Estrous Cycle Regulation of Extrasynaptic δ -Containing GABA_A Receptor-Mediated Tonic Inhibition and Limbic Epileptogenesis

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ABSTRACT

The ovarian cycle affects susceptibility to behavioral and neurologic conditions. The molecular mechanisms underlying these changes are poorly understood. Deficits in cyclical fluctuations in steroid hormones and receptor plasticity play a central role in physiologic and pathophysiologic menstrual conditions. It has been suggested that synaptic GABA_A receptors mediate phasic inhibition in the hippocampus and extrasynaptic receptors mediate tonic inhibition in the dentate gyrus. Here we report a novel role of extrasynaptic δ -containing GABA_A receptors as crucial mediators of the estrous cycle–related changes in neuronal excitability in mice, with hippocampus subfield specificity. In molecular and immunofluorescence studies, a significant increase occurred in δ -subunit, but not α 4- and γ 2-subunits, in the dentate gyrus during diestrus. However, δ -subunit upregulation was not evident in the CA1 region. The δ -subunit expression was undiminished by age

and ovariectomy and in mice lacking progesterone receptors, but it was significantly reduced by finasteride, a neurosteroid synthesis inhibitor. Electrophysiologic studies confirmed greater potentiation of GABA currents by progesterone-derived neurosteroid allopregnanolone in dissociated dentate gyrus granule cells in diestrus than in CA1 pyramidal cells. The baseline conductance and allopregnanolone potentiation of tonic currents in dentate granule cells from hippocampal slices were higher than in CA1 pyramidal cells. In behavioral studies, susceptibility to hippocampus kindling epileptogenesis was lower in mice during diestrus. These results demonstrate the estrous cycle–related plasticity of neurosteroid-sensitive, δ -containing GABA_A receptors that mediate tonic inhibition and seizure susceptibility. These findings may provide novel insight on molecular cascades of menstrual disorders like catamenial epilepsy, premenstrual syndrome, and migraine.

Introduction

The menstrual cycle influences many brain conditions. Estradiol is secreted in the second half of the follicular phase and increases to a peak at midcycle, whereas progesterone is elevated during the luteal phase and declines before menstruation begins (Reddy, 2009, 2013). Fluctuations in steroid hormones, deficits in their cyclical availability, and receptor plasticity play a central role in susceptibility to menstrual conditions such as premenstrual syndrome, migraine, and catamenial epilepsy (Backstrom et al., 2003; Reddy, 2009). Catamenial epilepsy is characterized by seizures that cluster most often during the perimenstrual or periovulatory period, when progesterone levels are low (Herzog and Frye, 2003; Herzog et al., 2004, 2011; Reddy et al., 2012). However, the molecular mechanisms underlying these changes remain unclear.

Progesterone is a precursor for the synthesis of neurosteroids such as allopregnanolone (AP) in the brain (Reddy and Mohan, 2011). These neurosteroids are increased in

parallel to their precursor progesterone during the menstrual cycle (Tuveri et al., 2008). Progesterone and neurosteroids have anxiolytic, anticonvulsant, and neuroprotective properties (Reddy, 2004; Reddy et al., 2005) and have been shown to play a significant role in epilepsy, anxiety, and depression (Smith et al., 1998b; Reddy, 2003; van Broekhoven and Verkes, 2003; Reddy and Jian, 2010). Neurosteroids rapidly alter neuronal excitability through direct interaction with GABA_A receptors (GABA_ARs) (Hosie et al., 2007). GABA_ARs are composed of five subunits from several classes (α _{1–6}, β _{1–4}, γ _{1–3}, δ , ϵ , θ , ρ _{1–3}). The major isoforms consist of 2α , 2β , and 1γ - or 2α , 2β , and 1δ -subunits. It has been reported that synaptic (mainly γ 2-containing) receptors mediate phasic inhibition in the hippocampus and extrasynaptic (mainly δ -containing) receptors mediate tonic inhibition in the dentate gyrus. Neurosteroids act on both synaptic and extrasynaptic receptors as positive GABA_AR allosteric modulators (Reddy, 2010; Uusi-Oukari and Korpi, 2010) but cause large effects on δ -containing extrasynaptic GABA_ARs, which are persistently activated by ambient GABA (Belelli et al., 2002; Stell et al., 2003). The resulting tonic conductance generates shunting inhibition that controls network excitability and behavior.

The ovarian cycle modulates behavior and seizure susceptibility (Reddy and Kulkarni, 1999; Molina-Hernandez et al.,

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; AD, afterdischarge; ANOVA, analysis of variance; AP, allopregnanolone; BIC, bicuculline; CA1PCs, CA1 pyramidal cells; DG, dentate gyrus; DGGCs, dentate gyrus granule cells; GABA_ARs, GABA_A receptors; G_{rms} , root-mean-square noise conductance; G_{tonic} , mean peak tonic conductance; NS, neurosteroid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PR, progesterone receptor; PRKO, progesterone receptor knockout; WT, wild-type.

2001; Maguire et al., 2005, 2009). Changes in neuronal excitability occur during changes in reproductive status such as puberty, pregnancy, or menopause. During puberty, the level of steroid hormone increases and behavioral changes begin. Because neurosteroids influence inhibition, behavioral and learning deficits observed during puberty are linked to changes in δ -containing GABA_ARs (Shen et al., 2007, 2010). Ovarian cycle-linked fluctuations in progesterone and neurosteroids have been proposed to alter GABA_AR-mediated tonic inhibition (Maguire et al., 2005; Gangisetty and Reddy, 2010; Reddy et al., 2012). However, there is little information on molecular mechanisms by which GABA_AR subunit composition and function in hippocampus subfields are regulated by the ovarian cycle. It is poorly understood how progesterone-derived neurosteroid AP modulates neuroplasticity and epileptogenesis during the ovarian cycle.

In this study, we determined the estrous cycle-related changes in GABA_AR δ -subunit expression and tonic inhibition in the hippocampus subfields, along with susceptibility to epileptogenesis in female mice. To simulate the luteal (high-progesterone) and follicular (low-progesterone) phases, we focused on diestrus (high-progesterone) and estrus (low-progesterone); these stages exhibit a hormonal milieu comparable to the human ovarian cycle. Our results demonstrate novel estrous cycle-related changes of δ -containing GABA_ARs expression in different hippocampal subfields and GABA_ARs δ -subunit-mediated tonic inhibition and seizure susceptibility, such as those that occur in catamenial epilepsy. These findings are also relevant to premenstrual syndrome and migraine in women.

Materials and Methods

Animals and Estrous Cycle Studies

Animals. Female adult (3–5 months old) wild-type (WT) and progesterone receptor knockout (PRKO) mice weighing 25–30 g were used in the study. The development of the PRKO mouse strain and genotyping procedures have been described previously (Lydon et al., 1995; Reddy et al., 2004). These mice were maintained on a hybrid C57BL6/129SV background. Basal levels of progesterone in serum were reported to be similar in the WT and PRKO groups (Chappell et al., 1997). The mice were housed in an environmentally controlled animal facility under a 12 hour/12 hour light/dark cycle and allowed free access to food and water, except during the experimental sessions. Genotype was confirmed by polymerase chain reaction (PCR) using mouse tail genomic DNA (Reddy et al., 2005). Young adult mice with regular cycles and aged mice (18 months old) that were acyclic were used in the study. Ovariectomized, young adult mice were used for experiments 2 weeks after the surgical procedure. All procedures were performed in strict compliance with the guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals under a protocol approved by the university's Institutional Animal Care and Use Committee.

Determination of Estrous Cycle and Plasma Progesterone Levels. Mice exhibit a 6-day ovarian/estrous cycle, which is subdivided into four stages that are associated with distinct hormonal milieu. Estrous cycle stage was determined by microscopic examination of vaginal smears with eosin staining (Maguire et al., 2005). Diestrus was characterized by the presence of many leukocytes. Proestrus was determined by largely small, round, nucleated epithelial cells. Estrus was confirmed by large, anucleated, cornified squamous epithelial cells. Metestrus was represented by both leukocytes and epithelial cells (Fig. 1). Estrus and diestrus were the stages chosen for further experiments. For studies of progesterone assay, mice were anesthetized

with isoflurane, and 0.5 ml of carotid blood was collected in heparinized tubes. The plasma was separated by centrifugation at 12,000g for 10 minutes at 4°C. Plasma concentrations of progesterone were analyzed by an immunoassay. The detection limit of the assay was <0.2 ng/ml. To simulate the luteal (high-progesterone) and follicular (low-progesterone) phases, we focused on diestrus (high-progesterone) and estrus (low-progesterone), stages that exhibit hormonal levels comparable to the human menstrual cycle.

