A Mouse Kindling Model of Perimenstrual Catamenial Epilepsy

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ABBREVIATIONS:
AD, afterdischarge; ADT, afterdischarge threshold; AP, allopregnanolone; GABA, \( \gamma \)-aminobutyric acid; HCG, human chorionic gonadotropin; P, progesterone, PMSG, pregnant mare’s serum gonadotropin

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ABSTRACT

Catamenial epilepsy is caused by fluctuations in progesterone-derived GABA_A receptor-modulating anticonvulsant neurosteroids such as allopregnanolone that play a significant role in the pathophysiology of epilepsy. However, there is no specific mouse model of catamenial epilepsy. In this study, we developed and characterized a mouse model of catamenial epilepsy using the neurosteroid withdrawal paradigm. It is hypothesized that seizure susceptibility decreases when neurosteroid levels are high (mid-luteal phase), and increases during their withdrawal (perimenstrual periods) in close association with GABA_A receptor plasticity. A chronic seizure condition was created using the hippocampus kindling model in female mice. Elevated neurosteroid levels were induced by sequential gonadotropin treatment, and withdrawal was induced by the neurosteroid synthesis inhibitor finasteride. Elevated neurosteroid exposure reduced seizure expression in fully-kindled mice. Fully-kindled mice subjected to neurosteroid withdrawal showed increased generalized seizure frequency and intensity, and enhanced seizure susceptibility. They also showed reduced benzodiazepine sensitivity and enhanced neurosteroid potency, similar to the clinical catamenial seizure phenotype. The increased susceptibility to seizures and alterations in antiseizure drug responses are associated with increased abundance of the α4- and δ-subunits of GABA_A receptors in the hippocampus. These findings demonstrate that endogenous neurosteroids protect against seizure susceptibility and their withdrawal, such as that which occurs during menstruation, leads to exacerbation of seizure activity. This is possibly due to specific changes in GABA_A receptor subunit plasticity and function, therefore providing a novel mouse model of human perimenstrual catamenial epilepsy that can be used for investigation of disease mechanisms and new therapeutic approaches.
Introduction

Catamenial epilepsy, the cyclical occurrence of seizure exacerbations during particular phases of the menstrual cycle in women with epilepsy, affects a high proportion of women of reproductive age with drug-refractory epilepsy. The word “catamenial” is derived from the Greek word katamenios meaning monthly. Catamenial seizure exacerbations are reported to affect up to 70% of women with epilepsy (Herzog et al., 2004; Bazan et al., 2005; Quigg et al., 2009). There are three forms of catamenial epilepsy: perimenstrual and periovulatory in normal cycles, and luteal in inadequate luteal phase cycles (Herzog et al., 1997; 2011). The most common form is perimenstrual, the cyclical seizure exacerbation during menstrual periods. It is a multifaceted neuroendocrine condition attributed to numerous causes. There is growing evidence from animal experiments suggesting that enhanced seizure susceptibility in perimenstrual catamenial epilepsy is due to the withdrawal of the progesterone (P)-derived neurosteroids as a result of the fall in P at the time of menstruation (Reddy, 2009; Pack et al., 2011). In women, there is evidence for seizure exacerbation following inadvertent inhibition of neurosteroid synthesis (Herzog and Frye, 2003).

Neurosteroids are endogenous regulators of seizure susceptibility. The prototype neurosteroid allopregnanolone (AP) is synthesized from P or other intermediate precursors. AP-like neurosteroids are broad-spectrum anticonvulsants and exhibit protective effects against seizures induced by pentylenetetrazol, pilocarpine, 6-Hz stimulation and kindling (Reddy, 2011). Neurosteroids rapidly alter neuronal excitability through direct interaction with GABA\textsubscript{A} receptors (Hosie et al., 2007). GABA\textsubscript{A} receptors are composed of five subunits from several classes (\(\alpha_{1-6}, \beta_{1-4}, \gamma_{1-3}, \delta, \varepsilon, \theta, \rho_{1-3}\)). The major isoforms consist of 2\(\alpha\), 2\(\beta\), and 1\(\gamma\) or \(\delta\)-subunits. Neurosteroids act on all GABA\textsubscript{A} -receptor isoforms, but cause large effects on extrasynaptic \(\delta\)-subunit receptors that mediate tonic inhibition (Belelli et al., 2002; Stell et al., 2003). Neurosteroids potentiate both synaptic and extrasynaptic GABA\textsubscript{A} receptors and thereby play a significant role in the pathophysiology of epilepsy (Reddy, 2011). Ovarian cycle-linked fluctuations in P and neurosteroids have been proposed to affect seizure susceptibility (Reddy et al., 2001; Maguire et al., 2005; Tuveri et al., 2008). Seizures decrease in the mid-luteal phase when serum P levels are high and increase premenstrually when P levels fall, causing the perimenstrual-type catamenial epilepsy. P protects against seizures by its conversion to neurosteroids, predominantly AP (Reddy et al., 2004). Consequently, perimenstrual seizure exacerbations may be due to withdrawal of the antiseizure effects of neurosteroids (Reddy, 2009). Neurosteroid withdrawal is associated with a marked upregulation of the \(\alpha_{4}\)-subunit in the hippocampus (Smith et al., 1998ab),
which is associated with an increase in seizure susceptibility and benzodiazepine resistance (Smith and Gong, 2005; Gangisetty and Reddy, 2010). Thus, neurosteroid withdrawal may be a key triggering factor for catamenial seizure exacerbations.

