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Glucocorticoid receptor-mediated regulation of *Rfrp* (GnIH) and *Gpr147* (GnIH-R) synthesis in immortalized hypothalamic neurons

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ABSTRACT

A novel RFamide peptide, gonadotropin-inhibitory hormone (GnIH) has emerged as a modulator of avian reproduction. However, the functional role of the mammalian homologue, RFRP-3 remains poorly understood. The RFRP-3 neuronal circuit is influenced by the stress axis. However, whether the *Rfrp* gene is under direct glucocorticoid (GC)-mediated transcriptional regulation, in the presence and absence of the gonadal steroid, 17β-estradiol, is unknown. We investigated the regulation of the *Rfrp* (GnIH) and *Gpr147* (GnIH-R) transcripts by steroids in a novel hypothalamic *Rfrp*-expressing cell model, rHypoE-23. The GC agonist, dexamethasone increased *Rfrp* and *Gpr147* mRNA levels. Dexamethasone acted directly on the nuclear GC receptor (GR) to mediate GC-dependent transcriptional changes, independently of *de novo* protein synthesis. 17β-estradiol had no significant effect on *Rfrp* or *Gpr147* biosynthesis in these neurons. This suggests that *Rfrp*-expressing neurons serve as potential upstream mediators of stress-induced effects through GR-dependent mechanisms.

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1. Introduction

Stress, whether acute or chronic, challenges the internal physiological homeostasis of an organism. The physiology of stress has been causally linked to the suppression of the hypothalamic–pituitary–gonadal (HPG) axis, eventuating in reproductive compromise (Du Ruisseau et al., 1977; Rivier and Rivest, 1991; Smith et al., 1971). Numerous studies in vertebrates, including humans have demonstrated that the activation of the hypothalamic–pituitary–adrenal (HPA) axis is dependent on the rapid release of neuropeptides, particularly corticotropin-releasing hormone (CRH) from the hypothalamus and adrenocorticotropic hormone (ACTH) from the pituitary. The activation of the HPA axis results in elevated levels of glucocorticoids (GCs) released from the adrenal cortex, leading to a global suppression of LH release (Collu et al., 1979; Du Ruisseau et al., 1979). Chronic administration of the GCs, cortisol or corticosterone, in humans and rodents, respectively results in the inhibition of cyclicity and ovulatory activity in female rats (Ramaley and Schwartz, 1980; Smith et al., 1971), as well as causes an inhibition of overall sexual maturation (Ramaley and Schwartz, 1980; Rivier and Rivest, 1991). The stress response, particularly the actions of CRH and GCs, act both directly on the hypothalamic level to inhibit GnRH biosynthesis and secretion (Attardi et al., 1997; Chandran et al., 1994, 1996; DeFranco et al., 1994; Tellam et al., 1998) and at the pituitary to suppress gonadotropic hormone synthesis and release into circulation (Gore et al., 2006; Tache et al., 1978).

In 2000, a previously unknown RFamide neuropeptide, gonadotropin-inhibitory hormone (GnIH) was characterized and isolated from the Japanese quail (*Coturnix japonica*), demonstrating a unique ability to suppress LH release (Tsutsui et al., 2000). In avian species, GnIH has a confirmed inhibitory role within the HPG axis *in vivo* (Bentley et al., 2006a, 2006b; Maddineni et al., 2008; Perfito et al., 2011; Ubuka et al., 2012a,b), though its role in mammals is still under investigation. The mammalian homolog to avian GnIH, RFamide-related peptide-3 (RFRP-3) demonstrates potent and sustained inhibitory actions on gonadotropic hormone secretion (Anderson et al., 2009; Clarke et al., 2008; Gibson et al., 2008; Johnson and Fraley, 2008; Kriegsfeld et al., 2006; Murakami et al., 2008; Ubuka et al., 2012a,b), as well as GnRH neuronal activation and signaling (Ducret et al., 2009; Son et al., 2012; Wu et al., 2009). Mammalian RFRP-3 is encoded by the *Rfrp* (also referred to as *Npvf*) gene and generates a preprohormone, which contains 2 physiologically active RFamide-related peptides, RFRP-1 and RFRP-3 (Hinuma et al., 2000). The receptor for GnIH/RFRP-3, GPR147 is a G protein-coupled receptor that is highly expressed in regions of the hypothalamus, pituitary, and gonads (Kriegsfeld et al., 2006; Ubuka et al., 2009; Ukena and Tsutsui, 2001).

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Immunohistochemical studies have further revealed that RFRP-3 cell bodies are concentrated in the dorsomedial nucleus of the hypothalamus (DMH) with terminals extending to the median eminence and midbrain regions, including the diagonal band of Broca, preoptic area (POA), and anterior hypothalamus (Kriegsfeld et al., 2006; Legagneux et al., 2009). Of interest, *Rfrp*-expressing neurons have been found to coexpress estrogen receptor α (ERα), the glucocorticoid receptor (GR), and CRH receptor 1 (CRH-R1) (Kirby et al., 2009; Kriegsfeld et al., 2006; Molnar et al., 2011; Poling et al., 2012). Therefore, as this body of evidence suggests RFRP-3 and GPR147 pose as a potential modulatory neuronal system operating at multiple levels of the HPG axis. To date, the regulation of *Rfrp* and *Gpr147* biosynthesis by GCs, in the presence or absence of gonadal steroids has not been directly assessed at the cellular level.

The central hypothalamic mechanisms involved in the stress-mediated suppression of GnRH release, whether direct or through innervating neuronal systems remains poorly understood. Due to the inhibitory nature of RFRP-3 and the co-expression of the *Gpr147* gene on GnRH neurons (Rizwan et al., 2012), this neuronal network poses as a potential mediator of stress-induced mechanisms directly influencing GnRH synthesis and release at the level of the GnRH neuron. Of interest, Kirby et al. demonstrated that 53% of RFRP-positive neurons co-express the GR and both acute and chronic stress cause a drastic increase in both *Rfrp* mRNA expression and protein levels (Kirby et al., 2009). Similarly, GnIH immunoreactive cell bodies are greatly induced by capture-handling stress in house sparrows (*Passer domesticus*) in a season-dependent manner over the avian reproductive cycle (Calisi et al., 2008).

