VALPROATE IS AN ANTI-ANDROGEN AND ANTI-PROGESTIN

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ABSTRACT

Anticonvulsant treatment is associated with a high prevalence of reproductive dysfunction compared with age-matched non-epileptics. The contributions from reproductive endocrine effects of anticonvulsant drugs relative to other factors including the convulsions, underlying neurological disease, and psychosocial concomitants remains difficult to disentangle. We examined the widely used anticonvulsants valproate (VPA) and carbamazepine (CBZ) for steroidal bioactivity using yeast-based steroid receptor-reporter system. In vitro bioassays for reproductive steroids were established by stably transforming yeast cells with human cDNA for androgen receptor (AR), progesterone receptor A (PR) or estrogen receptor α (ER) together with a reporter plasmid containing a ß-galactosidase gene under transcriptional control of an androgen (ARE), progestin (PRE) or estrogen (ERE) reporter element. Bioassays were performed by culturing yeast in the absence (agonist bioassay) or presence (antagonist bioassay) of cognate ligands (5 nmol/L testosterone for AR, 1.6 nmol/L progesterone for PR and 5 nmol estradiol for ER) for 4 (PR) or 16 (AR, ER) hours using ß-galactosidase activity in yeast cell lysates as the end-point. VPA showed dose-dependent inhibition of progesterone-induced PR- and testosterone-induced AR activity. VPA had no ER antagonist bioactivity nor any AR, PR or ER agonist bioactivity. CBZ had no significant agonist or antagonist AR, PR or ER bioactivity. We conclude that VPA is a non-steroidal antagonist for human AR and PR but not ER. VPA's androgen and progesterone antagonism at concentrations much lower than therapeutic blood levels (350-700 μM) seems likely to contribute to the frequency of reproductive endocrine disturbances among patients treated with VPA.

INTRODUCTION

Epilepsy is associated with a high prevalence of reproductive dysfunction in men (1) and women (2, 3). These disorders include subfertility, anovulation, menstrual disturbance, hyperandrogenism, lowered effectiveness of oral contraception, adverse pregnancy outcomes in women (2, 3) and sexual dysfunction, androgen deficiency symptoms, testicular atrophy, impaired spermatogenesis and subfertility in men (1) compared with age-matched non-epileptics. These effects appear to be multifactorial with reproductive endocrine effects due to anti-convulsant drugs and epilepsy itself as well as psychosocial factors all contributing to the high prevalence of reproductive disorders.

Among anticonvulsants, carbamazepine (CBZ) and valproate (VPA) are among the two most widely used. In epileptic patients, increased blood steroid hormone binding globulin (SHBG) concentrations are the most consistent change observed in reproductive endocrine parameters with anticonvulsant drugs (4, 5). Increased SHBG reduces metabolic clearance of testosterone (6) and could thereby impair endogenous sex steroid action. It is unlikely, however, this is sufficient to explain the diversity of reproductive disorders of patients on long-term anticonvulsants (1-3) and other pathogenic mechanisms are likely to be involved.

The possibility that CBZ and VPA may have reproductive endocrine effects due to interaction of these drugs with the sex hormone receptors, androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PR) has not been evaluated. All three receptors belong to a large superfamily of nuclear hormone receptors that share a well-conserved DNA-binding domain (DBD), a structurally conserved ligand-binding domain (LBD) and an N-terminal domain with no homology between the different receptors (7). After ligand binding, the receptor dimerizes and binds to hormone response elements located within the promoters of hormone-responsive genes to act as a ligand-activated transcription factor. Drugs can interfere with this receptor-mediated process by many potential mechanisms including altering blood hormone levels (via changes in synthesis, distribution, metabolism or clearance), modifying steroid receptor expression levels, directly binding to the receptor to mimic or block steroid function or interacting with post-receptor co-regulator proteins. So far only the first possibility has been considered in any detail. We therefore utilised a system

whereby each of the mammalian sex steroid receptors are stably introduced into the yeast strain *Saccharomyces cerevisae* to function as steroid-dependent transcription activators. This yeast reporter assays can then serve as useful tools for studying mammalian steroid receptor function. In the present study, therefore we used yeast-based AR, PR or ER in-vitro bioassays to investigate potential interactions of CBZ or VPA with these sex steroid hormone receptors. We show that VPA, but not CBZ, antagonizes both AR and PR action without effects on ER.

