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MATERIALS & METHODS

ANIMAL HOUSING AND MAINTENANCE

Adult female Charles Foster rats (2-3 months old; 180-220 g) were housed in controlled conditions of temperature (25±1 ºC), relative humidity (40-50%) and light (photoperiod cycle of 12 h light:12 h dark) with food (Sai Durga feeds and foods, Bangalore, India) and water made available *ad libitum*. Animals were allowed to acclimatize for one week before starting the experiment. All experimental protocols were approved by the Institutional Animal Ethical Committee and experiments were performed as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (938/PO/ReBi/S/06/CPCSEA).

PCOS RAT MODEL

PCOS was induced in the animals using two different drugs. Each will be discussed one by one below.

1) Letrozole induced PCOS rat model:

PCOS rat model was developed using Letrozole, a non-steroidal aromatase inhibitor (Kafali et al., 2004). The procedure was as follows:

Reagents:

i) 1% Carboxy methyl cellulose (CMC): 1 gm of CMC in 100 ml of distilled water was boiled in a water-bath with continuous stirring until the CMC powder was completely dissolved. The solution was cooled at room temperature and stored at 15°C till further use. This was used as the vehicle.

ii) Letrozole dose preparation: Letronat™ tablets containing 2.5 mg letrozole were purchased from Natco Pharma Ltd., Hyderabad, India. The tablets were crushed using mortar-pestle and the powder was weighed and mixed with 0.5 ml of 1% CMC and administered orally to animal at a final dose of 0.5 mg letrozole/kg body weight.

Procedure: Rats were randomly assigned to two different groups. A daily treatment regime of 21 days included oral administration of 0.5 ml 1% CMC for control group and 0.5mg/kg body weight of letrozole dose for PCOS group. After 21 days of treatment, a series of biochemical assays was performed to check for induction of PCOS.

2) Testosterone propionate induced PCOS rat model

Reagents:

i) Testosterone propionate (TP) and Propylene glycol were purchased from HiMedia Laboratories, Mumbai, India.

ii) TP dose preparation: TP (10mg/kg body weight) concentration was calculated for each animal; appropriate amount of TP was dissolved in 0.1 ml of propylene glycol and subcutaneously injected into the animals.

Procedure: PCOS rat model was developed by using testosterone propionate (Beloosesky et al. 2004). For PCOS induction by TP, freshly weaned (21 days old) female rats were randomly assigned to two different groups. Control group received 0.1 ml of propylene glycol as vehicle whereas PCOS groups were injected with 10mg/kg body weight of testosterone propionate (TP) dissolved in 0.1ml of propylene glycol. The treatment regime consists of subcutaneous injection of either vehicle or TP daily, for 35 days. After 35 days of treatment, a series of biochemical assays was performed to check for development of PCOS.

For PCOS validation, Oral glucose tolerance test, estrus cyclicity, serum hormone profile and ovarian histology was performed. Following validation, animals were sacrificed in their diestrus stage by decapitation. Pituitary, hypothalamus, hippocampus, frontal cortex, ovary and adrenals were dissected out and used for further experiments.

ORAL GLUCOSE TOLERANCE TEST

OGTT was performed according to the method of Buchanan et al. (1991). 12 hour fasting blood plasma was collected from orbital sinus in vials containing sodium fluoride, followed by oral administration of 1 gm/kg body weight of glucose. Blood was then collected every 30

minutes till 2 hours and plasma glucose levels were estimated using Glucose oxidase-peroxidase (GOD-POD) kit (Reckon diagnostics, Vadodara, India).

Glucose (GOD-POD method)

Principle:

The method is an enzyme-coupled reaction for the estimation of blood glucose. Glucose Oxidase (GOD) catalyses the oxidation of aldehyde group of glucose in the presence of oxygen to form gluconic acid with liberation of H_2O_2. Peroxidase (POD) converts H_2O_2 into water and active oxygen which reacts with chromogen 4-amino anti-pyrine to form a pink colored complex which can be estimated colorimetrically at 505nm.

Sample Preparation: Blood was collected in fresh vials coated with 6mg/ml Sodium flouride (NaF). NaF is an anticoagulant and it inhibits glycolysis. For the separation of plasma, the tubes containing blood were centrifuged at 3000 g for 15 minutes at 4°C. The clear supernatant (plasma) was transferred into tubes and immediately used for glucose estimation.

$$\text{Glucose} + O2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4-amino antipyrene} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red colored quinol} + H_2O$$

Reagents:

i) Enzyme GOD-POD

ii) Glucose standard (100mg/ml)

iii) Chromogen

Procedure:

Reagent	Blank	Standard	Test
Enzyme	1.0 ml	1.0 ml	1.0 ml
Glucose Standard	-	0.01 ml	-
Sample	-	-	0.01 ml

Mix well and incubate for 5 Min at 37°C. Mix & read absorbance at 505 nm against blank.

Calculation:

$$\text{Glucose concentration (mg/dl)} = \frac{[\text{Absorbance of Sample} \times \text{Conc. of Std (100)}]}{\text{Absorbance of Std}}$$

Unit: mg/dl. **Normal Values:** Fasting: 70-100 mg/dl; 2 h post prandial: up to 140 mg/dl. OGTT profile was analyzed by plotting the graph of glucose concentration in mg/dl vs. time in minutes.

ESTRUS CYCLE

Estrus cyclicity was monitored during the whole period of treatment by examining vaginal lavage. Every morning between 8:00 and 9:00 AM, vaginal smears were obtained with a swab soaked in normal saline, placed on a standard slide and immediately observed under 10X magnification. The stage of estrus cycle was confirmed by the presence of different cell types. Proestrus stage consists of mainly round, nucleated epithelial cells; Estrus smear predominates with anucleated cornified cells; Metestrus smear comprises of equal proportion of leukocytes, cornified cells and nucleated epithelial cells; and Diestrus stage primarily contains leukocytes.

HORMONE ESTIMATION

For estimation of hormones from serum as well as tissues, commercially available enzyme-linked immune sorbent assay (ELISA) kits were used.

- Estradiol, testosterone and Insulin ELISA kits - Dia-metra, Italy
- Progesterone kits- Diagnostics Biochem, Canada.
- FSH, LH and ACTH Chemiluminescence immunoassay (CLIA) kits - Elabscience Biotechnology Co., Ltd, USA.

Sample preparation:

Serum: Animals were fasted overnight and blood was collected at diestrus stage in the morning between 8 and 9 AM. Serum was separated by centrifuging tubes at 5000 g for 15 minutes and supernatant was used for ELISA or CLIA.

Tissue: Tissue homogenate (5% for pituitary and adrenal; 10% for other tissues) was prepared in ice-cold PBS followed by centrifugation at 3000g for 10 minutes. The supernatant was collected in fresh tubes and utilized immediately for hormone estimation or stored at -80°C until use. ELISA and CLIA were performed according to manufacturer's protocol.

HISTOLOGICAL ANALSYSIS

Reagents:

10% neutral buffered formalin (NBF):

Formalin : 10 %
$NaH_2PO_4.H_2O$: 0.4 %
Na_2HPO_4 : 0.65 %

Ovaries from 4 different animals from each group were collected and fixed in NBF. For histological examination, 5 µm thick sections were cut on a microtome and stained with Hematoxylin-Eosin. Histo-anatomical changes were screened for and micrographs were taken on a Leica DM2500 microscope through a Leica EZ camera.

PROTEIN ESTIMATION

Principle: The method of Lowry et al. (1951) was followed. Tyrosine & Tryptophan present in tissue homogenates react with Folin Ciocalteau reagent (FC reagent) to give blue coloured complex. The colour is formed due to the reaction of alkaline copper with protein and reduction of phospho-molybdate by the protein. The intensity of colour depends on the amount of aromatic acids present, whose absorbance is measured at 660 nm.

Reagents:

i) Lowry's Reagent:
   Reagent A: 2 % Sodium Carbonate 0.1N NaOH
   Reagent B: 1 % Copper Sulfate
   Reagent C: 2 % Sodium Potassium Tartarate

   To make 100 ml of Lowry's reagent (Reagent-D), add 98 ml of reagent A, 1 ml of reagent B & 1 ml of reagent C (freshly prepared).

ii) Folin Ciocalteau reagent (FC reagent) (Freshly prepared): Mix equal volumes of FC reagent and distilled water (1:1 ratio).

iii) BSA Standard concentration: 1mg/ml.