In the present study, we sought to investigate whether GCs alone or in the presence of the gonadal steroid, 17β-estradiol can directly act on *Rfrp*-expressing neurons to regulate transcriptional changes and to further delineate the receptor-mediated pathways involved. Due to heterogeneous nature and complex architecture of the *in vivo* hypothalamus, dissecting the direct effects of GCs on a particular neuronal systems is confounded and hence, very challenging. Therefore, to ascertain GC-mediated effects on *Rfrp* and *Gpr147* gene expression, we employed a rat embryonic-derived immortalized hypothalamic cell line, embryonic rat hypothalamic-23 (rHypoE-23). This cell line has been previously characterized for the expression of neuron-specific markers (Belsham et al., 2004; Gingerich et al., 2009), as well as demonstrates strong endogenous expression of *Rfrp* mRNA and other key reproductive receptors. We establish that the GC agonist, dexamethasone has direct effects on *Rfrp* and *Gpr147* expression in rHypoE-23 neurons. Furthermore, we elucidate the transcriptional mechanisms and GR-mediated changes in the Rfrp/Gpr147 system *in vitro*. Taken together, these findings provide the first line of evidence that dexamethasone directly augments GR-mediated transcriptional changes of *Rfrp* and *Gpr147* genes in an *Rfrp*-expressing hypothalamic cell line.

2. Materials and methods

2.1. Cell culture and reagents

Immortalized hypothalamic cells were grown in monolayer in Dulbecco's Modified Eagles Medium (DMEM; Sigma–Aldrich, St. Louis MO, USA), supplemented with 5% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA). Furthermore, rHypoE-23 cell lines were grown in 37 °C with 5% CO_2, a methodology previously described (Jones et al., 1989; Titolo et al., 2006). Dexamethasone and 17β-estradiol (Sigma–Aldrich, St. Louis MO, USA) were dissolved in absolute ethanol to a stock concentration and stored at −20 °C prior to mRNA studies. RU-486 (Sigma–Aldrich), a GR antagonist and cycloheximide (CHX; Sigma–Aldrich), a protein synthesis inhibitor, were reconstituted to a stock concentration in absolute ethanol or water, respectively. By using water in subsequent dilutions, the final ethanol concentration in vehicle controls and treated neurons was minimized to nanomolar concentrations. Finally, the transcription inhibitor, 5,6-Dichlorobenzimidazole riboside (DRB; Tocris Bioscience, Ellisville, MO, USA) was reconstituted in dimethyl sulfoxide (DMSO; Sigma–Aldrich) to a stock concentration.

2.2. Single-step reverse transcriptase PCR (RT-PCR)

Cells were grown to approximately 85–90% cell confluence in 100 mm culture plates. Total RNA was isolated by the guanidium isothiocyanate phenol chloroform extraction method. Subsequent to isolation, RNA concentration and purity ratios were measured using the NanoDrop 2000c spectrophotometer, followed by a DNAse-treatment using the TURBO DNase kit, according the manufactures protocol (Ambion, Life Technologies, Carlsbad, CA, USA) prior to amplification. Single-step reverse transcriptase PCR was performed using the Qiagen OneStep kit, according to manufacturers protocol (Qiagen Sciences, Inc., Germantown, MD, USA). In brief, 200 ng of template DNase treated RNA was combined with buffer, dNTPs, enzyme mix and gene-specific primers (Supplemental Table 1). PCR products were separated using a 2% agarose (BioShop Canada Inc.) gel containing 0.5 μg/mL ethidium bromide and run alongside a 100 bp ladder (Fermentas Life Science, Canada), which was visualized with UV light. To verify PCR products, bands were excised from the gel and purified using the MiniElute Gel Extraction kit (Qiagen Sciences Inc.), followed by sequencing at The Centre for Applied Genomics (TCAG, Toronto, ON, Canada).

2.3. Quantitative RT-PCR (qPCR)

rHypoE-23 cells were grown in monolayer in 60 mm culture plates to 85–90% confluence. Cell culture media was changed to phenol red free DMEM (HyClone, Thermo Fisher Scientific), supplemented with 5% charcoal-stripped FBS, protocol previously described (Roy et al., 1999; Titolo et al., 2006, 2008; Wetsel and Negro-Vilar, 1989), and 1% penicillin/streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA) for a minimum of 2 h prior to steroidal treatments. Hypothalamic neurons were treated with vehicle, dexamethasone (20 nM), and/or 17β-estradiol (10 nM). Total RNA was harvested at the indicated time points. For antagonist and inhibitor studies, rHypoE-23 neurons were pretreated with 50 μM RU-486, 1 μg/mL CHX, 60 μM DRB, or vehicle for one hour prior to dexamethasone (20 nM) treatment and RNA was isolated at 8 and 24 h time points. RNA was isolated by the guanidium isothiocyanate phenol chloroform extraction method, followed by measurements of RNA concentration and purity with the NanoDrop 2000c spectrophotometer. Subsequently, 2 μg of total RNA was DNAse-treated using the TURBO DNase kit (Ambion, Life Technologies, Carlsbad, CA, USA) and reverse transcribed according to manufacturer's protocol using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies). 50 or 100 ng of cDNA template were amplified using Platinum SYBR green qPCR master mix for real-time RT-PCR (Invitrogen, Burlington, Canada). The primer sequences for the primary transcripts investigated are as follows: Rfrp-SYBR Sense, TTA TTG ACT TTA GCA ACT TCA AGC; and Rfrp-SYBR Anti-sense, AGT GAC ACT TCT TTC CTT TAC CCC; Gpr147-SYBR Sense: AAC ACC CTG GTC TGC TTC ATT GTG; and Gpr147- SYBR Anti-Sense: TGA CGG CCA GGT TGA GGA TAA ACA. Samples were run in triplicates on the Applied Biosystems Prism 7000 real-time PCR machine, according to

manufacturers guidelines. All gene values were normalized to the internal control, 18S and analyzed using a standard curve method.

2.4. Statistical analysis

Data are presented as the mean ± SEM for the number of independent experiments indicated. Data analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) or SigmaStat (Systat Software, Inc., Chicago, IL). Statistical significance was determined by one- or two-way ANOVA, as indicated, following a Bonferroni's post hoc test.

