ORIGINAL ARTICLE

Valproate inhibits GnRH-induced gonadotropin release from anterior pituitary cells of male rat *in vitro*

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Abstract

OBJECTIVE: Valproate (VPA) a potent antiepileptic drug has been claimed to induce reproductive disturbances in men. Long-term VPA treatment can affect sperm morphology and induce testicular atrophy in non-epileptic rats. It has been reported that VPA reduced testosterone secretion stimulated by hCG in isolated rat Leydig cells. These results suggest direct effect of VPA on testes in rats. However centrally mediated effects at hypothalamo-pituitary level can therefore not be excluded. This study focused on the dose and time-dependent effects of VPA on basal and GnRH-induced LH and FSH release from the primary anterior pituitary cells culture of male rats.

MATERIAL AND METHODS: The dose-dependent effect of 10 nM–100 mM of VPA on basal LH release from anterior pituitary cells after 3h of incubation was examined. To determine the time-dependent effects on LH, FSH, TSH and PRL release short (3 h) and long-term (24 h) incubations in the presence of 10 nM, 100 nM and 1 μM of VPA were maintained.To assess whether VPA can affect GnRH-induced LH and FSH release, cells were incubated for 3 h with 10 nM, 100 nM and 1 μM of VPA in the presence of GnRH. The concentration of rLH, rFSH, rPRL and rTSH in incubation medium was determined by RIA method.

RESULTS: VPA did not affect the basal LH, FSH, PRL and TSH release from the primary anterior pituitary cells culture of male rats. VPA in concentration 1μM significantly suppressed GnRH-induced LH secretion. However VPA at all tested doses diminished GnRH-induced FSH release.

CONCLUSIONS: VPA may diminish gonadotropin release *in vitro* but this effect can only be achieved after GnRH-dependent specific receptor activation. Both gonadotropins differ in their pattern of response for increasing doses of VPA.

INTRODUCTION

Valproate (VPA) a potent antiepileptic drug has been claimed to evoke a severe reproductive endocrine disturbances in both sexes in humans (Isojarvi *et al.* 2005). In men, therapeutic VPA treatment induced smaller testicular volumes with an increase in sperm abnormalities (Isojarvi *et al.* 2004) as well as an elevated androstendione and lowered LH and FSH serum concentration (Roste *et al.* 2005). In women lower birth rates and a greater risk of the syndromes associated with infertility, such as an anovulatory cycles and hypothalamic pituitary axis disruption were observed (Kaplan 2004). Moreover over 50% of women taking VPA for epilepsy were found to have hyperandrogenism (Svalheim *et al.* 2003) and polycystic ovaries (Isojarvi *et al.* 2001).

Studies on male animal experimental models revealed that prolonged VPA treatment significantly affected their fertility through changes in sperm morphology and the generation of testicular atrophy (Roste *et al.* 2001; Krogenaes *et al.* 2008). It has also been reported that VPA reduced hCG-stimulated testosterone secretion from isolated rat Leydig cells (Kuhn-Velten *et al.* 1990). Although most available data indicate that the anti-reproductive effects of VPA are primarily exerted at the gonadal level, chronic treatment with VPA has been associated with a variety of endocrine related side effects which suggest the actions of this drug are centrally mediated. Indeed, 90 days long VPA administration resulted in a significant reduction of serum estradiol and LH concentration in females whereas in males serum testosterone levels were not affected, however a significant increase in FSH and LH concentrations at the high VPA dose was observed (Roste *et al.* 2002). One of the possible mechanisms responsible for the centrally mediated inhibitory impact of VPA on reproductive development and function may occur via its action on the GABA-ergic inhibitory neurotransmission in the brain. 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 (Czapinski *et al.* 2005). Since GABA is crucial inhibitory neurochemical regulator of GnRH neurons and VPA is suggested to influence on GABA-ergic activity, it is possible that VPA may mimic and/or elevate the activity of GABA-ergic system that in consequence leads to disturbances occurring within the GnRH neuronal population activity.

Despite VPA action occurring in neurons, its effects on gonadotropins activity exerted directly at the pituitary level cannot be ruled out. Therefore this study focused on the dose and time-dependent effects of VPA on basal and GnRH-induced LH and FSH release from the primary culture of anterior pituitary cells male rats.