Procedure:

Reagent	**Blank**	**Test**
Reagent-D	1.0 ml	1.0 ml
Distilled Water	0.1 ml	0.09 ml
Sample	-	0.01 ml
Keep at Room Temperature for 10 minutes		
FC Reagent	0.1 ml	0.1 ml
Mix well & Keep at Room Temperature for 20 minutes (In Dark)		
Read absorbance at 660 nm		

Calculation: Protein was quantified using a standard graph. **Unit:** mg/ml

CORTICOSTERONE ESTIMATION

The method described by Katyare & Pandya (2005) was followed and is described below.

Reagents:

i) Chloroform-Methanol (2:1) (Freshly prepared)

ii) Chloroform

iii) 0.1N NaOH

iv) 30N H_2SO_4

v) Isolation medium:
   0.25 M Sucrose
   10mM Tris-HCl buffer (pH-7.4)
   1 mM EDTA
   0.25 mg BSA/ml

Sample Preparation:

Serum: Blood was collected between 8 and 9 AM in the morning. After blood collection, tubes were kept at room temperature for 15-20 minutes, followed by centrifugation at 3000g for 15 minutes at room temperature. Supernatant containing serum was transferred to a fresh vial and stored at -80°C until use.

Tissue: Tissue homogenates were prepared in isolation medium for pituitary (2.5% w/v), and for hypothalamus, hippocampus, frontal cortex, ovary and adrenal gland (10% w/v). The tubes were centrifuged at 10,000g for 30 minutes at 4˚C and supernatant was used directly for corticosterone estimation or stored at -80°C until use.

Procedure: For corticosterone estimation, 0.2 ml of serum or 0.2 ml of 1:10 diluted tissue sample was mixed with equal amount of freshly prepared chloroform-methanol (2:1), followed by 3 ml of chloroform. The samples were vortexed for 30 seconds and centrifuged at 2000 g for 10 minutes. The chloroform layer was carefully transferred to a fresh tube containing 0.3 ml of 0.1 N NaOH. The mixture was vortexed and NaOH layer was immediately removed. The samples were vortexed vigorously with 3 ml of 30 N H_2SO_4. After phase separation, the chloroform layer was removed carefully and discarded. The tubes containing H_2SO_4 were kept in dark for 30-60 minutes. Fluorescence measurements were carried out with excitation and emission wavelengths set at 472 and 523.2 nm, respectively using Hitachi F-7000 florescence spectrophotometer.

Standard Graph: Stock solution – 10 μg/ml of corticosterone dissolved in chloroform

Standard range – 20 ng to 400 ng corticosterone

Calculations: A standard curve of corticosterone was used for quantification.

GLUTAMATE AND GABA ESTIMATION (Bhattacharyya et al., 2009)

Reagents:

i) Phosphate buffered saline

ii) 0.17M Perchloric acid

iii) Mobile Phase:
0.1M monosodium phosphate
0.5mM EDTA
25% (v/v) Methanol
Adjust the pH to 4.5

iv) Derivatization mixture:
50mM sodium sulphite
90mM tetraborate buffer (pH-10.4)
37mM orthopthaldehyde
5% (v/v) methanol

v) Standard: Glutamate – 2ng/ml
GABA – 2ng/ml

The amino acid standard was prepared by spiking known amount of mixed standards (10, 20, 40, 80, and 160 ng/ml of each glutamate and GABA).

Sample preparation: Tissues were washed thoroughly with ice cold PBS and 10% (w/v) homogenates were made in 0.17M perchloric acid. For complete protein precipitation, homogenates were kept for at least 30 minutes in cold condition, followed by centrifugation at 12000g for 20 minutes at 4°C. The supernatant was immediately used for neurotransmitter estimation or kept at -80°C until use.

Procedure: The amount of glutamate and GABA was assessed by reverse phase HPLC (RP-HPLC) coupled with electrochemical detector (Waters 2465; Waters Corporation, Milford, USA). Estimation was performed according to the previously described method with minor modifications in the derivatization step. Briefly, a Sunfire® C18 column (4.6 × 150 mm, particle size 5 µm) was used, and separation was carried out using mobile phase containing a solution of 0.1 M monosodium phosphate and 0.5 mM EDTA, 25% (v/v) methanol; pH was adjusted to 4.5, at a flow rate of 1.2 ml/min and an operating potential of 0.85 V. The solution was made in degassed deionized water. The derivatization reagent mixture consisted of 37 mM orthopthaldehyde (OPA), 50 mM sodium sulfite, 90 mM tetraborate buffer (which was set to pH 10.4 with sodium hydroxide, prior to addition of

OPA), and 5% methanol. For preparation of standard calibration curve, 20 µl of derivatization reagent was mixed with 1 ml of amino acid standard for 10 min. After incubation, 20 µl of sample was injected in HPLC. For sample analysis, 20 µl of the supernatant was mixed with 0.4 µl of the derivatizing reagent and incubated at room temperature for 10 min. Of the mixed supernatant, 20 µl was injected in HPLC. The standard curves were used to quantify the amount of glutamate and GABA in each sample by calculating the area under curve (AUC).

NOREPINEPHRINE, DOPAMINE AND SEROTONIN ESTIMATION (Bhattacharyya et al., 2009)

Reagents:

i) Phosphate buffered saline

ii) 0.17M Perchloric acid

iii) Mobile Phase:
32 mM Citric acid
12.5mM Disodium hydrogen orthophosphate
0.5mM Octyl sodium sulphate
0.5mM EDTA
2mM KCl
15% (v/v) Methanol
adjust the pH to 4.2

iv) Derivatization mixture:
50mM sodium sulphite
90mM tetraborate buffer (pH-10.4)
37mM orthopthaldehyde
5% (v/v) methanol

v) Standard: Norepinephrine (NE) – 1ng/ml
Dopamine (DA) – 1ng/ml
Serotonin (5HT) – 1ng/ml

The amino acid standard was prepared by spiking known amount of mixed standards (10, 20, 40, 80, and 160 ng/ml of each Norepinephrine, Dopamine and Serotonin).

Sample preparation: For Norepinephrine (NE), Dopamine (DA) and Serotonin (5HT) estimation, sample preparation was same as mentioned for glutamate and GABA estimation.

Procedure: 20 µl of deproteinized sample was injected in HPLC with Sunfire® C18 column (4.6 × 150 mm, particle size 5 µm), and separation was carried out using mobile

phase containing methanol (15% v/v) in 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, and 2 mM KCl, at a flow rate of 1.2 ml/min, an operating pressure of 3000 psi, and an operating potential of 0.61 V. The internal standard curves were prepared by spiking known amounts of mixed standard (10, 20, 40, 80, and 160 ng/ml of NE, DA and 5HT) in 1 ml. The standard curves were used to quantify the amount of neurotransmitter in each sample by calculating the AUC.

EPINEPHRINE (Ghosh et al., 1951)

Reagents:

i) 10% Trichloroacetic acid

ii) 10% Sodium Carbonate

iii) 5% Sodium Hydroxide

iv) Folin's Reagent: Mixture of equal volumes of FC reagent and D/W (1:1 ratio)

Sample preparation: Tissues were homogenized in 10% Trichloroacetic acid (5% w/v homogenate for pituitary and adrenal; 10% w/v homogenate for hypothalamus and ovary; 20% w/v homogenates for hippocampus and frontal cortex). The tubes containing homogenates were centrifuged at 10,000g for 10 minutes and the supernatant was used for epinephrine estimation.

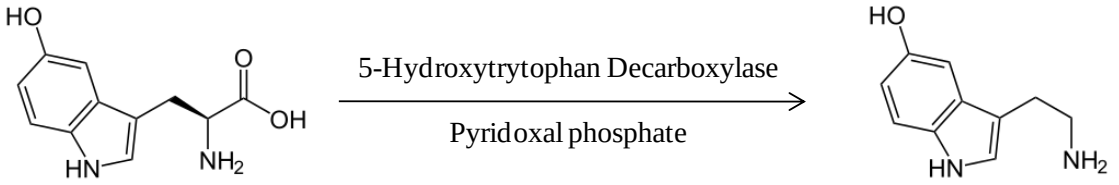
Procedure:

Reagents	**Blank**	**Test**
All the labelled test tubes were cooled at 15°C for 5 minutes		
10% sodium carbonate	0.5 ml	0.5 ml
10% TCA	0.5 ml	-
Tissue homogenate	-	0.5 ml
Keep at room temperature for 30 minutes		
Folin's reagent	0.250 ml	0.250 ml
5% NaOH	0.750 ml	0.750 ml
Mix well and measure the absorbance within one and half minute at 486 nm		

Calculation: Calculation was done according to standard graph. **Units:** µg/mg of tissue

5-HYDROXYTRYPTOPHAN DECARBOXYLASE (Sangwan et al., 1998)

Principle:



5-Hydroxytryptophan

5-Hydroxytryptamine (Serotonin)

Serotonin produced by TDC was measured fluorimetrically by extracting it into ethyl acetate.

