Stress Levels of Glucocorticoids Inhibit LHβ-Subunit Gene Expression in Gonadotrope Cells

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Increased glucocorticoid secretion is a common response to stress and has been implicated as a mediator of reproductive suppression upon the pituitary gland. We utilized complementary in vitro and in vivo approaches in the mouse to investigate the role of glucocorticoids as a stress-induced intermediate capable of gonadotrope suppression. Repeated daily restraint stress lengthened the ovulatory cycle of female mice and acutely reduced GnRH-induced LH secretion and synthesis of LH β-subunit (LHβ) mRNA, coincident with increased circulating glucocorticoids. Administration of a stress level of glucocorticoid, in the absence of stress, blunted LH secretion in ovariectomized female mice, demonstrating direct impairment of reproductive function by glucocorticoids. Supporting a pituitary action, glucocorticoid receptor (GR) is expressed in mouse gonadotropes and treatment with glucocorticoids reduces GnRH-induced LHβ expression in immortalized mouse gonadotrope cells. Analyses revealed that glucocorticoid repression localizes to a region of the LHβ proximal promoter, which contains early growth response factor 1 (Egr1) and steroidogenic factor 1 sites critical for GnRH induction. GR is recruited to this promoter region in the presence of GnRH, but not by dexamethasone alone, confirming the necessity of the GnRH response for GR repression. In lieu of GnRH, Egr1 induction is sufficient for glucocorticoid repression of LHβ expression, which occurs via GR acting in a DNA- and dimerization-independent manner. Collectively, these results expose the gonadotrope as an important neuroendocrine site impaired during stress, by revealing a molecular mechanism involving Egr1 as a critical integrator of complex formation on the LHβ promoter during GnRH induction and GR repression. (Molecular Endocrinology 26: 1716–1731, 2012)

NURSA Molecule Pages†: Ligands: Corticosterone.

Stress profoundly disrupts reproductive function. Whether the nature of the stressor is physical (e.g., foot-shock, exercise), immunological (e.g., infection, administration of cytokines or endotoxins), or psychological (e.g., isolation, mental performance tasks), each has been shown to decrease circulating levels of gonadotropins in mammals (1–5). Associated with this reproductive disturbance is an activation of the hypothalamic-pituitary-adrenal axis and an elevation in circulating glucocorticoids from the adrenal cortex, the final hormonal effectors of the hypothalamic-pituitary-adrenal axis. Evidence that the glucocorticoid receptor (GR) antagonist, RU486, attenuates the inhibitory effect of immobilization stress on LH secretion in male rats or psychosocial stress on pituitary responsiveness to GnRH in ovariectomized ewes implies a physiological role for glucocorticoids in mediating the inhibitory effects of stress on LH secretion, although RU486 can also block the effects of progesterone (6–8).

Although there is little doubt that glucocorticoids suppress gonadotropin secretion, the neuroendocrine mechanism underlying this effect is not well understood. Inhibi

† Annotations provided by Nuclear Receptor Signaling Atlas (NURSA) Bioinformatics Resource. Molecule Pages can be accessed on the NURSA website at www.nursa.org.

Abbreviations: ChIP, Chromatin immunoprecipitation; DBD, DNA-binding domain; Egr1, early growth response factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GR, glucocorticoid receptor; GST, glutathione-S-transferase; αGSU, glycoprotein hormone alpha-subunit; SF1, steroidogenic factor 1; TK, thymidine kinase.
bition at the hypothalamic level is supported by evidence that glucocorticoids reduce the frequency of LH pulses in ovary-intact female sheep, ovariectomized female rats, and women during the follicular phase of the ovulatory cycle (9–11). Because LH pulse frequency is generally modulated by the GnRH neurosecretory system, these findings suggest an action of glucocorticoids to suppress the frequency of GnRH pulses. A recent study in follicular phase sheep provides the first definitive evidence that glucocorticoids inhibit GnRH pulses in pituitary portal blood (12). GR is expressed within hypothalamic neurons implicated in GnRH regulation (13), and such neurons provide a potential indirect target by which glucocorticoids may inhibit GnRH secretion or GnRH synthesis (14); however, a direct action within the GnRH neuron itself is supported by evidence that glucocorticoids blunt GnRH synthesis and release from immortalized GnRH neurons, GT1–7 cells (15, 16). Thus, the mechanism whereby glucocorticoids suppress GnRH and LH remains unclear and may involve direct actions upon the GnRH neuron itself, indirect actions via another neuronal cell type, or actions upon an extrahypothalamic site.

With regard to a site outside of the central nervous system, the most obvious possibility is that glucocorticoids act via GR located within gonadotrope cells of the anterior pituitary gland. Evidence that glucocorticoids reduce the amplitude of the LH response to a GnRH challenge in rodents, pigs, cows, and women is consistent with this possibility (17–20). Further, suppression of responsiveness to GnRH in vitro has been observed in rodent, porcine, and bovine pituitary cell cultures, indicating that glucocorticoids can act directly upon the gonadotrope cell to inhibit responsiveness to GnRH (19–21). Consistent with an action upon the gonadotrope cell, GR has been identified in rat gonadotropes (22), and studies in rat and pig primary cells suggest that glucocorticoids inhibit signaling mechanisms downstream of the GnRH receptor, including protein kinase C and cAMP (20, 23). It is not known, however, whether these nongenomic actions of glucocorticoids that inhibit intracellular signaling pathways ultimately lead to a reduction in LH release. Alternatively, evidence suggests that glucocorticoids can act genomically to suppress gonadotrope responsiveness by regulating transcription and translation of the GnRH receptor gene (24, 25).

Another potential mechanism whereby glucocorticoids could diminish GnRH responsiveness of the gonadotrope is via regulation of gonadotropin synthesis. At the molecular level, LH and FSH are glycoprotein hormones that exist as heterodimers, consisting of a common and abundantly expressed glycoprotein hormone alpha-subunit (αGSU) complexed with a unique β-subunit that confers biological specificity (26). Synthesis of the β-subunit gene of each hormone is the rate-limiting step in the overall production of LH and FSH (26, 27). Because expression of each β-subunit is tightly controlled by endocrine, paracrine, and autocrine actions, including hypothalamic GnRH, the activin-inhibin-follistatin system, and steroid hormones of gonadal origin (27, 28), it is possible that GR regulation of transcriptional activity underlies the inhibitory effects of stress on the gonadotrope.

Transcriptional effects of steroid hormones within the gonadotrope have been shown for the gonadotropin genes, including Cga, Fshb, and Lhb (29, 30). With regard to Lhb, androgen repression of Lhb involves protein-protein interactions between the androgen receptor and steroidogenic factor 1 (SF1) and is localized to a bipartite SF1 element within the Lhb proximal promoter, critical for mediating GnRH responsiveness (31, 32). Progesterone repression also involves indirect receptor binding but differs from androgen repression of Lhb gene expression in that, rather than SF1 elements, progesterone repression involves two novel promoter regions located upstream of the SF1 sites. Similar to progestins and androgens, glucocorticoids have been shown to inhibit Lhb gene expression (29, 33), although the mechanism is unclear, raising the possibility that stress impairs fertility by way of disruption of gene expression within the gonadotrope cell.

