4

ROLE OF HYPOTHALAMIC-PITUITARY-ADRENAL AXIS IN PCOS RAT MODEL

Reproduction is a fundamental process regulated by several intrinsic factors such as steroids, inhibin, kisspeptin (Christensen et al., 2012; Pinilla et al., 2012) as well as extrinsic factors such as stress, opioids and neurotransmitters (Vrekoussis et al., 2010; Bhattarai et al., 2014). Amongst the other factors, stress is a predominant factor increasing due to lifestyle alterations, which may affect several body functions. Stress can be defined as a state of real or perceived threat to homeostasis (Smith & Vale, 2006). To combat stress and maintain normal body function, activation of endocrine, nervous and immune systems is required; which is collectively known as stress responses (Chrousos & Gold, 1992; Carrasco & Van de Kar, 2003). The stress-mediated adaptation of homeostasis is principally achieved through hypothalamic-pituitary-adrenal (HPA) axis.

Overview of Hypothalamic-Pituitary-Adrenal (HPA) axis

Corticotropin releasing hormone (CRH) is the principle regulator of Hypothalamic-Pituitary-Adrenal (HPA) axis. Parvocellular neurons of the paraventricular nucleus of hypothalamus secrete CRH in presence of stress stimuli. CRH reaches the anterior pituitary through hypophysial portal vessels and stimulates the release of adrenocorticotropic hormone (ACTH) from pituitary through activation of CRH receptor. ACTH released by corticotropes of anterior pituitary enters the blood stream and stimulates adrenal cortex to release cortisol, which then binds to its receptor (glucocorticoid receptor GR) on target tissues and culminates

into stress response. Further, cortisol regulates ACTH and CRH release from pituitary and hypothalamus, respectively via short and long feedback loops.

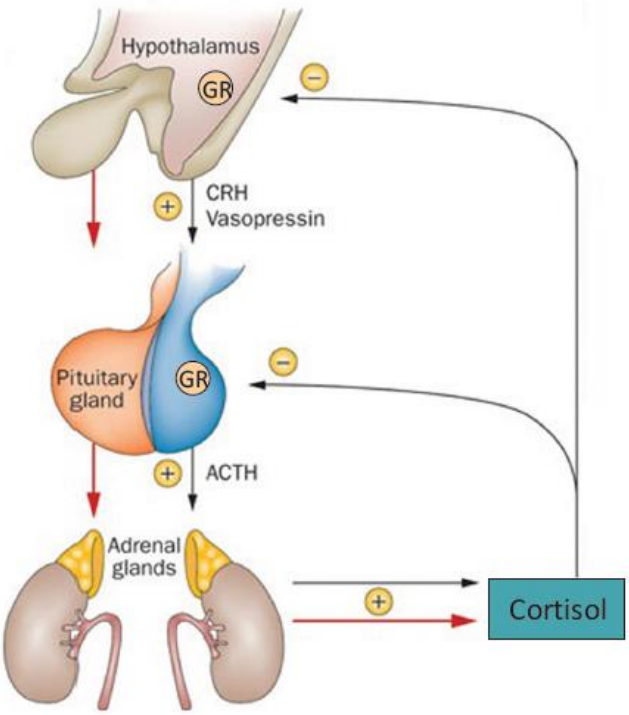

Figure 4.A: Hypothalamic-pituitary-adrenal axis

Corticotropin releasing hormone (CRH) and related peptides

CRH is a 41-amino acid peptide that is synthesized by hypophysiotropic neurons localized in the medial parvocellular subdivision of paraventricular nucleus (PVN) of the hypothalamus. The principle function of CRH includes stimulation of ACTH release from anterior pituitary as well as regulation of autonomic nervous system, food intake, learning and memory and reproductive behaviour (Chatterton, 1990; Contarino et al., 2000; Croiset et al., 2000). CRH is localized throughout the central nervous system including olfactory bulb, preoptic area, lateral hypothalamus, amygdala and motor cortex (Sawchenko et al., 1993). In addition, CRH is also synthesized in various peripheral tissues such as adrenal glands, testes, placenta, gastrointestinal tract and skin (Smith & Vale, 2006). Recently, three different peptides demonstrating structural similarities to CRH have been identified that include Urocortin (Ucn) 1, Ucn2 and Ucn3. The localization of Ucn1 is prominent in Edinger-Westphal nucleus, cerebellum, hippocampus, neocortex, basal ganglia, amygdala and various nuclei of hypothalamus that include supraoptic, ventromedial and paraventricular nuclei (Hillhouse & Grammatopoulos, 2006). Similarly, Ucn2 mRNA or immunoreactivity has also been

reported in various brain regions including paraventricular, supraoptic and arcuate nuclei of hypothalamus, locus coeruleus, brain stem, stria terminalis, medial amygdaloid nucleus as well as anterior and intermediate lobe of pituitary (Hillhouse & Grammatopoulos, 2006). Ucn3 is also distributed widely in brain areas such as ventromedial and preoptic nuclei of hypothalamus, lateral septum, stria terminalis, and amygdala (Chen et al., 1993).

The actions of CRH family of peptides are mediated by their binding to two subtypes of GPCRs – CRH receptor 1 (CRHR1) and CRHR2. CRHR1 receptors are predominantly found in anterior pituitary, olfactory bulb, hippocampus, cerebral cortex and cerebellum with low expression in peripheral tissues such as adrenal glands, ovaries and testes (Van Pett et al., 2000; Smith & Vale, 2006). The expression of CRHR2 is less prominent in brain, localized to lateral septum, ventral medial hypothalamus and raphe nuclei while high expression is reported in heart, skeletal muscle, skin and gastrointestinal tract (Van Pett et al., 2000; Kishimoto et al., 1995). Both CRHR1 and CRHR2 have different binding affinity for CRH family peptides. CRH has higher specificity for CRHR1 than CRHR2 while Ucn1 binds to both CRHR1 and CRHR2 with same affinity. Unlike CRH and Ucn1, Ucn2 and Ucn3 have high affinity for CRHR2 and a very little affinity for CRHR1 (Smith & Vale 2006; Hillhouse & Grammatopoulos 2006). CRH and urocortins play varied physiological functions owing to their widespread distribution. CRH and CRHR1 are the principle regulator of pituitary ACTH release whereas Ucn1, Ucn2 and Ucn3 are involved in energy balance, reproductive behaviour, immune response and other stress mediated alteration in physiology (Hillhouse & Grammatopoulos 2006). Among all, the direct role of Ucn2 in GnRH and gondotropin synthesis and release is emerging (Kageyama, 2013).

Adrenocorticotropic hormone (ACTH)

Binding of CRH to its receptor CRHR1 on anterior pituitary corticotropes stimulates cAMP mediated response through activating $G\alpha_s$ subunit (Hillhouse & Grammatopoulos, 2006), resulting into production and secretion of ACTH. The synthesis of ACTH occurs from the precursor peptide pro-opiomelanocortin (POMC). Along with ACTH, β-endorphin, β-lipotropic hormone and melanocortins are also processed from POMC (Raffin-Sanson et al., 2003). ACTH is a peptide of a single chain of 39 amino acids that is secreted throughout the day in irregular pulses (Mitrovic, 2003). The pulsatile release of ACTH and cortisol regulates sleep-wake cycle and circadian rhythm. ACTH mediates its action through binding to the ACTH receptor (ACTHR) or melanocortin receptor 2 (MC_2 receptor). ACTH receptors are

GPCRs of the melanocortin receptor family, principally localized to zona fasciculata of adrenal cortex, skin and adipocytes. Activation of ACTHR by ACTH stimulates $G\alpha_s$ subunit leading to increased cAMP production through adenylate cyclase (Mountjoy et al., 1992). Elevated cAMP induces Protein kinase A (PKA), which increases adrenal steroid production via activating ERK (extracelluar signal-regulated kinase) and CREB (cAMP-response element binding protein) signalling molecules (Carbajal et al., 2011; Manna & Stocco, 2011).