Reagents:

i) Homogenate buffer: 0.1M Sodium Phosphate buffer (pH-7.5) containing 5mM thiourea, 1mM EDTA & 5mM β-Mercaptoethanol.

ii) Assay buffer: 0.1M Sodium Phosphate buffer (pH-8.5) containing 10% glycerol & 5mM β-Mercaptoethanol.

iii) Substrate (freshly prepared):10mM 5-Hydroxytryptophan (5-HTP) (final 1mM)

iv) Co-factor (freshly prepared): 10mM Pyridoxal Phosphate (PLP) (final 1mM)

v) 4N NaOH

vi) Ethyl Acetate

Sample preparation: 10% w/v homogenates were prepared in homogenate buffer. The tubes were centrifuged at 10,000g for 30 minutes at 4˚C and supernatant was used as enzyme source and for protein estimation.

Procedure:

Reagents	**Blank**	**Test**
Assay buffer	0.7 ml	0.7 ml
Homogenate buffer	0.1 ml	-
10mM 5-HTP	0.1 ml	0.1 ml
10mM PLP	0.1 ml	0.1 ml
Enzyme source	-	0.1 ml
The tubes were incubated at 37°C for 40 minutes		
The reaction was terminated by adding 2 ml of 4N NaOH		

Serotonin formed by the reaction was mixed with 3.5 ml of ethyl acetate. The tubes were vortexed thoroughly to ensure complete serotonin extraction. For phase separation, tubes

were centrifuged at 1000g for 10 minutes and upper organic phase was collected. Fluorescence measurement of serotonin was taken at 350nm with prior excitation at 280nm.

Calculations:

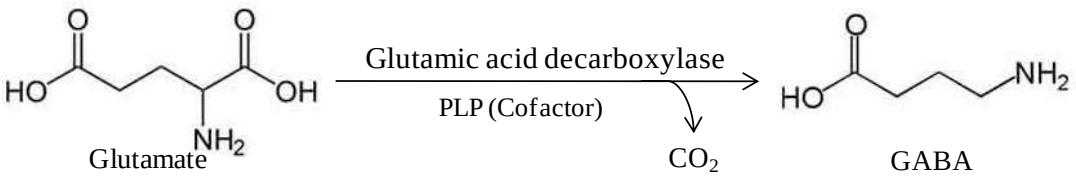
$$\text{Activity} = \frac{\text{Fluorescence units of test} \times \text{dilution factor}}{\text{Slope} \times \text{incubation time in minutes} \times \text{mg Protein}}$$

Slope was calculated from standard curve for which 1 µmol/ml Serotonin standard was prepared and fluorescence was estimated.

Units: nanomoles of product formed/min/mg of protein. **Range:** 2-40 nmoles/assay

GLUTAMIC ACID DECARBOXYLASE (GAD) (MacDonnell and Greengard, 1975)

Principle:



Reagents:

i) Homogenate solution: 0.15M KCl containing 5mM EDTA

ii) Assay buffer: 80mM Potassium Phosphate buffer (pH-6.2)

iii) 0.25M Sodium Glutamate

iv) 5mM Pyridoxal Phosphate

v) 0.014M Ninhydrin dissolved in 0.5M Sodium Carbonate Buffer (pH-9.95)

vi) 15% TCA (final 0.5%)

vii) Triton X-100

viii) Copper Tartarate reagent (100 ml)

Sodium Bicarbonate	: 160 mg
Copper Sulphate	: 30 mg
Tartaric acid	: 33 mg

Sample preparation: Tissues were homogenized (10% w/v) in homogenate solution containing 0.5% Triton-X. The homogenates were kept on ice for 30 minutes and then centrifuged at 3,000 g for 10 minutes and supernatant was used as enzyme source and for protein estimation.

Reagents	Blank	Test
Assay buffer	0.7 ml	0.7 ml
Homogenate buffer	0.1 ml	-
0.25M Na-glutamate	0.1 ml	0.1 ml
5mM PLP	0.1 ml	0.1 ml
Enzyme source	-	0.1 ml

All the tubes were incubated at 37°C for 30 minutes. The reaction of GAD was terminated by addition of 0.5 ml of 15% TCA. Following the termination, tubes were centrifuged at 5000g for 10 minutes. The GABA produced in the reaction was measured spectrofluorimetrically. 1 ml of GABA containing supernatant was mixed with 0.5 ml of 0.014M Ninhydrin solution and the tubes were kept in a water-bath set at 60ºC for 30 minutes. The samples were allowed to cool at room temperature and incubated with 5 ml of Copper tartarate reagent for 15 minutes. The fluorescence emission was measured at 451 nm with prior excitation at 377nm using Hitachi F-7000 fluorescence spectrophotometer.

Calculations:

$$\text{Activity} = \frac{\text{Fluorescence units of test} \times \text{dilution factor}}{\text{Slope} \times \text{incubation time in minutes} \times \text{mg Protein}}$$

Slope was determined from standard curve for which following procedure was employed.
GABA Stock solution: 0.1mg/ml in 10% TCA
Glutamic acid Stock solution: 15 µM glutamic acid in 10% TCA

For standard curve, 1 to 50 µg of GABA was mixed with 100 µl of 15 µM glutamic acid in a final volume of 1 ml. The GABA in the solution was estimated as described above.
Units: nanomoles of product formed/min/mg of protein.

MONOAMINE OXIDASE ACTIVITY (MAO-A and MAO-B) (Yu et al., 2002)**:**

Reagents:

i) Homogenate buffer: 0.25 M Sucrose- 0.1M Tris- 0.02M EDTA Buffer (pH-7.4)
ii) Resuspension buffer: 0.01M Sodium Phosphate buffer with 320mM sucrose (pH-7.4)
iii) Assay buffer: 0.01M Sodium Phosphate buffer (pH-7.4)
iv) Substrate: MAO-A: 4mM 5-Hydroxytryptamine (Serotonin)
MAO-B: 0.1M Benzylamine
v) 1N HCl
vi) Butyl acetate
vii) Cyclohexane

Sample Preparation: 10% w/v homogenates were prepared in homogenate buffer and cell debris were removed by centrifugation at 800 g for 10 min at 4˚C. The supernatant was collected into a fresh vial and again centrifuged at 12,000g for 20 minutes at 4˚C. The remaining pellet was dissolved in resuspension buffer and kept on ice for 20 minutes with intermittent shaking. The tubes were centrifuged at 3000g for 10 minutes at 4˚C. The supernatant obtained was used as enzyme source.

Procedure:

	MAO-A		**MAO-B**	
Reagents	**Blank**	**Test**	**Blank**	**Test**
0.1M Sodium phosphate buffer (pH 7.4)	800 µl	800 µl	800 µl	800 µl
4mM 5-Hydroxytryptamine	100 µl	100 µl	-	-
0.1M Benzylamine	-	-	100 µl	100 µl
1M HCl	200 µl	-	200 µl	-
Enzyme source	100 µl	100 µl	100 µl	100 µl

The tubes were incubated at 37ºC for 20 minutes and afterwards reactions were terminated by adding 200 µl of 1M HCl. The reaction product was extracted by vortexing for 5 minutes with 2 ml of butyl acetate for MAO-A or cyclohexane for MAO-B. Tubes were centrifuged at 3000g for 5 minutes 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.

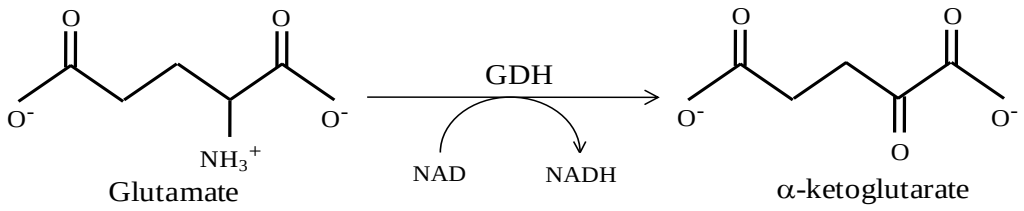
Calculations:

$$\text{Activity} = \frac{(\text{OD of Test} - \text{OD of Blank}) \times \text{Dilution factor}}{\text{Molar extinction coefficient} \times \text{incubation time (min)} \times \text{mg protein}}$$

Units = µmoles of product formed/min/mg of protein.