We initiated two lines of investigation in the mouse to tease apart the mechanisms whereby elevated glucocorticoids inhibit gonadotrope responsiveness during episodes of stress. First, we tested the hypothesis that restraint stress, and/or an elevation in glucocorticoids mimicking the level induced by restraint stress, can disrupt reproductive function and inhibit gonadotrope production of LH in female mice. Second, we conducted a series of studies to examine the molecular mechanisms underlying glucocorticoid regulation of the Lhb promoter utilizing the immortalized LβT2 gonadotrope cell line.

Materials and Methods

Animals

Female C57Bl/6 mice (6 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME), and housed in a UCSD vivarium animal facility under standard conditions. All animals were housed under a 12-h light, 12-h dark cycle (lights on at 0700 h) and provided with food and water ad libitum. Mice were group housed (four females per cage) for 2 wk of acclimatization. All experimentation was performed between 0900 and 1300 h in a room within the vivarium. Care was taken to minimize pain and discomfort for the animals. Mouse colonies were maintained in agreement with protocols approved by the Institutional Animal Care and Use Committee at the University of
California, San Diego. All procedures were approved by the University of California, San Diego IACUC.

**Determination of phase of estrous cycle**

At 8 wk of age, vaginal lavage was performed daily (at 0900 h) by flushing the vagina with distilled H$_2$O. Collected smears were mounted on glass slides and examined microscopically for cell type (34). The smears were classified into one of three phases of estrus: diestrus, proestrus, or estrus. Female mice exhibiting two consecutive 4- to 6-d estrous cycles, including positive classification of diestrus, proestrus, and estrus, were used in animal experiments. Estrous cycle length was calculated as the length of time between two successive occurrences of estrus. The time spent in each cycle stage was calculated as the proportion of time classified in each cycle phase during the observation period, and values were analyzed by two-way repeated measures ANOVA, group (no stress vs. stress) × time (d 1–d 18 vs. d 19–d 36). All statistics were performed using JMP 7.0 (SAS Institute, Cary, NC), and significance was established as $P < 0.05$.

**Restraint stress protocol**

After vaginal lavage and estrus classification, mice were either returned to their group home cage (no stress) or placed individually into clear plastic restraint tubes (stress). The ventilated tubes (Harvard Apparatus, Holliston, MA) are designed to be small enough to restrain a mouse so that it is able to breathe but unable to move freely. The restraint devices were cleaned between uses with soap, water, and ethanol (70%). Mice are continually observed by experienced personnel during the 180-min restraint period. After the restraint period, stress mice are returned to individual home cages or killed by decapitation, and trunk blood or pituitary tissue was collected from individual animals. Hormone values were analyzed by one-way ANOVA followed by Tukey's post hoc test or two-way ANOVA, group (no stress vs. stress) × time (vehicle vs. GnRH).

**Corticosterone response protocol**

Female mice (8 wk of age) were ovarioectomized and allowed to recover for 2 wk before experimentation. To test the LH response to a stress level of corticosterone, animals received a bolus injection of corticosterone (200 ng/kg, sc) or vehicle. After 90 min, animals were killed by decapitation, and trunk blood was collected from individual animals. Hormone values were analyzed by one-way ANOVA followed by Tukey’s post hoc test.

**Hormone analysis**

Trunk blood was collected and serum separated by centrifugation and stored frozen at −20 C before analysis at the Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia (Charlottesville, VA). Corticosterone concentrations were determined by RIA in single 25- to 50-μl aliquots of serum. Assay sensitivity averaged 20.0 ng/ml. LH concentrations were determined by two-site sandwich immunooassay in duplicate 50-μl aliquots of serum. Assay sensitivity averaged 0.07 ng/ml.

**Plasmids**

The −1800 rat LHβ-luc in pGL3 was kindly provided by Dr. Mark Lawson. The 5′-truncations of the −1800-bp LHβ-luc reporter plasmid were created by inserting fragments between KpnI and HindIII in pGL3 have been partially described by Thackray et al. (35): −400, −300, −200, −150, −87. The 5′- and 3′-mutations of either Egr1 or SF1 binding sites in the −200 rat LHβ-luc have been reported previously (35). The reporter plasmids with the multimerized consensus SF1 site (TGACCT-TGA) or consensus Egr1 site (CGCCCCCGCG) were created by ligating an oligonucleotide, containing four copies of the indicated site, between KpnI and NheI in pGL3, upstream of the 81-bp thymidine kinase (TK) promoter. The sequences of all promoter fragments were confirmed by dideoxynucleotide sequencing.

The mouse Ptx1 pcDNA3 expression vector has been previously described (36). The rat Egr1 and mouse SF1 cDNA were kindly provided by Dr. Jacques Drouin and Dr. Bon-chu Chung, respectively. The cDNA were cloned into the pCMV expression vector using the Clal/XbaI restriction sites of both plasmids. The human Egr1 cDNA was provided by Dr. Hermann Pavenstadt and was cut using Xbol/EcoRI and inserted into pGEX-5X expression vector using the SmaI restriction site by blunt end cloning. Dr. Douglass Forbes provided the green fluorescent protein (GFP) expression plasmid. The wild-type rat GR pSG5 plasmid was provided by Dr. Keith Yamamoto. Both GR mutants are in pSG5 and have been previously described; the GRdim4 mutant contains four point mutations that prevent dimerization and DNA binding of the receptor (29), and the GR DNA-binding domain (DBD) mutant contains a mutation in the DNA-binding domain (33).

**Cell culture and transient transfection**

LβT2 cells, cultured as previously described (29), were seeded into 12-well plates at 3 × 10$^5$ cells per well and incubated overnight at 37 C. Each well was transfected with 400 ng of the luciferase-reporter plasmid or control pGL3 vector and 100 ng of a β-galactosidase reporter gene regulated by the TK promoter (TK-βgal) as a control for transfection efficiency using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). In experiments utilizing Egr1, SF1, or Ptx1 to induce promoter activity, cells were also transfected with 100 ng Egr1 (unless indicated otherwise) or empty pCMV vector, 100 ng SF1 or empty pCMV vector, 50 ng Ptx1 or empty pcDNA3 vector. Eighteen hours after transfection, cells were transferred to serum-free DMEM (supplemented with 0.1% BSA, 5 mg/liter transferrin, and 50 μM sodium selenite) containing either the natural glucocorticoid, corticosterone (100 μM, Sigma Aldrich, St. Louis, MO), synthetic glucocorticoid, dexamethasone (100 μM, Sigma Aldrich), or vehicle (0.1% ethanol). When cells were treated with GnRH (10 nM; Sigma Aldrich), treatment with GnRH or vehicle (0.1% BSA) began 6 h before harvest. Cells were harvested and extracts were prepared for assay of luciferase and β-galactosidase activity as previously described (33).

