INTRODUCTION

The term epilepsy encompasses a number of different syndromes including cerebrovascular disease, degenerative disease, neoplasm, infection, trauma and the cardinal feature of which is a predisposition to recurrent unprovoked seizures (Hauser et al., 1993). Seizures, in turn, are sudden, brief attacks of altered consciousness; motor, sensory, cognitive, psychic, or autonomic disturbances; or inappropriate behaviour caused by abnormal excessive or synchronous neuronal activity in the brain. Insights in the etiology of epileptogenesis are limited. Genetic predisposition has been implicated in a vast number of epilepsies, and several genes have been identified underlying distinct forms of seizure disorders, but defects in these genes account for only a minority of patients afflicted with epilepsy. Although the mechanism of epileptogenesis is still hazy, we have plethora of drugs for the treatment of the enigmatic syndrome called epilepsy which are available according to the available symptoms of the syndrome. These drugs are called anti-epileptic drugs (AEDs) or anticonvulsants. Just like the mechanism of the disease, the mechanism of some drugs is also unsolved, but because of the clear anti-seizure effects they are prescribed widely ignoring their side effects. AEDs used for the cure of epilepsy acts just to mitigate the seizures rather than healing epileptogenesis which further highlights the importance of studies on AEDs.

Since the nature of epilepsy requires long-term and sometimes lifelong treatment mostly applied as polytherapy, occurrence of several adverse effects is inevitable. In a European multi-center study, at least one drug-related adverse effect has been reported in 88% of the patients (Toledano and Gil-Nagel, 2008). Endocrine disorders are of major concern for clinicians who treat patients with epilepsy. These disorders include subfertility, anovulation, menstrual disturbance, hyperandrogenism, lowered effectiveness of oral contraception, adverse pregnancy outcomes in women (Yerby, 2000; Bauer et al., 2002) and sexual dysfunction, androgen deficiency symptoms, testicular atrophy, impaired spermatogenesis and sub-fertility in men (Herzog, 2002). While epilepsy per se may be associated with the development of reproductive disorders (Herzog et al., 1986; Bilo et al., 1988), there is accumulating data suggesting that anti-convulsant drugs play a significant role in the development of these disorders and that they may be more harmful
than epilepsy itself (Isojarvi et al., 1993; Isojarvi et al., 1995a; Isojarvi et al., 1995b; Rattya et al., 2001a; Rattya et al., 2001b). Many of the side-effects of anti-convulsant drugs on reproductive endocrine functions have been confirmed in animal models (Tauboll et al., 1999; Roste et al., 2001; Roste et al., 2002).

Women with epilepsy (WWE) face a host of challenges due to use of AEDs and reproductive endocrine disturbances are a major health problem in these patients (Morrell et al., 2002; Betts et al., 2003; Kaplan, 2004). They also have lower birth rates and greater risk for the syndromes associated with infertility, such as hypothalamic pituitary axis disruption, polycystic ovary syndrome and anovulatory cycles. There is an extremely complex interaction between epilepsy, AEDs and the reproductive endocrine system, which still is not fully elucidated (Isojarvi, 2008). Fertility is lower in both men and women with epilepsy than in the general population (Wallace et al., 1998; Artama et al., 2006). Moreover reproductive endocrine disorders are more common among patients with epilepsy than among the population in general (Herzog et al., 1986; Isojarvi et al., 1993; El-Khayat et al., 2004). These disorders have been attributed both to epilepsy itself and to use of AEDs. AEDs alter endocrine function in both men and women with epilepsy and this alteration may lead to clinically significant reproductive endocrine disorders (Isojarvi et al., 2005). An increased frequency of reproductive endocrine disorders, in particular of polycystic ovary syndrome (PCOS), has been described in women with temporal lobe epilepsy (Herzog et al., 1984) and idiopathic generalized epilepsy (Bilo et al., 1988). A pathogenic role of epilepsy itself has been proposed by some researchers (Herzog et al., 1986; Nappi et al., 1994), whereas others suggest that the use of AEDs, in particular valproic acid (VPA), may mediate this association (Isojarvi et al., 1993; Isojarvi et al., 1996). Despite extensive clinical and experimental research, the underlying pathogenic mechanism remains unclear (Herzog, 2008; Cansu et al., 2008).

