DISCUSSION

The present study was aimed to investigate whether AED, VPA disrupts reproductive neuroendocrine function in female rats through its GABAergic action on GnRH system. The study initially explored the VPA mediated changes in estrous cyclicity, ovarian histology and decrease in serum levels of gonadal hormone estrogen. The GABAergic mechanism of action of VPA was also elucidated by studying the expression of GABA as well as GAD, the rate limiting enzyme in GABA synthesis. We further studied the changes in structural plasticity in GnRH neurons and their axon terminals along with the expression of neuron-glial plasticity markers PSA-NCAM and GFAP both at translational and transcriptional levels. Chronic VPA treatment has been associated with reproductive endocrine disorders and complications with reproductive health in VPA medication have been reported in both non epileptic women (O’Donovan et al., 2002; McIntyre et al., 2003) and women with epilepsy (Morrell et al., 2003; Isojarvi, 2008). These findings suggest that the effects are drug specific, which have been further, demonstrated in animal and cell culture studies (Nelson-DeGrave et al., 2004, Tauboll et al., 2009). Our study investigated independent effects of AED, VPA without other confounding factors of epilepsy on reproductively mature female wistar rats undergoing normal cyclicity. This study demarcates the VPA mediated effects on reproductive system, and ascertains the reproductive abnormalities to be the result of VPA medication and not a complication caused exclusively by epilepsy or a combination of epilepsy and the use of AEDs.

5.1 VPA mediates disturbance in cyclicity and ovarian histology:

Daily vaginal smear morphology observation showed that after 10–12 days of VPA treatment, estrous cycle was disturbed which continued upto six weeks of drug treatment and thereafter the animals entered into constant diestrous phase. VPA mediated disturbances in menstrual cycle have been reported to be a result of epilepsy itself or a combination of epilepsy and VPA treatment (Tauboll et al., 2008). Prevalence of menstrual disorders has been reported in 45% of women receiving VPA monotherapy and to have atleast one anovulatory cycle in 38.1% as compared to 10.7% of women not on
Clinical reports also suggest disturbance in menstrual cycle and hormonal changes in women with epilepsy undergoing VPA medication (Kaplan, 2004; Isojarvi et al. 2005). The data presented in these studies showed prevalence of menstrual disorders in 45% among women taking VPA monotherapy for epilepsy, and they were frequently associated with polycystic ovaries and/or hyperandrogenism, which were seen in 9/10 (90%) of the subjects. PCOS and hyperandrogenism is especially common, if VPA medication is started before the age of 20. Moreover, the serum mean androgen levels are increased in women on VPA (Isojarvi et al., 1993). On the comparative account of relation between reproductive function and AEDs, menstrual disorders have been reported in 59% of women on VPA as compared to 12% of carbamazepine (CBZ) treated women and 15% of control women. Hyperandrogenism and/or PCOS were detected in 70% of VPA-treated women as compared to 20% in the CBZ-treated women and 19% among the control women. Moreover, a short-term (3 months) prospective study in newly diagnosed women starting treatment with VPA suggested that an increase in serum testosterone and androstendione levels can be seen in approximately half of the women within 3 months after starting VPA (Isojarvi et al., 2005). Betts et al. (2003) reported 30% prevalence of PCOS in women ever treated with VPA only as compared to 6% in women ever treated with either CBZ or lamotrigine (LTG) only or to 14% among healthy control women. Development of menstrual abnormalities along with an increase in testosterone levels were reported in VPA treated bipolar patients which further establish implication of VPA in reproductive disturbances independent of epilepsy (Rasgon et al., 2005).

In the current study VPA mediated changes in ovarian histology are apparent from a significantly higher number of ovarian cysts per mid ovarian section in VPA treated group as compared to vehicle treated control rats (Fig. 1). An increase in ovarian weight and increased number of cystic formations in the ovaries of intact, non epileptic animals are in general agreement with observations in humans of PCOS and endocrine changes in women (Prabhakar et al., 2007). Use of VPA is associated with a frequent occurrence of reproductive endocrine disorders characterized by polycystic changes in the ovaries, high serum testosterone concentrations (hyperandrogenism) and menstrual disorders (Isojarvi et al. 2005). Similar findings have been reported in a three centre
study conducted in European countries where 59% of women on VPA reported menstrual disorders and 70% came up with hyperandrogenism and polycystic ovaries (Isojarvi 2008). These reports confirm the role of VPA in the development of menstrual irregularities and cystic formations in ovaries as observed in our study (Fig. 1B, 1C) as well. The appearance of disturbed cyclicity in VPA treated group at around third menstrual cycle (10-12 days) is further supported by a recent report where PCOS and menstrual irregularities in VPA treated women are reported to be independent of duration of VPA treatment suggesting possibility of VPA related reproductive dysfunction emerging early in the first several months of therapy (Gorkemli et al., 2009). Moreover a short term prospective study also suggested hormonal disturbances in about 50% of newly diagnosed women starting treatment with VPA (Isojarvi et al., 2005).

