ज्ञान-विज्ञान विमुक्तये

UNIVERSITY GRANTS COMMISSION
BAHADURSHAN ZAFAR MARG
NEW DELHI- 110002

Project completion report

1.	UGC Reference No. &Date	**No. F. 37-185/2009 (SR)**
2.	Name of the Principal investigator	**Prof. Gurcharan Kaur**
3.	Department and University/College where the project has undertaken	Department of Biotechnology, Guru Nanak Dev University, Amritsar
4.	Title of the Project	**Reproductive neuroendocrine dysfunction in cycling female rats treated with antiepileptic drugs.**
5.	Date of Implementation	March 2010
6.	Tenure of the Project	Three Years
7.	Grants Received	Details submitted by Grants section
8.	Objective of the Project	Appendix I
9.	Methodology	Appendix II
10.	Work done so far (Please give details)	Appendix III
11.	Has the progress been according to original plan of work and towards achieving objectives,	Yes
12.	Whether Project work was delayed.	No
13.	Please indicate the difficulties, if any, experienced in implementing the project	None
14.	Collaboration, if any (with Department, University, Industry etc.)	No
15.	Ph.D Enrolled	YES
16.	Details of the Publication resulting from the project work (please attach re-prints) letter of Acceptance of paper communicated	Dinesh Lakhanpal, Hardeep Kataria and Gurcharan Kaur (2011) Neuroendocrine plasticity in GnRH release is disrupted by valproic acid treatment of cycling rats. Acta Neurol. Belg. 111, 121-129.
17.	Any other information which could help in evaluation of work done on the project	Nil

Reproductive neuroendocrine dysfunction in cycling female rats treated with antiepileptic drugs.

Appendix I

OBJECTIVES

To test whether Anti Epileptic Drugs (AEDs) which exert anticonvulsive effects through GABAergic system affect reproductive health by their effects on the hypothalamo-hypophyseal-gonadal (HPG) axis at the level of the hypothalamic GnRH synthesis and/or release, the following parameters were proposed to be studied after treating young adult female rats with different AEDs:

I. Estrous cyclicity and ovarian histology
II. Immunofluorescence staining for GnRH, GABA and PSA-NCAM and western blotting for GAD and PSA-NCAM in Median eminence-arcuate region of hypothalamus
III. GnRH mRNA expression in Median eminence-arcuate region of hypothalamus
IV. ELISA based assay of plasma LH and estrogen levels
V. Hypothalamic explant cultures to study the effect of AEDs on GnRH release under *in-vitro* conditions

Appendix II

Methodology:

Experimental animals:

Wistar strain young adult virgin cycling female albino rats in the age group of 3-4 months and weighing 150-200 gm were used for these experiments. Animal care and procedures were followed in accordance with the guidelines of Animal Ethical Committee, Guru Nanak Dev University, Amritsar. The estrous cycle was monitored by daily inspection of vaginal cytology in female rats and the animals were treated with AEDs, Sodium Valproate (300 mg/Kg body weight) and Phenytoin (100 mg/Kg) at 10.00 am every day for 2 months. The rats of age matched control group were treated with 0.9N saline and used as control.

Ovarian Histology:

After 2 months of drug treatment, these animals (n=5) along with control female rats in proestrous phase were sacrificed via anesthetic overdose with sodium pentobarbital (100mg/Kg). The animals were weighed before sacrificing and ovaries were removed thereafter. Fat tissue attached to ovaries was removed and ovaries were weighed before putting them into formalin solution for fixation. For microscopical evaluation of ovaries, the

mid-ovarian sections were cut for studying follicular morphology by Eosin and Haematoxylin staining.

LH and Estradiol ELISA:

Blood sample was collected from each group (n=5 each) i.e. treated and control and allowed to clot for 30 min. The samples were then centrifuged for 15 min at 5000 rpm. Serum was separated and immediately assayed for LH and Estradiol ELISA according to the protocol given with ELISA kit (Cusabio).

Immunofluorescent staining of GABA, GAD, GnRH, PSA-NCAM and GFAP:

For immunofluorescence labeling of GABA, GAD, GnRH, PSA-NCAM and GFAP rats (n=5 each) were perfused after 2 months of treatment with Phenytoin (100 mg/Kg). The control animals were treated with 0.9N saline. 30µm thick coronal sections of brain were cut on cryotome and processed free floating. The sections were treated in following manner: three 15 min washes in 0.01M PBS, pH 7.4; 30 min in 0.3% TritonX-100 in 0.1M PBS for permeabilization. The sections were washed with 0.1% PBST for 15 min. After washing, sections were preincubated for 1h at room temperature in a blocking solution (5% NGS in PBS with 0.3% Triton X-100). The sections were then incubated with primary antibodies, which were mouse monoclonal anti-GABA (sigma) and rabbit monoclonal anti-GAD (Sigma), monoclonal anti-PSA-NCAM (AbCys), mouse monoclonal anti-GFAP (Sigma) and rabbit monoclonal anti-GnRH (Sigma) antibody with appropriate dilution of anti-GABA (1:500), anti-GAD (1:2000), anti-PSA-NCAM (1:500), anti-GFAP (1:500) and anti-GnRH (1:2000) in 0.1% Triton X-100 and 5% NGS for 48 hours at 4°C. Specificity of staining was determined by negative staining control procedures without adding primary antibodies to incubation buffer. The sections were washed for 5 min with three changes of 0.1% PBST at room temperature. The sections were incubated with specified secondary antibodies anti-mouse IgG for GABA (Alexafluor 488, Invitrogen with dilution of 1:500), anti-rabbit IgG for GAD (Alexafluor 488, Invitrogen with dilution of 1:500), anti-mouse IgM for PSA-NCAM (Alexafluor 488, Invitrogen with dilution of 1:500), anti-rabbit IgG for GFAP (Alexafluor 488, Invitrogen with dilution of 1:500), anti-rabbit IgG for GnRH (Alexafluor 568, Invitrogen with dilution of 1:500), in 0.01M PBS for 2 hours at room temperature. Sections were washed for 15 min with three changes of 0.1% PBST. Tissue sections were then mounted on glass slides and coverslipped using the Fluoromount (Sigma) anti-fading mounting medium for fluorescent detection.

