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Pituitary CRH-binding protein and stress in female mice

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Abstract

The CRH-binding protein (CRH-BP) binds CRH with very high affinity and inhibits CRH-mediated ACTH release from anterior pituitary cells *in vitro*, suggesting that the CRH-BP functions as a negative regulator of CRH activity. Our previous studies have demonstrated sexually dimorphic expression of CRH-BP in the murine pituitary. Basal CRH-BP expression is higher in the female pituitary, where CRH-BP mRNA is detected in multiple anterior pituitary cell types. In this study, we examined stress-induced changes in CRH-BP mRNA and protein expression in mouse pituitary and assessed the *in vivo* role of CRH-BP in modulating the stress response. Pituitary CRH-BP mRNA was greater than 200-fold more abundant in females than males, and restraint stress increased pituitary CRH-BP mRNA by 11.8-fold in females and 3.2-fold in males as assessed by qRT-PCR. In females, restraint stress increased CRH-BP mRNA levels not only in POMC-expressing cells, but also in PRL-expressing cells. The increase in female pituitary CRH-BP mRNA following stress resulted in significant increases in CRH-BP protein 4–6 h after a 30-minute restraint stress as detected by [^{125}I]-CRH:CRH-BP cross-linking analyses. Based on this temporal profile, the physiological role of CRH-BP was assessed using a stressor of longer duration. In lipopolysaccharide (LPS) stress studies, female CRH-BP-deficient mice showed elevated levels of stress-induced corticosterone release as compared to wild-type littermates. These studies demonstrate a role for the pituitary CRH-BP in attenuating the HPA response to stress in female mice.

Keywords

CRH-binding protein; stress; pituitary; female; LPS; restraint

Introduction

Corticotropin releasing hormone (CRH), a 41-amino acid peptide, is the primary neuroendocrine mediator of the mammalian stress response (1). Following stress, CRH is

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synthesized in the paraventricular nucleus of the hypothalamus (PVN) and secreted into the pituitary portal vasculature. CRH binds to CRH receptors on anterior pituitary corticotropes, stimulating the release of adrenocorticotropic hormone (ACTH) (reviewed in (2)). ACTH, in turn, increases the production and release of glucocorticoids from the adrenal cortex. Glucocorticoids initiate the cascade of metabolic and physiologic changes associated with the classic stress response and feed back on the hypothalamic-pituitary-adrenal (HPA) axis to assist in the return to homeostasis. In addition to the well-characterized function of CRH in the HPA axis, CRH is expressed in numerous sites within the central nervous system where it acts as a neurotransmitter mediating the behavioral, autonomic, and metabolic responses to stress (2, 3).

The CRH family of ligands, including CRH, urocortin I (4), urocortin II/Stresscopin-related peptide (5, 6) and urocortin III/Stresscopin (5, 7), bind and signal via two cognate seven transmembrane G-protein coupled receptors, CRH receptor type I (CRH-R1) and CRH receptor type II (CRH-R2). CRH-R1 has been shown to mediate the classic neuroendocrine response to stress (8, 9). In the rodent, CRH-R1 is expressed in numerous locations within the central nervous system and the anterior and intermediate lobes of the pituitary (10, 11). Within the anterior pituitary, CRH-R1 has been localized largely to a subset of corticotropes, a target site of stress-induced CRH signaling (10, 12). More recently, CRH-R1 has also been detected in lactotropes, gonadotropes and thyrotropes in mouse pituitary (12). CRH-R2, which preferentially binds to the urocortins, is thought to modulate the effects of central CRH-R1 signaling (reviewed in (13)). In the pituitary, CRH-R2 has been localized to a subset of gonadotropes in male rats (14).

CRH-binding protein (CRH-BP), a 37kDa secreted glycoprotein, can also bind CRH and modulate its signaling at CRH receptors (15–17). While CRH-BP is structurally and functionally distinct from the CRH receptors, it binds CRH with equal or higher affinity than the receptors, placing it in an ideal position to regulate CRH activity (18, 19). CRH-BP is expressed at numerous sites throughout the central nervous system, including sites of CRH and/or CRH-receptor expression, as well as in the anterior pituitary, the target site of hypophyseal CRH and a site of CRH-BP/CRH-R1 co-localization (20–22). Recombinant CRH-BP attenuates CRH-induced ACTH secretion in primary anterior pituitary cells and AtT-20 cells (23, 24). These studies suggest that the function of pituitary CRH-BP is to inhibit CRH signaling at its receptors by binding and sequestering the ligand or targeting it for degradation.

In vivo studies using transgenic and knockout CRH-BP mouse models are consistent with the *in vitro* findings, and support the hypothesis that CRH-BP is a negative regulator of CRH activity (25–27). Other studies have examined the regulation of the CRH-BP promoter or endogenous CRH-BP expression. Numerous factors activated by the stress response, including CRH, downstream members of CRH-activated signaling pathways (i.e., cAMP), and glucocorticoids are all important regulators of CRH-BP gene expression and protein secretion (28–33). *In vivo* studies showed that restraint stress increased CRH-BP gene expression 3-fold in male rat pituitary, while adrenalectomy decreased pituitary CRH-BP mRNA to 8% of control levels (31). Restraint stress and predator odor stress also increased CRH-BP gene expression in the basolateral amygdala, a central target site of CRH signaling

(34–36). Together, these data demonstrate that stress and glucocorticoids are important regulators of CRH-BP expression and suggest that increased CRH-BP may be an important homeostatic regulator of CRH activity following stress.