Glucocorticoids

Upon ACTH stimulation, adrenal produces glucocorticoids cortisol and corticosterone from zona fasciculata of adrenal cortex. Similar to other steroid hormones, glucocorticoids are also synthesised from cholesterol by steroidogenic pathway. Steroidogenic enzymes are localized in to three different zones of adrenal cortex leading to synthesis of various products namely aldosterone and corticosterone from zona glomerulosa, cortisol from zona fasciculata and androgens from zona reticularis. ACTH stimulates cholesterol transport through increasing the number of LDL receptors on adrenocortical cells (Mitrovic, 2003). Once inside the cells, the cholesterol is transported to inner mitochondrial membrane by the steroid acute regulatory protein (StAR) and processed further by enzyme Cytochrome P450 side-chain cleavage (CYP11A1), the rate limiting step in steroidogenesis.

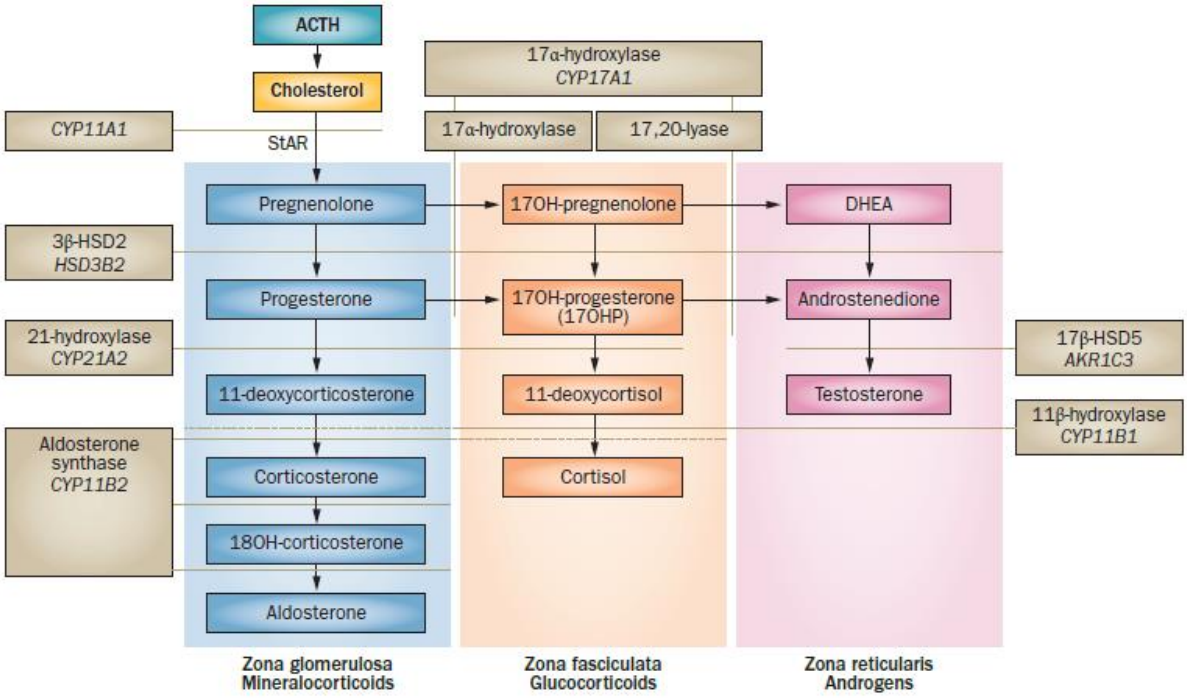

Figure 4.B: Steroidogenesis in adrenals (Han et al., 2013)

As shown in the Figure 4.B, several enzymatic reactions convert pregnenolone into aldosterone, cortisol or androgens (Han et al., 2013). The important enzymes for aldosterone and cortisol synthesis are P450 cytochrome family members 21-hydroxylase and 11β-hydroxylase. Progesterone and 17-hydroxyprogesterone are converted to 11-deoxycorticosterone and 11-deoxycortisol by the enzyme 21-hydroxylase, respectively. Further, hydroxylation reactions convert these substrates into corticosterone and cortisol. In humans and other primates, cortisol is released from the adrenal gland and serves as the major glucocorticoid hormone. Rodents lack the enzyme17α-hydroxylase in the adrenal gland and are thus unable to produce cortisol. Instead, corticosterone is the principle glucocorticoid in these species (Mitrovic, 2003). Under the influence of angiotensin, corticosterone is converted into aldosterone through the enzyme aldosterone synthase. In addition to glucocorticoids and mineralocorticoids, androgens are also produced upon ACTH stimulation and all these together combat stress condition.

Glucocorticoids are the major class of steroid hormones that regulate metabolic, cardiovascular, immune and behavioural processes (Smith & Vale, 2006). The functionality of glucocorticoid is dependent on its binding to glucocorticoid receptor (GR) on various target organs. GR is a member of the nuclear receptor superfamily that belongs to ligand-dependent transcription factor receptors (Oakley & Cidlowski, 2013). GR is transcribed by the gene NR3C1 localized on chromosome 5 in humans. Upon stimulation of glucocorticoid, GR is activated, resulting into transcriptional induction or suppression of 10-20% of human genome suggesting the widespread effect of glucocorticoids (Galon et al., 2002; Oakley & Cidlowski, 2013). GR is predominantly present in various brain regions such as hippocampus, hypothalamus enterorhinal cortex, frontal cortex and paraventricular nucleus where it functions in neuronal and glial metabolism, neurotransmittion, learning and memory

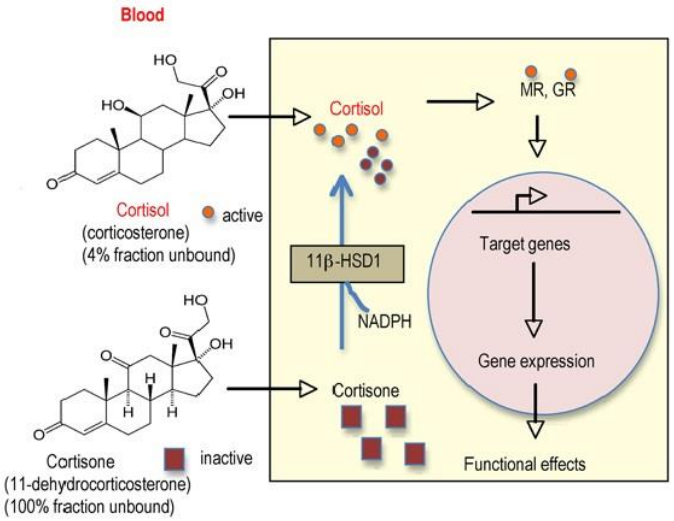

Figure 4.C: Mechanism of action of glucocorticoids (Yau & Seckl, 2012)

and mood regulation (Webster et al., 2002). Also, GR is widely distributed in peripheral tissues including heart, ovary, testis, liver, kidney and adipose tissue and help in stress-mediated responses.

