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GnRH dysregulation in polycystic ovarian syndrome (PCOS) is a manifestation of an altered neurotransmitter profile

Nirja Chaudhari, Mitali Dawalbhakta and Laxmipriya Nampoothiri*

Abstract

Background: GnRH is the master molecule of reproduction that is influenced by several intrinsic and extrinsic factors such as neurotransmitters and neuropeptides. Any alteration in these regulatory loops may result in reproductive-endocrine dysfunction such as the polycystic ovarian syndrome (PCOS). Although low dopaminergic tone has been associated with PCOS, the role of neurotransmitters in PCOS remains unknown. The present study was therefore aimed at understanding the status of GnRH regulatory neurotransmitters to decipher the neuroendocrine pathology in PCOS.

Methods: PCOS was induced in rats by oral administration of letrozole (aromatase inhibitor). Following PCOS validation, animals were assessed for gonadotropin levels and their mRNA expression. Neurotrasnmitter status was evaluated by estimating their levels, their metabolism and their receptor expression in hypothalamus, pituitary, hippocampus and frontal cortex of PCOS rat model.

Results: We demonstrate that GnRH and LH inhibitory neurotransmitters – serotonin, dopamine, GABA and acetylcholine – are reduced while glutamate, a major stimulator of GnRH and LH release, is increased in the PCOS condition. Concomitant changes were observed for neurotransmitter metabolising enzymes and their receptors as well.

Conclusion: Our results reveal that increased GnRH and LH pulsatility in PCOS condition likely result from the cumulative effect of altered GnRH stimulatory and inhibitory neurotransmitters in hypothalamic-pituitary centre. This, we hypothesise, is responsible for the depression and anxiety-like mood disorders commonly seen in PCOS women.

Keywords: PCOS, GnRH, LH, FSH, Neurotransmitters

Background

The reproductive system is governed by the hypothalamic-pituitary-gonadal axis (HPG), wherein a pulsatile release of GnRH from the hypothalamus stimulates anterior pituitary gonadotropes to release LH and FSH, leading to steroid production from the ovaries. The regulation of HPG axis is quite complex, involving several intrinsic factors (estrogens, progesterone, inhibin, activin, etc) [1] as well as extrinsic factors (neurotransmitters, neuropeptides, stress, etc) [2]. However, any abnormality that prevents or interferes with the function of these factors may culminate into reproductive endocrine anomalies. One of the most prevalent reproductive endocrinopathies is polycystic ovarian syndrome (PCOS), affecting 6–10% of women worldwide [3]. The key features of PCOS include hyperandrogenemia, oligo–/ano-vulation and peripheral cyst formation in ovaries [4]. In addition, PCOS is a disorder underpinning neuroendocrine abnormalities, characterized by increased GnRH and LH: FSH ratio [5]. However, in spite of its widespread occurrence and profound implications, the etiology of this disease remains poorly understood.

While reduced norepinephrine, dopamine and serotonin has been reported in the serum of PCOS women [6], their levels in GnRH regulatory regions of the brain are unknown due to the obvious difficulties in obtaining these tissues from patients. A tissue-specific understanding of the neurotransmitters would help us gain an insight into the pathogenesis of PCOS. Thereby, the objective of the current study was to evaluate the status of GnRH-regulatory neurotransmitters in a PCOS rat model.

* Correspondence: lpnmsubaroda@gmail.com
Reproductive-Neuro-Endocrinology Lab, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India

BMC

Chaudhari et al. Reproductive Biology and Endocrinology (2018) 16:37

Page 2 of 13

To address the above objective, Letrozole, an aromatase inhibitor, was used to induce PCOS in rats [7]. Evaluation of the neurotransmitter levels was performed from hypothalamus, pituitary as well as from hippocampus and frontal cortex. The reasons for selecting these areas of brain mainly include i) presence of GnRHR in the described tissues, which contributes to regulation of reproduction and reproductive behaviour [8, 9]; ii) active steroidogenesis occurring in these regions [10, 11] and iii) them being important sites of neurotransmitter synthesis [12]. The rates of neurotransmitter synthesis and clearance were monitored by estimating the activities of neurotransmitter metabolizing enzymes. Gene expression analysis of specific neurotransmitter receptors that profoundly influence pulsatile release of GnRH/LH and other reproductive processes was performed in PCOS and normal rats.

Methods
Animals
Charles Foster female rats (2–3 months old) were housed in controlled conditions of temperature, humidity and light with ad libitum availability of food and water. Animals were allowed to acclimatize for one week before treatment. All experimental protocols listed herein were approved by the Institutional animal ethical committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, India and they are in accordance with the ethical standards of the Committee For the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Induction of PCOS in rats
PCOS was induced in rats by oral administration of letrozole, a non-steroidal aromatase inhibitor [7]. For PCOS induction, 100 rats were randomly assigned to two different groups ($n = 50$ per group). A daily treatment regime of 21 days included oral administration through gavage of 0.5 ml of 1% carboxymethyl cellulose (CMC) for control group and 0.5 mg/kg body weight of letrozole dissolved in CMC for PCOS group. After 21 days of treatment, body weight, oral glucose tolerance, estrus cyclicity, serum estrogen, progesterone and testosterone levels and ovarian histology profile were analysed to check for development of PCOS.

Estrus cyclicity
Estrus cyclicity was monitored daily before (for 14 days) and also during (for 21 days) the course of treatment by microscopic examination of the predominant cell type in vaginal smears [13]. Animals showing regular cycles of 4–5 days complete with the proestrus, estrus, metestrus and diestrus stages were defined as normal cyclic rats, whereas animals in which the estrus cycle was found arrested in any one of the stages for 4 consecutive days were termed as acyclic rats.

Oral glucose tolerance test (OGTT)
OGTT was performed according to the method of Buchanan et al. [14], wherein 12 h fasting blood plasma was collected from orbital sinus into vials containing sodium fluoride and EDTA, followed by oral administration of glucose at 1 g/kg body weight. The blood was then collected every 30 min for 2 h and plasma glucose levels were estimated using Glucose oxidase-peroxidase (GOD-POD) kit (Reckon diagnostics, Vadodara, India).

Blood and tissue collection
Blood and tissue collection was performed during diestrus stage of estrus cycle and between 8 and 9 AM in the morning. Blood was withdrawn through orbital sinus in a tube and centrifuged at 5000 g for 15 min at room temperature. Supernatant containing serum was separated and immediately stored at – 80 °C. Following blood collection, animals were euthanized by cervical dislocation. Pituitary, hypothalamus, hippocampus and frontal cortex were dissected out and stored at – 80 °C.

Hormone profile
For estimation of hormones, commercially available ELISA kits were used (17β Estradiol ELISA kit-DKO003; Testosterone ELISA kit-DKO002 and Insulin ELISA kit-DKO076; Diametra. Italy). Progesterone was measured through ELISA kit (CAN-P-35) from Diagnostics Biochem Canada Inc., Canada. FSH and LH were estimated in serum using ELISA kits (rat FSH ELISA kit-E-EL-R0391; rat LH ELISA kit-E-EL-R0026) from Elabscience Biotechnology Co., Ltd., USA. Assays were performed according to manufacturers' protocols. Sensitivity of methods are 8.68 pg/ml (17β Estradiol), 0.01 ng/ml (Testosterone), 0.1 ng/ml (Progesterone), 0.025 μIU/ml (Insulin), 0.38 ng/ml (FSH) and 0.19 ng/ml (LH) at 95% confidence limit.

Histology
Ovaries of 6 different animals from each group were removed and fixed in Bouin's fixative. For histological examination, 5 μm thick sections were prepared and stained with Hematoxylin-Eosin and histo-anatomical changes were observed under light microscope [15].