MATERIALS AMD METHODS

Three-month-old Wistar male rats (240±10 g) were maintained under standard laboratory conditions on 12/12 h light/dark cycle (lights on at 07.00 a.m). Standard pelleted food (Murigran, Poland) and tap water were available *ad libitum*. All experiments were approved by the IVth Local Ethical Committee for the care and use of experimental animals.

The procedures of pituitary tissue dissection and primary cell culture preparation were based on previously described methods (Baranowska *et al.* 2003). Briefly, ketamine anesthetized rats were decapitated and isolated anterior pituitary lobes were washed twice with DMEM supplemented with 10% FCS and antibiotic/antimycotic. Then, collected pituitaries were enzymatically dispersed for 15 min at 37 °C in the presence of DMEM containing 0.1% trypsin and this was followed by incubation in DMEM with 10% FCS and 0.1% DNA-se I (15 min at 37°C). Pre-digested tissue was also dispersed through a sieve (50 mesh) and the viability of isolated cells (>97%) was assessed by trypan blue staining. Finally, the dispersed cells were dispensed on 24-well culture plates (5×10^5 cells/ml) and were then preincubated for 48h in DMEM containing10%FCS and finally subjected to the three independent experiments.

The dose-dependent effect of 10 nM–100 mM of VPA on basal LH release from anterior pituitary cells during 3h of incubation was examined To determine the time-dependent effects on LH, FSH, TSH and PRL release short (3 h) and log-term (24 h) incubations in the presence of 10 nM, 100 nM and 1 μM of VPA were maintained. Finally, to assess whether VPA can affect GnRH-induced LH and FSH release, cells were incubated for 3h with 10 nM, 100 nM and 1 μM of VPA in the presence of 100 nM GnRH (Blitek *et al.* 2005). Each assay condition was implemented in triplicate within an experiment, and each experiment was performed three to four times.

All media and chemicals were purchased from Sigma (Sigma Aldrich , Germany) and culture dishes were from Nunc (Thermo Fisher Scientific, Denmark).

Rat LH, FSH, TSH and PRL medium concentrations were measured by RIA using antibodies and standards provided by the National Pituitary Agency and Dr. A. F. Parlow (Harbor-UCLA Research and Education Institute c/o Los Angeles Biomedical Research Institute Los Angeles, CA90060). Respective values were expresses in terms of the LH-RP3, FSH-RP2, PRL-RP-3 and TSH-RP-3 of the reference standard. The detection limit for LH, FSH, PRL and TSH were 0.1, 1.25, 0.39 and 0.5 ng/ml respectively. The intra and inter-assay coefficients of variation were below 8% and 10%, respectively. Changes of LH, FSH, TSH and PRL concentration were presented as the percentage of the control value which was set at 100% and expressed as mean ± S.E.M.

Statistical analysis was performed with the Kruskall-Wallis test followed by Mann-Whitney U test (program STATISTICA™ 7.1 PL (StatSoft). Differences in mean values were considered significant if $p \leq 0.05$

RESULTS

In the first experiment the dose-dependent (10 nM–100 mM) effect of the short-term VPA incubation on basal LH release from anterior pituitary cells *in vitro* was examined. As shown in Figure 1, no effect on LH release at any applied d was observed within 3h of the VPA treatment.

Next, to evaluate whether VPA may exert its effects after a prolonged time of treatment as well as whether it can affect anterior pituitary releasing activity, cells were incubated for 3 and 24 h in the presence of 10 nM, 100 nM and 1 μM of VPA. RIA analysis revealed that in comparison to the respective control groups, VPA treatment did not induce any direct significant change in LH, FSH, TSH as well as PRL releasing activity regardless of dose as well as the time of the incubation test (Figures 2 and 3).