The activity of cortisol depends upon the bioavailability of the active molecule in the tissue. Cortisol and corticosterone are the active glucocorticoids whereas cortisone is inactive glucocorticoid. Cortisone can be converted to cortisol or corticosterone by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and the reverse reaction is carried out by 11β-HSD2 enzyme. Most of the cortisol/corticosterone is bound with corticosteroid binding globulin (CBG) and around 4% is freely available in blood circulation whereas all inactive cortisone molecules are unbound (Yau & Seckl, 2012). Only, freely available cortisol and cortisone can enter the tissue. As shown in figure 4.C, the cortisone is converted into cortisol by 11β-HSD1 and activates GR, which translocates to nucleus and either activates or suppresses the transcription of various genes.

Regulation of Hypothalamic-Pituitary-Adrenal axis

Stress response is very crucial for maintenance of normal homeostasis and survival. Thereby, regulation of HPA axis activity is achieved through several mechanisms, which mainly include feedback control by glucocorticoids. In addition, glucocorticoid receptors present in discrete brain regions also influences CRH and ACTH secretion via numerous neurotransmitter and neurosteroid interactions.

Feedback regulation of HPA axis

Glucocorticoids can influence HPA axis activity through two mechanisms: i) slow feedback regulation through genomic alteration and ii) fast non-genomic feedback regulation. The delayed feedback regulation involves participation of GR localized in a number of stress-responsive brain regions that include hippocampus, frontal cortex, pituitary and amygdala (Herman et al., 2016). Active glucocorticoids directly enter into these tissues or inactive cortisone is converted into active cortisol and then it binds to cytosolic GR. Activated GR translocates into nucleus and binds to DNA-binding motif glucocorticoid response elements (GRE) in the promoter region of genes and regulates expression of CRH and ACTH from hypothalamus and pituitary respectively (Keller-Wood & Dallman, 1984; Bamberger et al., 1996). It has been reported that glucocorticoid has higher affinity for mineralocorticoid receptors (MR) than for GR and hence, the basal HPA secretion is regulated via MRs, while

GRs participate in stress-induced feedback regulation of HPA axis (De Kloet et al., 1998; Smith & Vale, 2006).

In contrast to slow feedback regulation, fast feedback regulation helps in rapid response to stressful conditions through involvement of several neurotransmitters. The major sites of fast feedback regulation are paraventricular nucleus (PVN), hippocampus and prefrontal cortex, which express high amount of GR and MR. *In vivo* and *in vitro* experiments have demonstrated that activation of GR in PVN results into rapid release of endocannabinoids which inhibit CRH neurons. Furthermore, neurotransmitters like GABA, glutamate, dopamine and serotonin regulate CRH release to CRH neurons via trans-synaptic inhibition (Herman et al., 2016) The involvement of neurotransmitters in HPA axis regulation will be discussed in the next chapter (Chapter 5).

In addition to its own regulation, the components of HPA axis influence other endocrine axes such as the hypothalamic-pituitary-ovarian axis and hypothalamic-pituitary-thyroid axis. Of these, interactions of HPA with HPO axis will be discussed in following section.

Crosstalk between adrenal and ovarian axis

It is well known that stress is the major inhibitor of reproductive processes and each component of the HPA axis has a potential to influence HPG axis (Figure 4.D).

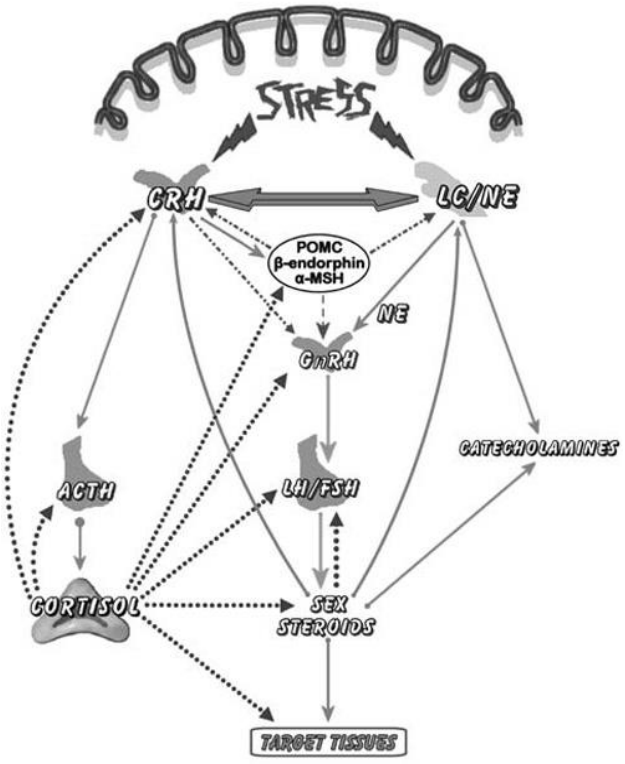

Figure 4.D: Crosstalk between adrenal and ovarian axes (Kyrou & Tsigos, 2008)

Amongst the HPA axis elements, glucocorticoids are the principal molecules affecting the reproductive axis. Various reports indicate that glucocorticoids inhibit GnRH neuronal activity through activation of GR in hypothalamus and hippocampus (Rabin et al., 1990; Kyrou & Tsigos, 2008; Whirledge & Cidlowski, 2010). Also, glucocorticoid suppresses GnRH mRNA and transcriptional activity as demonstrated with experiments on hypothalamic cell line (Chandran et al., 1994; Whirledge & Cidlowski, 2010). In addition to hypothalamus, GR is expressed in pituitary gonadotropes where it differentially regulates gonadotropin secretion. Glucocorticoid treatment in immortalized mouse gonadotrope cell line significantly reduced GnRH-stimulated LH release (Breen et al., 2012). Along with brain regions, GR, 11bHSD1 and 11bHSD2 have been shown present in ovaries of rats, pigs, cattle and humans (Amweg et al., 2013; Zhu et al., 2016). The expression pattern of these molecules changes during estrus cyclicity suggesting their role in ovarian folliculogenesis (Amweg et al., 2013).

ACTH and CRH families of peptides also influence HPA axis. Intravenous injection of ACTH in sow resulted in increased plasma cortisol levels with no change in LH concentration (Zhu et al., 2016). Also, follicular wall of ACTH-treated sow demonstrated significant decrease in mRNA expression of LHR, Cyp17A1, 3bHSD and Cyp19A1, thereby indicating the role of ACTH in ovarian response to gonadotropins and in ovarian steroidogenesis (Zhu et al., 2016). Furthermore, CRH can directly suppress GnRH release from hypothalamus or it can indirectly inhibit GnRH through increasing the secretion of β-endorphin from arcuate neurons (Rabin et al., 1990; Kyrou & Tsigos, 2008). In addition to classic HPA axis moieties, role of urocortin 2 (Ucn2) in regulation of reproduction is emerging. Studies on hypothalamic N39 cells suggest that Ucn2 stimulates GnRH mRNA levels via the CRH2 receptor (Kageyama et al., 2012). Also, Ucn2 reduces the expression as well as release of LH from pituitary gland (Nemoto et al., 2010). Ucn2 has also been detected in ovary and its expression changes with the ovarian cycle indicating the role of Ucn2 in ovarian folliculogenesis (Xu et al., 2006).