The potential pathophysiology underlying a VPA mediated increase in PCOS prevalence is unknown, however it appears to be multifactorial and several factors act simultaneously to cause this reproductive endocrine disorder. Abnormality of HPG axis, hyperinsulinemia, local factors in the ovary and weight gain in some patients may be critical for the development of PCOS (Genton et al., 2001). VPA mediated increase in GABA synthesis and release may block NMDA type glutamate receptors (Loscher, 1999) which finally results in disruptions in central regulation of reproduction at the level of HPG axis. Neuroendocrine abnormalities caused by VPA may be other plausible factor which may make the WWE susceptible for PCOS. An alternative explanation for why VPA increases the risk of PCOS is related to the association between VPA use and weight gain, which increases insulin resistance and, potentially, the risk for PCOS (Isojarvi et al., 1996). However, obesity alone is unlikely to fully explain the association of VPA use with PCOS as some VPA treated women are lean (Bilo et al., 2001). Another pharmacodynamic property of VPA that might contribute to its association with PCOS is lack of its effect on induction of hepatic cytochrome P450 (CYP) enzymes which is otherwise associated with other AEDs (Herzog, 1996, Anderson, 1998a). CYP isoenzyme induction facilitates clearance of gonadal steroids and reduces circulating testosterone levels (Anderson, 1998b) and because this mitigating action against hyperandrogenism is lacking in VPA treated patients, which may contribute selectively to the increased risk of PCOS.
We further analysed the blood serum estrogen levels associated with VPA mediated reproductive cycle disruption and found significant decrease in blood estrogen level in VPA treated group. Estrogen was found to be 19.4±2.1 pg/ml in VPA treated group as compared to vehicle treated control proestrous rats 100.1±6.9 pg/ml (Fig. 1F). These results are supported by in vivo study in rats where VPA treatment was found to increase the testosterone/estradiol ratio, mainly because of marked reduction of serum estradiol levels (Roste et al. 2002). VPA is an inhibitor of histone deacetylases (HDACs), enzymes that remove the acetyl group from histones in nucleosomes and thus modulates transcription of specific genes (Gottlicher, 2004; Saha and Pahan, 2006). Increase in VPA induced androgen synthesis has been correlated to the HDAC-inhibitor activity of VPA and associated with increased expression of the P450 side-chain cleavage (CYP11A) and 17α-hydroxylase, 17-20 lyase (CYP17) mRNAs (Wood et al., 2004). Subsequent gene expression profiling of normal theca cells treated with VPA demonstrated that they more closely resembled the gene expression profile of PCOS theca cells (Wood et al., 2005), thus establishing a direct link between VPA treatment and increased ovarian androgen biosynthesis, and the genesis of PCOS-like symptoms. Because CYP17 is the key enzyme required for androgen production in theca cells, earlier studies have proposed that excess androgen production in PCOS results from dysregulation of CYP17 enzyme activity due to an intrinsic ovarian defect (Rosenfield et al. 1990; Jakubowicz and Nestler, 1997; Nestler, 1997) and suggested that androgen production per theca cell is increased in PCOS (Gilling-Smith et al., 1994). Another in vitro study has suggested that VPA diminishes steroid hormone production by modulating the transcription factor SF-1 thereby suppressing the early steroidogenic gene CYP11A (Chen et al., 2007) which encodes the enzyme for cholesterol side chain cleavage and is associated with PCOS and hyperandrogenism (Gharani et al., 1997). VPA has been shown to alter steroidogenesis and increase testosterone to estradiol ratios in porcine ovarian follicles (Gregoraszczuk et. al. 2000). Similar studies have demonstrated that VPA treatment directly augments CYP17 gene expression and androgen biosynthesis in ovarian theca cells propagated in long-term culture (Nelson-Degrave et al. 2004). Higher number of ovarian cysts in VPA treated rats in our study could be a result of drug mediated modulation of key genes implicated in the synthesis of androgens. Overexpression of the genes in response to VPA
increase the androgen levels thus resulting in hyperandrogenism which itself is a PCOS marker.

A recent study on estrogen levels in VPA exposed cells in human adrenal carcinoma cell line reported decrease in estradiol levels and a general downregulation of expression of genes encoding for enzymes early in steroidogenesis in a dose-dependent manner (Gustavsen *et al.*, 2009). VPA effect on human granulosa cell culture showed significant and concentration dependent decrease in basal and FSH-stimulated estradiol secretion (Tauboll *et al.*, 2009). Even short-term VPA treatment has been shown to disrupt follicular steroidogenesis in isolated ovarian follicular cells resulting in increased testosterone and progesterone and decreased estradiol secretion, although it was not possible to reverse the steroidogenic effects of VPA by removing the drug from the cell cultures (Tauboll *et al.*, 2002). In a prospective study an increase in serum androgen concentration was documented in women with newly diagnosed epilepsy who were started on VPA (Rattya, 2001a). In girls treated with VPA for a mean of two years, higher serum testosterone levels were found than in untreated controls (Vainionpää *et al.*, 1999). AED withdrawal resulted in reversal of endocrine changes in sex steroid hormone levels in both sexes (Lossius *et al.*, 2007). VPA reduces the effectiveness of P450 aromatase, which converts testosterone to estradiol and a withdrawal of VPA may unblock the P450 aromatase and thus increase estrogen (Tauboll *et al.* 2003). Our preliminary data on abnormalities in reproductive cycles, morphological changes and cyst formation in the ovaries in female rats after long-term VPA treatment is also in line with clinical reports of VPA effects which have shown prevalence of menstrual disorders, polycystic ovaries and/or hyperandrogenism in WWE under VPA medication (Isojarvi, 2008). An increased number of cysts formation in the ovaries of non-epileptic animals and increased ovarian weight after long term VPA treatment as observed in current study (Fig. 1) confirms that the reproductive dysfunction in epileptic subjects on VPA treatment is the result of AED use.

5.2 GABAergic mechanism of VPA action and its consequences on reproductive plasticity:

GABA, one of the most important inhibitory neurotransmitter, important to the timing and amplitude of the GnRH induced LH surge on proestrus (Cashion *et al.*, 2004),
plays key role in the pubertal activation (Terasawa and Fernandez, 2001) and pulsatility, and gonadal steroid feedback regulation (Herbison, 1998) of the GnRH neurons. Since VPA enhances inhibitory events through GABAergic system (reviewed in Czapinski et al. 2005) we extended our study to explore the role of GABAergic mode of action by VPA in reproductive neuroendocrine disfunction in rats. Our results showed significant increase in GABA-ir and decrease in GnRH-ir in mPOA and median-eminence region of hypothalamus in VPA treated test rats as compared to the vehicle treated control proestrous rats (Fig. 2, Fig. 3) which may be responsible for the disruption of estrous cycle observed after few days of VPA administration. Bilger et al. (2001) have reported a temporally controlled GABA availability increase to LHRH nerve terminals in median-eminence region of the hypothalamus to disrupt estrous cyclicity in the rat. Another study reported that antagonists to GABA receptors resulted in LH surge almost similar to controls while GABA infused animals did not exhibit any LH surge in rats (Herbison and Dyer, 1991) and GABA treatment also affects the electrical activity of GnRH neurons, predominantly by hyperpolarizing effect upon GnRH neurons (Han et al., 2004).