MATERIALS AND METHODS

Materials

Hormones, valproate and carbamazapine were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) and dissolved in ethanol for stock concentrations.

Plasmids and Reporter Gene Constructs

The full-length hPR cDNA plasmid and the PRE-ß-galactosidase reporter plasmid were kindly provided by Professor DP McDonnell. Yeast strain YPH500 (MATa, *ura*3-52, *lys*2-801, *ade*2-101, *trp*1-Δ63, *his*3-Δ200, *leu*2-Δ1) was co-transformed with both plasmids by standard alkali-transformation (Alkali cation yeast transformation kit, BIO101 systems, Qbiogene Inc., Carlsbad, CA, USA). Co-transformant yeast strains were selected by tryptophan and uracil auxotrophy. Yeast strains (1) YPH500 transformed with YEpE22 and YRpE2 and (2) YPH500 transformed with YEpAR and YPpG2 were also kindly provided by Professor DP McDonnell.

Yeast culture

Yeast transformants were grown overnight at 30°C with vigorous orbital shaking at 300 rpm in CSM-leu-ura (ER,AR BIO101) or CSM-trp-ura (PR, BIO101). Following overnight culture, the yeast culture was subcultured in fresh medium and allowed to grow until early-mid-log phase (OD600nm ~1.0).

Estrogen, progesterone and androgen receptor assays

For AR and ER bioassays, yeast from early-mid-log phase growth were diluted (to OD600=1.0) in selective medium (CSM-leu-ura) plus 100 μM $CuSO_4$ to induce receptor production. For the PR bioassay, yeast were diluted (to OD600=0.7) in selective medium (CSM-trp-ura). Diluted yeast were aliquoted into 24-well culture plates (500 μl/well) and 5 μl doses of steroid or drug were added. For antagonism experiments, 5 μl of testosterone ($5X10^{-9}$ M) or progesterone ($1.6X10^{-9}$ M) or estradiol (5X10-9 M) and 5 μl of valproate or carbamazapine ($1X10^{-7}$ M) were added. Each assay included testosterone (AR), progesterone (PR) or estradiol (ER) standard curve as well as a vehicle (0.1% ethanol) control. Multiwell plates were incubated at 30°C with shaking for either overnight (AR & ER) or 4 hr (PR). After incubation, the yeast culture samples were washed in assay buffer, lysed and extract assayed for β-

galactosidase. Dose-responses were fitted to a 4 parameter sigmoid curve using nonlinear regression option in Sigmaplot version 8.

ß-galactosidase assay

After incubation, the yeast culture samples were transferred to 1.7 ml microcentrifuge tubes, centrifuged at 3,000 rpm, and the cells resuspended in 250 μl of assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$,). Cells were incubated on ice for 15 mins and then lysed by vortexing in the presence of 4.5 μl 0.1% SDS and 9 μl chloroform. Lysate was then warmed to 30°C (5 mins) before 30 μl ONPG (4mg/ml) added and incubated at 30°C until formation of yellow color. Reaction was stopped with addition of 75 μl sodium carbonate (1M). The exact time was recorded. The yellow color (ONPG cleavage) was measured at OD_{420} and OD_{550}. ß-galactosidase activity was determined by [1000*OD_{420}-((1.75*OD_{550})/(time*volume*OD_{600}))]

Statistical analysis

Results of the experimental studies are reported as mean±SE compared to controls. Unpaired Student's t-tests were used to determine the significance of changes between groups. A value of $P<0.05$ was regarded as significant.

RESULTS

The dose-response characteristics of the yeast PR, AR and ER bioassays were established by measuring the response to the receptor's cognate ligands progesterone, testosterone and estradiol, respectively, over the steroid concentration range from 10^{-6} M to 10^{-16} M (Figure 1).

The potential agonist activity of carbamazepine and valproate were evaluated at 10 μM concentration. Neither drug produced nonspecific yeast cell toxicity at this dose. Neither carbamazepine nor valproate displayed agonist activity for any of the 3 steroid hormone receptors (Figure 2).