GLUTAMATE DEHYDROGENASE (Lee et al., 2000)**:**

Principle:



Glutamate dehydrogenase activity was measured in the direction of oxidative deamination of glutamate into α-ketoglutarate. The reaction utilizes NAD and converts it into NADH which is measured spectrophotometrically at 340 nm.

Reagents:

i) Homogenate buffer: 0.25 M Sucrose-10mM HEPES, pH-7.4

ii) Assay buffer: 50mM Tris buffer (pH-9.5)

iii) 500mM Glutamate (Freshly prepared)

iv) 50mM EDTA

v) 28mM NAD (Freshly prepared)

vi) 20mM ADP (Freshly prepared)

Sample preparation: Tissues were homogenized in 10 volume of 0.25 M Sucrose-10mM HEPES, pH-7.4. The homogenates were centrifuged at 1000g for 10 minutes at 4°C. The supernatant was collected in a fresh vial and centrifuged at 12000g for 30 minutes at 4°C to yield a mitochondrial pellet and post-mitochondrial supernatant. The pellet was resuspended in homogenate buffer and used as enzyme source.

Procedure:

Reagents	**Volume**
Tris Buffer (pH-9.5)	800 µl
500mM Glutamate	50 µl
50mM EDTA	50 µl
28mM NAD	50 µl
20mM ADP	50 µl
Absorbance of blank was measured at 340 nm	
Enzyme source	50 µl
The reaction was started as soon as the enzyme was added and increase in absorbance was monitored at 340 nm for every 10 seconds for 1minute	

Calculation:

$$\text{Activity} = \frac{(\text{Absorbance of Test} - \text{Absorbance of Blank}) \times \text{Dilution factor}}{\text{Molar extinction coefficient} \times \text{mg protein}}$$

Dilution factor: total assay volume (ml) / volume of enzyme source (ml)

Molar extinction coefficient of NADH: 6220 $M^{-1}cm^{-1}$

Unit: µmoles of NAD^+ reduced / minute/ mg protein

GABA TRANSAMINASE (GABA-T) (Basu et al., 2010)

Reagents:

i) Homogenate buffer: Na/K Buffer (50mM NaH_2PO_4, 5mM KCl, 120mM NaCl, pH-7.4)

ii) Assay buffer: (freshly prepared)
100mM potassium pyrophosphate buffer (pH-8.6) containing
   a. 5mM α-ketoglutarate
   b. 4mM NAD
   c. 3.5mM β-mercaptoethanol
   d. 10μM pyridoxal-5'-phosphate

vii) 100mM GABA (Freshly prepared)

Sample preparation: Tissue homogenates (5% w/v for pituitary and 10% w/v for other tissues) were prepared in cold Na/K buffer (pH-7.4) and centrifuged at 12,000g for 15 min at 4°C. The pellet was resuspended in Na/K buffer was used for enzyme assay.

Procedure: Tissue homogenates (50 μl) were incubated with 1 ml of assay buffer (100mM potassium pyrophosphate buffer containing α-KG, NAD, β-ME and PLP) for 15 minutes at 37°C. The absorbance of blank was measured at 340nm. 100 μl of 100mM GABA was added to the cuvette and absorbance was immediately monitored at 340nm every 10 seconds for 2 minutes.

Calculation:

$$\text{Activity} = \frac{\text{Slope} \times \text{Dilution factor}}{\text{Molar extinction coefficient} \times \text{mg protein}}$$

Slope: Change in absorbance per minute

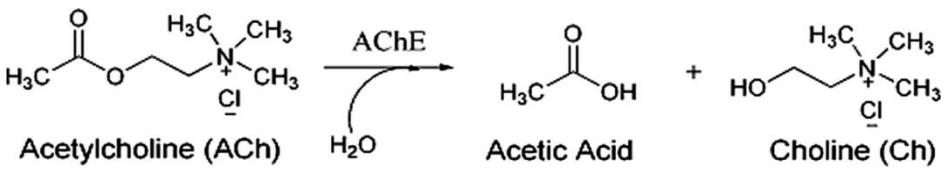
Dilution factor: total assay volume (ml) / volume of enzyme source (ml)

Molar extinction coefficient of NADH: 6220 $M^{-1}cm^{-1}$

Unit: μmoles of NAD^+ reduced / minute/ mg protein.

ACETYL CHOLINESTERASE ASSAY (Ellman et al., 1956)

Principle:



Acetylcholine is hydrolyzed by Acetycholine esterase (AChE) to acetyl Co-A and thiocholine. The catalytic activity is measured by following the increase of the yellow anion, 5-thio-2-nitrobenzoate, produced from thiocholine when it reacts with DTNB, which can be measured spectrophotometrically at 405 nm.

Reagents:

i) Assay Buffer: 0.1 M Sodium phosphate buffer pH-8.0

ii) Buffered Ellman's Reagent (Freshly prepared): 10mM DTNB, 17.85 mM $NaHCO_3$ in 0.1 M Sodium phosphate buffer pH-7.0

iii) Substrate (Freshly prepared): 75mM Acetylthiocholine Iodide in distilled water

Sample Preparation: Tissues were homogenized by adding appropriate amount of 0.1M sodium phosphate buffer (pH-8.0) to make 2.5% (w/v) homogenate of pituitary, 10% of hypothalamus and ovary, 20% of frontal cortex and hippocampus and 5% of adrenal homogenates. Homogenized samples were centrifuged at 12000g for 5 minutes and the supernatant was used for enzyme assay.

Procedure: For estimation of AChE activity, kinetic method was employed which is as follows. The tubes containing 1.5 ml of phosphate buffer (pH-8.0), 10µl of substrate and 50µl of Ellman's reagent were incubated at 25°C for 10 minutes and then absorbance of blank was measured at 405nm. Absorbance was monitored at every 2 minutes for 10 minutes after addition of 200µl of enzyme. The specific activity was calculated as follows:

Calculations:

$$\text{Specific Activity} = \frac{[\text{slope} \times \text{total volume in cuvette (µl)}]}{\text{Molar extinction coefficient} \times \text{Volume of homogenate (µl)} \times \text{mg protein}}$$

Specific activity = µmoles of substrate hydrolyzed / minute / mg of protein

Slope = Change in Absorbance per minute

Molar extinction coefficient of DTNB = 1.36×10^4

SUPEROXIDE DISMUTASE ASSAY (Marklund and Marklund, 1974)

Principle:

The autoxidation of pyrogallol was investigated in the presence of EDTA in the pH range 7.9–10.6. The rate of autoxidation increases with increasing pH. At pH 7.9 the reaction is inhibited to 99% by superoxide dismutase (SOD), indicating an almost total dependence on

the participation of the superoxide anion radical, $O_2 \cdot^-$, in the reaction. Up to pH 9.1 the reaction is still inhibited to over 90% by superoxide dismutase, but at higher alkalinity, $O_2 \cdot^-$ - independent mechanisms rapidly become dominant.

Reagents:

1. 100 mM Phosphate buffer
2. 0.2 mM Pyragallol (freshly prepared)
3. 0.1 M Phosphate buffer saline (PBS) (pH-7.4)

Sample preparation:

Hemolysate: Blood was collected in EDTA-coated vials (1mg per ml of blood) and centrifuged at 3000g for 15 minutes at 4°C. Supernatant containing plasma was separated and pellet containing erythrocytes was washed with PBS. Ice-cold distilled water was added to pellet to make hemolysate and haemoglobin was measured by Drabkin's reagent. For superoxide dismutase 0.5 gm % Hb was used for assay.

Tissue: Homogenates of pituitary, adrenal (5% w/v); hypothalamus, hippocampus, frontal cortex, ovary (10% w/v) were made in PBS and centrifuged at 3000 rpm for 10 minutes. The supernatant collected was analysed for SOD activity.

Procedure:

Reagents	**Volume**
100mM Phosphate buffer	1000µl
4mM Pyragallol	50µl
Set as Blank	
Hemolysate/tissue sample	20µl
Absorbance was monitored at 420 nm for 3 min continuously for every 30 sec.	