Considering both the inhibitory role of GABA as a neurochemical regulator of GnRH neurons, as well as VPA’s proposed GABAergic mechanism of action, it is possible that VPA may mimic and/or elevate the activity of GABAergic system to interfere with normal activity of GnRH neurons and thus disrupts reproductive function in patients treated with VPA. GnRH-ir as well as its mRNA expression in VPA treated rats was downregulated when compared with vehicle treated controls. This disruption in GnRH synthesis and/or release is very important from the perspective of central regulation of reproduction. Pulsatile GnRH stimulus is required to maintain gonadotropin synthesis and secretion and to determine gonadotropin subunit gene expression and secretion of pituitary LH and FSH (Marshall et al., 2001) to regulate the reproductive cycle. Moreover a significant increase in the GABA-ir cell bodies was seen in the present study in the subventricular zone in the median-eminence region which is a site of neurogenesis (Fig. 3D) in the adult brain. This observation indicates that one important mechanism of VPA action may be to induce the differentiation of endogenous precursor cells in the adult brain to GABAergic neurons. The results are supported by in vitro study by Laeng et al. (2004), which reported that VPA treatment of rat forebrain stem cell
cultures stimulated GABAergic neuron numbers as well as neurite outgrowth of these neurons and decreased the number of astrocytes in these cultures.

GABA synthesis is carried out by two anabolic enzymes GAD65 and GAD67, both isoforms and their mRNAs are localized within neuronal cell bodies as well as their axon terminals (Esclapez et al. 1994). A recent study has demonstrated GAD65 and GAD67 mRNA in embryonic POA region with GAD67-ir cells located nearby GnRH neurons (Fujioka et al., 2007), suggesting GABA synthesis in cells adjacent to GnRH neurons in the POA starting from embryonic development. Ultrastructural data also provide evidence that GABAergic neurons synapse directly onto GnRH neurons located within the mPOA (Leranth et al. 1985). GAD67 mRNA expression is high during the early morning hours of proestrous and then declines around the time of the GnRH-induced LH surge (Cashion et al. 2004). A morning rise and an afternoon fall in GAD67 mRNA levels marks two E2-dependent signals required for GnRH and LH surge release (Curran-Rauhut and Peterson, 2002). Present data shows that GAD-ir was significantly higher (Fig. 4B, 4D) and GnRH-ir (Fig. 2D) as well as its mRNA expression (Fig. 2E) was significantly lower in VPA treated test group in comparison to control proestrous rats. Both protein (Fig. 5 A, B, C) and mRNA expression (Fig. 6 A, B, C, D, E and Fig. 7 A, B, C, D, E) of GAD were higher in VPA treated test rats, which supports the hypothesis that GABA elevating effect of VPA may be due to enhanced activity of GAD which is the rate limiting enzyme for GABA synthesis. VPA is an inhibitor of HDACs, enzymes that remove the acetyl group from histones in nucleosomes and thus modulating transcription of specific genes (Gottlicher, 2004; Saha and Pahan, 2006). Moreover VPA has been shown to upregulate GAD67 mRNA expression via HDAC inhibition mechanism as VPA hyperacetylates the brain nucleosomal histones (Tremolizzo et al., 2002, Dong et al. 2005). Thus enhanced GAD expression by VPA induced HDAC inhibition may be another plausible mechanism to explain the VPA mediated increase in GABAergic neurotransmission, which in turn may decrease GnRH release and reproductive failure in rats given long term VPA treatment.

GABAergic mechanism of VPA action is thus unequivocal in disrupting the function of HPG-axis. Along with GABAergic system, it is appreciable to explore other key players in the central regulation of reproduction which in orchestration with GABA,
regulate the reproductive cycle. Astroglial cell population is one such candidate, having close interactions with GnRH neurons in hypothalamus. We extended our work on astroglial interactions with GnRH neurons because these neuronal-glial interactions are regulated by estrogens, which were seen to decrease in VPA treated group.

5.3 Effect of VPA on astro-glial regulation of GnRH release is mediated by decrease in estrogen levels:

The present results of dual immunofluorescence staining of GnRH and GFAP in the mPOA (Fig. 9) and median-eminence region of hypothalamus from VPA treated test and vehicle treated control rats have shown reduced glial ensheathment of GnRH cell bodies (Fig. 8A) and their terminals in external zone of median-eminence (Fig. 8A) in control rats, whereas in the VPA treated animals, GFAP staining was seen in both the external and internal zone of median-eminence region (Fig. 8D). In a previous study from our laboratory Parkash and Kaur (2005) have shown increase in apposition of glial processes to the axon terminals of GnRH neurons in the external zone of median-eminence region and a corresponding decrease of ensheathment in EBP-OVX rats suggesting that alterations in the circulating gonadal steroid levels influence the morphology and function of glial cells. These observations suggest that the decrease in glial ensheathment on GnRH neuron terminals in the external zone of median-eminence region in control proestrous rats facilitate GnRH release via reduced glial apposition to GnRH axon terminals.