Anovulation has been reported to be more frequent in women with temporal lobe epilepsy taking polytherapy than in those receiving a single AED (Cummings et al., 1995). Lowered sex hormone levels in epilepsy may be, in part, accounted for by the AED therapy, which involves a decrease in estrogen production and conversion from androgens in liver and peripheral tissues. In addition, both seizures and AEDs may affect
hormone secretions and gonadotropin ovulatory surge by directly targeting a number of anatomic substrates, including the limbic system, hypothalamus, pituitary, and gonads (Herzog, 2008).

Hormonal changes that have been associated with the use of AEDs in women include reduced serum levels of free testosterone (FT), progesterone, free estrogen index, and/or estradiol (E), and increased levels of testosterone, sex hormone-binding globulin (SHBG), luteinizing hormone (LH), follicle-stimulating hormone (FSH), cortisol and cortisol/dehydroepiandrosterone sulfate (DHEAS) ratios indicating hyperandrogenism (Isojarvi et al., 1990; Isojarvi et al., 1993, Isojarvi et al., 1995b; Rattya et al., 2001a,b; Luef et al., 2002; Morrell et al., 2008). VPA in particular and wide range of other conventional and novel AEDs now available for epilepsy are being increasingly used for the treatment of migraine, bipolar disorder, pain and other conditions. Such use creates a greater need for knowledge regarding the effects of these medications on reproductive health.

1.1 GABAergic mechanisms in epilepsy and AEDs:

Excitation and inhibition of neurons is mediated by many different neurotransmitters. γ–Aminobutyric acid (GABA) is now recognized as the principal inhibitory neurotransmitter (Schwartz, 1988). GABA is localized primarily in short-axon interneurons that synapse on cell bodies and axons, and serve to maintain inhibitory tone that counterbalances neuronal excitation which if perturbed results in seizures. Ultrastructural data also provide evidence that GABAergic neurons synapse directly onto gonadotrophin releasing hormone (GnRH) neurons located within the median preoptic area (mPOA) of the hypothalamus (Leranth, 1985).

GABA is formed within GABAergic axon terminals by transamination of α-ketoglutarate to glutamic acid, which is then decarboxylated by glutamic acid decarboxylase (GAD) to GABA. It is released into synapse and then acts at one of two types of GABA receptors: GABA_A and GABA_B receptors, which hyperpolarize (inhibit) the target neurons by different mechanisms. Evidence from a number of experimental and clinical sources provide strong support for role of GABA in the mechanism and treatment of epilepsy. A number of GABA agonist drugs are anticonvulsant in experimental
animals (muscimol and progabide), whereas, GABA antagonists are proconvulsant (Treiman, 2001). Inhibition of GABA synthesis is epileptogenic (bicuculine and picrotoxin). Benzodiazepine and barbiturates enhance GABA-mediated inhibition by modulating the chloride channel and they also are anticonvulsants (Macdonald, 1983, Barker and Owen, 1986). Vigabatrine and Tiagabine increase the synaptic GABA through different mechanisms and have been shown to be potent antiepileptic drugs (Beran et al., 1996; French et al., 1996; Sachdeo et al., 1997; Uthman et al., 1998).

