Radhika Nagamangalam Shridharan, Harshini Krishnagiri, Vijayakumar Govindaraj*, SitiKantha Sarangi and Addicam Jagannadha Rao

Neonatal exposure to estradiol-17β modulates tumour necrosis factor alpha and cyclooxygenase-2 expression in brain and also in ovaries of adult female rats

DOI 10.1515/hmbci-2015-0072
Received December 8, 2015; accepted January 14, 2016

Abstract: The sexually dimorphic organization in perinatal rat brain is influenced by steroid hormones. Exposure to high levels of estrogen or endocrine-disrupting compounds during perinatal period may perturb this process, resulting in compromised reproductive physiology and behavior as observed in adult In our recent observation neonatal exposure of the female rats to estradiol-17β resulted in down-regulation of *TNF-α*, up-regulation of *COX-2* and increase in SDN-POA size in pre-optic area in the adulthood. It is known that the control of reproductive performance in female involves a complex interplay of the hypothalamus, pituitary, and ovary. The present study was undertaken to understand the possible molecular mechanism involved in changes observed in the ovarian morphology and expression of selected genes in the ovary. Administration of estradiol-17β (100 μg) on day 2 and 3 after birth revealed up-regulation of *ER-α*, *ER-β*, *COX-2* and down-regulation of *TNF-α* expression. Also the decrease in the ovarian weight, altered ovarian morphology and changes in the 2D protein profiles were also seen. This is apparently the first report documenting that neonatal estradiol exposure modulates *TNF-α* and *COX-2* expression in the ovary as seen during adult stage. Our results permit us to suggest that cues originating from the modified brain structure due to neonatal exposure of estradiol-17β remodel the ovary at the molecular level in such a way that there is a disharmony in the reproductive function during adulthood and these changes are perennial and can lead to infertility and changes of reproductive behavior.

Keywords: *COX-2*; estradiol-17β; neonatal; ovary; *TNF-α*.

***Corresponding author: Dr. Vijayakumar Govindaraj,** Research Associate, Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India, Phone: +91-80-22932308, Fax: +91-080-23600814, E-mail: vijaygovindaraj@biochem.iisc.ernet.in; vijaygovindaraj@gmail.com
Radhika Nagamangalam Shridharan and SitiKantha Sarangi: Department of Microbiology and Biotechnology, Bangalore University, Bangalore, India
Harshini Krishnagiri and Addicam Jagannadha Rao: Department of Biochemistry, Indian Institute of Science, Bangalore, India

Introduction

In rodents, the sexual differentiation of the brain occurs during critical period (first 2 weeks after birth) [1]. The organization of brain sex differences during this critical period is important for adult reproductive function [1, 2]. Studies have shown that administration of estradiol-17β or compounds with estrogenic activity to newborn rats during critical period results in irreversible masculinization as well as defeminization in the brain resulting in abnormal sexual behavior as adults [1, 3–6]. Recently we observed that neonatal exposure to estradiol-17β in the female rat resulted in up-regulation of *COX-2* and increased size of SDN-POA compared to control during adulthood [7]. Our data supported the fact that estradiol-17β plays critical role in the defeminization and masculinzation of the rat brain. It is imperative to study the contributing factors to monitor the expression of selected genes associated with synaptogenesis, an important process committed to the structural changes in the brain, in order to understand the long-term ramifications on the ovarian dysfunction [8, 9]. Our recent study showed that neonatal estradiol exposure modulate synaptogenesis related genes such as *TNF-α* and *COX-2* in female rat POA during adulthood [7]. It is important to note that these genes are not only involved in synaptogenesis and sex difference, but also shown to have a role in oocyte maturation and ovulation. Considering the fact that the final target is the ovary for the hormonal action in terms of anatomic integrity as well as sexual

receptivity, it was of interest to examine the changes in the ovary following neonatal administration of estradiol-17β. In the present work, we monitored the effect of neonatal administration of estradiol-17β to new born female rats on day 2 and 3 after birth as assessed on postnatal day 120 on ovarian histology and expression of selected genes and proteomic profile.