Finally, the third experiment focused on VPA-dependent effects on GnRH-stimulated LH and FSH release *in vitro*. To answer a question concerning a possible VPA involvement in the modulation of gonadotropin axis activity, cells were incubated for 3h with 100 nM of GnRH in the presence of three different (10 nM, 100 nM and 1 μM) doses of VPA. Similarly to previous results, no dose-dependent effects of VPA on LH and FSH release from non-stimulated male anterior pituitary cells *in vitro* were detected (Figures 4 and 5). In contrast, VPA effectively diminished GnRH-induced LH and FSH release although both gonadotropins differed in their pattern of response for increasing doses of VPA. In comparison to GnRH-activated cells, co-treatment with 100 nM of GnRH and different doses of VPA resulted in a significant reduction of FSH release in all VPA doses tested (Figure 5; $p<0.05$) However, when compared to the control group, FSH release from GnRH –stimulated and 10 nM as well as 100 nM VPA treated cells remained still significantly elevated (Figure 5; $p<0.05$). In contrast, GnRH-dependent LH release was unaffected both by 10 nM as well as 100 nM of VPA whereas only in cells co-incubated in the presence of 1 μM VPA was LH release completely inhibited and reached the control level (Figure 4; $p<0.01$). Moreover, 1 μM dose of VPA completely reduced also GnRH-stimulated FSH release which in consequence did not differ from that observed in control cells (Figure 5).

DISCUSSION

The obtained results demonstrate that VPA, a potent antiepileptic drug can affect gonadotropin release *in vitro* but its effect requires GnRH receptor activation.

Although the mechanism(s) involved in mediating VPA effects in the pituitary gland are not well recognized, its potential impact on intracellular calcium metabolism cannot be excluded. That VPA may affect intracellular calcium balance was demonstrated mostly in neuronal cells. Indeed, VPA was shown to depress low calcium field bursts in hippocampal neurons (Agopyan *et al.* 1985) while in rat nodose neurons to

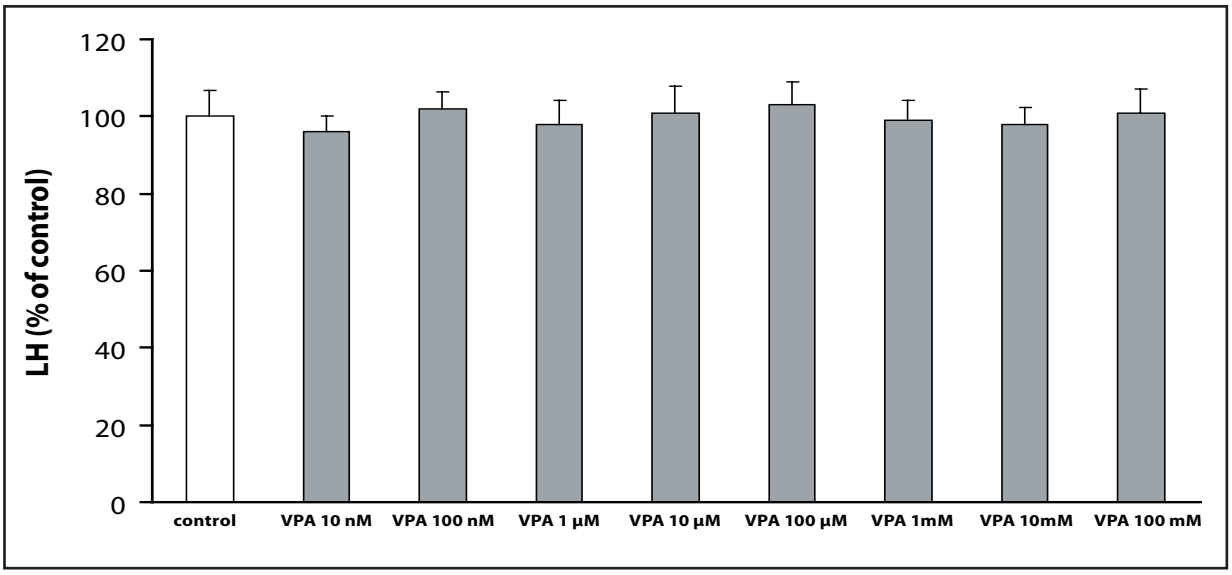

Fig. 1. The dose-dependent effect of VPA on LH release from primary culture of anterior pituitary cells of rats. 5×10^5 cells/well were incubated with 10 nM–100 mM VPA for 3 h (n =12 wells/treatment group). In comparison to the control group no significant differences in LH release at any applied dose of VPA were stated. Control value of LH was 8.98± 0.47 ng/ml. Each bar represents the mean ± S.E.M. with the percentage of the control value.