Rationale

All the above literature demonstrates the functioning of HPA axis during normal stress condition. During chronic stress exposure, modulation of baseline HPA function occurs with alterations in the responsiveness towards stress stimuli which may further hinder various body functions including metabolism (Herman et al., 2016). Such instances exert deleterious effects on metabolically active tissues like liver and adipose leading to development of

metabolic syndrome (Kyrou & Tsigos, 2008). As mentioned in Chapter 1, one of the emerging metabolic syndromes in women is polycystic ovarian syndrome and many researchers believe that increased prevalence of PCOS is, in part, due to changing lifestyle and increased stress levels (Bruner et al., 2006; Diamanti-Kandarakis et al., 2006). In this regard, the involvement of HPA axis dysregulation in PCOS pathology has been postulated. More than 50% of PCOS women demonstrate increased adrenal androgen production (Lanzone et al., 1995). The mechanism for adrenal hyperandrogenemia may arise from either altered adrenal responsiveness to ACTH or abnormal adrenal stimulation by factor(s) other than ACTH. Dexamethasone treatment to women with PCOS resulted in increased 17-hydroxyprogesterone (17-OHP) responses to ACTH, suggesting the alteration of 17α-hydroxylase enzyme (Ehrmann et al., 1992; Strauss III & Barbieri., 2014). Contrary to this, other studies failed to replicate the effect of dexamethasone (Azziz et al., 1995; Ditkoff et al., 1995). Thereby, two theories have been postulated to indicate the role of HPA axis in the pathophysiology of PCOS. According to one theory, increased peripheral cortisol metabolism results in a compensatory increase of ACTH secretion via a decrease in the negative feedback signal, maintaining normal serum cortisol levels at the expense of adrenal androgen excess (Tsilchorozidou et al., 2003). The recent study by Blumenfield and colleagues (2016) has demonstrated altered 5α- & 5β-reductase (cortisol degrading enzymes) activity in PCOS women supporting the hypothesis of dysregulated cortisol metabolism in PCOS. In contrast to this, another theory suggests an exaggerated adrenal response to ACTH which results into increased production of adrenal androgens in women with PCOS (Goodarzi et al., 2015). However, neither of the theories completely explicates the neuroendocrine alterations of PCOS condition. Although the influence of stress regulators including CRH, glucocorticoids and Ucn2 on reproduction is well known, a detailed understanding of these elements in PCOS pathology has yet to be obtained. Hence, an aim of the present study was to understand the status of the hypothalamic-pituitary-adrenal axis and its interaction with the ovarian axis in PCOS condition using letrozole-induced PCOS rat model.

Materials and Methods

The methods employed in this study have been discussed in detail in Chapter 2. Briefly, PCOS induction in animals was performed using letrozole. After validation of PCOS characteristics, PCOS positive animals were used for analysis of HPA axis activity. For all the experiments, blood and tissue collection was carried out between 8:00 and 9:00 am in diestrus stage of estrus cycle to negate any variation in the sample due to diurnal rhythm. For

estimation of ACTH, chemiluminescence immunoassay (CLIA) kits were used, whereas corticosterone was estimated by spectrofluorimetric method (Chapter 2). Transcript analysis of genes encoding CRH (*CRH*), CRHR1 (*CRHR1*), Ucn2 (*Ucn2*), CRHR2 (*CRHR2*), ACTHR (*ACTHR*), GR (*GR*) and 11βHSD1 (*11βHSD1*) was performed using real-time PCR. Also, adrenal steroidogenesis was measured using mRNA expression of steroidogenic enzymes- StAR (*StAR*), 3βHSD (*3βHSD1*), 21α-hydoxylase (*Cyp21a1*), 11β-hydroxylase (*Cyp11b1*), and Aromatase (*Cyp 19a1*). To confirm the alteration in adrenal steroidogenesis, western blot analysis of key signalling molecules phospho-ERK1/2, phospho-CREB and steroidogenic proteins StAR and Aromatase was performed. Also, estimation of steroid hormones progesterone, testosterone and estradiol was performed in adrenal gland using ELISA kits.

Results

HPA axis in PCOS

CRH is a peptide hormone transcribed from the gene CRH. Real-time PCR data revealed no difference in hypothalamic *CRH* and pituitary CRH receptor1 (*CRHR1*) in control and PCOS groups (Figure 4.1). However, expression of hypothalamic *CRHR1* was significantly reduced in PCOS group as compared to control ($P<0.01$). Binding of CRH to its receptor on pituitary leads to release of ACTH, which enters the blood stream and reaches the target organ adrenal. When analysed for serum ACTH levels, no difference was observed between the two groups. Further, ACTH binds to ACTH receptor on adrenal which stimulates steroidogenesis to form corticosteroids. Transcript analysis of *ACTHR* in adrenal demonstrated a significant increase in the *ACTHR* expression ($P<0.001$) with elevated serum corticosterone in PCOS ($P<0.001$).

Adrenal steroidogenesis in PCOS

Adrenal steroidogenesis is similar to the ovarian process wherein binding of ACTH to its receptor results into activation of steroidogenesis via increasing cAMP-mediated pathway. To understand the status of adrenal steroidogenesis, gene expression of key steroidogenic enzymes was analysed (Figure 4.2). PCOS rats demonstrated marked increase in expression of *StAR* ($P<0.01$), *3βHSD* ($P<0.05$), *Cyp21a1* ($P<0.05$), and *Cyp11b1* ($P<0.01$) with no change in *Cyp19a1* expression. Transcription of steroidogenic enzymes is regulated by the activation of downstream signalling cascade of ACTHR. Hence, to confirm any alteration of signalling pathway, western blot analysis of activated form of key signalling molecules phospho extracellular signal–regulated kinase (pERK1/2), phospho cAMP response element-

binding protein (pCREB) and steroidogenic proteins StAR and Aromatase was performed. PCOS rats demonstrated significantly increased protein levels of pERK1/2 ($P<0.001$), pCREB ($P<0.001$) and StAR ($P<0.01$) in adrenal gland with no such difference in Aromatase expression. This change in the transcript and protein levels could be well correlated with the adrenal steroid content. As depicted in Table 4.1, PCOS rats exhibited increased testosterone ($P<0.001$) and corticosterone ($P<0.001$) levels in adrenal gland, while no difference was observed for the progesterone and estradiol content between two groups.

HPA regulation

HPA axis is regulated by the feedback regulation of corticosteroids through glucocorticoid receptor (GR) and 11-β Hydroxysteroid dehydrogenase enzyme. Hence, mRNA expression studies for *GR* and *11βHSD1* was carried out in hypothalamus, pituitary, hippocampus, frontal cortex, adrenal and ovary (Figure 4.3). PCOS rats had significantly reduced expression of *GR* and *11βHSD1* in hypothalamus ($P<0.001$), pituitary ($P<0.01$), hippocampus ($P<0.05$) and ovary ($P<0.05$), whereas no change was observed in adrenal and frontal cortex *GR* and *11βHSD1* expression.

