SUMMARY

Women with epilepsy face a host of challenges due to use of AEDs and reproductive endocrine disturbances are a major health problem in these patients along with lower birth rates and greater risk for the syndromes associated with infertility, such as hypothalamic pituitary axis disruption, PCOS and anovulatory cycles. These disorders have been attributed both to epilepsy itself and to use of AEDs. Despite extensive clinical and experimental research, the underlying mechanism remains unclear. However a pathogenic role of epilepsy itself has been proposed by some researchers, whereas others suggest that the use of AEDs, in particular VPA, may mediate this association. Furthermore chronic treatment with VPA has been associated with a variety of endocrine related side effects, suggesting that VPA acts at multiple levels within CNS. VPA administration has been implicated in reproductive function by decreasing secretion of anterior pituitary hormones such as LH, FSH and mean serum estrogen levels.

Valproic acid (2-propylpentanoic acid) is an extremely potent and widely prescribed anticonvulsant agent and is generally agreed upon that VPA acts to potentiate GABA mediated postsynaptic inhibition within the CNS via some, as of yet, unknown cellular mechanisms. Several mechanisms have been postulated including inhibition of several degradative enzymes in the GABA shunt pathway; as well as an increase in the activity of GAD the rate limiting enzyme in the synthesis of GABA. Although VPA does not directly interact with postsynaptic GABA receptors, it does increase regional neuronal concentrations of GABA by both inhibiting its metabolism and increasing its synthesis.

The GnRH system in the hypothalamus constitutes the final common pathway for the central regulation of pituitary gonadotrophin release. Mammalian female reproductive cycle neuroendocrine control involves complex and well-orchestrated interactions among gonads, pituitary and hypothalamus. GnRH is synthesized in neuronal cell bodies distributed diffusely in the hypothalamic mPOA and secreted from the external zone of median eminence region by the increase in the length of axons. In females, the preovulatory surge of LH is initiated by an abrupt increase of GnRH release, which is preceded by GnRH axon terminals contact with the perivascular space directly. This
functional remodelling in GnRH neurons in hypothalamus involves participation of cell adhesion molecules, astrocytes and gonadal hormones to render the pulsatile secretions into the perivascular space at median-eminence region. Ultrastructural data indicate that GABAergic neurons establish inhibitory synapses directly onto the GnRH neurons within the mPOA. Moreover, additions of GABA and GABA agonists directly into the mPOA have been shown to reduce GnRH gene expression, suppress the release of LH from the anterior pituitary, and block the estrogen-induced LH surge that normally occurs at the time of proestrous.

The morphological plasticity in the adult mammalian CNS has been correlated with the enhanced expression of the NCAM and its polysialylated form PSA-NCAM. PSA is a polymer of highly charged α-2,8 sialic acid, which is post-translationally added to NCAM by a specific polysialyltransferase enzyme. The amount of PSA on the NCAM molecule increases its anti-adhesion properties and supports structural plasticity both in the developing and in the adult nervous system. The median-eminence region of hypothalamus expresses high levels of PSA-NCAM and maintains a high capacity for neuroplastic changes in the adult brain to facilitate dynamic transformation of individual GnRH axon terminals during proestrous phase, and is orchestrated by endocrine events occurring in a cyclic manner.

Classically considered as supporting cells of neurons, astrocytes display rapid electrical, metabolic, and transcriptional responses to neural activity. Astrocytes participate in synaptic patterning via responsiveness to steroid hormones in steroid concentrating brain regions in mammalian brain. Specialized glial cells play key role in GnRH release by extension and retraction of their processes which are found interposed between GnRH axon terminals and the hypophyseal portal vasculature and this neuronal-glial plasticity is an important factor regulating GnRH cyclicity. Astroglial processes withdrawal in external zone of median-eminence region facilitates sprouting of GnRH neuron terminals to enhance their physical contact with the pericapillary space during proestrous phase when estrogen levels are high in comparison to diestrous. Earlier study from our laboratory has reported astro-glial processes association with the GnRH neuron terminals in median-eminence region which vary otherwise in conjunction with cyclic changes in GnRH axon terminals. VPA action through GABAergic mechanism is
unequivocal but VPA mediated decrease in gonadal steroid levels may play synergistical role in disruption of glial plasticity in median-eminence region and ultimately the GnRH cyclicity.