Methods

Animals and treatment

Pups born to timed pregnant Wistar rats were housed at the Central Animal Facility, Indian Institute of Science, Bangalore, India, under light: dark (12/12 h) cycle at 24 °C, 50% average relative humidity and access to food and water ad libitum. Animals were maintained in accordance with the guidelines issued by Institutional Animal Ethics Committee (IAEC), Indian Institute of Science and the procedures employed in the study have been approved by the IAEC. New born female rats were administered 100 µg of estradiol-17β (E2; Sigma, St. Louis, MO, USA, Cat No.E8875, dissolved in 0.05 mL of sesame oil) by subcutaneous route after birth on day 2 (first injection) and day 3 (second injection) with 24 h interval and control littermates were administered 0.05 mL of sesame oil [7]. The rats were allowed to grow normally and were sacrificed on day 120 during metestrus period by using excess CO_2 in a euthanasia chamber and ovaries were dissected, weighed and stored at –80 °C until further use.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from the ovary was extracted using TRI reagent (Sigma Chemicals Co, MO, USA) according to manufacturer's instructions. The integrity of the RNA was checked on 1% MOPS-HCHO agarose gel and the quantity of RNA was estimated spectrophotometrically. Reverse transcription of RNA and subsequent PCR were performed as described earlier [10]. The list of highly specific forward and reverse primers is given in Table 1. Gel electrophoresis was carried out by taking 30 µL of the PCR product on a 1.5% agarose gel containing 0.5 µg/mL of Ethidium bromide, in 1X TBE buffer containing 45 mM Tris-borate and 0.5 mM EDTA. The difference in the intensities of the products following electrophoresis was analyzed using the Kodak Electrophoresis and Gel Documentation Analysis System (EDAS-120) (Eastman Kodak Co., New York, USA). The expression levels of specific transcripts were inferred upon normalizing their signal intensities to that of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), which served as an internal control in this semi-quantitative analysis.

Table 1: List of primers used in the study.

	Gene	Sequence 5′ →3′ (F-Forward; R-Reverse)	Length	Annealing temperature (°C)
1	CBR1	F- AAGGAGTCCATGCGAAAGAA	20	60 °C
		R- GCTCCTTCTTCTGGGCTTTT	20	
2	MK12	F- CCTGGAAAAGATGCTGGTGT	20	
		R- GAGGCTTGAAGCTGAGCACT	20	
3	GAPDH	F- CTCATGACCACAGTCCATGC	20	
		R- TTCAGCTCTGGGATGACCTT	20	

Western blot analysis

Protein lysates were prepared from control and E2 treated ovarian samples by homogenization of the tissues in a hand held Teflon-glass homogenizer using RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100 and protease inhibitor cocktail from Roche Diagnostics GmbH, Mannheim, Germany). The homogenate was centrifuged at 2500 *g* at 4 °C for 10 min and the total protein in the supernatant was estimated spectrophotometrically by Lowry's method [11]. From each group, 40 µg of protein was electrophorsed on 10% sodium dodecyl sulfate polyacrylamide gel and Western blotting was carried out as described [10]. The primary antibodies used were anti-ER-α (Cell Signalling Technology, Denver, MA, USA, Cat No. 8644, dilution- 1:500, MW 66 kDa), anti-ER-β (Abnova, Taipei City, Taiwan, Cat No. H00002100-M01, dilution-1:500, MW 37 kDa), anti-COX-2 (Cell Signalling Technology, Denver, MA, USA, Cat No.12282, dilution-1:500, MW 74 kDa), anti-TNF-alpha (17 kDa) (a kind gift from Prof. Anjali Karande, Indian Institute of Science, Bangalore), and anti-GAPDH (Cell Signalling Technology, Denver, MA, USA, Cat No. 2118, dilution-1:2000, MW 37 kDa). Goat anti-rabbit (Cat No. NA934, GE Healthcare, NJ, USA) and goat anti-mouse (Cat No. NA931, GE Healthcare, NJ, USA) were used as secondary antibodies at the dilution of 1:5000. Signal intensities of various bands were determined by using the Kodak Electrophoresis and Gel Documentation Analysis System (EDAS-120, Eastman Kodak Co., New York, USA). The expression levels of the amplified products of interest were inferred upon normalising their signal intensities to that of GAPDH. Values so obtained were used to determine mean±SEM for a graphical representation.

Two-dimensional gel electrophoresis (2DE) and image analysis

Equal quantity of protein (250 µg) from control and treated ovaries were subjected to two dimensional electrophoresis as described [12]. The acetone precipitated protein was re-suspended in 250 µL of sample rehydration buffer (7 M Urea, 4% CHAPS, IPG buffer (Catalogue No.17600088, GE Healthcare, Piscataway, NJ, USA). Immobiline pH gradient (IPG) gel strips –13 cm (Catalogue No.17600088, GE Healthcare, Piscataway, NJ, USA) were rehydrated overnight in 250 µL of rehydration buffer containing protein sample. Isoelectric focusing (IEF) was carried out in Ettan IPGPhor 3 (GE Healthcare, Piscataway, NJ, USA) at 500 V (Step and Hold) for 1 h, at 1000 V (Gradient) for 1 h, at 8000 V (Gradient) and at 8000 V (Step and Hold) for 2 h and 20 min, respectively. After first dimension, the gel strips were equilibrated for 15 min in SDS equilibration buffer (6 M urea, 50 mM Tris (pH 8.8), 30% v/v glycerol, 2% w/v SDS, 2% w/v DTT). Second dimension electrophoresis was performed using 10% sodium dodecyl sulfate polyacrylamide gel. Gels were stained with coomassie blue R-250 and stained gels were scanned by HP ScanJet G3110 scanner and images were analyzed by Dymension 2D gel image analysis software (Syngene, Cambridge, UK).