Based on the neurosteroid withdrawal approach, we have developed a rat model of perimenstrual catamenial epilepsy (Reddy et al., 2001). Rodents have a 4 to 5 day estrous cycle, and studies of fluctuations in seizure susceptibility in cycling female rats have not led to results that are relevant to the human menstrual cycle. In order to provide a model that more closely mimics the human situation, a condition of elevated P was created in rats by gonadotropin treatment. This resulted in prolonged high circulating levels of P similar to those that occur in the luteal phase of the menstrual cycle. Then, to simulate the withdrawal of AP that occurs at the time of menstruation, the animals were treated with finasteride 11 days after the initiation of gonadotropin treatment. Withdrawal of neurosteroids had led to decreased seizure threshold and increased seizure activity (Reddy et al., 2001). This paradigm was also verified in female epileptic rats with spontaneous seizures (Reddy and Zeng, 2007; Lawrence et al., 2010). Although rat models are useful to investigate therapies for catamenial epilepsy (Reddy and Rogawski, 2001), there is currently no mouse model that recapitulates neuroendocrine and clinical features of catamenial epilepsy for use in molecular and genetic investigations.

In this study, we developed a mouse model of catamenial epilepsy based on the emerging neuroendocrine mechanisms. The model is based on the premise that seizure susceptibility decreases when neurosteroid levels are high (luteal phase) and increases during their withdrawal (perimenstrual periods) in females in association with specific changes in the GABA\(_A\) receptor subunit plasticity.
Materials and Methods

Animals. Female adult mice of C57BL6 strain weighing 25 to 30 g were used in the study. Mice were housed four to a cage with free access to food and water. The mice were housed in an environmentally controlled animal facility under a 12 h light/dark cycle. The animals were cared for in strict compliance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Gonadotropin-induced Neurosteroid Synthesis and Withdrawal Paradigm. A state of elevated neurosteroid was induced in female mice by a sequential gonadotropin regimen (Brooke et al., 2007). To produce prolonged elevated levels of P and neurosteroids that more closely model the luteal changes in women, mice were treated with pregnant mare’s serum gonadotropin (5 IU, s.c.) at 3 PM followed 48 h later by human chorionic gonadotropin (5 IU, s.c.) at 1 PM. The day of the second gonadotropin injection was considered day 1 of elevated neurosteroids. On the morning of the 9th day, mice were injected with the 5α-reductase and neurosteroid synthesis inhibitor finasteride (50 mg/kg, i.p.) to produce an abrupt decline in neurosteroid levels to more closely model perimenstrual changes in women. Animals were tested 24 h after finasteride administration (neurosteroid withdrawal). The control group received saline injections. This protocol is similar with standard P treatment approaches used previously for induction of neurosteroid withdrawal (Smith et al. 1998ab; Moran and Smith, 1998), and is also comparable to the pseudopregnancy model in rats (Reddy et al., 2001; Reddy and Rogawski, 2001). Although it is not practical to replicate the actual endocrine milieu of the menstrual cycle in mouse models, this endocrine state may be physiologically similar to the perimenstrual period. We did not utilize the gonadectomy model because of potential problems of interpretation associated with complete deficiency of ovarian-derived hormones, and such animals need hormone replacements that may have variable effects on seizures depending on the age, dose, and duration of treatment (Scharfman et al., 2005).

Determination of Plasma AP Levels. Animals were anesthetized with isoflurane and ~0.5 ml carotid blood was collected in heparinized tubes. The plasma was separated by centrifugation at 12,000 × g for 10 min and stored at −20 °C in 10 ml glass tubes coated with 7.5% EDTA solution. The concentration of AP was analyzed by liquid chromatography-mass spectrometry as previously described (Reddy et al., 2004). A 0.2 ml plasma sample was added to a tube containing evaporated internal standard. The steroid and internal standard were extracted with 4 ml hexane. Each sample
was analyzed using the APCI technique under acidic conditions. A standard curve was plotted using pure AP in methanol mixed with 0.2 ml of blank mouse plasma.

**Hippocampus Kindling Model of Epilepsy.** To determine the effect of neurosteroid exposure and withdrawal on seizure susceptibility, we utilized the hippocampus kindling model, which is a model of human complex partial seizures (Goddard et al., 1969). Kindling is the repetition of stimuli that initially evoke afterdischarges but not seizures. A mild focal, non-convulsant electrical stimulus to the hippocampus on a daily basis leads to the development of a kindled state exhibiting electrographic and behavioral seizures. Once an animal has been kindled, the heightened response to the stimulus is permanent and seizures occur upon stimulation even after several months (Reddy and Mohan, 2011).