Western blotting of GAD, GFAP and PSA-NCAM:

Tissue from ME-ARC region of hypothalamus (n=5 each) was pooled from treated and control young female rats to isolate protein using homogenizing buffer from three different sets of animals for control and test each. The lysate was centrifuged at 10,000Xg for 10 min at 4oC. Supernatant was used for protein estimation by Bradford's method and supernatant was mixed with 6X sample buffer. Protein lysate (20-30 µg) was resolved on one-

dimensional 10% SDS-PAGE for GAD and GFAP and and 8% SDS-PAGE for PSA-NCAM. The separated proteins were then blot transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech) using the semidry Novablot system (Amersham Pharmacia) at 25 V for 2 hours. Subsequently, membranes were blocked for 2h at room temperature with 5% skimmed milk solution in TBST buffer (13.3 mM Tris. 0.8% NaCl (w/v); pH 7.6) containing 0.1% Tween-20 (Sigma) and incubated with rabbit anti-GAD and mouse anti-GFAP (1:5000), and mouse anti-PSA-NCAM (1:2000) monoclonal antibodies overnight at 4^{o}C. After three washes of 10 minutes each in 0.1% TBST, conjugated horseradish peroxidase (HRP) secondary antibodies anti-rabbit IgG, anti-mouse IgG and anti-mouse IgM (1:7000) was added for 2h at RT. Immunoreactive bands were visualized using ECL Plus western blot detection system (Amersham Biosciences) according to the manufacturer's instructions and further exposed to Hyper film ECL. The films were developed and the antibody-labeling intensity (ROD) analyzed using Gel documentation system (AlphaEaseTM, Alpha Innotech Corporation). In order to account for potential variations in protein estimation and sample loading, expression of each protein was compared to that of α-tubulin in each sample by stripping the blot in 62.5 mM Tris, 2% SDS and 100 mM 2-mercaptoethanol (pH 6.7) for 30 min at 50ºC and re-probing with an anti- α -tubulin antibody (1:5000).

GnRH, PST AND GAD mRNA expression by RT-PCR:

The expression values of PST, GFAP, GnRH, NCAM, GAD65 and GAD67 was quantified by semi-quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) analysis, using β-actin mRNA as an internal standard. Tissue from hypothalamus (n=5) was pooled from AEDs treated and control rats to isolate RNA using TRI reagent (Sigma). For GnRH mRNA analysis whole of hypothalamus was used (n=3 for each group). RNA concentration and purity were determined based on measurement of the absorbances at 260 and 280 nm. The integrity of the isolated RNA was checked by nondenaturing agarose gel electrophoresis. Equal amounts of RNA were used for cDNA synthesis. cDNAs were synthesized in 20µl reactions containing 200 units M-MLV reverse transcriptase, 4µl 5Xfirst strand buffer, 2µl DTT (0.1M) (Invitrogen), 5 µg of total RNA, 1 mM each of dNTPs (Amersham), 20 units of ribonuclease inhibitor (Sigma), and 250ng pd(N)6 random hexamers (MBI, Fermentas). 2 µl of cDNA was amplified in a 50 µl PCR reaction mixture containing two units Taq polymerase, 5 µl 10X PCR buffer, 1.5µl of 50mM $MgCl_2$ (Invitrogen), 1µl of 10mM dNTP mix (Amersham), and 20pM respective primers as listed in Table 1. Cycling conditions were comprised for an initial denaturation of 3 min at 94°C followed by 35 cycles of amplification (at 94°C for 40 sec, 55°C for 45 sec and 72°C for 1 min) and final elongation step at 72°C for 10min. To control the PCR reaction components and the integrity of the RNA, 2 µg of each cDNA sample was amplified separately for β-actin specific primer. The primer sequences and product sizes were as follows:

Table-2 : RT-PCR Primer Sequence

Marker	Product size (in bp)	Sequence
PST	500	forward 5' TAA GGT GCA ATC TAG CTC CTG TGG TGG 3' reverse 5' GCA TCC TGT GAG GAC TGG CGT TGG AAA 3'
GnRH	124	forward 5'GGC AAG GAG GAG GAT CAA A 3' reverse 5'CCA GTG CAT TAC ATC TTC TTC TG3'
GAD65	437	forward 5' GGC TCT GGC TTT TGG TCC TT 3' reverse 5' TGC CAA TTC CCA ATT ATA CTC TTG A3'
3'GAD67	302	forward 5'GCT GGA AGG CAT GGA AGG TTT TA 3' reverse 5'AAT ATC CCA TCA CCA TCT TTA TTT GAC C 3'
β-actin	291	forward 5'TCA CCC ACA CTG TGC CCA TCT ACG A 3' reverse 5'CAG CGG AAC CGC TCA TTG CCA ATG G 3'

A 6µl aliquot from each PCR reaction was electrophoresed in a 2% agarose gel containing 0.2 µg/ml ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, the PCR bands on the photograph of the gel was scanned by a densitometer linked to a computer analysis system. PST, GnRH, GAD65 and GAD67 signal was normalized relative to the corresponding β-actin signal from the same sample and the data was expressed as the PST/ β-actin ratio.

