secretion. These observations were supported by statistical analysis. Repeated measures ANOVA revealed a main effect of time ($F(2,68) = 35.148$, $p < 0.0001$). Post hoc tests revealed that ACTH levels were higher at the 0.5 h time point than at either the 1.5 ($p < 0.0001$) or 2.5 ($p < 0.0001$) h time points.

Quartered anterior pituitaries from HCC rats responded to in vitro CRH stimulation with increased secretion of ACTH. However, this response was impaired in anterior pituitaries from rats previously exposed to IS (fig. 4). ANOVA revealed a significant dose \times stress interaction ($F(3,88) = 2.886$, $p < 0.05$). Post hoc tests revealed that quartered anterior pituitaries from HCC rats and rats previously exposed to IS did not respond differently to media alone ($p > 0.05$). Furthermore, in vitro ACTH levels from quartered anterior pituitaries taken from HCC rats and subsequently stimulated with 10 ($p < 0.005$) and 100 ($p < 0.001$) nM CRH were elevated as compared to media alone. However, in vitro ACTH levels from quartered anterior pituitaries taken from rats previously exposed to IS and subsequently stimulated with 10 ($p > 0.05$) and 100 ($p > 0.05$) nM CRH were not significantly different as compared to media alone.

Experiment 5 – Effects IS on the ACTH Response to in vivo CRH Challenge

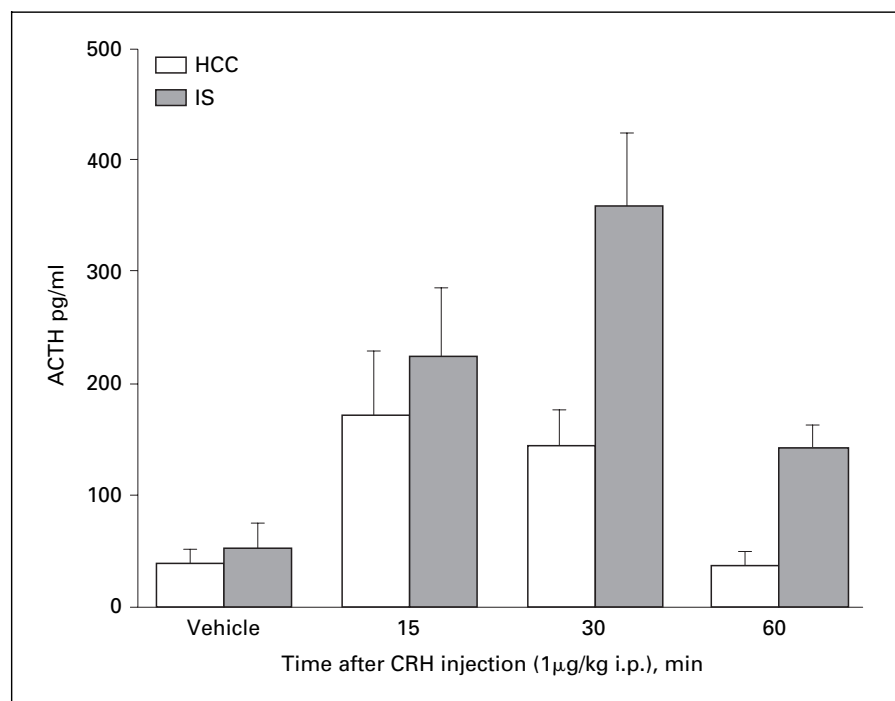
Methods and Rationale

The results of experiment 4 indicate that, in rats previously exposed to IS, the anterior pituitary is less responsive to in vitro stimulation with CRH. To test whether rats previously exposed to IS are also less sensitive to the ACTH-secreting effects of CRH in vivo, rats ($n = 100$; 5–20/group) were exposed to IS or remained in their home cages. 24 h after this treatment, rats were injected i.p. with 0 or 1 $\mu\text{g}/\text{kg}$ CRH. Unanesthetized rats were decapitated 15, 30, or 60 min following injection and trunk blood was collected for measurement of ACTH levels.