CV-1 cells, a monkey kidney cell line that does not express detectable endogenous GR (37), were seeded into 12-well plates at 1.5 × 10$^5$ cells per well as previously described (38) and treated as indicated above with the following addition. Each
well was transfected with 50 ng of the GR expression vector (wild type or mutant) or empty pG55 vector.

Luciferase reporter experiments were performed in triplicate and were repeated at least three times. To normalize for transfection efficiency, all luciferase values were divided by β-galactosidase, and the triplicate values were averaged. To control for interexperimental variation, the control pGL3 reporter plasmid was transfected with Tk-βgal and any relevant expression vectors, and the average pGL3/βgal value was calculated. Average luc/βgal values were divided by the corresponding pGL3/βgal value. Individual values obtained from each independent experiment were averaged and analyzed by Student’s t test or one-way ANOVA followed by Tukey’s post hoc test.

**Quantitative real-time PCR**

Preparation of cDNA from mouse pituitary or LβT2 cells was performed as previously described (39). Briefly, RNA was extracted with Trizol reagent (Invitrogen/GIBCO, Carlsbad, CA) according to the manufacturer’s instructions, treated to remove contaminating DNA (DNA-free; Ambion, Austin, TX), and reverse transcribed using Superscript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed in an iQ5 Real-Time PCR instrument (Bio-Rad Laboratories, Inc., Hercules, CA) and used iQ SYBR Green Supermix (Bio-Rad Laboratories) with specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LHβ, or Egr1 cDNA.

LHβ forward: CTGTCACAGGCAACTCTGG

LHβ reverse: ACAGGAGGCAAACGACG

Egr1 forward: ATTTTTCTCTAGGCCCCAAGC

Egr1 reverse: ATGGGAACTCCTGAAGACGCC

GAPDH forward: TGCACCACAACATGCTTAG

GAPDH reverse: GGATGCAGGGATGATGTTTC

The iQ5 real-time PCR program was as follows: 95 C for 15 min, followed by 40 cycles at 95 C for 15 sec, 55 C for 30 sec, and 72 C for 30 sec. Within each experiment, the amount of LHβ or Egr1 and GAPDH mRNA was calculated by comparing a threshold cycle obtained for each sample with the standard curve generated from serial dilutions of a plasmid containing GAPDH, ranging from 1 ng to 1 fg. All samples were assayed (in triplicate) within the same run, and the experiment was conducted three times. Values were analyzed by one-way ANOVA followed by Tukey’s post hoc test or two-way ANOVA, group (no stress vs. stress) × time (vehicle vs. GnRH).

**Dual immunofluorescence**

Adult mouse pituitary paraffin tissue sections (Zyagen, San Diego, CA) were dewaxed in xylene, rehydrated through a series of graded ethanol baths, and washed in H2O. Sections were immersed in 10 mm sodium citrate buffer (pH 6.0), heated in a standard microwave twice for 5 min, and allowed to stand for 20 min at room temperature. After a brief wash in PBS, nonspecific binding was blocked with 5% goat serum/0.3% Triton X-100 for 60 min at room temperature. Dual fluorescence labeling was tested on the same section with a guinea pig antirat LHβ primary antibody (anti-rLH-1C-2, NIDDK NHPP; 1:200 dilution in 5% goat serum/0.3% Triton X-100) plus a rabbit antimouse GR primary antibody (sc-1004, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:500 dilution) for 48 h at 4 C. LHβ- and GR-containing cells were revealed using a goat rhodamine antiguinea pig IgG secondary antibody (ab6905, 1:300 dilution; Abcam, Cambridge, MA) plus a goat fluorescein anti-rabbit IgG secondary antibody (FI-1000; Vector Laboratories, Burlingame, CA; 1:300 dilution) for 60 min at room temperature. After rinsing with PBS, sections were coverslipped with Vectashield HardSet mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories). Exclusion of each primary antibody was run as a negative control, and each antibody was run separately to confirm that each immunostaining pattern was similar to published reports (40, 41). Specificity of the GR antibody was confirmed by immunoblot analysis of LβT2 and CV-1 cell lysates, cell lines previously shown to contain and lack GR (29, 37), respectively, which detected a single band of the expected size.

Sections were viewed using an inverted fluorescence microscope (Nikon Eclipse TE 2000-U; Nikon, Tokyo, Japan), and digital images were collected using a Sony CoolSNAP EZ cooled charge-coupled device camera (Roper Scientific, Trenton, NJ) and analyzed with Nikon Imaging System—Elements image analysis system (version 2.3; Nikon).

**Western blot analysis**

Nuclear extracts were prepared as previously described (36) from LβT2 cells treated with dexamethasone (100 nm, 1 h), GnRH (10 nm, 45 min), GnRH + dexamethasone or vehicle (0.1% BSA/0.1% ethanol). Nuclear extract (20 μg) was boiled for 5 min in 5× Western-loading buffer, fractionated on a 10% SDS-PAGE gel, and electrophoreted for 90 min at 300 mA onto polyvinylidene difluoride (Millipore Corp., Billerica, MA) in 1× Tris-glycine-sodium dodecyl sulfate/20% methanol. Blots were blocked overnight at 4 C in 3% BSA and then probed for 1 h at room temperature with rabbit antihuman Egr1 antibody (sc-110, Santa Cruz Biotechnology) diluted 1:750 in blocking buffer. Blots were then incubated with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology), and bands were visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL). Bio-Rad Pre-stained Protein Ladder Plus serves as a size marker.

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed as previously described (29, 35). Briefly, confluent LβT2 cells in 10-cm plates were treated with dexamethasone (100 nm, 1 h), GnRH (10 nm, 1 h), GnRH + dexamethasone (cotreatment 1 h), or vehicle (0.1% BSA/0.1% ethanol) and cross-linked with 1% formaldehyde. The nuclear fraction was obtained, and chromatin was sonicated to an average length of 300–500 bp using a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT). Protein-DNA complexes were incubated overnight with nonspecific rabbit IgG (sc-2027, Santa Cruz Biotechnology) or rabbit antihuman GR (ab3579, Abcam) and precipitated with protein A/G beads. Immunoprecipitated DNA and DNA from input chromatin were analyzed by quantitative PCR using primers specific for a 220-bp sequence of the mouse LHβ proximal promoter (–180 LHβ/+40 LHβ). Primers specific to the mouse FSHβ promoter (–223 FSHβ/+57 FSHβ) and FSHβ coding region were used as positive and negative controls, respectively. DNA from immunoprecipitated samples was quantified relative to a standard curve representing percent of input chromatin. For ChIP assays com-
paring chromatin from hormone-treated LβT2 cells, the fold enrichment of antibody signal over IgG was calculated for each primer set, and data from each independent experiment were normalized to the indicated negative control gene. ChIP and input samples were quantified using a standard curve made from ChIP input DNA. ChIP samples were normalized to their appropriate input samples and are expressed as fold enhancement over nonspecific IgG.