Molecular and Cellular Studies

TaqMan Real-Time PCR. The GABA_AR subunit mRNA expression was determined by the TaqMan real-time PCR assay as described previously (Gangisetty and Reddy, 2009). The hippocampus was rapidly dissected for total RNA extraction by using a Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was prepared using the Superscript II first-strand cDNA synthesis kit (Invitrogen). The PCR primers and TaqMan probe sequences specific for GABA_AR subunits, such as $\alpha 4$ and δ -subunits, and glyceraldehyde-3-phosphate dehydrogenase genes were designed and optimized for real-time PCR analysis using the Primer Express software (Applied Biosystems Inc., Foster City, CA) (Gangisetty and Reddy, 2009). 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, uracil-N-glycosylase (to prevent contamination from previous qPCR reactions), dNTPs with dUTP, and optimized buffer components. Briefly, each sample was run in triplicate design and each 25- μ l reaction mixture consisted of 12.5- μ l of TaqMan Universal PCR Master mix, 400 nM primers, and 300 nM TaqMan probe for each targeted genes as described previously (Gangisetty and Reddy, 2009). The real-time PCR run consisted first of one cycle of 50°C for 2 minutes (AmpErase activation), then one cycle of 95°C for 10 minutes (Taq activation), and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute (denaturation, annealing, and extension). The GABA_AR subunit mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase expression as a percent change in the same samples.

Western Blot Analysis. Western blot analysis of GABA_AR δ -subunits was performed with modification of the published protocol (Maguire et al., 2005; Gangisetty and Reddy, 2010). Membrane protein (50 μ g) from the hippocampus was loaded on to 12% Tris-HCl gel (Bio-Rad, Hercules, CA) and subjected to electrophoresis at 75 V for 3 hours under denaturing conditions. The proteins were transferred to polyvinylidene fluoride membrane (Bio-Rad) and then blocked in 5% nonfat milk at room temperature for 1 hour. Membranes were then incubated with a rabbit polyclonal antibody specific for GABA_AR δ -subunits (1:500; PhosphoSolutions, Aurora, CO) and mouse monoclonal β -actin antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for overnight. Membranes were then washed three times with 1X Tris-buffered saline and Tween 20 at room temperature for 20 minutes. The blots were then incubated with anti-rabbit antibody (1:2500) conjugated to horseradish peroxidase for 1 hour at room temperature. Blots were washed three times for 20 minutes in 1X Tris-buffered saline and Tween 20. Immuno-reactive GABA_AR δ -subunit bands (52 kDa) were detected using enhanced chemiluminescent reagents (Pierce/Thermo Scientific, Rockford, IL), and then membranes were stripped and reprobed with the mouse monoclonal antibody for β -actin. Protein bands were quantified using Alpha Imager software (Alpha Innotech, San Leandro, CA). β -Actin was used as internal control. Changes in δ -subunit expression were expressed as percent change from control values.

Immunocytochemistry and Confocal Microscopy. The δ -subunit distribution in the hippocampal neurons was determined by immunocytochemistry and confocal microscopy according to the methods published previously with slight modifications (Mangan et al., 2005; Wu et al., 2010; Tangney et al., 2013). A suspension of freshly

Fig. 1. Histologic features of the murine estrous cycle and progesterone levels in female mice. (A) Cellular profile of eosin-stained vaginal smears obtained at various stages. Diestrus is characterized by abundant leukocytes with very few parabasal cells, whereas estrus is typified by the presence of irregularly shaped, highly cornified nucleated epithelial cells. Proestrus is characterized by small, irregularly shaped, noncornified epithelial cells, whereas the hallmark of metestrus is the abundance of both epithelial cells and leukocytes. Bar = 50 μ m. (B) Plasma progesterone levels during estrus and diestrus. Progesterone levels were significantly higher in diestrus compared with levels during estrus. Values represented as mean $\pm$ S.E.M. ($n = 6$ mice per group). * $P < 0.05$ versus estrus stage.

dissociated hippocampal CA1 pyramidal cells (CA1PCs) and dentate gyrus granule cells (DGGCs) from adult female mice in diestrus and estrus was plated onto thin glass-bottom dishes for 2 hours. The cells were fixed with 4% paraformaldehyde for 15 minutes followed by four washes in phosphate-buffered saline (PBS) containing 0.1 M glycine. Cells were permeabilized with ice-cold methanol at 4°C for 1 minute followed again by four rinses with PBS solution. Cells were then incubated with blocking solution containing 1% bovine serum albumin (Vector Laboratories, Burlingame, CA), 2.5% normal goat serum, 5% 20 $\times$ saline-sodium citrate (Sigma-Aldrich, St. Louis, MO), and 0.1% Triton X-100 for 1 hour. After the blocking phase, the cells were incubated with the primary rabbit GABA_AR δ -subunit antibody (N terminus, 1:100; PhosphoSolutions) or control rabbit IgG (1:100; Santa Cruz Biotechnologies) for 1 hour at room temperature. Cells were rinsed with PBS solution and then incubated with Alexa Fluor 555 labeled secondary antibody of goat-anti-rabbit IgG (1:200; Molecular Probes/Invitrogen) for 1 hour in the dark. Cells were washed with PBS solution four times, treated with one or two drops of ProLong AntiFade (Molecular Probes/Invitrogen), and covered with coverslip. Serial image sections through focus with a step size of 0.1–0.3 μ m thickness were collected and analyzed using Nikon confocal microscope with NIS-Elements software suite (Nikon Instruments, Inc., Tokyo, Japan). The normalized mean intensity was calculated from the ratio from the mean intensity of GABA_AR δ -subunits minus background to the mean density of the control antibody minus background. The normalized integrated mean intensity was used to compare density from different experimental groups.

Electrophysiologic Studies

Hippocampal Slices Preparation. Transverse slices (300–400 μ m thickness) of hippocampus were prepared using standard techniques from adult female mice in diestrus and estrus. Mice were anesthetized with isoflurane, and brains were excised rapidly. The brains were placed in cold (4°C) artificial cerebrospinal fluid (ACSF) buffer containing 0.3 mM kynurenic acid (Tocris Bioscience, Minneapolis, MN). ACSF buffer was composed of (in mM): 126 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 11 glucose (pH adjusted to 7.35–7.40 by gassed with 95% O₂–5% CO₂, osmolarity, 305–315 mOsm/kg). Several hippocampus slices were cut with a Vibratome (model 1500 with 900 Refrigeration System; Leica Microsystems, Inc.,

Bannockburn, IL). Hippocampus were dissociated from the microdissected subfield tissues CA1, CA3, and dentate gyrus (DG), respectively. The collected tissue samples were rapidly frozen for RNA and protein extractions. For electrophysiology and immunocytochemistry studies, the microdissected subfield hippocampal slices were equilibrated in ACSF at 24°C on a mesh surface in a small beaker in a water bath (ThermoFisher Scientific, Waltham, MA) continuously bubbled with oxygen (95% O₂ and 5% CO₂).

Dissociation of Neurons. The dissociation of hippocampal CA1PCs or DGGCs was prepared by the standard dissociation technique described previously (Kay and Wong, 1986; Reddy and Jian, 2010) from adult female mice in diestrus and estrus. The hippocampal pieces of the CA1 or DG region were microdissected carefully under the microscope (model SMZ 647; Nikon) and incubated in ACSF for 1 hour at 24°C. The isolated slices were transferred into an enzymatic solution consisting of ACSF with protease XXIII (3 mg/ml; Sigma-Aldrich). The slices were then incubated for precisely 23 to 25 minutes at 24°C. The remaining slices were rinsed twice with ACSF and gently triturated through three different sizes of fire-polished Pasteur pipettes to release single cells. For this step, three fire-polished Pasteur pipettes with increasingly smaller tips were used. For each batch, slices were triturated five or six times with each pipette with approximately 1 ml of ACSF in it. Then the solution was allowed 1 minute for the tissue to settle down, and the suspensions of freshly dispersed cells were carefully plated onto the recording chamber for electrophysiology and immunocytochemistry experiments (Warner Instruments, Hamden, CT).