Electrode implantation and stimulation procedures for mouse hippocampus kindling were performed as described previously (Gangisetty and Reddy, 2010). Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). A twisted bipolar stainless steel wire electrode (model MS303/1; Plastic One, Roanoke, VA) was stereotaxically implanted in the right hippocampus (2.9 mm posterior, 3.0 mm lateral, and 3.0 mm below dura) (Franklin and Paxinos, 1997) and anchored with dental acrylic to three jeweler's screws placed in the skull. A period of 7 to 10 days was allowed for recovery. The stimulation paradigm consisted of 1 ms-duration, bipolar, square current pulses delivered at 60 Hz for 1 s using a kindling stimulator (A-M Systems, Sequim, WA). The afterdischarge (AD) threshold was determined by stimulating at 5 min intervals beginning with an intensity of 25 μA and increasing in increments of 25 μA until an AD of at least 5 s was obtained. Stimulation on subsequent days used an intensity 125% of the threshold value. Seizure activity following each stimulation was rated according to the criterion of Racine (1972) as modified for the mouse: stage 0, no response or behavior arrest; stage 1, chewing or head nodding; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, falling. The AD was recorded from the hippocampus electrode with a Grass CP511 preamplifier (Astro-Med, West Warwick, RI) and stored in digital form using Axoscope 8.1 (Axon Instruments, Foster City, CA). AD duration was the total duration of hippocampus electrographic spike activity (amplitude > 2 x baseline) occurring in a rhythmic pattern at a frequency > 1Hz. The day of AD threshold determination was considered day 1 of kindling. Kindling stimulation was delivered daily until stage 5 seizures were elicited on three consecutive days. Stimulation was continued on a five-day per week schedule each afternoon. Mice were used for the neurosteroid withdrawal paradigm when they consistently exhibited stage 5 seizures with stimulation, which is considered the “fully kindled” state.
Test Drug Administration and Stimulation Protocol. To examine the ability of test drugs to suppress the expression of kindled seizures, fully-kindled animals were tested 24 h after the induction of neurosteroid withdrawal. Two different drugs, diazepam and AP, were selected for pharmacological evaluation in neurosteroid-withdrawn animals. Diazepam is a benzodiazepine-site agonist at $\alpha_1$, $\alpha_2$- or $\alpha_3$-containing GABA$_A$ receptors with potent antiseizure activity, but is insensitive as an agonist at $\alpha_4$-containing GABA$_A$ receptors expressed in the hippocampus (Smith et al., 1998a; Gulinello et al., 2001). The neurosteroid AP binds to all isoforms but has enhanced sensitivity at $\delta$-subunit containing GABA$_A$ receptors. In the kindling studies, animals were tested for drug sensitivity 24 h after neurosteroid withdrawal. On the day of testing, animals were injected intraperitonially with diazepam (0.1-1 mg/kg) or AP (1-10 mg/kg) 15 min before kindling stimulations. Control animals were injected similarly with vehicle (15% cyclodextrin). During each stimulation session, the behavioral seizure score and the AD duration were noted.

TaqMan Real-Time PCR Assay. The GABA$_A$ receptor subunit mRNA expression was determined by the TaqMan real-time PCR assay as described previously (Gangisetty and Reddy, 2009). Mice were anesthetized with isoflurane and the hippocampus was rapidly dissected for RNA isolation. The total RNA was extracted from the hippocampus using a Trizol reagent and cDNA was prepared using the Superscript II first-strand cDNA synthesis kit (Invitrogen Inc., Carlsbad, CA). The PCR primers and TaqMan probe specific for GABA$_A$ receptor subunits and GAPDH genes were designed using the Primer Express software (Applied Biosystems Inc., Foster City, CA). Primer set and probe for the $\alpha_4$-subunit were composed of the following sequences: forward, 5'-AGA-ACT-CAA-AGG-AGA-AAT-TGT-3'; reverse, 5'-TTC-ACT-TCT-GTA-ACA-GGA-CCC-C-3'; and sequence-specific TaqMan probe: 5'-6-FAM-ACG-CAG-CCT-GTT-GTC-ATA-ACC-ATC-CAG-C-TAMRA-3'. A set of primers and sequence specific TaqMan probes were designed and optimized for real-time PCR analysis of the $\delta$-subunit gene. TaqMan PCR reactions were carried out in an AB 7500 fast real-time system (Applied Biosystems). Real-time PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems), which contained AmpliTaq Gold DNA Polymerase, AmpErase, UNG, dNTPs with dUTP, and optimized buffer components. Each sample was run in triplicate design and each 25-µl reaction mixture consists of 12.5-µl TaqMan Universal PCR Master mix, 400 nM primers, and 300 nM TaqMan probe for the target genes as described previously (Gangisetty and Reddy, 2009). The real-time PCR run consisted first of 1 cycle of 50 °C for 2 min, then 1 cycle of 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. The target input amount for each target gene was normalized to GAPDH.
expression in the same samples to control for loading variability, and then expressed as a percent change with respect to mean control values in the same run.

**Data Analysis.** Group data are expressed as the mean± standard error of the mean (SEM). Differences in kindling seizure stage between groups were compared with the nonparametric Kruskal-Wallis test followed by the Mann-Whitney *U*-test. Comparison of means of the AD duration between groups was made with one-way analysis of variance, followed by unpaired two-tailed Student’s *t*-test. Comparison of the mean percentage inhibition of seizure stage and AD duration in fully kindled animals was made by Wilcoxon signed ranks test and paired two-tailed Student’s *t*-test, respectively. To construct dose-effect curves, diazepam and AP were tested at several doses spanning the dose producing 50% protection (ED$_{50}$) in the kindling model. The GABA$_{A}$ receptor α4- and δ-subunit expression was analyzed based on the relative quantification approach as described previously (Gangisetty and Reddy, 2009). In all statistical tests, the criterion for statistical significance was $p < 0.05$.

**Drugs.** Gonadotropins (Sigma, St. Louis) were dissolved in saline. Stock solutions of AP (Steraloids Inc., Newport, RI) and other drugs for injection were made in 15% β-cyclodextrin in saline, and additional dilutions were made using sterile saline. Diazepam solution (Hospira, Lake Forest, IL) was diluted in sterile saline. Drug solutions were administered subcutaneously or intraperitonially in a volume equaling 1% of the animal’s body weight.
Results

Progression of Hippocampus Kindling Development in Female Mice. To create a background model for developing catamenial epilepsy in female mice, we utilized the hippocampus model of complex partial seizures. As shown in Fig.1, daily kindling stimulation was associated with a steady progression of behavioral seizures (Fig.1A) and AD duration (Fig.1B). Mice were subjected to once-daily kindling via an implanted electrode in the dentate gyrus region until they exhibited stage 5 seizures for 3 consecutive days, which is considered the “fully kindled” state. Mice reached the fully kindled state with consistent stage 5 seizures after ~14 stimulations (Fig.1A).

