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Cyclic Withdrawal From Endogenous and Exogenous Progesterone Increases Kainic Acid and Perforant Pathway Induced Seizures

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FRYE, C. A. AND L. E. BAYON. *Cyclic withdrawal from endogenous and exogenous progesterone increases kainic acid and perforant pathway induced seizures.* PHARMACOL BIOCHEM BEHAV **62**(2) 315–321, 1999.—Antiseizure effects of progesterone (P) and its metabolite, 5α-pregnan-3α-ol-20-one (3α, 5α-THP) were investigated following continuous vs. discontinuous P exposure. In Experiments 1, 32 cycling Long–Evans rats were administered kainic acid (32 mg/kg SC), ictal behavior was examined, and plasma 3α,5α-THP levels were measured by radioimmunoassay. Proestrus/estrus rats showed less ictal activity and had elevated 3α,5α-THP levels prior to kainic acid compared to diestrus/metestrus subjects. In Experiment 2, 49 ovariectomized (ovx) rats were SC injected with estradiol benzoate (EB; 10 μg) and P (500 μg), to mimic estrus, or sesame oil vehicle (0.2 cc); all subjects were administered kainic acid. Rats tested with EB+P showed a reduced mean duration of full seizures and increased 3α,5α-THP, whereas those tested 24 h following EB+P had more tonic clonic seizures and lower 3α,5α-THP concentrations, comparable to ovx control animals. In Experiment 3, 49 ovx rats were stereotaxically implanted with bipolar electrodes into the perforant pathway. Prior to perforant pathway stimulation, rats received cholesterol or EB+P capsules for 1 month, continuously or intermittently. Irrespective of continuous or intermittent EB+P, the presence of progestins at the time of perforant pathway stimulation reduced partial seizure activity. Continuous EB+P capsules resulted in increased 3α,5α-THP levels compared to all other conditions, and less damage in the hilus of the hippocampus, compared to intermittent EB+P. These data confirm that P and 3α,5α-THP have antiseizure effects, and further suggest that repeated cycles of endogenous or exogenous P and/or 3α,5α-THP withdrawal influences seizure threshold and/or hippocampal integrity. © 1999 Elsevier Science Inc.

Progesterone 5α-pregnan-3α-ol-20-one (3α,5α -THP) Neuroprotection Hippocampus Extragenomic Neurosteroid Antiseizure

STEROID hormones' alteration of seizure susceptibility is well documented. Over a hundred years ago, it was suggested that seizure frequency was related to a specific phase of the menstrual cycle (24). Extensive research in people and animals has supported the notion that cyclic patterns of seizures, as in catamenial epilepsy, are a result of hormonal effects (21). Increases in circulating estradiol exacerbate catamenial epilepsy, particularly complex partial and tonic clonic seizures (1,2,21). Physiological levels of estrogens decrease the threshold and increase the magnitude of seizure activity in experimental animals (6,27). Increases in endogenous or exogenous progesterone (P) increase the threshold and decrease the magnitude of seizure activity (47). Cyclic increases in P during the luteal phase are associated with decreases in tonic–clonic seizures in women with catamenial epilepsy (2). Thus, in general, estradiol has proconvulsant effects and P has antiseizure actions.

Although estradiol increases and P decreases seizures, interactions between ictal activity and sex hormone function are complex. Of particular interest is the growing evidence that P's antiseizure actions may be attributable to its metabolite 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP). 3α,5α-THP is a more potent anticonvulsant for bicuculline, pentylenetetrazol (PTZ), picrotoxin, and penicillin-induced convulsions (3,10, 23) than is P. Fluctuations in P and 3α,5α-THP levels over the menstrual cycle, estrous cycle, pregnancy, and postparturition seem to influence seizure activity (2,13,36).