Recording of GABA-Evoked Currents. Electrophysiologic recordings were performed in the whole-cell patch-clamp configuration (Reddy and Jian, 2010). All electrophysiologic experiments were performed at 22–24°C. The recording chamber was fixed into the stage of an inverted microscope with phase-contrast and differential interference contrast optics (model IX71; Olympus, Tokyo, Japan). The physiologic bath solution for isolated single-cell recording had the following composition (in mM): 140 NaCl, 3 KCl, 10 HEPES, 2 MgCl₂, 2 CaCl₂, and 16 glucose (pH adjusted to 7.4 with NaOH, osmolarity, 315–325 mOsm/kg). Cells were visualized and images were acquired through video camera CCD-100 (Dage-MTI, Michigan City, IN) with FlashBus Spectrum 1.2 software (Pelco, Clovis, CA). Recording pipettes were pulled from capillary glass tubes (King Precision Glass, Claremont, CA) using a P-97 Flaming-Brown horizontal puller (Sutter Instrument Company, Novato, CA). The pipette tip resistances were

2–4 M Ω for single-cell recording and 4–6 M Ω for slices recording. The pipettes were dipped for 2–3 seconds in a Cs⁺ pipette solution (high Cs⁺ for isolated single-cell and slices recording) containing (in mM) 124 CsCl, 20 tetraethylammonium, 2 MgCl₂, 10 EGTA, 10 HEPES, 0.1 GTP, 4 ATP (pH adjusted to 7.2 with CsOH, osmolarity, 295–305 mOsm/kg). Pipettes were back-filled with the same solution. Currents were recorded by using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The membrane capacitance, series resistance, and input resistance of the recordings were monitored by applying a 5-mV (100-millisecond) depolarizing voltage step from a holding potential of –70 mV. Signals were low-pass filtered at 1 kHz and digitized at 2 kHz with Digidata 1440A system (Molecular Devices). The current values were normalized to cell capacitance (an index of cell size) and expressed as current density (pA/pF). For whole-cell current from isolated single cells, fractional potentiation produced by test drugs was calculated as I_{NS}/I_{GABA} , where I_{GABA} was the response of peak amplitude at the application of GABA (3 μ M) and I_{NS} is the response of peak amplitude at the coapplication of GABA and the neurosteroid AP (10 nM to 1 μ M). For fast application of test drugs, the perfusion pipette was positioned <100 μ m away from the cell in the dish. GABA, allopregnanolone, and GABA_AR competitive antagonist bicuculline (BIC; 10 μ M) were applied using a multichannel perfusion system (Automate Scientific, Berkeley, CA).

Tonic Current Recording and Analysis. The GABA_AR-mediated tonic current recording and analysis were made as described previously (Mchedlishvili and Kapur, 2006). Hippocampal slices (300 μ m) were maintained in continuously oxygenated ACSF at 32°C in a holding chamber for 60 minutes and then back to room temperature in a recording chamber. Hippocampal CA1PCs and DGFCs were visually identified using an Olympus BX51 microscope equipped with a 40 $\times$ water-immersion objective, infrared-differential interference contrast optics, and video camera (Kay and Wong, 1986). Tonic currents of GABA_ARs were recorded in the presence of tetrodotoxin (0.5 μ M, Na⁺ channel blocker; Calbiochem/Millipore Corporation, Billerica, MA), *d*,-2-amino-5-phosphonopivalic acid (40 μ M, *N*-methyl-D-aspartate channel blocker; Sigma-Aldrich), and 6,7-dinitroquinoxaline-2,3-dione (10 μ M, non-*N*-methyl-D-aspartate glutamate receptor blocker) and confirmed by GABA_AR inhibitor BIC (10 μ M). Off-line analysis of tonic currents were analyzed with Mini-Analysis software (Synaptosoft, Leonia, NJ) (Mchedlishvili and Kapur, 2006). To study the tonic inhibition, transient events were manually removed from the current trace so that it consisted of only membrane current at the voltage-clamp mode. To account for cell-to-cell variability, the tonic current was expressed as a conductance [G (pS) = tonic current/membrane potential] normalized to membrane capacitance (pS/pF). Averaged amplitude of tonic current conductance (G_{tonic} , pS/pF) and root-mean-square noise amplitude (G_{rms} , pS/pF) were measured (Fig. 6). G_{tonic} is the current conductance from the difference in holding potential (HP = –60 mV) before and after application of BIC. G_{rms} is the noise conductance from chloride ions passing through the opened channels and in proportion to the chloride driving force. G_{tonic} was measured and averaged in 100 ms each epoch with 1-second interval between epochs for 30 epochs. The measurements were taken 30 seconds before and 3 minutes after application of a drug. G_{rms} was studied in 50 milliseconds each epoch with 500-millisecond interval between epochs for 30 epochs before and after drug application in each cell. To assess the effect of a drug on G_{rms} in an individual neuron, the distribution of G_{rms} in 30 epochs before the application of a drug (during the baseline period) was compared with that after drug application by means of Kolmogorov-Smirnov test. To compare data obtained from a group of neurons, G_{rms} values in individual epochs before and after drug application were averaged.

Behavioral Studies

Hippocampus Kindling Epileptogenesis. The rapid kindling model of epileptogenesis was used for assessment of seizure susceptibility over the estrous cycle. Rapid kindling allows accelerated evaluation of experimental manipulations during the progression of epilepsy

induction (Lothman and Williamson, 1993; Reddy and Mohan, 2011). The rapid kindling procedure was similar to the conventional kindling with daily stimulations except that stimulations were applied every 30 minutes until mice exhibited consistent stage 5 seizures. This procedure has been used extensively as a model of compressed epileptogenesis. A mild focal, nonconvulsant electrical stimulus to the hippocampus on a repeated basis leads to development of a kindled state exhibiting electrographic and behavioral seizures. In mouse kindling, the focal electroencephalographic afterdischarge models complex partial seizures, and the behavioral motor seizure stages 4/5 models generalized seizures.

Electrode implantation and stimulation procedures for mouse hippocampus kindling were performed as described previously (Reddy and Mohan, 2011). Mice were anesthetized by an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg i.p.). A stimulation-recording bipolar electrode (model MS303/1; Plastic One, Roanoke, VA) was stereotactically implanted in the right ventral hippocampus (2.9 mm posterior, 3.0 mm lateral, and 3.0 mm below dura) using the Franklin and Paxinos atlas (Franklin and Paxinos, 1997). The electrode was anchored with dental acrylic to three small screws placed in the skull. After a postoperative recovery period of at least 1 week, the electrographic afterdischarge (AD) threshold was determined by an application of 1-ms duration of biphasic rectangular pulses at 60 Hz for 1 second, beginning at 25 μ A by using an isolated pulse stimulator (A-M Systems, Sequim, WA). AD duration was the total duration of hippocampus electrographic spike activity (amplitude >2 $\times$ baseline) occurring in a rhythmic pattern at a frequency >1 Hz. Additional stimulations increasing in increments of 25 μ A were given at 5-minute intervals until an electrographic AD duration lasting at least 5 seconds was detected using the digital electroencephalography system (Astro-Med, West Warwick, RI). Mice were stimulated at 125% AD threshold (1-millisecond duration pulse, 60-Hz frequency for 1 second) at 30-minute intervals until they showed stage 5 seizure, which is considered the fully kindled state (Reddy and Mohan, 2011). Stimulations were delivered every 30 minutes until stage 5 seizures were elicited on three consecutive trials. The electrographic activity and AD duration were acquired from the hippocampal electrode using Axoscope 8.0 software with Digidata 1322A interface (Molecular Devices) through a Grass CP511 pre-amplifier (Astro-Med). Behavioral seizures were rated according to Racine's scale (Racine, 1972) as modified for the mouse: stage 0, no response or behavior arrest; stage 1, chewing or facial twitches; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, falling. Kindling is a permanent phenomenon, and an intense seizure can be elicited weeks or months after kindling development. Mice with electrode implanted but nonstimulated were used as sham controls. To determine the effect of AP on kindling progression, animals were injected with AP (3 mg/kg s.c.) 15 minutes before kindling sessions. Control animals were injected similarly with vehicle (15% cyclodextrin solution). During each stimulation session, the behavioral seizure score and the AD duration were noted. The rate of kindling development, that is, the number of stimulation required to induce stage 5 seizures, was determined in mice in estrus and diestrus, with or without AP treatment. Cumulative AD duration was calculated as an index of total seizure activity for reaching stage 5 seizure. At the end of the study, mice were anesthetized and perfused transcardially with paraformaldehyde for Nissl staining to verify the electrode placement.