The antagonist activity of carbamazepine and valproate were evaluated at 10 μM concentration in the presence of mid-range doses (~EC_{50}) of the appropriate hormone (5 nmol/L testosterone, 1.6 nmol/L progesterone, 5 nmol/L estradiol). Maximal effects of valproate (100 μM) achieved blockade of AR activity to 40%- and PR activity to ~22% of EC_{50}-induced PR activity. Carbamazepine showed no antagonist effects. Neither valproate or carbamazepine demonstrated antagonism at the ER (Figure 3).

We further characterized the antagonist activity of valproate by determining the effects of lower concentrations of valproate on testosterone-, dihydrotestosterone- and progesterone- inducing activity (Figure 4). VPA demonstrated a dose-dependent blockade of AR activity with first significant effect at 30 μM for both testosterone and dihydrotestosterone. VPA also showed dose-dependent inhibition of progesterone-induced PR activity with first detectable effects at 10 μM.

DISCUSSION

Reproductive dysfunction including subfertility (8-10) and disruption of reproductive endocrine function including menstrual disturbances and anovulation, hyperandrogenism and polycystic ovary (PCO)-like syndrome in women (11-13) and sexual dysfunction and androgen deficiency in men (1) are common in patients with epilepsy. The role of anticonvulsant drugs in these diverse reproductive disorders remains unclear as most clinical studies have been observational and relatively small. Nevertheless, prospective studies demonstrate endocrine effects of antiepileptic drugs the most consistent being increased blood SHBG concentration (4, 5, 14, 15), although the mechanism remains unknown. Other endocrine changes are not readily explained solely by increased blood SHBG concentrations.

Valproate is among the most frequently prescribed anticonvulsant drugs used by more than 2 million people daily (16). Its role in the reproductive endocrine disturbances frequently present in men and women with epilepsy is controversial (1, 11, 13, 16). As well as a broad spectrum of antiepileptic drug, valproate is also prescribed as a mood stabiliser for bipolar disorders, for neuropathic pain including headache and for migraine prophylaxis (17, 18). The present study shows that VPA, at concentrations much lower than therapeutic blood levels, blocks AR and PR, but not ER, action in an in-vitro bioassay. By contrast, carbamazepine had no detectable agonist or antagonist steroidal bioactivity and neither VPA nor CBZ had any sex steroid agonist activity in vitro. These findings indicate that valproate may produce clinical effects as an anti-androgen and anti-progestin when used as an anticonvulsant.

The low fertility of women with epilepsy (9, 10, 19) has many contributing factors including social as well as biological factors. The strong PR antagonism by valproate, detectable at 10 μM and nearly complete at 100 μM compared with blood therapeutic concentrations of 350-700 μM, suggests some biological effects in women may be at least partly due to impaired PR-mediated progesterone action on reproductive tissues notably the uterus, breast and ovary. The anti-progestin effects of valproate may be a previously unrecognised factor contributing to the low fertility of women with epilepsy, notably to their otherwise unexplained higher rates of apparent miscarriage (8). In addition, anti-progestin effects may contribute to the higher frequency of anovulation among valproate-treated women with epilepsy (20) as mice null for PRA, but not PRB,

fail to ovulate (21, 22). Further studies are required to clarify the contribution and mechanism of valproate in lowering reproductive potential of women with epilepsy or bipolar mood disorder. Apart from progesterone's role as a universal precursor on the steroidogenic pathway, there is no known role of progesterone mediated via a progesterone receptor in men so there are no known or likely male reproductive health effects of the anti-progestin effects of valproate.

The anti-androgenic effects of valproate are most likely to be manifest in men with epilepsy. These effects might contribute to the delayed puberty (23), impaired reproductive function and androgenic effects (1) reported in males with epilepsy. The most characteristic biochemical effects expected for a drug with pure anti-androgen properties would be increases in blood testosterone and LH (and to a lesser extent FSH) concentrations (24) comparable with the findings in androgen insensitivity due to an inactivated androgen receptor (25) or treatment with non-steroidal anti-androgen (26, 27). Such findings are reported in hormonal studies of men with epilepsy with control non-epileptic populations (5, 28-30). The alternative interpretation of such anti-androgenic effects is attributing them to increased blood SHBG concentrations, and consequently lowered "free" or biologically active testosterone, remains speculative.