Emerging evidence strongly suggested an important role for hormonally responsive glia which until now were considered relatively passive supporters of nerve function, providing structural, metabolic and trophic support to the communicating neurons. These cells have been shown as active participants in the processes of synaptic patterning and transmission by their responsiveness to steroid hormones through changes in their morphology (Mong and Blustein, 2006). These steroid induced morphological changes have been suggested to affect neuronal function by regulating synaptic connections via ensheathment and de-ensheathment of the synapses (Garcia-Segura and McCarthy, 2004). Over the past decade, it has been established that fluctuating physiological conditions during the ovarian cycle can indeed reversibly alter structural relationships among the various cell types of the median eminence that specifically
interact with axon terminals containing GnRH (Prevot et al., 1999 and Yamamura et al, 2004). Increased GnRH neuro-terminal contact with the perivascular space have been reported in the median-eminence region on the day of proestrous by Prevot et al., (1999) without clear explanation of underlying neuro-glial plasticity. Furthermore the glial cells express receptors for gonadal hormones, metabolize gonadal steroids, and participate in the synthesis of endogenous steroids by the nervous system (Garcia-Segura and Melcangi 2006). Our results suggest that VPA mediated decrease in estrogen levels may be one of the factor to enhance glial apposition in the external zone of median-eminence region as estrogen facilitates GnRH release via withdrawal of glial apposition in control rats, further suggesting role of glial cells in regulation of reproductive axis. A report from non-human primates by Witkin et al., (1991) underlines the role of gonadal-steroids in regulation of GnRH neuro-glial plasticity, reporting ovariectomy mediated decrease in synaptic inputs to GnRH neurons and reversion of this effect by gonadal steroid replacement. Interestingly synaptic contact loss accompanied increase in glial ensheathment of GnRH perikarya, also shown in our results (Fig. 9B) where enhanced glial ensheathment of GnRH cell body is seen in VPA treated group and could be linked to VPA mediated decrease in estrogen level. This report indicates the control and regulation of GnRH plasticity in non-human primates via ovarian cycle-dependent plasticity and our results further underlines the VPA mediated anomalies in the central regulation of reproduction.

Glial cells are known to regulate GnRH release either by direct interactions with GnRH neuronal somas and terminals or by modulation of the neuronal circuits that regulate the activity of GnRH neurons. Neurons, glia and brain capillaries are organized into well structured neuro-glio-vascular units, in which individual astroglial cells support the function of specific neuronal populations and territories and communicating with associated segments of the microvasculature (Haydon and Carmentnito, 2006). These neuron-glia-vascular units make microfunctional domains which likely play important role in maintaining a precisely regulated microenvironment for reliable neuronal signaling in an ever changing physiological context. Median eminence dynamics involve neurosecretory axons, special emendymoglial cells called tanycytes and the basal lamina having fenestrated endothelium that allows for the bidirectional passage of
macromolecules between the portal blood and nervous tissue (Ciofi et al., 2009 and Rodriguez et al., 2010). As the dynamics of these microfunctional domains is regulated by steroid hormones and our results of VPA mediated decrease in estrogens is likely to interfere with the normal functioning of regulation of reproductive axis.

GnRH neuronal activity is modulated by non-neuronal cell population of glial cells along with the transsynaptic regulatory mechanisms (Ojeda et. al, 2000). GnRH neuron terminals rarely form neuro-haemal junctions at the vascular wall of the portal system (Kozlowsky and Coates, 1985), rather these nerve endings are separated from pericapillary space by astroglial processes (Prevot, 2002). The apposition of GnRH axon terminals by astrocytes in the median-eminence region is minimum (when estrogen levels peak) due to retraction of astroglial endfeet (Prevot et al., 1999) resulting in the release of the engulfed axon terminals and establishment of a direct neurohaemal relationship between GnRH neuroendocrine neurons and the pituitary portal blood. This presumably results in greater GnRH release into the portal blood however in diestrous (when estrogen levels are at nadir) the glial appositions extend which further establishes a diffusion barrier between terminals and capillaries (King and Rubin, 1996; Prevot, 2002). Our immunofluorescent results also show the extension of astroglial processes into external zone of median-eminence region (Fig. 8D) and also increase in GFAP is clearly reflected at protein level in our Western blot studies (Fig 10 D) as well as in mRNA level in our FISH and RT-PCR results (Fig. 10B, Fig. 10F).

The mechanism behind the regulation of GnRH secretion by astroglial cells is still elusive. The underlying cellular mechanisms and signaling pathways are implicated which involve hypothalamic astrocytes being continuously exposed to diverse stimuli ranging from neurotransmitter release, signaling molecules produced by neighboring cells and paracrine factors to hormones. Astrocyte mediated integration of these extracellular signals is important to generate appropriate responses capable of supporting the function of the particular neuronal populations and territories composing the GnRH neuroendocrine network (Prevot et al., 2010). Rise in circulating levels of estrogens at the onset of GnRH/LH surge and expression of estrogen receptors in glial cells in median eminence (Langub and Watson, 1992) suggests that estrogens are likely to be key humoral factors involved in the orchestration of the glia to neuron communication
processes that allow GnRH neurons to directly contact the pituitary portal blood vessels at proestrus when estrogen levels are at peak.

Hypothalamic astroglial cells synthesize and release several growth factors which are able to regulate GnRH neuronal function, including transforming growth factor (TGF)\(\beta\)1, basic fibroblast growth factor (bFGF), insulin like growth factor-1 (IGF-1) and members of epidermal growth factor (EGF) family of trophic factors (Ojeda et al., 2006; Mahesh et al., 2006). IGF-1 is required for the estradiol induced release of gonadotrophins (Etgen et al., 2006) to act on GnRH cell bodies to enhance its gene transcription (Daftary and Gore, 2005) and on median eminence to stimulate GnRH release (Hiney et al., 1991). Astrocytes deliver this peptide to the sites of action within the hypothalamus (Garcia-Segura et al., 1999) enhancing strikingly at the time of the preovulatory surge of gonadotrophins presumably due to estrogen dependent upregulation of IGF-1 receptors (Hiney et al., 1996). This IGF-1 mediated upregulation of GnRH synthesis and release could have been downregulated due to VPA mediated decrease in estrogen levels as indicated by lower GnRH-ir both in mPOA and median-eminence region of hypothalamus in VPA treated rats.