Data Analysis. Group data were expressed as mean $\pm$ S.E.M. The GABA_AR subunit expression was analyzed based on the relative quantification approach as described previously (Gangisetty and Reddy, 2010). For electrophysiology, we used data only from cells in which stable gigaseals were maintained. In most analyses, the raw current value was normalized to cell capacitance (an index of cell size) and expressed as current density (pA/pF) or current conductance (pS/pF). Statistical comparisons were performed with repeated-measures analysis of variance (ANOVA) followed by post hoc tests or with an independent two-tail *t* test, as appropriate. Comparison of the mean

percentage inhibition of seizure stage in animals was made by Wilcoxon signed ranks test. Significant differences in the rate of kindling and AD durations between groups were assessed by repeated measures ANOVA or one-way ANOVA followed by Dunnett's *t* test. Differences were considered statistically significant at $P < 0.05$.

Drugs and Treatments. Stock solutions of finasteride and 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. Drug solutions were administered s.c. or i.p. in a volume equaling 1% of the animal's body weight. β -Cyclodextrin and other reagents were procured from Sigma-Aldrich unless otherwise noted.

Results

Determination of the Estrous Cycle and Progesterone Levels in Mice. To model the menstrual cycle-related changes in ovarian hormones, we investigated the mouse estrous cycle. Different stages of the cycle were determined by microscopic examination of vaginal smears (Fig. 1A) and by estimating plasma progesterone levels (Fig. 1B). The average length of the cycle was 6 days. Among the four different stages, diestrus 1 is considered similar to the luteal phase, and estrus is similar to the perimenstrual phase of the menstrual cycle (Fig. 1A). Plasma progesterone levels were significantly higher in diestrus 1 (10.3 ± 2.0 ng/ml) relative to estrus (2.5 ± 0.3 ng/ml) (Fig. 1B). Diestrus 2 is not associated with a significant increase in progesterone levels. For the purposes of this study, diestrus 1 is referred to simply as diestrus. Estrus and diestrus were selected for further characterization.

Cyclical Alterations in GABA_A Receptor Subunit Expression during the Estrous Cycle. To determine the cyclical changes in GABA_AR subunit plasticity in the hippocampus, we carried out TaqMan real-time PCR assay and Western blot analysis of receptor subunit expression in the hippocampus subfields of the CA1, CA3, and DG regions. The expressions of GABA_AR $\alpha 1$ -subunit mRNA in CA1 and DG, as well as GABA_AR $\alpha 2$ - and δ -subunits mRNA in CA3 and DG, showed significant increases in diestrus compared with estrus (Fig. 2). Quantification of δ -subunit mRNA copy number revealed a considerably greater expression within DG compared with the other hippocampus subfields (Fig. 2F). Expression of δ -subunit in CA1 pyramidal layer increased but did not reach statistical significance (Fig. 2A). In addition, no significant changes were seen in the expression levels of GABA_AR $\alpha 4$ - and $\gamma 2$ -subunits in any of the hippocampus subfields (Fig. 2). To determine whether changes in δ -subunit mRNA levels translate to increased neuronal δ -subunit protein, we determined the δ protein levels from DG during estrus and diestrus. Western blot analysis indicated a significant increase in total δ -subunit protein expression in diestrus compared with that during estrus (Fig. 2, D and E).

To confirm the presence of GABA_AR δ -subunits in neurons of hippocampus, the single-cell distribution of the δ -subunit was determined by fluorescent immunocytochemistry using a primary antibody specific for the δ -subunit. Staining with the δ -subunit antibody showed broad distribution on the soma, axon, and dendritic regions of freshly dissociated CA1PCs and DGGCs acquired from estrus or diestrus (Fig. 3A). The normalized δ -subunit staining intensity, expressed as percentage of change in fluorescence intensity relative to the value for control IgG, was 2.23-fold greater in DGGCs in

diestrus compared with that in estrus (Fig. 3B, $P < 0.05$). The δ -subunit protein was concentrated on cell membrane, as shown in images from X-Z and Y-Z axes obtained from confocal microscopy (Fig. 3C). Our data were consistent with previous study that AP-induced increase in δ -subunit expression was most likely via insertion of receptors in the cell membrane (Kuver et al., 2012). Taken together, these results demonstrate dynamic variations in GABA_AR subunits composition, especially the δ -subunit, over the estrous cycle, whereby elevated progesterone levels in diestrus correlated with increased GABA_AR δ -subunit expression.

Influence of Age and Ovariectomy on the GABA_A Receptor δ -Subunit Expression. We next investigated whether aging and ovarian hormones alter GABA_A receptor δ -subunit expression in the hippocampus. TaqMan reverse-transcription PCR analysis of δ -subunit expression was carried out in the hippocampus subfields CA1, CA3, and DG regions in ovariectomized and aged mice compared with young adult females. It has been reported that ovariectomized mice and aged, acyclic mice show compensative progesterone production from other places, such as the adrenal cortex (Lu et al., 1979; Steger and Peluso, 1982). In aged, acyclic female mice, GABA_AR δ -subunit mRNA expression was significantly increased in CA3 and DG regions compared with that in young adult mice in estrus. No significant difference was found in the expression of GABA_AR δ -subunit in CA1 regions between young and aged mice (Fig. 4A); a significant increase was seen in the expression of δ -subunit in DG from ovariectomized mice compared with mice in estrus; however, no significant difference was seen in the expression of the δ -subunit from ovariectomized mice compared with mice in diestrus in the CA1, CA3, and DG regions (Fig. 4A). These results suggest that ovarian cycling is essential and closely associated with the regulation of GABA_AR δ -subunit expression.

Role of Progesterone Receptors and Neurosteroids on the GABA_A Receptor δ -Subunit Expression during the Estrous Cycle. We next extended our study to determine a potential mechanistic pathway involving progesterone receptors (PR) and neurosteroids in δ -subunit expression. To determine whether the PR pathway is involved in regulating the δ -subunit expression during the estrous cycle, we used female PRKO mice, which lack both PR-A and PR-B receptor subtypes in the brain (Reddy et al., 2005), as a robust genetic model. We previously reported GABA_AR $\alpha 4$ -subunit expression in the hippocampus of WT and PRKO mice does not differ significantly (Gangisetty and Reddy, 2010). In PRKO mice, δ -subunit mRNA expression was significantly increased in CA3 and DG regions compared with WT estrous stage. No significant change occurred in δ -subunit mRNA expression in CA1 region (Fig. 4B), which suggests that PRs may not be necessary in the cycle regulation of GABA_AR δ -subunit plasticity.

To explore the relative role of the neurosteroid AP in GABA_AR δ -subunit expression, we used finasteride, a 5α -reductase inhibitor that blocks the synthesis of progesterone-derived AP (Fig. 4D). After treatment with finasteride (50 mg/kg i.p.) twice daily for 1 week, GABA_AR δ -subunit expression was decreased by 67 and 38% in control (WT-diestrus) and PRKO mice, respectively (Fig. 4C). These results demonstrate a role of progesterone-derived AP and related neurosteroids, independent of PRs, in regulating GABA_AR δ -subunit expression in the hippocampus.

Fig. 2. Changes in GABA_AR subunit expression in the hippocampus CA1, CA3, and DG subfields over the ovarian cycle. (A–C) TaqMan RT-PCR analysis of GABA_AR subunits mRNA expression in the hippocampus. (D and E) Western blot analysis of δ -subunit protein in estrus (E) and diestrus (DE). (E) Densitometric quantification of DG δ -subunit protein levels. (F) mRNA copy number quantification for δ -subunit according to subfield region. Data represent the mean $\pm$ S.E.M. ($n = 8$ animals per group). * $P < 0.05$ versus estrus group.