Appendix III

WORK DONE SO FAR

Experimental data of Sodium valproate (VPA):

Estrogen levels in VPA treated test group: Serum estradiol concentrations measured by ELISA assay shows significant decrease in VPA treated group (19.4±2.1 pg/ml) as compared to vehicle treated control proestrous phase rats (100.1±6.9 pg/ml).

Effect of VPA treatment on GnRH and GFAP at protein and mRNA level in hypothalamus: Immunofluorescence data indicated that GnRH expression was significantly higher in the ME-ARC region of the hypothalamus in the vehicle treated control proestrous group as compared with the VPA treated test rats (Fig. 1C, 1G). GnRH axons were co-distributed with the glial elements in the internal zone of the ME-ARC region in the control group, whereas GFAP staining was observed in both internal and external zones of the ME-ARC region in test group rats (Fig. 1A, 1E), thus indicating reduced glial apposition with GnRH axon terminals in the parenchymatous space to facilitate GnRH release in the control group and higher glial apposition in the test group. Using quantitative immunofluorescence analysis of staining intensity measurements, we showed a statistically significant increase in the GFAP staining in the outer zone of ME-ARC region in the test group (Fig. 1B, 1F). The quantitative analysis of immunoblots also revealed a significantly higher GFAP protein content from the ME-ARC region of VPA treated test rats (Fig. 1H). FISH and RT-PCR data indicated that GFAP mRNA expression was significantly higher in the VPA treated rats (Fig.2A, B, C and D). GnRH mRNA expression quantified by RT-PCR showed higher expression in the vehicle treated proestrous rats (Fig. 2E).

Fig

GAD65 and GAD67 protein and mRNA expression in hypothalamus: GABAergic neurons were found to show significantly higher immunoreactivity for GAD in ME-ARC region of the hypothalamus (Fig.3D) and lower GnRH-ir (Fig.3E) in VPA treated test group as compared to vehicle treated control proestrous rats. A statistically significant increase ($P<0.05$) in the GAD staining was observed in the ME-ARC region from the VPA treated test rats (Fig.3C). Western blot hybridization signals from ME-ARC region revealed a significantly higher GAD65 and GAD67 protein content from test rats (Fig. 3F) which was further supported by FISH data (Fig. 4). GAD65 and GAD67 mRNA expression quantified by semi quantitative RT-PCR analysis also showed higher expression of GAD in the ME-ARC region of VPA treated test rats (Fig. 4D, 4H).

PST mRNA expression using FISH and RT-PCR: FISH data indicated that PST mRNA expression was significantly higher in the ME-ARC region in the vehicle treated control proestrous group (Fig. 5A) as compared to the VPA treated test rats (Fig. 5B). Quantitative immunofluorescence data is presented in Fig. 5C. RT-PCR data further confirmed higher expression of PST in the ME-ARC region of hypothalamus in control proestrous rats (Fig. 5D).

Experimental data of Phenytoin (PHT)

Body Weight, Estrous Cyclicity, Ovarian Weight and Histology

The drug treated animals showed increase in body weight as compared to saline treated animals (Fig 1C, D). AEDs treatment of cycling female rats disrupted estrous cycle after 12–14 days of daily drug treatment. The ovarian weight of drug treated animals was

observed to be little higher than control group (Fig 1E, F). The AEDs treated rats were found to have significantly higher number of ovarian cysts per mid-ovarian section as compared to vehicle treated control rats (Fig 1G-J).

LH and estradiol level in Phenytoin treated and control rats:

Serum LH and estradiol levels were assayed from test and control rats. Serum LH level was significantly reduced after 2 months of drug treatment as compared to control animals (Fig 1A). Further, Serum estradiol concentration in drug treated female rats was also found to be reduced as compared to saline treated control rats (Fig 1B).