Finally, CRH-BP is expressed in a striking sexually dimorphic pattern in the murine pituitary, with significantly greater expression in females (37). In male pituitary, CRH-BP was exclusively localized to a subset of corticotropes (21, 37). In contrast, dual *in situ* hybridization analysis localized CRH-BP mRNA expression to multiple cell types in the female murine pituitary including proopiomelanocortin (POMC)-, prolactin (PRL)-, and luteinizing hormone β (LHβ)- expressing cells (corticotropes, lactotropes and gonadotropes, respectively) (37). Surprisingly, the majority of CRH-BP transcript in female pituitary, nearly 80% at proestrus, was localized to PRL-expressing cells. Cell-specific functional roles for CRH-BP remain unclear; data suggest that the abundant levels of CRH-BP, constitutively secreted from multiple anterior pituitary cell types, could have a profound effect on stress-induced CRH signaling at the corticotrope. While stress has been shown to increase CRH-BP expression in the male rat pituitary (31), it is unknown whether CRH-BP in murine pituitary is similarly regulated by stress and whether the regulation involves multiple cell types in females. Since multiple anterior pituitary cell types express glucocorticoid and CRH receptors and are responsive to changes in glucocorticoid levels and/or stress, we hypothesized that stress would increase CRH-BP expression in multiple cell types in the female anterior pituitary and that increased pituitary CRH-BP after stress would contribute to attenuation of HPA axis activation in the female mouse.

Materials and Methods

Animals and sample collection

Eight to twelve-week old male and female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for all restraint stress experiments. Female CRH-BP knockout mice (26) were bred in our facility and backcrossed to C57BL/6J mice for >15 generations. CRH-BP knockout mice and wild-type (wt) controls for LPS studies were 4–10 months old at time of experiments. Mice were maintained on a 14 h light/10 h dark schedule with lights on between 0600 h and 2000 h and had access to food and water *ad libitum*. Mice were habituated to single housing for >1 week prior to stress experiments. All animal experiments were conducted according to NIH guidelines for animal care and were approved by the University of Michigan Committee on Use and Care of Animals.

Estrous cycle staging was performed as described by Speert et al. (37). For stress studies, vaginal smears were either collected 4 h prior to the stress experiments or determined postmortem. Cycling female mice in estrus, metestrus, and diestrus were used for time course (mRNA and protein) and dual *in situ* hybridization studies. Proestrus mice were excluded, as CRH-BP is known to be increased at proestrus (37).

Restraint stress

Mice were gently restrained for 30 minutes in Teflon wraps, fastened with Velcro for a snug fit. Mice were then returned to their home cages until the appropriate time point (60, 120, or

240 minutes after initiation of 30 minute stress for mRNA analyses or 1, 2, 3, 4, or 6 h after initiation of 30 minute stress for protein analyses). Unstressed controls were removed from their cage and immediately euthanized. Mice were euthanized by rapid cervical dislocation; trunk blood was collected and pituitaries were removed and immediately placed at −80°C for subsequent experiments.

Lipopolysaccharide (LPS)

Lipopolysaccharide (Escherichia Coli 0111:B4 (Sigma)) was dissolved in pyrogen-free sterile saline and administered at 2 mg/kg ip. Mice were returned to their home cage after injection and left undisturbed for 6 h. Mice were euthanized and trunk blood and pituitaries collected.

Blood collection and corticosterone assay

Trunk blood was collected into chilled tubes containing 0.5M EDTA and aprotinin (50 KIU). Plasma was collected after centrifugation at 3000 rpm for 10 minutes. Plasma corticosterone was measured using the double antibody corticosterone RIA kit (MP Biomedicals) according to manufacturer's instructions.

Real Time RT-PCR

Restraint stress experiment—Total cellular RNA was isolated from single pituitaries by homogenization with a Polytron (Kinematica, Inc. Johnson City, TN) in Trizol reagent (Invitrogen, Carlsbad, CA). Total pituitary RNA was treated with RNase-free DNase according to the manufacturer's protocol (DNAfree-Turbo; Ambion, Austin, TX). Each individual pituitary yielded approximately 10μg of RNA. Five micrograms of DNased RNA was used for first-strand cDNA synthesis using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). PCR reactions (25μl) contained 1 or 2μl cDNA template (female and male, respectively), 12.5μl 2xSYBR Green I Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA), and 250nM forward and reverse primers for mCRH-BP or TATA-binding protein (TBP) (38). Reactions were carried out in a Bio-Rad iCycler as described (38) with the following modifications. The cycling conditions included 3 minutes at 95°C, 34 cycles of 95°C for 20 seconds, 62°C for 20 seconds and 72°C for 20 seconds, followed by melt-curve analysis. mCRH-BP gene-specific expression was normalized in parallel reactions with TBP gene expression, which was not regulated by gender or restraint stress. Changes in mCRH-BP gene expression following stress were calculated using $2^{-\Delta\Delta CT}$ method (39). Statistical significance was determined using Student's *t* test. *P* values < .05 were considered statistically significant for all experiments.

Time-course experiment after stress—Total cellular RNA was extracted from single female pituitaries and reversed transcribed to cDNA as described above. PCR was as described above with the following minor modifications: 1) 2 ul of cDNA was used per sample; 2) 2X RT^2 SYBR green qPCR master mix (SABiosciences, Frederick, MD) was used requiring a 10-minute hot start. Cycling conditions continued for 40 cycles at the temperatures and times described above followed by melt-curve analysis. Statistical significance was determined using ANOVA followed by Fisher's LSD post-hoc analysis.