Cyclical Alterations in Neurosteroid-Sensitive GABA_A Receptor-Mediated Whole-Cell Cl[−] Currents during the Estrous Cycle. To study the effect of GABA_AR subunits, especially δ -subunit plasticity on receptor function, we used patch-clamp electrophysiology to assess inhibitory currents in neurons. The effect of allosteric modulators on GABA_AR function is markedly influenced by the presence of the δ -subunit (Maguire et al., 2005; Mtchedlishvili and Kapur, 2006; Rajasekaran et al., 2010). We used electrophysiology to confirm δ -subunit-specific pharmacological effects in the dissociated neurons during estrous cycle. In this study, GABA-gated, whole-cell Cl[−] currents were recorded in acutely dissociated CA1PCs and DGGCs (Fig. 5). Acutely dissociated DGGCs were identified from DG layer as small- and medium-sized neurons with typical oval-shaped soma and single process, and CA1PCs were identified from CA1 layer as typical pyramidal cell body and clear primary apical dendrite (Fig. 5A).

Application of GABA (0.1–10,000 μ M) for 5 seconds induced inward currents in a concentration-dependent manner and peaked at 1000 μ M in DGGCs from estrus and diestrus (Fig. 5, A and B). First, we determined the effect of the estrous cycle-related subunit plasticity of GABA_A receptors on GABA sensitivity. The full GABA concentration-response curves (0.1–10,000 μ M) in DGGCs from estrus and diestrus are illustrated in Fig. 5B. The GABA EC₅₀ values were not significantly different between DGGCs in animals at diestrus (18.4 ± 3.1 μ M, $n = 6$) and estrus (20.6 ± 2.9 μ M, $n = 6$). Although concentration responses from both stages appear similar, the DGGCs from diestrus were 12% more sensitive to GABA currents than those from estrus, suggesting the subtle alterations in GABA sensitivity across the estrous cycle. These results are consistent with our previous results of concentration response curves for GABA (0.1–1000 μ M)-evoked currents in CA1 neurons (EC₅₀ of 15 μ M) (Reddy and Jian, 2010).

It has been suggested that the extracellular concentration of GABA in the hippocampus ranges from around 2.1 to 3.8 μM (Shin et al., 2002). At 3 μM GABA ($\sim\text{EC}_{10}$), channel desensitization is minimal at 30-second intervals between applications (Reddy and Jian, 2010). In these experiments, we selected a 3- μM concentration of GABA to examine the characteristics of GABA currents that mediate mainly extrasynaptic (δ -subunit) but including synaptic (γ -subunit) GABA_ARs. Application of 3 μM GABA for 5 seconds evoked brief inward currents at a holding potential of -70 mV (Fig. 5A). The inward currents evoked by GABA were completely blocked by the GABA_AR antagonist BIC (10 μM). The average response to 3 μM GABA is summarized in Fig. 5A (lower panel), where the data for each cell have been normalized to cell capacitance (an index of cell size) and expressed as current density (I_{GABA} , pA/pF). Average I_{GABA} from DGGCs during estrus and diestrus differed significantly. Previous studies in human embryonic kidney 293T cells with specific recombinant GABA_ARs expressed have shown that the cells with expression of γ 2-subunit-containing receptors produce larger currents compared with cells with expression of δ -subunit containing receptors (Bianchi and Macdonald, 2003). Consistent with this finding, I_{GABA} in CA1PCs were 2-fold higher than in DGGCs (Fig. 5A; $n = 12$ –20).

We next assessed the ability of AP to potentiate currents on GABA_ARs gated by 3 μM GABA. In acutely dissociated CA1PCs and DGGCs from estrus or diestrus, neurons were preapplied with AP for 5 seconds, and then GABA was coapplied with AP for 5 seconds. AP is an allosteric modulator of GABA_ARs and produces little direct effect at nanomolar concentrations (Reddy, 2009). AP caused a concentration-dependent increase (I_{NS}) in peak-current responses evoked by GABA (Fig. 5C). At 10 nM AP, the mean fractional potentiation (ratio of AP + GABA to GABA alone; presented as $I_{\text{NS}}/I_{\text{GABA}}$) were 1.47- and 1.15-fold in DGGCs from diestrus and estrus stages, respectively ($n = 9$ to 10, $P > 0.05$). At 1 μM AP, the peak amplitudes obtained were 7.14- and 4.25-fold greater in DGGCs from diestrus and estrus stages and were 4.51- and 3.01-fold greater in CA1PCs from diestrus and estrus, respectively (Fig. 5D; $n = 5$ –8, $P < 0.05$). The data could not be Hill-curve fit because no response plateau was achieved even at high concentrations. However, it is highly unlikely that the AP responses illustrated in Fig. 5, C and D, are due to direct activation of GABA_ARs because direct activation occurs at concentrations higher than 1 μM used in this study. We directly tested this possibility with 1 μM AP in both DGGCs and CA1 cells without GABA (not shown). The baseline changes observed were less than 5% when compared with 1 μM AP + 3 μM GABA's response. Moreover, traces in Fig. 5C, at 1 μM AP perfused for 5 seconds before GABA, indicate lack of significant chloride current. In addition, the potentiating effects of AP in female mice were comparable with the testosterone-derived neurosteroid androstenediol in male mice (Reddy and Jian, 2010), with the exception of higher potentiation in DGGCs of diestrus. The latter might be related to higher δ -subunit in DGGCs of diestrus versus DGGCs of male mice. Thus, these results are consistent with greater sensitivity of δ -subunit containing GABA_A receptors to neurosteroids.

Cyclical Alterations in Neurosteroid-Sensitive Extrasynaptic GABA_A Receptor-Mediated Tonic Currents during the Estrous Cycle. To investigate further the effect

of elevated levels of δ -subunit expression on the functional changes in perisynaptic or extrasynaptic GABAergic inhibition, we recorded GABA-gated tonic currents in CA1PCs and DGGCs in hippocampal slice preparations, in which most synapses and dendrites remain intact. CA1PCs and DGGCs are distinct neuronal populations in which tonic inhibition is mediated by α 5-, γ -containing GABA_ARs in CA1PCs where δ -subunits are scarce, and by δ -containing receptors in DGGCs (Stell et al., 2003; Caraiscos et al., 2004; Maguire et al., 2005). For analysis of tonic currents, mean peak tonic conductance (G_{tonic}) and holding root-mean-square noise conductance (G_{rms}) were measured (Fig. 6). The control G_{tonic} was measured as the difference between initial current before GABA application and current in the presence of BIC. Control G_{tonic} in DGGCs in diestrus was 3-fold larger compared with estrus at a holding potential of -60 mV (Fig. 6, A and E; $n = 9$ –11 cells, $P < 0.05$). However, no significant difference was seen in the tonic conductance recorded in CA1PCs in diestrus compared with those in estrus. Perfusion of 3 μM GABA was used to model extracellular GABA concentration in vivo (Timmerman and Westerink, 1997; Shin et al., 2002; Rajasekaran et al., 2010), which is believed to activate selectively GABA_AR-mediated tonic inhibition without largely altering synaptic currents; 3 μM GABA significantly increased tonic current (Fig. 6A). The G_{tonic} in DGGCs of diestrus after the application of GABA was significantly greater compared with CA1PCs of diestrus, as well as CA1PCs and DGGCs in estrus (Fig. 6E; $n = 5$ –12). The lack of G_{tonic} changes in the CA1 region between estrus and diestrus, in which GABA_AR δ -subunit expression was low, is consistent with the hypothesis that selective regulation in tonic current is mediated by δ -subunits during the ovarian cycle (Pirker et al., 2000; Wei et al., 2003; Maguire et al., 2005). In addition, during the application of 3 μM GABA, the inward tonic current reached the peak (G_{tonic}) and then decayed to persistent, low desensitized current, which did not return to the baseline in the given time. The desensitization/decay of DGGCs of diestrus was 8% from the peak of G_{tonic} and was significantly smaller than the decrease of 17% in estrus DGGCs, 27% decrease in diestrus CA1PCs, and 45% decrease in estrus CA1PCs.