3. Results

3.1. rHypoE-23 neurons express Rfrp and Gpr147 mRNA, in addition to ERs, GR, and other key reproductive receptors

We have previously reported the generation of an array of clonal hypothalamic neuronal cell lines that demonstrate unique expression profiles of neuropeptides and relevant receptors (Belsham et al., 2004; Gingerich et al., 2009). With the aim of characterizing a representative *Rfrp*-expressing cell line, we conducted single-step RT-PCR to further determine the gene expression profile of the rHypoE-23 neurons. In accordance with immunohistochemical studies, we demonstrate that rHypoE-23 neurons show high expression of *Rfrp* mRNA (Fig. 1B), as well as synthesize the GR, both nuclear ERs and the G-protein coupled estrogen receptor, GPR30 (Fig. 1A). We also report the presence of the androgen receptor (AR) mRNA in rHypoE-23 neurons. The expression of *Rfrp*, as well as the presence of steroid receptors, suggests that this cell line is suitable for the study of direct hormonal regulatory mechanisms involved in *Rfrp* biosynthesis at the cellular level. Moreover, coexpression of *Rfrp* and *Gpr147* in rHypoE-23 neurons indicates the potential for *Rfrp* autoregulatory mechanisms, a regulatory pathway that has not been explored, to date.

3.2. Dexamethasone, but not 17β-estradiol has stimulatory effects on Rfrp and Gpr147 mRNA expression in rHypoE-23 neurons

The presence of GR, as well as both nuclear ERs prompted investigation into the potential direct regulation of *Rfrp* and *Gpr147* genes by the GR agonist, dexamethasone and/or the main gonadal steroid, 17β-estradiol in this neuronal model. Neurons were treated with vehicle, 20 nM dexamethasone, 10 nM 17β-estradiol, or co-treatment of dexamethasone with 17β-estradiol over a 24 h time course. We found that dexamethasone significantly induces a 2.98 and 2.47-fold change in *Rfrp* mRNA levels alone or in the presence of 17β-estradiol, respectively, following 24 h treatment: vehicle (0.49 ± 0.05) vs. dexamethasone (1.48 ± 0.22), $P < 0.05$; vehicle (0.49 ± 0.05) vs. dexamethasone + estradiol (1.23 ± 0.37), $P < 0.05$ (Fig. 2A). Similarly, *Gpr147* levels are significantly augmented after 8 and 24 h following 20 nM dexamethasone treatments [8 h, vehicle (0.78 ± 0.06) vs. dexamethasone (1.44 ± 0.20), $P < 0.01$; 24 h, vehicle (0.80 ± 0.14) vs. dexamethasone (1.47 ± 0.05), $P < 0.01$] (Fig. 2B). In this study, we found 17β-estradiol to have no significant effects on either *Rfrp* or *Gpr147* mRNA levels (Fig. 2A and B). These results clearly demonstrate that both *Rfrp* and *Gpr147* genes are regulated directly by GCs and not by the gonadal steroid, 17β-estradiol in the hypothalamic rHypoE-23 neuronal cell line.

3.3. Potential autocrine regulation of Rfrp and Gpr147 mRNA expression by RFRP-3

The co-expression of *Rfrp* and *Gpr147* in rHypoE-23 neurons prompted the investigation into potential autoregulatory mechanisms involved in the fine-tuning of the RFRP/GPR147 system. Neurons were treated with vehicle, 10 nM RFRP-3, or 100 nM RFRP-3 over a 24 h time course and RNA was isolated at indicated time points (1, 2, 4, 8, and 24 h). We found that RFRP-3 did not exert any significant changes on *Rfrp* gene expression, however a moderate dose-dependent increase was observed at 1 h following 100 nM RFRP-3 treatment: vehicle (0.70 ± 0.14) vs. 10 nM RFRP-3 (1.14 ± 011) vs. 100 nM RFRP-3 (1.21 ± 0.10), $P > 0.05$ (Fig. 3A). *Gpr147* mRNA levels were not significantly changed upon 10–100 nM RFRP-3 treatment over a 24 h time course (Fig. 3B). These results demonstrate that RFRP-3 at the indicated doses does not independently participate in any autoregulatory mechanisms within 24 h in this *Rfrp*-expressing neuronal cell model.

3.4. GR antagonist, RU-486 abolishes the dexamethasone-mediated mRNA expression changes in rHypoE-23 neurons

We further investigated whether the induction of *Rfrp* and *Gpr147* mRNA levels by dexamethasone in rHypoE-23 neurons was a GR-mediated mechanism. RU-486, a GR antagonist was used to block endogenous GR activity. rHypoE-23 neurons were pre-incubated with 50 nM RU-486 or water vehicle for 1 h prior to 20 nM dexamethasone treatment. Neurons pre-treated with RU-486 failed to demonstrate modified levels of *Rfrp* mRNA upon dexamethasone treatment after 24 h: 50 nM RU-486 (0.585 ± 0.14) vs. RU-486 + dexamethasone (0.72 ± 0.16), $P > 0.05$ (Fig. 4A). Contrary to these findings, control neurons exhibit a 2.75-fold increase of *Rfrp* mRNA levels in response to dexametha-

A

	Hypothalamus	rHypoE-23
RFRP	+	+
Kiss-R (GPR54)	+	-
GnIH-R (GPR147)	+	+
ERα	+	+
ERβ	+	+
GPR30	+	+
GR	+	+
AR	+	+
Galanin	+	+

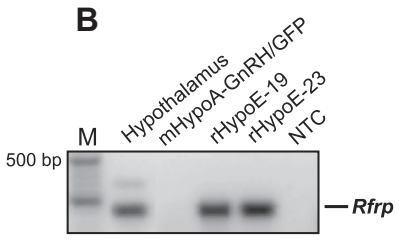


Fig. 1. Characterization of the gene expression profile of Rfrp-expressing, rHypoE-23 neurons. (A) RT-PCR results of relevant reproductive neuropeptides and receptors in the whole hypothalamus (positive control) and indicated hypothalamic cell line. (+) indicates the presence of a gene, and (−) indicates the absence or weak expression of a gene. (B) Representative RT-PCR screening of Rfrp, amplicon size is 150 bp. Total RNA was isolated and used in the One-Step RT-PCR Qiagen kit with gene-specific primers. Products were visualized on a 2% agarose gel with whole hypothalamus (positive control), two Rfrp-expressing cell lines (rHypoE-19 and rHypoE-23), a non-Rfrp-expressing cell line (mHypoA-GnRH/GFP) and a non-template control (NTC; negative control).