Neurotransmitter estimation
Neurotransmitters were estimated using reverse phase HPLC coupled with electrochemical detector (Waters 2465; Waters corporation, Milford, USA) [16]. Tissues were homogenized in 0.17 M perchloric acid, centrifuged at 12000 g for 20 min at 4 °C and supernatant was immediately used for neurotransmitter estimation or kept at – 80 °C until use. For HPLC analysis, tissue samples as well as neurotransmitter standards were mixed with derivatization reagent (37 mM orthopthalaldehyde, 50 mM sodium sulfite, 90 mM tetraborate buffer-pH 10.4 and

5% methanol) for 10 min and 20 μl of sample was injected in HPLC. Glutamate and GABA were separated using a Sunfire® C18 column containing 0.1 M monosodium phosphate, 0.5 mM EDTA and 25% (*v*/v) methanol (pH 4.5) as mobile phase. For separation of norepinephrine, dopamine and serotonin, mobile phase containing a solution (pH 4.2) of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 0.5 mM octyl sodium sulfate, 0.5 mM EDTA, 2 mM KCl and 15% (v/v) methanol was used. Standard curves were used to quantify the amount of neurotransmitter in each sample by calculating area under the curve (AUC).

Epinephrine estimation
For epinephrine estimation, a colorimetric method was used [17]. Tissues were homogenized in 10% trichloroacetic acid followed by centrifugation at 10000 g for 10 min at 4 °C. Supernatant (0.2 ml) was added to a tube containing 0.25 ml of 10% (*w*/v) sodium carbonate and incubated for 30 min at room temperature followed by addition of 0.125 ml of Folin's reagent and 0.375 ml of 5% (w/v) NaOH. Absorbance of epinephrine was recorded at 486 nm within 90 s of incubation.

5-Hydroxy tryptophan decarboxylase (TDC)
TDC was measured spectroflourimetrically as described by Sangwan and group [18]. Tissue homogenates were prepared in 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM thiourea, 1 mM EDTA and 5 mM β-mercaptoethanol. Tubes containing homogenates were centrifuged at 10000 g for 10 min at 4 °C, supernatant was separated and used as an enzyme source. For TDC assay, 0.1 ml of homogenate was added to a tube containing 0.7 ml of assay buffer (0.1 M sodium phosphate buffer-pH 8.5, 10% glycerol and 5 mM β-mercaptoethanol), 0.1 ml of 10 mM 5-hydroxytryptophan and 0.1 ml of 10 mM pyridoxal phosphate (PLP). The solutions were mixed properly and incubated at 37 °C for 40 min, followed by termination of enzyme reaction by adding 2 ml of 4 N NaOH. Serotonin formed was extracted by adding 3.5 ml of ethyl acetate followed by centrifugation at 1000 g for 10 min. Fluorescence measurement of upper organic layer was taken at 350 nm with prior excitation at 280 nm using a Hitachi F-7000 fluorescence spectrophotometer.

GABA-transaminase (GABAT)
GABAT was estimated by kinetic method using spectrophotometer [19]. Tissues were homogenized in phosphate buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) and centrifuged at 12,000 g for 15 min at 4 °C. The pellet was resuspended in phosphate buffer and was used for enzyme assay. Tissue homogenates (50 μl) were incubated with 1 ml of 100 mM potassium pyrophosphate buffer (pH 8.6) containing 5 mM α-ketoglutarate, 4 mM NAD, 3.5 mM β-mercaptoethanol and 10 μM pyridoxal

phosphate for 15 min at 37 °C. The absorbance of blank was measured at 340 nm followed by addition of 0.1 ml of 100 mM GABA. The absorbance was immediately monitored at 340 nm for every 10 s for 2 min.

Glutamic acid decarboxylase (GAD)
GABA formed by the enzyme GAD was estimated using spectroflourimetry method [20]. Tissues were homogenized in 0.15 M KCl containing 5 mM EDTA and 0.5% triton-X, incubated for 30 min on ice and centrifuged at 3000 g for 10 min. Supernatant (0.1 ml) was incubated with a solution containing 80 mM potassium phosphate buffer (pH 6.2), 25 mM sodium glutamate and 0.5 mM pyridoxal phosphate for 30 min at 37 °C. The reaction of GAD was terminated by addition of 0.5 ml of 15% TCA followed by centrifugation at 5000 g for 10 min. GABA containing supernatant was mixed with 0.5 ml of 14 mM ninhydrin solution and the tubes were kept in a water-bath set at 60 °C for 30 min. The samples were incubated with 5 ml of copper tartarate reagent (160 mg sodium bicarbonate, 30 mg copper sulphate and 33 mg tartaric acid dissolved in 100 ml of distilled water) for 15 min. The fluorescence emission was measured at 451 nm with prior excitation at 377 nm using a Hitachi F-7000 fluorescence spectrophotometer.

Monoamine oxidase (MAOA & MAOB)
Spectrophotometric method was employed for estimation of MAOA and MAOB activity [21]. Tissue homogenates were prepared in homogenate buffer (0.25 M sucrose, 20 mM EDTA and 0.1 M tris- pH 7.4) and cell debris was removed by centrifugation at 800 g for 10 min at 4 °C. The supernatant was centrifuged at 12000 g for 20 min at 4 °C and resultant pellet was dissolved in 0.01 M sodium phosphate buffer (pH 7.4) containing 320 mM sucrose by keeping in ice for 20 min. The tubes were again centrifuged at 3000 g for 10 min at 4 °C and supernatant obtained was used as enzyme source. The assay mixture for MAOA included 0.1 M sodium phosphate buffer (pH 7.4) containing 0.4 mM 5-hydroxytryptamine whereas for MAOB 10 mM of benzylamine was used as a substrate. The reaction was terminated by addition of 0.2 ml of 1 M HCl after 20 min of incubation at 37 °C. Product formed was extracted by vortexing for 5 min with 2 ml of butyl acetate for MAOA or cyclohexane for MAOB. Tubes were centrifuged at 3000g for 5 min and upper organic layer was measured at wavelength of 280 nm for MAO-A activity and 242 nm for MAO-B activity with spectrophotometer, respectively.

Glutamate dehydrogenase (GDH)
GDH activity was measured in the direction of oxidative deamination of glutamate into α-ketoglutarate [22]. Tissues were homogenized in 10 volume of 0.25 M sucrose-10 mM HEPES, pH -7.4 and centrifuged at 1000 g for

Chaudhari *et al. Reproductive Biology and Endocrinology* (2018) 16:37

Page 4 of 13

10 min at 4 °C. The supernatant was collected in a fresh vial and centrifuged at 12000 g for 30 min at 4 °C to yield mitochondrial pellet which was dissolved in homogenate buffer and used as enzyme source. The enzyme (0.05 ml) was added to assay mixture (50 mM tris buffer-pH 9.5, 1 M glutamate, 0.1 M EDTA, 56 mM NAD and 40 mM ADP) and absorbance was monitored immediately at 340 nm for every 10 s for 1 min.

Acetylcholine esterase (AChE)

Acetylcholine is degraded by enzyme AChE, which was estimated by kinetic method [23]. Tissue homogenates were prepared in 0.1 M sodium phosphate buffer (pH 8.0), centrifuged at 12000 g for 5 min at 4 °C and resulting supernatant was used as enzyme source. Tubes containing 1.5 ml of 0.1 M phosphate buffer (pH 8.0), 0.01 ml of freshly prepared substrate (75 mM acetylcholine iodide in distilled water) and 0.05 ml of freshly made Ellman's reagent (10 mM DTNB and 17.85 mM NaHCO$_3$ dissolved in 0.1 M sodium phosphate buffer – pH 7.0) were incubated at 25 °C. Absorbance of blank was measured at 405 nm followed by addition of 0.2 ml of enzyme. The change in absorbance was monitored thereafter for 10 min at every 2 min-interval.