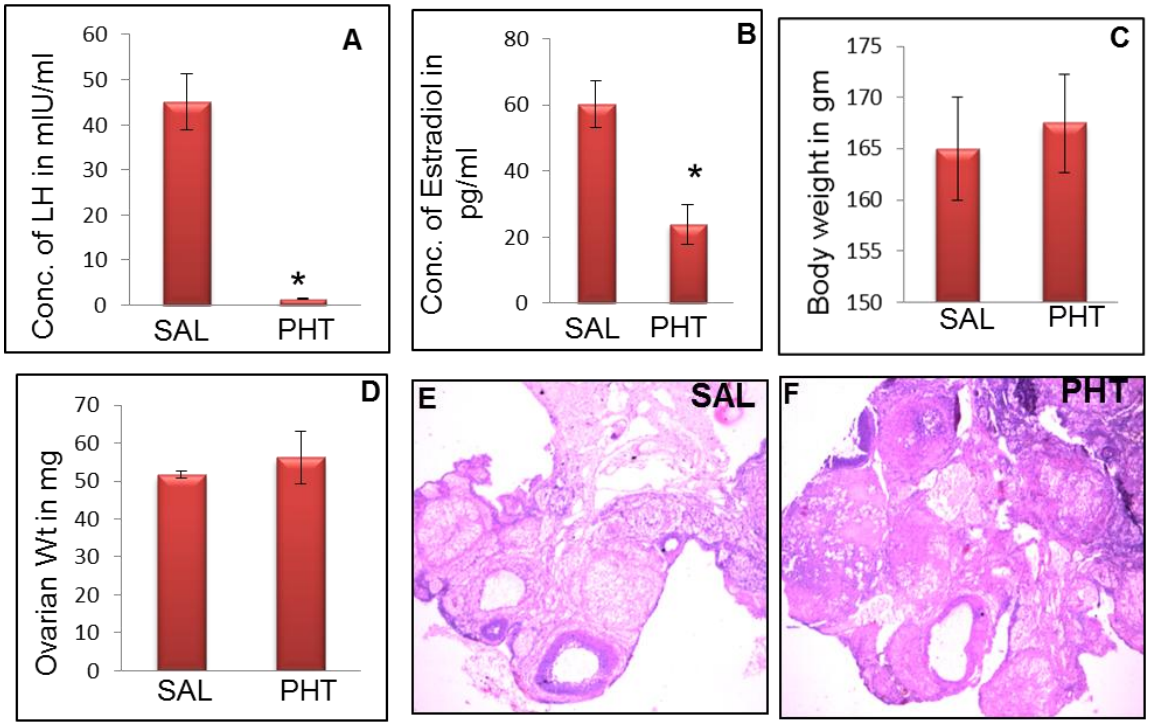

Fig 1. Comparison of Body weight gain (in gm), ovarian histology, ovarian weight (in mg), Serum estradiol (in pg/ml), LH (in mlU/ml) in saline (SAL) and drug treated (TPM and GBPT) female rats (n=5 each). (A and B) Serum LH and estradiol level was reduced in test animals than control group. (C and D) Increased ovarian weight and body weight in drug treated rats. (E-F) Ovarian sections show normal follicles in control rats as compared to large cyst in drug treated animals.

GnRH and PSA-NCAM Expression in AEDs Treated Test and Control Proestrous Rats:

GnRH-ir was found to be reduced in test group as compared to control animals (Fig 2A and B). Double staining for GnRH and PSA-NCAM in ME-ARC region from control group revealed that heavy PSA-NCAM expression was co-localized with GnRH immunoreactivity in co-localized areas as compared to low staining intensity in test group (Fig 2E and F). Dense PSA-NCAM immunoreactivity was identified along the lateral border of the external zone of the ME-ARC region from control group, as compared to test group (Fig 2C and D).

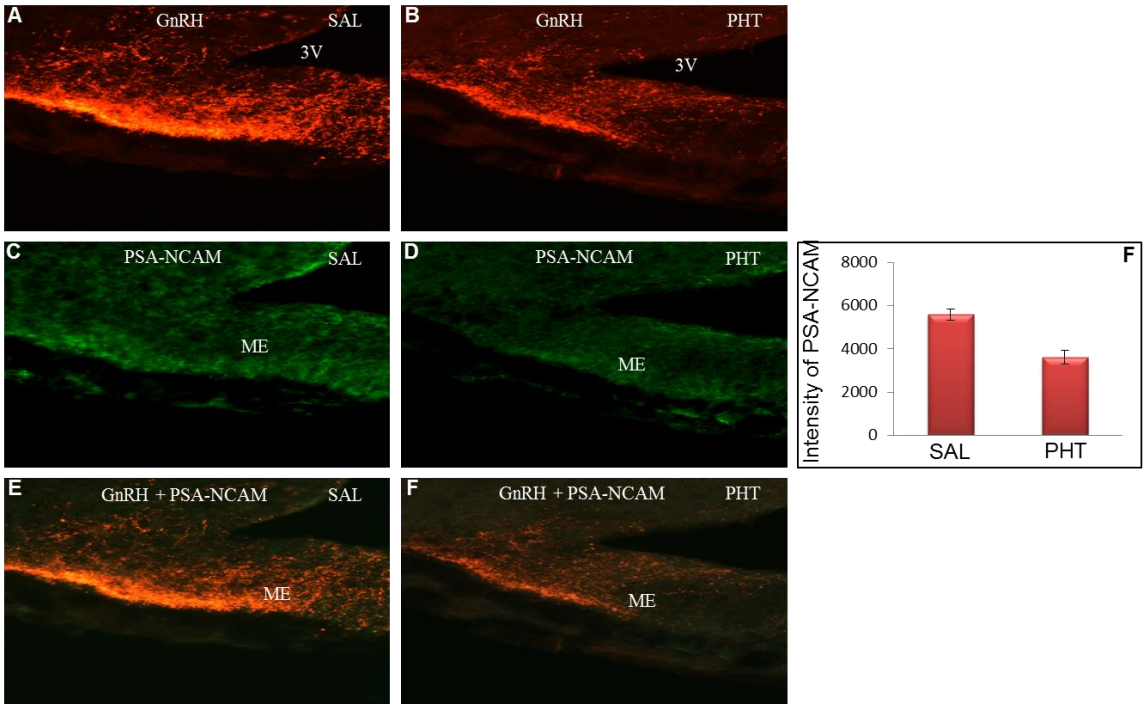

Fig 2. Immunofluorescent images of 30 µm thick coronal section through the ME-ARC region of the hypothalamus; double immunostaining for GnRH and PSA-NCAM is shown for the (E) vehicle treated control proestrous (F) PHT treated test rats. The intense PSA-NCAM and GnRH dual immunostaining was located along the external zone of the lateral portion of the ME-ARC region from control as compared with test rats. Both immunoreactivities appear to be co-localized in the ventral arcuate nucleus and in the internal and external zone of the ME-ARC region. (C-D) show single immunostaining for PSA-NCAM and (A-B) for GnRH. Intensity measurement data of the PSA-NCAM (F) immunoreactivity in the ME-ARC region of vehicle treated control proestrous and drug treated test. For each type of data analysis, measurements were performed on five to eight sections each from four to five test and control rats. All statistical analyses were performed using Student's t-test. Values are means ±S.E.M; * = $P < 0.05$.