Gonadotropin-induced Neurosteroid Synthesis and Withdrawal Paradigm. To create a model of the perimenstrual endocrine milieu to simulate the menstruation associated neurosteroid withdrawal, a state of prolonged neurosteroid levels was induced in mice by sequential gonadotropin regimen (Fig.2A). Neurosteroid withdrawal was induced by treatment with finasteride (50 mg/kg, ip), a 5α-reductase inhibitor that blocks the conversion of P into AP. As shown in Fig.2B, plasma AP levels, measured by LC-MS/MS assay, were increased following gonadotropin treatment (mean level, 95 ng/ml on day 3 vs. vehicle control, 5 ng/ml). The levels of AP were gradually elevated from the baseline after gonadotropin treatment. Plasma AP was significantly reduced (~80%) 24 h after finasteride treatment (Fig.2B). Thus, the elevated plasma AP concentrations following gonadotropin treatment mimics the high levels of neurosteroids during the luteal phase of the menstrual cycle, whereas the finasteride-induced withdrawal is similar to the marked decrease in neurosteroid concentrations that occur before the start of menstruation.

Elevated Endogenous Neurosteroids Decrease Seizure Expression in Fully-Kindled Mice. To determine the effect of elevated neurosteroid level on kindled seizures, fully-kindled mice were given gonadotropin treatment to boost endogenous AP levels (Fig.2A) and then the animals were subjected to once-daily stimulation sessions, and seizures were measured as electrographic AD and behavioral seizures. As shown in Fig.3, the severity of generalized (stage 4/5) seizures was markedly reduced during the period of gonadotropin-induced elevation in neurosteroids, indicating endogenous neurosteroids reduce or check seizure susceptibility.

Neurosteroid Withdrawal Increases Seizure Susceptibility in Fully-Kindled Mice. To determine whether the neurosteroid withdrawal is associated with heightened seizure susceptibility, we analyzed the stimulation-evoked seizure activity in animals undergoing neurosteroid withdrawal. Fully-kindled
mice were subjected to neurosteroid withdrawal protocol as described in Fig.1A. Four parameters were assessed as indices of seizure propensity: (a) AD threshold (ADT) current for generalized seizures; (b) stimulation-induced electrographic AD duration, (c) behavioral seizure intensity measured as per the Racine scale, and (d) duration of generalized seizures. Consistent with heightened excitability, there was a marked decrease in the ADT current to induce generalized seizures at 24 h after neurosteroid withdrawal (mean ADT value, 105 and 60 μV for control and withdrawal, respectively) (Fig.4A). The mean duration of the individual generalized seizures was longer in withdrawal than in control animals (Fig.4B). The total duration of AD was significantly higher in withdrawal animals (Fig.4C). The number of animals exhibiting generalized seizures at 50% ADT current was significantly higher after neurosteroid withdrawal than in the control group (Fig.4D). This response was significantly higher 12 and 24 h after neurosteroid withdrawal and returned to control level by 48 h after withdrawal (Fig.4D), indicating a transitory period for seizure exacerbation following neurosteroid withdrawal. The electrographic events are illustrated in Fig.5. Neurosteroid-withdrawn animals showed continuous bursts of spikes that progressively increased in amplitude and duration, indicating heightened epileptiform activity (Fig.5). The electrographic AD duration was increased markedly 12 h after withdrawal, reached maximal level 24 h after withdrawal, and declined to almost control level by 48 h after withdrawal (Fig.5). Finasteride did not cause such seizure exacerbations in fully-kindled control (non-withdrawing) animals, indicating the specificity of neurosteroid withdrawal on the exacerbation of seizure activity in fully-kindled mice.

**Neurosteroid Withdrawal Alters GABA<sub>A</sub> Receptor Subunit Plasticity.** To investigate the potential molecular mechanisms underlying the seizure exacerbations, we determined the changes in GABA<sub>A</sub> receptor subunit mRNA expression during neurosteroid withdrawal in the hippocampus, which has previously been shown to exhibit neurosteroid-dependent plasticity (Smith et al., 1998a; Maguire and Mody, 2007; Gangisetty and Reddy, 2010). Twenty-four hours after neurosteroid withdrawal, the levels of α4-subunit were significantly increased compared with its expression in control group (Fig.6A). The abundance of δ-subunit mRNA in the hippocampus was also significantly increased in withdrawn animals (Fig.6A). In contrast, no changes in levels of β2- and γ2-subunit expressions were observed 24 h after neurosteroid withdrawal (Fig.6A). Overall, these findings indicate a robust increase in the expression of α4- and δ-subunit expression in the hippocampus during neurosteroid withdrawal in the mouse catamenial paradigm (Fig.6B). The localization of GABA<sub>A</sub> receptor subunits is not known with the TaqMan PCR technique. Although dentate gyrus normally has high expression of α4 and δ-subunits, they are also expressed in CA1 hippocampus in response to fluctuations in neurosteroids (Smith et al., 1998b).
Neurosteroid Withdrawal Induces Diazepam Insensitivity in Fully-Kindled Mice. To further investigate if neurosteroid withdrawal is associated with alterations in the antiseizure profile of benzodiazepines, diazepam was characterized pharmacologically in fully-kindled mice at 24 h after neurosteroid withdrawal (Fig.7). Diazepam produced a dose-dependent suppression of behavioral seizure activity (Fig.7A) and AD duration with significant effects at 0.1, 0.3 and 1 mg/kg in control (non-withdrawal) animals (Fig.7B), confirming diazepam protection against hippocampus kindling-induced seizures. In contrast, mice undergoing neurosteroid withdrawal had significantly decreased seizure protection by diazepam (Fig.7AB). At 1 mg/kg dose, diazepam produced an average of a 95% and 30% decrease in seizure expression in control and withdrawal groups, respectively. Taken together, these results are consistent with the notion that neurosteroid withdrawal causes relative insensitivity to diazepam due to increased $\alpha_4$-containing GABA$_A$ receptor expression in the hippocampus.