Status of Urocortin 2 in PCOS

Urocortin 2 is the major molecule to influence HPA and HPG axis. When analysed for expression, significant reduction in *Ucn2* and its receptor *CRHR2* was observed in only pituitary ($P<0.001$) of PCOS animals whereas no difference in expression was seen in hypothalamus, hippocampus, frontal cortex, ovary and adrenal gland as compared to control animals (Figure 4.4).

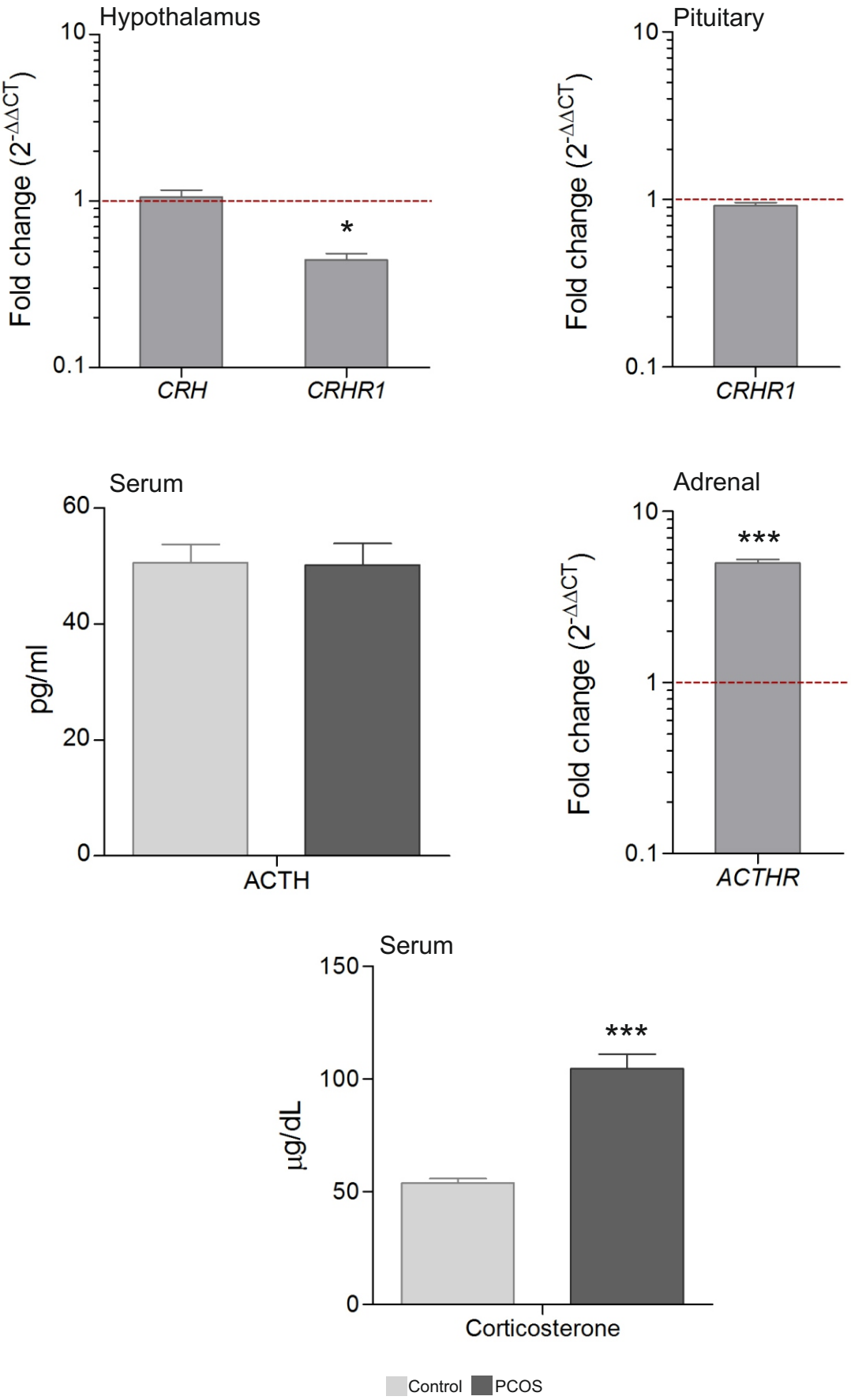

Figure 4.1: Status of components of HPA axis. Transcript levels of hypothalamic CRH1, CRHR1 and adrenal ACTHR. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). ACTH and corticosterone levels in serum. Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

