Reproduction in women is characterized by cyclic changes in the ovary and uterus which is required for ovarian follicle development, ovulation and preparation of endometrium for implantation. This pattern of repeated ovarian cycle is regulated by interactions of several stimulatory and inhibitory signals from hypothalamus, pituitary and ovary forming a reproductive axis (Hypothalamic-Pituitary-Ovarian axis). Gonadotropin releasing hormone (GnRH), the master regulator of reproduction is released from the hypothalamus in a pulsatile manner which stimulates anterior pituitary gonadotrops to release follicle stimulating hormone (FSH) and lutenizing hormone (LH). These gonadotropins, through circulation, reach the ovaries and bind to their receptors present on ovarian cells. Under the influence of FSH and LH, ovarian follicular development and ovulation proceeds with secretion of estrogens, progesterons and androgens. Further, these steroid hormones regulate the HPO axis through classic feedback loops as well as with the participation of various neuropeptides, neurosteroids and neurotransmitters from higher centers of the brain. Synchronous activity of all these components is obligatory for the proper functioning of reproductive processes and any alteration therein may culminate into a reproductive endocrine disorder such as the polycystic ovarian syndrome.

Polycystic ovarian syndrome is the most common endocrine disorder, affecting 5-15% of women in their reproductive age (Azziz et al., 2004; Joshi et al., 2014). The characteristic features of PCOS include oligo-/anovulation, clinical or biochemical hyperandrogenemia and presence of many peripheral cysts in the ovary (Goodarzi et al., 2011). In addition to reproductive anomalies, PCOS is also associated with several metabolic complications such as hyperinsulinemia, dyslipidemia, insulin resistance, obesity and cardiovascular disorders.
Furthermore, psychological comorbidities such as anxiety, depression and mood disorders are common in PCOS women (Azziz et al., 2016). Though the disorder is prevalent and the discomfort caused due to it is very high, its etiology remains elusive. In this context, studies suggest that an altered neuroendocrine status, characterized by increased GnRH pulsatility and elevated LH/FSH ratio may underpin the pathology. However, regulation of GnRH/LH release is complex and involves crosstalk between a range of intra- and extra-ovarian factors such as neuropeptides, neurotransmitters and stress-regulators. Thereby, the present work was taken up with an aim to understand the status of various regulatory molecules of reproductive axis to decipher the neuro-endocrine pathology in PCOS, using rodent model.

As PCOS is a complex endocrinopathy involving dysfunction of multiple organ systems, logistic and ethical limitations on human patients demand the need of animal models for better understanding of pathogenesis. With this reference, the present study has used two different animal models for PCOS induction that include treatment with letrozole (a non-steroidal aromatase inhibitor) and with testosterone propionate (TP). Both the letrozole- and TP-treated rats were arrested in diestrus stage of estrus cycle, along with increased testosterone levels and presence of peripheral cysts in the ovaries which are the 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 (Belosesky et al., 2004; Kafali et al., 2004; Maharjan et al., 2010). Although TP was able to develop human PCOS like condition, being a steroid, testosterone can directly modulate various other body functions. Thus, the effects observed may not be solely due to hyperandrogenic condition, making it an unsuitable model to understand androgen-mediated mechanisms of PCOS pathology (Walters, 2015). Therefore, to decipher the underlying pathogenesis of PCOS, all further experiments were performed using letrozole-induced PCOS rat model.

As stated above, the functioning of HPO axis is altered in PCOS pathology (Blank et al., 2006; Solorzano et al., 2012). Thereby, initial studies were performed to understand the status of reproductive axis in letrozole-induced PCOS model. Data obtained clearly demonstrated increased GNRH1, LHβ and FSβ expression in PCOS condition which resulted in an increased serum LH/FSH ratio. These results can be well correlated with increased GnRH/LH release, commonly seen in PCOS women (Banaszewska et al., 2003; Azziz et al., 2016). The
release of LH and FSH stimulates steroidogenesis and follicular growth and development through binding to their receptors on the ovary. In this regard, increased transcripts of *LHR*, *StAR*, *Cyp17a1* while decreased expression of *Cyp19a1* was observed in ovaries of PCOS animals suggesting the overstimulation of steroidogenesis. This was further confirmed by elevated levels of testosterone and reduced levels of progesterone and estradiol in PCOS ovary. Similar to our results, women with PCOS have shown increased transcript and protein levels of steroidogenic enzymes (Nelson et al., 1999; 2001). The altered ovarian steroids result into development of immature follicles and cyst formation in PCOS ovaries (Jonard and Dewailly, 2004; Chang, 2007).