Neurosteroid Withdrawal Confers Enhanced AP Sensitivity in Fully-Kindled Mice. Additionally, we investigated the efficacy of the prototype neurosteroid AP in mice 24 h after neurosteroid withdrawal (Fig.8). Control and neurosteroid-withdrawn mice were tested in the kindling model with three doses of AP (1, 5 and 10 mg/kg, ip). At these doses, AP exerted dose-dependent suppression of the behavioral seizures (Fig.8A) and AD duration (Fig.8B) in control (non-withdrawal) mice. In contrast, after neurosteroid withdrawal, AP produced greater suppression of behavioral seizures (Fig.8A) and AD duration (Fig.8B) with significant effects at 1 and 5 mg/kg when compared with control group. Moreover, plasma levels of AP achieved at various doses of AP treatment were similar between control and withdrawn groups, especially without significant drug accumulation in withdrawn animals (Fig.8C), indicating that AP sensitivity was not due to pharmacokinetic factors. The synthetic neurosteroid ganaxolone (1, 3 and 10 mg/kg) also produced enhanced (~60%) efficacy in fully-kindled neurosteroid withdrawn animals (figure not shown), confirming the enhanced sensitivity to neurosteroids in the neurosteroid withdrawal model of catamenial epilepsy (Reddy and Rogawski, 2001).
Discussion

The principal findings of the study are that acute inhibition of neurosteroid synthesis or neurosteroid withdrawal in female mice caused exacerbation of seizure activity as evident by an increase in kindling seizure severity, AD duration and generalized seizure duration. The AD threshold for evoking generalized seizures was markedly decreased 24 h after withdrawal, suggesting vulnerability to seizures. Neurosteroid withdrawal was associated with increased GABA$\alpha$4- and $\delta$-subunit expression, reduced antiseizure sensitivity to diazepam, and enhanced antiseizure sensitivity to AP. This potential pathophysiological profile is consistent with clinical catamenial seizure features (Fig.6B). Taken together, these results suggest that endogenous neurosteroids protect against seizures and their withdrawal, such as that which occurs during menstruation, which may exacerbate seizure activity, providing a novel mouse model of catamenial epilepsy that can be used for the investigation of disease mechanisms and evaluation of novel therapeutic approaches. Our data on $\delta$- and $\alpha$4-subunit expression provide a potential molecular mechanism for the enhanced seizure susceptibility and drug sensitivity in this epilepsy model. Alterations in ovarian hormones and modification of GABAergic inhibition are an intensely investigated hypothesis guiding research into pathophysiological mechanisms underlying catamenial epilepsy (Scharfman and McLusky, 2006; Tuveri et al., 2008; Reddy, 2009). The main concern is the lack of a suitable mouse model to investigate the pathophysiological mechanisms of catamenial seizure exacerbations. Using the neurosteroid withdrawal paradigm, we developed a rat model and successfully utilized for pharmacological testing of agents to inhibit catamenial seizures (Reddy et al., 2001; Reddy and Rogawski, 2001). Catamenial seizures are observed in women with preexisting epilepsy. Therefore, animal models should mimic this key criterion. Kindling provides a suitable background seizure model for developing a perimenstrual catamenial epilepsy model in female mice. Hippocampus kindling is a widely used model of human complex partial seizures. Unlike the pilocarpine-induced epilepsy model, the kindling model does not result in neuronal loss and maintains reproductive function in female animals. During the menstrual cycle, circulating P levels are low in the follicular phase, but rise in the midluteal phase for approximately 10 to 11 days before declining in the late luteal phase. Circulating AP levels parallel those of its parent P (Tuveri et al., 2008). Although the dynamics of brain AP during the menstrual cycle have not been studied, it is likely that local synthesis of GABA$\alpha$ receptor modulating neurosteroids occur in regions relevant to epilepsy such as the hippocampus and amygdala (Reddy, 2011). The results from the present study demonstrate a striking
increase in seizure activity following neurosteroid withdrawal in the kindling model. The seizure exacerbation was maximal 12 to 24 h after withdrawal and declined to control value at 48 h following withdrawal onset. Thus, neurosteroid withdrawal associated seizure exacerbation may represent a transient surrogate marker for perimenstrual catamenial epilepsy.