The more prominent effects of valproate compared with carbamazepine is consistent with the clinical evidence that valproate treatment is associated with greater disruption of reproductive endocrine function than other anticonvulsant drugs (11, 15, 31). Similarly, valproate treatment for other conditions such as bipolar mood disorders and migraine is also associated with similar disruption of reproductive endocrine function to a greater extent than other treatments (32, 33). Nevertheless, the available clinical studies are limited in explanatory power due to their mostly observational design and small sample size. Previous mechanistic studies have shown valproate has endocrine effects such as activation of PPARδ (34), demethylation of DNA in an *in vitro* cell model (35), modulation of GABA-ergic neuronal input to the hypothalamus (36, 37) and a range of metabolic effects notably increasing blood leptin and insulin, but decreasing IGFBP1, levels (11, 32, 38, 39).

Whether the anti-androgenic effects of valproate have any clinical significance in women, where blood androgens are normally at levels equivalent to castrate men or children remains doubtful. At the level of the ovarian follicle these effects may

stimulate ovarian androgen synthesis (38) as well as inhibiting follicular aromatization (40, 41). In normal non-epileptic rats, long-term VPA treatment induced endocrine changes and increased the number of ovarian follicular cysts (42). Similarly, in male rats high doses of valproate retards fertility, inhibit fertility, testicular weight and spermatogenesis as well as epididymal and prostate weight in rodents and larger mammals (43-48). While these changes are likely to be due to the lowered blood testosterone concentrations, valproate does not directly inhibit androgen biosynthesis (49, 50). The findings of increased LH and FSH in some (42) but not all (46) studies is consistent with valproate's anti-androgen action in an in-vitro bioassay.

In conclusion, we have shown using a yeast-based in-vitro bioassay for steroid hormones that VPA, a commonly used anticonvulsant for treatment of epilepsy and bipolar disorder, is an antiandrogen and antiprogestin but has no antiestrogen effects. These effects of VPA are evident at concentrations well below therapeutic blood concentrations of valproate when used to treat epilepsy or bipolar mood disorder. By contrast, carbemazapine had no sex steroid hormone receptor bioactivity. These findings suggest a new reproductive endocrine mechanism for valproate in the frequent reproductive dysfunction reported in valproate treated patients.

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Figure Legends

Figure 1: Yeast bioassay for AR, PR and ER activity. (A) Dose response curve for testosterone induced AR activity. Testosterone (dose range 10^{-6} to 10^{-16} M) was added to yeast cultures for an incubation period of 24 hours before the culture media assayed for β-galactosidase activity. Values represent the mean ± SE of 6 separate experiments. (B) Dose response curve for progesterone induced PRA activity. Progesterone (dose range 10^{-6} to 10^{-16} M) was added to yeast cultures for an incubation period of 4 hours before culture media assayed for β-galactosidase activity. Values represent the mean ± SE for 6 separate experiments. (C) Dose response curve for estradiol induced ERα activity. Estradiol (dose range 10^{-6} to 10^{-16} M) was added to yeast cultures for an incubation period of 24 hours before culture media assayed for β-galactosidase activity. Values represent the mean ± SE of 3 separate experiments. All curves are presented as the % of maximal β-galactosidase activity.

Figure 2: The effects of CBZ and VPA on androgen, progesterone and estrogen bioassays. (A) AR agonist activity of CBZ and VPA relative to EC_{50} testosterone dose (5 X 10^{-9} M). Yeast cultures were treated with either 10 X 10^{-6}M CBZ or VPA or 5 X 10^{-9} M testosterone and incubated for 24 hours. Following incubation, β-galactosidase activity was assayed and values represent the mean ± SE of 3 separate experiments. Values are expressed as % of 5 X 10^{-9} M testosterone-induced β-galactosidase activity. (B) PR agonist activity of CBZ and VPA relative to EC_{50} dose of progesterone (1.6 X 10^{-9} M). Yeast cultures were treated with either 10 X10^{-6} M CBZ or VPA or 1.6 X 10^{-9} M progesterone and incubated for 4 hours. Following incubation, β-galactosidase activity was assayed and values represent the mean ± SE of 3 separate experiments. Values are expressed as % of 1.6 X 10^{-9} M progesterone-induced β-galactosidase activity. (C) ER agonist activity of CBZ and VPA relative to EC_{50} dose of estradiol (5 X 10^{-9} M). Yeast cultures were treated with either 10 X 10^{-6} M CBZ or VPA or 5 X 10^{-9} M estradiol and incubated for 24 hours. Following incubation, β-galactosidase activity was assayed and values represent the mean ± SE of 3 separate experiments. Values are expressed as % of 5 X 10^{-9} M estradiol-induced β-galactosidase activity.