Taking into consideration both the inhibitory role of GABA as a neurochemical regulator of GnRH, as well as VPA proposed GABAergic mechanism of action, we hypothesized that VPA may mimic and/or elevate the activity of GABAergic system in hypothalamus and disrupt the GnRH pulse generator. The present study was aimed to test whether VPA’s anticonvulsive effects via key molecules of GABAergic system may be having inhibitory effects on the HPG axis at the level of GnRH synthesis and/or release and thereby affect reproductive health of subjects taking VPA. Since VPA is also reported to cause cognitive and memory dysfunctions so the neuronal plasticity and GABAergic function was also studied in hippocampus and piriform cortex regions which are associated with memory and cognitive function.

Objectives:

- The effect of chronic treatment with VPA was studied on estrous cyclicity and ovarian histology in female rats to illustrate the correlation between VPA use and cyst formation in ovaries thus causing PCOS.
- Further to elucidate whether VPA disturbs reproductive function at the level of the hypothalamic GnRH synthesis and/or release, expression of GnRH and GABA were studied in mPOA and median-eminence region having GnRH cell bodies and axon terminals, respectively, after VPA treatment of cycling female rats.
- To ascertain whether VPA increases GABA levels in hypothalamus by upregulating its synthesis rate, the expression of GAD was studied in median-eminence region of hypothalamus in control and VPA treated animals.
- To further test whether VPA treatment alters neuroendocrine plasticity, the expression of PSA-NCAM and glial fibrillary acidic protein (GFAP), neuronal and glial plasticity markers respectively were studied in median-eminence region in control and VPA treated animals. GFAP and PST the enzyme responsible for polysialylation of NCAM were also studied at transcriptional levels.
- To test whether VPA treatment affects the neuronal plasticity and GABAergic
function in brain regions known to be associated with learning and memory, we further extended our study to hippocampus and piriform cortex regions of the brain.

**Brief Methodology:**

Wistar strain female albino rats in the age group of 3-5 months were housed three per cage in a temperature humidity controlled environment on a 12-h light –12-h dark cycle with free access to food and water. The estrous cycle was monitored by daily inspection of vaginal cytology. To study the antifertility effects of VPA, rats were given intraperitoneal injection of VPA (300mg/kg) and control animals were injected vehicle solution (double distilled water) between 9.00 AM to 10.00 AM for 12 weeks. Rats were sacrificed in proestrous phase for control while VPA treated rats which were in diestrous phase after 12 weeks of treatment between 2.00 to 4.00 PM. Animal care and procedures were followed in accordance with the guidelines of Institutional Animal Ethical Committee, Guru Nanak Dev University, Amritsar.

VPA effects on ovarian histology were studied by microscopical evaluation of ovaries, three mid-ovarian paraffin sections were cut for studying cyst morphology by Eosin and Haematoxylin staining. Serum estrogen levels were determined in using competitive ELISA assay kit from Cayman Chemicals Company, USA. To further elucidate effect of VPA on dynamic regulation of HPG axis and GnRH axon terminal’s structural plasticity, co-expression of GnRH was also studied with PSA-NCAM, NCAM, GFAP, GABA and GAD by immunofluorescent staining. PSA-NCAM, NCAM, GFAP and GAD expression was further studied by Western blotting. Expression of GFAP, PST and GAD in median-eminence region of hypothalamus was also studied at mRNA level by fluorescent in-situ hybridisation and RT-PCR. PSA-NCAM and GAD were also studied from hippocampus and piriform cortex using immunohistofluorescence, RT-PCR and Western blotting.

**Results and Conclusions:**

Chronic treatment with VPA resulted in disturbances in estrous cyclicity upto 6 weeks and thereafter the rats were in permanent diestrous phase. The ovarian histology showed higher number of cystic formations and increased ovarian weight in VPA treatment group indicating VPA mediated effect on ovarian morphology and physiology.
This was further confirmed by serum estradiol concentrations showing significant decrease in VPA treated test group as compared to vehicle treated control proestrous rats (100.1±6.9 pg/ml).