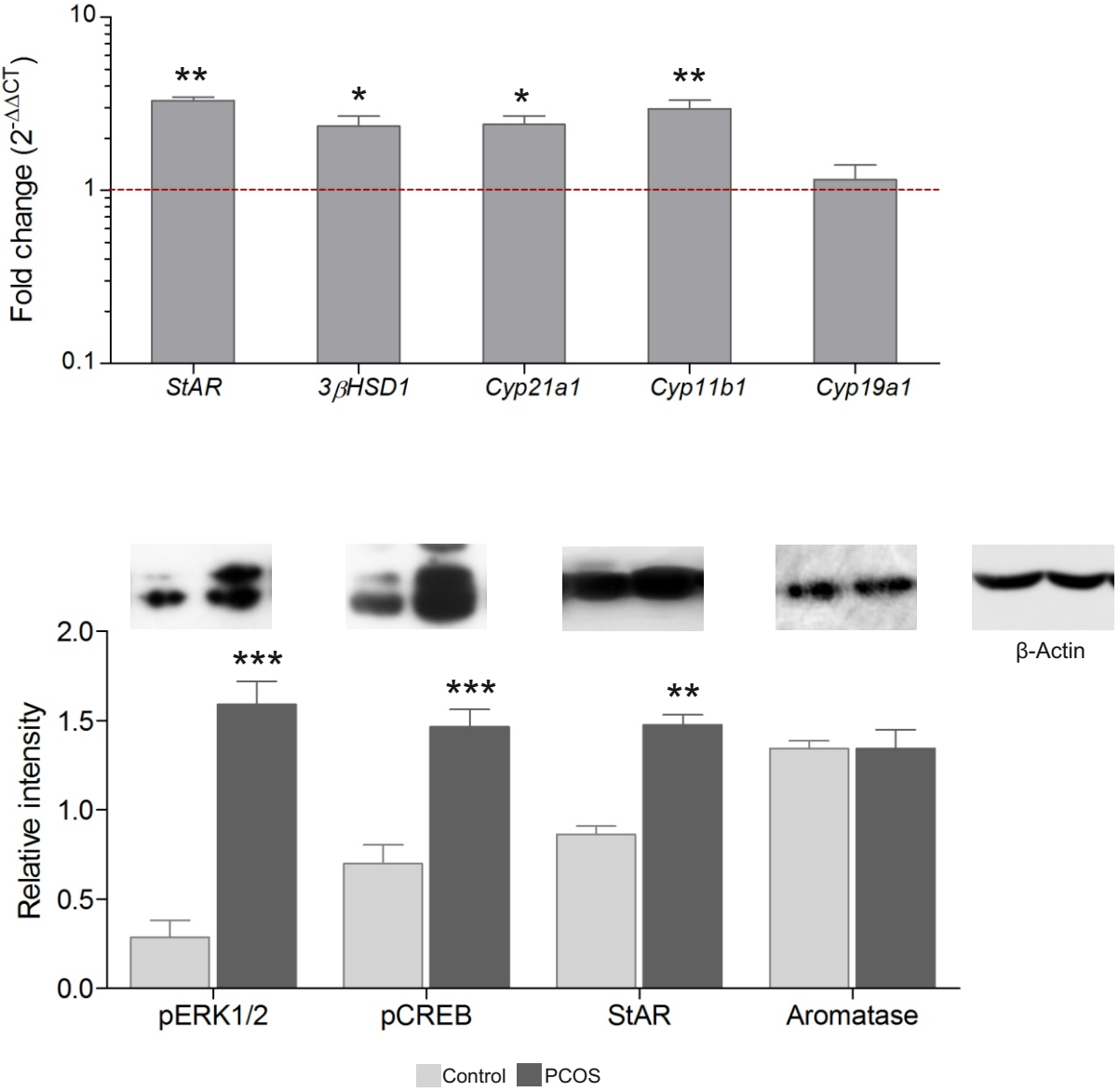

Figure 4.2: Steroidogenesis markers in adrenal glands. Upper: Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Lower: Relative intensities of bands obatined on western blot. Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

Table 4.1: Steroid levels in Adrenal

	Progesterone (pg/g)	**Testosterone (ng/g)**	**Estradiol (pg/g)**	**Corticosterone (ug/g)**
Control	0.673 ± 0.046	1.350 ± 0.098	175.44 ± 19.37	2.21 ± 0.175
PCOS	0.725 ± 0.051	2.524 ± 0.11 ***	167.58 ± 21.10	5.89 ± 0.260 ***

All values are Mean±SEM of four individual experiments. ***P<0.001 as compared control values.

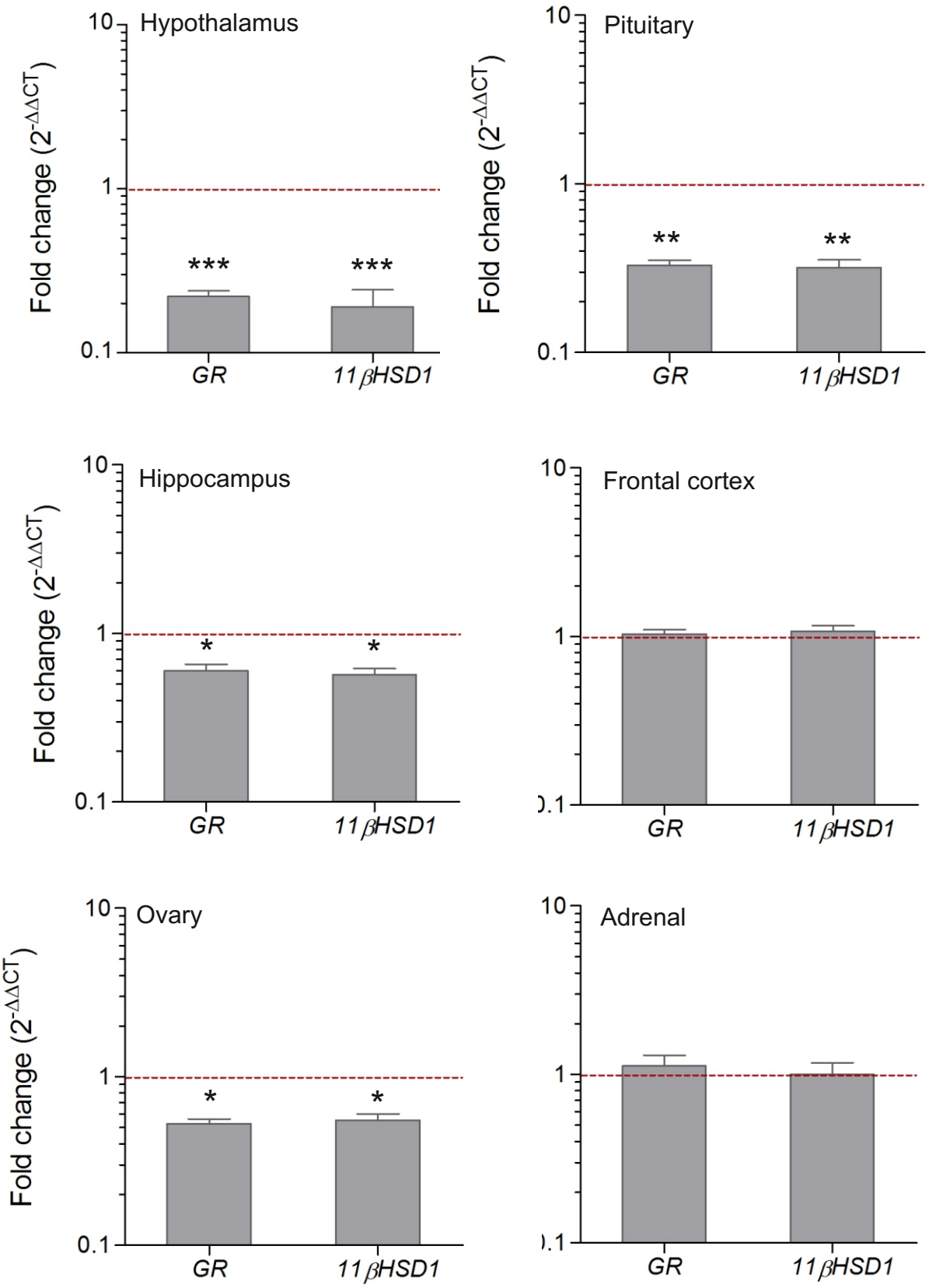

Figure 4.3: Gene expression of *GR* and *11βHSD1*. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