Dual in situ hybridization analysis

In situ hybridization riboprobes— ^{35}S-UTP and digoxigenin-labeled riboprobes were generated as previously described (37). CRH-BP was always labeled with ^{35}S-UTP whereas rat PRL and mouse POMC riboprobes were labeled with digoxigenin-11-UTP. A 4:6 digoxigenin-11-UTP: UTP ratio was used for the transcription reactions.

In situ hybridization— Pituitaries from female mice were cryosectioned and stored at 80°C until use (12 μm thick sections, 2 sections/slide, each pituitary yielded approximately 50 sections). The procedure for dual *in situ* hybridization followed Speert et al. (37) with minimal modifications. Briefly, 2–3 slides per mouse were post-fixed for 1 h in 4% paraformaldehyde (in sodium phosphate buffer) and washed three times in 2X SSC. Sections were incubated in 0.25% acetic anhydride in 0.1M triethanolamine for 10 minutes with stirring, rinsed in dH2O, dehydrated and air-dried. Slides were hybridized with a ^{35}S-labeled CRH-BP riboprobe (2×10^6 cpm/slide) and a digoxigenin-labeled riboprobe (PRL or POMC) in 50% formamide hybridization buffer overnight at 55°C. After hybridization, excess unhybridized probe was removed by 2X SSC washes and by incubating slides in RNase A (37 C, 1 h). Slides were washed in decreasing salt solutions (2X, 1X, and 0.5X SSC) before a final high-stringency wash in 0.1X SSC (65°C, 1 h). Slides were cooled to room temperature in 0.1X SSC, followed by equilibration in Buffer 1 (100 mM Tris, pH 8.0, 50 mM NaCl). Slides were blocked in Buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum. Slides were incubated overnight with anti-digoxigenin-AP (Roche Diagnostics, Indianapolis, IN) diluted 1:10,000 in fresh block buffer. Excess antibody was removed by washing in Buffer 1 followed by equilibration in ASB buffer (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM $MgCl_2$). Digoxigenin-labeled products were revealed in a color reaction with NBT/BCIP solution (Roche) diluted in ASB buffer with 1 mM levamisole (to block endogenous alkaline phosphatase). Reactions were stored in the dark until the appropriate color product developed (dark purple stain in cells). Reactions were terminated with several washings in deionized water. Slides were stripped of antibody (0.1 M glycine, pH=2.5) and fixed in 2.5% gluteraldehyde to preserve color. For ^{35}S-CRH-BP detection, slides were dipped in Ilford K5 nuclear emulsion and stored for 3 days in the dark at 4°C. Slides were developed in Kodak D19 developer (2 minutes) and Rapid Fixative (3 minutes). Unstressed and stressed sections were processed together for direct comparison.

Dual in situ hybridization analysis— Since the abundance of each hormone differs, 2 strategies were employed to determine relative counts of labeled cells. For lower abundance POMC-cells, the entire section was scanned and positive cells were counted (4 sections/mouse, n=4 mice). PRL signal was much more abundant, therefore the section was subdivided into fields. Three to five fields per section were analyzed for each mouse (2–4 sections/mouse, n=4–5 mice). This yielded 500–1450 PRL-positive cells counted per section. Dual-labeled cells were digoxigenin-positive and contained silver grains (CRH-BP signal). The percentage of POMC- or PRL-expressing cells that expressed CRH-BP mRNA (3 or more grains per cell) was calculated, and control and stressed values were compared by Student's t-test.

Silver grain counting—Silver grains were counted for double-labeled cells only. As grain number/cell cannot be accurately discerned above approximately 20 grains, the numbers of grains were grouped into bins of 3–6, 7–10, 11–20, and greater than 20.

[^{125}I]-CRH:CRH-BP chemical cross-linking

Pituitaries were isolated from experimental animals as described above. Individual pituitaries were lysed with a glass dounce homogenizer in 50 μl cold lysis buffer (50 mM Tris (pH 7.4), 6 mM $MgCl_2$, 1 mM EDTA, 10% sucrose, 2X Protease Inhibitor cocktail (Sigma Chemical Co., St. Louis, MO)) and centrifuged for 10 minutes, 10,000 rpm, at 4°C. The supernatant was removed and protein content was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). CRH-BP protein levels were determined by chemically cross-linking equivalent amounts of protein lysate (50 μg) to (2-[^{125}I]iodohistidyl^{32}) human CRH (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, UK) using disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) as previously described (23). Cross-linked products were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, dried, and exposed to a phosphorimager screen for 4–7 days. Intensities of [^{125}I]-CRH:CRH-BP cross-linked product (41 kDa) were measured using ImageQuant analysis software and integrated densities were analyzed using one-way ANOVA followed by Fisher's LSD post hoc analysis to determine significance between time points. Previous studies have demonstrated that the cross-linking assay provides a quantitative measure of CRH-BP protein levels ((40) and data not shown).

Results

Restraint stress increases CRH-BP mRNA expression in both male and female mouse pituitary

Acute restraint stress is a potent activator of the HPA axis, stimulating peak secretion of ACTH and corticosterone within 20–40 minutes after the initiation of the stress (25, 26). CRH-BP mRNA expression following acute restraint stress in male and female mouse pituitaries was determined. Control animals (no stress) were sacrificed prior to the start of the experiments, while experimental mice (stress) were restrained for thirty minutes, returned to their home cages for thirty minutes, and sacrificed. Total RNA from individual stressed or control pituitaries was used for quantitative real time RT-PCR (qPCR). Basal expression of CRH-BP mRNA was higher in female pituitaries as compared to males (>200-fold), consistent with our earlier observations of sexually dimorphic expression (37). Restraint stress induced a 3.2-fold increase in CRH-BP mRNA in male pituitary (Table 1, $t(4) = -3.29$, $P < .05$). Strikingly, pituitary CRH-BP mRNA expression increased 11.8-fold after stress in female mice (Table 1, $t(4) = -5.87$, $P < .01$).