Expression of GnRH and GFAP in drug treated and proestrous control rats:

The astro-glial cells were seen to lose their star shaped structure which may be due to retraction of their processes surrounding GnRH axon terminals. Co-expression of GnRH and GFAP in drug treated and control rats are shown in Fig 3. The present data showed extended processes of astro-glial cells surrounding the GnRH axon terminals in median eminence region of test group (Fig 3E-F). GnRH axons were co-distributed with the glial elements in both internal and external zone of median eminence in the PHT treated rats (4D), whereas, GFAP expression was highly reduced and restricted mainly to the internal zone of median eminence region in control rats (Fig 4C).

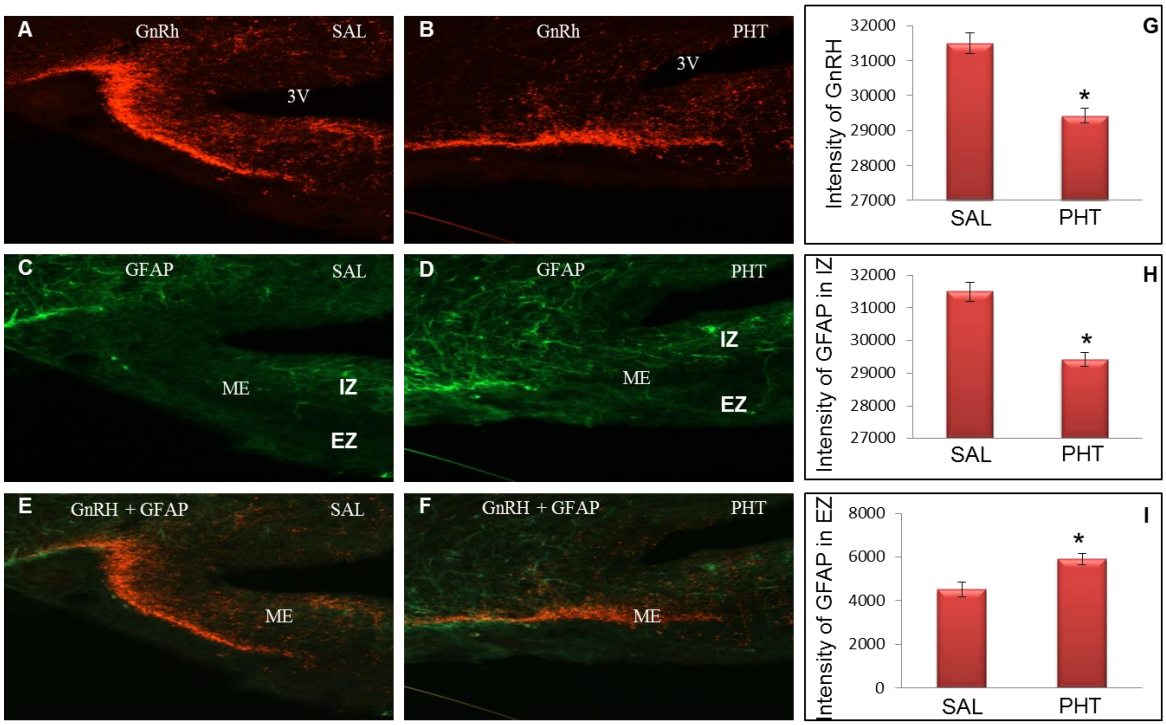

Fig 3. Immunofluorescent images of 30µ thick coronal sections from median eminence (ME) region of vehicle treated control (SAL) and Phenytoin (PHT) female (n=5 each) rat brain. The immunostaining in the ME region for GnRH and GFAP is shown for GnRH (A-B), GFAP (C-D) as well as dual immunofluorescence of GnRH and GFAP (E-F) for SAL and PHT treated animals, respectively. GnRH-ir was reduced in test rats as compared to their corresponding control rats (G). GFAP immunostaining is visible in both internal and external zone of ME in PHT treated rats (D), whereas, GFAP-ir is reduced and restricted to internal zone of median eminence in SAL (C). (H and I) depict relative intensity measurement ±SEM of GFAP immunofluorescence from internal and external zone of ME. *=pValue<0.05.

Expression of GnRH and GABA in drug treated and proestrous control rats:

In AED treated test group, GABAergic neurons were found to showed higher immunoreactivity in ME-ARC region of the hypothalamus as compared to vehicle treated control proestrous rats (Fig 4 C and D). Dual immunofluorescent staining for GnRH and GABA in ME-ARC region has shown decrease in immunoreactivity in AEDs treated test group rats as compared to vehicle treated control proestrous rats (Fig 2C, D and 3C, D). Furthermore, Western blot results show decreased expression of GAD in median eminence region of drug treated animals (Fig 2G and 3G).