RNA isolation and PCR

Total RNA was extracted using TRIsoln reagent (GeNei, India) and 2 μg of RNA was reverse-transcribed using Verso cDNA synthesis kit with Oligo-dT primers (ThermoScientific, USA). Real-time quantitative polymerase chain reaction (qPCR) was performed using Quantstudio Real Time PCR system (Life Technologies, USA). Primers were procured from IDT (CA, USA) and their sequences are given in Table 1. All the samples were run in triplicate and accompanied by a non-template control. PCR was performed with SYBR select PCR Master Mix (Applied Biosystems, USA) according to manufacturer's protocol. Thermal cycling conditions included initial denaturation in one cycle of 15 min at 95 °C, followed by 45 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. The fold changes in the mRNA were calculated for each sample group using the $2^{-\Delta\Delta CT}$ method [24]. Fold changes in expression of less than 0.5 and greater than 2 were considered biologically significant.

Statistical analysis

Statistical analysis was performed using Student's t-test and Two-way ANOVA, followed by Bonferroni *post-hoc* test using GraphPad Prism 5 software. P values of < 0.05 were deemed to be statistically significant.

Table 1 Primer sequences of rat targeted genes

Targeted Genes	Primer sequence	Accession number
GnRH1	F: 5′ CCGCTGTTGTTCTGTTGACTG 3′ R: 5′ TCACACTCGGATGTTGTGGA 3′	NM_012767
GnRHR	F: 5′ TCTGCAATGCCAAAATCATC 3′ R: 5′ GTAGGGAGTCCAGCAGATGAC 3′	NM_031038.3
FSHβ	F: 5′ AGGAAGAGTGCCGTTTCTGC 3′ R: 5′ GCTGTCACTATCACACTTGC 3′	NM_001007597.2
LHβ	F: 5′ CTGTCCTAGCATGGTTCGAGT 3′ R: 5′ AGTTAGTGGGTGGGCATCAG 3′	NM_012858.2
TH	F: 5′ CATTGGACTTGCATCTCTGG 3′ R: 5′ GTTCCTGAGCTTGTCCTTGG 3′	NM_012740.3
COMT	F: 5′ GACGCGAAAGGCCAAATCAT 3′ R: 5′ ACGTTGTCAGCTAGGAGCA 3′	NM_012531.2
5HT1A	F:5′CCCCCCAAGAAGAGCCTGAA3′ R:5′GGCAGCCAGCAGAGGATGAA3′	NM_012585.1
α1AR	F: 5′ ACCAGCTCCGGTGAACATTT 3′ R: 5′ GCCGCCCAGATATTGCAGAA 3′	NM_017191.2
D2R	F:5′ TGAACCTGTGTGCCATCAGCA 3′ R:5′ TTGGCTCTGAAAGCTCGACTG 3′	NM_012547.1
GABAB1	F:5′CGCTACCATCCAACAGACCA3′ R:5′TGTCAGCATACCACCCGATG3′	NM_031028.3
M2-AchR	F:5′CACAGTTTCCACTTCGCTGG 3′ R:5′ CACCTTTTTGGGCCTTGGTG 3′	NM_031016.1
NMDAR	F:5′ ACACCGACCAAGAAGCCATC 3′ R:5′ GGACTCATCCTTATCCGCCA 3	NM_012574.1
β-Actin	F: 5′ AGGCCCCTCTGAACCCTAAG 3′ R: 5′ GGAGCGCGTAACCCTCATAG 3′	NM_031144.3

Results

Induction of PCOS in rats

Testosterone levels were significantly elevated in serum of letrozole treated animals ($P < 0.001$) with a decrease in progesterone levels ($P < 0.05$) and no change in serum estradiol levels (Table 2). Figure 1 demonstrates the ovarian histology. Control sections showed follicles in various stages of development (Fig. 1a) whereas treatment group sections had numerous peripheral cysts along with low number of corpus lutea (Fig. 1b, c and d). Treatment group also had a high percentage of acyclic rats (disturbed estrus cycle) (Fig. 1e); mainly arrested in diestrus stage. Further, there was a significant increase in the body weight (Fig. 1f) ($P < 0.01$), glucose intolerance (Fig. 1g), area under the curve for glucose (Fig. 1h), serum insulin level ($P < .001$) and HOMA-IR index (Table 2) of letrozole treated group as compared to the CMC control group. All the animals of letrozole-treated group exhibited hormonal and structural alterations and were considered as PCOS positive rats for further experiments.

Gonadotropin status in PCOS

GnRH1 plays a pivotal role in stimulating pituitary release of FSH and LH. When analysed for gene expression (Fig. 2a) , PCOS rats demonstrated significantly increased transcripts of hypothalamic *GnRH1* ($P < 0.01$) and pituitary *GnRHR* ($P < 0.01$), while hypothalamic *GnRHR* expression was reduced ($P < 0.001$) as compared to control rats. GnRH released from the hypothalamus stimulates gonadotropin secretion from anterior pituitary. Gonadotropin estimations revealed no change in FSH levels among both the groups while a significant increase was observed in LH levels of PCOS rats ($P < 0.001$), leading to an elevated LH:FSH ratio (Table 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 (Fig. 2b).

Neurotransmitter levels in PCOS rats

GnRH and LH release are mainly influenced by various neurotransmitters secreted by discrete brain regions. When estimated, serotonin levels (Fig. 3a) were significantly reduced in all the tissues analysed with greatest decrease in hypothalamus ($P < 0.001$) and pituitary ($P < 0.001$) of PCOS group rats as compared to control. A similar trend was observed for norepinephrine (Fig. 3b) content. Epinephrine

levels (Fig. 3c) were also decreased in hypothalamus ($P < 0.01$) and pituitary (P < 0.01) of PCOS animals; however no difference was observed in hippocampus and frontal cortex. Furthermore, notably low levels of dopamine (Fig. 3d) and GABA (Fig. 3e) were also seen in all tested brain tissues of PCOS animals as compared to control rats. In contrast to all these neurotransmitters, the amount of glutamate was significantly elevated (Fig. 3f) in hypothalamus ($P < 0.001$), pituitary ($P < 0.001$), hippocampus ($P < 0.01$) and frontal cortex ($P < 0.01$) of PCOS rats as compared to control.

Neurotransmitter synthesizing enzymes in PCOS rats

The turnover of neurotransmitters is tightly regulated by the activities of their metabolising enzymes. Serotonin synthesizing enzyme Tryptophan decarboxylase (TDC) was reduced in all analysed brain tissues of PCOS animals ($P < 0.01$), except in the hippocampus (Fig. 4a). GABA-T, which acts as a synthesizing enzyme for glutamate and degrading enzyme for GABA, showed heightened activity in hypothalamus (P < 0.01), pituitary (P < 0.01), hippocampus ($P < 0.05$) and frontal cortex (P < 0.05) of PCOS rats as compared to control animals (Fig. 4b). Glutamic acid decarboxylase (GAD) enzyme catalyses the conversion of glutamate into GABA. GAD activity exhibited notable decrease in hypothalamus (P < 0.01) and pituitary (P < 0.01) of PCOS rats but no change was observed in other tissues (Fig. 4c). Furthermore, gene expression of tyrosine hydroxylase *(TH)*, a rate limiting enzyme for all catecholamine synthesis, was reduced in hypothalamus ($P < 0.01$) and pituitary ($P < 0.05$) of PCOS rats as compared to control (Fig. 4d).