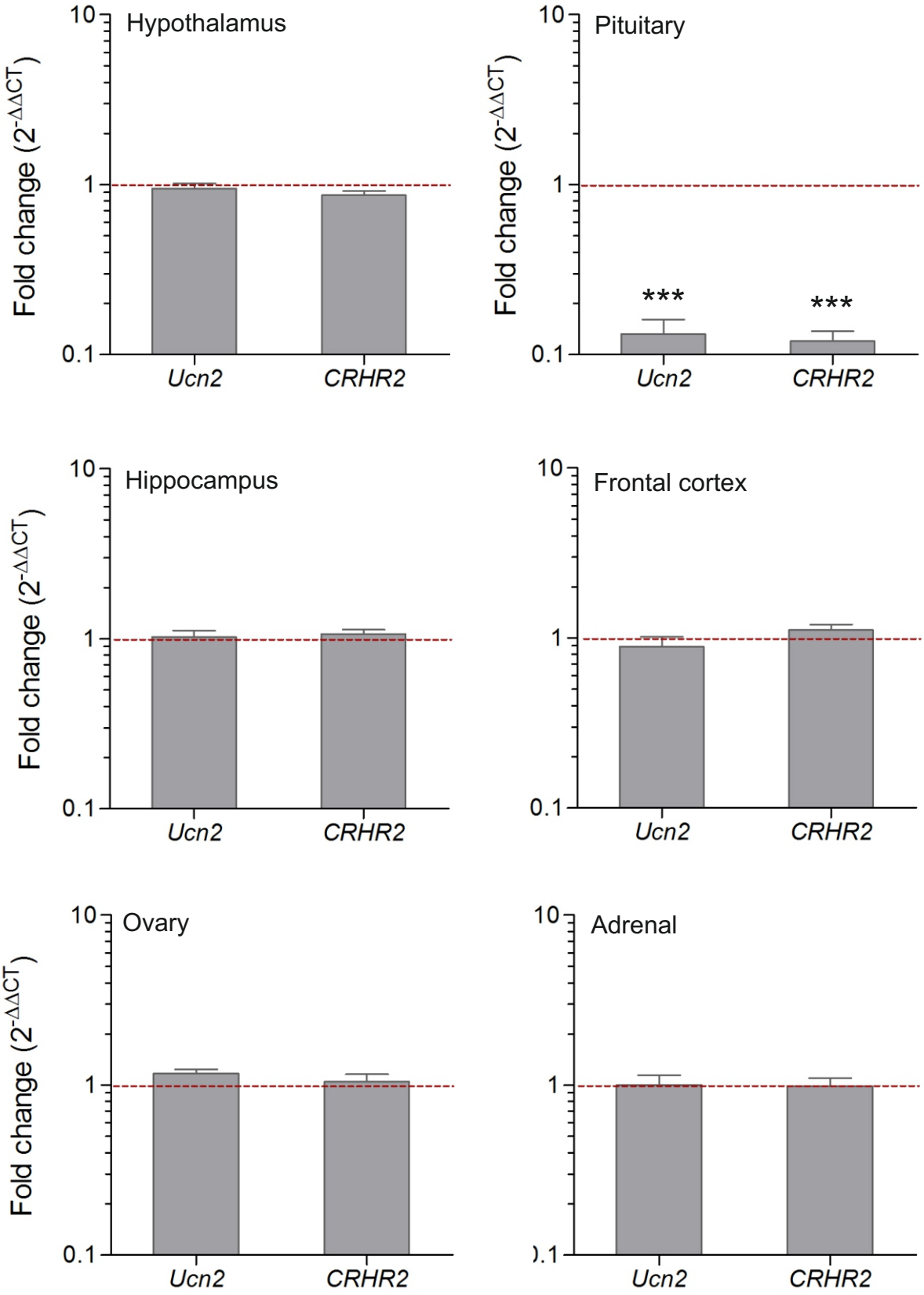

Figure 4.4: Gene expression of stress regulators *UCN2* and *CRH2*. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.

Discussion

Studies have postulated the role of HPA axis in pathophysiology of PCOS (Stener-Victorin et al., 2005). It is estimated that over 50% of PCOS women have excess adrenal androgens; however, the cause for the same is not known. The elevated adrenal androgen can be due to heightened ACTH release in response to CRH or hyper-responsive adrenal gland towards ACTH stimulation or both (Lanzone et al., 1995; Feng et al., 2009). In this regard, various studies have been carried out involving exogenous administration of CRH or ACTH to PCOS women (Carmina et al., 1992; Mongioi et al., 1988; Luboshitzky et al., 2003). However, only one study has demonstrated *CRH* mRNA expression in hypothalamus of DHT-induced PCOS rat model (Feng et al., 2009). Similar to our result, this study could not find any difference in CRH immunoreactivity between control and PCOS group. Furthermore, expression of pituitary *CRHR1* and serum ACTH levels in letrozole-induced PCOS rats was similar to control group indicating that increased androgen production from adrenal of PCOS women may not be associated with hypothalamic CRH and pituitary ACTH alterations (Azziz et al., 1998). There is heterogeneity regarding the cortisol synthesis and metabolism in PCOS condition. Basal cortisol levels were normal in PCOS women as observed in studies, while there are also reports indicating that PCOS women have higher plasma cortisol levels (Lanzone et al., 1995; Luboshitzky et al., 2003; Milutinovic et al., 2011). The increased serum corticosterone in PCOS rats could be due to hyper-responsiveness of adrenal gland to ACTH stimulation as suggested by a few studies (Azziz et al., 1998; Uncu et al., 2007; Cinar et al., 2012). However, the reason for increased adrenal response to ACTH stimulation in PCOS has yet to be understood. In this context, detailed analysis of adrenal steroidogenesis was performed in the present study. Binding of ACTH to its receptor ACTHR results in activation of a signalling cascade leading to increased steroidogenesis. Activated ACTHR increases cAMP and cAMP-protein kinase A (PKA) activity that helps in cholesterol internalization through elevating StAR expression (Clark, 2016). Furthermore, increased PKA activity results into phosphorylation of ERK1/2 and CREB which leads to transcriptional activation of *StAR, Cyp21a1* and *Cyp11b1* and hence increased steroidogenesis (Ruggiero & Lalli, 2016). For the first time, present data demonstrated increased *ACTHR* expression in adrenal gland of PCOS. Furthermore, the increase in *ACTHR* expression was well correlated with the expression of signalling molecules, steroidogenic enzymes transcripts as well as with steroid hormone profile of PCOS. Thus, we support the

hypothesis of adrenal hyper-responsiveness in PCOS condition, which results into heightened adrenal steroid production.

Similar to other endocrine axes, HPA axis is also regulated by a feedback system. The end product of the axis, *viz.*, corticosterone, acts on the hypothalamus as well as on pituitary through glucocorticoid receptors to regulate its functioning (Munck et al., 1984). Also, 11βHSD1 in different tissues convert cortisone to active corticosterone which then binds to GR and stimulates further downstream signalling (Tasker & Herman, 2011). The present study demonstrated reduced expression of *GR* and *11βHSD1* in hypothalamus and pituitary suggesting the inhibition of negative feedback to CRH and ACTH release in PCOS. This result explains the reason that although the corticosterone levels are very high, CRH and ACTH levels remain normal in PCOS condition due to disrupted negative loop.

As hypothalamus is the master regulator of endocrine function, alteration in one element is bound to affect the other endocrine axis. Also, interaction between HPA-HPG axes occurs at various levels to influence one another (Chand & Lovejoy, 2011). There are several reports which indicate the inhibitory role of CRH in GnRH release (Kageyama et al., 2012; Kageyama, 2013). However, the current study did not find any role of CRH in increased Gnrh1 expression in letrozole-induced PCOS model. Along with CRH, Urocortins also influence reproduction, behaviour, etc. with binding to their receptor CRHR2. Intracerebroventricular injection of Ucn2 reportedly suppresses LH pulsatile secretion and intracerebroventricular injection of CRHR2 antagonist blocks stress-induced suppression of LH secretion in ovariectomized and estrogen-replaced rats (Nemoto et al., 2010; Li et al., 2011). For the first time, the current study has revealed decreased expression of Ucn2 and Crhr2 in pituitary of PCOS animals with no change in hypothalamic transcripts. Hence, it can be postulated that transcriptional down-regulation of Ucn2-Crhr2 (inhibitory signals) in pituitary may result into increased *LHβ* expression leading to increased LH to FSH ratio (as outlined in Chapter 3), a characteristic of PCOS women. Along with the neuropeptides, glucocorticoid also regulates reproduction through binding to its receptor GR. GR and 11β HSD1 are localized in GnRH neurons of hypothalamus and they primarily, inhibit GnRH release via transcriptional inhibition (Whirledge & Cidlowski, 2013). Furthermore, GR is expressed in mouse gonadotropes and treatment with glucocorticoids reduces GnRH-induced LHβ expression in immortalized mouse gonadotrope cells (Breen et al., 2012). In the present study, expression of *GnRHR1* in PCOS was increased with increased *LHβ* and *FSHβ*

expression, which could be attributed by the decreased expression of *GR* and *11βHSD1* in hypothalamus and pituitary.