In VPA treated test group, GABA-ir was found to be significantly higher both in mPOA and the median-eminence region of the hypothalamus. VPA mediated GABA upregulation was further shown at the level of GABA synthesising enzyme with significantly higher GAD-ir both in mPOA and median-eminence regions of the hypothalamus. GABA-ir cell bodies were identified throughout the median-eminence region on the lateral walls of third ventricle in VPA treated rats. These results were further supported by quantitative analysis of immunoblots from test group revealing increase in GAD-65 and GAD-67 protein expression in median-eminence region. GAD stained GABAergic cell bodies in mPOA also showed higher immunoreactivity in test group rats. VPA mediated effects on GABAergic circuitry were also noted at the transcriptional level as is evident from fluorescent in situ hybridisation (FISH) and semi quantitative RT-PCR analysis, which indicated that GAD65 and GAD67 mRNA expression was significantly higher in the median-eminence region from VPA treated test rats as compared to control group.

GnRH-ir showed significantly higher intensity and cell number in vehicle treated control proestrous rats as compared to VPA treated test rats. Increase in length and branching of GnRH axon terminals in the median-eminence region was observed in the control rats while VPA treated rats had lower GnRH-ir and the axon terminals scarcely branched. Thin GnRH axon terminals in median-eminence region were seen to emit very thin processes in the direction of the perivascular space which is the site of active GnRH secretion. GABAergic mode of VPA action was confirmed in support of our hypothesis that VPA mediated increase in GABA may mimic/and or elevate GABA mediated inhibition of GnRH neurons and ultimately result in disruption of normal HPG axis functioning.

Double immunostaining for GnRH and PSA-NCAM in median-eminence region from control group revealed heavy PSA-NCAM expression which was co-localized with GnRH immunoreactivity. GnRH cell bodies in mPOA from control group were seen to distinctly express PSA-NCAM on the cell surface as compared to VPA treated test group.
Dense PSA-NCAM immunoreactivity was identified along the lateral border of the external zone of the median-eminence region from control group. GnRH-ir in mPOA cell bodies as well as their terminals in median-eminence region from control group was higher in comparison to VPA treated group. Results of co-localization analysis further illustrated that GnRH and PSA-NCAM were being expressed together with significantly lower intensity in test group as compared to control group. PSA-NCAM co-expression with GnRH axon terminals in median-eminence region illustrates its functional importance in structural remodeling of the GnRH neurosecretory system axis. These results were further supported by quantitative analysis of immunoblots from the VPA treated test group revealing lower PSA-NCAM expression in median-eminence region of VPA treated test rats. VPA mediated effects were also evident at transcriptional level in PST mRNA expression FISH and RT-PCR analysis revealed significantly higher expression in the median-eminence region of the hypothalamus in the control proestrous group as compared to the VPA treated rats.

Further it was observed that GnRH axons terminals co-distributed with the glial elements only in the internal zone of the median-eminence region in the control group, whereas GFAP-ir was seen in both internal and external zones of the median-eminence region in VPA treated rats, 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 GFAP staining in the outer zone of the median-eminence region in the VPA treated group. The quantitative analysis of immunoblots from the VPA treated group also revealed higher GFAP protein expression in median-eminence region of VPA treated rats as compared to control proestrous group. FISH data also indicated that GFAP mRNA expression was significantly higher in the external zone of the median-eminence region of the hypothalamus in the VPA treated group. This increase was further confirmed by expression level of GFAP mRNA quantified by semi quantitative RT-PCR from the median-eminence, where it showed higher expression in the VPA treated test rats.

Hippocampus in the adult mammalian brain continues to generate new neurons throughout life. Proliferation of neural stem cells within the subgranular zone of the
dentate gyrus, within the hippocampus, continuously produces new granule cell neurons which are incorporated into the dentate gyrus. Our results showed decrease in PSA-NCAM-ir in hippocampus and piriform cortex regions in VPA treated rats, which may contribute to loss of learning and memory in subjects on AEDs treatment. Higher GAD expression in hippocampus and piriform cortex further highlights the VPA mediated upregulation of inhibitory circuitry leading to neuro-suppression in these areas and further disrupting memory functions.

Alterations in GnRH, GABA, GAD, PSA-NCAM, PST and GFAP expression after VPA treatment as well as changes in ovarian histology along with decrease in serum estrogen levels in VPA treated rats suggests that VPA causes disturbance in the reproductive function by disrupting HPG through GnRH pulse generator in non-epileptic rats. These results encourage further work to study the central regulation of reproductive endocrine side effects of AEDs. Alternations in neuronal plasticity in the regions of brain associated with learning and memory further suggests careful prescription of VPA as AED.