To determine the temporal profile of increased CRH-BP mRNA following stress, female mice were stressed by restraint for 30 minutes, returned to their homecage, and euthanized 60, 120 or 240 minutes after the initiation of stress (Figure 1). Consistent with the previous study, qPCR analysis showed a >10-fold increase in CRH-BP mRNA in female pituitaries following stress. One-way ANOVA revealed a significant effect of time ($F(3,8)$, $F=27.2$, $P < .001$); post-hoc analysis indicated that CRH-BP mRNA was elevated above control at 60

and 120 minutes after initiation of 30 minute restraint stress (*P*s < .01, Figure 1), with CRH-BP mRNA levels decreased to control levels by 240 minutes. Similar results were obtained with ribonuclease protection assays (data not shown), showing 10-fold increases in CRH-BP mRNA at 30 minutes and 60 minutes after the initiation of 30 minute restraint stress in female pituitary. The very low levels of CRH-BP mRNA in male pituitary led us to focus our *in situ* hybridization studies on CRH-BP mRNA in female pituitary.

Stress increases CRH-BP mRNA expression in multiple pituitary cell types

CRH-BP mRNA is expressed in multiple cell types in the female pituitary, including a sub-set of POMC-, PRL-, and LHβ-positive cells (37). The abundant increase in total CRH-BP mRNA in the female pituitary following stress led us to hypothesize that stress positively regulated CRH-BP expression in several pituitary cell types. Dual *in situ* hybridization analysis was used to examine changes in the cell-specific distribution of CRH-BP mRNA before and after restraint stress. CRH-BP mRNA co-localized with a sub-set of POMC- and PRL-positive cells in the basal state, consistent with earlier studies (37), and restraint stress elevated CRH-BP expression in both POMC- and PRL-positive cells in the female anterior pituitary (Figures 2A and 2B). The percentage of POMC- and PRL-positive cells with detectable levels of CRH-BP significantly increased after stress (Figure 2C, POMC, $P < .05$; Figure 2D, PRL, $P < .001$). To determine if the amount of CRH-BP transcript per cell increased following stress, the number of silver grains (CRH-BP signal) per POMC:CRH-BP or PRL:CRH-BP double-positive cell was counted. Stress increased the levels of CRH-BP mRNA per POMC:CRH-BP or PRL:CRH-BP double-labeled cell as illustrated in the shift in the histogram profiles before and after stress (Figures 2E and 2F). Due to the small percentage of LHβ-positive cells that expressed CRH-BP basally, we were unable to reliably quantify stress-induced changes in CRH-BP expression in this cell type. These data support roles for both lactotrope-derived and corticotrope-derived CRH-BP in the female stress response.

Time course of pituitary CRH-BP protein levels after stress in female mice

To examine CRH-BP protein levels following stress, pituitaries were collected from female mice either directly before (0, control) or 1, 2, 3, 4, or 6 h after the initiation of a 30-minute restraint stress. Equal amounts of individual pituitary extracts were cross-linked to [^{125}I]-CRH and resolved by SDS-PAGE. Quantitative analysis of the [^{125}I]-CRH:CRH-BP complex in the cross-linked pituitary extracts revealed a 3-fold increase in CRH-BP protein over 6 h in response to the 30 minute restraint stress (Figure 3). One way ANOVA showed a significant time-dependent increase in functional CRH-BP protein levels in female pituitaries (Figure 3, $F(5,12)=6.9$, $P < .01$); post-hoc analysis revealed that 4 h and 6 h levels were increased over 0 and 1 h (*P*s < .05). No [^{125}I]-CRH:CRH-BP complex was detected using male pituitary extracts (data not shown), consistent with the low levels of CRH-BP mRNA in male pituitary.

LPS stress

After acute restraint stress, ACTH and corticosterone levels peak within 20–40 minutes after the initiation of the stress and return to baseline by 90 minutes (25, 26). In contrast, CRH-BP

protein levels increase at a slower rate and remain significantly elevated for at least 6 h. These results suggest that a stressor of longer duration would more readily reveal the role of CRH-BP in modulation of the stress response. Lipopolysaccharide (LPS) is an immune stressor that increases both cytokine and HPA activity for extended periods of time after administration. Previous studies with LPS have shown significant increases in corticosterone (CORT) and PVN CRH mRNA levels and decreased pituitary CRH receptor levels up to 6 h after LPS administration (41–44). We have detected increased pituitary CRH-BP cross-linking activity levels in female mice at 6 h after LPS administration compared to saline vehicle control (data not shown), indicating a robust increase in CRH-BP protein levels after LPS stress. To test the role of CRH-BP in the return of the HPA axis to homeostasis after this immune stressor, plasma CORT levels were measured 6 h after LPS injection in female wild-type (wt) and CRH-BP-deficient (CRH-BP KO) mice. LPS increased plasma CORT levels in female CRH-BP KO and wt mice compared to saline injection, as indicated by a significant effect of treatment ($F(1,34)=122.9$, $P < .0001$). The treatment x genotype interaction ($F(1, 34)= 4.1$, $P = .05$) indicated CORT levels were significantly higher at 6 h after LPS in CRH-BP KO mice compared to wt littermates ($P < .01$; Figure 4), suggesting that the normal increase in CRH-BP levels after LPS stress plays an important role in the termination of the stress response in female mice.