The G_{rms} , represented as noise conductance from chloride ions passing through the opened channels before and after the application of GABA with BIC, was examined (Fig. 6). In response to BIC, a slow outward current occurred, as mentioned previously herein, accompanied by reduction in baseline noise. The G_{rms} in DGGCs of diestrus was 9.22 ± 0.98 pS/pF during baseline period, 19.28 ± 1.16 pS/pF at 3 minutes after the application of 3 μM GABA, and 8.27 ± 0.93 pS/pF at 2 minutes after the application of GABA + 10 μM BIC (Fig. 6, B and F). The distribution of G_{rms} measurements after the application of GABA in DGGCs of diestrus was significantly increased as determined by the Kolmogorov-Smirnov test (Fig. 6C). To understand more fully the impact of 3 μM GABA on G_{rms} , a G_{rms} amplitude-events distribution histogram was created, which demonstrates that GABA increased the frequency of large G_{rms} (Fig. 6D). The summarized data of G_{rms} in DGGCs of diestrus after the application of GABA were significantly greater compared with DGGCs of estrus and CA1PCs of diestrus and estrus (Fig. 6F; $n = 5$ –12). These results further confirm that ovarian cycle-related GABA_AR δ -subunit regulation contributes to tonic inhibitory current.

Fig. 3. Immunocytochemical distribution of GABA_A δ -subunit in dissociated hippocampal CA1PCs and DGGCs over the ovarian cycle. (A) Immunofluorescence labeling using anti- δ -subunit primary antibody with Alexa Fluor 555-labeled IgG (blue). The δ -subunit staining intensity in DGGC was markedly greater than in cells from CA1. Bar = 10 μ m. (B) Normalized mean integrated densities to control IgG staining in each group before comparing between groups ($n = 6$). (C) Isolated DGGC body of diestrus is labeled blue for the GABA_A δ -subunit in X-Y, Y-Z, and X-Z axes. The δ -subunit staining was evident at cell membrane surface (arrows). Bar = 5 μ m. * $P < 0.05$ versus DGGC in estrus; # $P < 0.05$ versus CA1 in diestrus.

The neurosteroid AP significantly enhanced GABA_AR-mediated currents in DGGCs (Fig. 6, G and H). AP is likely to be found slightly higher in the hippocampus in vivo and δ -GABA_ARs are highly sensitive to AP (Maguire et al., 2005; Mchedlishvili and Kapur, 2006; Maguire and Mody, 2007). We investigated the modulation of extrasynaptic tonic current by 300 nM AP in the presence of 3 μ M GABA. The G_{tonic} and G_{rms} were enhanced by 300 nM AP in DGGCs of diestrus (Fig. 6G). Compared with GABA alone, AP enhanced G_{tonic} by 2.6-fold and G_{rms} by 1.2-fold in DGGCs of diestrus ($P < 0.05$). However, no significant differences in tonic inhibition were found between GABA alone and GABA + AP groups in CA1PCs (Fig. 6H). We employed 300 nM AP to uncover the δ -subunit-specific effects of neurosteroids on tonic conductance because δ -containing GABA_ARs display greater sensitivity to neurosteroids at physiological or supraphysiological levels. Although the exact physiological levels of AP in the extracellular space within the hippocampus are unknown, the levels may range from 10 to 120 nM, depending on the physiological or other conditions such as estrous cycle or pregnancy (Murri and Galli, 1997; Bernardi et al., 1998a,b; Concas et al., 1998; Genazzani et al., 1998; Tuveri et al., 2008;

Reddy, 2009). Even at low or physiological levels (30 nM), AP can elicit similar greater response in diestrus than in estrus per the standard linear concentration-response relationship (fractional response of maximal peak current). These results clearly suggest that tonic inhibition is modulated by GABA_A δ -subunit plasticity and can be significantly potentiated by the neurosteroid AP during the diestrus stage.

Alterations in Susceptibility to Limbic Epileptogenesis during the Estrous Cycle. To investigate whether the cycle-related plasticity of GABA_ARs mediating phasic and tonic inhibition affects neuronal network excitability in a seizure model, we studied the susceptibility of mice to epileptogenesis in rapid hippocampus kindling (Reddy and Mohan, 2011; Reddy et al., 2012). The progression of rate of kindling, electrographic mean AD threshold, and cumulative AD activity time for kindling criterion were recorded as main indices of epileptogenesis (Fig. 7). Mice in diestrus showed a significant resistance to the development of kindling epileptogenesis, as evident in the increased number of stimulations required to elicit behavioral seizures compared with estrous mice. The rate of kindling, expressed as the number of stimulations required to induce stage 4/5 seizures,

Fig. 4. Effect of aging, ovariectomy, and finasteride treatment on δ -subunit expression in the hippocampus subfields. (A) δ -Subunit mRNA expression in young (cycling), ovariectomized, and aged (acyclic) mice. (B) δ -Subunit mRNA expression in PRKO mice. (C) δ -Subunit mRNA in finasteride (50 mg/kg i.p.)-treated WT (diestrus) and PRKO mice. (D) Schematic illustration of finasteride blockade of 5α -reductase enzyme involved in the biosynthesis of AP and related neurosteroids. Values represent the mean $\pm$ S.E.M. ($n = 8$ animals per group). * $P < 0.05$ versus young (estrus) or WT (estrus) group; ** $P < 0.05$ versus vehicle control group (C).

was significantly slower in mice in diestrus compared with mice in estrus (Fig. 7A). No significant differences in the current required to trigger the initial AD were evident between mice in estrus and those in diestrus (Fig. 7B). In addition, no difference was seen in the total electrographic activity between the groups in estrus and those in diestrus (Fig. 7C). Representative AD traces are illustrated in Fig. 7D. We found that mice in estrus showed high-amplitude electrographic discharges during kindling development compared with those in diestrus (Fig. 7D). To ascertain the influence of drug-induced elevations in tonic currents on epileptogenesis, we examined the effect of AP on kindling epileptogenesis progression at estrus and diestrus. During kindling sessions, animals were injected with AP (3 mg/kg s.c.) 15 minutes before stimulations. The AP-treated mice in diestrus showed significant suppression of behavioral seizure activity compared with those in estrus (Fig. 7A). Similarly, AP-treated mice in diestrus required protracted electrographic seizure activity for reaching the kindling criterion compared with mice in estrus (Fig. 7C). The amplitude of AD spike activity was markedly reduced in AP-treated mice in diestrus (Fig. 7D). Thus, these results are consistent with the increased expression of δ -subunit during diestrus, which confers greater sensitivity to neurosteroid suppression of seizures and epileptogenesis.

Discussion

The ovarian cycle is generally recognized as a key regulator of GABA_ARs plasticity and function. The present study shows that cyclical elevations in progesterone levels in diestrus are accompanied by subfield-specific, increased extrasynaptic GABA_A δ -subunit expression in the hippocampus. Increase in tonic inhibition and decrease in seizure susceptibility are functional consequences of this plasticity. AP strongly

potentiated tonic inhibition and has powerful protective activity in the hippocampus kindling model of epileptogenesis during diestrus. Collectively, these novel findings provide molecular and cellular bases of neurosteroid dynamics in estrous cycle-regulated neuronal excitability and seizure susceptibility, strongly implicated to play an important role in menstrual cycle-related brain conditions such as catamenial epilepsy, premenstrual syndrome, and migraine.