### Neurotransmitter degrading enzymes in PCOS rats

Neurotransmitters are quickly metabolized through degrading enzymes secreted into the synaptic cleft. Serotonin, norepinephrine, dopamine and epinephrine are metabolized by monoamine oxidase (MAO). When analysed for activity, a significant increase in MAO-A was observed in hypothalamus ($P < 0.001$), pituitary ($P < 0.001$), hippocampus ($P < 0.01$) and frontal cortex ($P < 0.01$) of PCOS rats as compared to control animals (Fig. 5a). MAO-B also followed a similar trend in all the tissues but it was less obvious as compared to MAO-A activity (Fig. 5b). Another major enzyme which degrades catecholamines dopamine, norepinephrine and epinephrine is Catechol-*O*-methyl transferase (COMT). The transcript level of *COMT* was markedly elevated in hypothalamus ($P < 0.01$) and pituitary ($P < 0.05$) of PCOS rats while no change was observed in

**Table 2** Serum hormone profile after 21 days of letrozole treatment

	Estradiol (pg/ml)	Testosterone (ng/ml)	Progesterone (ng/ml)	Insulin(μIU/ml)	HOMA-IR
Control	132.3 ± 17.37	0.315 ± 0.05	12.25 ± 0.54	7.42 ± 1.04	1.56 ± 0.14
Let-treated	139.3 ± 19.10	1.177 ± 0.07***	9.95 ± 0.39*	15.54 ± 1.27***	4.40 ± 0.21***

All values are presented as Mean ± SEM; $n = 6$ per group; *$P < 0.05$; ***$P < 0.001$ as compared to control

$$HOMA - IR = \frac{[Glucose\ (mg/dl)\ \times\ Insulin\ (\mu IU/ml)]}{405}$$
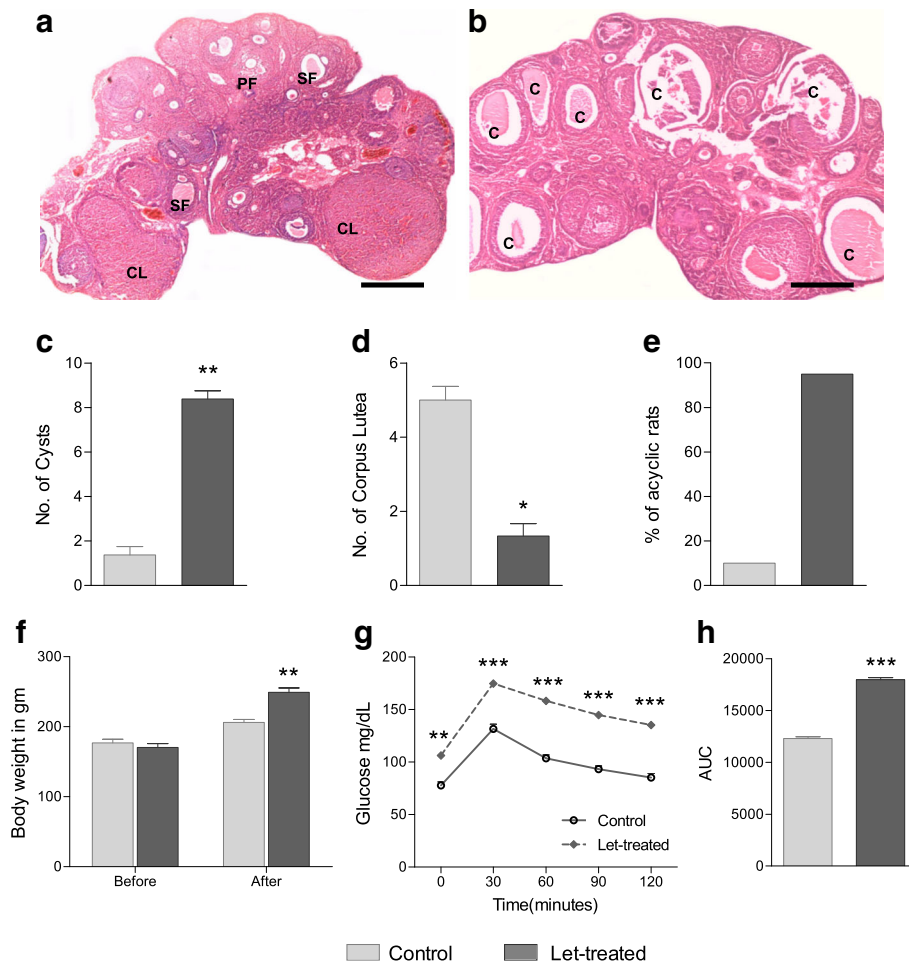
Fig. 1 Validation of PCOS rat model after 21 days of letrozole treatment. Hematoxylin-Eosin stained ovarian sections of Control group (**a**) and letrozole treatment group (**b**) under 4X magnification (Scale bar = 500μm). PF: Primary follicle; SF: secondary follicle; CL: Corpus luteum; C: Cyst. **c** Number of cystic follicles, **d** number of CL, and **e** percent of females that were acyclic after letrozole treatment. Change in body weight (**f**); Oral glucose tolerance test profile (**g**) and area under the curve (AUC) for glucose tolerance (**h**) in control and treated rats. Error bars represent SEM; n = 6–10 per group; **$P < 0.01$; ***$P < 0.001$ as compared to control group
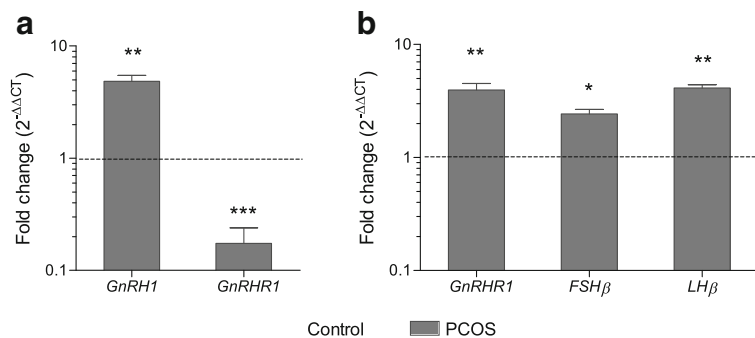
Fig. 2 Gonadotropin status in letrozole-induced PCOS animals. **a** Expression profile of hypothalamic *GnRH1, GnRHR1*, and **b** pituitary *GnRHR1, FSHβ* and *LHβ*. *β-Actin* was used as internal control and fold change in expression was calculated by $2^{-\Delta\Delta CT}$ method. Values are mean fold change in gene expression of PCOS group samples as compared to control samples (represented by black dashed line). Error bars represent SEM; n = 6 per group; **$P < 0.01$; ***$P < 0.001$ as compared to control group

Chaudhari *et al. Reproductive Biology and Endocrinology* (2018) 16:37

Page 7 of 13

Table 3 Serum Gonadotropin levels in letrozole induced PCOS rat model

	FSH (ng/ml)	LH (ng/ml)	LH: FSH
Control	2.20 ± 0.099	2.40 ± 0.138	1.08: 1
PCOS	2.29 ± 0.180	6.76 ± 0.132 ***	2.97: 1***

All values are presented as Mean ± SEM; n = 6 per group; ***P < 0.001 as compared to control values

other tissues (Fig. 5c). In addition, metabolizing enzymes glutamate dehydrogenase (GDH) and acetylcholine esterase (AChE), which degrade glutamate and acetylcholine respectively, were also assessed. Acetylcholine esterase activity (Fig. 5d) was also higher in PCOS animals as evident in hypothalamus (P < 0.01), pituitary (P < 0.01) and hippocampus (P < 0.05). In contrast to other metabolizing enzymes activity, that is increased in PCOS animals, GDH activity (Fig. 5e) was significantly low in hypothalamus (P < 0.01), pituitary (P < 0.01), hippocampus (P < 0.05) and frontal cortex as compared to control tissues.