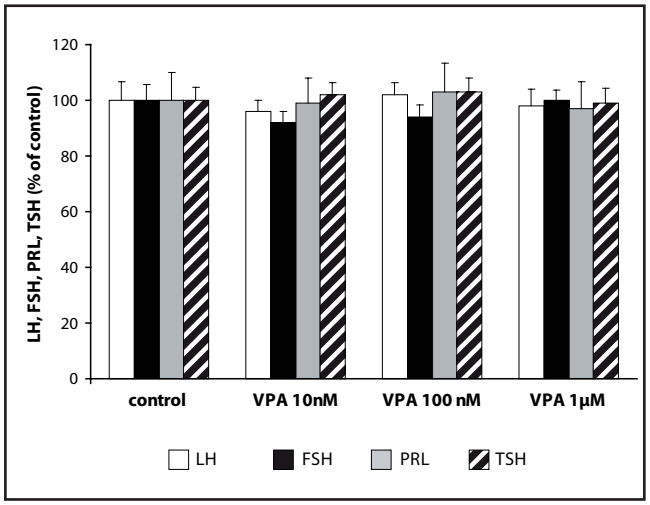

Fig. 2. Short-term incubation effect of VPA on LH, FSH, PRL and TSH release from primary of anterior pituitary cells of rats. 5×10^5 cells/well were incubated with 10 nM, 100 nM and 1 μM of VPA for 3 h (n = 9–12 wells/treatment group). In comparison to their control groups no significant differences in LH, FSH, PRL and TSH release at any applied dose of VPA were stated. Control values of LH was 14.3±0.43ng/ml, FSH – 4.56±0.26 ng/ml, PRL – 63.4±4.16 ng/ml and TSH – 3.06±0.08 ng/ml respectively. Each bar represents the mean ± S.E.M. with percentage of the control value.

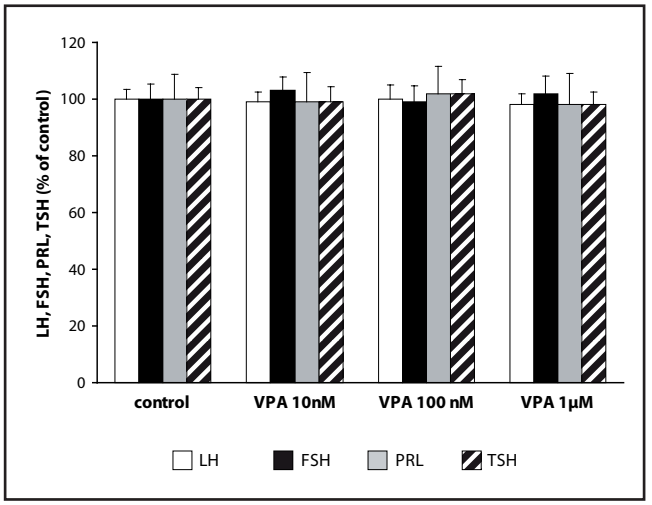

Fig. 3. Long-term incubation effect of VPA on LH, FSH, PRL and TSH release from primary culture of anterior pituitary cells of rats. 5×10^5 cells/well were incubated with 10 nM, 100 nM and 1 μM of VPA for 24 h (n=9–12 wells/treatment group). In comparison to control groups no significant differences in LH, FSH, PRL and TSH release at any applied dose of VPA were stated. The control values of LH were 11.1±0.55 ng/ml, FSH – 4.75±0.31 ng/ml, PRL – 137.8±9.5 ng/ml and TSH – 3.88±0.48 ng/ml respectively. Each bar represents the mean ± S.E.M. with percentage of the control value.

reduce the low-threshold calcium current (Kelly *et al.* 1990). It also blocked SMA type I mouse motor neurons excitability and reduced spontaneous Ca^{2+} influx both in cell bodies and in distal axons in concentration-dependent manner (Rak *et al.* 2009) as well as suppressed L-calcium channel activity in cultured human neuroblastoma cells (Kito *et al.* 1994). Since nifedipine, a selective L-calcium channel blocker, diminished GnRH-activated LH release from gonadotropic cells (Stojilkovic 2006) also VPA *via* its action on L-channel activity might – at least partially – exert a modulatory action on gonadotropin release. Moreover, it cannot be excluded that VPA could affect gonadotropins release by an impact exerted on intracellular second messenger system activity. Indeed, a depletion of inositol as well as an attenuation of phosphoinositide signaling through VPA inhibitory action on myo-inositol-1 phosphate synthase activity was shown in the frontal cortex of mouse brains (Shaltiel *et al.* 2004). Recent studies on amoeba *Dictyostelium discoideum* have shown that VPA treatment diminished PIP and PIP_2 phosphorylation as well as rapidly attenuated phospholipid signaling through an inhibition of phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) production that reduced endocytic trafficking. Moreover, in the same study a depolarization-dependent neurotransmitter release in rat nerve terminals was also examined and this process was also suppressed upon application of VPA (Xu *et al.* 2007).