GR expression has also been demonstrated in the ovary, where glucocorticoids directly regulate steroid biosynthesis (Michael et al., 1993). In rat and porcine granulosa cells maintained in culture, glucocorticoids enhanced gonadotropin-stimulated production of progesterone (Schreiber et al., 1982). In a study, the effects of glucocorticoids on the steroidogenesis of ovarian granulosa cells were investigated, wherein cortisol and dexamethasone inhibited the increase in aromatase activity induced by FSH in cultured rat granulosa cells (Hsueh & Erickson, 1978). Also, in cultured human granulosa cells, cortisol and dexamethasone inhibited LH-stimulated steroidogenesis indicating that *in vitro* glucocorticoids exhibit inhibitory effects on the ovary (Michael et al., 1993). In this regard, data from current study has demonstrated decreased expression of *GR* and *11βHSD1* in polycystic ovary. Hence, reduced *GR* expression in PCOS ovary could result into altered ovarian steroidogenesis leading to hyperandrogenic condition. In addition to influence on steroidogenesis, glucocorticoid also helps in follicle maturation by decreasing inflammation during ovulation (Andersen, 2002; Witorsch, 2016). However, stress-induced concentration of glucocorticoid has opposite effect on the oocyte development. In this direction, cortisol injection to mice resulted in increased apoptosis of oocyte by triggering secretion of death signal Fas from ovarian cells (Yuan et al., 2016). Interestingly, the expression of ovarian *GR* in these mice was significantly reduced as compared to control animals suggesting a GR-independent role of glucocorticoid in ovary. Thus, the observed increase in serum corticosterone levels in PCOS may have deleterious effects on the ovarian cells which could be responsible for increased granulosa cell death in PCOS condition as reported earlier by our group (Maharjan et al., 2010).

Furthermore, the direct role of ACTH in ovarian steroidogenesis has been emerging recently. Studies on cattle have demonstrated that ACTH stimulation to ovary not only influences steroidogenesis but also enhances local or *de novo* cortisol production from the ovary (Amweg et al., 2013; Amweg et al., 2017). It was observed that this action of ACTH is mediated by the ACTH receptor present on the ovary. Also, the stimulation of ACTHR by ACTH may result in increased testosterone production from the tertiary ovarian follicle whereas it can reduce aromatase activity as seen in cystic ovarian disease (COD) in cattle (Dobson & Smith, 1995; Amweg et al., 2013). As, COD in cattle show similarities with

ovarian impairment in PCOS, further studies are required to understand any role of ACTH signalling in PCOS condition which may hinder ovarian function.

Direct influence of Ucn2 on ovarian folliculogenesis was also observed in various models (Xu et al., 2006; Geraghty et al., 2016). Presence of Ucn2 in ovary has been detected in rats, primates and humans and changes in its expression have been observed during follicular development and maturation (Xu et al., 2006). Even so, we were unable to find any change in *Ucn2* and *CRHR2* expression in PCOS ovary suggesting that Ucn2 might not be involved in ovarian steroidogenic abnormalities in PCOS condition. However, further interaction of regulatory molecules needs to be assessed to decipher the function of other Ucn molecules in PCOS pathology.

In addition to classical regulation of HPA axis, this axis is also modulated by other brain regions including hippocampus and frontal cortex (Morimoto et al., 1996; de Kloet et al., 2005). The presence of GR was observed in the discrete regions of brain and it is believed that glucocorticoids exert regulatory effects through binding to GR in these regions. Neurotransmitters and neuropeptides in hippocampus and frontal cortex can directly influence CRH release. Activation of GR in these regions of brain demonstrated inhibition of the HPA axis activity whereas other studies provided evidence for the stimulatory effect on HPA axis upon GR activation (van Haarst et al., 1997; Juruena et al., 2006; Furay et al., 2008; Wang et al., 2012). It has been also suggested that modulation of GR in hippocampus not only affects HPA axis but is also involved in stress resistance, anxiety and depression (de Kloet et al., 2005). Results from the current study reflect a significant decrease in *GR* and *11βHSD1* expression while no change was observed in frontal cortex of PCOS rats. Although the *GR* was reduced in hippocampus, *CRH* mRNA was unchanged in PCOS condition suggesting involvement of other regulatory molecules in maintaining normal CRH levels. Also, low levels of GR in hippocampus could be related to anxiety and depression like behaviours which are commonly seen in PCOS women (Goodarzi et al., 2011; Azziz et al., 2016).

In addition to neuroendocrine effects on reproduction, glucocorticoid functions in metabolic signalling of the body. Glucocorticoids increase blood glucose levels by activating gluconeogenesis in hepatic cells. They also inhibit or potentiate insulin actions on skeletal muscle and adipose tissue respectively, ultimately promoting visceral adiposity and the metabolic syndrome (Chrousos, 2000). Furthermore, various reports indicate that increased

cortisol levels and dysfunction of HPA axis act as causative agents for the development of visceral obesity and insulin resistance (Chrousos, 2000; Rosmond, 2003). Thus, it can be suggested that elevated corticosterone observed in the present study may aggravate insulin signalling in PCOS condition leading to increased insulin resistance as seen by a higher HOMA-IR index.

Conclusion

The present work is the first of its kind to demonstrate the interactions of stress regulators and gonadal loops in PCOS. Results presented herein reveal that increased androgen production in PCOS is associated with hyper-responsiveness of adrenal towards ACTH stimulation, which was evident by heightened expression of adrenal *ACTHR*, steroidogenic signalling molecules and steroidogenic enzymes expression. Furthermore, down-regulation of *GR* and *11βHSD1* in hypothalamus and pituitary suggests the disruption of negative feedback loop to HPA axis which results in normal secretion of CRH and ACTH even in presence of increased corticosterone in PCOS condition. Besides the components of HPA axis, involvement of stress regulator Ucn2 in PCOS was also elicited in present data which might result into increased LH pulsatility. Along with hypothalamus and pituitary, ovary also expresses *GR* and *11βHSD1* and it mainly functions in inhibition of LH-stimulated steroidogenesis. The reduced expression of ovarian *GR* and *11βHSD1* in letrozole-induced PCOS model indicates that low levels of inhibitor (GR) could result into increased ovarian steroidogenesis and hyperandrogenemia in PCOS Also, the increased concentration of glucocorticoid may exert deleterious effect on ovarian folliculogenesis, which may result in ovarian cyst formation in PCOS. As glucocorticoid increase is associated with metabolic aberrations, dysfunctional HPA axis seen in this study may increase the risk of metabolic syndrome such as insulin resistance and obesity in PCOS. Furthermore, glucocorticoids also regulate mood and behaviour through interactions of neurotransmitters with GR in hippocampus. The present study demonstrated reduced levels of *GR* and *11βHSD1* in hippocampus of PCOS rat, which may suggest the involvement of stress-related behavioral alterations seen in PCOS condition. However, a detailed understanding of neurotransmitters with the HPA axis is required gain an insight into the regulation of mood in PCOS that may manifest in anxiety and depression.