Neurotransmitter receptor profile in PCOS rats

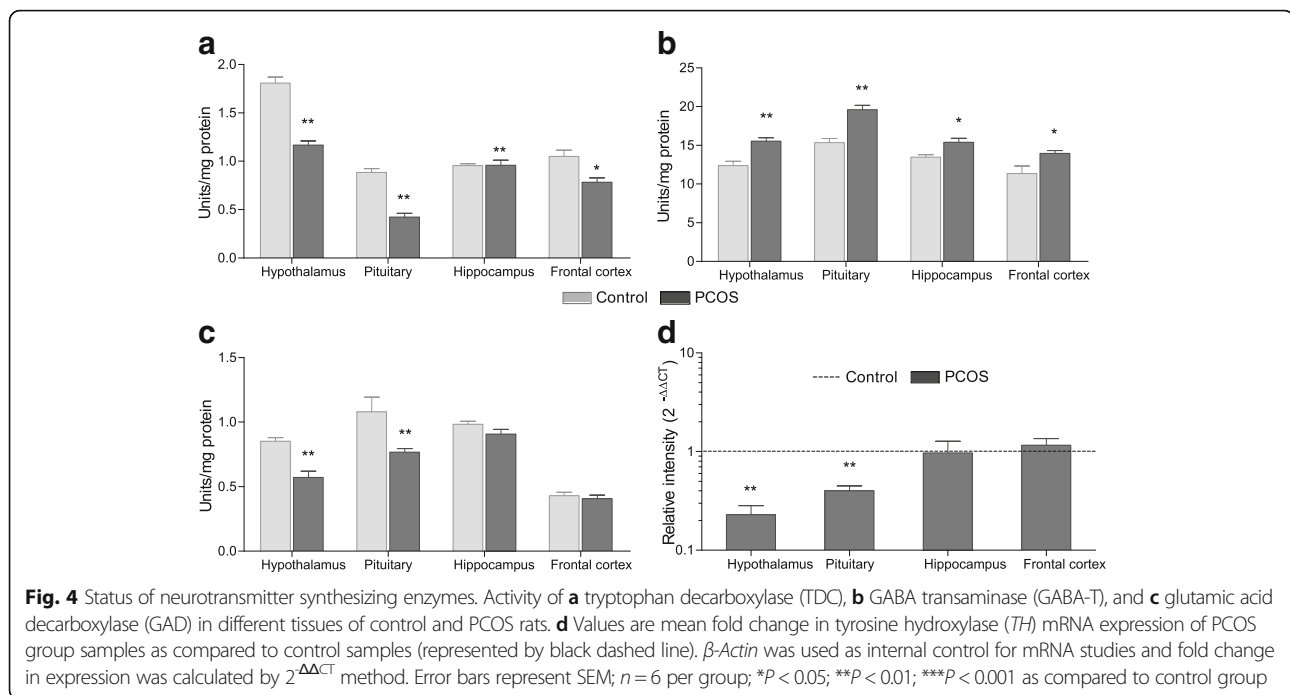Neurotransmitter receptors expressed on pre- or postsynaptic neurons relay their biological action. An mRNA expression profile of various neurotransmitter receptors was thus generated using quantitative real-time PCR. Transcript levels for serotonin receptor *5HT1A*, adrenergic alpha1 receptor *(α1AR)*, dopamine D2 receptor *(D2R)* and *GABAB1* receptor declined significantly in all tissues tested in PCOS rats as compared to control (Fig. 6a-d). Transcriptional down-regulation of muscarinic acetylcholine 2 receptor *(M2AchR)* receptor (Fig. 6e) was also observed in hypothalamus (P < 0.05) and pituitary (P < 0.05) of PCOS animals. Contrary to all these and in line with glutamate content, *NMDA* receptor expression (Fig. 6f) was found markedly high in hypothalamus (P < 0.01), pituitary (P < 0.001), hippocampus (P < 0.05) and frontal cortex (P < 0.05) of PCOS animals when compared with control tissues.

Discussion

PCOS is a very common endocrine disorder in women of reproductive age and is characterized by increased androgen production and abnormal gonadotropin secretion, resulting in chronic anovulation. To understand the etiopathology of PCOS, the present study employed a letrozole-treated rat model, which exhibits hormonal,
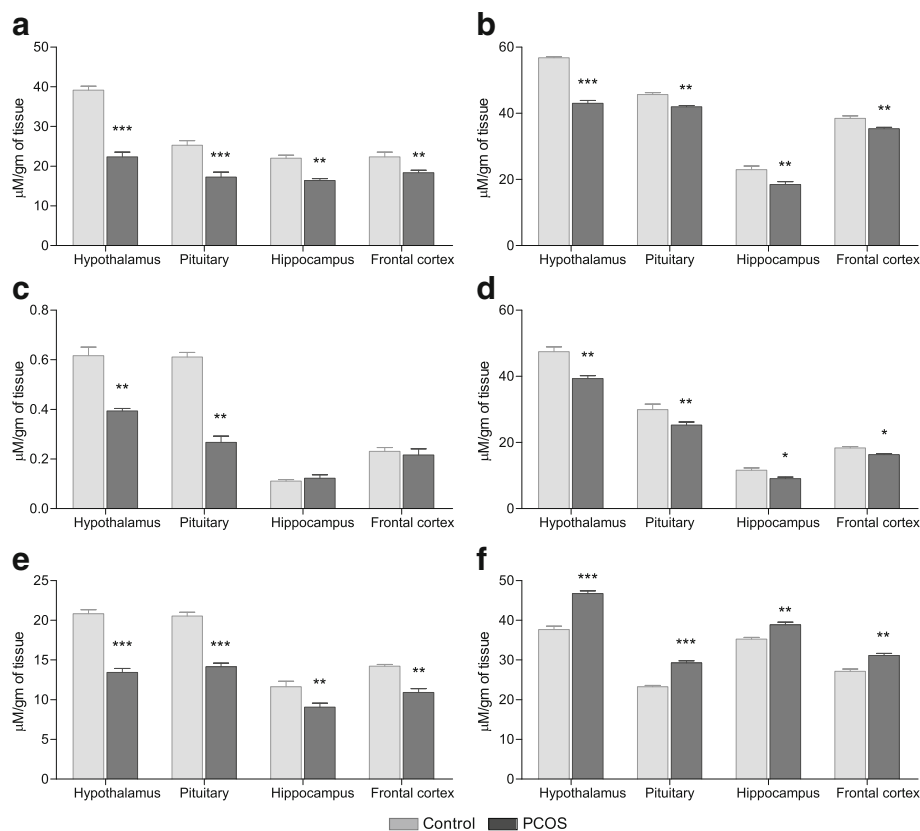
Fig. 3 Neurotransmitter levels in control and PCOS animals. **a** Serotonin, **b** norepinephrine, **c** epinephrine, **d** dopamine, **e** GABA and **f** glutamate levels in different tissues of control and PCOS rats. All values are represented as Mean ± SEM; n = 6 per group; *P < 0.05; **P < 0.01; ***P < 0.001 as compared to control group