Estrogens have been shown to activate erbB-mediated signaling events by stimulating the expression of both TGF\(\alpha\) and erbB1 and to enhance the effect of PGE2 receptors (Ma et al., 1997; Ma et al., 1999) in hypothalamic GnRH neurons (Rage et al. 1997). TGF\(\alpha\) is a potent gliatrophin member of epidermal growth factor family (EGF) which is capable of inducing high degree of plasticity in astrocytes (Sharif et al., 2006; Sharif et al., 2007; Dufour et al., 2009) and to stimulate GnRH release (Ojeda, 1990). The underlying mechanism in median-eminence employs TGF\(\alpha\) which acts on erbB1 receptors to promote outgrowth of their processes, and then within 12h elicits a PGE2 dependent production of TGF\(\beta\)1, which in turn induces retraction of the astroglial processes (Ma et al., 1997; Ma et al., 1999; Prevot et al., 2003). A second mechanism is also speculated which involve induction of acute actin cytoskeleton remodeling in ependymoglial cells mediated by nitric oxide where increase in endogenous NO release results in evaginations of basal parenchyma and establishment of direct contact between GnRH nerve endings and the endothelial wall of the portal vasculature (De Seranno et al., 2004). The possibility of involvement of NO in the process by which estrogen stimulates
GnRH secretion is further supported by estrogen mediated increase in release of NO and GnRH (Prevot et al., 1999). As astrocytes and tanycytes in median-eminence contain estrogen receptors of the alpha type which appear to mediate stimulatory effects of the steroid on TGFα and TGFβ formation (Ma et al., 1992; Buchanan et al., 2000). This intertalk between estrogen and key signaling molecules is interesting because VPA mediated decrease in estrogen levels is likely the underlying factor behind the disruption in reproductive function in drug treated animals.

It emerged from highly sensitive microarray analysis that estrogen affects neuronal plasticity in hypothalamic neurons not only by transcription of new membrane proteins (e.g., receptors and channels), but also by altering expression of downstream signaling molecules and proteins involved in neurosecretory pathways (Malyala et al., 2004). Since coordinated activity of GnRH neurons is critical for reproductive success, these transcription and ensuing protein dependent changes may play a role in the synaptic remodeling observed in the hypothalamus on the day of proestrous under the gonadal hormone influence. This observation underlies the importance of estrogen in the central regulation of reproductive function. The current results of decrease in serum estrogen levels may have implications in reproductive function disruption at central level highlighting the VPA’s role in reproductive function disruption at HPG axis. We further explored VPA mediated effects on the cell surface molecule PSA-NCAM which plays key role in structural remodelling of the GnRH neurons.

5.4 VPA disrupts PSA-NCAM mediated structural remodelling of GnRH neuron:

The mechanism of structural remodeling in GnRH neurons in mPOA and their axon terminals in median-eminence region is still not clear, but the role of one molecule which is an important marker of neuronal plasticity, PSA-NCAM in structural remodelling is quite evident from our results and previous study from our lab (Kaur et al., 2002; Parkash and Kaur, 2005). PSA-NCAM immunostaining was found on the surface of GnRH perikarya (fig. 11) in control proestrous rats while in VPA treated rats, PSA-NCAM-ir was very weak. PSA-NCAM expression is strategic from the perspective of its increasingly attractive proposed mechanism which accounts for the degree and nature of adhesive interactions between cells and their immediate environment. The large volume
of this polymer presumably via heterophilic interactions with other cell surface and extracellular matrix components produces hindrance between adjacent membranes and interferes with NCAM mediated adhesion or adhesion induced by other adhesion receptors (Kleene and Schachner, 2004) and thus facilitating axon growth, remodeling of cell contacts and functional synaptic plasticity (Dityatev et al., 2004).

Previous studies have shown dynamic transformation of individual GnRH terminals in the median-eminence region of the hypothalamus as a function of different phases of the estrous cycle in rats (Prevot, 2002; Parkash and Kaur, 2005; Parkash and Kaur, 2007a). The adult pattern of synaptic connections could be altered in the rat hypothalamus in relation to endocrine events as shown by synaptic restructuring of the median-eminence region during the proestrous phase of the estrous cycle as is evident from GnRH axon sprouting (Fig.12A) which is absent in VPA treated rats (Fig.12B). Median-eminence region provides a common point of convergence for neuroendocrine GnRH neurons which are diffusely distributed throughout the hypothalamus, the control of GnRH neuronal activity and hence plasticity is also regulated at median-eminence region. The present results have further shown that the median-eminence region region of hypothalamus which has GnRH neuron terminals, retains the capacity to express PSA-NCAM in the control group but VPA treated rats showed downregulation of PSA-NCAM-ir (12B, F). PSA-NCAM expression in the median-eminence region in control proestrous rats may be responsible for the structural reorganization of the GnRH neuron terminals.