It is interesting to note that these ovarian steroids signal to the brain to regulate GnRH and LH release through a feedback loop (Evans et al.1994; Couse et al. 2003). However, GnRH neurons in the hypothalamus expresses only Estrogen receptor-β (ERβ) (Temple et al. 2004; Christian et al. 2008), 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 in glutamatergic and GABAergic neurons which are in vicinity of GnRH neurons (Stumpf and Jennes, 1984; Leranth et al., 1992; Smith et al., 2005; Franceschini et al., 2006; Kenealy and Terasawa, 2012). With this reference, neuropeptide- Kisspeptin and its receptor- GPR-54, Neurokinin B, Dynorphin, RFRP3 (mammalian homolog of GnIH) and its receptor-GPR147 along with insulin were studied.

In rodents, 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 (ARC) kisspeptin neurons regulate estrogen- and progesterone-mediated negative feedback that controls GnRH and LH pulsatility (Li et al., 2009). Also, ARC Kisspeptin neurons co-express NKB and Dyn and are thereby termed as KNDy neurons, which are responsible for differential effects of kisspeptin on GnRH regulation (Maeda et al., 2010). As PCOS is a disorder of altered GnRH pulsatility, we hypothesize a role for these neuropeptides in developing PCOS pathology. In support of our speculation, hypothalamic *Kiss1/Kiss1r* and *Nkb* mRNA levels were significantly increased while *Dyn* expression was markedly reduced in letrozole-induced PCOS rats. Estradiol through ERαreduces expression of *Nkb* while Progesterone receptor activation increases*Dyn*(Foradori et al., 2005; Navarro et al., 2009). In this line, reduced expression of
*Esr1* and *Pgr*in hypothalamus of PCOS is well correlated with *Nkb*, *Dyn* and *Kiss1*expression, suggesting the impairment of steroid-mediated negative feedback in PCOS condition which could result into increased GnRH pulsatility. Although steroid mediate-feedback regulation was demonstrated to be altered in prenatally androgenised PCOS mice model (Moore et al., 2013), this is the first study to have demonstrate such derangement using letrozole-induced PCOS model. Recent studies also demonstrate the role of Androgen receptor (AR) in GnRH regulation (Hu et al., 2004; Walters et al., 2007; 2009). AR 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). Thereby, an increase in AR expression both at protein and mRNA levels in the present study may lead to increase in Kiss1 expression, resulting into increased GnRH and LH release.

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 more potent inhibitor of GnRH release (Johnson et al., 2007). The present data has shown significant down-regulation of *RFRP3* and *GPR147* in hypothalamus and pituitary of PCOS rats, suggesting the reduction in the major inhibitory molecule which could increase GnRH and gonadotropin secretion as observed in our PCOS model. Furthermore, 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 tissue (Salvi et al., 2005). Interestingly, present study has demonstrated significantly increased insulin level in serum as well as in hypothalamus, pituitary and ovary of letrozole-induced PCOS rats which may further enhance the pituitary sensitivity towards GnRH, resulting into increased LH secretion.

For the first time, the present study has 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.

It should also be noted that these neuropeptides are also expressed in the ovary, where they participate in follicular development (Laoharatchatathanin et al., 2015; Oishi et al., 2012).
Present study clearly demonstrated hyperandrogenic and hyperinsulinemic condition in the PCOS rat ovary. Also, the expression of Kiss1/Kiss1r, RFRP3 and GPR147 was significantly reduced in the ovary, which was simultaneous with steroidogenesis and steroid receptor expression. This suggests that modulation of several intra-ovarian factors could result into increased steroidogenesis and premature follicular development, leading to follicular arrest and cyst formation in PCOS. Thereby, present study aptly demonstrates the role of several neuropeptides and steroids in PCOS condition which could result into increased GnRH/LH release, leading to ovarian cyst formation.

Additionally, the role of stress has been implicated in regulation of reproduction 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). Stress response in the body is executed by activation of Hypothalamic-Pituitary-Adrenal axis wherein hypothalamus secretes corticotrophin releasing hormone (CRH) that stimulates release of adrenocorticotropin (ACTH) hormone from pituitary, which in turn results into production of corticosteroids from adrenal gland. The end products of the axis regulate the axis via feedback regulation through binding to glucocorticoid receptors. Studies have postulated the role of HPA axis in patho-physiology 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. 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). 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 Urocortin2 (Ucn2) on reproduction is well known, the detailed understanding of these elements in PCOS pathology has yet to be elucidated. Hence, goal of the present study was to understand the status of the hypothalamic-pituitary-adrenal axis and its interaction with ovarian axis in PCOS condition using letrozole-induced PCOS rat model.