The notion that P's antiseizure actions may be due to 3α,5α-THP suggests that progestins' antiseizure actions may be a

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consequence of interaction with γ-aminobutyric acid (GABA)/benzodiazepine receptor complexes (GBRs), rather than actions at intracellular progestin receptors (PRs). Evidence for this includes the following. First, P has a high affin- ity and 3α,5α-THP a very low affinity for intracellular PRs (20), while the opposite relationship is seen for the steroid's actions at GBRs. 3α,5α-THP is about 50 times more effective than benzodiazepines, and 500 times more effective than P itself at modulating GBRs (29,39,40). Second, localization of PRs and GBRs reveals greater concentration of GBRs than PRs in the hippocampus (8,30,34), which is a substrate involved in seizure disorders (44–45). Third, although Selye reported over 50 years ago that P, as well as 3α,5α-THP, protected against PTZ-induced seizures, the prolonged latency of P's anticonvulsant effects compared to 3α,5α-THP implies that P may be metabolized for its antiseizure activity (41). Fourth, blocking P's metabolism to 3α,5α-THP by administration of 5α-reductase inhibitors, such as finasteride (16) or 4MA (15), attenuate P's antiseizure actions in kainic acid and perforant pathway seizure models. Fifth, concentrations of 3α,5α-THP over the estrous cycle, pregnancy, and postpartum are more directly related to seizure activity than are P levels (13,36). In addition, levels of 3α,5α-THP associated with changes in ictal activity are well within the range of concentrations shown previously to potentiate the in vitro actions of GABA at GBRs (17,18,22,26,29). Seizure threshold is decreased when endogenous plasma levels of 3α,5α-THP fall below approximately 5 ng/ml (13). Together, these data suggest that P's antiseizure actions may, in part, be due to actions of 3α,5α-THP at GBRs.

In extreme cases, withdrawal from chronic exposure to GABAergic ligands can produce seizures (5,19,28). The purpose of the present study was to examine whether withdrawal from sustained exposure to P, and subsequently its GABA active metabolite, 3α,5α-THP, may precipitate alterations in seizure activity.

METHOD

All methods and procedures described below were preapproved by the Institutional Animal Care and Use Committee.

Subjects and Housing

Female Long–Evans rats (n = 115), were obtained from Charles River Laboratories (Kingston, NY) at approximately 55 days of age, and housed in hanging stainless steel cages (24 × 18 × 19 cm) in a temperature-controlled room (21 ± 1°C). Rats were maintained on a 12-h light:12-h dark cycle (lights off at 0800 h) with continuous access to Purina Rat Chow and water.

Surgery

For Experiments 2 and 3, all subjects were ovariectomized (ovx) under Rhompun (12 mg/kg IP) and Ketaset (80 mg/kg IP) anesthesia. All animals were allowed a 1 week recovery period prior to inclusion in the experiment.

Kainic Acid-Induced Ictal Activity

For Experiments 1 and 2, animals were placed in a glass arena (26 × 30 × 50 cm) for 10 min of habituation on the day of testing, followed by kainic acid (32 mg/kg SC) injection. Immediately after injection, ictal behaviors were recorded for a 2-h period. In our lab, kainic acid-induced ictal activity is used as a model of tonic–clonic epileptic seizures. This seizure model is characterized by cycles of grooming, wet-dog shakes, chewing, drooling, and partial seizures, which culminate in prolonged periods of tonic–clonic seizures, characterized by rearing on the hind legs and complete loss of balance (13,15–16). Administration of kainic acid has been reported to readily cross the blood–brain barrier, to be specifically neurotoxic to excitatory amino acid neurons in the hippocampus, and to produce seizure activity and brain damage similar to those seen in epileptic patients (44).

Ictal Activity Induced by Perforant Pathway Stimulation

For Experiment 3, animals received 1 h of constant perforant pathway stimulation with biphasic pulses with a duration of 0.1 ms per phase, at 20 Hz with 12.5 V. During this stimulation, ictal activity was measured. Perforant pathway tends to be a more subtle model of seizure activity, marked by behaviors similar to those seen following kainic acid administration (chewing, drooling, grooming, and wet-dog shakes), but culminating in prolonged periods of partial seizures rather than tonic–clonic seizures (12,16).

Radioimmunoassay

Radioimmunoassay for 3α,5α-THP was done according to the methods of Purdy and colleagues (33), Finn and Gee (10), and Frye et al. (16). Steroids were extracted from serum with ether, ether was evaporated in a savant, and the remaining pellets were reconstituted in bovine serum albumin (BSA) assay buffer (pH = 7.4). The standard curve was prepared in duplicate to give a range of six concentrations from 100 to 4,000 pg. The standards were added to BSA assay buffer, followed by addition of [^{3}H]-3α,5α-THP (NET-1047, 51.3 ci/mmol; New England Nuclear, Boston, MA, 8,000 dpm/100 μl) and antibody (250 μl) for a total volume of 950 μl. The polyclonal antibody was purchased from Dr. Robert Purdy (Veterans Medical Affairs, La Jolla, CA), and is very specific to 3α,5α-THP (10). A 1:5,000 dilution of this antibody was used, which bound between 40–60% of [^{3}H]-3α,5α-THP (approximately 20,000 cpm or 100 pg).