Figure 3: VPA inhibitory action in androgen and progesterone, but not estrogen, bioassays. (A) VPA antagonism of testosterone-induced AR activity. Yeast cultures were incubated for 24 hours with 5 X 10^{-9} M testosterone in the presence of 10 X 10^{-6} M valproate or 10 X 10^{-6} M carbamazepine. Values represent mean $\pm$ SE of 4 separate experiments and are presented as % of 5 X 10^{-9} M testosterone-induced β-galactosidase activity. *$p<0.05$. (B) VPA antagonism of progesterone-induced PR activity. Yeast cultures were incubated for 4 hours with 1.6 X 10^{-9} M progesterone in the presence of 10 X 10^{-6} M valproate or 10 X 10^{-6} M carbamazepine. Values represent mean $\pm$ SE or 4 separate experiments and are presented as % of 1.6 X 10^{-9} M progesterone-induced β-galactosidase activity. *$p<0.005$. (C) No antagonism of estradiol-induced ER activity by VPA or CBZ. Yeast cultures were incubated for 24 hours with 5 X 10^{-9} M estradiol in the presence of 10 X 10^{-6} M valproate or 10 X 10^{-6} M carbamazepine. Values represent mean $\pm$ SE or 4 separate experiments and are presented as % of 5 X 10^{-9} M estradiol-induced β-galactosidase activity.

Figure 4: Dose-dependent inhibition by increasing concentrations of valproate of progesterone (●), testosterone (■)or dihydrotestosterone (▲) stimulated yeast cells. Yeast were cultured in the presence of 5 X 10^{-9} M testosterone or dihydrotesterone or 1.6 X10^{-9} M progesterone together with 1 - 10 X 10^{-6} M valproate. Values represent mean $\pm$ SE from 3 separate experiments and are presented as % of maximal β-galactosidase activity.