Results

Since ACTH levels were not different 15, 30, or 60 min after vehicle injection in either HCC or IS, these groups were combined into single HCC-vehicle and IS-vehicle groups. CRH injection elevated ACTH levels in both HCC and IS animals. However, CRH-induced elevations in ACTH levels were significantly more pronounced in animals previously exposed to IS (fig. 5). These observations were supported by statistical analysis. ANOVA revealed a significant drug \times stress interaction ($F(3,92) = 3.904$, $p < 0.05$). Post hoc tests revealed that prior exposure to IS did not alter ACTH levels after i.p. vehicle injection or 15 min after i.p. CRH injection (both $p > 0.05$). However, as compared to HCC rats, rats previously exposed to IS had significantly increased ACTH levels 30 ($p < 0.05$) and 60 ($p < 0.0005$) min after i.p. CRH injection.

Fig. 5. ACTH response to in vivo CRH challenge in inescapable tail shock (IS) rats. Rats were exposed to IS or remained in their home cages as controls (HCC). 24 h later, rats were injected intraperitoneally with vehicle of 1 $\mu\text{g}/\text{kg}$ CRH. Unanesthetized rats were rapidly decapitated 15, 30, or 60 min following injection. Prior exposure to IS sensitized the ACTH response to in vivo CRH challenge. Values are means \pm SE.



Discussion

One obvious explanation for the increased responsiveness of the HPA axis after IS exposure is diminished effectiveness of GC negative feedback. While IS animals are resistant to the effects of DEX [16], there is no change in GR expression in the pituitary, hypothalamus, or hippocampus after IS exposure [15]. Data presented here reveal that the complete elimination of adrenal GCs (and thus GC negative feedback) did *not* eliminate the differences in HPA responsiveness between HCC and IS rats. While this finding does not exclude the possibility that IS exposure produces an impairment in the effectiveness of GC negative feedback, it does indicate that differences in the efficiency of GC negative feedback alone cannot account for the observed differences in HPA axis output between rats previously exposed to HCC or IS treatment. The stimulatory drive on some component of the HPA axis must be increased by IS, in addition to any changes in negative feedback. Indeed, assessment of *c-fos* mRNA and POMC hnRNA reveals that the anterior pituitary is more strongly activated by subsequent challenge in rats previously exposed to IS. Moreover, IS-induced sensitization of the *c-fos* mRNA response in the pPVN clearly indicates that stimulatory input to the pPVN and/or the response of the pPVN to that input is enhanced in rats

previously exposed to IS. Such alterations could undoubtedly mediate the sensitization of the HPA axis observed after IS.

AVP is a factor that could contribute to the sensitization of the HPA axis in rats previously exposed to IS, as it not only potently enhances the ACTH-releasing effects of CRH, but also has been implicated as a mediator of sensitization of the HPA axis in a variety of animal models [17, 24, 26, 33]. While a number of challenges that produce hyper-reactivity of the HPA axis are associated with a phenotypic shift of CRH-containing cells of the pPVN toward the co-production and co-secretion of AVP [34], this shift is certainly not a precondition for sensitization of the HPA axis as neither amphetamine- nor social defeat-induced sensitization of the HPA axis are associated with increased AVP levels [14, 35].

However, data presented here do not support an involvement of AVP in IS-induced HPA sensitization. First, IS exposure does not result in elevated levels of AVP mRNA in the pPVN. Of course, mRNA expression may not always directly reflect peptide expression because changes in peptide expression can be caused by changes in translation, posttranslational processing or protein degradation, and need not be preceded by changes in transcription or mRNA stability. Thus, IS exposure could produce changes in AVP peptide expression in the pPVN indepen-

dent of changes in AVP mRNA expression. However, another finding presented here suggests that changes in AVP peptide do not contribute to the IS-induced HPA sensitization. These data show that IS exposure sensitizes the rapid increase in POMC hnRNA levels caused by subsequent challenge. This is important because AVP does not induce POMC gene transcription and, in fact, has a negative effect on POMC hnRNA expression [36, 37]. Moreover, while AVP undoubtedly synergizes with CRH to enhance ACTH secretion, this synergism does not apply to induction of POMC gene transcription [36, 38, 39]. Thus, it appears that a factor(s) other than AVP contributes to the sensitization of the HPA axis induced by IS exposure.