Calculation:

1 Unit = 50% inhibition of pyrogallol auto-oxidation/g Hb/ minute or

50% inhibition of pyrogallol auto-oxidation / mg protein/minute

CATALASE ESTIMATION (Aebi et al., 1984)

Principle: Catalase (CAT) is a heme-containing enzyme, which catalyzes dismutation of hydrogen peroxide into water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240nm, since hydrogen peroxide absorbs UV light maximally at this wavelength.

Reagents:

i) Phosphate buffered saline (pH-7.4)

ii) 50mM Phosphate Buffer (pH 7.0)

iii) 0.59M Hydrogen peroxide

Sample preparation:

Hemolysate and tissue homogenates were made as described for SOD activity. For Catalase assay 0.25 gm % Hb was used.

Procedure:

0.05M Potassium Phosphate buffer	100µl
0.59M H_2O_2	20µl
Set as Blank	
hemolysate, frontal cortex and adrenal Pituitary, hypothalamus, hippocampus, ovary	20µl 40 µl

Immediately after adding H_2O_2, the decrease in the absorbance was measured for every 5 second interval for 60 seconds at 240nm.

Calculation:

$$\text{CAT activicy} = \frac{\Delta \text{ OD } \times \text{ Dilution factor}}{\text{Molar extinction coefficient } \times \text{mg protein}}$$

Δ OD: Change in OD/min

Dilution factor: Total assay volume/ volume of hemolysate or homogenate

Molar extinction coefficient of H_2O_2: 43.6 $M^{-1}cm^{-1}$

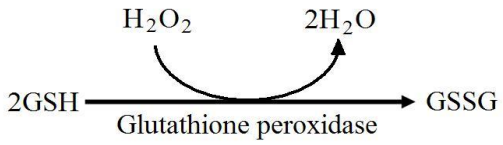
Unit: µmoles of H_2O_2 decomposed/g Hb/ min or

µmoles of H_2O_2 decomposed/ mg protein/ min

GLUTATHIONE PEROXIDASE ASSAY (Paglia and Valentine, 1967)

Principle:

Utilization of GSH by Glutathione peroxidase (GPx) enzyme is measured by means of the colored substrate produced by reaction with DTNB at 412nm.



Reagents:

i) Precipitating agent: Glacial metaphosphoric acid (1.67g), EDTA (0.2g), NaCl (30g); Make up to the total volume to 100ml with distilled water (Freshly prepared)

ii) 15mM GSH

iii) 0.4M Phosphate buffer (pH -7.0)

iv) 15mM sodium azide

v) 10mM H_2O_2

vi) 0.4M Na_2HPO_4

vii) 0.04% (W/V) DTNB (Freshly prepared)

Sample preparation: Hemolysate and tissue homogenates were made as described for SOD activity. For GPx activity 0.25 gm % Hb was used.

Procedure:

Reagent	**Blank**	**Control**	**Test**
0.4M Phosphate buffer (pH -7.0)	200 µl	200 µl	200 µl
15mM GSH	-	200 µl	200 µl
15mM sodium azide	200 µl	200 µl	200 µl
Sample	-	-	100 µl
D/DW	400 µl	200 µl	100 µl
Incubated at 37°C for 3 min.			
10mM H_2O_2	200 µl	200 µl	200 µl
Incubated at 37°C for 3 min.			
Precipitating reagent	800 µl	800 µl	800 µl
Centrifuged at 3000 g for 10 min.			
supernatant	0.6 ml		0.6 ml
0.4M Na_2HPO_2	0.6 ml		0.6 ml
0.04% (W/V) DTNB	0.03 ml		0.03 ml
Absorbance of supernatant was measured at 412 nm			

Unit: µmoles of GSH utilised/g Hb/minute or µmoles of GSH utilized/mg protein/minute.

REDUCED GLUTATHIONE ESTIMATION (Beutler et al., 1963)

Principle: 5-5' dithiobis -2-nitrobenzoic acid (DTNB) is a disulfide which is reduced by sulfhydryl compound forming an intense yellow coloured anion which can be measured at 412 nm.

Reagents:

i) Precipitating agent: Glacial metaphosphoric acid (1.67g), EDTA (0.2g), NaCl (30g) were dissolved in 100ml distilled water (Freshly prepared)

ii) 0.3M Na_2HPO_4

iii) 0.04% (w/v) DTNB (Freshly prepared)

iv) Phosphate buffered saline (PBS) (pH-7.4)

v) Standard (Freshly prepared): 20mM reduced glutathione (GSH)

Sample preparation: Hemolysate and tissue homogenates were made as described for SOD activity. For reduced glutathione (GSH) estimation, 0.5 gm % Hb was used.

Procedure:

Reagents	**Blank**	**Sample**
STD/hemolysate/tissue	-	100 µl
PBS	100 µl	-
Precipitating reagent	1.0 ml	1.0 ml
Incubated in ice for 10 mins and then Centrifuge at 3000g for 10 mins		
Supernatant	0.4 ml	0.4 ml
0.3M Na_2HPO_4	0.6 ml	0.6 ml
0.04% (W/V) DTNB	0.03 ml	0.03 ml
Measured absorbance at 412 nm		

Standard range: 10-100 µg.

Unit: µg of GSH /gm Hb or µg of GSH /mg of protein**.**

LIPID PEROXIDATION (Ohkawa et al., 1978)

Principle: Lipid Peroxidation leads to the formation of an endoperoxide - malondialdehyde (MDA), which reacts with Thiobarbituric acid (TBA) to form pink colored Thiobarbituric reactive substance (TBARS). This can be measured at 532 nm.

Reagents:

i) Phosphate buffered saline (pH-7.4)

ii) Thiobarbituric acid reagent: (Freshly prepared)
Thiobarbituric acid : 100 mg
EDTA : 46 mg
20% TCA : 10 ml
2.5N HCl : 5 ml
Total volume was made up to 20 ml with distilled water

iii) Standard**:** 10mM 1,1,3,3-TMP (Tetramethoxipropane).

Sample preparation: Hemolysate and tissue homogenates were made as described for SOD activity. For estimation of lipid peroxidation, 0.5 gm % Hb was used.

Procedure:

Reagents	**Blank**	**Standard**	**Sample**
Sample	-	100 µl	100 µl
PBS	1 ml	900 µl	900 µl
Thiobarbituric acid Reagent	1 ml	1 ml	1 ml
Kept in boiling water bath for 20 min			
Tubes were centrifuged at 3000g for 15 min			
Absorbance of the supernatant was measured at 532 nm			

Calculation: Plot the standard graph with standard TMP solution and extrapolate results of unknown sample from graph.

Standard range: 1-10 nmoles

Unit: nmoles of TBARS formed/gm Hb or nmoles of TBARS formed/mg protein.

ESTIMATION OF HEMOGLOBIN (Cook, 1985)

Principle: Hemoglobin is oxidized to methaemoglobin with alkaline ferricyanide reagent giving an intense yellow colored cyanmethaemoglobin, which is measured at 546nm. Hemoglobin estimation was carried out using Drabkin reagent kit according to manufacturer's instructions (Reckon diagnostics, Vadodara, India).

Reagents:

i) Drabkin's Reagent
ii) Standard methaemoglobin vial 60 mg %

Procedure: 1.25 ml of Drabkin's Reagent was mixed with 5µl of blood sample. The solution was mixed thoroughly and incubated for 5 minutes at room temperature. The absorbance of sample was read at 540 nm against distilled water blank.

Note: The Drabkin's standard (60mg/dl) was for single use only and it needs no further dilution. Turbidity in standard indicates contaminant and such standard should be discarded.

Calculation:

$$\text{Hb gm/dl} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \frac{\text{Total assay volume}}{\text{Volume of blood used}} \times \frac{1}{10}$$

Unit: gm Hb/dl or % gm Hb.

NITRIC OXIDE ESTIMATION (Moshage et al., 1995)

Principle: It is an indirect method to estimate NO wherein nitrite present in the sample reacts with the acidic sulphanilic acid to form a transient diazonium salt. This intermediate then reacts with the coupling agent N-naphthyl-ethylenediamine (NED) to form a stable azo-compound. The absorbance of this compound can be measured at 540nm.