Assay tubes were vortexed and incubated at 4°C for 24 h. Separation of bound and free was done by the rapid addition of 200 μl of ice-cold dextran-coated charcoal. Following a 15-min incubation on ice, samples were centrifuged at 1200 × g for 10 min. The supernatant (500 μl) was pipetted into a scintillation vial and a volume of 6 ml of scintillation cocktail was added. Sample tube concentrations were calculated using the logit-log method of Rodbard and Hutt (35), interpolation of the standards and correction for recovery. The minimum detectable limit of the assay was 100 pg. The intraassay and interassay coefficients of variance were 12.1 and 15.6%, respectively.

Histological Analysis

Following testing in Experiment 3, animals were intracardially perfused with 0.9% saline followed by 10% formalin. Fixed brains were sectioned at 40 μm with a freezing cryostat and stained with cresyl violet for neuron counting. The most medial section of the hilar region was visualized using a light microscope at 400× magnification. Two observers, uninformed of the experimental condition of each subject, counted the number of neurons present (concordance between two observers >80%).

Statistical Analysis

In all experiments, multiple one-way analyses of variances (ANOVAs) were used to examine effects of hormone condi-

tion on various measures of ictal activity, the number of neurons in the hilar region, and/or 3α,5α-THP levels. Where appropriate, ANOVAs that revealed significant differences at the $p < 0.05$ level were followed by Duncan's post hoc tests and least-squares mean comparisons between groups.

PROCEDURE

Experiment 1

Vaginal smears were obtained from all animals ($n = 32$) to determine the day of the estrous cycle. After two normal cycles (4–5 days/cycle), animals were randomly assigned to be tested for kainic acid-induced seizure activity during either proestrus/estrus ($n = 8$) or metestrus/diestrus ($n = 8$). Immediately following testing, all animals were killed by rapid decapitation, and trunk blood collected for radioimmunoassay of 3α,5α-THP. Additional proestrus/estrus ($n = 8$) and metestrus/diestrus ($n = 8$) animals were killed via rapid decapitation at what would have been the time of kainic acid injection. Trunk blood was collected, and plasma was assayed for 3α,5α-THP.

Experiment 2

Ovariectomized ($n = 34$) rats were randomly assigned to receive systemic estradiol benzoate (EB: 10 μg) or sesame oil vehicle (0.2 cc) at hour 0 and P (500 μg) or vehicle (0.2 cc) 44 h later. Rats were primed with EB prior to P administration to produce an estrus-like state. Kainic acid was administered to rats at hour 48, 4 h following vehicle (ovx, $n = 8$) and P injections (EB+P, $n = 8$), or at hour 68, 24 h following P injection (withdrawal, $n = 9$). Additional animals ($n = 3$/group) were put through the same hormone conditions, but were killed via rapid decapitation at what would have been the time of kainic acid injection. Trunk blood was collected for later radioimmunoassay of plasma 3α,5α-THP.

Experiment 3

Ovariectomized rats ($n = 49$) were anesthetized with 12 mg/kg IP Rhompun/80 mg/kg IP Ketaset, and stereotaxically implanted with a bipolar electrode (Plastics One, Roanoke, VA) into the perforant pathway (−4.4 mm AP, ±7.5 mm ML, and −4.0 mm DV). A skull screw and cranioplastic dental cement were used to secure the electrode. Rats were allowed to recover for 1 week prior to testing.

All animals were randomly assigned to receive EB and P or cholesterol implants. Animals were sedated using Ketaset (80 mg/kg) and received silastic implants [SC; see (23) for detailed methods]. All animals, regardless of implant condition, were sedated to ensure that there was no effect of repeated anesthesia use. EB implants (0.062 ID, 0.125 OD) contained estradiol-17-benzoate (Sigma, St. Louis, MO, β-Estradiol 3-Benzoate, 30 mg/mm; 10 mm/100 g b. Wt.), and P implants (0.132 ID, 0.183 OD) contained crystalline P (Sigma, 4-Pregnene-3, 20,dione, 10 mm/animal). This treatment provides levels of EB and P in the range observed during estrus in naturally cycling animals. Control animals were implanted with two cholesterol implants. Rats were tested for perforant pathway-induced ictal activity in the following conditions: continuous cholesterol (ovx, $n = 8$) for 1 month, continuous EB and P implants (EB+P, $n = 8$) for 1 month, intermittent withdrawal of EB and P implants and tested 2 days following the fourth implant removal (withdrawal, $n = 8$), or tested while the EB and P implants were still in place after three removal cycles (intermittent EB+P, $n = 8$). Following perforant pathway stimulation, animals were perfused and brains collected for histological analysis.