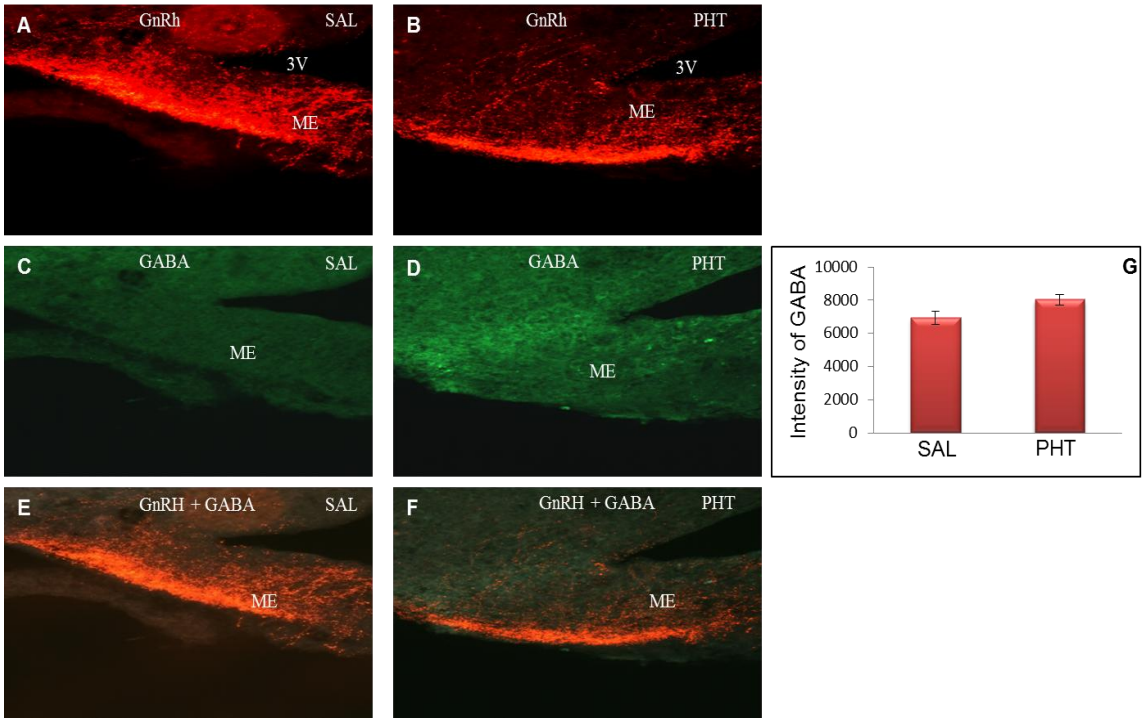

Fig 3. Immunofluorescent images of 30µ thick coronal sections from median eminence (ME) region of vehicle treated control (SAL) and Phenytoin (PHT) female (n=5 each) rat brain. The immunostaining in the ME region for GnRH and GABA is shown for GnRH (A-B), GABA (C-D) as well as dual immunofluorescence of GnRH and GABA (E-F) for SAL and PHT treated animals, respectively. GnRH-ir was reduced in test rats as compared to their corresponding control rats (G). G depict relative intensity measurement ±SEM of GABA immunofluorescence from ME region. *=pValue<0.05.

Expression of GABA and GAD in AEDs Treated Test and Control Proestrous Rats:

In AED treated test groups, Immunofluorescent staining for GAD in ME-ARC region has shown increase in immunoreactivity in AEDs treated test group rats as compared to vehicle treated control proestrous rats (Fig 4A, B and E). Furthermore, Western blot results show increased expression of GAD in median eminence region of drug treated animals (Fig 4D and E).

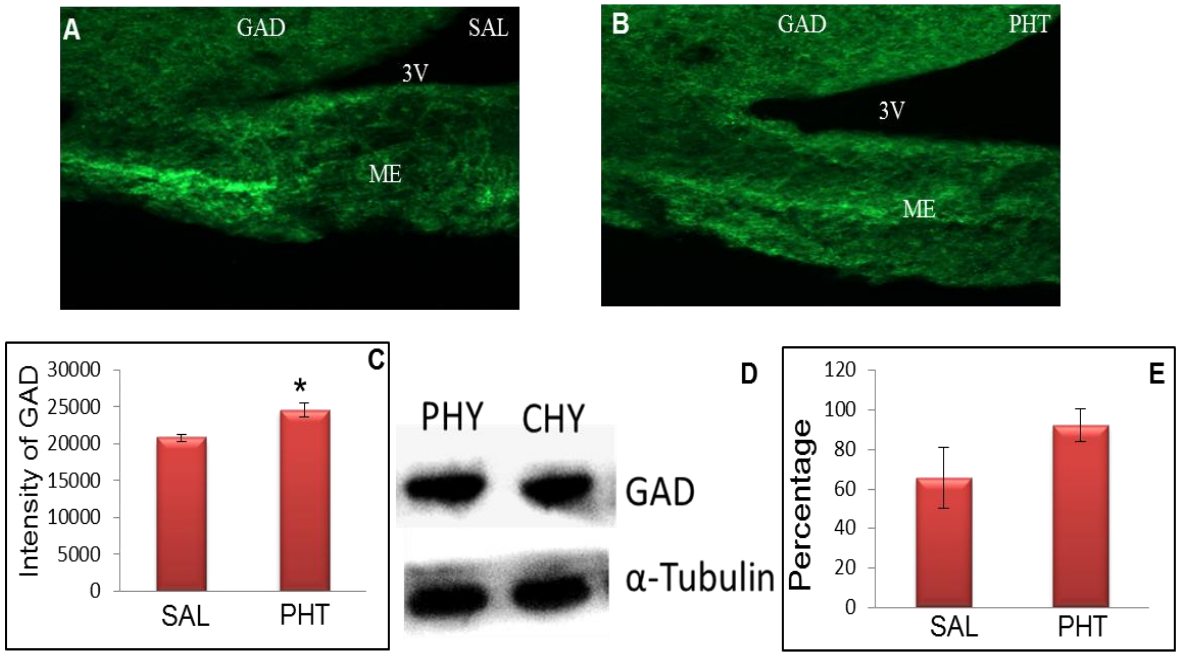

Fig 4. Immunofluorescent images of 30 µm thick coronal section through the ME-ARC region of the hypothalamus GAD is shown for the (A) vehicle treated control proestrous and (B) PHT treated test rats. Intensity measurement data of the GAD in the ME-ARC region of vehicle treated control proestrous (SAL) and PHT treated test (E). (D and E) Western blot results are shown for GAD in ME region. For each type of data analysis, measurements were performed on five to eight sections each from four to five test and control rats. All statistical analyses were performed using Student's t-test. Values are means ± S.E.M; d = P < 0.05. ME- median eminence. SAL- Saline, GAD- Glutamic acid decarboxylase, CHY- Control Hypothalamus, THY- Test Hypothalamus.