The gonadotropin-induced endocrine state is more physiologically similar to the hormonal milieu of the menstrual cycle than the paradigms that employ exogenous P or AP treatment (Shen et al., 2005; Gangisetty and Reddy, 2010). Seizure exacerbation observed in the present study is due to reduced neurosteroid levels in the brain. Finasteride blocks the synthesis of neurosteroids such as AP and related 5α-reduced pregnane analogs that modulate GABA_A receptor function (Mukai et al., 2008). Consistent with the time-course for finasteride inhibition of neurosteroid synthesis, we noted a latent period (12 to 24 h) between finasteride administration and the onset of seizure propensity. Moreover, the seizure susceptibility returned to baseline within 48 h, likely reflecting the time to regenerate 5α-reductase enzyme activity in the brain. Our measurements of plasma AP levels are consistent with the enzyme inhibition effect of finasteride. We have previously published similar results obtained after neurosteroid withdrawal in the pseudopregnancy model (Reddy et al., 2001), and a transitory increase in the frequency of spontaneous seizures in epileptic rats (Reddy and Zeng, 2007). Such heightened seizure susceptibility is consistent with previous reports in related models of neurosteroid withdrawal (Smith et al., 1998ab; Moran and Smith, 1998). Furthermore, seizure exacerbation as a result of finasteride inhibition of P metabolism has been observed in women with epilepsy (Herzog and Frye, 2003). Patients treated with finasteride had significantly reduced levels of neurosteroids (Duskova et al., 2009). These findings suggest that endogenous neurosteroids in the brain play a key role in controlling seizure propensity, most likely due to their ability to enhance synaptic and tonic inhibition in the hippocampus. It is suggested that inhibition of neurosteroids may accelerate the putative mechanisms that promote epileptic seizures (see Fig.6B).

The molecular basis for the enhanced seizure susceptibility in perimenstrual catamenial epilepsy is not completely understood, but may be due to specific alterations in GABAergic inhibition in the hippocampus. The GABA_A receptor composition undergoes dynamic plasticity in response to physiological signals, the hormonal (neurosteroid) milieu, and exogenously administered agents such as benzodiazepines or ethanol (Smith et al., 1998b; Smith et al., 2007; Reddy, 2009). The precise changes in brain GABA_A receptor subunit composition occurring during the human menstrual cycle or in animal models of catamenial epilepsy have not been determined. There is strong evidence that ovarian cycle-linked fluctuations in neurosteroids modulate the GABA_A receptor plasticity (Maguire et
al., 2005; Maguire and Mody, 2007). Our observation that neurosteroid withdrawal increases the α4-subunit expression in the hippocampus is consistent with the established premise that prolonged exposure to P followed by withdrawal in female rats causes upregulation of the α4-subunit (Smith et al., 1998b; Gulinello et al., 2001). Similar increases in α4-subunit levels have been observed previously in response to withdrawal from AP (Smith et al., 1998a; Follesa et al., 2000) and synthetic neurosteroids (Mascia et al., 2002). The α4-subunit can coassemble with γ2 to form synaptic GABAA receptors. The key consequence of the incorporation of the normally low abundance α4-subunit into synaptic GABAA receptors is that currents generated by these receptors have accelerated decay kinetics, so that there is less total charge transfer, which likely results in reduced net synaptic inhibition and a state of hyperexcitability (Smith and Gong, 2005). The increase in α4 when coassembled with either γ2- or δ-subunits in the hippocampus may result in benzodiazepine-insensitive receptors (Shen et al., 2005; Maguire et al., 2005). Therefore, when neurosteroids are withdrawn at the time of menstruation, the α4-subunit is upregulated and synaptic inhibition is diminished, resulting in enhanced excitability, which, among other effects, causes a predisposition to catamenial seizures.

We observed that neurosteroid withdrawn mice were strikingly less sensitive to the antiseizure effects of diazepam in the kindling model. These findings are highly consistent with the enhanced abundance of α4-subunit in vivo in the hippocampus. GABAA receptors containing the α1/2/3/5-subunits in combination with any of the β-subunits and the γ2-subunit are most prevalent in the brain (Mohler et al., 2002). These receptors are sensitive to benzodiazepine modulation. Moreover, receptors containing the α4-subunit are highly expressed in the dentate gyrus, but are benzodiazepine insensitive (Whiting et al., 2000; Mohler et al., 2002). This mechanism may underlie diazepam’s reduced protection (insensitivity) against seizures during neurosteroid withdrawal. Similar increases in α4-subunit and its pharmacological properties have been described after withdrawal from P or neurosteroids (Smith et al., 1998ab).

In the present study, we found increased expression of δ-subunit in the hippocampus following the neurosteroid withdrawal paradigm. The δ-subunit preferentially coassembles with α4-subunit to form perisynaptic/ extrasynaptic GABAA receptors (Sur et al., 1999). The δ-containing receptors are predominantly expressed in the dentate gyrus, the gateway that controls hippocampus excitability. Tonic inhibition in dentate gyrus neurons is mainly mediated by δ-subunit-containing receptors (Stell et al., 2003). Furthermore, both δ and α4-subunits are increased in the hippocampus of neurosteroid withdrawn animals. Therefore, the compensatory changes such as increased expression of α4δ-
subunit-containing receptors appear to preserve GABAergic inhibition in the dentate gyrus around the perimenstrual period associated with heightened excitability. These changes could lead to alterations in tonic inhibition and neurosteroid sensitivity.