An additional four to five animals per condition (ovx, $n = 4$; EB+P, $n = 4$; withdrawal, $n = 5$; intermittent withdrawal, $n = 4$) that did not undergo perforant pathway stimulation were killed by rapid decapitation, and trunk blood collected at what would have been the start time of perforant pathway stimulation for radioimmunoassay of plasma 3α,5α-THP.

RESULTS

Experiment 1: Kainic Acid-Induced Seizure Duration Was Increased in Metestrus/Diestrus Animals Compared to Proestrus/Estrus Animals

Animals tested during proestrus/estrus had significantly shorter total duration of tonic clonic seizures compared to metestrus/diestrus animals, $F(1, 14) = 9.57$, $p \leq 0.01$ (Fig. 1, top). Proestrus/estrus animals tended to have a shorter mean duration of tonic–clonic seizures compared to animals tested in metestrus/diestrus, $F(1, 14) = 3.63$, $p \leq 0.10$ (Fig. 1, middle). Following kainic acid testing, plasma 3α,5α-THP levels were elevated irrespective of the phase of the estrus cycle (proestrus/estrus = 10.272 ± 1.31 ng/ml; metestrus/diestrus = 8.24 ± 1.94 ng/ml), $F(1, 14) = 0.76$, $p \geq 0.20$. Prior to kainic acid testing, plasma levels of 3α,5α-THP were significantly higher in proestrus/estrus animals compared to metestrus/diestrus animals, $F(1, 14) = 6.72$, $p \leq 0.05$ (Fig. 1, bottom).

Experiment 2: Twenty-Four-Hour EB+P Withdrawal Produces Tonic–Clonic Seizure Comparable to Ovx Controls Following Kainic Acid

As has previously been shown, EB+P had antiseizure effects in a kainic acid seizure model. Interestingly, 24 h following discontinuation of EB+P, seizure activity was increased to levels comparable to those of ovx control animals. EB+P animals had significantly shorter total durations of tonic–clonic seizures compared to EB+P withdrawal and ovx control animals, $F(2, 22) = 73.25$, $p \leq 0.01$ (Fig. 2, top), and tended to have shorter mean duration of tonic–clonic seizures compared to the EB+P withdrawal animals, $F(2, 22) = 2.943$, $p \leq 0.10$ (Fig. 2, middle).

Circulating levels of 3α,5α-THP in animals that did not undergo kainic acid testing were inversely related to seizure activity. EB+P animals had significantly higher plasma 3α,5α-THP levels compared to ovx and EB+P withdrawal animals, $F(2, 6) = 8.24$, $p \leq 0.05$ (Fig. 2, bottom).

Experiment 3: Cyclic EB and P Withdrawal Does Not Increase Ictal Activity if EB+P Are Present in a Perforant Pathway Seizure Model

As was demonstrated in Experiment 2, acute EB+P reduced ictal activity compared to cholesterol and EB+P withdrawal conditions. Notably, cycles of EB+P withdrawal did not decrease seizure threshold if EB+P capsules were present at the time of perforant pathway stimulation. There was not a statistical difference for the total duration of partial seizures between the four groups, $F(3, 28) = 1.38$, $p \geq 0.10$; however, there was a difference between groups in the mean durations of partial seizures, $F(3, 28)2.89 = p \leq 0.05$ (Fig. 3, top and middle). Post hoc tests revealed that subjects that had cholesterol had mean durations of partial seizures that were greater than continuous EB+P and intermittent EB+P conditions.