Spot intensity was quantified by the total spot volume normalization which involved triplicates for each group. The volume of protein spots with a change >1.5 ($p \leq 0.05$) was considered to be significant.

Statistical analysis

Data in each experiment are presented as mean±SEM (n=5 per group). Differences between groups were analyzed by the two-tailed Student's t-test and p-values <0.05 were considered statistically significant.

Results

Morphology of the ovary

Administration of estradiol-17β to neonatal rats resulted in significant decrease in ovarian weight with no significant change in the overall body weight (Figure 1A and B). Ovarian morphology was distinctly different across treatment groups. Female rats neonatally exposed to estradiol-17β had very smaller size ovary with no clear signs of active oogenesis or ovulation. Ovaries of neonatally exposed to E2 had numerous large antral-like follicles and degenerating oocytes. Microscopic examination of ovarian sections revealed that while the ovary of the control group had several follicles and corpora lutea the ovary from the treated group is small in size and no corpora lutea were seen (Figure 1C).

Analysis of mRNA levels of selected genes by RT-PCR

It can be seen from the results presented in Figure 2 that there was a significant increase in the expression of *ERα*, *ERβ*, *COX2* and a significant decrease in the expression of *TNF-α* mRNA levels in the ovaries of E2 treated rats as observed on postnatal day 120 as assessed by RT-PCR. It can also be seen from the results presented in Figure 3 that there was a significant increase in the expression of ER-α, ER-β, COX-2 and a significant decrease in the expression of TNF-α protein levels as assessed by Western blot.

Proteomic analysis by two dimensional electrophoresis

Two-dimensional electrophoresis (2DE) was carried out as described in the methods section to identify differentially

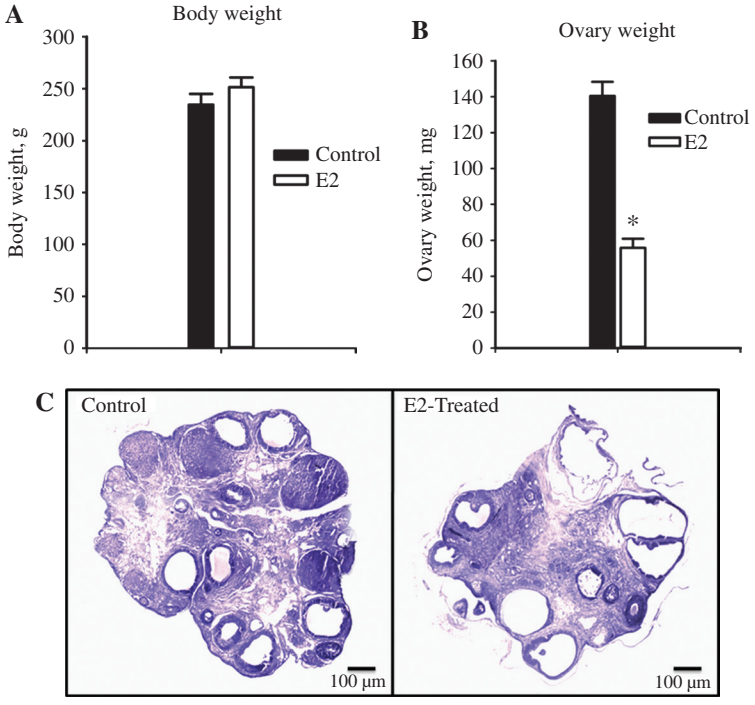

Figure 1: Effect of neonatal exposure to oestrogen on body weight (A), ovarian weight (B) and morphology of ovary (C).
The data of the body and the ovarian weights from five animals per group are expressed as mean±SEM (*p<0.05). There was no significant change in the body weight (A). Compared to controls, rats that were neonatally exposed to estradiol-17β had very small ovaries (B) with no clear signs of active oogenesis or ovulation (C). This was characterized by absence of corpus luteum, presence of cystic follicles with thickened thecal layer and atretic granulosa cells and abundant stroma.

