Reproduction in women is characterised by cyclic changes in the ovary and uterus, which are required for normal ovarian follicle development, ovulation and preparation of endometrium for implantation. This pattern of repeated ovarian cycle is of absolute necessity for the proper functioning of reproductive processes and is regulated by interactions of several stimulatory and inhibitory signals from the hypothalamus, pituitary and the ovary itself. The reproductive axis is initiated by the pulsatile secretion of hypothalamic gonadotropin releasing hormone (GnRH). GnRH released into hypophyseal portal system stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from anterior pituitary into the blood stream through binding to its receptor present on gonadotrops of pituitary. Under the influence of FSH and LH, ovarian follicular development and ovulation proceeds with secretion of estrogens, progesterons and androgens. Moreover, these steroid hormone regulate the HPO axis through classic feedback regulatory loops as well as with participation of various neuropeptides from higher centers of brain. Each component of the axis will be discussed individually in the following section.

**Hypothalamic Gonadotropin Releasing Hormone (GnRH)**

**GnRH Localization**

GnRH is a decapeptide, isolated and characterized in 1971 (Conn and Crowley, 1994). Earlier, it was also known as luteinizing hormone-releasing hormone (LHRH). In human,
there are approximately 7000 GnRH expressing neurons in areas of the brain linked to gonadotropin regulation (Rance et al., 1994). Unlike neurons secreting other hypothalamic releasing factors, GnRH neurons do not exist in a defined nucleus, but are scattered throughout the medial basal hypothalamus, with an additional population in the preoptic area (Dudas and Merchenthaler, 2006; Strauss III & Berbeiri, 2014).

**GnRH isoforms and their receptors**

There are mainly three isoforms of GnRH present in mammals – GnRH1, GnRH2 and GnRH3. GnRH1 is encoded by the *Gnrh1* gene, located on human chromosome 8. GnRH1 binds specifically to its receptor GnRHR1, expressed primarily by pituitary gonadotrophs in mammals and mediates central control of reproduction. GnRH2 is located on chromosome 20 and works through its own receptor (GnRHR2). GnRH2 is widely expressed both within and outside the brain. It likely plays a role in reproductive behaviour in lower animal species. It is a potent stimulator of LH and FSH *in vitro* and *in vivo* in animal models, but its role in humans is unknown. GnRH3 has been identified in human brain by immunohistochemistry and HPLC. It has a similar hypothalamic distribution to that of GnRH, and may act through the GnRH receptor. However, its consensus sequence has not been found in the human genome and thus, it is unlikely that GnRH3 plays a role in human reproduction (Neill, 2002; Strauss III & Berbeiri, 2014).

**Pulsatile secretion of GnRH**

A prominent feature of the reproductive system is the absolute requirement for pulsatile secretion of GnRH into the pituitary portal system for normal gonadotropin secretion. The classical studies of Knobil (1992) on rhesus monkeys have demonstrated the importance of GnRH pulsatility. In the experiment, LH and FSH secretion increased when intermittent GnRH was given to hypothalamic-lesioned monkeys, whereas constant GnRH stimulation resulted in gonadotropin inhibition. The pulsatile secretion of GnRH from normal and immortalized hypothalamic GnRH neurons is highly calcium-dependent and is also stimulated by cAMP (Krsmanovic et al., 2009). In addition to intrinsic GnRH pulsatility, GnRH neurons are also influenced by external cues such as neuropeptides, neurotransmitters, and steroids, which affect both the amplitude and the frequency of GnRH secretion (Wetsel et al., 1992; Strauss III & Berbeiri, 2014).
Pituitary Gonadotropins

Localization of FSH and LH

LH and FSH are synthesized in gonadotropes, which comprise between 7% and 15% of the cells in the pituitary. Immunohistochemical studies in the rat indicate that approximately 70% of gonadotropes stain for both LH and FSH while the remainder stains for LH or FSH with approximately equal numbers of cells staining for each (Childs et al., 1994; Strauss III & Berbeiri, 2014).

Structures of gonadotropins and their receptors

Both FSH and LH are glycoprotein dimers each consisting of two subunits α and β. The α-subunit is common in FSH, LH, hCG (human chorionic gonadotropin hormone) as well as in thyroid stimulating hormone (TSH). The β-subunit is distinct and hormone-specific, which allows the differential function of each hormone. The α-subunit of these hormones consists of 92 amino acids. The β-subunit of FSH consists of 118 amino acids with five sialic acid residues, whereas the β-subunit of LH includes one to two sialic acid residues in a total 121 amino acids peptide. Sialic acid residues are responsible for the half-life of the hormone, where higher the sialic acid content the longer the half-life of that molecule (Beshay & Carr, 2013). Thereby, FSH has a half-life of several hours while LH has a shorter half-life of approximately 20 min. Because of this shorter half-life, LH needs to be rapidly synthesized and typically has pulses higher in amplitude than FSH (Weiss et al., 1995; Besecke et al., 1996).

FSH and LH receptors both belong to the GPCR family. FSH receptors exist exclusively on the membrane of granulosa cells, while LH receptors are found on membranes of theca cells. In the presence of estradiol, FSH induces LH receptors on granulosa cells. LH receptor activity primarily stimulates androstenedione production from theca cells, which is transported to neighboring granulosa cells, aromatized to estrone and eventually converted to estradiol. This is the basis of the two-cell theory of the ovary (Beshay & Carr, 2013).

Release of Gonadotropins

Binding of GnRH to its receptor GnRHR1 on anterior pituitary gonadotropes activates a signaling cascade culminating into the release of gonadotropins – FSH and LH. Stimulation of GnRHR primarily activates guanosine triphosphate (GTP)-binding protein (Goαq/11), which stimulates phospholipase Cβ, protein kinase C (PKC), calcium-calmodulin kinase II
and increases calcium influx (Haisenleder et al., 2003; Thackray et al., 2010). Activation of PKC further results into signal transduction of various mitogen activated protein kinases (MAPK) like ERK1/2, JNK and P38 MAPK. GnRH can also directly stimulate MAPK pathway through activation of \( G_\alpha \) subunit (Tsutsumi et al., 2010). A recent study has demonstrated that FSH\( \beta \) induction depends predominantly on \( G_{aq/11} \) while LH\( \beta \) expression depends on \( G_\alpha_s \) (Choi et al., 2012; Strauss III & Barbieri, 2014).

Synthesis and secretion of LH and FSH are differentially controlled by the amplitude and frequency of GnRH stimulation (Marshall et al., 1991; Kaiser, 1998). FSH is secreted along with LH in response to acute stimulation by GnRH, but the relative role of GnRH in the overall control of FSH synthesis is much less than for LH. Blockade of the GnRH receptor using a specific GnRH receptor antagonist results in 90% inhibition of LH secretion, but only 40-60% inhibition of FSH (Hall et al., 1990). LH secretion is highly responsive to increases in the dose of GnRH, while FSH is relatively insensitive to GnRH dose. A physiological frequency of GnRH results in synthesis and secretion of all three gonadotropin subunits. Slow frequencies of GnRH stimulation favor the synthesis and secretion of FSH \textit{in vitro} and are associated with an increase in FSH \textit{in vivo} in settings in which gonadal feedback is low (Gross et al., 1987). In GnRH-deficient men and women, an increase in the frequency of GnRH stimulation results in an increase in mean levels of LH with no appreciable change in FSH (Spratt et al., 1987; Hall et al., 1998). The direct effect of GnRH pulse frequency on GnRH receptor number and the downstream effects of modulation of receptor number underlie the frequency modulation of LH and FSH secretion (Bedecarrats & Kaiser, 2003). While increased GnRH pulse frequency increases the synthesis and mean levels of LH, the LH pulse amplitude is inversely related to GnRH pulse frequency (Finkelstein et al., 1988; O’Dea et al, 1989). Thus, studies in GnRH-deficient men and women have shown that slower GnRH pulse frequencies are associated with higher LH amplitude in response to GnRH while faster frequencies are associated with a decrease in the amplitude response to GnRH (Spratt et al., 1987; Hall et al., 1998; Strauss III & Berbeiri, 2014).

Thus, GnRH pulse generator is the central player for cyclic changes in gonadotropin levels which is essential for the ovarian follicular development cycle, better known as the menstrual cycle in humans.
Ovarian cycle

Under the influence of pituitary gonadotropins, primordial ovarian follicles are progressively converted into mature tertiary follicles, which are then released into the fallopian tube during ovulation. This process of ovarian follicle development is known as folliculogenesis. As described in Chapter 1, the ovarian cycle can be divided into three phases: i) Follicular phase comprises of the time during which follicle matures and it is mainly dependent on the FSH secretion; ii) Ovulatory phase wherein the fully developed ovarian follicle releases oocyte under the influence of pituitary LH and iii) Luteal phase which starts after ovulation and is mainly involved in the maintenance of corpus luteum. During all these cyclic changes, various, ovarian cells produce steroid hormones such as androgens, progesterone and estrogens through activation of the steroidogenic pathway.