Discussion

In this study, we examined stress-induced changes in CRH-BP mRNA and protein expression in murine pituitary and assessed the physiological role of CRH-BP in modulating the stress response. Based on previously demonstrated positive regulation of CRH-BP by both CRH and glucocorticoids (28, 29, 31), we hypothesized that stress would increase CRH-BP expression in the male and female murine pituitary. CRH-BP mRNA was detected by qPCR in both male and female mouse pituitaries in the unstressed state, with CRH-BP mRNA levels much more abundant in females compared to males. Restraint stress increased CRH-BP gene expression 3.2-fold in the male mouse pituitary, whereas the same stress increased CRH-BP mRNA levels by 11.8-fold in the female mouse pituitary. In female mice, restraint stress increased CRH-BP mRNA in multiple pituitary cell types, increasing expression not only in POMC-expressing cells (the sole site of CRH-BP mRNA pituitary expression in males), but also in PRL-expressing cells. The dramatic increase in pituitary CRH-BP mRNA after stress resulted in elevated CRH-BP protein levels as well, with significant increases in CRH-BP protein observed in female pituitaries at 4–6 h after a 30 minute restraint stress. Physiological experiments implicate a role for the pituitary CRH-BP in attenuating the HPA response to LPS stress in females, as female CRH-BP KO mice exhibit higher levels of LPS stress-induced CORT release as compared to wt littermates.

The increased basal expression of CRH-BP in female mouse pituitary compared to male, as measured by qPCR, was consistent with previous ribonuclease protection assays and *in situ* hybridization studies from our laboratory showing the sexually dimorphic expression of CRH-BP in mouse pituitary (37). This work had also shown that multiple cell types including corticotropes, lactotropes and gonadotropes express CRH-BP in female mice, while male mice and ovariectomized female mice exhibit detectable CRH-BP mRNA only in corticotropes (37). Estrogen positively regulates basal CRH-BP expression *in vivo* (37)

and the induction is thought to involve classic ERE half-sites in the 5′ proximal promoter (45). Expression of ERα and ERβ in multiple anterior pituitary cells types including abundant expression of ERα in lactotropes and corticotropes, major sites of CRH-BP expression, suggests a mechanism for direct estrogen regulation of CRH-BP transcription (46).

Our results also clearly demonstrate increased pituitary CRH-BP expression in response to stress. In the male mouse pituitary, restraint stress increased CRH-BP mRNA levels by 3.2-fold, consistent with our earlier observations in the male rat (31). In the current study, restraint stress also increased CRH-BP mRNA expression in the female mouse pituitary, inducing a 11.8- fold increase over control levels. Stress-mediated increases in CRH-BP expression involved multiple cell types in the female anterior pituitary. Restraint stress increased both the percentage of POMC-positive cells expressing CRH-BP as well as the amount of CRH-BP expressed per POMC:CRH-BP co-labeled cell. Similarly, both the number of PRL-positive cells expressing detectable CRH-BP as well as the amount of CRH-BP per PRL:CRH-BP double positive cell increased significantly in female pituitary following stress.

Stress increases CRH and glucocorticoid levels, and both factors could directly influence pituitary CRH-BP expression. Corticosterone has been shown to increase pituitary CRH-BP mRNA in adrenalectomized male rats (31), suggesting a positive regulation of pituitary CRH-BP by corticosterone. This effect is likely mediated by the glucocorticoid receptor (GR) which is expressed in multiple anterior pituitary cell types, including corticotropes (47). CRH also increases CRH-BP transcription and protein expression (29) and CRH-BP promoter activity (28). In the pituitary, this effect is likely mediated by CRH-R1, and *in situ* hybridization experiments have shown that CRH-R1 mRNA is expressed in multiple anterior pituitary cell types (12). While the majority of CRH-R1 expression is localized to POMC-positive cells (50% females and 70% males), a significant percentage of the CRH-R1-positive cells in female pituitary express PRL (40%) (12). CRH and stress have also been shown to stimulate PRL secretion (48–53) and CRH-R1 appears to mediate this effect. CP-154,526, a CRH-R1 selective antagonist, reduced immobilization stress-induced PRL release (51) and hypoxia stress-induced increases in PRL mRNA (53), supporting interactions between CRH, the stress system, and lactotrope function. Thus, CRH and CORT release following stress are likely key factors that increase CRH-BP expression in corticotropes in male and female mice and lactotropes in female mice.

Based on the increased CRH-BP mRNA after stress, we predicted that CRH-BP protein levels would also be elevated after stress. CRH-BP protein levels were assessed using the previously characterized [^{125}I]-CRH:CRH-BP cross-linking assay (23). CRH-BP could not be detected in male pituitary extracts from basal or stressed mice with the cross-linking assay, consistent with the very low levels of CRH-BP mRNA in male pituitary. In contrast, the stress-mediated increases in pituitary CRH-BP protein levels in female mice were readily detected using the cross-linking assays. This result is consistent with previous studies showing increased CRH-BP protein in media from astrocyte or mixed neuronal cultures or total cellular protein from fetal amygdalar cultures after 12–24 h treatment with CRH or forskolin (29, 30, 54). While pituitary CRH-BP mRNA levels returned to baseline by 4 h

after restraint stress, female CRH-BP protein levels were significantly increased by 4 h and remained elevated at 6 h. The temporal profile of stress-mediated increases in pituitary CRH-BP protein levels shown in Figure 3 suggested that CRH-BP may not function to alter CRH-mediated HPA activation immediately following a brief moderate intensity stress (e.g. 30 minute restraint stress), but may have significant biological effects on CRH activity after repeated brief stressors or a more prolonged stressor. Preliminary experiments with female CRH-BP-deficient mice support this hypothesis, as no significant differences were detected in CORT levels at 30–60 minutes after 30 minute restraint stress in CRH-BP KO and wt mice. Male CRH-BP KO mice also show no significant difference from wt littermates in HPA response to 30 minute restraint stress (26).