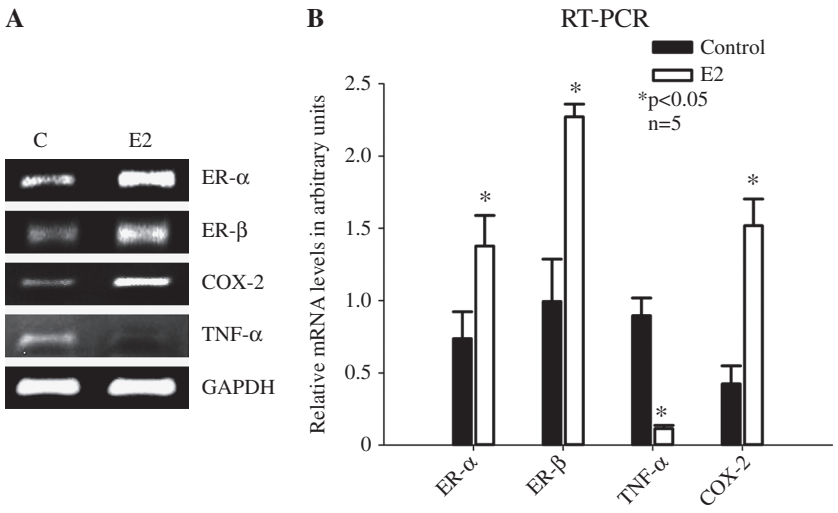

Figure 2: mRNA expression patterns of *ER-α*, *ER-β*, *TNF-α* and *COX-2* genes in the ovary.
RNA isolated from ovary of control and neonatally estradiol-17β treated female rats was reverse transcribed to cDNA and subjected to RT-PCR analysis with GAPDH as an internal control. Data from five animals per group was expressed as arbitrary densitometric units (mean±SEM, *p <0.05 compared with control).

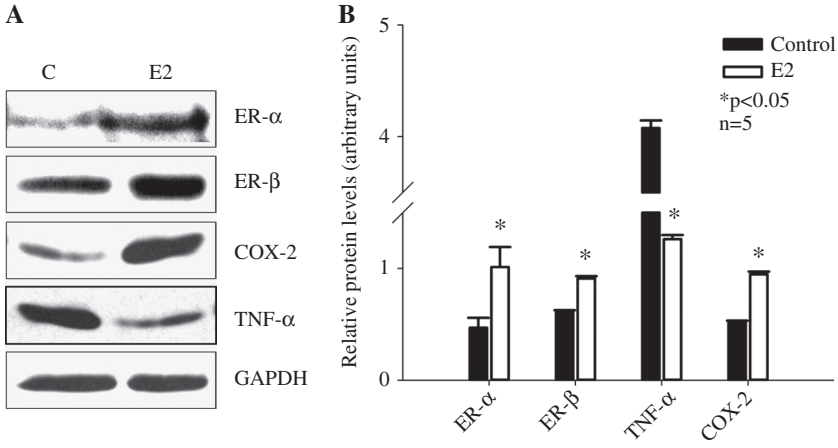

Figure 3: Western blot analysis for ER-α, ER-β, TNF-α and COX-2 in the ovary.
A total of 40 μg of protein each from control and estradiol-17β treated ovary was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to western blotting as mentioned in materials and methods section. Data from five animals per group was expressed as arbitrary densitometric units (mean±SEM).

expressed proteins in control and neonatally estradiol-17β administered ovary samples. A representative gel image has been given in Figure 4A. Analysis of gel images by Dymension 2D gel image analysis software revealed approximately 270 protein spots in each gel. There were no protein spots which were expressed as only specific for either control or E2 treated ovary. There were six protein spots (marked with the number) differentially expressed between control and E2 treated ovary in which three (spots 1–3) were down-regulated and three (spots 4–6) were up-regulated in E2 treated compared to control. The close-up images of differentially expressed proteins are presented in Figure 4B. The differentially expressed protein spots were subjected to in-gel digestion and MALDI-TOF/TOF analysis, and mass spectral data were submitted to a database search against the NCBInr database for protein identification using the Mascot search programme (http://www.matrixscience.com). The identity of down-regulated and up-regulated proteins is presented in Table 2. The RT-PCR validation of two differentially expressed proteins such as carbonyl reductase 1 (CBR1) and mitogen-activated protein kinase 12 (MK12) is presented in Figure 5.