In addition to ovary and adrenal, steroidogenesis also occurs in various other organs of body including brain. The steroids synthesized in the brain are also known as neurosteroids or neuroactive steroids due to their property of modulating different brain functions such as learning and memory, anxiety, depression, sleep, endocrine regulation, sexual activity and food consumption (Bates et al., 2005; Gleason et al., 2005). The well known neurosteroids allopregnanolone, dehydroepiandrosterone (DHEA), progesterone, estadiol and androstanediol are biosynthesized via steroidogenesis in hypothalamus, pituitary, hippocampus, neocortex, cerebellum and pons (King et al., 2002; Meethal et al., 2009).

Figure 3.A: Steroidogenesis in brain

Steroidogenic enzymes have been identified in neurons, astrocytes and oligodendrocytes of these regions and it has been observed that the rate of steroidogenesis is highest in neurons compared to the other two cell types (King et al., 2002; Meethal et al., 2009).
Like in other tissues, steroidogenesis in brain is initiated by the transport of cholesterol into inner mitochondrial membrane by translocator protein (TSPO) and steroidogenic acute regulatory protein (StAR). Cytochrome P450 side-chain cleavage (P450scc) enzyme converts cholesterol into pregnenolone, a rate-limiting and hormonally-regulated step of steroidogenesis (Do Rego et al., 2009). Pregnenolone can be utilized for synthesis of various neurosteroids such as dehydroepiandrosterone (DHEA) by the enzyme cytochrome P450 17α-hydroxylase/C17, 20-lyase (P450C17) or it can be converted into progesterone by 3β-hydroxysteroid dehydrogenase/D5-D4 isomerase (3β-HSD). Progesterone is further converted into allopregnanolone by subsequent reactions of 5α-reductase (5α-R) and 3α-hydroxysteroid oxidoreductase (3α-HSOR) or it can form androstenedione by P450C17. Furthermore, cytochrome P450 aromatase (P450arom) and 17β-hydroxysteroid dehydrogenase (17β-HSD) can form estradiol from androstenedione or it can be converted into dihydrotestosterone and androstenediol through 5α-reductase (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD) respectively. The exact mechanism of steroidogenesis activation has not yet been clearly understood, however, like in other steroidogenically active tissues, neurosteroidogenesis is stimulated by a cAMP signal. Also, increasing evidence suggests the role of several neurotransmitters including Glutamate, GABA and melatonin in regulation of steroidogenesis in brain (Do Rego et al., 2009).

**Regulation of Hypothalamic-pituitary-Ovarian (HPO) axis**

Reproductive axis is regulated by various intra- and extra-ovarian factors. Intra-ovarian factors mainly comprise gonadal steroids and peptide hormones that control HPO axis via feedback mechanism. Neuropeptides, opioids and neurotransmitters are the extra-ovarian factors that modulate GnRH and gonadotropin synthesis and secretion.

**Steroid-mediated feedback regulation of HPO axis**

During the early follicular phase, FSH concentration is modestly elevated whereas concentrations of LH and ovarian hormones estradiol and progesterone are low which foster follicular growth. Estradiol and inhibin level gradually increases, leading to decreased FSH secretion through negative feedback loop, while secretion of LH starts to increase. This suppression of FSH is very crucial for the selection of pre-ovulatory dominant follicle. As the cycle progresses, the estrogen feedback effect becomes positive at around 36-48 h before ovulation. At this time, estradiol exerts differential effects on gonadotropins which is characterized by a robust increase in LH level (LH surge) and to a lesser extent in FSH level.
that causes rupture of follicles, resulting in ovulation. The elevated levels of estradiol, progesterone and inhibin during the luteal phase, suppress pituitary gonadotropin secretion. Thereby, indicating the dual effect of estradiol on LH release. This effect of estradiol was explained by the experiments on primates wherein 300% increase in circulating estradiol for 24 h resulted in negative feedback to LH whereas positive feedback effect of estradiol was only observed after 36 h of estradiol incubation (Knobil, 1974). As the luteal phase advances, progesterone secretion from corpus luteum increases with increase in estradiol secretion, which is required for endometrial receptivity and successful implantation. In absence of pregnancy, the secretion of estradiol, progesterone and inhibin from the corpus luteum ceases, which removes the feedback inhibition of the gonadotropins, leading to gradual increase in FSH and LH secretion resulting into a new ovarian cycle (Barret et al., 2016).

As mentioned above, estradiol is the principle steroid hormone that regulates the feedback loop through interacting with its receptors (ERα and ERβ) on pituitary and hypothalamus. Various studies have been employed to understand the role of these receptors in GnRH/LH regulation. Transgenic null mice of ERα (ERαKO) demonstrated gonadal impairment, altered feedback regulation and were infertile (Couse et al., 1995; Dupont et al., 2000). Also, there was a significant increase in the serum LH levels in ERαKO female mice as compared to control (Wersinger et al., 1999), indicating the role of ERα in estrogen-mediated negative feedback to hypothalamus and pituitary (Radovick et al., 2012). On the other hand, knockout mice of ERβ (ERβKO) were subfertile with normal levels of serum gonadotropins (Dupont et al., 2000; Antal et al., 2008). However, gonadotropin levels were significantly elevated in ERαβ-double knockout (ERαβKO) mice as compared to ERαKO mice, implicating that ERβ may also be important in negative feedback regulation (Couse et al., 2000). Along with estradiol, progesterone and androgen also facilitate steroid-mediated feedback to HPO axis. Estrogen increases expression of progesterone receptors (PRα & PRβ) in hypothalamus and preoptic area. When PRKO mice (PR knockout) were treated with estrogen, no surge of GnRH and LH was observed (Chappell et al., 1999). Similarly, GnRH and LH surge was absent in rats treated with progesterone antagonist (Chappell & Levine, 2000), thereby indicating that progesterone receptors are essential for successful release of GnRH surges (Radovick et al. 2012). In addition, role of androgen receptor is emerging as an important regulator for female reproductive cycle. Estradiol-induced LH surge was inhibited in prenatally androgenized animals (Foecking et al., 2005b) and this effect of androgen was reversed when animals were treated with androgen receptor blocker-flutamide (Sullivan &
Moenter, 2004). Furthermore, androgen receptor knockout (ARKO) female mice demonstrated reduction in ovulatory LH surge with decreased ovarian steroidogenesis (Cheng et al., 2013), implying that AR is obligatory for the GnRH/LH surge. Although reports indicate the importance of steroids in GnRH regulation, various immunolocalization studies indicate that these steroid receptors are not co-localized with GnRH neurons (Herbison 1998; Wintermantel et al., 2006; Christian et al., 2009) except ERβ (Temple et al., 2004). Instead these steroids influence GnRH/LH release through several interneurons containing kisspeptin, neuropeptide Y, catecholamines, glutamate and GABA (Stumpf & Jennes, 1984; Leranth et al., 1992; Smith et al., 2005; Franceschini et al., 2006; Kenealy & Terasawa, 2012). In addition to gonadal steroid feedback, a recent study also demonstrates a role of neurosteroids in GnRH/LH release. Meethal and colleagues (2009) have suggested that neurosteroids regulate HPG axis via modulating GnRHR signaling and thereby affect gonadotropin release. This indicates that crosstalk of conventional HPO-axis components and neurosteroids is essential for GnRH/LH release and ovarian function.

Neuromodulators of GnRH release
In addition to classic steroid feedback regulation, GnRH axis is influenced by several factors including kisspeptin, Neurokinin B, Dynorphin, Gonadotropin-inhibitory hormone and insulin. These molecules will be discussed in detail in following section.

Kisspeptin
Kisspeptin, a product of gene Kiss1 was originally known as metastatin due to its capability to suppress metastatic potential of malignant melanoma (Lee et al., 1996). Experimental mutations in gene encoding kisspeptin receptor in human and rodents prevented puberty and caused infertility (de Roux et al., 2003; Seminara et al., 2004). These experiments opened an era of research, aiming towards the role of kisspeptin as a regulator of reproduction (Pinilla et al., 2012; Simonneaux et al., 2013). The expression of Kiss1 gene is prominent in the areas of arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) (Gottsch et al., 2004; Clarkson et al. 2009) with lesser expression in amygdala (Cravo et al., 2011). The kisspeptin released from these areas of brain project to preoptic area, median eminence and limbic area (d’Anglemont de Tassigny et al., 2008; Clarkson et al., 2009) and binds to its GPCR Kiss1R/GPR-54. It was demonstrated that more than 90% of GnRH neurons express GPR-54, suggesting a direct role of kisspeptin in GnRH release (Irwig et al., 2004).
Kisspeptin acts as a potent stimulator of GnRH neuronal activity (Han et al., 2005; Piet et al., 2015). The brief exposure to nanomolar concentrations of kisspeptin is able to evoke long-lasting electrical activity of GnRH neurons as observed by electrophysiological studies on acute brain slices (Han et al., 2005; Dumalska et al., 2008). Similar activation of GnRH neurons by kisspeptin was also demonstrated in vivo (Constantin et al., 2013). Furthermore, treatment of kisspeptin antagonist blocks pulsatile LH secretion as well as GnRH neuron firing (Roseweir et al., 2009), whereas kisspeptin-mediated LH release was completely inhibited by GnRH agonist, acycline (Gottsch et al., 2004; Irwig et al., 2004). Although GPR-54 (Kisspeptin receptor) is localized to pituitary, these studies confirm that kisspeptin directly acts upon the hypothalamic GnRH neurons (Smith et al., 2008; Clarke, 2011).