The precise subunit plasticity in the hippocampus during the estrous cycle is not clearly understood. However, there is evidence that cyclical fluctuations in steroid hormones across the estrous cycle regulate several GABA_A subunits (Maguire et al., 2005; Gangisetty and Reddy, 2010; Reddy et al., 2012). Previous studies reported that compared with estrus, the diestrus δ -subunit is increased, the $\gamma 2$ -subunit is decreased, and the $\alpha 4$ -subunit exhibits no change (Maguire et al., 2005). Another study demonstrated δ -subunit response to progesterone treatment in an isolated culture system (Mostallino et al., 2006). The current study uses endogenous fluctuations in progesterone and neurosteroids across the estrous cycle to examine expressional and functional subunit plasticity in the hippocampal subfields. We used highly sensitive, optimized assays for subunit mRNA expression and subsequent Western blot analysis (Gangisetty and Reddy, 2009). Furthermore, we focused on individual hippocampus subfields, in which we report regulation of GABA_A expression to be subfield specific. We found greater $\alpha 1$ expression in CA1 and DG and higher $\alpha 2$ and δ expression in CA3 and DG during diestrus compared with estrus. Moreover, there was no change in $\alpha 4$ and $\gamma 2$ expression in diestrus (Fig. 2). Our data on δ and $\alpha 4$ expression are consistent with previous studies (Maguire et al., 2005; Maguire and Mody, 2007). In contrast to previous reports, we observed no significant change in subfield $\gamma 2$ expression. Differences could be due to previous studies' protein analysis in the whole hippocampus as measure of

Fig. 5. Whole-cell GABA-gated Cl^- currents (I_{GABA}) in acutely dissociated CA1PCs and DGGCs during diestrus and estrus. (A) Morphology of acutely dissociated hippocampal principal cells used for whole-cell recording of current, voltage-clamped at -70 mV. Bar = 10 μ m. Representative recordings (middle panel) from cells of CA1 and DG subfields. GABA (3 μ M) activated Cl^- currents (I_{GABA}) were larger in CA1 than in DG. Lower panel: I_{GABA} peak values. Current is normalized to cell capacitance (an index of cell size) and expressed as current density (pA/pF, $n = 12$ – 20 cells). * $P < 0.05$ versus estrus group. (B) Concentration response curves for GABA (0.1 – $10,000$ μ M) evoked currents in DGGCs from estrus and diestrus. Current amplitude (I_{GABA}) values were normalized to the maximum values ($I_{GABA-MAX}$) of GABA responses in the same cells. (C) Representative traces showing AP potentiation (I_{NS}) of I_{GABA} at varying concentrations (10 nM to 1 μ M) in DGGC. AP was preapplied for 5 seconds before the onset of the GABA coapplication. Gray thin bars indicate application of 3 μ M GABA. Solid bars indicate application of AP. (D) Concentration-response curves for AP derived from experiments similar to those shown in (C). Peak amplitude values during coapplication of GABA and neurosteroid were compared with the amplitude of GABA responses in the same cells (as fractional potentiation, I_{NS}/I_{GABA}). AP ($n = 5$ – 10) produced a concentration-dependent enhancement of GABA-activated currents with no response plateau. Value bars or points represent the mean $\pm$ S.E.M. * $P < 0.05$ versus CA1 cells from estrus; $\&P < 0.05$ versus DGGC from estrus; $\#P < 0.05$ versus CA1 cells from diestrus.

change, whereas we investigated mRNA in individual subfields, allowing greater signal sensitivity. The $\alpha 4$ - and δ -subunits preferentially coassemble in DGGC extrasynaptic membranes (Mostallino et al., 2006; Maguire and Mody, 2009). Since diestrus results in an increase of δ , but not $\alpha 4$, other α -subunits may increase to coassemble with the δ subunit.

In the present study, diestrus subunit increases of $\alpha 1$ and $\alpha 2$ in DG, $\alpha 1$ within CA1, and $\alpha 2$ within CA3 are novel observations of primarily synaptic α -subunit plasticity in the estrous cycle. $\alpha 1\beta\delta$ receptors possess low desensitization kinetics and neurosteroid sensitivity similar to $\alpha 4\beta\delta$ (Bianchi and Macdonald, 2003; Zhelezova et al., 2008). The $\alpha 1$ could plausibly assemble with δ extrasynaptically. Emerging evidence suggests that $\alpha 2$ is involved in synaptic targeting; moreover, $\alpha 2\beta 3\delta$ receptors have very low GABA potency (Wu et al., 2012). Therefore, it is unlikely that $\alpha 2$ -containing receptors assemble extrasynaptically with δ -subunit or respond to low micromolar extracellular GABA to gate tonic inhibition. Nevertheless, substantial increases in synaptic $\alpha 2$ could affect overall neuronal excitability. The $\alpha 2\beta\gamma 2$ receptor has faster activation kinetics, prolonged open duration and

bursting activity, and slower deactivation/decay time than $\alpha 1\beta\gamma 2$ (Lavoie et al., 1997). A large switch from $\alpha 1$ - to $\alpha 2$ -containing receptors in the hypothalamus has been previously shown to be coupled with slower postsynaptic current decay (Brussaard et al., 1997; Brussaard and Herbison, 2000). Therefore, an increased $\alpha 2$ -subunit proportion could result in greater net inhibitory postsynaptic current. More investigation is required to understand whether cycle-related α -subunit plasticity within the hippocampus modulates synaptic activity.

We observed that a diestrus δ -subunit increase within DG has positive modulatory effects on tonic inhibition. Furthermore, increases in AP potentiation of whole-cell and tonic currents during diestrus are mainly δ -directed. The δ -subunit has potent transduction properties to increase the opening of GABA $_A$ channels in the presence of AP (Brown et al., 2002). There is a subtle effect of the estrous cycle-related δ -subunit plasticity on GABA sensitivity in DGGCs with a marginal 12% greater sensitivity at diestrus (Fig. 5). Our observations of increased δ -subunit expression (Figs. 2 and 3) in diestrus DGGCs may contribute to increased GABAergic currents (Fig.

Fig. 6. Characterization of AP-potentiated GABA_AR tonic currents in CA1PCs and DGGCs from hippocampal slices of adult female mice in diestrus and estrus. (A) (upper panel) Morphology of CA1PC and DGGC recorded from hippocampal slice. Bar = 10 μ m. (Lower panel) Tonic current trace (I_{tonic}) recorded baseline, during 3 μ M GABA (gray bar), and with 10 μ M BIC (solid bar). Recordings in whole-cell mode, voltage-clamped at -60 mV. BIC decreased I_{tonic} and eliminated extrasynaptic currents. a: basal I_{tonic} (control); b: peak of GABA-enhanced I_{tonic} . (B) Current traces expanded from (A) display baseline root-mean-square noise (I_{rms}) before the application of GABA (control), in the presence of GABA alone (+GABA) or with BIC (+GABA + BIC). Note the larger I_{rms} in +GABA condition compared with the control and +BIC. (C) Kolmogorov-Smirnov test was used to compare I_{rms} before and after application of GABA in DGGCs. I_{rms} in the presence of GABA (dotted line) was more than during baseline (solid line). (D) Distribution of I_{rms} in the cell during the baseline period (open bars) and in the presence of GABA (black bars). The frequency distribution histogram demonstrates the rightward shift of I_{rms} in the presence of GABA in DGGC of diestrus. (E) The averaged tonic conductance (G_{tonic} , normalized I_{tonic} for cell capacitance, pS/pF, $n = 4-12$) and (F) the averaged baseline noise conductance (G_{rms} , pS/pF). In the absence of GABA, G_{tonic} and G_{rms} were significantly higher in DGGC of diestrus than in estrus, whereas no difference was detected in CA1PCs. The G_{tonic} and G_{rms} peaks were enhanced in the presence of GABA application in all groups. G_{rms} was decreased in all conditions during BIC application. (G) (left panel) Representative current traces of tonic current activated by GABA (gray bar), potentiation by AP (light gray bar), and blocked by BIC (solid bar). (Right panel) Expansions from traces in the left panel display I_{rms} before application of GABA (control), in the presence of GABA alone (+GABA), with AP (+GABA + AP), and with BIC (+GABA + AP + BIC) from DGGC of diestrus. (H) Summarized data of G_{tonic} and G_{rms} after application of AP in cells from DG and CA1 of diestrus ($n = 3-7$). * $P < 0.05$ versus control within group; & $P < 0.05$ versus control at DGGC of diestrus group; # $P < 0.05$ versus in the presence of GABA (+GABA).

Fig. 7. Ovarian cycle-related changes in susceptibility to hippocampus kindling epileptogenesis in mice. (A) Rate of kindling epileptogenesis (number of stimulations required to induce stage 4/5 seizures) in control and AP (3 mg/kg s.c.)-treated animals at diestrus and estrus stages. AP was administered 15 minutes before kindling session. (B) Mean afterdischarge (AD) threshold values in mice at diestrus and estrus stages. (C) Cumulative AD duration for the total time spent in electrographic seizure activity in control and AP-treated mice in diestrus and estrus. (D) Representative traces of the electrographic afterdischarge seizure activity in control and AP-treated mice at diestrus and estrus stages. Traces show depth recordings from a right hippocampus stimulating/recording electrode. Arrows indicate onset of the 1-second kindling stimulus, which is followed by the stimulus artifact. Values represent mean $\pm$ S.E.M. ($n = 6$ –9 mice per group.) * $P < 0.05$; versus estrus group; # $P < 0.05$ versus control diestrus group.