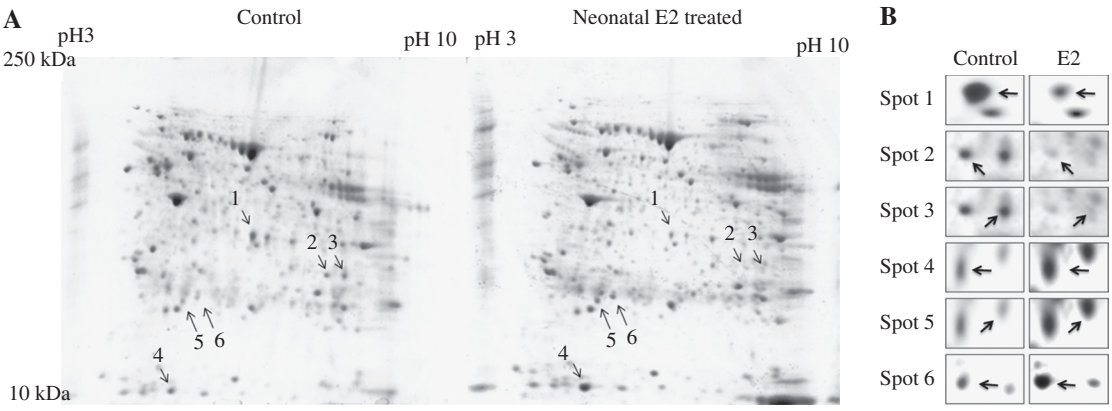

Figure 4: A representative 2-DE gel image of proteins profile of control and neonatally estradiol-17β treated rats (A). Proteins were subjected to iso-electric focusing in the first dimension using 13 cm IPG strips (pH 4–7), followed by separation on 10% SDS-PAGE gels in the second dimension (molecular weight 200–10 kDa range). The 2-DE gel was stained with Coomassie Brilliant Blue. Magnified images of differentially expressed protein spots indicated by dark rectangles with arrow mark (B).

Discussion

We previously demonstrated that estradiol-17β exposure during early postnatal life disrupts the expression of synaptogenesis related genes in preoptic area [7]. In the present study, using the same animal model, the increase in expression of *ER-α, ER-β, COX-2* and down-regulation of *TNF-α*, alterations in ovarian morphology in the ovary of neonatally estradiol-17β treated female rats was observed. In our previous study the decreased expression of *ER-α* and *ER-β* was observed in pre-optic area of neonatally estradiol-17β treated animals [7]. It is known that both *ER-α* and *ER-β* are critically important for sexual differentiation of the brain [13] and *ER-β* which is important in inducing a luteinizing hormone (LH) surge by oestrogen, typical of female estrous cycle, was decreased significantly in the hypothalamus, which is the main tissue in initiating cyclicity [7]. Our unpublished data show that serum estradiol level was not significantly different between control (28.31±1.30 pg/mL) and estradiol treated groups (24±4.30 pg/mL) and are comparable to values reported in the literature (25–35 pg/mL) for the adult animals [14]. The reason for this discrepancy between increased expression of receptor and no significant change in serum estradiol levels may be due to epigenetic or other post transcriptional mechanisms. Studies have shown that epigenetic regulation of genes is involved in the control of sexual differentiation of the female brain [15]. During postnatal development, the neonatal estradiol treatment may have lasting effect on oestrogen receptors expression mediated by DNA methylation of its promoter through long-term transcriptional control by altering protein-DNA interactions. It is also possible that when the level of ligand is drastically reduced the system responds to sequester whatever the ligand is available by increasing the expression of the corresponding receptor [16].

The control of reproductive cyclicity and ovulation is based on interaction of oestrogen with hypothalamus and pituitary via feedback mechanisms by influencing the cyclic secretion of LH and FSH [17]. Some studies have reported that neonatal estradiol or androgen induced sterility with increased prolactin secretion [18, 19]. We wish to point out that the level of estradiol (100 μg) administered in this study is not so high as to completely abolish FSH and LH producing cells and replaced by prolactin (PRL) producing cells. The level of estradiol administered is high but the duration of start of treatment and sacrifice of animals is 120 days and considering the long interval it is unlikely that high concentration of serum estradiol remains high. There is an alteration in responsiveness of hypothalamus and pituitary and it is unlikely the effects seen are due to high levels of PRL. Also it is essential to note that the alpha foeto-protein concentration is high in new born animals [20] will sequester E2 and only small percentage of injected estradiol enters in to the brain. And this is the reason why such high concentration of estradiol had to be injected. It is very important to note that studies which showed the effects of estrogenization and androgenization on both the dopaminergic and serotoninergic control of prolactin secretion are qualitatively different. The effect of drugs (dopaminergic and serotoninergic blockers) that alters dopaminergic versus serotoninergic effects on prolactin secretion varies depending on whether the sterility was induced by testosterone or estradiol [20]. Also studies demonstrated that more than two to three times as many prolactin-expressing neurons from female hypothalamus

Table 2: List of differentially expressed protein identified by MALDI-TOF/TOF.

Spot number on the gel	Protein identity	UniProtKB Accession number #	Nominal mass, Da	Calculated pI	Mascot score, %	Greater than this score is significant (p<0.05)	No of matched peptides	Sequence coverage, %
Down-regulated in E2 treated ovary								
1	Aldo-keto reductase family 1	P51652 (AKC1H_RAT)	37,740	5.90	73	52	17	51
2	Carbonyl reductase 1	P47727 (CBR1_RAT)	30,849	8.22	63	52	14	45
3	Gastrotropin (fatty acid binding protein family6-FABP6)	P80020 (FABP6_RAT)	14,592	5.52	55	52	10	67
Up-regulated in E2 treated ovary								
4	LIX1-like protein	Q5PQQ7 (LIX1L_RAT)	36,921	8.80	58	52	10	16
5	Mitogen-activated protein kinase 12	Q63538 (MK12_RAT)	42,132	5.91	68	52	6	18
6	40s ribosomal protein S15a	P62246 (RS15A_RAT)	14,944	10.1	58	52	4	44

survive when compared to those derived from male hypothalamus, and this indicate that the changes are independent of gonadal steroids [21]. The current understanding neuronal basis of prolactin secretion in sexual differentiation require further investigations.