Figure 3.B: Kisspeptin regulation of GnRH release (Skorupskaite et al. 2014)
It has also been demonstrated that steroid-mediated feedback regulation of reproductive axis is mediated through kisspeptin neurons (Revel et al., 2006; Ansel et al., 2010). Immunohistochemical studies have demonstrated that around 90% of the AVPV kisspeptin neurons of the female rat brain express Estrogen receptor-α (ERα) and Progesterone receptor (PR) (Adachi et al., 2007; Clarke, 2011). Similarly, co-expression of ERα, PR and androgen receptor was observed in arcuate nucleus kisspeptin neurons of ovine brain (Franceschini et al., 2006). It has been suggested that steroid-mediated positive feedback regulation of HPG axis is through activation of AVPV kisspeptin neurons (Pinilla et al., 2012; Skorupskaite et al., 2014). A recent report indicates that estradiol increases the synthesis of neuroprogesterone which together with estradiol enhances kisspeptin activity in AVPV neurons that is responsible for LH surge (positive feedback) (Meethal et al., 2009). On the other hand, kisspeptin neurons of ARC are involved in estradiol and progesterone-mediated negative feedback regulation which is responsible for pulsatile release GnRH/LH (Clarke, 2011; Pinilla et al., 2012; Simonneaux et al., 2013; Piet et al., 2015).

**Neurokinin B (NKB) and Dynorphin (Dyn)**

Kisspeptin neurons in arcuate nucleus coexpress Neurokinin B (NKB) and Dynorphin (Dyn) and hence, this population is also termed as KNDy neurons (Goodman et al., 2007; Navarro et al., 2009). Both NKB and Dyn can potentially influence GnRH release. Blockade of NKB receptor resulted in a similar hypogonadism and pubertal delay as seen with mutation of kisspeptin receptor-GPR-54 (Guran et al., 2009; Topaloglu et al., 2009). In contrast, Dyn is an endogenous opioid peptide and central administration of its receptor antagonist increases GnRH/LH secretion (Sandoval-Guzman and Rance, 2004). Although, these peptides are expressed separately in various regions of brain, the colocalization of kisspeptin, NKB and Dyn in ARC is conserved in different species and it plays a central role in GnRH regulation (Burke et al., 2006; Goodman et al., 2007; Navarro et al., 2009; Lehman et al., 2010). Similar to kisspeptin neurons of AVPV, KNDy neurons of ARC demonstrated a very high degree of colocalization with estrogen receptor-α, progesterone receptor and androgen receptor (Foradori et al., 2002; Smith et al., 2005b; Franceschini et al., 2006; Lehman et al., 2010). Furthermore, KNDy neurons project to preoptic area and median eminence wherein they directly form contact with GnRH neurons and regulate estradiol and progesterone mediated GnRH secretion (Lehman et al., 2010; Wakabayashi et al., 2010). Increasing evidence also suggests the role of KNDy neurons in estradiol mediated positive feedback, required for LH surge (Goodman & Inskeep, 2006; Lehman et al., 2010). However, this response was highly
species-specific, and observed in sheep; whereas in rats and mice KNDy neurons are solely involved in steroid-nediated negative feedback regulation of GnRH/LH (Lehman et al., 2010).

**Gonadotropin inhibitory hormone (GnIH)/RF-amide related peptide-3 (RFRP-3)**

In contrast to kiss1 and NKB, GnIH is an important inhibitor of GnRH and gonadotropin release which was first isolated from the avian brain (Tsutsui et al., 2000). Similar to birds, the presence of GnIH homolog RF-amide related peptide-3 (RFRP-3) was identified in various species (Hinuma et al., 2000; Yano et al., 2003; Ubuka et al., 2009). RFRP expressing neurons are mainly found in the mediobasal hypothalamus and project to multiple brain regions that include preoptic area, arcuate nucleus, lateral septum, anterior hypothalamus and bed nucleus of the stria terminalis (Mason et al., 2010; Simonneaux et al., 2013). The co-localization of RFRP3 with 40-80% of GnRH neurons suggests the direct or indirect role of RFRP3 in GnRH regulation (Bentley et al., 2003; Johnson et al., 2007; Clarke, 2011). In addition to inhibition of GnRH cell firing, RFRP3 localized to median eminence inhibits gonadotropin release from the pituitary (Clarke et al., 2008) as well as it suppresses sexual behaviour in birds and rats (Bentley et al., 2006; Johnson et al., 2007).

![Figure 3.C: RFRP3 regulation of GnRH release (Kriegsfeld et al. 2010)](image)
The actions of RFRP3 are mediated through binding to its receptor-GPR147 which is also known as neuropeptide FF receptor 1 (NPFF1R). As the name suggests, GPR147 is a GPCR associated with adenylate cyclase via $G_i$ subunit (Mollereau et al., 2002; Simonneaux et al., 2013). Although GnIH/RFRP3 has been implicated in suppression of reproductive function, the putative role of this peptide in ovarian function is increasing. Immunohistochemistry studies have revealed the presence of RFRP3 and GPR147 in ovarian cells of rodents and humans (Sihgh et al., 2011b; Oishi et al., 2012). The expression pattern of these molecules changes with the ovarian cycle, implicating the importance of RFRP3 system in ovarian folliculogenesis (Singh et al., 2011a). However, the role of this peptide in governing cyclic fluctuations of gonadotropins during ovarian cycling in mammals is still not well understood (True et al., 2011).

**Insulin**

Insulin is primarily known for the control of glucose uptake and storage and thus as a keeper of energy homeostasis (Xu et al., 2006). Also, increasing evidence suggests the role of insulin in regulation of reproduction through maintaining the energy balance (Tanaka et al., 2000; Gamba & Pralong, 2006; Pralong, 2010; Fontana & Torre, 2016). In this regard, studies have demonstrated insulin’s and its receptor’s immunoreactivity in many areas of CNS that are involved in the control of reproduction such as arcuate nucleus, ventromedial nucleus and preoptic area implying a direct action of insulin in GnRH release (Bruning et al., 2000; Plum et al., 2005).

![Figure 3.D: Insulin regulation of GnRH release (Modified from Acosta-Martinez, 2012)](image-url)
Furthermore, studies using Gnv-3 hypothalamic cell-line observed the presence of insulin receptor in GnRH-expressing neurons and this cell-line possesses many characteristics of insulin-sensitive tissues (Salvi et al., 2005). Upon insulin stimulation, Gnv-3 cell line increases GnRH expression through increasing the expression of c-fos (Gamba & Pralong, 2006). The regulatory role of insulin in reproduction is also evident by several in vivo studies. Low levels of circulating insulin in Type 1 diabetic (T1D) patients as well as in T1D animal model are associated with hypogonadotropic hypogonadism, characterized by reduced LH pulsatility and decreased sexsteroid concentrations (Arias et al., 1992; Griffin et al., 1994; Tanaka et al., 2000). In addition to direct influence on hypothalamic-pituitary centre, insulin can act as a co-gonadotropin in theca cells and modulate ovarian steroidogenesis through increasing the expression of steroidogenic enzymes StAR and 17α-hydroxylase (Nestler et al., 1998; Munir et al., 2004; Fontana & Torre, 2016). However, the molecular mechanism involved in the insulin-mediated stimulation of steroidogenesis has not been completely elucidated.

**Rationale**

It is thus a logical surmise that reproductive function is regulated by the Hypothalamic-Pituitary-Ovarian (HPO) axis, wherein pulsatile release of GnRH from the hypothalamus stimulates the anterior pituitary to release FSH and LH (Caraty et al., 1989; Levine et al., 1990), which further results into release of sex-steroids like estradiol and progesterone from the ovaries. Regulation of this axis is carried out by several positive and negative feedback mechanisms (Evans et al., 1994; Couse et al., 2003). In addition to feedback regulation, several neuropeptides upstream of the hypothalamic GnRH neurons influence GnRH/LH secretion, including kisspeptin, NKB, Dyn, RFRP3, etc. Any alteration in the regulatory loop of HPO axis may result in adverse reproductive conditions such as the polycystic ovarian syndrome (PCOS), one of the most common endocrinopathies in women.

PCOS is characterised by of hyperandrogenism, oligo-/anovulation and presence of many cysts in the ovarian periphery. A disturbance in the neuroendocrine component is a hallmark of PCOS and includes increased GnRH pulsatility leading to increased LH/FSH ratio, further showing up as increased androgen levels and cysts in the ovary. Many researchers suggest the involvement of altered HPO axis in PCOS pathology. Women with PCOS demonstrate abnormal reproductive function with altered sensitivity to steroid feedback regulation (Sullivan & Moenter, 2004; Cernea et al., 2015). Furthermore, in utero exposure to
testosterone of sheep resulted in delayed or absent LH surge, indicating attenuation of estradiol-mediated negative and positive feedback in prenatally androgenised sheep model of PCOS (Robinson et al., 1999; Sharma et al., 2002; Unsworth et al., 2005). However, prenatal androgen treatment may interfere with developmental brain circuits to reproduction. Thereby, another model of PCOS is required to understand the neuroendocrine pathology of PCOS.