We further tested this hypothesis using LPS, an immune stressor that increases both cytokine and HPA activity for extended periods of time after administration. Six hours after LPS administration, CORT and PVN CRH mRNA levels remain significantly increased (44, 55), and pituitary CRH-BP protein levels are also elevated (data not shown). As shown in Figure 4, female CRH-BP-deficient mice exhibit significantly increased CORT levels compared to wt mice at 6 h post-injection, suggesting that the CRH-BP in wt mice is acting to decrease HPA activation at 6 h post-injection. In the absence of CRH-BP, the HPA response is less restrained, resulting in increased CORT levels. Consistent with our results, transgenic mice overexpressing CRH-BP in many tissues demonstrated a blunted ACTH response at 2–3 h post- LPS injection (27). Thus, the elevated levels of CRH-BP produced in response to stress likely work to reduce HPA activation, providing an additional feedback mechanism for blunting the stress response and allowing the return to homeostasis. This feedback mechanism appears to be especially important in female mice, where stress-mediated CRH-BP regulation and expression is key not only in corticotropes but also in lactotropes. It is possible that CRH-BP is synthesized and released from the lactotrope (a major site of CRH-BP expression in females) principally to attenuate CRH-mediated HPA activation, especially after more intense or longer-acting stressors. However, the regulated CRH-BP expression in lactotropes may also play a novel role in modulating the actions of CRH on CRH-R1-expressing lactotropes, a putative site for integration between stress and prolactin-mediated effects on maternal and immune function (56).

Together these data illustrate that the stress-induced temporal pattern of CRH-BP protein expression in the pituitary may be important for regulating the HPA response to enduring stressors, especially in female mice. Future studies will further examine the role of pituitary CRH-BP in response to various stressor modalities (processive and systemic stressors) and in response to chronic stress. Based on the studies presented here, we propose that the CRH-BP may play a gender-specific role in modulation of the organism’s response to prolonged or chronic stress, potentially regulating stress-sensitivity or resilience to affective disorders.

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Highlights

- Pituitary CRH-BP mRNA and protein are up-regulated by stress.
- In female mice, stress increased CRH-BP in corticotropes and lactotropes.
- Female CRH-BP knockout mice have a greater CORT response to lipopolysaccharide.

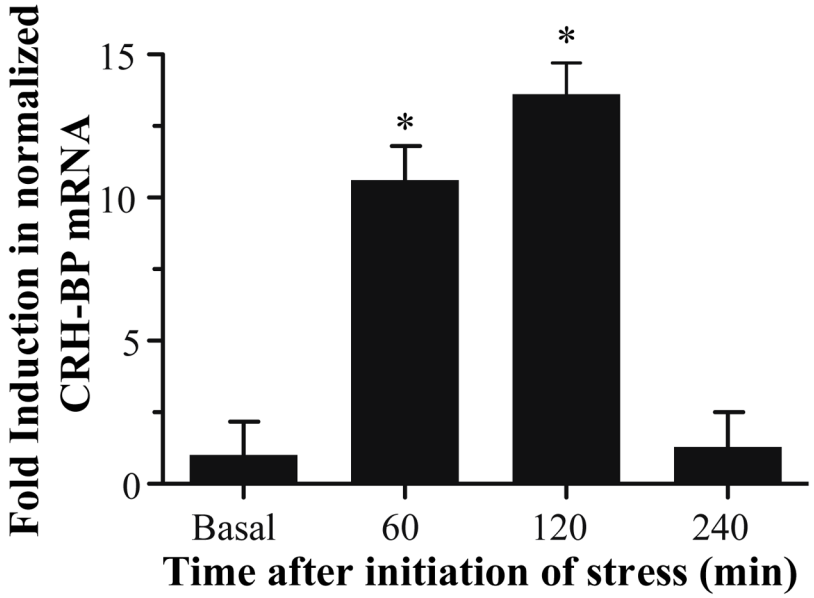

Figure 1.

Restraint stress increases CRH-BP mRNA in the mouse pituitary. Time-course of CRH-BP mRNA expression in female mouse pituitaries following restraint stress. Data represent mean +/− SEM for n= 3–4 mice per time point (* $P < .01$ compared to Basal and 240 minutes).