GAD65 and GAD67 Protein expression quantification using Western Blotting and mRNA expression quantification using FISH and RT-PCR:

GAD65, GAD67 and α-tubulin labelling in the representative samples from VPA treated test and control proestrous groups is shown in Fig 5C, Fig 5D. The mean value for the percentage of GAD65, GAD67 to α-tubulin ROD for the two groups is also illustrated. The quantitative analysis of immunoblots from the VPA treated test group revealed higher GAD-65 and GAD-67 protein expression in ME-ARC region of VPA treated test rats ($p \leq 0.05$) as compared to control proestrous group (Fig 5C, Fig 5D). GAD stained GABAergic cell bodies in mPOA shows higher immunoreactivity in test group rats (Figure 5B).

FISH data also indicated that GAD65 mRNA expression was significantly higher in the ME-ARC region from VPA treated test rats (Fig. 6B) as compared to control proestrous group (Fig. 6A). Using quantitative immunofluorescence analysis of staining intensity measurements, we showed a statistically significant increase in the GAD65 mRNA expression ($p<0.05$) in the ME-ARC region in the test group (Fig. 6C). Expression level of GAD65 mRNA quantified by semi quantitative RT-PCR analysis further showed higher expression of GAD65 ($p<0.05$) from the ME-ARC region of VPA treated test rats (Fig. 6D).

FISH data for GAD67 mRNA also indicated significantly higher expression in the ME-ARC region from VPA treated test rats (Fig. 7B) as compared to control proestrous group (Fig. 7A). Using quantitative immunofluorescence analysis of staining intensity measurements, we showed a statistically significant increase in the GAD67 mRNA expression ($p<0.05$) in the ME-ARC in the test group (Fig. 7C). Expression level of GAD67 mRNA quantified by semi quantitative RT-PCR analysis further showed higher expression of GAD67 ($p<0.05$) from the ME-ARC region of VPA treated test rats (Fig. 7D).

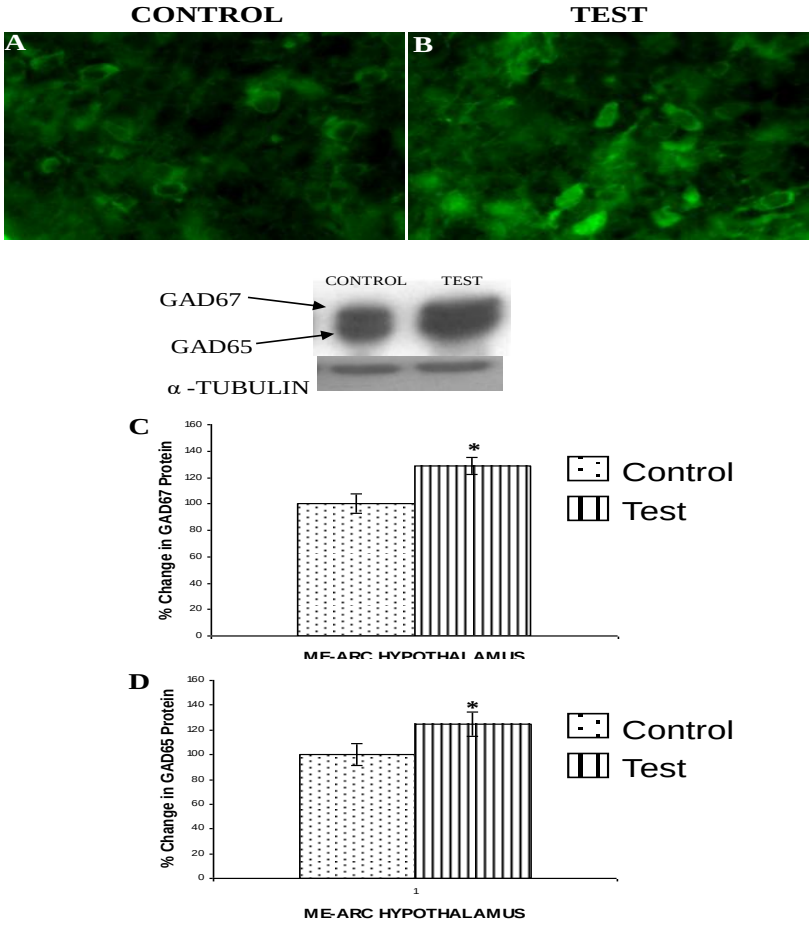

Fig. 5: Photomicrograph of 40µm thick coronal section through the mPOA region of the hypothalamus; immunostaining of the section for GAD is shown for control proestrous (A) as compared to VPA treated test group (B). Representative Western blot hybridisation signals of GAD65 and GAD67 from median-eminence region in hypothalamus from control proestrous and test rats are shown (C, D). Mean values of GAD65 and GAD67 levels for each group is expressed as percentage of α-tubulin labeling (H). All statistical analyses were performed using Student's t-test, values are means ±S.E.M, * $P<0{\cdot}05$, Magnification x100.