A major aim of the current study was to investigate the role of steroids as well as neuropeptides such as kisspeptin, NKB, Dyn, RFRP-3 and insulin which may result into increased GnRH/LH pulsatility leading to PCOS pathology. As discussed in the introduction, complex regulation of the HPO is acquired through interactions of several neuropeptides and steroids in steroidogenically active tissues of brain, ovary and adrenal. Thereby, understanding the contribution from these tissues such as hypothalamus, pituitary, hippocampus, frontal cortex and ovary in HPO regulation may enhance our understanding of the intricate regulation of neuroendocrine axis in PCOS pathology. To achieve this goal, the present study uses two different animal models for PCOS induction that include treatment with letrozole and with testosterone propionate.

**Materials and methods**

PCOS was induced in animals as described in detail in Chapter 2 using two different drugs – letrozole (an aromatase inhibitor) and testosterone propionate (TP). For letrozole-induced PCOS rat model, adult virgin female rats (2-3 months old) were either treated with carboxymethyl cellulose (Control) or with 0.5mg/kg body weight of letrozole (PCOS group) for 21 days, orally. On the other hand, for testosterone induced PCOS model, young female rats (21 days old) received propylene glycol (Control) or 10mg/kg body weight of testosterone propionate (PCOS group) by subcutaneous injection for 35 days. Following the treatment, animals were tested for incidence of PCOS characteristics by examining body weight, oral glucose tolerance, estrus cyclicity, serum profile of steroid hormones and histology of the ovaries. Following validation, animals were sacrificed in diestrus stage by decapitation. Pituitary, hypothalamus, hippocampus, frontal cortex and ovaries were dissected out and used for further analyses.

For evaluation of HPO axis, real-time PCR expression studies of Gnrh1 (GnRH1), Gnrhr1 (GnRHR1), Fshb (FSHβ), Lhb (LHβ), Fshr (FSHR) and Lhr (LHR) were performed. Steroidogenesis in various tissues was analysed using mRNA expression profile of steroidogenic markers Star, Cyp17a1, Cyp21a1, Cyp19a1 and by performing western blots of
StAR, Aromatase, pCREB and pERK proteins. Further, steroid (Progesterone, estradiol and testosterone) and insulin levels in each tissue were estimated using ELISA kits. To study the feedback regulation of HPO axis, mRNA and protein expression of estrogen receptors (ERα and ERβ) and androgen receptor (AR) was tested. In addition to feedback regulation, the HPO axis is also regulated by several molecules including kisspeptin, NKB, Dynorpin, RFRP3 and Insulin. Thereby, real-time expression studies of Kiss1 (kisspeptin), GPR-54, Nkb, dyn, Npvf (RFRP3), Npff (GPR-147) and Insr (Insulin receptor) genes encoding these molecules were performed.

Results

PCOS induction using letrozole and testosterone propionate

PCOS is a disorder of reproductive and metabolic complications. To validate PCOS rat model, reproductive parameters like estrus cyclicity, blood steroid levels, ovarian histology were studied. As demonstrated in Table 3.1, the estrus cycle of both the letrozole-treated and testosterone propionate (TP)-treated animals were found disturbed and most of the animals were arrested in diestrus stage as opposed to control animals. Testosterone levels were significantly increased in letrozole (P<0.001) and TP-treated animals (P<0.001) as compared to control rats whereas estradiol levels remained unchanged in all groups (Table 3.2). Also, a decrease in progesterone content was observed in letrozole-treated rats (P<0.05) while no change was observed in progesterone of TP-treated rats as compared to control. Since it is known that maintenance of the steroid milieu is a prerequisite for the ovarian structure and function, the ovaries were studied for their histology profile using Hematoxylin-Eosin stain. Control sections demonstrated follicles in various stages of development (Figure 3.1 A and B), whereas both letrozole (Figure 3.1 C) and TP-treated (Figure 3.1 D) animals demonstrated numerous fluid-filled cysts in the ovary. Also, rats treated with letrozole demonstrated significantly increased number of peripheral cysts as compared to both control and TP-treated groups, while the number of corpus lutea were reduced in letrozole and TP-treated rats as compared to control animals. For metabolic complications, body weight changes were monitored along with oral glucose tolerance and insulin levels. PCOS model demonstrated increased bodyweight (Figure 3.2) and glucose intolerance (Figure 3.3) as compared to control groups with increased insulin levels and HOMA-IR, although the changes were more significant for letrozole-treated group as compared to TP-treated animals.
Both letrozole and TP treatment were able to induce PCOS-like characteristics in rats. However, testosterone is a steroid hormone which influences various body functions and hence, it may not be able to replicate the exact PCOS pathology. Thereby, all the further experiments were carried out using letrozole-induced PCOS rat model, since it exhibited characteristics similar to the human PCOS phenotype.

**Altered hypothalamic-pituitary-ovarian axis in PCOS**

GnRH is transcribed by the genes *GnRHI* and *GnRH2*, wherein *GnRHI* plays a pivotal role in stimulating pituitary release of FSH and LH and *GnRH2* functions in control of puberty, feeding and energy balance. Hence, gene expression study of *GnRHI* was carried out and increased mRNA was observed in the hypothalamus of PCOS rats as compared to control (P<0.01). GnRH acts upon its target tissue through its receptor GnRHR but there are no reports addressing the status of GnRHR expression in PCOS condition. The current study demonstrated increased *GnRHR1* expression in pituitary (P<0.01) and decreased hypothalamic *GnRHR1* expression (P<0.001) in PCOS rats (Figure 3.4).

GnRH released from the hypothalamus stimulates gonadotropin secretion from anterior pituitary. Thereby, serum gonadotropin levels were measured using a CLIA kit and it was found that serum FSH levels remained same in both the groups whereas a significant increase was observed in LH levels of letrozole-treated animals (P<0.001) with elevated FSH:LH ratio (Table 3.3). To examine whether the origin of this alteration lies at the genetic level, transcript analysis was carried out. In present study, both the *FSHβ* (P<0.05) as well as *LHβ* (P<0.01) mRNA were found significantly increased in the pituitary of PCOS rats as compared to control (Figure 3.4). Gonadotropins act on the ovaries via their receptors. Hence, mRNA expression of *FSHR* and *LHR* in ovaries were studied. As shown in Figure 3.4, there was no significant difference in *FSHR* mRNA between the experimental groups, whereas a marked increase was seen in the expression of *LHR* mRNA in PCOS rats as compared to control (P<0.05).

**Steroidogenesis in PCOS**

The binding of gonadotropin to their receptors on ovary results in the activation of a signalling cascade, ultimately leading to increased steroid production. mRNA expression profile of steroidogenic enzymes revealed significant increase in the expression of *StAR* (P<0.01) and *Cyp17a1* (P<0.01) while the expression of *Cyp19a1* (P<0.01) was notably reduced in ovary of PCOS animals (Figure 3.5). These alterations were well correlated with
the change in protein expression of StAR and aromatase and with the increase in pERK1/2 and pCREB contents of PCOS animals. Furthermore, these results were in line with the local production of steroids as measured by ELISA, wherein testosterone content was significantly elevated (P<0.001), while reduced levels of progesterone (P<0.01) and Estradiol (P<0.01) were observed in letrozole-induced PCOS rat ovary as compared to controls (Table 3.4). As already mentioned earlier, steroidogenesis also occurs in various other tissues such as hypothalamus, pituitary, hippocampus and frontal cortex. However, the status of steroid production in these tissues has not been studied in PCOS. Like in the ovary, steroid alteration was observed in all the brain regions studied with less significant results for hippocampus and frontal cortex. Real-time PCR studies revealed a marked increase in expression of StAR (P<0.01) and Cyp17a1 (P<0.01), while Cyp19a1 was significantly reduced (P<0.01) in PCOS hypothalamus (Table 3.5; Figure 3.6). Also, the change in the mRNA expression was well correlated with the protein expression of the steroidogenic regulators as well as the steroid concentration in the hypothalamus. In accordance with this, pituitary of the PCOS animals also demonstrated hypoestrogenic (P<0.01) and hyperandrogenic (P<0.001) condition in the brain with significantly decreased progesterone content (P<0.05) as compared to control animals (Table 3.6; Figure 3.7). This altered steroid status is congruent with the results obtained for mRNA and protein expression pattern. In hippocampus, similar steroid profile was observed with decreased estrogen (P<0.05) and increased testosterone (P<0.05) levels, which was concurrent with the increased StAR (P<0.05) and Cyp17a1 (P<0.05) and decreased Cyp19a1 (P<0.05) expression (Table 3.7; Figure 3.8). No notable change was observed in protein expression as compared to control animals. Although frontal cortex demonstrated similar steroid hormone profile, the change was not significant as compared to frontal cortex of control animals (Table 3.8; Figure 3.9).