Reagents:

i) 2.5% meta-phosphoric acid
ii) Sulphanilic acid
iii) N-naphthyl-ethyldiamine (NED)
iv) Phosphate buffered saline (PBS)
v) Standard stock solution: 1M sodium nitrite
vi) Griess reagent (Freshly prepared)
Sulphanilic acid : 1 g
NED : 0.1 g
Dissolved in 100ml of 2.5% meta-phosphoric acid

Sample preparation:

Serum: Blood was collected in a vial and allowed to stand at room temperature for 20-30 minutes. The serum was separated by centrifugation at 3000g for 15 minutes.

Tissues: Tissue homogenates were prepared in cold PBS with following (w/v) percentage: Pituitary-2.5%; Hypothalamus, ovary and adrenal- 10%; Hippocampus and frontal cortex – 20%. Homogenates were centrifuged at 3000g for 10 minutes and clear supernatant was used for NO estimation.

The assay for NO was carried out in 96 well plate and absorbance was measured using a microplate reader.

Procedure:

Reagents	**PBS**	**Serum or homogenate**	**Griess Reagent**
Blank	150 µl	-	150 µl
Serum	100 µl	50 µl	150 µl
Pituitary	50 µl	100 µl	150 µl
Hypothalamus	-	150 µl	150 µl
Hippocampus	-	150 µl	150 µl
Frontal cortex	-	150 µl	150 µl
Ovary	-	150 µl	150 µl
Adrenal	50 µl	100 µl	150 µl

Color was allowed to form for 10 minutes at room temperature after which absorbance was measured at 540 nm

Standard range: 1.5 to 100 nmol of sodium nitrite. **Unit:** nmol of NO per ml of serum or nmol of NO per mg protein**.**

PROTEIN CARBONYLATION (Levine et al., 1990)

Reagents:

i) Protein lysis buffer
   a. 62.5mM Tris (6.8 pH)
   b. 6M Urea
   c. 2% SDS
   d. 10% glycerol
   e. D/W

ii) 20% Trichloroacetic acid (TCA)

iii) 10mM DNPH (dinitrophenyl hydrazine) in 2M HCl

iv) 2M HCl

v) 6M Guanidium hydrochloride

vi) Ethanol

vii) Ethyl acetate

Sample Preparation: Tissues were homogenized in cold protein lysis buffer with different concentrations (w/v): 2.5% for pituitary, 5% for adrenal, 10% for hypothalamus, hippocampus and ovary and 20% for frontal cortex. The homogenates were centrifuged at 10,000g for 10 minutes and supernatant was used for estimation of carbonyl groups.

Procedure: For both blank as well as test, 50 µl of homogenate was mixed with equal volume of 20% TCA. The tubes were centrifuged at 3000g for 10 minutes at room temperature and the supernatant was discarded. 1.5ml of 2M HCl was added to the pellet of blank tube whereas 1.5 ml of DNPH solution was added into test vials. The solution was vortexed every 10 minutes for 1 hour followed by addition of 1.5 ml of 20% TCA. After 15 minutes of incubation at room temperature, tubes were centrifuged at 3400g for 10 minutes. The supernatant was discarded and pellet was washed thrice with 1 ml of ethylacetate-ethanol (1:1) mixture to remove excess of DNPH. The pellet was dissolved in 1.25 ml of 6M guanidine hydrochloride and absrorbance was measured at 370 nm.

Calculation:

$$\text{Carbonyl groups} = \frac{(\text{Absorbance of Test} - \text{Absrorbance of Blank}) \times \text{Dilution factor}}{\text{molar extinction coefficient} \times \text{mg protein}}$$

Unit: nmoles of carbonyl groups/ mg protein

Molar extinction coefficient of DNPH: 22000 $M^{-1}L^{-1}$

RNA ISOLATION

1. **Diethyl pyrocarbonate (DEPC) water**

   1 ml of DEPC was added to 1 litre of distilled water and kept overnight on magnetic stirrer for complete homogeneous solution. The solution was autoclaved and used for preparation of all the regents in RNA isolation.

2. **Tris Borate EDTA Buffer (TBE) (10X; 1L)**

Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

   Volume made to 1 litre with DEPC water

3. **RNA loading dye (5X; 1 ml)**

500 mM EDTA	8 µl
40% Formaldehyde	72 µl
Glycerol	200 µl
Formamide	30 µl
2.5% Bromophenol blue	30 µl
10X TBE	400 µl

   Volume made to 1 ml with DEPC water

4. **Other reagents used**

   TRIzol reagent (Phenol + GITC)

   Chloroform

   2-Propanol

   75% Ethanol prepared in DEPC water

   1% Agarose in TBE buffer

   25 mM Ethidium bromide

   All glassware and disposables were sterile and all surfaces involved were cleaned prior to the experiment. The isolation was carried out using a clean pair of gloves.

RNA isolation protocol

Isolation: Tissue was homogenized in TRIzol reagent (1 ml per 100 mg tissue), followed by addition of 200µl Chloroform. Tubes were allowed to stand on ice for 15 minutes with occasional gentle shaking. The mixture was then centrifuged at 12,000 g for 20 minutes at 4°C. The organic and aqueous phases separated with a visible white interphase. The upper layer was carefully taken into a new microcentrifuge tube (strictly without contamination from the lower layers) containing 500 µl isopropanol. This was gently mixed and refrigerated for at least 1 hr to allow precipitation. The tube was then centrifuged at 12,000 g for 15 min. at 4°C. The supernatant was discarded and the pellet, which contains RNA, was washed with 1 ml of 75% ethanol and centrifuged again. After a total of three such washes, the tube was opened and left on ice for the pellet to dry (Overdrying was avoided since it can lead to trouble with dissolution of the RNA in water). After excess ethanol had dried off, the pellet was dissolved in 30 µl DEPC water.

Quantification: RNA was quantified using UV spectrophotometer. 1 µl RNA solution obtained from the above isolation step was diluted in 1 ml DEPC water and read at 260 nm and 280 nm on a UV-Vis spectrophotmeter. The following formula was used to analyse the quantity of RNA:

$$RNA\ (\mu g/\mu l) = 40 \times OD_{260}$$

A ratio of the OD_{260} to OD_{280} was used to check for quality of the RNA preparation. A ratio of around 2 was accepted as indicative of good purity of the RNA solution. Further, 2 µg of the RNA was electrophoresed on a 1% Agarose gel containing Ethidium bromide. Three distinct and sharp bands reflected good integrity of the RNA.

cDNA SYNTHESIS

Isolated RNA was reverse transcribed using Verso cDNA synthesis kit (ThermoScientific, USA). The cDNA reaction was carried out for each tissue in a total volume of 20 µl as follows:

RNA (1 µg/ µl)	1 µl
Oligo dT primers	1 µl
dNTP mix	2µl
DEPC water	6 µl
Samples were heated at 65°C for 10 minutes to remove any 2° structure of RNA	
Kept on ice for 2 minutes and following components were added	

5x RT Buffer	4 µl
Reverse Transcriptase (RT)	1 µl
RT enhancer	1 µl
DEPC water	4 µl
Samples were heated at 42°C for 30 minutes	
The reaction was terminated by heating the samples at 95°C for 2 minutes	

The cDNA synthesized was stored at -20°C till further use.

REAL-TIME PCR

For the current study, all quantitative gene expression analyses were carried out by real time PCR using SYBR Green based master-mix (Takara Bio, Japan) on a QuantStudio Real Time PCR system (Life Technologies, USA). Primers were procured from IDT (CA, USA).

Real-time PCR protocol

For each reaction mix, a mix with the following composition was prepared:

Component	**Volume**
2X SYBR Green master-mix	5 µl
Forward primer (5 µM)	0.5 µl
Reverse primer (5 µM)	0.5 µl
cDNA template	1 µl
Nuclease-free water	4 µl

For every sample, each gene was assayed in triplicate. The reactions were run in 96-well plates (ABI) sealed with clear sealing films. The following program was used for amplification:

Temperature	**Time**	
95˚C	100 sec	
95˚C	10 sec	45 cycles
60˚C	30 sec (acquisition)	
72˚C	30 sec	

This was followed by a melt-curve analysis with the following program:

Temperature	**Time**
95˚C	10 sec
65˚C	60 sec
97˚C	1 sec

Data analysis: Cq values were obtained for each well; normalized Cq values were calculated by subtracting the Cq values of internal control gene or reference gene (β-Actin) from those of the target gene. Mean of these normalized Cq values were plotted.

$$\Delta Cq = Cq\ of\ target\ gene - Cq\ of\ reference\ gene$$

Moreover, fold change in expression was calculated by the ΔΔCq method of Livak and Schmittgen (2001). For fold change of gene expression in sample 2 as compared to that in sample 1:

$$\Delta\Delta Cq = \Delta Cq\ of\ gene\ in\ sample\ 2 - \Delta Cq\ of\ gene\ in\ sample\ 1$$

ΔΔCq values were plotted on a graph with vertical axis following a logarithmic scale.