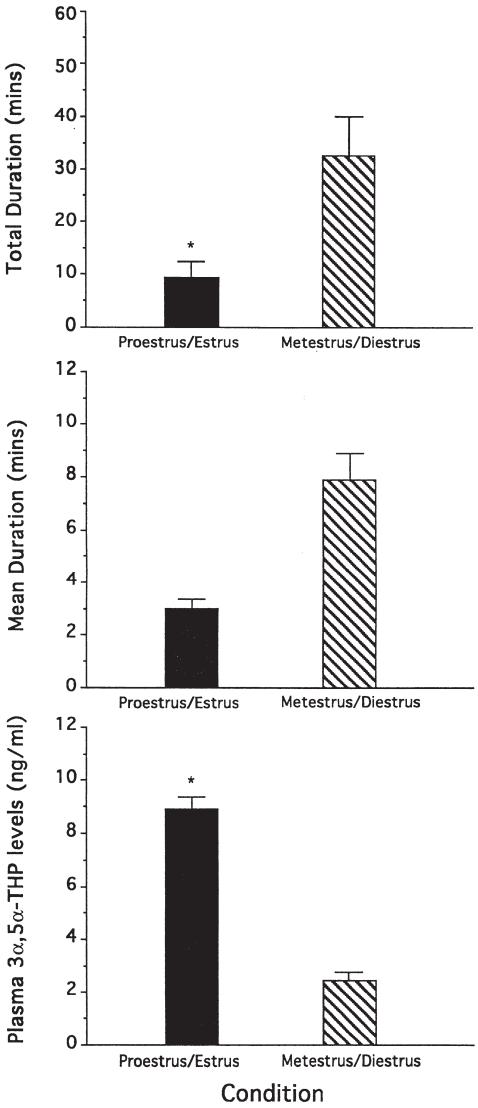

FIG. 1. The top and middle panels depict the total duration and mean duration of individual tonic clonic seizures (min ± SEM) following kainic acid administration for animals in proestrus/estrus or metestrus/diestrus. The bottom panel depicts the mean plasma level of 3α,5α-THP in proestrus/estrus and metestrus/diestrus animals that did not undergo kainic acid testing. *Indicates a significant difference ($p \leq 0.05$) between proestrus/estrus and metestrus/diestrus groups.

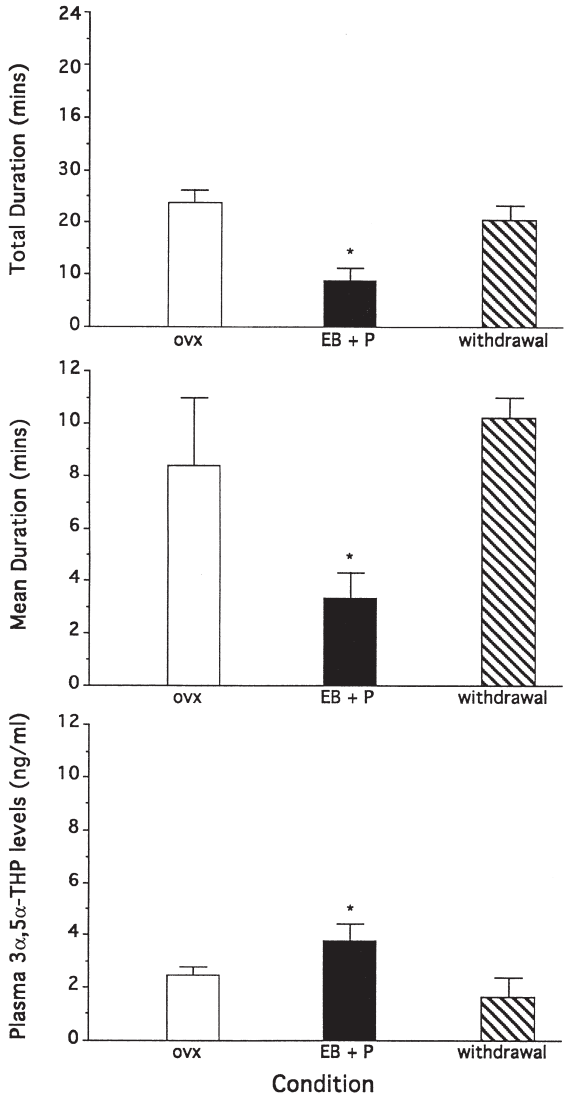

FIG. 2. The top and middle panels depict the total duration and mean duration of individual tonic clonic seizures (min ± SEM) following kainic acid administration for ovx/control, EB+P, or withdrawal animals. The bottom panel depicts the mean plasma level of 3α,5α-THP in ovx/control, EB+P, or withdrawal animals that did not undergo kainic acid testing. *Indicates a significant difference ($p \leq 0.05$) from ovx control.