-180 LHβ-forward: CGAGTGAGGCCAATTCACTGGG
+40 LHβ-reverse: GGCCTTACCACATCTTCAGGAGC
-223 FSHβ-forward: GTGCTGCTGCCATATCAGATTCGG

+57 FSHβ-reverse: GCATCAAAGTCTGCTACCTCACC-TGTG

FSHβ-coding forward: GCCGTTTCTGCAATAAGC
FSHβ-coding reverse: CAATCTTACGGTCTCGTATACC

The iQ5 real-time PCR program was as follows: 95°C for 15 min, followed by 40 cycles at 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. All samples were assayed within the same run, and the experiment was conducted three times. Individual values obtained from each independent experiment were averaged and analyzed by one-way ANOVA followed by Tukey’s post hoc test.

Glutathione-S-transferase (GST) interaction assay

35S-labeled proteins were produced using the TnT Coupled Reticulolysate System (Promega Corp., Madison, WI). Bacteria transformed with the GST plasmids were grown to an OD of 0.6 and then induced with isopropyl-β-d-thiogalactoside overnight at 30°C. The bacterial pellets were sonicated in 0.1% Triton X-100 and 5 mM EDTA in 1× PBS and centrifuged, and the supernatant was bound to glutathione Sepharose 4B resin (Amersham Pharmacia Biotech, Piscataway, NJ). The beads were washed four times in PBS and then in HEPES/Nonidet P-40/EDTA buffer (10 mg/ml BSA, 20 mM HEPES (pH 7.8), 50 mM NaCl, 5 mM dithiothreitol, and 0.1% Nonidet P-40). For the interaction assay, 35S-labeled protein and -translated GR (20 μl), SF1 (5 μl), or GFP (5 μl) was added to the beads with 400 μl HND buffer. The beads were incubated for 1 hr at 4°C and then washed twice with HND buffer and twice with 0.1% Nonidet P-40 in PBS. Thirty microliters of 2× Laemmli load buffer were added, and the samples were boiled and then electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel. One tenth of the 35S-labeled in vitro-transcribed and -translated product was loaded onto the gel as input. The gel was dried, and the proteins were visualized by autoradiography.

Results

Chronic restraint stress compromises estrous cyclicity

To evaluate the mechanism whereby elevated glucocorticoids impair reproduction, we developed a model to assess whether daily restraint stress disrupts estrous cyclicity. Vaginal cytology was examined daily by vaginal lavage in a cohort of female mice during an 18-d control period (d 1–d 18). After this period of observation, mice were randomly assigned to either the stress or no stress group (n = 9/group), and estrous cyclicity was monitored for an additional 18 d (d 19–d 36). Figure 1A illustrates profiles of vaginal histological classification during the prestress and stress period of three female mice exposed to 180 min of daily restraint stress. In mice not subjected to stress, cycle length was not significantly different between the two periods of assessment (P > 0.05; d 1–d 18 vs. d 19–d 36, 5.33 ± 0.31 min vs. 4.81 ± 0.45 min, Fig. 1B). In contrast, stressed mice exhibited a significant increase in average cycle length in the stress period compared with the prestress period (P < 0.05; d 1–d 18 vs. d 19–d 36, 5.25 ± 0.35 min vs. 6.75 ± 0.55 min). Specifically, exposure to daily restraint stress significantly increased the time spent in diestrus during the stress period, d 19–d 36, without altering the time spent in estrus or proestrus as compared with the prestress period, d 1–d 18 (P > 0.05; Fig. 1C).

Acute restraint stress disrupts gonadotrope function

Having found that repeated exposure to stress compromises reproductive neuroendocrine activity as evi-
enced by a disruption in estrous cyclicity in female mice, the next step was to focus on the role of the pituitary gland and test whether gonadotrope responsiveness, as assessed by GnRH-induced LH synthesis or secretion, is diminished by acute restraint stress. Separate cohorts of female mice, in which estrus cyclicity was confirmed and diestrus females selected, were used to assess gonadotrope responsiveness to GnRH in the absence or presence of stress. In the first study, GnRH-induced LH secretion was monitored in groups of control mice (no stress) or mice exposed to 180 min of restraint stress (Fig. 2A). Blood was collected before stress, as well as 30 and 180 min after the initiation of the control or stress period for measurement of corticosterone (n = 7 per time point per group). To assess LH release in response to GnRH, female diestrus mice were administered GnRH (200 ng/kg, sc; n = 7 per group per treatment) or saline vehicle and killed 10 min after injection. Blood was collected and processed for measurement of LH at 180 min after the initiation of stress. This GnRH dose was selected because it has been shown to produce a significant, yet submaximal, LH response, that peaks 10 min after administration in a mouse model of diestrus in which female mice are ovariectomized and estrogen primed (42, 43). Serum levels of corticosterone remained low in no stress control animals (Fig. 2B, open circles); corticosterone levels were significantly increased in stressed mice at 30 min (P < 0.05; stress vs. no stress, 588.6 ± 26.4 vs. 108.8 ± 35.5 ng/ml) and remained significantly elevated 180 min after initiation of restraint (Fig. 2B, gray circles). Stress did not significantly induce serum levels of progesterone at either 30 min or 180 min after the initiation of restraint (data not shown). Mean LH in the no stress diestrus females receiving vehicle was not significantly different from values in the vehicle-treated stress animals, indicating that stress does not significantly impair responsiveness to endogenous GnRH in this animal model (P > 0.05; stress Veh vs. no stress Veh, Fig. 2C). In the no stress group, exogenous GnRH caused a robust increase in circulating LH levels as compared with vehicle-treated animals (P < 0.05; 1.83 ± 0.23 vs. 0.56 ± 0.11 ng/ml, Fig. 2C). GnRH also significantly increased LH in stressed animals (P < 0.05, Fig. 2C). However, the LH response to exogenous GnRH was significantly blunted in restraint-stressed animals compared with the response in no stress controls (P < 0.05; stress GnRH vs. no stress GnRH, 1.19 ± 0.18 vs. 1.83 ± 0.23 ng/ml; Fig. 2C), suggesting that stress diminishes the ability of the pituitary to respond to GnRH. Taken together, these experiments reveal an interplay between GnRH and stress and implicate responsiveness of the pituitary gonadotrope as a potential neuroendocrine site of LH suppression.
We further investigated the response of the gonadotrope by analyzing GnRH-induced LHβ mRNA transcript level in mice exposed to restraint stress. Initially, diestrous female mice received saline vehicle or GnRH (200 ng/kg, sc; n = 7 per group per treatment) and were subsequently sorted into no stress or stress treatment groups (Fig. 2D). Animals were killed and the pituitary glands were collected for mRNA analysis by quantitative RT-PCR. Stress did not alter basal LHβ transcript levels compared with expression in no stress vehicle controls (P < 0.05; stress Veh vs. no stress Veh; Fig. 2E). In the absence of stress, exogenous GnRH resulted in a 4-fold increase in LHβ mRNA compared with values in vehicle-treated animals (P < 0.05; Fig. 2E). In stressed mice, the LHβ transcript level in response to GnRH was significantly reduced by approximately 45% (P < 0.05; no stress GnRH vs. stress GnRH, 16.5 ± 2.3 vs. 8.9 ± 1.1; Fig. 2E). Collectively, these observations raise the possibility that stress can interfere with ovarian cyclicity by disrupting the synthesis and secretion of LH at the level of the anterior pituitary gonadotrope cell.