SDS-PAGE AND WESTERN BLOT

Reagents:

1. **RIPA Buffer**

Tris base	50 mM
NaCl	150 mM
Triton X-100	1%
SDS	0.1%
Sodium deoxycholate	0.5%

pH set to 8.

Protease inhibitor (Sigma, USA) was added freshly before use as per manufacturer's instructions.

Tissue homogenates (5% for pituitary, ovary and adrenal; 10% for hypothalamus, hippocampus, frontal cortex) were prepared in RIPA buffer containing protease inhibitors. The samples were kept in cold condition for 1 to 2 hours to ensure complete lysis, followed by centrifugation at 10000g for 20 minutes at 4°C. Protein was estimated from the supernatant by Lowry's method.

SDS-PAGE

1. **Sample Buffer/Loading Buffer (5X)**

Tris base	250 mM
SDS	10%
Glycerol	50%
Bromophenol blue	0.1%

pH set to 6.5.

β-Mercaptoethanol was added freshly during sample preparation at a final concentration of 100 mM.

2. SDS-PAGE running Buffer (1x)

Tris base	25 mM
Glycine	250 mM
SDS	0.2%

3. Gel stock (30%)

Acrylamide	29%
Bis-acrylamide	1%

Solution was kept in dark overnight at room temperature followed by filtration through Whatman paper no. 1 before use.

4. Resolving gel buffer (12%; 20 ml)

30% Gel stock	8 ml
Distilled water	6.6 ml
1.5 M Tris Cl (pH 8.8)	5 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.012 ml

APS solution must be prepared fresh. APS and TEMED must be added just before pouring the resolving gel buffer in the PAGE assembly.

5. Stacking gel buffer (4%; 7 ml)

30% Gel stock	1.162 ml
Distilled water	4.8 ml
1 M Tris Cl (pH 6.8)	0.882 ml
10% SDS	0.07 ml
10% APS	0.07 ml
TEMED	0.007 ml

APS solution must be prepared fresh. APS and TEMED must be added just before pouring the stacking gel buffer in the PAGE assembly.

6. Coommassie stain

CBB-R250	0.1%
Methanol	40%
Acetic acid	10%

7. Destaining solution

Methanol	30%
Acetic acid	5%

Protocol for SDS-PAGE

Gel casting: Resolving gel buffer was poured between PAGE plates in the casting assembly, immediately followed by butanol. The gel was left undisturbed and allowed to fully polymerise for half an hour. The butanol was removed and gel was washed thoroughly with distilled water and stacking gel buffer was poured on top of it. The comb was inserted immediately.

Sample preparation: Sample buffer (Loading buffer) at final working concentration, protein sample as per the required loading amount and water were added to prepare the final sample. Solution was heated at 80˚C for 10 min. and placed on ice till sample was loaded onto the gel.

CBB staining: Gel was stained in CBB staining solution overnight. This was followed by destaining for 2 to 4 hours.

WESTERN BLOT

1. **Transfer Buffer**

Tris base	25 mM
Glycine	192 mM
Methanol	20%

pH set to 8.3. Methanol added fresh before use. Buffer made ice-cold for use.

2. **Tris buffer saline (10x; 1 litre)**

Tris base	24 g
NaCl	88 g

Adjust the pH to 7.4 and make up the volume upto 1 litre with distilled water

3. **Washing buffer (TBS-T)**

0.1% Triton X-100 in TBS (1x)

4. **Blocking buffer (TBS-MT)**

5% skimmed milk powder in TBS-T

5. **Anitbody dilution buffer**

BSA and Sodium azide added to TBS-T to a final concentration of 5% and 0.02% respectively.

Western blot protocol

After PAGE, the gel was equilibrated in transfer buffer for 30 min. A transfer stack was then prepared with the gel and membrane sandwiched between two Whatman filters papers (no. 1)

and a sponge pad of the same dimensions on either side. Transfer was carried out in cold condition in a transfer tank at 100 V for 90 min.

Membrane was stained in 0.5% Ponceau stain to check the quality of transfer. The reversible stain was washed off with TBS and the membrane processed for antibody probing.

Antibody probing: Membrane was blocked with blocking buffer at room temperature for 1 hour. It was then incubated in primary antibody at 4˚C overnight. The membrane was washed with washing buffer thrice for 10 minutes each time. This was followed by incubation with HRP-conjugated secondary antibody for 1 hour. Membrane was washed again in washing buffer thrice as in the previous step. Bands were developed by treating the membranes with ECL reagent (Immobilon Western Chemiluminescent HRP substrate, Millipore, USA) as per the manufacturer's instructions, followed by exposing it to the X-ray film for 30 seconds. The bands on the X-ray film was developed by immersing the X-ray film into developer solution for 1 minute followed by dipping it into fixer solution as per the instructions (Fujifilm, Japan). The protein bands were quantified using ImageJ software.

BEHAVIOUR ANALYSIS

1. **Tail suspension test** (Castagne et al., 2010)

   In this test, the animals were suspended at a height by three-fourths length of their tail (Figure 2.1) and assessed for the amount of time that they were immobile. If an animal has depression like symptoms it will remain immobile while a normal animal will try to free itself. The animals are subjected to a pre-test 24 hrs prior to the final test so as to acclimatize them to the novel environment. The behaviour of the animal was videographed for 5 minutes and time spent immobile was calculated.

2. **Forced swimming test** (Castagne at al., 2010)

   Requirements: Transparent cylinder of Height 60 cm; Diameter 24 cm; Video Camera.

   In this test, the animals were subjected to an inescapable cylinder filled with water and assessed for the amount of time spent immobile or floating as compared to swimming behavior (set-up shown in Figure 2.2). If an animal is depressed, it exhibits more of immobility or a floating behavior to just keep it alive rather than a normal animal that will show swimming behavior to try to escape from the situation. The animals were subjected to a pre-test of 5 minutes, 24hrs prior to the final test. The behaviour of the animal was videographed for 15 minutes and time spent immobile was calculated.

3. **Open field test** (Bailey et al., 2009)

   Requirements: Wooden cubicle of Height 35 cm and length and breadth 75 X 75 cm; Video Camera. The field was divided into 16 squares of equal area (Figure 2.3).

   The animals were assessed for their exploratory behaviour. This test is used to study exploratory as well as anxiety like behaviour in rodents. The animals when exposed to a novel environment, try to explore it. However, an anxious or depressed animal will not exhibit such behaviour. The animal was kept in the centre square of the field and behaviour was videographed for 10 minutes. Animals were scored on the basis of line crossing and rearing abilities.

4. **Sucrose preference test** (Monteggia et al, 2007)

   Requirements: Sipper-bottles; 1% Sucrose solution in drinking water

   The sucrose preference test was used to assess anhedonia (lack of interest to reward stimuli). Each animal was housed in a separate cage, containing two drinking water bottles, for one week for acclimatization. Animals were fasted for 12 h prior to the test. The test was conducted over a 48-hour period with using a two-bottle test, one with 1% sucrose solution and the other with water. To prevent potential side preference of drinking, the position of the bottles was changed after 24 hours. The consumption of water or sucrose solution was measured daily between 8:00 to 9:00 a.m. for the duration of test.

   Calculation:

$$\text{Sucrose preference (\%)} = \frac{[100\times \text{volume of sucrose consumed}]}{\text{total volume consumed}}$$

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. For each experiment 4 to 6 animals were used from both the groups. All values are presented as Mean$\pm$SEM. P values $\leq$ 0.05 were deemed to be statistically significant.

Figure 2.1: Set-up for tail suspension test

Figure 2.2: Apparatus for forced-swimming test

Figure 2.3: Arrangement for open-field test.