Feedback regulation of HPO axis

The regulation of HPO axis occurs via feedback regulation of steroid hormone which is mediated via activation of their receptors on various brain regions. The mRNA expression of estrogen receptors – ERα (P<0.01), ERβ (P<0.05) and progesterone receptor PR (P<0.05) was significantly reduced in hypothalamus of PCOS animals with marked increase in androgen receptor AR expression (P<0.01) as compared to controls (Figure 3.10). The protein expression profile of these steroid receptors was concomitant with the transcript levels demonstrating decreased ERα (P<0.05) and ERβ (P<0.05) expression with increased AR (P<0.05) expression in the hypothalamus of PCOS animals. The expression profile of
pituitary followed similar pattern of change as observed in case of hypothalamus of PCOS animals (Figure 3.11). The expression of $ER_\alpha$ was significantly reduced in hippocampus with decreased $ER_\alpha$ (P<0.05) content and increased AR (P<0.05) protein and gene expression, while the expression of $ER_\beta$ remained unchanged from control (Figure 3.12). There was no significant difference found in the expression of steroid receptors in frontal cortex (Figure 3.13). When analysed for ovarian steroid receptors, elevated levels of AR protein (P<0.05) with increase in its transcripts (P<0.05) was observed in PCOS animals (Figure 3.14). On the other hand, the transcripts of $ER_\alpha$ (P<0.05), $ER_\beta$ (P<0.05) and $PR$ (P<0.05) were significantly decreased with reduced expression of $ER_\alpha$ (P<0.05) and $ER_\beta$ (P<0.05) in ovary (Figure 3.14) as compared to control animals. It is also important to note that steroid receptor activation depends upon the availability of steroid ligands and the change in steroid receptor can be well correlated with the respective steroid levels in that particular tissue in PCOS.

**HPO axis regulators in PCOS**

In addition to feedback regulation, HPO axis is regulated by various neuropeptides which include kisspeptin, NKB, Dyn, RFRP3 and Insulin. Expression profile of the genes encoding these peptides was generated in our system (Figure 3.15). The mRNA expression of $KISS1$, $GPR54$, and $NKB$ was significantly elevated (P<0.05), while expression of $Dyn$, $RFRP3$ and $GPR147$ (P<0.05) demonstrated marked decrease in hypothalamus of PCOS animals as compared to control animals. Furthermore, the pituitary demonstrated no significant change in the expression of $KISS1$, $GPR54$, $NKB$, $Dyn$ and $IR$, whereas the expression of $RFRP3$ and $GPR147$ (P<0.05) was reduced in PCOS animals as compared to control animals. Also, PCOS rats demonstrated significantly elevated levels of insulin in hypothalamus (P<0.01), pituitary (P<0.05) and ovary (P<0.05) suggesting hyperinsulinemic condition in these tissues of PCOS animals. In addition to hypothalamic regulation, these peptides are also synthesized in the ovary and help in ovarian steroidogenesis and follicle maturation. The expression of $RFRP3$ and $GPR147$ (P<0.05) was significantly reduced while no change was observed in expression of $KISS1$, $GPR54$, $NKB$, $Dyn$ and $IR$ in ovary of PCOS animals.
Table 3.1: Estrus cycle profile after letrozole or Testosterone propionate treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Letrozole treated</th>
<th>Control</th>
<th>TP-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cycle</td>
<td>100%</td>
<td>-</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>Extended Proestrus</td>
<td>-</td>
<td>5%</td>
<td>-</td>
<td>5%</td>
</tr>
<tr>
<td>Extended Estrus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Extended Metestrus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5%</td>
</tr>
<tr>
<td>Extended Diestrus</td>
<td>-</td>
<td>95%</td>
<td>-</td>
<td>90%</td>
</tr>
</tbody>
</table>

Figure 3.1: Ovarian histology to show induction of PCOS. Controls (A, B) show normal histoarchitecture, while Letrozole-group ovaries (C) and TP-group ovaries (D) demonstrate presence of multiple cysts (Arrows). Magnification 4X.
Table 3.2: Serum hormone profile

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Letrozole treated</th>
<th>Control</th>
<th>TP-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (pg/ml)</td>
<td>132.3 ± 17.37</td>
<td>139.3 ± 19.10</td>
<td>125.3 ± 20.4</td>
<td>136.4 ± 18.62</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>0.315 ± 0.05</td>
<td>1.177 ± 0.07***</td>
<td>0.357 ± 0.09</td>
<td>1.058 ± 0.11**</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>12.25 ± 0.54</td>
<td>9.95 ± 0.39*</td>
<td>14.37 ± 0.66</td>
<td>13.24 ± 0.87</td>
</tr>
<tr>
<td>Insulin (mIU/ml)</td>
<td>7.42 ± 1.04</td>
<td>15.54 ± 1.27***</td>
<td>8.61 ± 1.17</td>
<td>12.48 ± 1.02**</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.56 ± 0.14</td>
<td>4.40 ± 0.21***</td>
<td>1.45 ± 0.29</td>
<td>3.08 ± 0.71**</td>
</tr>
</tbody>
</table>

All values are Mean±SEM of four individual experiments. HOMA-IR=Glucone (mg/dL)*Insulin (IU/ml)/405. *P<0.05, **P<0.01, ***P<0.001 as compared to control.

Figure 3.2: Change in body weight after Letrozole and TP treatment of rats. Values represent mean±SEM; N=10 per group. **P<0.01 as compared to control group.

Figure 3.3: OGTT profile after Letrozole and TP treatment of rats. Values represent mean±SEM; N=10 per group. **P<0.01, ***P<0.001 as compared to control group.
Table 3.3: Gonadotropin levels in letrozole induced PCOS rat model

<table>
<thead>
<tr>
<th></th>
<th>FSH (ng/ml)</th>
<th>LH (ng/ml)</th>
<th>LH:FSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.20 ± 0.099</td>
<td>2.40 ± 0.138</td>
<td>1.08</td>
</tr>
<tr>
<td>PCOS</td>
<td>2.29 ± 0.180</td>
<td>6.76 ± 0.132***</td>
<td>2.97***</td>
</tr>
</tbody>
</table>

All values are presented as Mean ± SEM of four individual experiments. ***P<0.001 as compared to control values.
Table 3.4: Steroid levels in Ovary

<table>
<thead>
<tr>
<th></th>
<th>Progesterone (pg/g)</th>
<th>Testosterone (ng/g)</th>
<th>Estradiol (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.871 ± 0.079</td>
<td>1.420 ± 0.072</td>
<td>451.8 ± 17.8</td>
</tr>
<tr>
<td>PCOS</td>
<td>3.096 ± 0.0448**</td>
<td>2.217 ± 0.118***</td>
<td>327.5 ± 15.5 ***</td>
</tr>
</tbody>
</table>

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

Figure 3.5: Steroidogenesis markers in ovary. 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 obtained 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 3.5: Steroid levels in Hypothalamus

<table>
<thead>
<tr>
<th></th>
<th>Progesterone (pg/g)</th>
<th>Testosterone (ng/g)</th>
<th>Estradiol (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.855 ± 0.036</td>
<td>1.088 ± 0.044</td>
<td>227.9 ± 15.4</td>
</tr>
<tr>
<td>PCOS</td>
<td>0.798 ± 0.043</td>
<td>1.40 ± 0.027 **</td>
<td>139.5 ± 16.7 **</td>
</tr>
</tbody>
</table>

All values are presented as Mean ± SEM of four individual experiments. **P<0.01; ***P<0.001 as compared to control values.
Figure 3.7: Steroidogenesis markers in pituitary. 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 obtained 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 3.6: Steroid levels in Pituitary

<table>
<thead>
<tr>
<th></th>
<th>Progesterone (pg/g)</th>
<th>Testosterone (ng/g)</th>
<th>Estradiol (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.33 ± 0.034</td>
<td>1.389 ± 0.056</td>
<td>340.3 ± 17.2</td>
</tr>
<tr>
<td>PCOS</td>
<td>0.833 ± 0.031*</td>
<td>1.817 ± 0.045***</td>
<td>237.6 ± 19.8**</td>
</tr>
</tbody>
</table>

All values are presented as Mean ± SEM of four individual experiments. *P<0.05; **P<0.01; ***P<0.001 as compared to control values.
Table 3.7: Steroid levels in Hippocampus

<table>
<thead>
<tr>
<th></th>
<th>Progesterone (pg/g)</th>
<th>Testosterone (ng/g)</th>
<th>Estradiol (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.251 ± 0.114</td>
<td>1.067 ± 0.066</td>
<td>221.7 ± 13.02</td>
</tr>
<tr>
<td>PCOS</td>
<td>1.790 ± 0.118</td>
<td>1.417 ± 0.044*</td>
<td>183.7 ± 11.67*</td>
</tr>
</tbody>
</table>

All values are presented as Mean ± SEM of four individual experiments. *P<0.05 as compared to control values.

Figure 3.8: Steroidogenesis markers in hippocampus. 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 obtained 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 3.8: Steroid levels in Frontal cortex

<table>
<thead>
<tr>
<th></th>
<th>Progesterone (pg/g)</th>
<th>Testosterone (ng/g)</th>
<th>Estradiol (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.67 ± 0.170</td>
<td>1.60 ± 0.124</td>
<td>211.6 ± 14.08</td>
</tr>
<tr>
<td>PCOS</td>
<td>1.659 ± 0.100</td>
<td>1.83 ± 0.082</td>
<td>197.4 ± 15.1</td>
</tr>
</tbody>
</table>

All values are presented as Mean ± SEM of four individual experiments.