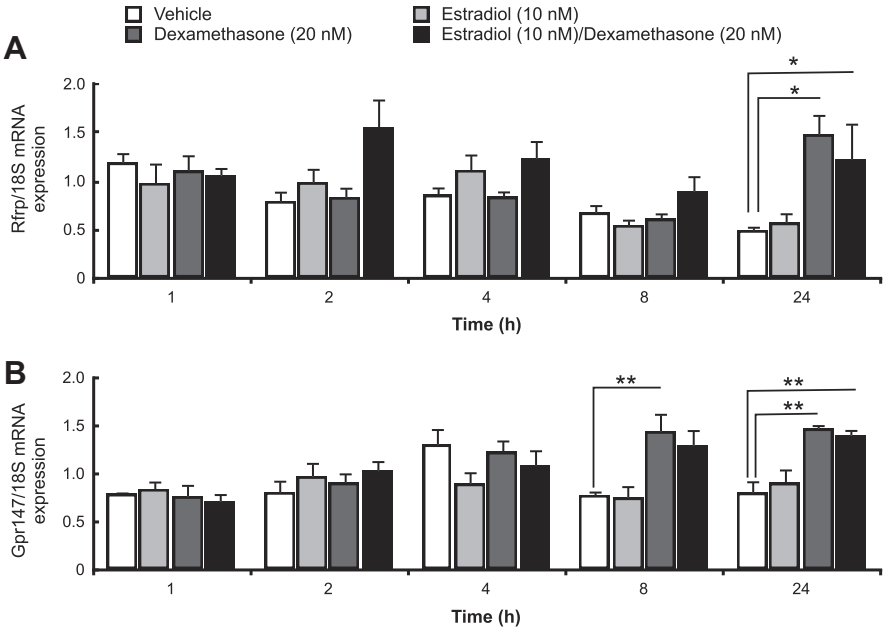


Fig. 2. Dexamethasone regulates the expression of Rfrp and Gpr147 genes in rHypoE-23 neurons. Cells were treated with 10 nM Estradiol, 20 nM Dexamethasone, co-treatment of estradiol (10 nM)/dexamethasone (20 nM), or vehicle. RNA was collected at specified time points and changes in Rfrp (A) or Gpr147 (B) mRNA expression were quantified using qRT-PCR. mRNA levels were normalized to 18S. Data is expressed as mean ± SEM (n = 5–6 independent experiments). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle control. Statistical significance was determined by two-way ANOVA with Bonferroni's post *hoc* test.

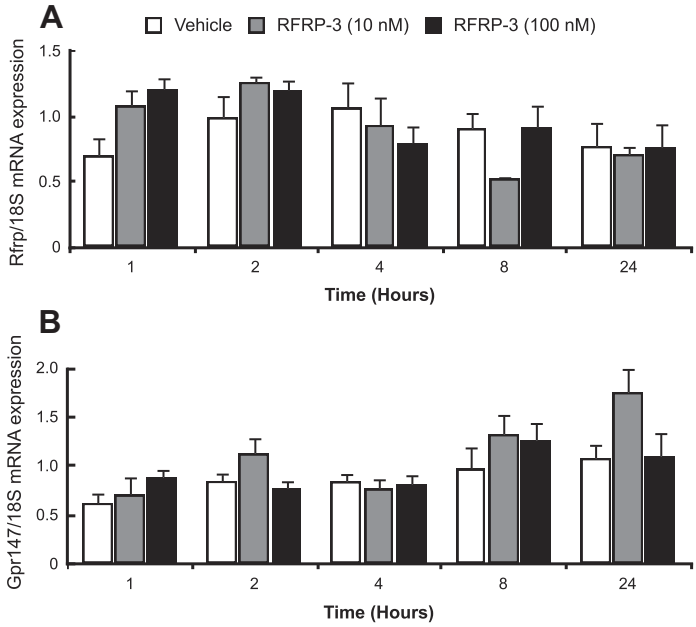


Fig. 3. Effect of RFRP-3 treatment on gene expression in the Rfrp-expressing, rHypoE-23. Cells were treated with vehicle, 10 nM RFRP-3, or 100 nM RFRP-3. Total RNA was collected over a 24 time course at specified time points and changes in Rfrp (A) or Gpr147 (B) mRNA expression were quantified using qRT-PCR. mRNA levels were normalized to the internal control, 18S. Data is expressed as mean ± SEM (n = 4–5 independent experiments). $^{*}P < 0.05$ vs. time-matched vehicle control. Statistical significance was determined by two-way ANOVA with Bonferroni's post *hoc* test.

sone treatment, as compared to controls (EtOH:H20, 0.63 ± 0.09 vs. dexamethasone, 1.75 ± 0.36; $P < 0.01$). Therefore, pretreatment with RU-486 significantly blocks the dexamethasone-mediated increases of *Rfrp* mRNA: dexamethasone: (1.75 ± 0.36) vs. RU-486 + dexamethasone (0.72 ± 0.16), $P < 0.05$. RU-486 exerted similar effects on dexamethasone-mediated upregulation of *Gpr147* mRNA levels, whereby total RNA was isolated at 8 and 24 h following dexamethasone treatment. qRT-PCR results demonstrate that pre-incubation of neuronal cells with RU-486 blocks the augmentation of *Gpr147* mRNA levels initially seen with dexamethasone treatment at 8 h: EtOH:H20 (0.77 ± 0.07) vs. dexamethasone (1.43 ± 0.14), $P < 0.01$; RU-486 (0.93 ± 0.14) vs. RU-486 + dexamethasone (0.92 ± 0.15), $P > 0.05$, as well as 24 h: EtOH:H20 (0.88 ± 0.05) vs. dexamethasone (1.66 ± 0.27) $P < 0.01$; RU-486 (0.78 ± 0.15) vs. RU-486 + dexamethasone (0.94 ± 0.05), $P > 0.05$ (Fig. 4B-C). RU-486 treatment alone has no effect on basal mRNA levels of *Rfrp* or *Gpr147*. These findings indicate that dexamethasone increases both *Rfrp* and *Gpr147* biosynthesis directly through a GR-dependent mechanism, and further demonstrates that the rHypoE-23 neuronal cell model is directly responsive to GCs.