Results of the current study 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 Mc2r (ACTHR), increased 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 into 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 11βHSD 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 11βHSD in hippocampus of PCOS rats, suggesting the involvement of stress-related behaviour alteration in PCOS condition.

In addition to classic endocrine regulation, reproductive function is also governed by the interactions of several neurotransmitters. In situ hybridization and immune-histochemical studies have identified presence of several neurotransmitter-containing neurons in the GnRH neurons of hypothalamus, suggesting a direct influence of these neurotransmitters in GnRH release (Hrabovszky & Liposits, 2013). Furthermore, many studies implicate that neurotransmitters interact with estrogens and progesterone receptors and facilitate steroid mediated feedback regulation of HPO axis (Christian et al., 2009; Barth et al., 2015). In this context, various studies have reported low levels of norepinephrine, dopamine and serotonin in serum of PCOS women (Shi et al., 2011). Also, catecholamine levels were altered in PCOS women follicular fluid (Musali et al., 2016). Several reports also indicate that low dopaminergic tone may be responsible for increased LH pulsatility in PCOS (Kalro et al., 2001; Gomez et al., 2011). All these studies indicate a putative role for neurotransmitters in neuro-endocrine aberrations of PCOS. However, due to limitations of obtaining human samples and the related ethical issues, the detailed understanding of neurotransmitter function...
in PCOS condition is lacking. With this reference, the status of GnRH regulatory neurotransmitters in PCOS condition was evaluated.

Present study clearly demonstrated a significant reduction in Serotonin, Nor-epinephrine, Dopamine, Epinephrine and GABA; whereas Glutamate content was markedly elevated in various regions of PCOS rat brain. The alteration of these neurotransmitters correlated with their biosynthesizing and metabolizing activities and suggested that, although neurotransmitter synthesis was decreased in PCOS condition, the reduced levels of neurotransmitters is mainly due to overactivity of their degrading enzymes such as Monoamine oxidase (MAO). Serotonin, Dopamine, Acetylcholine and GABA inhibit GnRH/LH release through binding to their receptors 5HT1A, D2, M2-Muscarinic acetylcholine receptor and GABAB1 respectively. A decrease in these neurotransmitter receptor expressions was observed in PCOS rats. In contrast to these, Glutamate is the potent stimulator of GnRH release and its receptor (NMDA) expression were significantly increased in PCOS animals. Thereby, present data suggests that increased stimulation to GnRH through elevated glutamate and the loss of GnRH inhibitory signals may result into increased GnRH pulsatility in PCOS condition. This is the first study wherein we have clearly demonstrated that neurotransmitter modulation may act as a key feature for development of PCOS pathology.