Plasma 3α,5α-THP levels, prior to perforant pathway stimulation, were increased in the continuous EB+P condition, compared to cholesterol implant, and intermittent EB+P implant conditions, $F(3, 12) = 7.61$, $p < 0.05$ (Fig. 3, bottom).

Despite EB+P protecting against seizures, examination of the neuronal counts in the hilar region of the hippocampus revealed that continuous EB+P prevented neuronal loss, but that intermittent withdrawal was less effective. There was no difference between withdrawal (mean ± SEM = 84.25 ± 5.59) and intermittent EB+P (mean ± SEM = 70.00 ± 5.46) animals. Continuous EB+P animals had the greatest number of preserved neurons (mean ± SEM = 96.13 ± 9.78) > withdrawal > cholesterol (mean ± SEM = 51.38 ± 7.60), $F(3, 28) = 6.92$, $p \leq 0.05$.

DISCUSSION

The hypothesis that withdrawal from sustained P, and subsequently 3α,5α-THP, affects seizure susceptibility was supported by the following findings. (A) Continued presence of P attenuated seizures and increased 3α,5α-THP levels, compared to control animals without hormone replacement. (B) Withdrawal from endogenous or exogenous EB+P, and subsequent reductions in 3α,5α-THP, returned seizure activity to that of control animals. (C) Restoration of 3α,5α-THP by intermittent exposure to EB+P did not return seizure activity to that of the continuous EB+P-treated animals. (D) Following perforant pathway stimulation, there was less neuronal damage in the hilar region of the hippocampus in animals treated with EB+P compared to cholesterol or withdrawal