Fig. 4 Status of neurotransmitter synthesizing enzymes. Activity of **a** tryptophan decarboxylase (TDC), **b** GABA transaminase (GABA-T), and **c** glutamic acid decarboxylase (GAD) in different tissues of control and PCOS rats. **d** Values are mean fold change in tyrosine hydroxylase (*TH*) mRNA expression of PCOS group samples as compared to control samples (represented by black dashed line). *β-Actin* was used as internal control for mRNA studies and fold change in expression was calculated by $2^{-\Delta\Delta CT}$ method. Error bars represent SEM; $n = 6$ per group; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ as compared to control group

reproductive and metabolic signs similar to the human PCOS condition [7, 25, 26]. Furthermore, it was observed currently that the PCOS rats had a high serum LH:FSH ratio, a characteristic feature of PCOS. This, we believe, must stem from increased transcription of the *GnRH* in the hypothalamus and *GnRHR* in the pituitary, as also observed by Kauffman and group [26]. Thus, letrozole-induced PCOS rat model possesses similar neuroendocrine traits as seen in PCOS women, making it a favourable model for use in PCOS research.
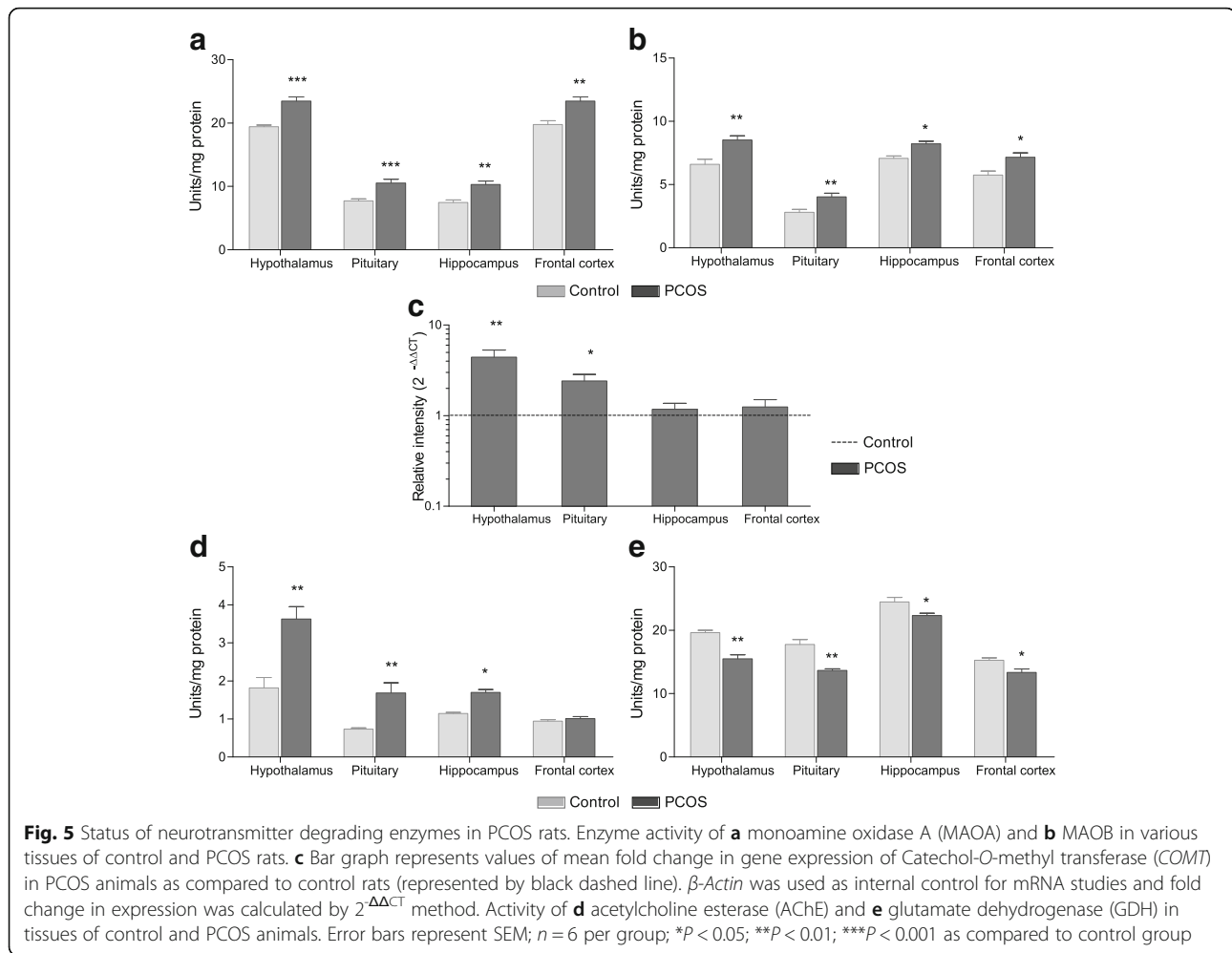
A number of studies using dual-label immunohisto-chemistry and in situ hybridization have shown that several neurotransmitter and neuropeptide receptors are expressed in GnRH neurons and they directly regulate GnRH, LH and FSH release [2]. The effect of serotonin on GnRH neurons is biphasic in nature wherein activation of 5-HT2A receptor increases GnRH neuronal activity via PKC (Protein kinase C) pathway, while activation of 5-HT1A receptor suppresses GnRH neuronal firing through adenylate cyclase [27, 28]. Serotonin content was found significantly reduced in all brain tissues of PCOS animals as compared to control, which can be well correlated with the observed decrease in TDC activity (serotonin synthesis) and heightened MAO activity. Also, the expression of 5HT1A receptor was decreased in PCOS animals. Based on this, and the above-cited references, increased GnRH and LH release in PCOS may result, at least partially, from reduced inhibition of GnRH by serotonin.

In addition to serotonin, the role of catecholamines is also known in GnRH regulation. Norepinephrine has been shown to rapidly increase GnRH mRNA levels in

ovariectomised rats [29]. It is also responsible for the pre-ovulatory LH surge through the α- and β-adrenergic receptors. Propranolol, an α-adrenergic receptor blocker stimulates NE-induced LH release, while treatment of β-antagonist blocked the release of pre-ovulatory LH surge [30, 31]. This indicates that the stimulatory effect of norepinephrine on LH release is mediated by β-adrenergic receptors while α-adrenergic receptor inhibits LH release. Moreover, α-adrenergic receptor is also involved in steroid mediated feedback regulation of GnRH [32]. In the case of epinephrine, although reports on GnRH regulation are rare, a positive relation is implied, where the former stimulates release of GnRH and LH, also through the α-adrenergic receptor [33, 34]. Whereas both epinephrine and norepinephrine were reduced in the brain of PCOS rats, the GnRH and LH were still elevated, pointing towards the involvement of other regulatory factors in this outcome.