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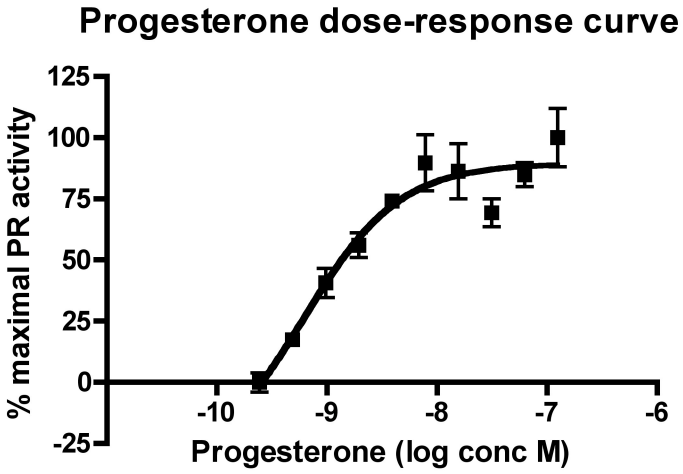
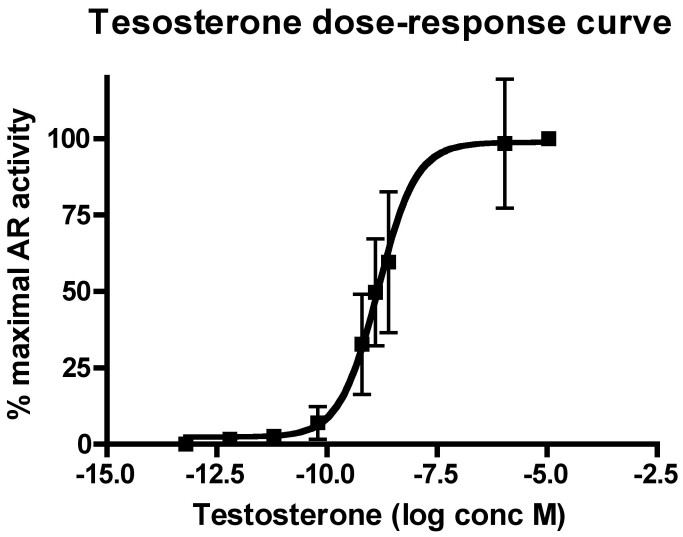
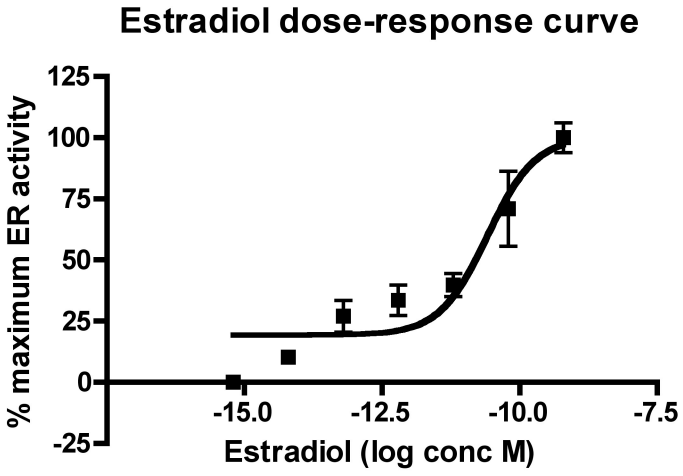
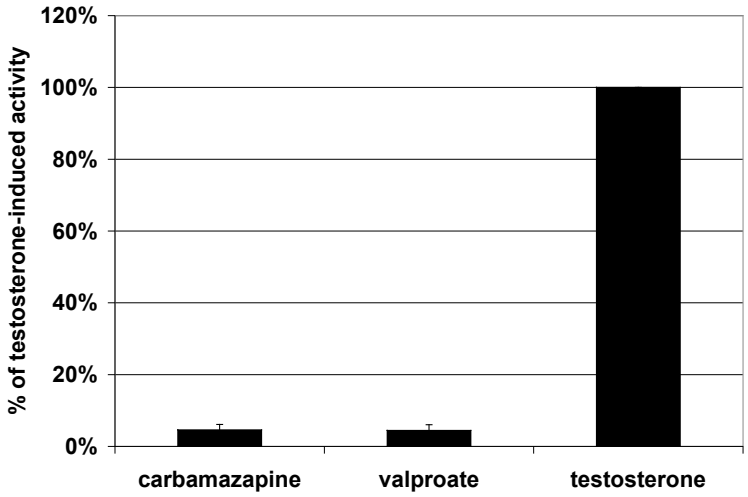

Progesterone dose-response curve
% maximal PR activity
125
100
75
50
25
0
-25
-10
-9
-8
-7
-6
Progesterone (log conc M)

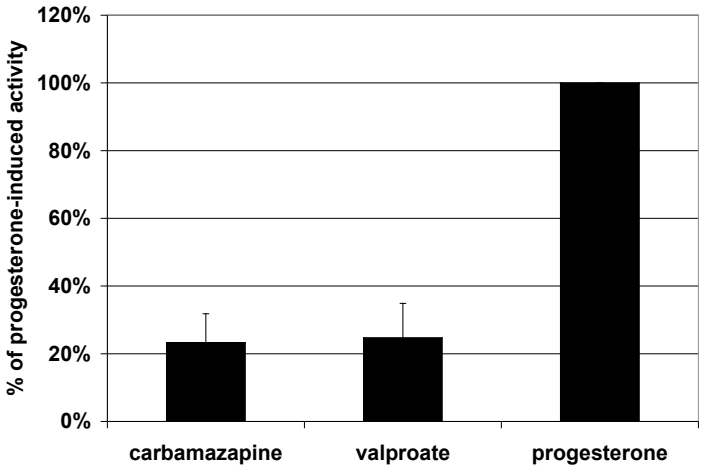

Tesosterone dose-response curve
% maximal AR activity
100
75
50
25
0
-15.0
-12.5
-10.0
-7.5
-5.0
-2.5
Testosterone (log conc M)