A very interesting observation in the present study was a drastic decrease in the expression of *TNF-α*. It is documented that E2 modulates the expression of *TNF-α* in the ovary and *TNF-α* has a very important role in the regulation of ovarian follicle atresia as well as in corpus luteum function (i.e. granulosa-cell proliferation and steroidogenesis) [22]. *TNF-α* is suggested to act as a survival/proliferating factor in the follicle of porcine ovaries. Also the expression of TNFR2 (*TNF-α* receptor) disappeared in ovarian follicles during follicular atresia [23]. Based on these findings it suggested that *TNF-α* acts as a survival factor for ovarian follicles. The increase in the expression of COX-2 gene in POA has been considered as a parameter to conclude that the animals have responded to estradiol-17β administration and also exhibit masculinization of spine density in the female brain [1, 8, 24]. Similar to the observation made in the brain, in the present study up-regulation of COX-2 expression was observed in the estradiol-17β treated ovary. It is known that COX-2 is a rate-limiting enzyme during prostaglandin (PGE2) synthesis and it is expressed in all female reproductive organs and COX-2 has been shown to play a key role in the regulation of ovulation [25]. COX-2 has been shown to be induced by factors such as tumour promoters, growth factors, cytokines, and mitogens in different cell types [26]. Over-expression of COX-2 has been associated with pathogenesis of many diseases such as multiple epithelial cancers and colon cancer and multiple sclerosis, neurodegenerative diseases, and heart infarction [25, 27]. In polycystic ovarian syndrome (PCOS), the elevated expression of COX-2 in theca layer of ovarian follicles has been reported with increased in the diameter of this layer due to the ability of COX-2 to stimulate cell proliferation, angiogenesis [28] and consequence of this may induce anovulation. Overexpression of COX-2 has shown to stimulate the loss of basement membrane of ovarian surface epithelia which is important for ovulation [29]. Studies on female COX-2 or PGE2-null mice showed diminished ovulation, and exhibit irregular cumulus expansion and animals were infertile [30, 31]. In addition to its important role in ovulation, experimental evidences show that COX-2 contributes to proliferation, invasion and metastasis in several tumors [32, 33] and it was found to be over expressed in pre-neoplastic changes of the ovarian surface epithelium [25, 29]. Based on these findings it suggested that the increased COX-2 and reduced TNF-α expression are associated with altered ovarian morphology.

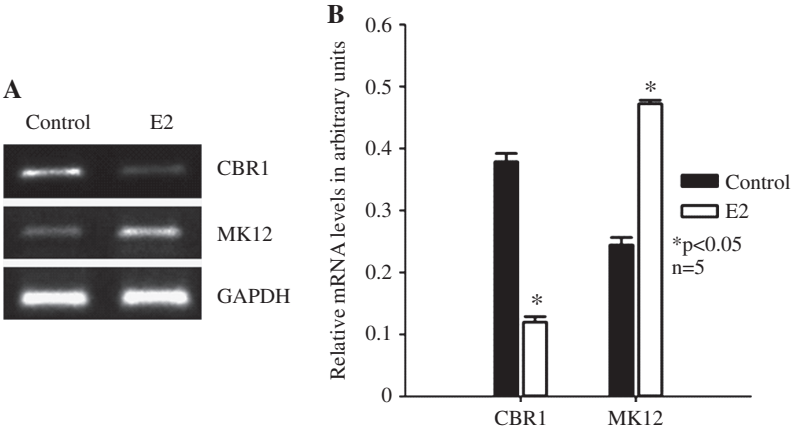

Figure 5: Valdiation of proteomic data for selected proteins by semi-quantitative RT-PCR.
RNA isolated from ovary of control and neo-natally estradiol-17β treated female rats was reverse transcribed to cDNA and subjected to RT-PCR analysis with GAPDH as an internal control. Data from five animals per group was expressed as arbitrary densitometric units (mean±SEM, *p<0.05 compared with control).