The molecular mechanism behind PSA mediated dynamic changes in GnRH neuron terminals is still elusive, however PSA is thought to intervene in axon terminal proliferation during development (reviewed in Kiss and Muller, 2001). It may serve a similar function in the GnRH terminals projecting to the median eminence, the hypothalamic region enriched in PSA-NCAM (Alonso et al., 1997) and undergoing dynamic plastic changes which facilitate the release of neuro-hormone into the pituitary portal blood (Prevot et al., 1999). Steroid hormones seem to be the inductive factors to enhance GnRH axon terminals sprouting and PSA-NCAM expression as evident from higher expression of PSA-NCAM and GnRH-ir axon terminals sprouting near perivascular space in EBP primed OVX rats as compared to OVX rats (Parkash and
Kaur, 2005). We observed greater expression of PSA-NCAM associated with GnRH terminals in the control proestrous rats as compared to VPA treated rats. These observations clearly suggested more prevalent PSA-NCAM expression under a stimulatory period preceding LH surge release in the afternoon of proestrus when estrogens peak which further supported by previous study from our laboratory, where ovariectomised rats primed with estradiol (EBP-OVX) have shown higher expression of PSA-NCAM in external zone of median-eminence region of hypothalamus which further indicates the permissive role of PSA-NCAM in GnRH release. Viguie et al. (2001) reported that PSA-NCAM might also promote rearrangement of cells in close vicinity to the GnRH neurons and their terminals besides influencing synaptic rearrangements. Earlier reports have also implicated PSA-NCAM in the remodeling of both the developing (e.g. prenatal GnRH migration) (Yoshida et al., 1999; Murakami et al., 2000) and adult neuroendocrine system (Theodosis et al., 2006). These findings together with the widespread role of PSA-NCAM in promoting neuroplasticity (Rutishauser and Landmesser, 1996) suggest that PSA-NCAM may provide a molecular substrate for structural remodeling of GnRH system and its surrounding astro-glial cells in adult cycling female rats.

Our results showed PSA-NCAM downregulation in VPA treated group in median-eminence region of hypothalamus, whereas PSA-NCAM-ir associated with GnRH neuron terminals was higher in external zone of median-eminence region in control proestrous group (Fig 12). VPA mediated decrease in PSA-NCAM in external zone of median-eminence may be interfering with the PSA mediated permissive role for the GnRH neuron terminals extension towards pericapillary space and thus disrupting the reproductive cycle in VPA treated rats as shown in the current study. However no such disruption was noticed in vehicle treated control rats. Other compelling evidence by Western blot analysis of membrane extract from the median-eminence region from control proestrous phase rats revealed a PSA immunoreactive band in the 150–250 kDa range, whereas VPA treated rats showed downregulation of PSA-NCAM-ir.

Seasonal mammals have shown variations in PSA-NCAM appositions on GnRH neurons with higher association during breeding season (Viguie et al., 2001). This association may also be important for the central control of reproduction in non-seasonal
mammals, where an upregulation of PSA-NCAM on GnRH cell bodies was reported in the proestrous phase of rats (Parkash and Kaur, 2007) suggesting permissive role of PSA-NCAM in the structural remodeling of GnRH neurons. These observations are also supported by previous studies which have demonstrated the existence of dynamic plasticity changes in the external zone of the median-eminence, which allows physical contacts between GnRH nerve endings and the perivascular space on the day of proestrus to facilitate the release of this neurohormone into pituitary portal blood (Alonso et al. 1997; Prevot et al., 1999).

Gonadal steroids are implicated in direct modulatory influence on the GnRH neurons and other neuronal networks involved in the control of GnRH secretion (Herbison and Pape, 2001). The functional link between PSA-NCAM expression in the median-eminence region in the modulation of GnRH release under the influence of gonadal hormones has also been suggested by previous studies from our lab. (Parkash and Kaur, 2005; Parkash and Kaur, 2007a). These hormones may be the inductive factors to enhance GnRH axon terminals sprouting as is also evident from higher expression of PSA-NCAM and GnRH-ir axon terminals sprouting near perivascular space in vehicle treated control rats (Fig. 10). Our results on blood serum analysis have shown significant decrease in serum estrogen levels in VPA treated group supported by in vivo study in rats reporting marked reduction of serum estradiol levels (Roste et al. 2002). Estrogen is one of the principal determinants of GnRH neuron functioning and acting as a classic homeostatic feedback molecule between gonads and brain enabling GnRH neurons to exhibit pulsatile pattern of biosynthetic and secretory activity (Herbison and Pape, 2001) which could be disrupted due to VPA mediated decrease in estrogen levels. During the proestrous phase of the cycle, PSA-NCAM is associated with axon terminals of GnRH neurons in the median-eminence and may be a consequence of an altered ovarian steroid milieu, which itself can induce hypothalamic neuroplasticity (Horvath et al., 1997, Parkash and Kaur, 2005). Moreover PSA appears to participate in estradiol-induced shape changes because neuraminidase treatment which specifically removes PSA from cell surface (Rutishauser et al., 1985) prevented the estradiol induced morphological changes furnishing direct evidence of PSA involvement in neuroplastic remodeling (Monlezum et al., 2005). Our results showed decrease in PSA-NCAM-ir in median-
eminence region in VPA treated group while its expression was higher in vehicle treated controls. Our study is supported by a recent report by Tan et al., (2009) which showed 25% increase in the PSA-NCAM protein content in Western blots from paraventricular region of hypothalamus in proestrous phase, the period of estrogen-induced synaptic plasticity compared with the metestrous phase where estrogen levels are minimum. Moreover PSA-NCAM staining was heavier in the median-eminence region in proestrous mice, at the time that the estrogens peak (Tan et al., 2009). The normal synaptic restructuring of the median-eminence region in vehicle treated proestrous rats further indicate that adult pattern of synaptic connections being altered in hypothalamus in relation to normal endocrine events could have been disrupted in VPA treated rats because of the VPA mediated decrease in serum estrogen levels.