The results discussed so far suggest that PCOS is state wherein hyperandrogenic and hypoestrogenic condition co-exists with increased corticosteroids, stress and altered neurotransmitters. It should be noted that these alterations increase ROS production, increasing the susceptibility oxidative stress and inflammation. Furthermore, PCOS is a metabolic syndrome having complications of hyperglycemia, hyperinsulinemia and dyslipidemia, which are predisposed to oxidative stress and inflammation. In this line, several reports have indicated decreased activity of antioxidant enzymes in serum of PCOS women (Murri et al., 2013; Piomboni et al., 2014). Also, circulatory inflammatory markers Interleukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α) and interferon-γ (IFNγ) are elevated in PCOS women (Gonzalez et al., 1999; Amato et al., 2003; Deligeoroglou et al., 2012), indicating that PCOS is a disorder of increased oxidative stress and inflammation. Although PCOS is a state of systemic inflammation, involvement of several organs and its contribution towards inflammation is not well documented. In this regard, antioxidant and inflammatory markers were studied in various organs of PCOS phenotype.
The present study clearly demonstrated reduction in antioxidant enzymes SOD, Catalase and GPx with decreased GSH levels in PCOS condition, further resulting in lipid peroxidation, protein carbonylation and caspase-3 activation (Chapter-6). Also, the expression of proinflammatory cytokines TNFα, IFNγ, IL1B, IL2 and IL6 were elevated whereas those of anti-inflammatory cytokine IL10 was decreased in PCOS animals. It is interesting to note that these changes were not only observed in the ovary but also in various areas of brain with the maximum alterations seen in hypothalamus and pituitary of PCOS animal. It is suggested that redox potential of the brain is weak, and thereby any alteration in brain may increase the susceptibility to oxidative stress (Miller et al., 2009). Data from the present study has revealed that PCOS is associated with hyperandrogenic and hypoestrogenic condition (Chapter 3). It is known that Estrogen acts as an antioxidant and low levels of Estrogen possess a risk of oxidative stress development (Bellanti et al., 2013; Unfer et al., 2015). Also, PCOS rats demonstrated increased corticosteroids and glutamate content with increased MAO activity. Although glucocorticoid has been known for anti-inflammatory effect, reports also indicate that increased glucocorticoids results into production of ROS, leading to oxidative stress. Similarly, glutamate in excess can lead to glutamate excitotoxicity whereas increased MAO activity is linked with elevated H2O2 production, both of which can lead to oxidative damage (Nagatsu & Sawada 2006; Balu et al. 2008). Thus, suggesting that imbalance of antioxidant (estradiol) and pro-oxidants (corticosteroids, glutamate & MAO) in PCOS could result into the observed oxidative stress; further causing inflammation in PCOS pathology. Additionally, reports also indicate that oxidative stress and inflammation affects various neuronal processes and imbalance of these factors may result into deleterious condition including psychological and neurodegenerative pathology. Increased oxidative stress and inflammation led to a decrease in serotonin levels with increased glutamate content as there was increased the expression of indolalmine-2,3-dioxygenase (IDO) and serotonin reuptake transporter (SERT) (Zhu et al. 2006). Furthermore, increased glutamate decreases Brain-Derived Neurotropic factor (BDNF) and its receptor TrkB expression which are the major neuro-protective agents (Dantzer et al. 2008; Koo & Duman 2008; Wu et al. 2007; Miller et al. 2009). The increased oxidative stress and inflammation in present study can be well correlated with the increased transcripts of IDO1 and SERT and decreased BDNF/TrkB expression. Also, these alterations are simultaneous with decreased serotonin content and increased glutamate and NMDA expression in present study.
Neurotransmitters are also involved in various functions including learning and memory, cognition, sleep-wake cycle, satiety and mood regulation and any alteration in their levels may precipitate into behavioural alterations. Low levels of neurotransmitters serotonin, dopamine and norepinephrine are observed in patients with major depressive disorders (MDD). Also, post-mortem studies on MDD patients have revealed decreased expression of BDNF in various regions of brain. Present data demonstrated similar pattern of alteration in brain of PCOS animals. It was therefore interesting to study any association of PCOS with depressive disorders. In this context, studies indicate increased incidence of anxiety, depression and mood disorders in PCOS women (Pasch et al., 2008; Kerchner et al., 2009; Goodarzi et al., 2011). Parallel to this, the current study demonstrated depression-like behaviour in animal model of PCOS which was evident by decreased mobility in behavioural tasks for depression and anxiety. This indicated that the altered steroid, neurotransmitter and redox status in PCOS could result into depressive condition, commonly observed in PCOS women.

Present study clearly demonstrates that PCOS is a complex disorder characterised by reproductive, metabolic and psychological aberrations, using a letrozole-induced PCOS rat model. For the first time, our work has depicted that the increased GnRH/LH release, a prominent feature of PCOS is a result of cumulative alterations of neuropeptides, neurosteroids, and neurotransmitters at several brain regions; further causing modulations of intra- and extra-ovarian factors which result into impaired follicular development and ovarian cyst formation in PCOS condition. Altogether, the observed aberrations in the PCOS brain are associated with increased oxidative stress and inflammation, leading to depression and mood disorders in PCOS pathology. Although the alterations observed in the present study are well correlated with the reproductive, neuroendocrine and psychological co-morbidities of PCOS, whether these are causes or consequences of PCOS remains obscure. In conclusion,

“PCOS is not just an ovarian dysfunction, but it is the disorder of an altered brain microenvironment”
Figure 1: HPO axis in normal and in PCOS condition

**Blue:** Stimulatory molecules; **Red:** Inhibitory molecules

**Bold:** Increased in PCOS condition; **Italics:** Decreased in PCOS condition

- 5HT: Serotonin
- DA: Dopamine
- NE: Norepinephrine
- Glut: Glutamate
- NKB: Neurokinin B
- Dyn: Dynorphin
- Kiss: Kisspeptin
- RFRP3: RF-amide related peptide 3
- E2: Estradiol
- P4: Progesterone
Figure 2: Brain micro-environment in PCOS condition

Blue: Neuroprotectants; Red: Pro-oxidants; Green: Mood regulatory neurotransmitters

↑: Increased in PCOS condition; ↓: Decreased in PCOS condition

MAO: Monoamine oxidase
BDNF: Brain-derived neurotropic factor
IDO: Indolamine2,3-dioxygenase
SERT: Serotonin reuptake transporter

5HT: Serotonin
DA: Dopamine
NE: Norepinephrine