Two-dimensional electrophoresis analysis of ovaries of control and neonatally estradiol treated rats revealed six differentially expressed proteins as shown in Table 2. One of the down-regulated proteins such as aldo-keto reductase family 1 (AKR1) is an enzyme which has been shown to play an essential role in the metabolism of all steroid hormones. This enzyme catalyses the reduction of progesterone to the inactive form 20-alpha-hydroxyprogesterone and it is expressed in the ovary throughout the estrous cycle [34]. Another down-regulated protein called carbonyl reductase 1 (Cbr1) is a NADPH-dependent oxidoreductase specific for carbonyl compounds such as quinines and xenobiotics. 20 beta-hydroxysteroid dehydrogenase (HSD)/carbonyl reductase-like (Cbr1) converts 17a-hydroxyprogesterone (17-P) to 17a,20beta-dihydroxyprogesterone [35]. Gastrotopin (fatty acid binding protein family6- FABP6) plays a role in fatty acid uptake, transport, and metabolism. FABP6 mRNA and protein has been found to be expressed in luteal cells of the rat ovary and FABP6 has been shown to have variety of function including steroid hormone metabolism [36]. FABP6 has been shown as one of the downstream targets of LH surge induced transcription factor called RUNX2 (runt-related transcription factor 2) [36]. The biological function of LIX1-like protein has not been elucidated, the other two up-regulated proteins mitogen-activated protein kinase and 40s ribosomal protein S15a are shown to be involved in acceleration of ovarian tumourigenesis and metastasis via the MAP kinase signal pathway [37]. Further studies are warranted to determine the significance of the effect of neonatal estradiol exposure on the up and down regulated proteins in the ovary.

In conclusion, our results suggest that although the main targets for the neonatal administration of E2 are the different regions of brain such as pre-optic area, hypothalamus and pituitary, the changes observed for the first time in *TNF-α* and *COX-2* levels suggest a possible role of these genes acting directly or indirectly on the adult ovary resulting in a remarkable change in morphology which subsequently leads to infertility.

Acknowledgments: The author A.J. Rao would like to thank the Indian National Science Academy for the award of INSA Senior Scientist Fellowship and Department of Science and Technology (DST-Government of India) for financial assistance (Grant No. SR/SO/HS-0016/2012) provided during the course of the work. Also the author A.J. Rao would like to thank, Department of Biochemistry, Indian Institute of Science, Bangalore, India.

Declaration of conflicting interests: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

1. McCarthy MM. Estradiol and the developing brain. Physiol Rev 2008;88:91–124.
2. de Vries GJ, Forger NG. Sex differences in the brain: a whole body perspective. Biol Sex Differ 2015;6:15.
3. Flanagan-Cato LM. Sex differences in the neural circuit that mediates female sexual receptivity. Front Neuroendocrinol 2011;32:124–36.