The data presented here suggest that IS exposure may produce a persistent increase in activity of the pPVN since CRH mRNA expression within the pPVN is persistently elevated after IS exposure. CRH related peptides in the pPVN have also been shown to respond independently to stress and adrenalectomy [40]. Furthermore, anterior pituitaries taken from rats 24 h following IS exposure exhibit an impaired ACTH response to *in vitro* CRH challenge. This impairment is consistent with enhanced central drive, as repeated or prolonged stimulation with CRH reduces CRH receptor binding and can cause reduced ACTH responsiveness or desensitization [29–31]. Thus, while IS-induced alterations in the response patterns of neurons within the pPVN to subsequent challenge have yet to be examined, the current data suggest that the proximate cause of IS-induced HPA sensitization is increased CRH release from the pPVN.

Prior exposure to a single session of IS enhances the ACTH response to *in vivo* CRH challenge, but impairs the ACTH response to *in vitro* CRH challenge. While these results are seemingly contradictory, the exaggerated *in vivo* CRH-induced ACTH release in rats previously exposed to IS might not depend upon direct action of exogenous CRH at the anterior pituitary, but rather activation of sensitized central circuits that impact the pPVN. CRH administration produces a number of peripheral and central changes in addition to the stimulation of ACTH release from the anterior pituitary. For example, peripheral injection of CRH increases colonic motility and rapidly induces neuronal activation in several brain regions, including the pPVN, as assessed by *c-fos* protein expression [41, 42]. This central activation is unlikely to be related to brain influx of CRH since the peptide does not cross the blood-brain barrier [43, 44]. Nonetheless, peripheral CRH injection may produce exaggerated ACTH release in rats previously exposed to IS

because peripheral CRH, like other stimuli that produce exaggerated ACTH release in IS rats (i.e. placement on an elevated pedestal), activates central circuits that project to the pPVN.

Whether stimulatory input to the pPVN and/or the response of the pPVN to that input is enhanced in rats previously exposed to IS remains unclear. Changes that mediate IS-induced HPA sensitization may be intrinsic to the pPVN and involve alterations in the responsiveness of local cells to input. Alternatively, afferent inputs to the pPVN may be sensitized, thereby producing more intense activation of the pPVN upon their own activation.

Some advances have been made recently in determining the neural circuitry and/or neurochemicals that contribute to sensitization of the HPA axis. For example, the posterior paraventricular thalamic nucleus has been implicated in the facilitation of the HPA axis response to restraint after chronic cold exposure [5]. However, the role of this pathway has not been examined in the context of IS-induced sensitization of the HPA axis. Additionally, in previously stressed rats, exogenous CORT administration facilitates the ACTH response to a novel stressor [45, 46]. An intriguing possibility is that the elevated levels of basal CORT produced by IS contribute to the IS-induced facilitation of the ACTH response to a subsequent challenge. However, ADX rats that were exposed to IS still exhibited an enhanced ACTH response to subsequent challenge, indicating that the presence of CORT is not necessary for the development or expression of IS-induced HPA sensitization. Finally, many stimuli that produce sensitization of the HPA axis are also associated with increased norepinephrine release in the hypothalamus [35, 47–49]. Noradrenergic input to the CRH neurons of the pPVN is derived principally from the brainstem nucleus tractus solitarius and the locus coeruleus [50, 51]. Moreover, administration of norepinephrine activates the HPA axis as assessed by CRH, ACTH, and CORT secretion [52–55]. Interestingly, the responsiveness of locus coeruleus neurons is increased following exposure to IS [56]. Further experiments would be needed to elucidate the role of norepinephrine in the IS-induced HPA sensitization.

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