Figure 3.9: Steroidogenesis markers in frontal cortex. 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 obtained 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.
Figure 3.10: Steroid receptor expression in hypothalamus. 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 obtained 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.
Figure 3.11: Steroid receptor expression in pituitary. 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 obtained 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.
Figure 3.12: Steroid receptor expression in hippocampus. 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 obtained 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.
Figure 3.13: Steroid receptor expression in frontal cortex. 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 obtained 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.
Figure 3.14: Steroid receptor expression in ovary. 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 obtained 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.
Figure 3.15: Status of HPO axis regulators. Left: Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by red dashed line). Right: Insulin levels. Error bars represent SEM; N=6 per group. *P<0.05; **P<0.01; ***P<0.001 as compared to control group.
Discussion

PCOS is a disorder linked with both reproductive and metabolic dysfunction. Although the neuroendocrine characteristic of PCOS is well known, the exact mechanism for such a derangement remains obscure. In this regard, two different rat models of PCOS were used to delineate the mechanism of PCOS. Both, letrozole- and TP-treated rats were arrested in diestrus stage of estrus cycle, and displayed hormonal imbalance and presence of peripheral cysts in the ovaries – common reproductive anomalies in human PCOS condition (Franks et al., 2008). Also, increased body weight, glucose intolerance and insulin resistance in both these animal models suggest development of metabolic syndrome in these rat models of PCOS which is similar to earlier reports (Belooesky et al., 2004; Kafali et al., 2004; Maharjan et al., 2010). However, it should be noted that letrozole-treated animals demonstrated profound changes in the reproductive and metabolic traits of PCOS as compared to TP-treated animals. Furthermore, in TP-treated rats, testosterone can be converted into estradiol and the effects observed may not be solely due to hyperandrogenic condition, making it an unsuitable model to understand androgen-mediated mechanism of PCOS pathology (Walters, 2015). Thereby, to decipher the underlying pathogenesis of PCOS, further experiments were performed using the letrozole-induced PCOS rat model.

Reports suggest that a change in the functioning of HPO axis is a cause of PCOS (Blank et al., 2006; Solorzano et al., 2012). In this context, the present study has demonstrated the aberration of HPO axis in PCOS condition using letrozole-based rat model. The increase in the expression of GnRH, FSHβ and LHβ mRNA in PCOS rats may result into the observed increase in serum LH/FSH ratio, which can be well correlated with the neuroendocrine abnormalities of PCOS that include increased GnRH pulsatility and elevated LH/FSH ratio (Banaszewska et al., 2003; Azziz et al., 2016). This increased LH release may result into increased ovarian steroidogenesis and elevated androgen secretion leading to hyperandrogenemia. In this regard, the current study demonstrated an increase in transcripts of ovarian LHR, StAR, Cyp17a1, with decreased Cyp19a1 expression in PCOS condition which suggest the overstimulation of ovarian steroidogenesis in PCOS. This was further confirmed by elevated levels of testosterone and reduced levels of progesterone and estradiol as observed in the letrozole-induced PCOS rat ovary. Similar to our results, women with PCOS have shown increased transcript and protein levels of steroidogenic enzymes (Nelson et al., 1999; 2001). This increased androgen and decreased estradiol and progesterone leads to thickening of theca cells and poor granulosa cells, which leads to development of immature
follicles and cyst formation in PCOS ovary (Jonard and Dewailly, 2004; Chang, 2007). It is also interesting to note that these ovarian steroids signal the brain to regulate GnRH and LH release through a feedback loop.

GnRH, a central player of reproduction, is secreted in distinct patterns of pulses and surge that differentially control the release of pituitary gonadotropins, which is mandatory for the cyclic changes occur during the female reproductive cycle (Belchetz et al., 1978). During most phases of the cycle, GnRH is released in a pulsatile manner that regulates folliculogenesis and corpus luteum, whereas surge mode of GnRH increases LH secretion, leading to ovulation (Maeda et al., 2010). Several experiments indicate that estrogen and/or progesterone negatively regulate pulsatile release of GnRH while surge mode is positively regulated by estrogen (Moenter et al., 2003; Sullivan & Moenter, 2005) through binding to steroid receptors in various regions of brain (Temple et al., 2004; Bashour and Wray, 2012). Transgenic studies wherein steroid receptors were knocked out (ERαKO, ERβKO and PRKO) demonstrated significantly increased LH release compared to wild-type mice (Chappell et al, 1997; Dorling et al. 2003), suggesting a role of these steroids as GnRH suppressors.

As mentioned in the introduction, GnRH neurons express only ERβ, indicating that steroid-mediated feedback regulation must occur through various interneurons. Immunohistochemical studies have revealed the presence of steroid receptors in neurons containing neuropeptides – kisspeptin, NKB, Dynorphin, RFRP-3, neuropeptide Y and also in glutamatergic and GABAergic neurons (Stumpf & Jennes 1984; Leranth et al. 1992; Smith et al. 2005; Franceschini et al. 2006; Kenealy & Terasawa, 2012). Among all the other factors, the role of kisspeptin in GnRH regulation has been extensively studied (Maeda et al., 2010; Simonneaux et al., 2013). Kisspeptin neurons of the AVPV regions are involved in estradiol-mediated positive feedback of GnRH surge release which results into subsequent LH surge and ovulation. In contrast to this, arcuate nucleus kisspeptin neurons regulate estrogen- and progesterone-mediated negative feedback that controls GnRH and LH pulsatility (Li et al., 2009). Neurokinin B and dynorphin are co-expressed in the arcuate nucleus kisspeptin neuron population, commonly known as KNDy neurons which are responsible for differential effects of kisspeptin on GnRH regulation. KNDy neurons of the arcuate nucleus project to GnRH terminals in median eminence and, at the same time, their collaterals and dendrites form a bilateral neural circuit connecting to their own cell body. This unique architecture of KNDy
neurons helps in autoregulation of kisspeptin release through NKB and Dyn (Maeda et al., 2010). In the KNDy neurons, stimulation of NKB and its receptor NK3 facilitates synchronized activation of kisspeptin neurons, whereas stimulation of Dyn/KOR (κ-opioid receptor) signalling terminates NKB mediated activation, generating a rhythmic oscillation of activity. This fluctuation in the activity results in the release of kisspeptin pulse in the median eminence, which in turn evokes GnRH release in a pulsatile manner (Maeda et al., 2010). The steroid-mediated regulation of KNDy neuron activity was demonstrated by several studies. In ovariectomized goat, GnRH pulse frequency decreased upon administration of low levels of estradiol and progesterone (Maeda et al., 2010; Wakabayashi et al., 2010). This further confirms that synchronous concert of steroids and neuropeptides governs the activity of GnRH pulse generator and thereby regulates the ovarian axis.

As PCOS is a disorder of altered GnRH pulsatility, we hypothesize a role for these neuropeptides in development of the related pathology. In support of our speculation, hypothalamic KISS1/GPR54 and NKB mRNA levels were significantly increased while Dyn expression was markedly reduced in letrozole-induced PCOS rats. Moreover, these neuropeptides are the target of steroid hormones. It was demonstrated that estradiol reduces expression of NKB and NK3 in arcuate nucleus leading to suppression of GnRH pulse release (Navarro et al., 2009). In addition, progesterone increases Dyn expression which may further decrease GnRH pulse frequency due to elevation of inhibitory Dyn (Foradori et al., 2005). In the current study, reduced levels of estradiol and progesterone with decreased mRNA expression of ERα and PR were observed in hypothalamus of letrozole-induced PCOS rats. This implicates that reduced levels of estradiol and progesterone may increase NKB expression and decrease Dyn expression respectively, leading to elevated KISS1 mRNA, further resulting into a greater GnRH pulse rate. Similar to our result, increased KISS1 and NKB mRNA was observed in hypothalamus of prenatally androginized (PNA) rat model of PCOS (Yan et al., 2014). Additionally, our assumption of impaired negative feedback in PCOS is also supported by the study in PNA mice, wherein reduced ERα and PR mRNA was observed (Moore et al., 2012).