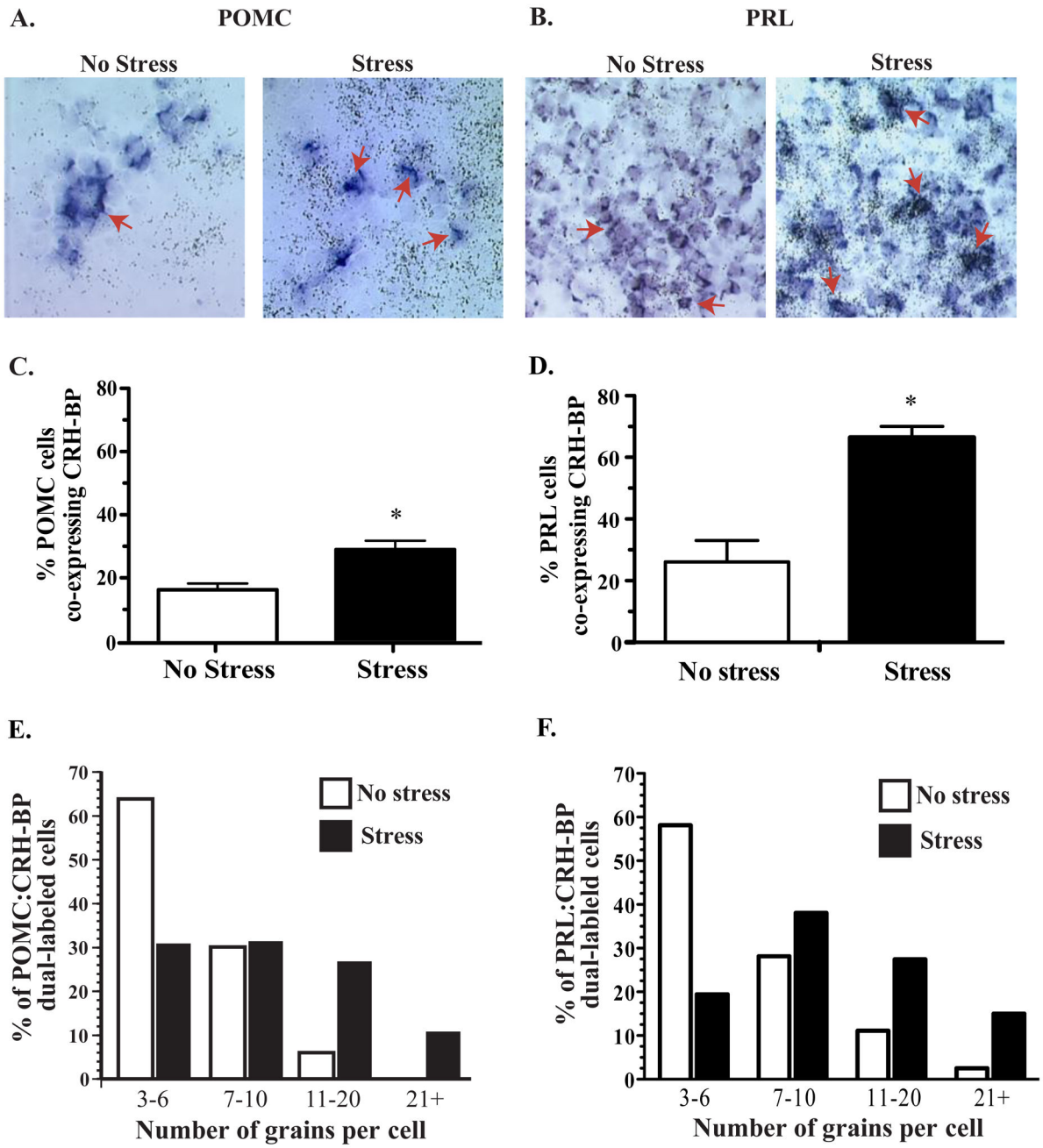

Figure 2.

Stress increases CRH-BP mRNA expression in POMC- and PRL-containing pituitary cells in the female pituitary. **A, B**. Representative brightfield images from dual *in situ* hybridization analysis of radiolabeled CRH-BP riboprobe (black grains) co-hybridized with a digoxigenin-labeled riboprobe (purple cells) for POMC (A) or PRL (B) mRNA. Examples of co-localized signals are represented by red arrows. **C, D**. Stress increased co-localization of CRH-BP with POMC (C) and PRL (D). Histograms illustrate an increased percentage of POMC- or PRL-positive cells that co-express detectable levels of CRH-BP mRNA after stress ($P < .05$). Data represent mean +/− SEM. **E, F**. Semi-quantitative measure of the

relative abundance of CRH-BP mRNA per POMC:CRH-BP or PRL:CRH-BP dual-labeled cell before and after stress. The data represent the percentage of POMC:CRH-BP (E) or PRL:CRH-BP (F) co-localized cells grouped by numbers of silver grains/cell. Hybridization of the unstressed and stressed samples was completed in the same experiment with identical riboprobes and emulsion exposure times.