Estradiol dose-response curve
% maximum ER activity
125
100
75
50
25
0
-25
-15.0
-12.5
-10.0
-7.5
Estradiol (log conc M)

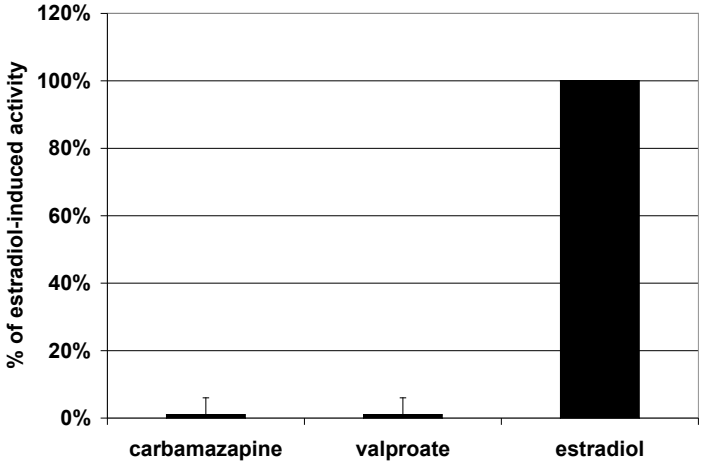
A


120%
100%
80%
60%
40%
20%
0%
% of testosterone-induced activity
carbamazapine
valproate
testosterone

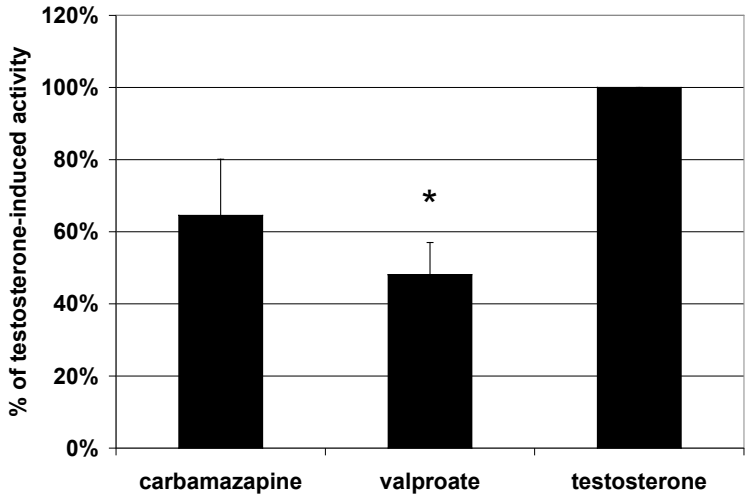
B


120%
100%
80%
60%
40%
20%
0%
% of progesterone-induced activity
carbamazapine
valproate
progesterone

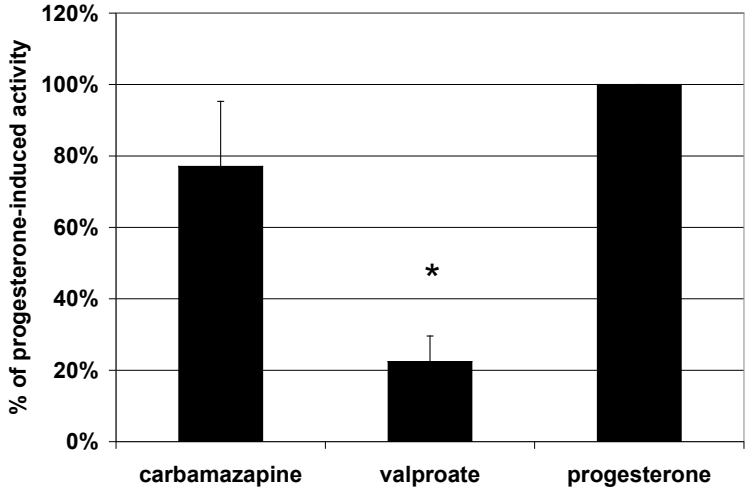
C


120%
100%
80%
60%
40%
20%
0%
% of estradiol-induced activity
carbamazapine
valproate
estradiol