5), tonic inhibition, persistent opening of GABA_AR channels (Fig. 6), and reductions in seizure susceptibility (Fig. 7). There were little alterations of tonic inhibition or AP sensitivity in CA1PCs at diestrus and estrus. Therefore, the enhanced neurosteroid sensitivity and reduced seizure susceptibility at diestrus are ascribed to the relative increase in δ -subunit in the dentate gyrus. Further, these responses may be due to either greater tonic current or higher levels of endogenous AP at diestrus. However, it is unlikely that differences in ambient GABA level at diestrus are the main factor for such amplified tonic current responses. First, the increase in δ -subunit is associated with greater current to both GABA and AP. We found this response in DGFCs but not in CA1 cells (Fig. 6E). Second, application of BIC blocked the tonic currents, and the resulting fractional blockade was equivalent to that of control (ambient GABA) values, indicating subtle differences in endogenous GABA.

Aging is associated with low levels of progesterone/neurosteroid in the brain and may profoundly affect anxiety and seizure susceptibility (Reddy, 2003, 2009; Reddy and Jian, 2010). GABA_AR δ -subunit expression in ovariectomized and aged, acyclic mice showed no differences compared with that of mice in diestrus (Fig. 4A), indicating that the changes in subunit plasticity are due to the cyclical changes instead of to the relative levels of different hormones. The observation that δ -subunit levels are high in aged and ovariectomized animals despite lower progesterone levels provides strong credence to the premise that in intact cycling females, the

distinct hormonal milieu at estrus may be restraining δ -subunit expression followed by disinhibition at diestrus. Therefore, undiminished δ -subunit expression in ovariectomized and acyclic aged mice might be related to compensatory mechanism or local neurosteroid synthesis.

Our results suggest that PRs are not responsible for the estrous cycle regulation of δ -subunit expression. Previously we showed that progesterone's antiseizure effects are preserved (Reddy et al., 2004) and development of epileptogenesis is delayed in PRKO mice (Reddy and Mohan, 2011). Progesterone-related upregulation of δ from estrus to diestrus was retained in PRKO mice (Fig. 4B). Increase in δ -subunit in both WT and PRKO was inhibited by finasteride block of AP synthesis. These results are consistent with the previous report on the role of PR (Maguire and Mody, 2007). Similar subunit plasticity, observed in GABA_AR expression upon neurosteroid withdrawal, is independent of the PR pathway (Gangisetty and Reddy, 2010). We conclude that PRs do not play a role in the regulation of δ -subunit expression, and this plasticity is neurosteroid dependent.

A key observation of this study is that finasteride can prevent the increase in δ -subunits on diestrus, suggesting that endogenous neurosteroids, such as AP, on diestrus may be the trigger for δ -subunit expression. However, the molecular mechanism of neurosteroid upregulation of δ -subunit expression remains unclear. A variety of mechanisms could contribute to the neurosteroid-mediated estrous cycle regulation of GABA_ARs plasticity (Joshi and Kapur, 2009; Abramian et al.,

2010; Kuver et al., 2012). The neurosteroid AP is synthesized de novo within neurons (Reddy, 2009, 2013), and circulating AP levels parallel those of precursor progesterone (Tuveri et al., 2008). Although the dynamics of brain AP during the menstrual cycle are not well studied, it is likely that local synthesis of neurosteroids occurs in key regions of the limbic system, including the hippocampus and amygdala (Agis-Balboa et al., 2006; Ebner et al., 2006; Mukai et al., 2008). GABA_AR heterogeneity confers different neurosteroid binding affinities and gating; $\alpha 1\beta 2\gamma 2$ and $\alpha 2\beta 2\gamma 2$ GABA_ARs are abundant in the hippocampus and have relatively low neurosteroid sensitivity. Both $\alpha 1\beta \delta$ and $\alpha 4\beta \delta$ compositions are present in DG and have high neurosteroid sensitivity/affinity. AP potentiation of I_{GABA} from DGGCs from diestrus was significantly larger than from diestrus CA1PCs, and estrus CA1PCs and DGGCs (Figs. 5 and 6). These changes contribute to reduced seizure susceptibility such as that occurring during the luteal phase in women with catamenial epilepsy.

Figure 8 depicts the proposed effects of the estrous cycle on GABA_AR plasticity and function. During estrus, progesterone is low; transcriptional regulation is shifted away from δ -subunit expression. During diestrus, progesterone is substantially increased systemically and within the brain, resulting in an appreciable increase in neurosteroid synthesis. Abundance of neurosteroid couples an increase in δ -subunit expression within the neuron, conferring greater production of δ -containing GABA_ARs at extrasynaptic sites within the membrane. These receptors are distinctively more sensitive to neurosteroid binding, and tonic inhibition is enhanced. Allopregnanolone produces greater positive modulation of tonic inhibition and thereby reduces seizure susceptibility during the diestrus. These observations are clinically relevant in women with epilepsy and other hormone-sensitive brain conditions such as premenstrual syndrome and migraine.

In perimenstrual catamenial epilepsy, evidence suggests that progesterone level changes play a key role in seizure exacerbations (Reddy, 2009; Reddy et al., 2012). Greater seizure activity within estrus is demonstrated in the hippocampus kindling model of epileptogenesis (Fig. 7). Heightened seizure susceptibility is consistent with previous reports in related models of neurosteroid withdrawal, pseudopregnancy, and ovarian cycle (Smith et al., 1998a,b; Reddy et al., 2001; Gangisetty and Reddy, 2010). The basis for enhanced seizure susceptibility in perimenstrual catamenial epilepsy may be the withdrawal of neurosteroids around the time of menstruation (Reddy et al., 2001, 2012). Although some studies show that progesterone and its metabolites regulate GABA_ARs to affect seizure susceptibility, they are limited to exogenous application of progesterone or studying a few subunits (Smith et al., 1998a,b; Maguire et al., 2005). Our present study shows overt changes in seizure susceptibility over the estrous cycle and in response to AP. Seizure susceptibility was decreased during diestrus as a result of enhanced GABA_AR tonic inhibition in which progesterone/AP levels were elevated and δ -subunit expression was increased. Specific δ -subunit regulation within the ovarian cycle is important to define the molecular mechanisms for catamenial epilepsy. Thus, δ -containing GABA_ARs may be a potential therapeutic target in epilepsy treatment. Neurosteroid agents that augment tonic inhibition and lack benzodiazepine-like side effects have shown promise in the treatment of epilepsy in women (Reddy, 2010; Pack et al., 2011).

Fig. 8. Working model of ovarian cycle-related alterations of synaptic ($\alpha\beta\gamma$) and extrasynaptic ($\alpha\beta\delta$) GABA_AR plasticity and function in the hippocampus. The diestrus stage is coupled with elevated progesterone and, consequently, higher neurosteroid allopregnanolone levels in the brain. This promotes upregulation of neurosteroid-sensitive, extrasynaptic δ -subunit-containing GABA_A receptor expression in the hippocampus dentate gyrus, which is a gateway for controlling hippocampus excitability. This molecular change could lead to increased tonic inhibition and thereby promote reduced susceptibility to seizures and other neurologic conditions.

In conclusion, this study demonstrates estrous cycle-related, neurosteroid-dependent cyclical alterations in extrasynaptic δ -subunit-containing GABA_AR plasticity and function in the hippocampus. Neurosteroid regulation of δ -containing GABA_ARs may represent rational molecular mechanisms for menstrual cycle-induced alterations in neuronal excitability and seizure susceptibility, especially in catamenial epilepsy and related menstrual conditions linked to deficiencies in tonic inhibition. These mechanisms are also relevant to premenstrual syndrome, migraine, and other menstrual cycle-related brain conditions in women.

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Authorship Contributions

Participated in research design: Reddy.
Conducted experiments: Reddy, Gangisetty, Wu, Carver.
Contributed new reagents or analytic tools: Reddy.
Performed data analysis: Reddy, Gangisetty, Wu, Carver.
Wrote or contributed to the writing of the manuscript: Reddy, Wu, Carver.

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