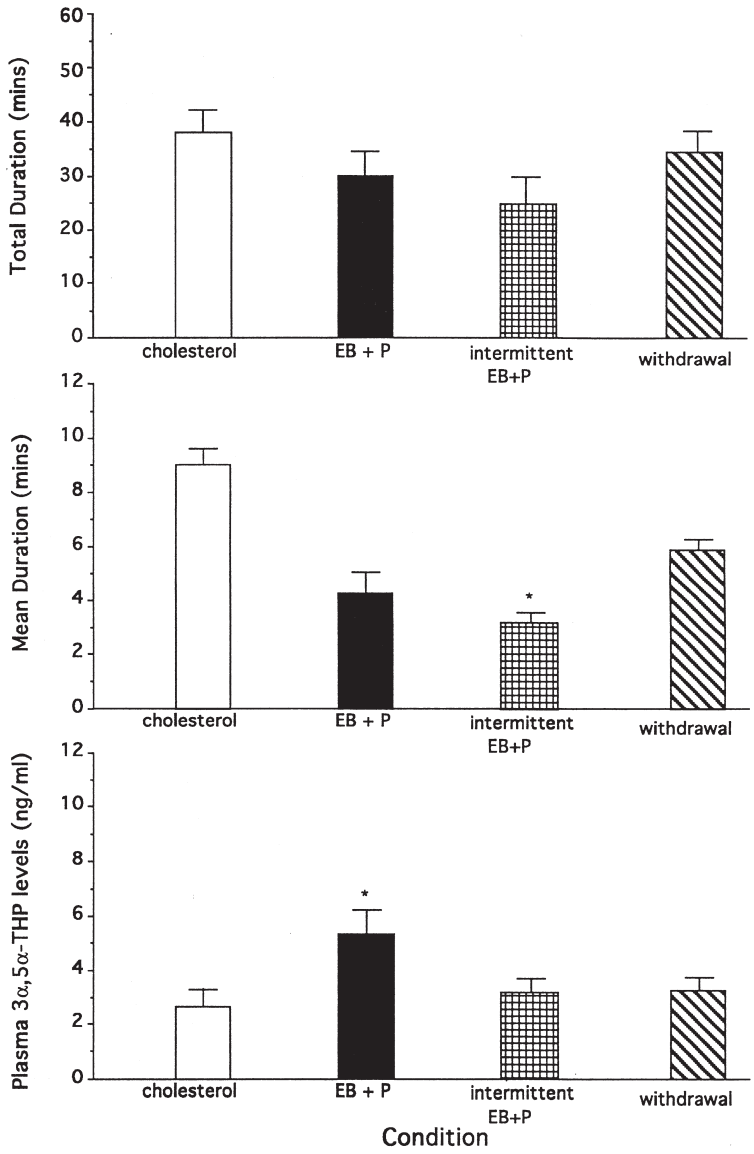

FIG. 3. The top and middle panels depict the total duration and mean duration of individual partial seizures (min ± SEM) following perforant pathway stimulation for cholesterol, EB+P, intermittent withdrawal, and withdrawal animals. The bottom panel depicts the mean plasma level of 3α,5α-THP in cholesterol, EB+P, intermittent withdrawal, and withdrawal animals that did not undergo perforant pathway stimulation. *Indicates a significant difference ($p \leq 0.05$) from cholesterol control.

animals. Together, these data suggest that 3α,5α-THP levels at the time of seizures have direct effects on seizure incidence and that history of endogenous or exogenous 3α,5α-THP withdrawal may have bearing on hippocampal integrity or the vulnerability of the hippocampus to seizure-inducing stimuli.

These data are consistent with past research from our laboratory that shows antiseizure actions of 3α,5α-THP. Males treated with 3α,5α-THP (2.5 mg/kg) prior to perforant pathway stimulation show a reduction in tonic–clonic and partial seizure activity (12). Progesterone and 3α,5α-THP (4.0 mg/kg SC) have comparable effects in reducing kainic acid and perforant pathway induced seizures in ovx rats (15), albeit P had a longer latency to reduce seizures than did 3α,5α-THP. Administration of 5α-reductase inhibitors blocks the antiseizure actions of exogenous P administration (15). 3α,5α-THP more directly correlates with endogenous variations in ictal activity than does P itself (13).

That proximate 3α,5α-THP concentrations and repeated withdrawal may influence seizure threshold and hippocampal integrity in response to perforant pathway stimulation is an important extension of past research demonstrating that P's antiseizure effects may be attributable, in part, to 3α,5α-THP. Precipitous decline in endogenous 3α,5α-THP, as occurs postparturition, can lower seizure threshold (13,14); however, it has

not previously been demonstrated that cycles of 3α,5α-THP withdrawal could result in decreased neuron integrity in the hippocampus, despite nonsignificant decreases in seizures and circulating 3α,5α-THP. That patterns of 3α,5α-THP withdrawal may have effects on hippocampal morphology is not surprising in light of other reports of (neuro) steroids altering hippocampal neurons. The extent to which the present findings relate to changes in hippocampal dendritic spine density over the estrous cycle (48,49), increased connectivity in response to 3α,5α-THP addition to cultured neurons (4), or enhancing effects of 3α,5α-THP that are seen on neuronal growth and plasticity (37,38), remains to be investigated.

The present findings indicate that seizure activity returns to ovx/control levels following withdrawal from P and/or 3α,5α-THP. Further, following repeated cycles of withdrawal, lower concentrations of 3α,5α-THP at the time of seizure induction are capable of producing antiseizure effects. There are several explanations for this effect. The most likely would seem that cycles of 3α,5α-THP withdrawal may alter GBRs. For example, chronic administration of 3α,5α-THP produces downregulation of GBR binding and function in cultured mammalian cortical neurons (51), as well as decreased chloride influx (52) and efficacy of positive modulators to potentiate GABA-induced currents (50). GBR receptor desensitization is also noted following prolonged infusion of GABA in the presence of 3α,5α-THP (7). Fluctuation in 3α,5α-THP over the estrous cycle also alter GBR sensitivity. Although in vitro and functional findings are not entirely consistent, these differences may be due, in part, to the sensitivity of the different GBR modulators, as well as the effects of specific subunits of GBRs; for example, the α4 subunit is very important (42,43). Some investigators have found that GBR-active neurosteroids are most potent on diestrus 1 (9), whereas others report greater sensitivity to benzodiazepines and barbiturates on proestrus and estrus (31,46). Hence, the aforementioned duration of exposure to ligands and their potency at GBR modulation, changes in subunit composition (32), endogenous GABA and hormone environment (25), and genetic differences (11) may underlie some of these apparent inconsistencies.

Clearly, the manner in which neurosteroids produce alterations in ictal activity is complex. However, additional research is warranted to elucidate the mechanism by which acute and chronic P and 3α,5α-THP modulate seizure threshold, as these differences may underlie endogenous variations in ictal activity.

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