1.2 Valproic Acid: Mechanism of action and effect on reproductive function:

Valproic acid (VPA, 2-propylpentanoic acid) is an extremely potent and widely prescribed anticonvulsant drug. Originally synthesized by Burton in 1882, VPA was later shown to have anticonvulsant properties (Meunier et al., 1963) and was licenced for use in the United States as an anticonvulsant in 1978. Since that time, the use of VPA in the clinical population has increased dramatically, making it one of the most frequently prescribed agents for the treatment of generalized seizure disorders. VPA has also become increasingly popular as a mood stabilizer in the treatment of several affective disorders, including major depression (Kemp 1992) and bipolar disorders (McElroy et al., 1992). Despite its widespread use, exact mechanism of VPA still remains relatively unclear. However it is generally agreed upon that VPA acts to potentiate GABA-mediated postsynaptic inhibition within the central nervous system (CNS) via some, as of yet, unknown cellular mechanisms (Lagace et al., 2004). The first major hypothesis for the cellular action of VPA was made by Godin et al. (1969), who proposed that VPA produces an increase in the level of GABA in the CNS. Since that time, several mechanisms have been postulated to explain the GABA-elevating effects produced by VPA, 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 (Faingold and Browning, 1987). Using isolated frog spinal cord it was found that low concentrations of VPA hyperpolarize dorsal roots, while higher concentrations produce depolarizations (Hackman et al., 1981). Thus VPA may retard rapid neuronal firing in a manner that mimics the actions of endogenous GABA (Faingold and Browning, 1987). In addition to the GABA hypothesis, some evidence suggests that VPA
may function to decrease excitatory amino acid and norepinephrine activity within the CNS (Chapman et al., 1983; Crowder and Bradford, 1987).

VPA has been associated with the occurrence of reproductive endocrine disorders characterized by high serum T, free androgen index, androstenedione, dehydroepiandrosterone sulfate concentrations, and with polycystic changes in ovaries and menstrual disorders (Verrotti et al., 2011). VPA has been shown to disrupt the secretion of several hormones, including growth hormone (GH), Adrenocorticotropic Hormone (ACTH), FSH, and LH (Lundberg et al., 1986; Invitti et al., 1988; Leškiewicz et al., 2008). In adults VPA administration has been shown to induce unresponsiveness to GnRH challenge, transient amenorrhea, polycystic ovarian syndrome and hyperandrogenism (Lundberg et al., 1986; Isojarvi et al., 1993). It has been suggested that VPA may slow pubertal maturation by altering the neurochemical system that normally plays an important role in timing the maturation of the GnRH pulse generator (Snyder and Badura, 1998; Illig et al., 2000; Dodge et al., 2000). A very high occurrence of hyperandrogenism and polycystic ovaries has been found among women taking VPA for epilepsy (Isojarvi et al., 2001a; Svalheim et al., 2003). These abnormalities were more frequent in women who had started the treatment before the age of 20 years than in women who had started the treatment later. In a study of non-epileptic patients taking VPA for bipolar disorder, about 50% women reported menstrual abnormalities in VPA treated group (O’Donovan et al., 2002).

Despite the widespread clinical complications however the definitive mechanism(s) mediating the side effects of VPA remains obscure. One possible mechanism to account for VPA’s inhibitory role on reproductive development and function may be via its action on the inhibitory neurotransmitter 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 (Owens and Nemeroff, 2003; Czapinski et al., 2005). Since VPA is one of the widely prescribed drug for epilepsy and other mood disorders like migraine and bipolar disorders, hence this study was aimed to explore its effect on neuroendocrine plasticity in female reproductive system.
1.3 **GABAergic regulation of GnRH release and AEDs mediated disruption:**

Primary afferent GABAergic neurons represent a predominantly inhibitory component of the GnRH neuronal network, which is important in the pulsatile secretion of gonadotrophins. Ultrastructural data also provide evidence that GABAergic neurons synapse directly onto GnRH neurons located within the mPOA of the hypothalamus (Leranth, 1985). Bilger et al. (2001) also reported that a temporally controlled increase in GABA availability to GnRH nerve terminals in the median eminence of hypothalamus disrupts estrous cyclicity in the rat. Additionally, administration of GABA and GABA agonists directly into the mPOA has been shown to reduce GnRH gene expression (Bergen et al., 1991), suppress the release of LH from the anterior pituitary (Lamberts et al., 1983; Scott and Clarke, 1993) and block the estrogen induced LH surge that normally occurs at the time of proestrous (Herbison and Dyer, 1991). Moreover infusion of GABA agonists into a superfusion medium containing nuclei from the mPOA has been found to directly decrease levels of GnRH secretion (Feleder et al., 1996).