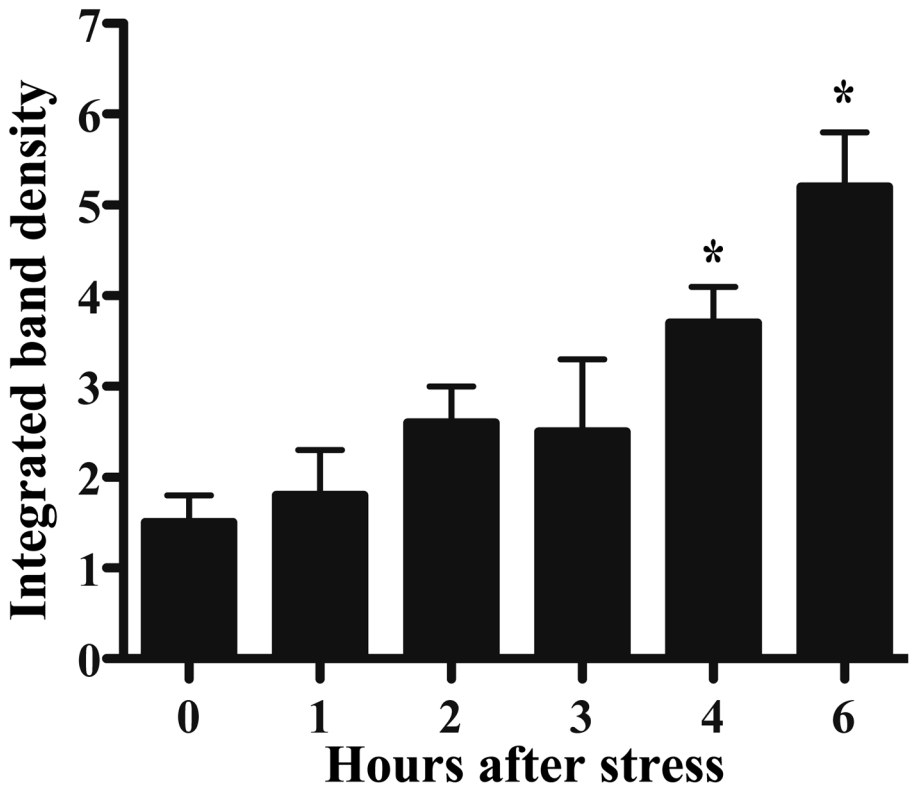

Figure 3.

Restraint stress increases CRH-BP protein levels in the female pituitary, as measured by [^{125}I]-CRH:CRH-BP chemical cross-linking and phosphorimager analysis. Time course of stress-induced CRH-BP protein levels as determined by integrated band density of [^{125}I]-CRH: CRH-BP complex in female pituitary protein extracts. Data represent the mean +/− SEM, n=3 pituitaries per time point (* $P < .05$ compared to baseline (0 h) and 1 h time points).

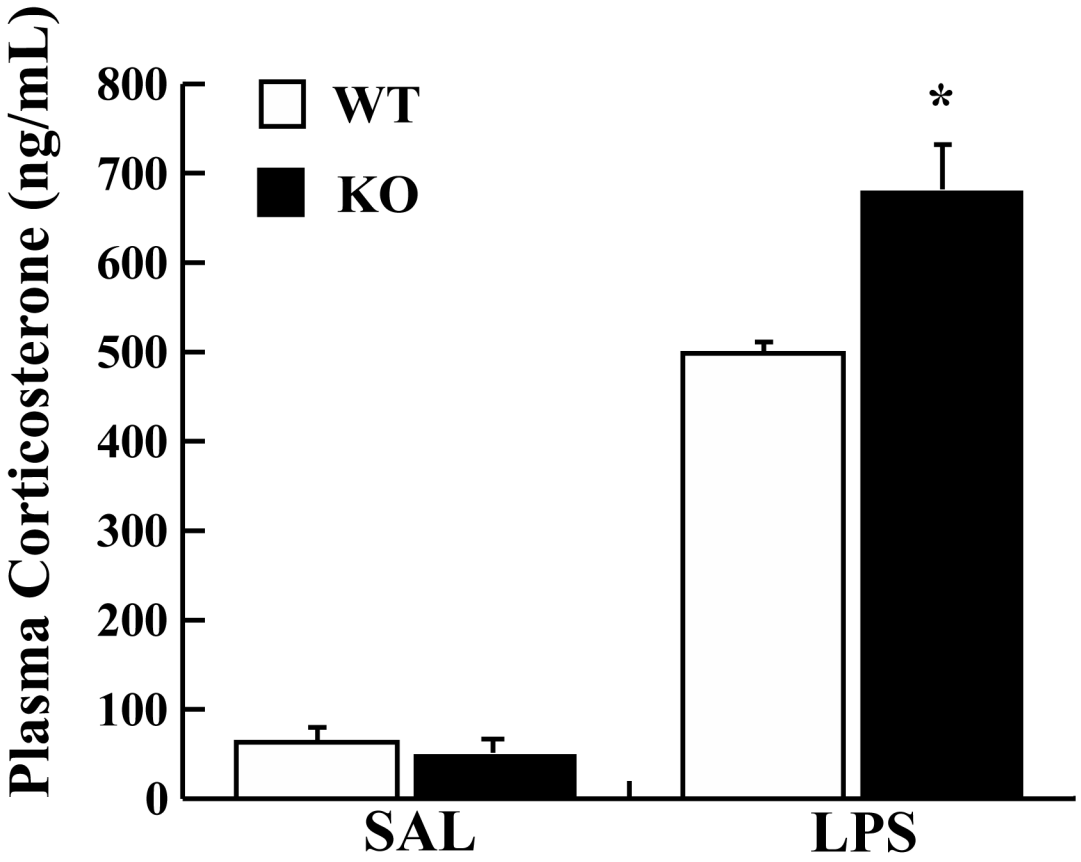

Figure 4.

Elevated CORT levels in female CRH-BP KO mice following LPS injection. Female mice were injected with LPS or saline (SAL) and trunk blood was collected 6 h later. Plasma CORT levels were significantly higher in LPS-injected CRH-BP KO mice vs. wt mice (* $P < .01$). Data represent mean +/− SEM, n=13–14 for LPS. There were no significant differences in basal plasma CORT levels between the genotypes.

Table 1

Quantitative Real-Time PCR Analysis

	CRH-BP average C_T	TBP average C_T	$2^{-\Delta\Delta CT}$	*P* value	Fold Change
FEMALE					
Control	22.08 ± 0.76	22.11 ± 0.50	1.03 ± 0.29		
Stressed	18.30 ± 0.55	21.90 ± 0.17	12.2 ± 3.27	<0.01	11.8
MALE					
Control	30.65 ± 0.24	20.70 ± 0.61	1.03 ± 0.32		
Stressed	29.16 ± 0.42	20.89 ± 0.09	3.35 ± 1.17	<0.05	3.2

Each group represents 3 biological samples assayed in triplicate.

Values shown are averages ± SD. TBP-TATA-binding protein.