Glucocorticoids impair LH secretion in vivo potentially via receptors expressed in mouse gonadotrope cells

Our studies thus far demonstrate that circulating glucocorticoids are increased within 30 min and remain significantly elevated for the 180-min stress paradigm (Fig. 2). Because stress likely induces a host of inhibitory intermediates, any of which could alter reproductive activity, we directly assessed the role of glucocorticoids by testing the hypothesis that a stress-like level of glucocorticoid in female mice reduces LH secretion. Pilot studies were conducted to identify a dose of glucocorticoid that approximated a stress level (~750 ng/ml) and an animal model that eliminated confounding effects of ovarian steroids. Blood was collected 90 min after a bolus administration of vehicle or corticosterone (200 ng/kg, sc; n = 6 per treatment) to ovariectomized female mice (Fig. 3A). Corticosterone remained low in mice treated with vehicle, yet values were markedly elevated after administration of corticosterone (P < 0.05; Fig. 3B). Treatment with corticosterone significantly reduced mean LH as compared with the value in mice treated with vehicle (P < 0.05; Cort vs. Veh, 3.4 ± 0.3 vs. 1.9 ± 0.6 ng/ml, Fig. 3C), demonstrating sufficiency of glucocorticoids to disrupt reproductive neuroendocrine function and relevancy as an inhibitory factor induced during stress.

Evidence that GR is expressed in gonadotrope cells in the rat (22) supports our hypothesis of a direct action of glucocorticoids via GR within mouse gonadotrope cells. To confirm the presence of this receptor in mouse gonadotrope, we used dual-label immunofluorescence of adult mouse anterior pituitary sections for LH and GR. As expected, immortalized pituitary gonadotrope cells, LβT2, responded to GnRH with a 2-fold increase in endogenous LHβ mRNA as measured by quantitative RT-PCR (Fig. 3A). Schematic depicting experimental events in which animals were administered corticosterone (200 ng/kg, sc; gray bar) or vehicle (white bar), 90 min before euthanasia and blood collection (Blood coll’n). B and C, Serum corticosterone (ng/ml, panel B) or LH (ng/ml, panel C) measured in animals administered vehicle (white bars) or corticosterone (gray bars). *, Significant (P < 0.05) effect of treatment. D–F, Photomicrographs of representative mouse pituitary sections subjected to two-color immunofluorescence staining using a fluorescein isothiocyanate-conjugated anti-GR (green fluorescent signal), followed by rhodamine-conjugated anti-LHβ (red fluorescent signal). Red (panel D), green (panel E), and merge (panel F) immunofluorescence images were taken of the same microscopic field using appropriate filters. White stars, GR-positive/LHβ-positive cells. Scale bar, 20 μm. Cort, Corticosterone; trt, treatment; Veh, vehicle.
4A). Treatment of LβT2 cells with a physiological, stress level of corticosterone decreased GnRH-induced LHβ mRNA expression \((P < 0.05); \text{GnRH vs. GnRH + Cort, 2.1 ± 0.3 vs. 1.6 ± 0.1; Fig. 4A}\); however, corticosterone alone did not reduce LHβ expression. These findings indicate that repression by glucocorticoids occurs in the presence of GnRH and confirm the LβT2 gonadotrope cell is an appropriate model for investigating the role of glucocorticoid-induced suppression of LHβ gene expression.

We began to dissect out the mechanism whereby glucocorticoids reduce LHβ-subunit induction in LβT2 cells, by investigating the regulation of an LHβ-luciferase reporter after incubation with either natural or synthetic glucocorticoids. For this purpose, LβT2 cells were transiently transfected with −1800 bp of the rat LHβ regulatory region fused upstream of a luciferase reporter gene (LHβ-luc) or pGL3 control plasmid and treated with corticosterone \((100 \text{ nM})\), dexamethasone \((100 \text{ nM})\), or vehicle \((0.1\% \text{ ethanol})\) for 24 h before harvest. Neither the natural glucocorticoid, corticosterone, nor the synthetic glucocorticoid, dexamethasone, significantly altered basal expression of the −1800-bp LHβ promoter \((P > 0.05); \text{Veh vs. Cort or Dex; Fig. 4B}\). In contrast, both glucocorticoids significantly blunted the robust increase in promoter activity induced by GnRH \((10 \text{ nM}, \text{final 6 h of glucocorticoid treatment}; P < 0.05; \text{Fig. 4B})\). Specifically, GnRH resulted in a 4.6-fold induction of LHβ-luc, which was reduced 35% by corticosterone, or 45% by dexamethasone. Although both glucocorticoids are capable of transcriptional repression of GnRH induction of the LHβ promoter, we focused on the synthetic glucocorticoid, dexamethasone, based on the intensity of its effect and evidence for its potent interaction with the endogenous steroid receptor, GR, expressed in LβT2 cells \((29)\) and identified in mouse gonadotrope cells (Fig. 3).

Using a promoter truncation approach, we identified regions of the LHβ gene that are functionally involved in glucocorticoid regulation. LβT2 cells were transiently transfected with a series of truncated LHβ reporter plasmids, ranging in length from −1800 to −87 bp of the 5′-regulatory sequence. Figure 4C illustrates the effect of treatment with GnRH in the presence or absence of dexamethasone on progressive 5′-LHβ promoter truncations. As observed previously, GnRH induction of the LHβ gene declined incrementally as the promoter was progressively truncated from −1800 to −87 \((\text{Fig. 4C and Ref. 44})\). Dexamethasone repressed LHβ promoter activity by approximately 40% when the region contained at least −150 bp of the proximal promoter. Interestingly, further truncation of the region from −150 to −87, which removed the proximal GnRH responsive elements \([\text{such as early growth response factor 1 (Egr1) and SF1}]\), resulted in a loss of GnRH induction and elimination of dexamethasone repression, indicating that GnRH responsiveness of the LHβ gene is required for glucocorticoid repression. Collectively, these data suggest that GR exerts its inhibitory effects upon the LHβ proximal promoter, likely via interactions with GnRH-responsive factors, such as Egr1 and SF1.
Proximal binding elements are important for glucocorticoid repression