Unlike norepinephrine and epinephrine, dopamine is a major suppressor for GnRH release [35]. It also inhibited the firing and anteroventral paraventricular (AVPV)-evoked GABA/glutamate postsynaptic currents in the GnRH neurons in vitro mediated by D1 and D2-like receptors in male and female mice [35]. Recent study in ewes also suggests that D2 dopamine receptor not only affects the GnRH release but also GnRH and GnRHR gene expression in hypothalamus. Also, LH pulse frequency increases upon local injection of Sulpride (D2R antagonist) in ewes, reflecting the potency of D2R in inhibiting GnRH and LH pulsatility [36]. In addition to its influence on GnRH/LH, it has an inhibitory effect on prolactin release. A positive association between PCOS, low dopamine and hyperprolactinemia has

Chaudhari *et al. Reproductive Biology and Endocrinology* (2018) 16:37

Page 9 of 13

Fig. 5 Status of neurotransmitter degrading enzymes in PCOS rats. Enzyme activity of **a** monoamine oxidase A (MAOA) and **b** MAOB in various tissues of control and PCOS rats. **c** Bar graph represents values of mean fold change in gene expression of Catechol-*O*-methyl transferase (*COMT*) in PCOS animals as compared to control rats (represented by black dashed line). *β-Actin* was used as internal control for mRNA studies and fold change in expression was calculated by $2^{-\Delta\Delta CT}$ method. Activity of **d** acetylcholine esterase (AChE) and **e** glutamate dehydrogenase (GDH) in tissues of control and PCOS animals. Error bars represent SEM; $n = 6$ per group; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ as compared to control group

been suggested [37]. Further, hyperprolactinemia exerts an inhibitory effect on the gonadotrophs [38]. In the present case, dopamine content in brain of PCOS rats was significantly decreased along with reduced expression of *D2R*, which may result into hypersecretion of prolactin in PCOS condition. Supporting our data, many studies suggest the role of reduced dopaminergic tone in increased LH release in PCOS [39, 40]. Additionally, treatment with bromocriptine, a D2 receptor agonist, can restore normal menstrual cycle and ovulation in PCOS women [39].

GABA is the major inhibitory neuron of the central nervous system. GABAB1 knockout mice demonstrated abnormal estrus cyclicity and reduced fecundity with significantly increased GnRH release as well as GnRH pulse frequency [41], whereas GABAA knockdown mice had normal estrus cycle and puberty onset [42]. In addition, treatment of GABA or muscimol, a GABAA/C receptor agonist, to cultured anterior pituitary cells results into increased secretion of LH through Ca2+ release [43]. However, when cultured pituitary cells were incubated with baclofen, a GABAB agonist, GnRH-induced LH

release was inhibited while basal LH secretion did not change [43, 44]. This suggests that GABAA/C stimulate basal LH secretion whereas GABAB suppresses GnRH-induced LH release. Reduced signalling of GABA through GABAB1 observed in our system may have contributed to an increased GnRH/LH pulse. Interestingly, a study in prenatally androgenised mouse model of PCOS demonstrated increased GABA innervations to GnRH neurons [45]. This disparity in the result is likely due to the fact that Moore and group have used arcuate nucleus for their study while we have used whole hypothalamus, which includes many such nuclei. Also, this group has used prenatally androgenised mouse model of PCOS [45] and in utero androgen exposure can lead to epigenetic changes, which could result in developmental alterations in neural circuits.

In contrast to GABA, Glutamate is the major excitatory neurotransmitter for GnRH release. GnRH neurons express both ionotropic glutamate receptors (AMPA, Kainate and NMDA) and metabotropic glutamate receptors. However, reports describing the role of metabotropic

Chaudhari *et al. Reproductive Biology and Endocrinology* (2018) 16:37

Page 10 of 13
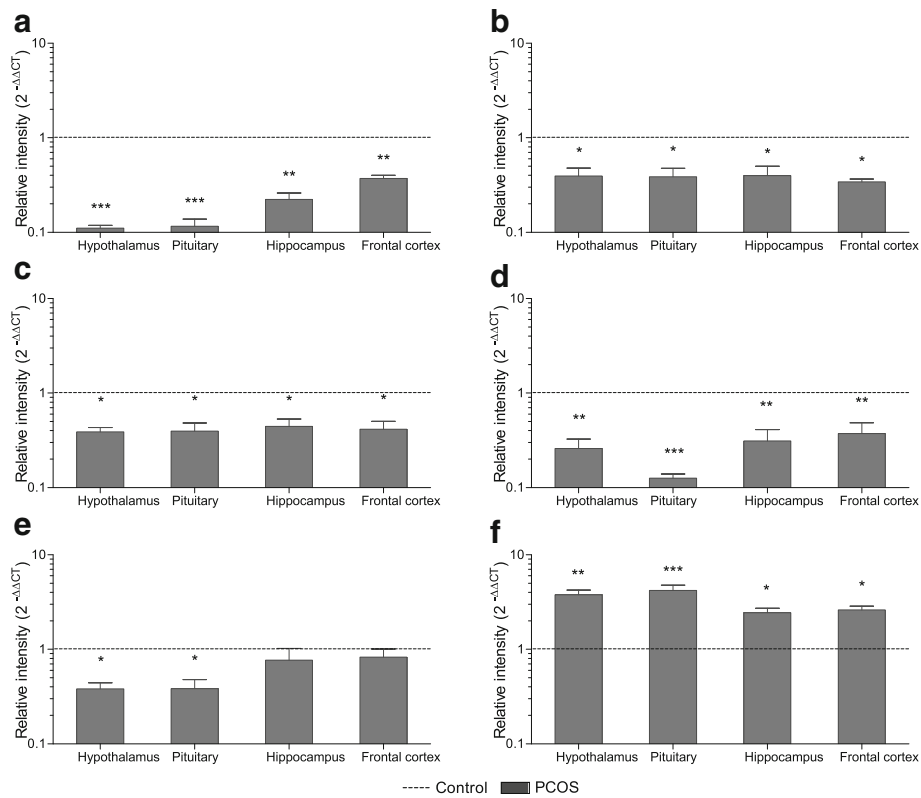
Fig. 6 Transcript analysis of neurotransmitter receptor in PCOS rats. mRNA expression profile of **a** serotonin 5HT1A receptor (*5HT$_{1A}$*); **b** alpha1-adrenergic receptor (*α$_1$AR*); **c** dopamine D$_2$ receptor (*D$_2$R*); **d** GABA B1 receptor (*GABA$_{B1}$*); **e** muscarinic acetylcholine 2 receptor (*M$_2$AchR*) and **f** NMDA glutamate receptor (*NMDA*) in control and PCOS brain tissues. Bar graph represents values of mean fold change in gene expression of PCOS animals as compared to control rats (represented by black dashed line). *β-Actin* was used as internal control for mRNA studies and fold change in expression was calculated by $2^{-\Delta\Delta CT}$ method. Error bars represent SEM; $n = 6$ per group; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ as compared to control group

glutamate receptors in GnRH regulation are scanty [46]. NMDA receptor antagonist-MK801 abolished endogenous pulses of GnRH secretion whereas pulsatile release of GnRH was not affected in the presence of 6,7-dinitroquinoxaline-2,3-dione (kainate receptor blocker) [47]. In addition, mRNA and protein expression study has revealed the presence of vesicular glutamate transporter in gonadotrophs of anterior pituitary and a stimulatory role for glutamate in LH release was also documented [48, 49]. In PNA-induced PCOS mouse model, no effect of glutamate was observed in GnRH pulsatility [45]. However, significantly high glutamate levels and NMDA receptor expression in PCOS animals were observed in the current study, suggesting direct overstimulation of GnRH and LH release. Further, the activities of GAD and GDH were significantly decreased in PCOS rats while that of GABA-T was markedly elevated suggesting that in PCOS condition the flux of reaction is towards the glutamate and not towards GABA.

Along with all the above-stated neurotransmitters, the role of acetylcholine in GnRH regulation is also emerging. In cultured GT1–7 cell line, acetylcholine stimulates GnRH release through activation of nicotinic receptor whilst an inhibitory effect of acetylcholine on GnRH activity was mediated by muscarinic receptor activation [50]. Also, in GT1–7 cells, acetylcholine treatment activates M2 muscarinic receptor that further reduces forskolin-induced cAMP production followed by suppression of GnRH release [51]. Similarly, treatment of exogenous acetylcholine to cultured anterior pituitary cells resulted in decreased response of GnRH-induced LH release. This response was counteracted by muscarinic receptor antagonist atropine [52]. Currently, activity of acetylcholine esterase (AChE), a hydrolytic enzyme of acetylcholine, was found elevated in the hypothalamus and pituitary of PCOS rats along with decreased expression of M2 muscarinic acetylcholine receptor (M2AChR), thus, suggesting reduced levels of acetylcholine in PCOS condition which may contribute to increased GnRH and LH pulse frequency in PCOS women.