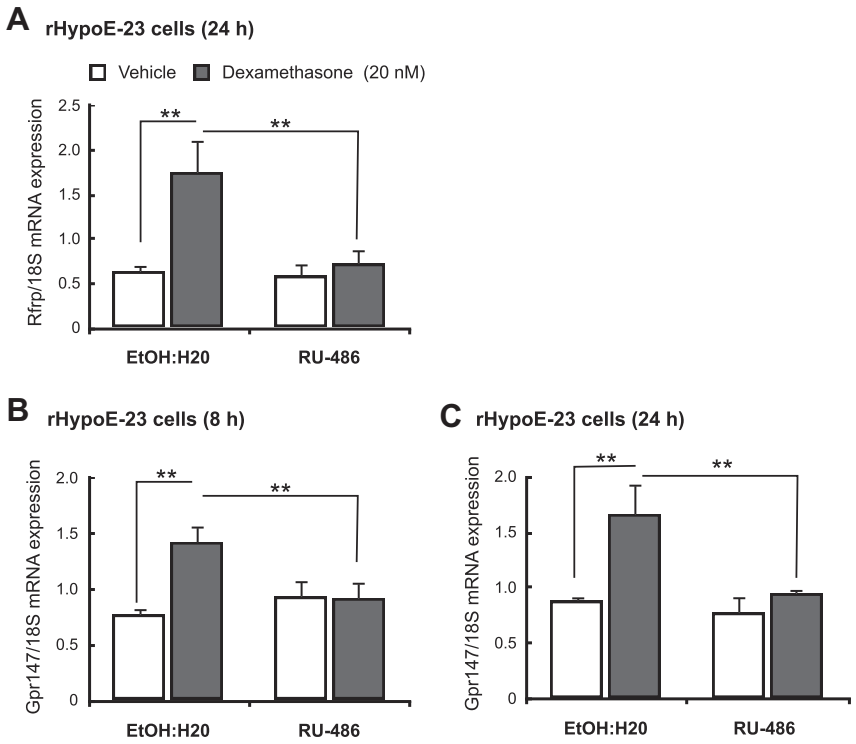


Fig. 4. Dexamethasone upregulation of Rfrp and Gpr147 mRNA expression in rHypoE-23 neurons is blocked by GR antagonist, RU-486. Relative mRNA levels for Rfrp (A) and Gpr147 (B and C) for 8 and 24 h with dexamethasone (20 nM) in the presence or absence of RU-486 (50 nM). mRNA levels were normalized to the internal control, 18S. Results shown are expressed as mean ± SEM (n = 4–5 independent experiments). $^{**}P < 0.01$ vs. control or dexamethasone. Statistical significance was determined by one-way ANOVA with Bonferroni's post *hoc* test.

3.5. Dexamethasone-mediated induction of Rfrp and Gpr147 genes is independent of de novo protein synthesis in hypothalamic neurons

To determine whether the upregulation of *Rfrp* and *Gpr147* levels is dependent on *de novo* protein synthesis, rHypoE-23 neurons were pre-exposed to CHX (1 μg/mL) for 1 h, followed by 20 nM dexamethasone treatments. CHX concentration was chosen due to minimal cell death, as determined after dose curve analysis. Total RNA was isolated at indicated time points and analyzed using qPCR. Despite the presence of CHX, dexamethasone significantly induces *Rfrp* expression after 24 h: CHX (0.34 ± 0.03) vs. CHX + dexamethasone (1.08 ± 0.12), $P < 0.05$ (Fig. 5A). Though, neurons treated with dexamethasone alone demonstrate significantly higher levels of *Rfrp* mRNA than those incubated with both CHX and dexamethasone: dexamethasone (2.47 ± 0.28) vs. CHX + dexamethasone (1.08 ± 0.12), $P < 0.01$. Similarly, CHX had no effect on the dexamethasone-mediated upregulation of *Gpr147* mRNA after 24 h in rHypoE-23 neurons: EtOH:H20 (0.65 ± 0.09) vs. dexamethasone (1.37 ± 0.23), $P < 0.01$; CHX (0.72 ± 0.07) vs. CHX + dexamethasone (1.27 ± 0.12), $P < 0.05$ (Fig. 5B). Our results indicate that CHX decreases the basal levels of *Rfrp*, suggesting that CHX may be inhibiting the synthesis of proteins, such as gene enhancers responsible for the stability and basal regulation of *Rfrp* mRNA levels. Furthermore, these findings demonstrate that *de novo* protein synthesis is not required for the effects of dexamethasone on *Rfrp* and *Gpr147* biosynthesis.

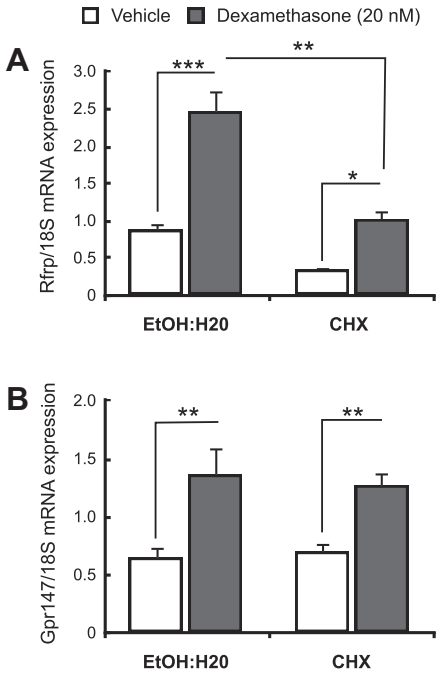


Fig. 5. Effects of protein synthesis blockade on dexamethasone-mediated mRNA expression changes in rHypoE-23 neurons. Cells were pre-treated with cycloheximide (CHX), a protein synthesis inhibitor (1 μg/mL) for 1 h and followed by dexamethasone or vehicle treatment for 24 h. RFRP (A) and GPR147 (B) mRNA expression was quantified using real-time RT-PCR and levels were normalized to the internal control, 18S. Results are expressed as mean ± SEM (n = 5–6 independent experiments). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle or CHX control. Statistical significance was determined by one-way ANOVA with Bonferroni's post *hoc* test.