In this study, we found that the neurosteroids AP and ganaxolone caused increased anticonvulsant effects in the mouse catamenial epilepsy model. The molecular mechanisms underlying enhanced neurosteroid sensitivity in the catamenial model remain unclear. Our Taqman PCR studies have revealed increased expression of δ-subunits in the hippocampus of withdrawing animals. This may be associated with enhanced neurosteroid sensitivity because the δ-subunit-containing GABA_A receptors have higher neurosteroid sensitivity (Mihalek et al., 1999; Wohlfarth et al., 2002) and are major contributors of tonic inhibition. Thus, neurosteroid withdrawal confers increased sensitivity to neurosteroids, possibly due to increased δ-containing GABA_A receptor expression in the hippocampus. Clearly, more needs to be learned about the regulation of δ-subunit expression to gain additional insights on tonic inhibition in the catamenial epilepsy model. Nevertheless, enhanced neurosteroid sensitivity in catamenial epilepsy has important therapeutic implications. Synthetic neurosteroids that augment tonic inhibition may provide a rational treatment strategy for controlling catamenial seizures at low doses that do not cause significant GABAergic side effects. Such neurosteroid replacement therapy may prevent seizure occurrence in women with epilepsy (Reddy and Rogawski, 2009).

More importantly, our data on the upregulation of hippocampal α4- and δ-subunits offer a molecular mechanism for the enhanced seizure susceptibility and drug sensitivity in this epilepsy model (Fig.6B). The enhanced seizure susceptibility subsequent to neurosteroid withdrawal may be related to increased expression of α4-containing synaptic GABA_A receptors that exhibit faster decay kinetics and confer benzodiazepine insensitivity. The enhanced potency of neurosteroids may be due to a relative increase after neurosteroid withdrawal in the expression of neurosteroid-sensitive δ-containing extrasynaptic GABA_A receptors. The α4-subunits which are increased during withdrawal could co-express with either or both δ and γ2. Although the increase in neurosteroid sensitivity may be due to δ-subunit, there is no reason to believe that this is mutually exclusive, because (1) the increase in δ is less than the increase in α4, and while γ2 is unchanged, α1 shows a slight decrease in expression; (2) flumazenil, a benzodiazepine antagonist which acts as an agonist at α4βγ2 (Wafford et al., 1996) produced a greater antiseizure effect during withdrawal (Gangisetty and Reddy, 2010); (3) α4βγ2-receptors exhibit a faster kinetics which likely results in reduced inhibition and increase in the seizure state; and (4) suppression of the α4-subunit prevents the withdrawal-induced increase in
seizures (Smith et al., 1998b). Thus, it is likely that seizure exacerbation is not only due to the relative lack of AP during withdrawal, but could also be due to increases in $\alpha 4\beta 2$-receptors.

In the present study, increased antiseizure activity of AP and ganaxolone is consistent with upregulation of $\delta$-subunit-containing GABA$_A$ receptors. The $\delta$-subunit increases may be transitory during withdrawal and followed by reduced expression with chronic exposures, as in pregnancy (Maguire and Mody, 2008) or in the prolonged luteal phase of the menstrual cycle. This change is believed to be a compensatory mechanism, which would avoid excessive sedation caused by high neurosteroid levels acting on $\delta$-subunit receptors. At the time of neurosteroid withdrawal, $\delta$ expression rapidly recovers. If recovery is not sufficiently fast, however, there could be an enhancement of excitability due to a reduction in tonic inhibition in the relative absence of neurosteroids.

There are a number of reports on epilepsy-associated plasticity in subunit expression and GABAergic inhibition (Joshi et al., 2012). There is evidence showing that epilepsy models result in reduced expression of $\delta$-subunit in the dentate gyrus (Zhang et al., 2007), although in a mouse model of temporal lobe epilepsy, $\alpha 4$ and $\gamma 2$ were increased (Peng et al., 2004). A marked decrease in neurosteroid sensitivity was observed in epilepsy rats (Tcheslishvili et al., 2001; Sun et al, 2007). High levels of $\delta$-expression, in contrast, would be expected to attenuate the seizure state. Thus, the relevance of the increased $\delta$-subunit expression in the catamenial model is unclear. It is suggested that these receptors are merely returning to their normal level of expression while the increase in $\alpha 4\beta 2$ would result in neuronal excitability. Further studies with more selective compounds that enhance tonic currents such as gaboxadol or DS2 (Wafford et al., 2009) may verify other possible mechanisms.

In conclusion, these findings demonstrate that endogenous neurosteroids protect against seizure susceptibility and their withdrawal, such as that which occurs during menstruation, leads to exacerbation of seizure activity. This is possibly due to specific changes in GABA$_A$ receptor subunit plasticity and function in female mice, providing a novel mouse model that has features of human catamenial epilepsy. The mouse model is helpful for investigation of disease mechanisms and also for developing novel treatments for catamenial epilepsy.

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Authorship Contribution

D.S.R. has participated in research design, conducted experiments, performed data analysis and wrote the manuscript. J.G. and O.G. have conducted experiments.
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Footnote to title page

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Figure Legends

Fig. 1. Progression of hippocampus kindling development in female mice. Panel A shows progression of behavioral seizure stage following each stimulation. Panel B shows progression of afterdischarge (AD) duration following each stimulation. Values represent the mean ± SEM (N=18 animals per group).

Fig. 2. Gonadotropin-induced neurosteroid synthesis and withdrawal paradigm. (A) Experimental protocol for the neurosteroid exposure and withdrawal model in mice. To mimic the prolonged exposure (like that of the luteal phase) followed by withdrawal (like that of menstruation) of progesterone and therefore the “neurosteroid” AP, mice were treated sequentially with gonadotropins. Mice were treated with pregnant mare’s serum gonadotropin (PMSG, 5 IU, s.c.) at 3 PM followed 48 h later human chorionic gonadotropin (HCG, 5 IU, s.c.) at 1 PM. Then, on day 9 they were given finasteride (50 mg/kg, i.p.), a 5α-reductase inhibitor that blocks the synthesis of P-derived neurosteroid AP. (B) Plasma AP levels in mice following treatment with gonadotropins and finasteride (50 mg/kg, i.p.) given as outlined in Fig.2A for induction of neurosteroid withdrawal. Thus, the dramatic decline in neurosteroid levels 24 h after finasteride (see Fig.1B) would create a state of acute neurosteroid withdrawal, which is proposed to partly model the neuroendocrine milieu commonly observed around the perimenstrual period. Values represent the mean ± SEM (n=6 animals per group). *p<0.01 gonadotropin vs. vehicle; #p<0.01 gonadotropin group (open circles) vs. gonadotropin+finasteride group.