It should be noted that along with neurotransmitters, HPG axis is governed by several neuropeptides of the like of kisspeptin, a major factor which directly or through its interaction with steroids and neurotransmitters, stimulates the release and expression of GnRH/LH [53, 54]. Various
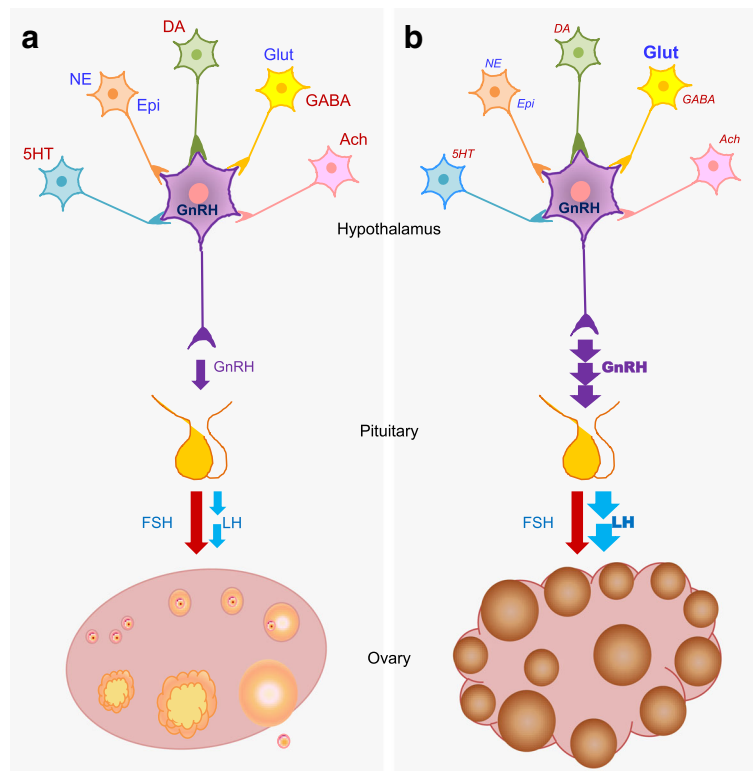
Fig. 7 Diagrammatic summary of results. Hypothalamic-pituitary-ovarian axis in normal (**a**) and in PCOS (**b**) conditions. Blue font: Stimulatory molecules; Red font: Inhibitory molecules; Bold font: Increased in PCOS condition; *Italic font*: Decreased in PCOS condition. Thick arrow: increased in PCOS condition as compared to control. 5HT: Serotonin; DA: Dopamine; NE: Norepinephrine; Epi: Epinephrine; Glut: Glutamate; Ach: Acetylcholine

immnohistochemical studies have demonstrated co-localization of GABAB, NMDAR glutamate receptor and D2 dopamine receptor in kisspeptin neurons. Furthermore, antagonists of GABAB and D2R dopamine receptors increase Kisspeptin-mediated GnRH and LH release [55, 56], whereas treatment with MK801-NMDA receptor antagonist blocks kisspeptin-dependent reinstatement of LH surge [57]. Data from our lab has revealed significant increases in expression of both *Kiss1* and its receptor *Gpr-54* in the hypothalamus of PCOS rats (manuscript under preparation) that also falls in line with a previous study [26]. Thus, alteration of neurotransmitters and neuropeptide together are likely to be responsible for the increased GnRH and LH pulsatility in PCOS condition.

Besides the regulation of endocrine axis, neurotransmitters are also implicated in several psychiatric manifestations. The vast majority of anti-depressants include inhibitors of monoamine oxidase and serotonin reuptake transporters (SSRI), indicating the role of serotonin, dopamine and norepinephrine in mood regulation [58–61]. Glutamate and GABA are also emerging candidates for depression and anxiety disorders [62, 63]. Alterations in acetylcholine signalling have also been shown to lead to symptoms of depression and anxiety wherein overactive or hyper-responsive muscarinic cholinergic system has

been documented [58]. All these references suggest that alteration in neurotransmitter profile seen in letrozole-induced PCOS model may result into development of depression and anxiety-like symptoms (manuscript communicated). In light of these references and our data, the occurrence of depression, anxiety and other mood disorders, which affect upto 40% of PCOS women [64, 65], can be linked to an altered neurotransmitter profile. We have indeed observed symptoms of depression and anxiety in behavioural experiments on letrozole-induced PCOS rat model (manuscript communicated).

Current study clearly demonstrated severe neurotransmitter modulation in letrozole-induced PCOS rat model. Likewise, in a study, rat treated with letrozole demonstrated decreased norepinephrine and dopamine content in hippocampus and frontal cortex [66]. However, the concentration and duration of letrozole treatment in that study was much higher as compared to our study. Furthermore, the possibility that the currently observed changes in neurotransmitters are due to other interactions of letrozole can be ruled out by our previous study wherein testosterone propionate-induced PCOS rat model also demonstrated similar neurotransmitter profile [67]. This thereby strengthens the result of present study indicating that neurotransmitter modulation is a pivotal attribute of PCOS condition.

Chaudhari et al. Reproductive Biology and Endocrinology (2018) 16:37

Page 12 of 13

Conclusion

Results from our study thus suggest the presence of heightened excitatory signal (glutamate) and decreased inhibitory currents (serotonin, dopamine, GABA and acetylcholine), which may be responsible for the increased pulsatility of GnRH and LH, leading to increased LH/FSH ratio as observed in PCOS (Fig. 7). It is also evident that the observed changes in neurotransmitter levels of the brain are mainly due to altered rates of their catabolism. Further, the dysregulated neurotransmitter profile in PCOS could also be the reason for low self-esteem, anxiety, frequent mood swings and depression, features closely associated with PCOS women. This is the first study which explicitly demonstrates that neurotransmitter modulation may act as a key feature in the development of PCOS pathology with increasing risk of other co-morbidities such as stress and mood.

Abbreviations
$5HT_{1A}$: Serotonin $5HT_{1A}$ receptor; AChE: Acetyl cholinesterase; COMT: Catecholamine-O-methyl transferase; D_2R: Dopamine D_2 receptor; FSH: Follicle stimulating hormone; GABA: γ-amino butyric acid; $GABA_{B1}$: $GABA_{B1}$ receptor; GABA-T: GABA transaminase; GAD: Glutamic acid decarboxylase; GDH: Glutamate dehydrogenase; GnRH: Gonadotropin-releasing hormone; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; HPG axis: Hypothalamic-pituitary-gonadal axis; LH: Luteinizing hormone; M_2AchR: Muscarinic acetylcholine 2 receptor; MAO: Monoamine oxidase; NMDA: N-methyl-D-aspartate glutamate receptor; OGTT: Oral glucose tolerance test; TDC: Tryptophan decarboxylase; TH: Tyrosine hydroxylase; $α_1AR$: $α_1$-adrenergic receptor

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
NC and LN designed and conceptualized this study. All experiments were performed by NC and MD. Data analysis, interpretation and manuscript writing was carried out by NC. LN critically reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval
All experimental protocols listed herein were approved by the Institutional animal ethical committee (IAEC) and were in accordance with the ethical standards of the Committee For the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Competing interests
The authors declare that they have no competing interests.

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Chaudhari *et al. Reproductive Biology and Endocrinology* (2018) 16:37

Page 13 of 13

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