3.6. Effect of transcriptional blockade on the dexamethasone-mediated induction of Rfrp and Gpr147 genes

To further define the mechanism through which dexamethasone mediates *Rfrp* and *Gpr147* mRNA levels, rHypoE-23 neurons were exposed to an RNA polymerase II gene transcription inhibitor, DRB (60 μM) 1 h prior to 20 nM dexamethasone treatments. Total

RNA from the rHypoE-23 neurons was collected at 8 and 24 h following treatments. Neurons pre-treated with EtOH:H20 or DMSO vehicle controls demonstrated a significant induction in *Rfrp* levels in the presence of dexamethasone at 24 h: EtOH:H20 (0.74 ± 0.08) vs. EtOH:H20 + dexamethasone (1.68 ± 0.18), $P < 0.001$; DMSO (0.78 ± 0.06) vs. DMSO + dexamethasone (1.54 ± 0.21), $P < 0.01$; however, in the presence of a transcriptional inhibitor, dexamethasone-mediated regulation of *Rfrp* expression was attenuated: DRB (0.54 ± 0.15) vs. DRB + dexamethasone (0.69 ± 0.89), $P > 0.05$ (Fig. 6A). Interestingly, DRB-mediated transcriptional blockade in the rHypoE-23 neurons still resulted in a significant induction of *Gpr147* levels upon dexamethasone treatment at 8 h: DRB (1.10 ± 0.07) vs. DRB + dexamethasone (1.52 ± 0.08), $P < 0.05$ (Fig. 6B). We observed that DRB treatment alone also elevated basal *Gpr147* levels at 8 h [EtOH:H20 (0.58 ± 0.04) vs. DRB (1.10 ± 0.07), $P < 0.01$; DMSO (0.75 ± 0.06) vs. DRB (1.10 ± 0.07), $P < 0.05$], suggesting that other transcriptional effectors or suppressors may be implicated in regulating basal *Gpr147* transcript stability in a time-dependent manner (Bradley and Cheatham, 1999; Dalvi et al., 2012). Similar to *Rfrp* regulation, dexamethasone failed to induce *Gpr147* mRNA levels in the presence of DRB at 24 h, as compared to controls: EtOH:H20 (0.63 ± 0.09) vs. EtOH:H20 + dexamethasone (1.10 ± 0.11), $P < 0.05$; DMSO (0.74 ± 0.08) vs. DMSO + dexamethasone (1.27 ± 0.06), $P < 0.01$; DRB (1.02 ± 0.13) vs. DRB + dexamethasone (1.31 ± 0.14), $P > 0.05$ (Fig. 6C).

4. Discussion

The RFRP neuronal circuit has emerged as an important mediator of reproductive function within the mammalian HPG axis, yet the direct regulation of *Rfrp* and *Gpr147* biosynthesis is not well defined. There is mounting evidence supporting the influential role of RFRP-3 in steroidal- and environmental-induced reproductive effects (Clarke and Smith, 2010; Kaewwongse et al., 2011; Kirby et al., 2009; Perfito et al., 2011; Poling et al., 2012; Soga et al., 2012); however, due to heterogeneity of the hypothalamus and numerous innervating neuronal networks, dissecting direct and indirect effects on the RFRP system *in vivo*, are challenging and confounded. In the present study, we used an embryonic hypothalamic cell line that demonstrates strong endogenous expression of *Rfrp* mRNA, as well as, relevant reproductive receptors, with the aim of delineating GC-mediated effects on the RFRP system, in the presence or absence of 17β-estradiol. The expression profile of rHypoE-23 neurons presently described is corroborated by *in vivo* studies demonstrating the co-expression of RFRP with the GR, ERs and AR (Kriegsfeld et al., 2006; Molnar et al., 2011; Poling et al., 2012; Smith et al., 2008); though, the latter has only been identified in male Syrian hamsters (Kriegsfeld et al., 2006) and at very low (3%) levels in male and female mice with no effect on *Rfrp* expression (Poling et al., 2012).

Furthermore, we describe for the first time that *Rfrp*-expressing neurons also synthesize *Gpr147*, indicative of a potential autoregulatory mechanism controlling *Rfrp* synthesis or secretion. This mechanism is substantiated by the presence of autocrine mechanisms in other neuropeptidergic systems, such as GnRH. The GnRH receptor (GnRH-R) has been found to be expressed on GnRH neurons *in vivo* and in GnRH-secreting cell models, allowing for a concentration and time-dependent ultra short feedback loop to mediate both the neuronal activity and peptide secretion in a subpopulation of neurons (Krsmanovic et al., 1993; Padmanabhan et al., 1995). The presence of *Gpr147* in *Rfrp*-expressing neurons warrants further investigation into potential autocrine feedback mechanisms responsible for the fine-tuning of *Rfrp* synthesis and peptide availability. Preliminary data suggests that RFRP-3 treatment only moderately increases *Rfrp* gene expression following 1 h of treatment; however no significant changes were reported