Table 2.1: Primer sequences of rat targeted genes

Target Genes	Primer sequence
α1-AR	F:5' ACCAGCTCCGGTGAACATTT 3' R:5' GCCGCCCAGATATTGCAGAA 3'
11βHSD1	F: 5' TGTCTCGCTGCCTTGAACTC 3' R: 5' AGTCCACCCAAGAGCTTTCC 3'
3βHSD1	F: 5' TCTACTGCAGCACAGTTGAC 3' R: 5' ATACCCTTATTTTTGAGGGC 3'
5HT1A	F:5'CCCCCCAAGAAGAGCCTGAA3' R:5'GGCAGCCAGCAGAGGATGAA3'
Actb	F: 5' AGGCCCCTCTGAACCCTAAG 3' R: 5' GGAGCGCGTAACCCTCATAG 3'
ACTHR	F: 5' GGCAGAAACTGGATCCTTCCG 3' R: 5' TCTTGCTGTGTTGTTGGTGTG 3'
AR	F: 5' GGAAGCACTGGAACATCT 3' R: 5' GTAGTCGCGATTCTGGTA 3'
BDNF	F: 5' CCATAAGGACGCGGACTTGT 3' R: 5' GAGGCTCCAAAGGCACTTGA 3'
CAT	F: 5' CTGACCAAGGTTTGGCCTCA 3' R: 5' CTGGGTAAGCAAAAAGGCGG 3'
COMT	F:5'GACGCGAAAGGCCAAATCAT 3' R: 5' ACGTTGTCAGCTAGGAGCA 3'
CRH	F: 5' CAGAACAACAGTGCGGGCTC 3' R: 5' CAAGGCAGACAGGGCGAC 3'
CRHR1	F: 5' TTCTGGATGTTCGGTGAGGG 3' R: 5' AAGGTACACCCCAGCCAATG 3'
CRHR2	F: 5' TCATTGGATGGTGCATACCC 3' R: 5' ATGATGGGGCCCTGGTAGAT 3'
Cyp11b1	F: 5' TGAAACCTGAGAGCGTGACA 3' R: 5' CGCGATACACATTCTTGATACAT 3'
Cyp17a1	F: 5' ATGATCCAAAACTGACCGCC 3' R: 5' AACCCTTTATCACCTCCAAGCC 3'
Cyp19a1	F: 5'GCTTCTCATCGCAGAGTATCCGG 3' R: 5'CAAGGGTAAATTCATTGGGCTTGG 3'
Cyp21a1	F: 5' TTTCGGAGACAAGCAGGACAG 3' R: 5' CCTGGATTGGGGAAGAACCT 3'
D2R	F:5' TGAACCTGTGTGCCATCAGCA 3' R:5' TTGGCTCTGAAAGCTCGACTG 3'
Dyn	F: 5' CCATCAACCCCCTGATTTGC 3' R: 5' TTGGTCAGTTCCGTGTAGCC 3'
ERα	F: 5' CCAGTCGAGCATCACTTA 3' R: 5' TGGCGTCGATTGTCAGAA 3'
ERβ	F: 5' TAGACAACCGCCATGAGT 3' R: 5' CACAACTGCTCCCACTAA 3'
FSHβ	F: 5' AGGAAGAGTGCCGTTTCTGC 3' R: 5' GCTGTCACTATCACACTTGC 3'
FSHR	F: 5' CTCATCAAGCGACACCAA 3' R: 5' GGAAAGGATTGGCACAAG 3'
GABAB1	F:5'CGCTACCATCCAACAGACCA3' R:5'TGTCAGCATACCACCCGATG3'
GnRH1	F: 5' CCGCTGTTGTTCTGTTGACTG 3' R: 5' TCACACTCGGATGTTGTGGA 3'
GnRHR	F: 5' TCTGCAATGCCAAAATCATC 3' R: 5' GTAGGGAGTCCAGCAGATGAC 3'

Target Genes	Primer sequence
GPR147	F: 5’ AGACAGTATGGAGGCGGAGC 3’ R: 5’ GTCTCCACATCACTCCCGTTC 3’
GPR54	F: 5’CTACATCGCTAACCTGGCGG 3’ R: 5’TCATGGCTGTCAAAGTGGCA 3’
GPx	F: 5’ CCGGGACTACACCGAAATGA 3’ R: 5’ TGCCATTCTCCTGATGTCCG 3’
GR	F: 5’ CCAAAGCCGTTTCACTGTCC 3’ R: 5’ AGCCGAAAGTCTGTTTCCCC 3’
IDO1	F: 5’ CTGGTGGGGACTGCGATAAA 3’ R: 5’ CACGAAGTCACGCATCCTCTT 3’
IFNγ	F: 5’ GGCAAAAGGACGGTAACACG 3’ R: 5’ GTTCACCTCGAACTTGGCGA 3’
IL1β	F: 5’ GCAGCTTTCGACAGTGAGGAG 3’ R: 5’ GCTTCTCCACAGCCACAATG 3’
IL10	F: 5’ GACGCTGTCATCGATTTCTCCC 3’ R: 5’ GTAGATGCCGGGTGGTTCAAT 3’
IL2	F: 5’ CCAAGCAGGCCACAGAATTGA 3’ R: 5’ GGCTCATCATCGAATTGGCAC 3’
IL6	F: 5’ TTCTCTCCGCAAGAGACTTCCAG 3’ R: 5’ ATACTGGTCTGTTGTGGGTGG 3’
IR	F: 5’ GCCTGGGCAACTGTTCAGA 3’ R: 5’ GTTTCGACAGGCCACACACTT 3’
Kiss1	F: 5’ CTACATCGCTAACCTGGCGG 3’ R: 5’ TCATGGCTGTCAAAGTGGCA 3’
LHβ	F: 5’ CTGTCCTAGCATGGTTCGAGT 3’ R: 5’ AGTTAGTGGGTGGGCATCAG 3’
LHR	F: 5’ GCTTTTACAAACCTCCCTCGG 3’ R: 5’ GCGAGATTAGAGTCGTCCCA 3’
M2-AchR	F:5’CACAGTTTCCACTTCGCTGG 3’ R:5’ CACCTTTTTGGGCCTTGGTG 3’
NKB	F: 5’ GAGGAACAGCCAACCAGACA 3’ R: 5’ GAGTGGAGTGCTTTTCTGCAC 3’
NMDAR	F:5’ ACACCGACCAAGAAGCCATC 3’ R:5’ GGACTCATCCTTATCCGCCA 3’
PR	F: 5’ CGCCCTACCTCAACTACCTG 3’ R: 5’ TTCTCAGACGACATGCTGGAC 3’
RFRP3	F: 5’ AGAGCAACCTAGGAAACGGG 3’ R: 5’ GAAGGACTGGCTGGAGGTTT 3’
SERT	F: 5’ AGACAGGGGTGTGGGTAGAT 3’ R: 5’ TGACGAAGCCAGAGACGAAG 3’
SOD1	F: 5’ AGGGCGTCATTCACTTCGAG 3’ R: 5’ CCTCTCTTCATCCGCTGGAC 3’
StAR	F: 5’ AGTGACCAGGAGCTGTCCTA 3’ R: 5’ GCGGTCCACCAGTTCTTCATA 3’
TH	F: 5’ CATTGGACTTGCATCTCTGG 3’ R: 5’ GTTCCTGAGCTTGTCCTTGG 3’
TNFα	F: 5’ ATGGGCTCCCTCTCATCAGT 3’ R: 5’ GCTTGGTGGTTTGCTACGAC 3’
TrkB	F: 5’ CACACACAGGGCTCCTTA 3’ R: 5’ AGTGGTGGTCTGAGGTTGG 3’
Ucn2	F: 5’ CGTTGGCATAACGCCTCAC 3’ R: 5’ GGACACAGAGCTGGGAGTTG 3’

Table 2.2: Details of antibodies used:

Primary Antibody for	Procured from	Dilutions used
β-Actin	Cell signaling technology # 4967	1:10000
Androgen receptor (AR)	Prof. Rakesh Tyagi, JNU, India	1:5000
Aromatase	Cell signaling technology # 14528	1:2500
Caspase-3	Sigma #SAB5600063	1:2000
Estrogen receptor-α (ERα)	Santacruz Biotech #543 (Dr. Anil Pillai, Medical college of Georgia)	1:2000
Estrogen receptor-β (ERβ)	Abcam #3577 (Dr. Anil Pillai, Medical college of Georgia)	1:5000
Phospho-CREB (Ser133)	Cell signaling technology # 9198	1:5000
Phospho-p44/42 MAPK (ERK1/2)	Cell signaling technology # 9101	1:5000
Steroid acute regulatory protein (StAR)	Prof. D. M. Stocco, Texas Tech University, Texas, USA	1:5000