In our *in vitro* study the lowest VPA concentration which effectively diminished GnRH-induced FSH release was 10 nM. In humans the therapeutic VPA plasma concentration range from 280 μM to 690 μM was due to brain-blood barrier which resulted in a 42 to 190 μM concentration detected in brain (Loscher 1999). Nevertheless, in fibroblast culture derived from spinal muscular atrophy patients, 0.5 μM VPA was shown to enhance SMN2 protein synthesis (Brichta *et al.* 2003). Therefore, if 1 μM of VPA completely inhibited GRH-stimulated LH as well as FSH release *in vitro*, a significant impact on gonadotropic axis activity at therapeutic concentrations applied *in vivo* might also be expected.

Despite the consistent suppressive effects of VPA on GnRH-induced LH and FSH release, the above data also revealed that both gonadotropins responded differently to applied VPA concentration: FSH release was reduced within the whole range (10 nM–1 μM) while LH release inhibition occurred only at the1 μM VPA concentration tested.

Although GnRH is a common pivotal regulator for both gonadotropins activity, a profound differences in terms of their patterns of biosynthesis and release exist. Indeed, after being sorted to a regulated secretory pathway in the gonadotrope cells, newly synthesized LH is packaged into secretory vesicles associated with chromogranin A and secretogranin II (Watanabe *et al.* 1991; Crafword *et al.* 2002; Crafword *et al.* 2002a,) and its release is subsequently GnRH-stimulated *via* the calcium-dependent mechanism. In contrast, newly synthesized FSH is sorted into a constitutive secretory pathway and the bulk of this hormone appears to be constitutively secreted thus the amount released

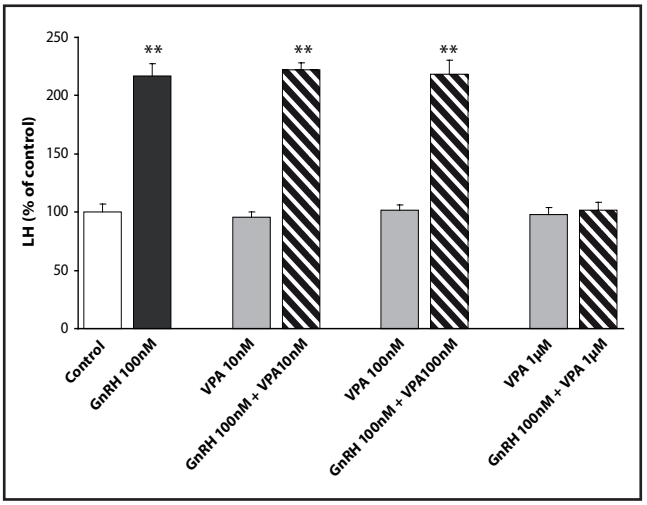

Fig. 4. Inhibitory effect of VPA on GnRH stimulated LH release from primary culture of anterior pituitary cells of rats.
5×10^5 cells/well were incubated with GnRH 100 nM , VPA10 nM, VPA100 nM, VPA 1 µM and GnRH 100 nM + VPA10 nM, GnRH 100 nM + VPA100 nM, GnRH 100 nM + VPA1 µM for 3 h (n = 9–12 wells/treatment group). GnRH stimulated LH release was inhibited by VPA 1 µM and unaffected by VPA 10 nM and VPA 100 nM. The control values of LH were 14.3±1.05 ng/ml. Each bar represents the mean ± S.E.M. with percentage of the control value. $^{**}p<0.01$ versus control. GnRH 100 nM versus GnRH 100 nM + VPA 1µM; $p<0.01$.