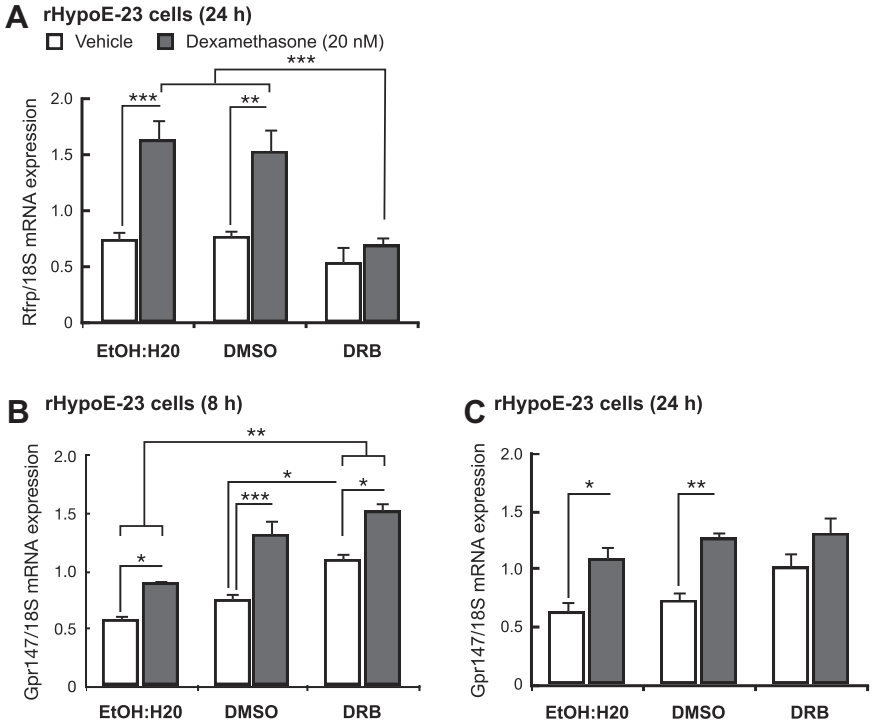


Fig. 6. Dexamethasone-mediated changes in Rfrp and Gpr147 mRNA levels are attenuated in the presence of a transcription inhibitor, 5,6-Dichlorobenzimidazole riboside (DRB) in rHypoE-23 neurons. Hypothalamic neurons were pre-treated for 1 h with DRB (60 μM) or vehicle, followed by dexamethasone (20 nM) treatment. Total RNA was isolated at 8 and 24 h following treatment and mRNA levels for Rfrp (A) and Gpr147 (B and C) were normalized to the internal control, 18S. Results shown are expressed as mean ± SEM ($n = 4–7$ independent experiments). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control or dexamethasone. Statistical significance was determined by one-way ANOVA with Bonferroni's post *hoc* test.

using the indicated doses within 24 h of treatment (Fig. 3). These results suggest that RFRP-3 may require other effectors to participate in autocrine mechanisms, a longer period of incubation, or may not directly regulate the RFRP system at the level of transcription in rHypoE-23 neurons. Further studies are required to investigate the role of RFRP-3 in any potential autoregulatory mechanisms in this hypothalamic cell model. Overall, given the co-expression of *Rfrp* with sex steroid and glucocorticoid receptors, the rHypoE-23 cell model is strongly representative of *Rfrp*-expressing neurons localized to the DMH.

17β-estradiol is the most potent estrogen and its main role is to regulate reproductive function at the level of the hypothalamus and pituitary (Herbison, 1998). Previous studies investigating the role of estradiol in the regulation of RFRP-3 have generally yielded conflicting results. It is noteworthy, that in the present study 17β-estradiol treatment alone did not exert any changes in *Rfrp* or *Gpr147* gene expression, nor did it demonstrate additive or antagonizing effects in the presence of dexamethasone in rHypoE-23 neurons. Initial studies in ovariectomized Syrian hamsters found that subcutaneous 17β-estradiol injections induce FOS activation in RFRP neurons after 3 and 6 h, suggesting a role of RFRP in estrogen feedback (Kriegsfeld et al., 2006). Subsequent studies report that estradiol facilitates the suppression of *Rfrp* mRNA levels in mice (Molnar et al., 2011; Poling et al., 2012), yet similar studies in ewes found no response (Smith et al., 2008) and investigations in prepubertal rats reported a stimulation of *Rfrp* mRNA (Iwasa et al., 2012). There are several possible explanations for these observed differences. 17β-estradiol-mediated effects can be highly sensitive to dose and developmental stage, whereby as reported by Iwasa et al. lower estradiol concentrations during the prepubertal stage can cause a stimulatory effect, while higher concentrations during adulthood, a time also marked by decreases in RFRP cell number, result in the suppression of *Rfrp* mRNA (Iwasa et al., 2012; Poling et al., 2012).

Another factor to consider is the existence of heterogeneous subpopulations within RFRP neurons, a common feature in neuropeptidergic systems both *in vivo* and *in vitro* (Attardi et al., 1997; Cheng et al., 2010; Dumalska et al., 2008; Goodman et al., 2007). *In vitro*, this notion is supported by observations that GT1-7 cells comprise of two morphologically distinct subpopulations of GnRH neurons, type I and II (Attardi et al., 1997). Moreover, the two populations of neurons display differential sensitivity and regulation of both GnRH mRNA and secretion upon GC exposure. Heterogeneity within *Rfrp*-expressing neurons is consistent with recent studies by Poling et al. showing the presence of two distinct high and low-expressing RFRP neuronal populations in the DMH, each revealing unique developmental and steroidal induction patterns (Poling et al., 2012). Interestingly, other studies have also shown that subpopulations of cells can vary in the ratios of ERs present on the cell surface in response to specific stimuli, which has been associated with alterations in both ER-responsiveness and signaling in a cell-specific manner (Jones et al., 1999; Titolo et al., 2006). It is therefore quite conceivable that functional heterogeneity exists among the RFRP neuronal population, particularly in regards to the sensitivity of cells to steroidal regulation. Moreover, this may also underlie the differences in ER expression profiles, reported by others and in the present study. Thus, rHypoE-23 neurons are likely representative of an *Rfrp*-expressing subpopulation indirectly mediated by estradiol, thereby supporting our findings that demonstrate no direct estradiol-mediated effect on gene expression profiles. Because we did not examine the effect of estradiol on protein synthesis, we cannot rule out the possibility that estradiol is regulating the peptide availability of RFRP or GPR147 in this hypothalamic cell model. Overall, it remains unclear whether the RFRP system serves as a direct target for the integration of sex steroid-dependent feedback.

In contrast to 17β-estradiol, previous reports examining the effects of stress on the RFRP/GPR147 neuronal system are convincing and indicate that *Rfrp*-expressing neurons are both responsive and direct targets of GCs (Calisi et al., 2008; Kirby et al., 2009). Similar to these findings, we report that dexamethasone evoked a significant increase in both *Rfrp* and *Gpr147* expression after 8 and 24 h following treatment in this hypothalamic cell model. It is well documented in literature that the physiological consequences of stress on the HPG axis, in humans and nonprimate species are regulated mainly by circulating levels of GCs. Administration of GCs decreases LH release in rats and humans (Collu et al., 1979; Du Ruisseau et al., 1979; Rivier and Rivest, 1991), as well as directly represses GnRH gene expression by acting at the level of the proximal promoter (Chandran et al., 1994, 1996). We report that the GR is required for the GC-induced transcriptional regulation of *Rfrp* and *Gpr147* genes, whereby a potent GR antagonist, RU-486 abolishes the initial effects observed upon dexamethasone treatment. The involvement of GCs in an *Rfrp*-mediated stress response aligns with evidence that adrenalectomized rats fail to demonstrate changes in *Rfrp* mRNA expression in response to stress, as compared to sham controls (Kirby et al., 2009).