In addition to ERα, estradiol rapidly affects GnRH neuron activity through binding to other estrogen receptors such as ERβ, STX-R (membrane estrogen receptor sensitive to diphenylacrylamide compound-STX) and GPR 30 (Temple et al., 2004; Sun et al., 2010; Zhang et al., 2010a). This rapid action of estradiol is achieved by modulation of voltage-
gated calcium channels, which increases calcium oscillation and thus increases GnRH neuron firing (Sun et al., 2010). Studies using several agonists and antagonists of these receptors indicate that high level of estradiol increases calcium oscillation and synchronous GnRH release, whereas low level had no such effect (Noel et al., 2009). In the present study, estardiol concentration was reduced in hypothalamus with no significant change in the \( \text{ER}\beta \) mRNA and protein, thereby indicating that \( \text{ER}\beta \) may not be involved in the increased GnRH release seen in PCOS. However, studies with other membrane-estrogen receptors are needed to confirm any role of rapid estradiol action in PCOS pathology. Furthermore, role of steroid receptors has also been implicated in regulation of pituitary gonadotropin. Estradiol is able to reduce GnRH-stimulated LH release as observed by injection of estradiol into pituitary tissue (Strobl & Levine, 1988). mRNA levels of FSH and LH were significantly elevated in \( \text{ER}\alpha \text{KO} \) female mice as compared to wild type mice (Scully et al., 1997, Lindzey et al., 1998). Also, transgenic female mice of pituitary-specific \( \text{ER}\alpha \text{KO} \) demonstrated significantly elevated serum LH levels (Gieske et al., 2007; Singh et al., 2009). However, this increase in serum LH was less compared to global \( \text{ER}\alpha \text{ KO} \) mice, suggesting that although pituitary is involved in estradiol-mediated feedback regulation, major feedback is attained through other neuronal locations including hypothalamus. In addition to estradiol, progesterone also influences LH release. mRNA expression of common \( \alpha \)-subunit of gonadotropin and LH\( \beta \) subunit is significantly elevated in ovariectomized animals and treatment of estradiol and progesterone in these animals notably reduces serum LH levels as well as mRNA expression of LH\( \beta \) subunit in the pituitary gland (Corbani et al., 1984; 1990; Plant & Zelenzink, 2014). Present data demonstrated marked reduction \( \text{ER}\alpha \) and \( \text{PR} \) in pituitary of letrozole-induced PCOS rats, implying the suppression of estradiol and progesterone-mediated negative feedback in PCOS condition at both hypothalamic and pituitary level.

Although the role of androgen in GnRH regulation in females has not been extensively studied, recent studies using androgen receptor knockout transgenic animals have open a new avenue in androgen mediated-GnRH control. Androgen receptor knockout (ARKO) female mice were sub-fertile with altered estrous cycles (Hu et al., 2004; Walters et al., 2007; 2009). Also, LH surge was decreased or absent in ARKO female mice with decreased expression of Kiss1 mRNA, suggesting the role of AR in control of kisspeptin-mediated GnRH/LH release (Cheng et al., 2013). Furthermore, mice with pituitary-specific AR knockout displayed low levels of FSH and LH, indicating the independent role of AR in pituitary gonadotropin release (Wu et al., 2014). Thereby, increase in \( \text{AR} \) expression both at protein and mRNA
levels in present study may lead to increase in KISS1 expression, resulting into increased GnRH and LH release. In addition, androgen can affect other steroid receptors and thereby interfere with the steroid mediated feedback regulation (Turgeon & Waring, 1999; Moore & Campbell, 2017). Hence, increase in AR expression in the present study may be one of the reasons for decreased PR expression in PCOS condition, which is in line with a study showing decreased expression of hypothalamic PR mRNA upon testosterone infusion (Foecking & Levine, 2005a). Further, a recent study using neuronal specific knockout of AR (NeurARKO) demonstrated the importance of brain AR in development of PCOS (Caldwell et al., 2017). In contrast to DHT-induced PCOS mice, NeurARKO mice exhibited no signs of PCOS when treated with DHT. Similarly, Eagleson and group (2000) have demonstrated that blockade of androgen action by flutamide restores estradiol- and progesterone-mediated feedback and increases GnRH sensitivity.

It is also important to note that these steroid receptors are co-expressed in several neurons containing neurotransmitters such as glutamate, GABA and serotonin, which directly connect to GnRH neurons in hypothalamus. Increasing evidence suggests that these neurotransmitters play very important roles in rapid as well as prolonged regulation of GnRH/LH release through binding to steroid receptors. However, the interactions of steroids with neurotransmitters will be discussed in another chapter (Chapter 5).

Along with ovary, steroid synthesis occurs in different regions of brain – pituitary (Vidal et al., 2000), hypothalamus (Ishunina et al., 2005) and frontal cortex (Do Rego et al., 2009) and they play important roles in regulation of HPG axis (Temple et al., 2004), sexual behavior (Carani et al., 1999) and other bodily functions (Zwain and Yen, 1999). Most studies have indicated that systemic androgen levels (Azziz et al., 2006) are elevated in PCOS but no correlation of this syndrome has been established with brain androgen levels. This is the first study wherein we have observed altered steroids in the brain of PCOS rats. As stated in this report, increased brain testosterone level can be due to increased mRNA expression of StAR and Cyp17a1. Increasing evidence suggests that neurosteroid synthesis is regulated by autocrine, paracrine and endocrine mechanism among which gonadotropin regulation of neurosteroidogenesis is widely accepted (Meethal et al., 2009). The presence of GnRHR and LHR has been reported in various regions of brain including hippocampus and cortex which act as a gonadotropin target for de novo steroidogenesis (Wilson et al., 2006; Liu et al., 2007). LH has ability to induce StAR expression in vitro as observed in human neuroblastoma and rat primary neurons (Liu et al., 2007). Also, treatment of leuprolide...
acetate, an antagonist of gonadotropins resulted in significant decrease in brain StAR expression (Wilson et al., 2008). Although the expression of LHR and GnRHR were not measured in hippocampus and cortex of the present study, increased mRNA expression of GnRH, LHβ and LH levels in PCOS condition may result into activation of StAR and Cyp17a1 in these regions of brain resulting into increased androgen levels. Furthermore, a recent study has demonstrated that estradiol and progesterone inhibit neurosteroidogenesis by suppressing StAR-mediated cholesterol transport into the mitochondria (Meethal et al., 2009). Thereby, low levels of serum progesterone as demonstrated in current study, suggest the suppression of inhibitory signal to neurosteroid synthesis, leading elevated testosterone in PCOS brain. In addition, reports also indicate the role of GnRH in neuro-estradiol synthesis. In hippocampal neuronal culture, estradiol synthesis increases when incubated at low doses of GnRH while a high dose of GnRH decreases estradiol synthesis (Prange-Kiel et al., 2008). Thus, increased GnRH pulsatility, a common feature in PCOS, might result into reduced brain estradiol levels as observed in the current study. Moreover, for PCOS induction, current study has used letrozole – an aromatase inhibitor. Letrozole is a competitive inhibitor of aromatase and a reduction in its mRNA expression was earlier reported only in the ovaries of letrozole-treated rats (Zurvarra et al., 2009). Our study additionally demonstrates a significant reduction in the mRNA and protein expression of aromatase in the brain regions of the PCOS rats. Reports suggest that brain aromatase plays an essential role in negative feedback of LH release via converting testosterone to estradiol (Couse and Korach, 1999; Roselli & Resko, 2001). This may be one of the reasons for increased testosterone and reduced estradiol levels in brain regions of PCOS animals, which ultimately results into decreased feedback to LH and hence, increased LH release. Thereby, the present work supports the notion that PCOS is a condition wherein co-existence of ovarian and brain steroidogenic modulations exacerbate the syndrome.

In addition to steroid-mediated feedback regulation of kisspeptin, role of RFRP3-a GnIH homolog is also emerging in regulation of reproduction (Ubuka et al., 2012). In contrast to kisspeptin, RFRP3 is a most potent inhibitor of GnRH release. GnRH release is profoundly inhibited when RFRP3 was administered centrally in rats (Johnson et al., 2007). Furthermore, cultured mouse brain slices decreased GnRH cell firing upon direct treatment of RFRP3, suggesting a direct role of RFRP3 in GnRH action. Similarly, central administration of RFRP3 receptor (GPR-147) antagonist RF9, increases gonadotropin secretion in rats (Pineda et al., 2010; Ubuka et al., 2012). Chronic GnIH treatment to mature male quail decreases
plasma LH concentration and the expressions of the common α, Lhb, and Fshb subunit mRNAs (Tsutsui et al. 2007; Tsutsui et al. 2015). Central administration of RFRP3 in female mice demonstrates differential effect on the LH depending upon the stage of estrus cycle. Intracerebroventricular injection of RFRP3 during estradiol negative feedback condition had no effect on LH secretion, whereas RFRP3 significantly reduced estradiol-mediated preovulatory LH surge (Ancel et al., 2017). Also, reduction in LH pulse amplitude and inhibition of GnRH elicited gonadotropin release and synthesis by mammalian GnIH (RFRP-3) in sheep and cattle (Clarke et al., 2008; Sari et al., 2009). Taken together, RFRP3 directly or indirectly inhibits GnRH and gonadotropin release. The present data has shown significant down-regulation of RFRP3 and GPR-147 transcripts in hypothalamus and pituitary of PCOS rats, suggesting a reduction in the major inhibitory molecule, which could increase GnRH and gonadotropin secretion as observed in our PCOS model. Furthermore, as GPR147 is coupled with Gaα subunit that inhibits adenylate cyclase and subsequent cAMP mediated-signaling cascade including ERK (Son et al., 2012). Increasing ERK1/2 phosphorylation in PCOS condition may be due, in part, to decreased expression of GPR147 observed presently. RFRP3 also increases neuroestrogen production through activating aromatase (Ubuka et al., 2014). In contrast, estradiol also increases RFRP3 expression through estrogen receptors (Clarke & Parkington, 2014). Co-localization of RFRP3 neurons with estrogen receptors ERα (Kriegsfeld et al., 2006; Molnar et al., 2011) and ERβ (Clarke et al., 2012) has been demonstrated in mice, hamster and sheep (Clarke & Parkington, 2014). The low levels of estradiol observed in the present study may decrease the expression of RFRP3 in PCOS which may in turn reduce estradiol due to loss of aromatase activation by RFRP3.