4. Rubin BS, Murray MK, Damassa DA, King JC, Soto AM. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ Health Persp 2001;109:675.
5. Krentzel AA, Remage-Healey L. Sex differences and rapid estrogen signaling: A look at songbird audition. Front Neuroendocrinol 2015;38:37–49.
6. McCarthy MM. The two faces of estradiol: effects on the developing brain. Neuroscientist 2009;15:599–610.
7. Radhika NS, Govindaraj V, Sarangi SK, Rao AJ. Neonatal exposure to 17β-estradiol down-regulates the expression of synaptogenesis related genes in selected brain regions of adult female rats. Life Sci 2015;141;1–7.
8. Schwarz JM, McCarthy MM. Cellular mechanisms of estradiol-mediated masculinization of the brain. J Steroid Biochem Mol Biol 2008;109:300–6.
9. McCarthy M. How it's made: organisational effects of hormones on the developing brain. J Neuroendocrinol 2010;22:736–42.
10. Govindaraj V, Basavaraju RK, Rao AJ. Changes in the expression of DNA double strand break repair genes in primordial follicles from immature and aged rats. Reprod BioMed Online 2015;30:303–10.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
12. Govindaraj V, Rao AJ. Comparative proteomic analysis of primordial follicles from ovaries of immature and aged rats. Syst Biol Reprod Med 2015;61:375–75.
13. Temple JL, Fugger HN, Li X, Shetty SJ, Gustafsson J-Ak, Rissman EF. Estrogen receptor β regulates sexually dimorphic neural responses to estradiol. Endocrinology 2001;142:510–3.
14. Montano M, Welshons W, Vom Saal F. Free estradiol in serum and brain uptake of estradiol during fetal and neonatal sexual differentiation in female rats. Biol Reprod 1995;53:1198–207.
15. Nugent BM, Wright CL, Shetty AC, Hodes GE, Lenz KM, Mahurkar A, Russo SJ, Devine SE, McCarthy MM. Brain feminization requires active repression of masculinization via DNA methylation. Nature Neurosci 2015;18:690–7.
16. Ing NH, Tornesi MB. Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. Biol Reprod 1997;56:1205–15.
17. Messinis IE. Ovarian feedback, mechanism of action and possible clinical implications. Hum Reprod Update 2006;12:557–71.
18. Barraclough CA. Production of anovulatory, sterile rats by single injections of testosterone propionate 1. Endocrinology 1961;68:62–7.
19. Mallampati R, Johnson D. Serum and pituitary luteinizing hormone, follicle-stimulating hormone and prolactin levels in gonadectomized male, female and androgenized female rats treated with oestradiol benzoate. J Endocrinol 1973;59:209–16.
20. De Mees C, Bakker J, Szpirer J, Szpirer C. Alpha-fetoprotein: from a diagnostic biomarker to a key role in female fertility. Biomarker Insights 2006;1:82.
21. Beyer C, Eusterschulte B, Pilgrim C, Reisert I. Sex steroids do not alter sex differences in tyrosine hydroxylase activity of dopaminergic neurons in vitro. Cell Tissue Res 1992;270:547–52.
22. Yan Z, Hunter V, Weed J, Hutchison S, Lyles R, Terranova P. Tumor necrosis factor-alpha alters steroidogenesis and stimulates proliferation of human ovarian granulosal cells in vitro. Fertil Steril 1993;59:332–8.
23. Nakayama M, Manabe N, Inoue N, Matsui T, Miyamoto H. Changes in the expression of tumor necrosis factor (TNF) α, TNFα receptor (TNFR) 2, and TNFR-associated factor 2 in granulosa cells during atresia in pig ovaries. Biol Rep 2003;68:530–5.
24. Amateau SK, McCarthy MM. Induction of PGE2 by estradiol mediates developmental masculinization of sex behavior. Nat Neurosci 2004;7:643–50.
25. Sirois J, Sayasith K, Brown KA, Stock AE, Bouchard N, Doré M. Cyclooxygenase-2 and its role in ovulation: a 2004 account. Hum Reprod Update 2004;10:373–85.
26. Liang Y-C, Huang Y-T, Tsai S-H, Lin-Shiau S-Y, Chen C-F, Lin J-K. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 1999;20:1945–52.
27. Hayes EC, Rock JA. COX-2 inhibitors and their role in gynecology. Obstet Gynecol Surv 2002;57:768–80.
28. Karimzadeh L, Nabiuni M, Kouchesfehani HM, Adham H, Bagheri A, Sheikholeslami A. Effect of bee venom on IL-6, COX-2 and VEGF levels in polycystic ovarian syndrome induced in Wistar rats by estradiol valerate. J Venom Anim Toxins 2013;19:32.
29. Roland IH, Yang W-L, Yang D-H, Daly MB, Ozols RF, Hamilton TC, Lynch HT, Godwin AK, Xu X-X. Loss of ovarian surface and cyst epithelial basement membranes and pre-neoplastic morphological changes in prophylactic oophorectomies. Cancer Res 2004;64(7 Supplement):173.
30. Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC, Langenbach R. Anovulation in Cyclooxygenase-2-Deficient Mice Is Restored by Prostaglandin E2 and Interleukin-1β 1. Endocrinology 1999;140:2685–95.
31. Hizaki H, Segi E, Sugimoto Y, Hirose M, Saji T, Ushikubi F, Matsuoka T, Noda Y, Tanaka T, Yoshida N. Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP2. Proc Natl Acad Sci 1999;96:10501–6.
32. Munkarah A, Ali-Fehmi R. COX-2: a protein with an active role in gynecological cancers. Curr Opin Obstet Gynecol 2005;17:49–53.
33. Menczer J. Cox-2 expression in ovarian malignancies: a review of the clinical aspects. Eur J Obstet Gynecol Reprod Biol 2009;146:129–32.
34. Seo K-S, Naidansuren P, Kim S-H, Yun S-J, Park J-J, Sim B-W, Park C-W, Nanjidsuren T, Kang M-H, Seo H, Ka H, Kim NH, Hwang SY, Yoon JT, Yamanouchi K, Min KS. Expression of aldo-keto reductase family 1 member C1 (AKR1C1) gene in porcine ovary and uterine endometrium during the estrous cycle and pregnancy. Reprod Biol Endocrinol. 2011;9:139.
35. Zapater C, Chauvigné F, Scott AP, Gómez A, Katsiadaki I, Cerdà J. Piscine follicle-stimulating hormone triggers progestin production in gilthead seabream primary ovarian follicles. Biol Reprod 2012;87:111.
36. Park E-S, Lind A-K, Dahm-Kähler P, Brännström M, Carletti MZ, Christenson LK, Curry Jr TE, Jo M. RUNX2 transcription factor regulates gene expression in luteinizing granulosa cells of rat ovaries. Mol Endocrinol 2010;24:846–58.
37. Wang Z, Hou J, Lu L, Qi Z, Sun J, Gao W, Meng J, Wang Y, Sun H, Gu H, Xin Y, Guo X, Yang G. Small ribosomal protein subunit S7 suppresses ovarian tumorigenesis through regulation of the PI3K/AKT and MAPK pathways. PLoS One 2013;8:e79117.