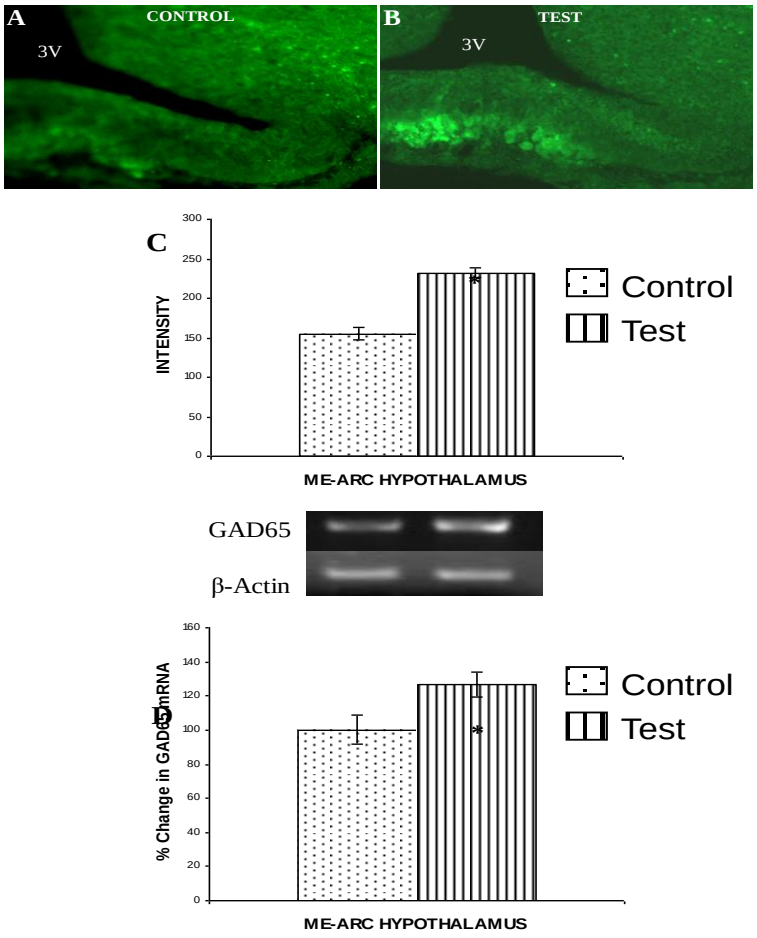

Fig. 6: Representative in-situ hybridization images of 30µm thick coronal sections showing staining for GAD65 mRNA in median-eminence region of the hypothalamus in brain. GAD65 mRNA expression is increased in median-eminence in test rats (B) as compared to control proestrous rats (A). Intensity measurement data also shows higher intensity in median-eminence in test rats (C). Representative semiquantitative RT-PCR product gel signals for GAD65 and β-actin from median-eminence region again shows increase in GAD65 mRNA expression in test rats (D). All statistical analyses were performed using Student's t-test, values are means ±S.E.M; * P<0·05, 3V Third Ventricle, Magnification x100.

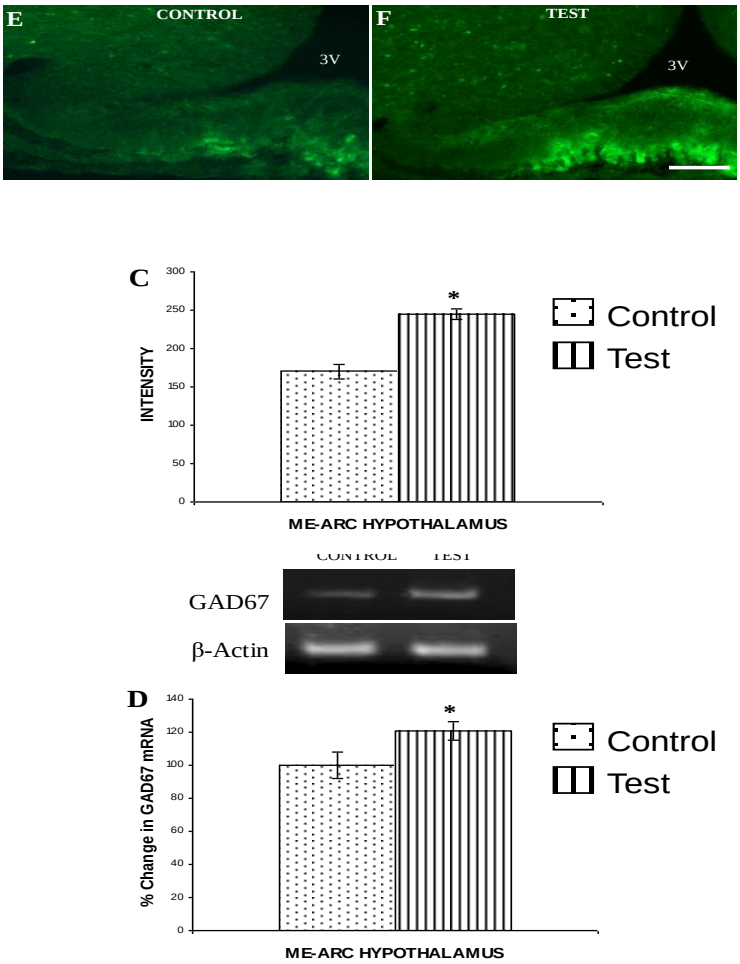

Figure 7: Representative in-situ hybridization images of 30µm thick coronal sections showing staining for GAD67 mRNA in median-eminence region of the hypothalamus in brain. GAD67 mRNA expression is increased in median-eminence in test rats (B) as compared to control proestrous rats (A). Intensity measurement data also shows higher intensity in median-eminence in test rats (C). Representative semiquantitative RT-PCR product gel signals for GAD67 and β-actin from median-eminence region again shows increase in GAD67 mRNA expression in test rats (D). All statistical analyses were performed using Student's t-test, values are means ±S.E.M; * P<0·05, 3V Third Ventricle, Magnification x100.