Given the parallel roles of RFRP-3 and stress in reducing responsiveness of the pituitary to GnRH (Son et al., 2012), leading to reductions in LH release (Johnson et al., 2007; Murakami et al., 2008; Smith et al., 2012), as well as acting at the level of the GnRH neuron to decrease neuronal activity (Ducret et al., 2009), it is plausible that this neuronal network serves to mediate GC-dependent suppression of the HPG axis. Interestingly, intracerebroventricular (ICV) injection of RFRP-3 was found to stimulate the release of ACTH and oxytocin, as well as induce anxiety-related behaviors during an open-field test in rats (Kaewwongse et al., 2011). Consistent with the RFRP-mediated stress-related behaviors, RFRP neurons have been shown to be in close apposition to CRH and oxytocin neurons (Qi et al., 2009), both of which are responsible for the maintenance of homeostatic control throughout the stress response (Onaka, 2004; Tellam et al., 1998). Therefore, *Rfrp*-expressing neurons may act directly on GnRH neurons or indirectly through other stress-related neuropeptidergic systems, like CRH. Presently, we cannot rule out the involvement of CRH in the direct regulation of *Rfrp* and *Gpr147* expression during the stress response, as we did not examine these effects; however, Kirby et al. found that 13% of RFRP neurons express the CRH-R1 and studies in hypothalamic N39 cells have shown that CRH (100 nM) increase *Gpr147* mRNA through the CRH-R1 (Kageyama et al., 2012; Kirby et al., 2009). Nevertheless, our results are consistent with a direct role for GCs in the regulation of *Rfrp* and *Gpr147* gene expression through a GR-dependent signaling mechanism in a hypothalamic *Rfrp*-expressing neuron model.

Gene regulation can occur at different levels, most commonly through transcriptional activation of the 5′ regulatory region, modulation of mRNA stability, and the rate of protein synthesis. To address which regulatory pathway predominates in the regulation of gene expression in rHypoE-23 neurons upon GC exposure, we further investigated the necessity of new protein synthesis and mRNA stability on GC-mediated changes in *Rfrp* and *Gpr147* levels. Using an inhibitor of protein synthesis, CHX, we are the first to report that the stimulation of gene expression of both *Rfrp* and *Gpr147* by dexamethasone is independent of *de novo* protein synthesis and likely due to transcriptional activation of specific promoter elements. This is consistent with results from other studies showing GR-mediated mechanisms in the hypothalamus. Delayed GC actions are generally mediated by the activation of the classical steroid hormone response involving the GR, a member of the nuclear receptor superfamily (Necela and Cidlowski, 2004). Ligand-bound GRs translocate into the nucleus and initiate an intracellular cascade leading to the activation or suppression of tar-

get genes. In line with this, the presence of a potent RNA polymerase II gene transcription inhibitor, DRB blocked the ability of dexamethasone to induce both *Rfrp* and *Gpr147* mRNA levels at 24 h.

Interestingly, transcription blockade did not attenuate the induction of *Gpr147* expression at 8 h; hence, the time-dependent activation of mRNA stabilizers cannot be ruled out for *Gpr147* expression in this hypothalamic model and requires further investigation. GR gene targets contain a glucocorticoid response element (GRE) or other DNA-binding transcription factor (TF) elements, allowing for direct DNA association or tethering mechanisms through associated TFs (Chandran et al., 1999; Johansson-Haque et al., 2008; Kriegsfeld et al., 2006). The GnRH-secreting, GT1-7 neurons show direct repression of GnRH following GC treatment by virtue of a GR-containing multiprotein complex at a distal and proximal negative GRE (Chandran et al., 1996, 1999). Similarly, studies in LβT2 gonadotrope cells found that dexamethasone upregulates mouse GnRH-R mRNA through the recruitment of GR to an AP-1 site, simultaneously bound with transcription factors, c-Jun and c-Fos (Kotitschke et al., 2009). Of interest, a search of the rat *Rfrp* promoter indicates the presence of a GRE, as well as, other commonly associated TF binding elements within the first 1500 base pairs (bp) of the 5′ flanking region. This observation is supported by similar findings by Kirby et al. in the rat *Rfrp* promoter (Kirby et al., 2009). Similarly, the rat *Gpr147* promoter reveals a classic GRE within 1500 bp of the transcriptional start site, supporting the direct GR-mediated transcriptional changes reported for both *Rfrp* and *Gpr147* genes in the present study. Future studies will map regions within the 5' regulatory regions of *Rfrp* and *Gpr147* to localize sequences that are both required and sufficient for the GR-mediated transcriptional changes in gene expression observed in the rHypoE-23 neurons.

Taken together with previous findings, the present study suggests that rHypoE-23 neurons are representative of functional *Rfrp*-expressing hypothalamic neurons originating from the DMH, which are directly regulated by GCs. Here we demonstrate for the first time that the synthetic GC, dexamethasone stimulates the transcription of *Rfrp* and *Gpr147* genes through a GR-dependent mechanism, as a result of transcriptional activation, rather than *de novo* protein synthesis in rHypoE-23 neurons. Because RFRP-3 has been found to regulate both neuroendocrine and behavioral mechanisms (Bentley et al., 2006a, 2006bClarke et al., 2008; Ducret et al., 2009; Johnson and Fraley, 2008; Perfito et al., 2011; Sethi et al., 2010; Smith et al., 2012), the stress-mediated transcriptional regulation of both *Rfrp* and *Gpr147* expression may further modulate or cross-talk with innervating neural pathways to exert effects within the HPG axis (Kirby et al., 2009; Kotitschke et al., 2009). Therefore, in line with the inhibitory role of RFRP-3 at the hypothalamic level (Ducret et al., 2009; Rizwan et al., 2012), we suggest that the RFRP/GPR147 system adds a new level of complexity to the fine-tuning of the mammalian HPG axis and is a direct target for stress-mediated transcriptional changes at the hypothalamic level, potentially altering reproductive function and status in response to a myriad of stressors.

Disclosure statement

The authors have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.12.015.

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