The proximal 150 bp of the rat LHβ promoter contain multiple binding elements that are critically important for GnRH regulation of LHβ transcription (Fig. 5A), including tandem elements for both Egr1 and SF1 that are arranged on either side of a homeobox element, previously shown to bind pituitary homeobox factor 1 (Ptx1). Ptx1 is expressed throughout pituitary development and plays a critical role in activation of a number pituitary genes, including Lhb (45). SF1 is specifically expressed in the gonadotrope of the anterior pituitary gland, and its importance for reproduction is underscored by a lack of gonads in SF1-null mice (46). Although highly important for LHβ transcription, SF1 and Ptx1 are not regarded as factors induced or regulated by GnRH (47). On the other hand, Egr1 is rapidly induced by GnRH and considered a critical regulator of LHβ gene expression (47), because Egr1 null mice lack LHβ expression in the pituitary gonadotropes (48). Therefore, we focused on the role of Egr1 in glucocorticoid repression of LHβ gene expression and tested the hypothesis that glucocorticoids interfere with GnRH induction of LHβ by blunting the GnRH-stimulated increase in Egr1 mRNA and protein. To investigate hormone regulation of Egr1 mRNA, LβT2 cells were treated with hormone, and after 45 min, RNA was harvested for Egr1 mRNA analysis by quantitative RT-PCR. Egr1 mRNA was low in vehicle-treated control cells, and expression was unchanged by treatment with dexamethasone alone (Fig. 5B). As expected, GnRH caused a dramatic 27.2-fold induction in Egr1 mRNA (P < 0.05 vs. Veh). This increase in GnRH-induced Egr1 transcript, however, was not significantly decreased by dexamethasone (P > 0.05; GnRH vs. GnRH + Dex, 27.2 ± 4.6 vs. 23.1 ± 4.5; Fig. 5B). Because we found that treatment with glucocorticoids does not suppress levels of GnRH-induced Egr1 transcript, we next tested the hypothesis that glucocorticoids regulate translation or degradation of Egr1 in LβT2 cells using Western blot analysis. Protein expression of Egr1 was undetectable in nuclear extracts of LβT2 cells after treatment with vehicle or dexamethasone (Fig. 5C). After treatment with GnRH, Egr1 protein was readily detected, yet protein expression did not significantly change after cotreatment with GnRH and dexamethasone, indicating that GR does not interfere with GnRH induction of Egr1 protein.

We further examined how glucocorticoids might influence GnRH-induced LHβ expression by performing ChIP assays on the endogenous mouse LHβ promoter in LβT2 cells. Cells were treated with dexamethasone or GnRH alone or in combination for 60 min before cross-linking. Sonicated chromatin was immunoprecipitated using either anti-GR or nonspecific IgG. Cross-linking was reversed and precipitates analyzed by quantitative PCR for 220 bp of the LHβ promoter (−180 to +40). Dexamethasone treatment alone did not increase GR binding to the proximal promoter over vehicle treatment (P < 0.05; Veh vs. Dex; Fig. 5D). However, we can only rule out a change in GR occupancy of the LHβ promoter at 60 min, and that later (or earlier) changes in the response to glucocor-
ticoids are still possible. In contrast, GnRH treatment increased GR binding 3.8-fold compared with vehicle (P < 0.05; Veh vs. GnRH), indicating that recruitment of GR to the LHβ promoter is not dependent on dexamethasone binding, but rather is dependent on a GnRH-responsive factor. No difference in GR binding was observed when the cells were concomitantly stimulated with dexamethasone and GnRH as compared with GnRH alone, suggesting that a change in GR conformation or recruitment due to ligand does not underlie the mechanism of glucocorticoid repression of LHβ transcription.

**Glucocorticoids interfere with Egr1 actions at the level of the promoter**

Having determined that GR recruitment is dependent on GnRH (i.e. a responsive factor such as Egr1), yet the inhibitory effect of glucocorticoids is downstream of GnRH-induced Egr1 mRNA or protein, we investigated the role of Egr1 at the level of the LHβ promoter. We began by testing the hypothesis that glucocorticoids reduce activity of the LHβ promoter when induced by Egr1 itself, in the absence of GnRH. Egr1 is a potent activator of LHβ transcription, and transfection of an Egr1 expression plasmid in LβT2 cells treated with vehicle caused a robust 35-fold increase in LHβ activity (P < 0.05; Veh: Empty Vec vs. Egr1; Fig. 6A). Treatment with dexamethasone significantly blunted the increase in LHβ activity induced by Egr1 compared with the response in cells treated with vehicle (P < 0.05; Egr1 [black bars]: Dex vs. Veh; Fig. 6A), indicating that glucocorticoids can interfere with activation by Egr1 at the level of the LHβ promoter in gonadotrope cells and that GR suppression does not require factors involved in GnRH signaling upstream of Egr1.

We next attempted to rescue the glucocorticoid repression of GnRH-induced LHβ activity in LβT2 cells. We hypothesized that if Egr1 were the sole factor affected by glucocorticoids, then titrating increasing amounts of Egr1 into LβT2 cells would restore full induction and prevent diminishment by glucocorticoids. LHβ-luc/βgal values are represented as fold induction of GnRH or GnRH + Dex relative to the vehicle control condition containing the same amount of Egr1. In the absence of exogenous Egr1, dexamethasone significantly blunted GnRH-induced LHβ activity (P < 0.05; empty vector: GnRH vs. GnRH + Dex; Fig. 6B). In the presence of increasing amounts of overexpressed Egr1 (50–200 ng), the percent repression significantly decreased (P < 0.05; empty vector vs. 100 or 200 ng Egr1; Fig. 6C), indicating that repression occurs, in part, via interfering with Egr1 function on the promoter.

We focused our attention on the action of glucocorticoids at the level of the LHβ promoter and analyzed the