5.5 VPA effects on expression levels of PST and NCAM in median-eminence region of hypothalamus:

We further explored the regulation of the sialylation of NCAM to understand the posttranslational changes in this molecule that occur during the normal ovarian cycle and in VPA treated group where ovarian cycle is disrupted. Two polysilyltransferases, ST8Sia II (STX) and ST8Sia IV (PST) regulate the synthesis of PSA in vertebrates, both differing markedly with respect to their spatial and temporal expression patterns (Wood et al., 1997). ST8Sia II (STX) is predominant in embryo, whereas ST8Sia IV (PST) persists at relatively higher levels in the postnatal brain (Hildebrandt et al., 1998; Ong et al., 1998). PST mRNA expression was studied in median-eminence region of hypothalamus by fluorescent in situ hybridization and RT-PCR. Our results from FISH data indicated that PST mRNA expression was significantly higher in the median-eminence region of the hypothalamus in the control proestrous group as compared to the VPA treated test rats (Fig. 13C). Expression level of PST mRNA quantified by semi quantitative RT-PCR analysis further confirmed decline in expression of PST mRNA in the median-eminence region in VPA treated test rats (Fig. 13G). A recent report by Tan et al. (2009), clearly indicates that increased PSA-NCAM in the paraventricular area is associated with increased expression of sialylation enzymes PST and STX during the proestrous phase when estrogen peaks.
The studies on the mRNA expression of the PST suggest that the biosynthesis of PSA is regulated at the transcriptional level (Eckhardt et al., 1995; Hildebrandt et al., 1998). The biosynthesis of PSA appears to occur directly on NCAM, as no other intermediate acceptor has been identified (Alcaraz and Goridis, 1991). NCAM exists in several structurally distinct isoforms that contain various amounts of 2-8-linked polysialic acid (PSA) on the extracellular domain of the membrane (Nelson et al., 1995). Mammalian neurons express three splice variants of NCAM (180, 140, and 120 kDa). Of these, NCAM-140 and NCAM180 are believed to localize to synapses (Schuster et al., 1998). While the stage and spatial specificity of expression of the multiple NCAM isoforms has been documented in a variety of tissues, the functional significance of the presence of these molecules is still speculative. It has been proposed that their structure allows them to differ in subcellular localization, turnover rate, ability to cluster at the cell surface, cell contact stabilization and ability to respond to cell signaling processes (Rutishauser and Landmesser, 1996; Yoshida et al., 1999). Doherty et al. (1990) reported that expression of NCAM-140 is associated with neurite growth promoting response from neurons isolated from cerebellum region of postnatal day 1, 3 and 4 rats. We observed decrease in hypothalamic content of the NCAM-180 and NCAM-140 isoforms (which are associated with PSA expression) in the VPA treated group compared to the vehicle treated control (Fig. 14) which further indicate disruption in normal function in VPA treated animals.

5.6 Effect of VPA on neuroplasticity marker PSA-NCAM and GAD: Possible link between VPA medication and Cognition impairment:
Children with epilepsy often show cognitive impairments (Addy, 1987) and a number of studies have suggested adverse effect of of AEDs on mental functioning (Seidel and Mitchell, 1999; Umka et al., 2010). Phenytoin, Phenobarbital and Carbamazepine treatment have serious concerns regarding mental health of the children and teenagers under medication, some of whom have shown cognitive dysfunction (Reinisch, 1995; Holmes, 2002). The published data so far suggests greatest concern about cognitive dysfunction in children exposed in utero to VPA (Ardinger, 1988; Moore, 2000). Previous studies specifically suggest the role of VPA in cognitive impairments (Wu and Wang, 2002; Umka et al., 2010). Our study was designed to
elucidate reproductive function anomalies caused by VPA treatment but we also observed VPA effect on the neuronal plasticity marker PSA-NCAM and the GABAergic neurons, the chief inhibitory interneuronal population in hippocampus. The hippocampal region (the CA fields, dentate gyrus, and subicular complex) is part of a system of anatomically related structures in the medial temporal lobe that are important for mammalian memory (Squire, 1992) and damage to this region impairs performance on a variety of tasks of learning and memory (Eichenbaum et al., 1989). Further, single-cell recording and neuroimaging techniques document changes in the hippocampal region during both learning and retention (Squire et al., 2004). Hippocampus is the final stage of convergence within the medial temporal lobe, receiving projections from the adjacent perirhinal and parahippocampal perirhinal cortices, as well as the entorhinal cortex (Lavenex and Amaral, 2000).

The NCAM being expressed by all cell types in the nervous system promotes neuron-neuron and neuron-glia adhesion via homophilic and heterophilic interactions with other cell adhesion and extracellular matrix molecules. It is also implicated in signal transduction, neurite outgrowth, fasciculation, synaptogenesis and synaptic plasticity (Dityatev et al., 2000; Maness and Schachner, 2007). Most of these functions depend on NCAM’s glycosylation, particularly on polysialylation, i.e., attachment of long homopolymers of α2,8-linked sialic acid residues, known as PSA (Rutishauser and Landmesser, 1996; Dityatev et al., 2004; Weinhold et al., 2005). In this study we observed marked decrease in PSA-NCAM expression in dentate gyrus and piriform cortex (Fig. 15, 16) which is required for correct axonal branching and fasciculation (Rutishauser and Landmesser, 1996) and promotes synaptic plasticity and spatial learning (Becker et al., 1996; Dityatev et al., 2004). The regulation of PSA expression varies in accordance with an age-, cell type- and activity-dependent manner, being highest during early development and in brain regions associated with neuroplasticity in the adult. A growing body of evidence has implicated NCAM and PSA as risk factors for major neuropsychiatric and neurodegenerative disorders, such as schizophrenia, bipolar disorder, depression, anxiety disorder, and Alzheimer's disease (Brennaman and Maness, 2008). This is in line with increasing appreciation of the roles played in brain malfunctions by the drastically decreased expression of polysialylated NCAM (PSA-
NCAM) in hippocampi of patients with schizophrenia (Barbeau et al., 1995) and a link between NCAM and neurocognitive changes in schizophrenia (Sullivan et al., 2007) suggesting the relationship between VPA mediated downregulation of PSA-NCAM and associated cognitive dysfunction. The transcriptional regulation of PSA-NCAM was also studied via PST expression levels in hippocampus and piriform cortex with the help of semi quantitative RT-PCR analysis which showed downregulation of PST mRNA expression in VPA treated rats (Fig. 16). Memory impairment is commonly associated with epilepsy, and the use of AEDs causes additional neuropsychologic deficits that are of particular concern in learning-age children and elderly patients. Moreover, the demonstration that VPA induces morphologic alterations and impairment in specific hippocampal-dependent memory task might explain the detrimental effects of antiepileptic treatment on cognition in human subjects (Sgobio et al., 2010).