Considering both the inhibitory role of GABA as a neurochemical regulator of GnRH cells, as well as VPA’s proposed GABAergic mechanism of action, it is possible that, VPA and other AEDs acting through GABAergic system may mimic and/or elevate the activity of GABAergic system to slow normal morphological maturational changes occurring within the GnRH neuronal population in children and reproductive function in adults. In a report by Laeng et al. (2004), VPA has been studied for its ability to promote neurogenesis from embryonic rat cortical or striatal primordial stem cells. 6 days of VPA treatment increased upto fivefold the number of neurons and their differentiation into GABAergic phenotype. These findings indicate that this ability of VPA to stimulate the genesis of GABAergic neurons or augmentation of neurite outgrowth of existing GABAergic neurons in epilepsy or bipolar disorder patients could compensate for GABAergic neuron atrophy or dendrite loss in these disorders.

Carbamazepine (CBZ) therapy is reported to increase levels of SHBG in women taking CBZ for epilepsy which results in decreased serum levels of bioactive estradiol and testosterone (Isojarvi et al., 1990; Isojarvi et al., 1995a; Rattya et al., 2001a). Enzyme-inducing AEDs, such as phenytoin and phenobarbital reduce the levels of biologically active sex steroid hormones (Isojarvi et al., 1990). Long-term levetiracetam
(LEV) treatment significantly affects reproductive endocrine function, as well as ovarian morphology, in non-epileptic rats (Svalheim et al., 2008). The AEDs mediated disruption in gonadal hormone levels suggests abnormality in normal hypothalamo-hypophyseal-gonadal (HPG) axis which is regulated by these hormones and thus disruption in normal GnRH cyclicity.

Taken together, all of this clinical evidence raises the possibility that chronic VPA and other AEDs administration may result in adverse effects on the neuroendocrine system, including disturbances of growth, sexual development, and fertility. Although a large body of literature has accumulated on the adverse effects of chronic VPA administration on the neuroendocrine system, its mechanism and sites of action for inhibition of the reproductive axis remain to be clarified.

1.4 GnRH neuro-glial plasticity:

The GnRH system in the hypothalamus constitutes the final common pathway for the central regulation of pituitary gonadotrophin release (Sarkar et al., 1976). 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 sprouting and extension of axons (King et al., 1995; Kaur et al., 2002). 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 (Prevot et al., 1999). This functional remodeling 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. Previous studies from our laboratory have shown expression of polysialic acid neural cell adhesion molecule (PSA-NCAM) on GnRH axon terminals and associated astro-glial cells in the median-eminence region of rats varying with estrous cycle phase by dual immunohistofluorescent staining (Parkash and Kaur, 2005; Parkash and Kaur, 2007a) and Western blotting (Kaur et al., 2002). In these studies, we also observed a link between the estrogen concentration and glial ensheathment of GnRH axon terminals in the median-eminence region (Parkash and
Kaur, 2005). The previous reports from our laboratory established that the neuro-glial plasticity at median-eminence region plays permissive role in regulating GnRH secretion mediated by retraction of astro-glial processes and thus removing physical barrier to GnRH axons contact with perivascular space to facilitate the release of neurohormone into perivascular capillary network (Parkash and Kaur, 2005).

The morphological plasticity in the adult mammalian CNS has been correlated with the enhanced expression of neural cell adhesion molecule (NCAM) and its polysialylated form PSA-NCAM (Seki and Arai, 1993). Cell-cell interactions mediated by NCAM play critical role in activity dependent synaptic plasticity in adulthood (Theodosis et al., 1999). NCAM exhibits structural diversity in the form of three major proteins (NCAM-180, -140, -120) that are generated from different mRNAs produced by alternative splicing of exons from a single gene composed of 26 exons (Goridis and Brunet, 1992). In addition to adhesive properties, NCAM is implicated in cell migration, neurite outgrowth, fasciculation, synaptogenesis and intracellular signaling, which are closely connected with the activation of secondary messangers (Walsh and Doherty, 1997).