**FIG. 6.** Glucocorticoids interfere with Egr1 actions at the level of the proximal promoter. A, To test whether glucocorticoids can interfere with Egr1-induced LHβ expression, Egr1 (black bars) or empty vector (Empty Vec, open bars) was transfected with the −1800-bp LHβ-luc reporter plasmid into LβT2 cells and subsequently treated with dexamethasone (Dex; 100 nM, 24 h) or vehicle (Veh; 0.1% BSA/0.1% ethanol). Data are shown relative to vehicle-treated in the absence of Egr1. * Significant induction by Egr1 vs. vector control; #, significant repression by glucocorticoid treatment on Egr1-induced LHβ expression. B, To determine whether titrating in increasing levels of Egr1 can rescue glucocorticoid repression of −1800-bp LHβ-luc activity, LβT2 cells were transfected with empty vector (EV, 200 ng) or increasing amounts of Egr1 [50 ng (plus 150 ng empty vector), 100 ng (plus 100 ng empty vector), 200 ng] and treated with GnRH (white hatched bars, 10 nM, final 6 h) or GnRH + dexamethasone (gray hatched bars, Dex (100 nM, entire 24 h); GnRH (10 nM, final 6 h)]. Results are depicted as LHβ-fold induction relative to the vehicle-treated condition in the presence of the same amount of Egr1 (dashed line) as indicated. *, Significant induction by GnRH; #, significant repression by glucocorticoid treatment on GnRH-induced LHβ expression. C, The effect of increasing Egr1 on the ratio of LHβ-fold induction in cells treated with GnRH alone vs. GnRH with dexamethasone was calculated and expressed as percent repression. * Significant difference in the ratio as compared with EV determined by Student’s t test. D, LβT2 cells were transfected with either the −200-bp LHβ-luc reporter plasmid (WT) or reporter plasmids containing the −200-bp LHβ region with mutations in 5′SF1 or Egr1 binding elements as indicated and cultured in the presence of GnRH (white hatched bars) or GnRH + Dex (gray hatched bars). Data are shown for each mutant promoter, relative to its own vehicle treatment. See Fig. 6B legend for more details. n.s., Not significant P = 0.08; WT, wild type.
necessity of known GnRH-responsive elements within the proximal 150 bp of the LHβ gene for glucocorticoid repression by creating cis mutations in the 5′-SF1, 3′-SF1, 5′-Egr1, and 3′-Egr1 binding elements in the context of a minimal −200 bp LHβ promoter (WT). We used this minimal LHβ promoter because it is sufficient for responsiveness to GnRH and glucocorticoids. cis mutation of either the 5′- or 3′-SF1 or the 5′-Egr1 site preserved significant GnRH induction and was sufficient for glucocorticoid repression, suggesting that these elements are not required for either GnRH induction or GR repression (P < 0.05; *, significant induction by GnRH; #, significant repression of GnRH induction; Fig. 6D). In contrast, the 3′-Egr1 cis mutation was the only mutation to abrogate glucocorticoid repression (Fig. 6D, 3′-Egr1 mutation). Of interest to our study, this Egr1 binding site has been shown to be critical for GnRH induction as well (49), and cis mutation of this element eliminated significant induction by GnRH in our hands, implying that GnRH responsiveness is necessary for repression by glucocorticoids.

Interaction and involvement of Lhb promoter proximal binding factors

Egr1 conveys GnRH induction of the LHβ promoter by interaction with other regulators of gene expression in the gonadotrope (47, 49–51). To assess the complex and cooperative roles of Egr1, SF1, and Ptx1, we used heterologous CV-1 cells. Unlike gonadotrope cells, CV-1 cells lack GnRH receptors, an Egr1 response to GnRH, and are devoid of endogenous SF1, Ptx1, and GR, which allowed us to reconstitute these factors and determine the necessity and sufficiency of proteins involved in suppression by dexamethasone. In addition to GR, CV-1 cells were cotransfected with 1800-bp LHβ-luc reporter plasmid or pGL3 control plasmid and Egr1, SF1, or Ptx1 alone, or in combination, and tested for activation of LHβ transcription in the presence of dexamethasone or vehicle. As shown in Fig. 7A (white bars), overexpression of Egr1, SF1, or Ptx1 alone induced small increases in LHβ transcription, with induction by Egr1 reaching significance. Dexamethasone significantly diminished induction by Egr1 (P < 0.05; Egr1: Dex vs. Veh). Interestingly, dexamethasone also significantly repressed LHβ induction when Egr1 was cotransfected with SF1 alone or SF1 plus Ptx1 (P < 0.05; Egr1: Dex vs. Veh, Egr1/SF1: Dex vs. Veh; Fig. 7A).

To address whether the Egr1-binding site is sufficient for glucocorticoid repression of LHβ transcription, reporter constructs containing four copies of the Egr1 consensus site ligated into pGL3 upstream of a minimal TK promoter were created (Egr1 multimer, Fig. 7B). CV-1 cells were cotransfected with the Egr1 multimer or control TKluc pGL3 plasmid, GR, and either Egr1 alone, or Egr1 in combination with SF1 and Ptx1, to test for sufficiency to activate transcription in the presence or absence of dexamethasone. Egr1 was sufficient to induce transcriptional activation of the Egr1 multimer, and this effect was blunted by glucocorticoids (P < 0.05; #, significant repression by Dex; Fig. 7B). Of interest, this site was sufficient to allow for enhanced activation by the combination of Egr1, SF1, and Ptx1, an effect that was also diminished by glucocorticoids. In contrast to the sufficiency of the Egr1 site, a consensus SF1-binding site multimer does not convey responsiveness to dexamethasone when induced by SF1 alone, but activation of the SF1 site by the combination of Egr1, SF1, and Ptx1 is disrupted by dexamethasone. Taken together, these findings indicate that the Egr1 site activated by Egr1 alone is sufficient for mediating repression. In contrast, the SF1 site activated
by SF1 alone is not sufficient although a complex with SF1, Egr1, and Ptx1 on the SF1 site can be repressed.

**Role of the GR at the level of the Lhb promoter**

Because CV-1 cells lack GR, this cell model allowed us to determine the necessity of DNA binding or dimerization by GR for repression in gonadotrope cells. In the absence of transfected GR in CV-1 cells, dexamethasone does not inhibit LHβ promoter activity (P > 0.05; no GR: Veh vs. Dex; Fig. 8A), confirming that GR is necessary for repression of LHβ by glucocorticoids. To determine whether direct DNA binding by GR plays a critical role in the repression of LHβ by glucocorticoids, we transfected CV-1 cells with two different GR mutants that are incapable of binding DNA. The first mutant, GR Dim mut, contains four point mutations in the dimerization domain of GR. These mutations prevent homodimerization of GR, but do not prevent indirect DNA binding through interactions with other transcription factors (52, 53). The second GR mutant, GR DBD mut, prevents direct DNA binding by the mutant. Dexamethasone elicited suppression in the presence of either transfected GR mutant, indicating that GR dimerization and DNA binding are not necessary for repression of GnRH-induced LHβ promoter activity (P < 0.05; Dim mut or DBD mut: Veh vs. Dex; Fig. 8A).

After demonstrating that GR does not require DNA binding to elicit repression upon the LHβ promoter, we investigated the ability of GR to be tethered to DNA by a GnRH-induced factor, by testing the hypothesis that GR is capable of binding Egr1. We asked whether in vitro-transcribed and -translated GR could bind bacterially expressed GST-Egr1 in pull-down experiments. Figure 8B demonstrates precipitation of 35S-labeled GR with GST-Egr1, as compared with GST alone, illustrating a physical interaction between GR and Egr1. As a positive control, we show that 35S-labeled SF1 binds GST-Egr1, a strong interaction that has been previously reported (54). In contrast, the interaction between 35S-labeled GFP with either GST construct was undetectable. Together with the ChIP results in Fig. 5D, these findings indicate that GR does not directly bind the LHβ promoter; rather it physically interacts with Egr1 and thus is recruited to the LHβ promoter as a complex with Egr1, identifying Egr1 as a critical factor mediating GnRH induction and GR repression of LHβ gene expression.