PCOS is a condition wherein metabolic aberration like hyperinsulinemia and insulin resistance co-exists. It has been suggested that insulin levels in circulation reflect its levels in various body organs including brain (Gamba & Pralong, 2006; Pralong, 2010). Interestingly, the present work shows significantly increased insulin levels in serum as well as in hypothalamus, pituitary and ovary of letrozole-induced PCOS rats. Increasing evidence suggests that insulin influences GnRH and LH release and thereby regulates reproduction. Insulin and its receptor expression have been observed in the GnRH neurons of hypothalamus and preoptic area (Bruning et al., 2000; Plum et al., 2005) and reports indicate that GnRH neurons exhibit many characteristics of insulin sensitive tissues (Salvi et al., 2006). Perfusion of insulin in hypothalamic pieces significantly increased GnRH release (Arias et al., 1992; Blázquez et al., 2014). Similarly, intracerebral infusion of insulin increased LH pulse

*HPO axis in PCOS* 106
frequency, suggesting stimulatory role of insulin in GnRH/LH release via activation of its receptor IR (Miller et al., 1995; Blázquez et al., 2014). Although no significant difference in insulin receptor transcripts (IR) was observed, insulin levels were elevated in hypothalamus and pituitary of PCOS rats in the present study. Binding of insulin to its receptor activates tyrosine phosphorylation of IR which then activates insulin receptor substrate (IRS) phosphorylation and stimulates various signaling pathways (Boucher et al., 2014). It has been noted that hyperinsulinemia and insulin resistance are associated with increased serine phosphorylation of IRS which inhibit the normal insulin signaling pathway (Boucher et al., 2014). Also, a recent study has demonstrated that estradiol can modulate insulin as well as insulin binding to its receptor (Root-Bernstein et al., 2014). The hypoestrogenic condition observed in the present study may interfere with insulin signaling. However, detailed understanding of estradiol interaction with insulin and its downstream signaling warrants further research. As PCOS is a metabolic syndrome with increased insulin levels, there is be possibility that there exists an insulin resistance-like condition in brain as observed in type 2 diabetes mellitus (Blázquez et al., 2014). However, supplementary studies are needed to delineate the exact role of brain insulin in PCOS pathology.

In contrast to insulin condition in brain, role of insulin in pathophysiology of ovarian changes are well known in PCOS. Insulin acts as a co-gonadotropin in ovarian theca cells during normal physiological conditions, while under hyperinsulinemic conditions, insulin increases gonadotropin-stimulated androgen synthesis (Diamanti-Kandarakis & Dunaif, 2012; Tosi et al., 2012). *In vitro* studies using theca cells from mammalian ovaries have demonstrated that insulin has a dose-dependent effect on cell proliferation, steroid production and expression of genes such as Star, Cyp17a1, Cyp11a1, overall promoting steroidogenesis (Morley et al., 1989; Mamluk et al., 1999; Young & McNeilly, 2010). There exists a selective insulin resistance in PCOS ovary which is characterized by normal insulin action on steroidogenesis, with its action on glucose metabolism significantly reduced (Fontana & Torre, 2016). Furthermore, insulin also increases hyperandrogenic condition through decreasing synthesis of sex-hormone binding globulin (SHBG) from liver (Diamanti-Kandarakis & Dunaif, 2012). Similar scenario of disrupted insulin signalling may also exist in our PCOS model due to hyperinsulinemia and an increased HOMA-IR.

It is noteworthy that hyperandrogenemia also induces hyperinsulinemia through activation of pro-inflammatory cytokines. Thus, in PCOS, hyperandrogenemia and hyperinsulinemia confer a vicious cycle. Hyperandrogenemia in ovary induces growth of primordial follicles
and increases the number of small antral follicles (Weil et al., 1999; Nisenblat & Norman, 2009). Furthermore, these increased small antral follicles increase the production of Anti-mullerian hormone (AMH) which aggravates follicular development by inhibiting FSH and increasing androgen synthesis (Pigny et al., 2003; Visser et al., 2012). Furthermore, testosterone also reduces the expression of GDF9, which is essential for ovarian follicular development (Yang et al., 2010). Thereby, the hyperandrogenic condition observed in the current study may induce AMH production and at the same time decrease GDF9 expression, leading to premature development of the ovarian follicle. Although estradiol levels in the ovary were reduced in the current study, increased androgen levels may increase the responsiveness of granulosa cells towards estradiol. Also, insulin and LH reduce estradiol and progesterone production as well as suppress proliferation of granulosa cells, thus resulting into decreased follicular maturation (Nisenblat & Norman, 2009; Dumesic et al., 2015).

In addition to steroidogenic regulation, several neuropeptides are also synthesized in ovary and facilitate ovarian follicle growth and maturation. Kiss1/GPR-54 mRNA and proteins have been identified in ovary of rats, monkey and humans (Castellano et al., 2006; Gaytan et al., 2009; Witchel & Tena-Sempere, 2013). Also, presence of NKB and Dynorphin has been recently detected in rat ovary (Laoharatchatathanin et al., 2015). Although the exact role of these peptides in ovary has not been elucidated, recent studies have demonstrated that expression of kiss1, Nkb and Dyn changes with the estrus cycle (Laoharatchatathanin et al., 2015; Uenoyama et al., 2016). Administration of LH significantly increases the expression of kiss1, Nkb and Dyn in rat ovary during late proestrus stage suggesting a putative role of these peptides in ovulation (Laoharatchatathanin et al., 2015). Furthermore, kisspeptin increases progesterone synthesis in rat luteal cells and helps in corpus luteum formation (Witchel & Tena-Sempere, 2013; Laoharatchatathanin et al., 2015). However, the present study could not find any correlation between the expression of these peptides in the ovary indicating involvement of other plausible targets in ovarian follicular impairment in letrozole-induced PCOS rodent model.

In addition, direct influence of RFRP3 on ovarian folliculogenesis was also observed in various models (Tsutsui et al., 2010; Oishi et al., 2012; Ancel et al., 2017). Treatment of RFRP3 in mice disrupted antral follicle development as well as inhibited lutenization through reducing LHR expression (Singh et al., 2011b). Furthermore, RFRP3 dose-dependently decreases expression of StAR and 3βHSD resulting in reduced production of progesterone (Singh et al., 2011b). Reduced expression of RFRP3 and GPR-147 was observed in ovary of
PCOS rats which is one of the probable reasons for increased ovarian steroidogenesis observed in the present study. Taken together, all this data indicates that in addition to gonadotropin abnormalities, alteration of several intra-ovarian factors may result in increased steroidogenesis and premature follicular development, leading to follicular arrest and cyst formation in PCOS.

In summary, the current piece of work demonstrates neuroendocrine alterations in PCOS using letrozole-induced rat model. Letrozole-treated animals demonstrated elevated serum testosterone levels, arrested estrus cyclicity and cyst formation in ovary. Also, these animals were glucose intolerant, hyperinsulinemic and showed increased HOMA-IR index, thereby indicating that letrozole was able to induce reproductive and metabolic characteristics of the human PCOS condition. Although steroid-mediated feedback regulation was known to be altered in PNA PCOS mice model, this is the first study to demonstrate such derangement using letrozole-induced PCOS model. Several lines of evidence indicate that in utero androgen exposure may lead to epigenetic changes in the neural circuitry and may alter brain sexual dimorphism in the offspring, implicating that the modulations observed in PNA mice may not actually be seen in PCOS women. Thereby, letrozole-induced PCOS model was employed herein to get an insight of the neuroendocrine pathologies in PCOS.

Our study clearly explicates the involvement of several modulations in brain regions that are responsible for the characteristic increase in GnRH pulsatility seen in PCOS. Current data revealed that estradiol- and progesterone-mediated negative feedback was impaired in PCOS condition, resulting in increased expression of Kiss1, Nkb and reduced Dyn mRNA. Also, the expression of RFRP3 was reduced in hypothalamus and pituitary of PCOS animals. All this, taken together, suggests that the decrease in the GnRH inhibitory RFRP3 and increase in the stimulatory Kiss1 may result into augmented GnRH pulsatility and thereby result into elevated LH/FSH ratio in PCOS. Moreover, heightened ovarian response to LH could be due to increased expression of LHR and insulin which increases ovarian steroidogenesis leading to increased androgen and at the same time reducing estradiol content. These altered steroids together with several intra-ovarian factors modulate ovarian folliculogenesis, resulting into formation of cysts in the ovary, a hallmark of PCOS. Along with the ovarian abnormalities, for the first time, it is hereby revealed that hypoestrogenic and hyperandrogenic conditions do exist in the brain of PCOS animals due to steroidogenic alterations in these regions. Furthermore, these steroids regulate GnRH axis via several interactions with neuropeptides and neurotransmitters. The changes observed in the steroid receptors are well correlated with
altered neuronal signals in PCOS animals. This is the first comprehensive report of its kind wherein we have illustrated a complex interaction of several factors collectively influencing the reproductive axis in PCOS. Using this model, we ably support our hypothesis that imbalances in the brain steroid status and their receptor expression are responsible for an altered feedback circuitry, thus contributing to PCOS etiology.