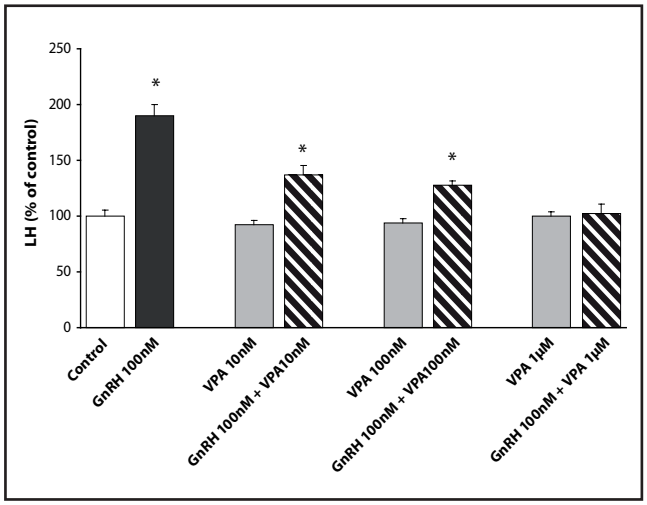

Fig. 5. Inhibitory effect of VPA on GnRH stimulated FSH release from primary culture of anterior pituitary cells of rats.
5×10^5 cells/well were incubated with GnRH 100 nM , VPA10 nM, VPA100 nM, VPA 1 µM and GnRH 100 nM + VPA10 nM, GnRH 100 nM + VPA100 nM, GnRH 100 nM + VPA1 µM for 3 h (n = 9–12 wells/treatment group). GnRH stimulated FSH release was inhibited by VPA 10 nM , VPA 100 nM and VPA 1 µM. The control values of FSH were 4.56±0.26 ng/ml Each bar represents the mean ± S.E.M. with percentage of the control value. $^{*}p<0.05$ versus control.
GnRH 100 nM versus GnRH 100 nM + VPA10 nM; $p<0.05$;
GnRH 100 nM versus GnRH 100 nM + VPA100 nM; $p< 0.05$;
GnRH 100 nM versus GnRH 100 nM + VPA1 µM; NS

is directly related to the rate of synthesis (Farnworth 1995; McNeilly *et al.* 2003; Nicol *et al.* 2004). Therefore, it cannot be excluded that separate intracellular mechanisms governing the patterns of LH and FSH release might be – at least in part – involved in an enhanced FSH *vs* LH sensitivity for VPA stimulation observed in our study in GnRH-treated cells.

So far, data concerning the direct effects of VPA on the release of anterior pituitary hormones release is very limited. Nevertheless, VPA-dependent inhibition of POMC derived peptides**:** IR – ACTH and IR-β-endorphin/LPH release was observed from cells not activated by CRF (Tominaga *et al.* 1989). In the same study, 3 h incubation of anterior pituitary cells of male rat with different doses of VPA resulted in attenuation CRH-induced IR-ACTH and IR-β-endorphin/LPH release.

In contrast the results of our study have shown that VPA did not affect basal releases of anterior pituitary hormones at any of doses tested. Moreover, VPA-induced effects observed in our study were exclusively dependent on parallel GnRH receptor activation. Therefore, a modulation of neuronal activity appears to be crucial for evoking this drug effects on pituitary activity. At the central level VPA affects GABA-ergic neurotransission by increasing GABA levels in the brain and this effect may reflect both an enhanced GABA synthesis (stimulation of glutamic acid decarboxylase) as well as a decreased GABA degradation (inhibition of GABA transaminase and succinic semialdehyde dehydrogenase) (Loscher 1999). Moreover, studies on several GABA transporters (h GAT1, mGAT3, mGAT4) have shown that VPA potentiates the turnover rate of GABA transporters proteins *via* an allosteric mechanism that generates an additional regulatory step facilitating GABA release from nerve ending (Whitlow *et al.* 2003). Also at the hypothalamic level, the anti-reproductive effects of VPA were shown to result from an enhanced GABA-ergic inhibition of the GnRH neuronal population within the medial preoptic area (Dodge *et al.* 2000**).** Since VPA treatment increased GABA expression, and reduced both GnRH and PSA-NCAM expression in mPOA and ME-ARC region of female cycling rats, it was supposed to disrupt the HPG axis at the level of GnRH pulse generator in hypothalamus (Lakhanpal & Kaur 2007).

In conclusion, the results of our study revealed that VPA may diminish gonadotropin release but this effect can only be achieved after GnRH-dependent specific receptor activation. Moreover, both gonadotropins differ in their sensitivity to VPA action since VPA was more effective in reducing GnRH-induced FSH than LH release from anterior pituitary cells.

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