**Discussion**

Utilizing a restraint stress paradigm that robustly stimulates corticosterone to investigate stress-induced suppression of gonadotrope function in female mice, we demonstrate that chronic exposure to stress impairs estrous cyclicity and reduces GnRH-induced LH synthesis and secretion in diestrous female mice. Whether the increase in glucocorticoids is induced by stress or exogenously administered, both conditions lead to repression of gonadotrope function, confirming the inhibitory role of glucocorticoids within the reproductive axis and demonstrating the capacity for suppression during stress. Although we acknowledge that stress likely increases a variety of mediators, including other hormones of the adrenal stress axis that have been shown to alter reproductive function during stress (2, 55, 56), we conclude, based on our current investigation, that glucocorticoids are sufficient to disrupt reproductive neuroendocrine function.

Seminal work by Dr. Neena Schwartz and colleagues (57) clearly identified inhibitory effects of glucocorticoids...
within the reproductive neuroendocrine axis and postulated that glucocorticoids may alter hypothalamic and pituitary function. Indeed, in vivo analyses demonstrated that glucocorticoids could prevent the postcastration rise in LH in rats but also blunt the response to GnRH in anterior pituitary fragments in culture (20, 57). Our present investigation expands upon these early studies by demonstrating that glucocorticoids can act directly upon the anterior pituitary gonadotrope cell to suppress GnRH induction of LHβ gene expression. Intriguingly, we observe a similar suppression in LHβ gene expression after stress, but it remains to be determined whether this is a direct effect of glucocorticoids within the pituitary gland. With regard to direct actions within the gonadotrope, we demonstrate a novel mechanism whereby GR is recruited to the 5′-region of the mouse LHβ gene in live LβT2 cells and blunts GnRH induction of LHβ by interfering with the genomic effects of Egr1 on the proximal LHβ promoter.

Glucocorticoid repression via GR maps to a highly active region of the LHβ promoter, which contains elements critical for GnRH induction. Promoter analyses revealed that glucocorticoid-mediated repression is lost upon 5′-truncation of the bipartite SF1 and Egr1 elements in the LHβ proximal promoter. Truncation of this region, however, also eliminated GnRH induction of the promoter (Fig. 4C, −87 LHβ-luc), suggesting that the ability of glucocorticoids to repress is closely tied to the highly coordinated and complex mechanism of GnRH action on Lhb. Not surprisingly, the only cis mutation that relieved suppression by glucocorticoids (Fig. 6D, the 3′-Egr site) also prevented significant induction of the LHβ promoter by GnRH, supporting the conclusion that glucocorticoid-induced repression is dependent on the response to GnRH and highlights the importance of Egr1 for repression by glucocorticoids.

Glucocorticoids have the potential to interfere with GnRH induction of LHβ via interference of GnRH receptor signaling upstream and downstream of Egr1 induction. For example, studies performed using rat and pig pituitary cell cultures suggest that chronic exposure to glucocorticoids can disrupt GnRH receptor-signaling mechanisms involved in gonadotropin release, including activation of protein kinase C and cAMP (20, 23). Further, GR has been shown to interact rapidly with c-Jun N-terminal kinase, a kinase implicated in mediating effects of GnRH in gonadotrope cells (58). In contrast to these actions of glucocorticoids on GnRH signaling, our findings support an action of glucocorticoids downstream of Egr1 induction by GnRH via GR acting within the LHβ chromatin. Glucocorticoids do not alter GnRH-induced Egr1 mRNA or protein in LβT2 cells, demonstrating that the pathway between GnRH binding its receptor and induction of the immediate early gene, Egr1, is intact. Furthermore, glucocorticoids blunt the ability of overexpressed Egr1 to induce LHβ activity, providing evidence that GR inhibition does not require elements upstream of Egr1 and focus our attention on a mechanism involving Egr1 at the promoter level. We show that titrating in increasing amounts of Egr1 partially overcomes glucocorticoid repression of Lhβ in LβT2 cells, solidifying a mediating role of Egr1.

With regard to the mechanism of repression, similar to other steroid hormone receptors, GR mediates transcriptional regulation of target genes via a host of direct and indirect mechanisms. For example, within the gonadotrope cell, induction of either the murine FSHβ or human αGSU gene occurs via direct GR binding to DNA at conserved glucocorticoid response elements (29, 30). In contrast, using cell models either devoid of or expressing GR (CV-1 vs. LβT2 cells, respectively), we find that neither an intact DBD nor dimerization domain is necessary for GR repression of the rat LHβ gene, implicating a genomic action that occurs via indirect GR binding. In addition, GR is recruited to the LHβ promoter chromatin by GnRH induction in the presence or absence of glucocorticoids. Coupled with our findings that GR physically interacts with Egr1 in GST-pulldown experiments, this provides evidence in support of our hypothesis that GR is tethered to the LHβ promoter via a GnRH-induced factor, and in particular by Egr1.

The requirement for a GnRH-induced factor may also contribute to the differential effect of glucocorticoids on transcription within the gonadotrope cell. On the one hand, genes encoding FSHβ, αGSU, and GnRH receptor are each induced by glucocorticoids alone (30, 33, 59). On the other hand, our data reveal that GR repression requires GnRH, likely due to the deficiency of GR recruitment to the proximal promoter in the absence of GnRH. On the GnRH receptor gene in gonadotrope cells, GnRH-induced GR recruitment has been shown to be involved in glucocorticoid induction of transcription (59). In that case, however, the recruitment of GR to the GnRH receptor promoter is dependent on rapid phosphorylation of GR by GnRH-signaling pathways, which differs from our finding that GR repression of LHβ transcription does not require GnRH signaling upstream of Egr1 activation. Rather, we conclude that GR is recruited by a factor induced by GnRH and hypothesize that Egr1 itself mediates the balance between GnRH induction and GR repression of the LHβ promoter.

In summary, the present study shows that restraint stress potently activates the adrenal stress axis in mice and interferes with gonadotropin synthesis and secretion. We
expand upon this finding by demonstrating that administration of a stress level of glucocorticoids inhibits LH secretion in female mice and detailing a mechanism whereby glucocorticoids repress activity of the anterior pituitary gland via regulation of LHβ gene expression in gonadotrope cells. We identify GR in native gonadotrope cells in the mouse and demonstrate that the recruitment of GR to the mouse LHβ promoter via Egr1 interferes with the tripartite transcriptional complex necessary for mediating responsiveness of this gonadotropin gene to GnRH. Utilizing dual in vivo and in vitro approaches, collectively, this work reveals new insights regarding the interaction between the adrenal stress and reproductive neuroendocrine axes by identifying a mechanism whereby LHβ expression is dampened after a stress elevation in glucocorticoid.

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