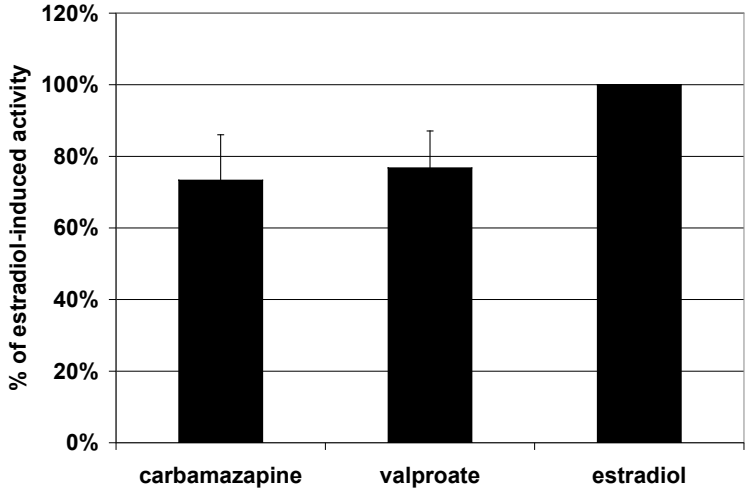
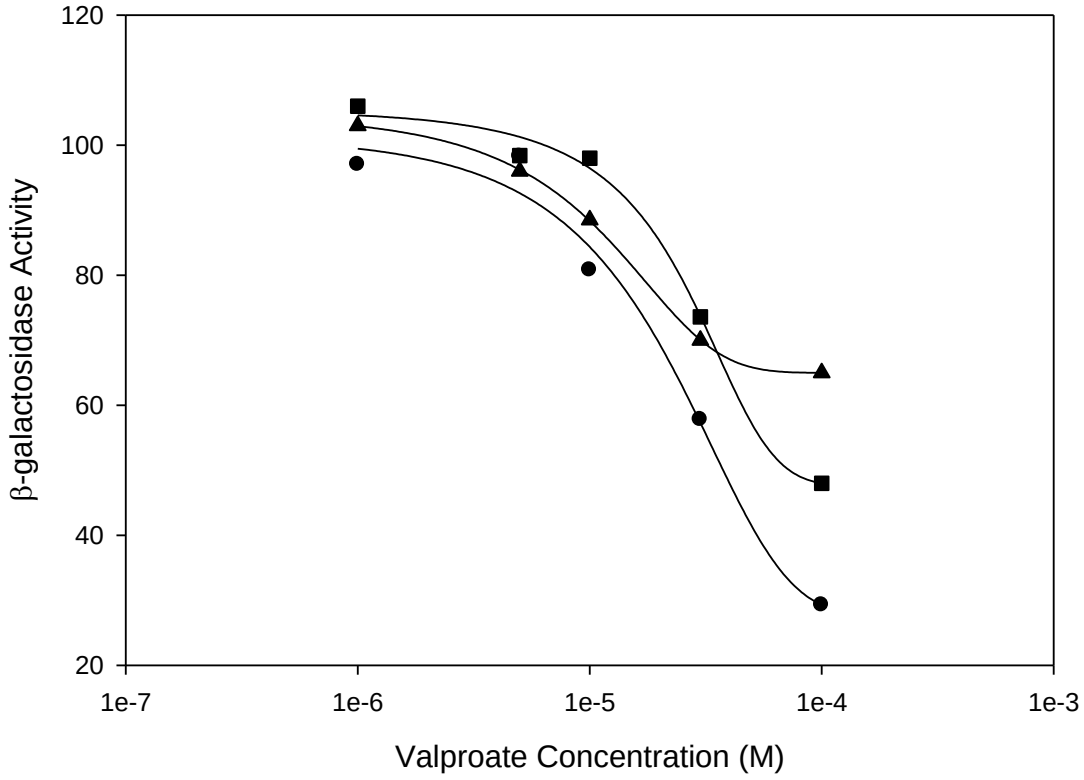
A


120%
100%
80%
60%
40%
20%
0%
% of testosterone-induced activity
*
carbamazapine
valproate
testosterone

B


120%
100%
80%
60%
40%
20%
0%
% of progesterone-induced activity
*
carbamazapine
valproate
progesterone

C


120%
100%
80%
60%
40%
20%
0%
% of estradiol-induced activity
carbamazapine
valproate
estradiol

VPA dose response curves