Fig. 3. Effect of elevated neurosteroids on seizure expression in fully-kindled mice. Panel A shows behavioral seizure stage during the 10-day period associated with elevated neurosteroids. Panel B shows AD duration during the study period. Values represent the mean ± SEM (N=9–15 animals per group). *p<0.05 vs. control group.

Fig. 4. Increased seizure activity during neurosteroid withdrawal in fully-kindled mice. Neurosteroid withdrawn animals exhibited seizure exacerbation as evident by a markedly decreased ADT current to induce generalized seizures (A), increased behavioral seizure duration (B), increased AD duration (C) and time-course of the percent of animals exhibiting generalized seizures (stage 4/5)
at 50% of regular ADT current (D). Values represent the mean ± SEM (N= 6–10 animals per group).

*p<0.05 vs. control group; #p<0.05, ##p<0.01 vs. control (non-withdrawn) group.

Fig. 5. Neurosteroid-withdrawal induced exacerbation of electrographic seizure activity in fully-kindled mice. Representative traces illustrating time-dependant exacerbation of electrographic seizure activity in a fully kindled mouse during neurosteroid-withdrawal period. Traces show depth recordings from a right hippocampus stimulating/recording electrode. Arrows indicate onset of the 1 s kindling stimulus, which is followed by the stimulus artifact. Control trace was obtained without neurosteroid withdrawal.

Fig.6. Neurosteroid withdrawal causes upregulation of GABA_A receptor α4- and δ-subunit expression in the hippocampus. (A) TaqMan real-time PCR analysis of GABA_A receptor subunit mRNA expression in the hippocampus in mice. The subunit expression was quantified in the hippocampus samples collected from control mice following neurosteroid withdrawal protocol as described in Fig.2A. Total RNA was extracted from the hippocampus and cDNA was prepared for TaqMan PCR analysis. Values represent the mean ± SEM (n = 6–8 animals per group). *p<0.01 vs. control group. (B) A working model indicating alterations in pharmacology due to neurosteroid withdrawal-induced changes in extrasynaptic GABA_A receptor plasticity. Neurosteroid withdrawal increase in α4-subunit may underlie the increased excitability due to reduced net inhibition and benzodiazepine-insensitivity, where as upregulation of extrasynaptic δ-subunit-containing receptors may promote neurosteroid sensitivity.

Fig.7. Neurosteroid withdrawal reduces the antiseizure sensitivity of diazepam in fully-kindled mice. Dose-response curve for diazepam (0.1–1 mg/kg, i.p.)-induced suppression of behavioral seizures (A) and AD duration (B) in control and neurosteroid withdrawn mice. Diazepam is a benzodiazepine-site agonist at α1/2-containing, but not α4-containing GABA_A receptors. The diazepam-insensitivity in mice undergoing neurosteroid withdrawal was consistent with increased α4-containing GABA_A receptor abundance in vivo. Vehicle or diazepam was injected intraperitonially 15 min before kindling stimulations. Values represent the mean ± SEM (N= 6–10 animals per group). *p<0.05 vs. diazepam-treated control (non-withdrawn) group.

Fig.8. Neurosteroid withdrawal confers enhanced antiseizure effects to AP in fully-kindled mice. Dose-response curve for AP (1–10 mg/kg, i.p.) inhibition of behavioral seizures (A), AD duration (B), and plasma AP levels (C) in control and neurosteroid-withdrawn mice. AP is a neurosteroid that acts on all GABA_A receptor isoforms, but has greater sensitivity at δ-containing GABA_A receptors. The AP’s
enhanced antiseizure efficacy in mice undergoing neurosteroid withdrawal was consistent with increased δ-containing GABA_A receptor abundance in vivo. Despite similar AP levels in control and withdrawal animals, AP had enhanced antiseizure effect in mice undergoing neurosteroid withdrawal that was not attributed to drug accumulation. Vehicle or AP was injected intraperitonially 15 min before kindling stimulations or plasma sample collection. Values represent the mean ± SEM (N= 6–8 animals per group). *p<0.05 vs. AP-treated control (non-withdrawn) group.
Figure 3: (A) Graph showing Seizure Stage over Number of Stimulations (days). The graph compares Control and NS Exposure groups. The y-axis represents Seizure Stage, ranging from 0 to 5, and the x-axis represents the number of stimulations (days) from 0 to 10. The control group is represented by open circles, and the NS Exposure group is represented by filled circles. Significant differences are indicated by asterisks (*).

(B) Graph showing AD Duration (sec) over Number of Stimulations (days). The graph compares Control and NS Exposure groups. The y-axis represents AD Duration (sec), ranging from 10 to 70, and the x-axis represents the number of stimulations (days) from 0 to 10. The control group is represented by open circles, and the NS Exposure group is represented by filled circles. Significant differences are indicated by asterisks (*).
Figure-7

(A)

Seizure Stage

- Control
- Withdrawal

Diazepam (mg/kg)

(B)

AD Duration (sec)

- Control
- Withdrawal

Diazepam (mg/kg)