Polysialic acid is a polymer of highly charged α-2,8 sialic acid, which is post-translationally added to NCAM by a specific polysialyltransferase (PST) enzyme (Nelson et al., 1995; Angata and Fukuda, 2003). The amount of PSA on the NCAM molecule modulates its adhesion properties and supports structural plasticity both in the developing and in the adult nervous system (Fryer and Hockfield, 1996; Theodosis et al., 2004). Two sialyltransferases ST8siaIV/ PST and ST8siaII/STX appear to be responsible for the synthesis of PSA-NCAM (Eckhardt et al., 2000; Muhlenhoff et al., 2001). These two enzymes seem to be regulatory factor for PSA synthesis and their activity is controlled at the mRNA level (Eckhardt et al., 1995; Hilderbrandt et al., 1998). The hypothalamus arcuate nucleus expresses high levels of PSA characterizing a highly sialylated isoform of the NCAM and maintains a high capacity for neuroplastic changes in the adult brain. PSA-NCAM is a key neuronal synaptic plasticity marker and when expressed in large quantities on NCAM, it renders modulation of adhesion properties between neighbouring neurons by decreasing NCAM homophilic binding and thereby attenuating cell adhesion.

At puberty, GnRH release changes from a low level, irregular pattern to one of
distinct pulses (Sisk et al., 2001; Harris and Levine, 2003). Pulsatile pattern of GnRH release drives pulsatile gonadotropin secretion that is critical for the activation and proper functioning of gonads (Belchetz et al., 1978). For carrying out this function of pulsatile release of GnRH, there occurs dynamic transformation of individual GnRH axon terminals in the median-eminence region of hypothalamus and this restructuring of median-eminence region during proestrous phase is orchestrated by endocrine events occurring in a cyclic manner (Parkash and Kaur, 2005). This earlier report from our laboratory had shown that median-eminence region where this synaptic restructuring occurs retains the capacity to express PSA-NCAM which may be responsible for the structural reorganization of the GnRH neuron terminals in proestrous phase of the reproductive cycle.

From early times glial cells have been considered passive supporters in nerve cell function, providing structural and metabolic support to the communicating neurons. Now, rapidly emerging evidence demonstrates that glial cells participate actively in the synaptic patterning and synaptic transmission (Oliet et al., 2001; McCarthy et al., 2002). Glial cells demonstrate a responsiveness to gonadal steroids that has been best characterized by physical changes in their morphology (Galbiati et al., 2003; Theodosis et al., 2004). Garcia-Segura and McCarthy (2004) has pioneered the study of hormonally responsive glial fibrillary acidic protein (GFAP) positive astrocytes and their relationship to neuronal and neuroendocrine functions in the arcuate nucleus of the adult rodent (Garcia-Segura and McCarthy, 2004). In the arcuate nucleus of the adult female rat, estrogen dependent extension of astrocytic processes transiently disconnects inhibitory axosomatic synapses during the proestrous and the estrous phase of the estrous cycle. The physical disruption of inhibitory synaptic inputs at the level of the arcuate nucleus has been suggested to facilitate the release of the GnRH by disinhibiting the excitatory inputs regulating its release (Galbiati et al., 2003; Garcia-Segura and McCarthy, 2004). Earlier study from our laboratory has reported astroglial processes association with the GnRH neuron terminals in median-eminence region which vary otherwise in conjunction with cyclic changes in GnRH neuronal terminals (Parkash and Kaur, 2005).

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

1.5 Objectives:
The current study was designed to address the following objectives:

- To ascertain the role of VPA in causing PCOS, the effect of chronic treatment with VPA was studied on estrous cyclicity and ovarian histology.
- Further to elucidate whether VPA exerts anticonvulsive effects through GABAergic system and 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 by immunofluorescence staining and Western blotting.
- To ascertain whether VPA increases GABA levels in hypothalamus by upregulating its synthesis rate, the expression of GABA synthesising enzyme GAD was also studied in median-eminence region of hypothalamus in control and VPA treated animals using immunostaining, Western blot and RT-PCR.
- To further test whether VPA treatment also alters neuroendocrine plasticity, co-expression of GnRH was studied with PSA-NCAM and GFAP, neuronal and glial plasticity markers respectively, in median-eminence region by immunohistofluorescence. The expression of these markers at transcriptional and translational levels in median-eminence region was further confirmed by Western blotting and RT-PCR.
- 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.