The underlying mechanism behind VPA mediated dysfunction in cognitive and memory function is not clear. However learning and synaptic plasticity in vitro and in vivo are altered in the CA1 and CA3 hippocampal subfields and/or in the dentate gyrus of mice deficient for NCAM (Cremer et al., 1994, Cremer et al., 1998; Muller et al., 1996; Senkov et al., 2006; Stoenica et al., 2006) suggesting the role of NCAM. This could be the possible underlying link between VPA mediated downregulation in PSA-NCAM leading to memory dysfunctions. Enzymatic removal of NCAM-associated PSA and genetic ablation of polysialyltransferase ST8SiaIV—required for polysialylation of NCAM in the adult hippocampus—lead to learning defects and impairment of LTP and LTD in the CA1 region of the hippocampus, but not in mossy fiber–CA3 synapses or in perforant path synapses of the dentate gyrus (Becker et al., 1996; Muller et al., 1996; Eckhardt et al., 2000; Senkov et al., 2006). Application of soluble PSA-NCAM to acute slices of NCAM-deficient mice restores normal CA1 LTP and improves contextual fear memory (Senkov et al., 2006). Since application of non-polysialylated NCAM is not effective in restoring normal CA1 LTP, the combined data indicate that PSA is both necessary and sufficient for normal induction of CA1 LTP. A recent study reported reduced cell proliferation in the sub granular zone of the dentate gyrus within the hippocampus of VPA treated rats and was linked to a significant impairment in their ability to perform a hippocampus-dependent spatial memory test (novel object location).
Memory formation involves making and breaking of new synapses and neuroplasticity in their contacts which is disrupted as shown by reduced PSA-NCAM-ir cell bodies in dentate gyrus (Fig. 15B, E). In addition, drug treatment caused a significant reduction in BDNF and Notch 1 levels within the hippocampus which further suggest that VPA may cause cognitive impairment and provide a possible mechanism for this by reducing neurogenesis within the hippocampus (Umka et al., 2010).

Another plausible mechanism which may be involved in VPA treated rats in the disruption of learning and memory function is the GABAergic mechanism of VPA action. VPA influences GABA synthesis and breakdown, leading to an increase in GABA concentrations in the brain (Loscher, 1999). Our results show significant upregulation of GAD-ir in hippocampus and piriform cortex (Fig. 17, 18). This upregulation has been shown to be regulated at transcriptional levels as shown by upregulation of GAD mRNA upregulation both in hippocampus and piriform cortex (Fig. 18). VPA increases GABA levels in the hippocampus in vivo (Rowley et al., 1995). A previous study has also shown VPA mediated inhibitory postsynaptic potential (Preisendorfer et al., 1987). LTP and LTD in the hippocampus is thought to be an important cellular mechanism for learning and memory (Malenka, 1994) and its induction requiring appropriate integration of GABAergic inhibitory and glutamatergic excitatory synaptic transmission (Davies and Collingridge, 1993). Our results showing upregulation of GAD both at transcriptional and translational level suggest disruption in integration of inhibitory and excitatory transmission which may lead to VPA mediated suppression in LTP. Zhang et al., (2003) have reported VPA mediated disruption in LTP and LTD in hippocampus of rats at different developmental stages. Synaptic plasticity is considered to underlie the neural basis of learning and memory includes LTP and LTD, and its induction is NMDA receptor-dependent (Malenka, 1994). The underlying mechanism in VPA mediated disruption in cognitive function could be explained based on a report indicating VPA mediated suppression on synaptic response mediated by NMDA receptors (Gean et al., 1994) and reducing the number of action potentials elicited by application of NMDA in neocortical neurons in the rat (Zeise et al., 1991). This reduction of NMDA receptor mediated responses may contribute to the impairment of LTP and LTD caused by VPA via disruption of the integration of excitatory/inhibitory synaptic transmission. VPA
mediated upregulation of inhibitory GABAergic circuitry as shown in our study could further deteriorate this integration and ultimately leading to impairment in cellular mechanisms underlying memory. Lee et al. (1996), have reported VPA mediated suppression in the expression of LTP in hippocampal slices. A large number of studies reported VPA mediated enhancement of GABAergic inhibitory neurotransmission and inhibitory postsynaptic potential (Loscher, 1999). Enhancement of GABA<sub>A</sub> receptor mediated hyperpolarising response will inhibit NMDA receptor activation and ultimately the suppression of LTP and LTD (Mott and Lewis, 1991).

5.7 Conclusions:

In the current study, we observed that VPA medication disrupted estrous cyclicity and reduced estrogen levels in cycling female rats. VPA potentiated GABAergic inhibitory neuronal circuitry by upregulating the synthesis of anabolic enzymes for GABA synthesis i.e. GAD65 and GAD67. VPA treatment of non-epileptic animals disrupted neuro-endocrine plasticity and normal functioning of GnRH pulse generator, which may be the underlying mechanism(s) for reproductive dysfunction in patients on AEDs treatment. Further VPA was also observed to affect the neuronal plasticity and GABAergic function in hippocampus and piriform cortex, brain regions known to be associated with learning and memory. Based on our observations of adverse effects of VPA on reproductive and cognitive health, it may be recommended that VPA and other AEDs acting through GABAergic mechanism should be prescribed with caution in case of pregnant and young women patients.

5.8 Future Directions:

There is need to analyse more such AEDs with GABAergic mechanism of action for their role in disruption of reproductive function and also to ascertain the safety levels to reduce their adverse effects on reproductive health and cognitive functioning.