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Ovarian cycle–linked changes in $GABA_A$ receptors mediating tonic inhibition alter seizure susceptibility and anxiety

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Disturbances of neuronal excitability changes during the ovarian cycle may elevate seizure frequency in women with catamenial epilepsy and enhance anxiety in premenstrual dysphoric disorder (PMDD). The mechanisms underlying these changes are unknown, but they could result from the effects of fluctuations in progesterone-derived neurosteroids on the brain. Neurosteroids and some anxiolytics share an important site of action: tonic inhibition mediated by δ subunit–containing $GABA_A$ receptors ($\delta GABA_A$Rs). Here we demonstrate periodic alterations in specific $GABA_A$R subunits during the estrous cycle in mice, causing cyclic changes of tonic inhibition in hippocampal neurons. In late diestrus (high-progesterone phase), enhanced expression of $\delta GABA_A$Rs increases tonic inhibition, and a reduced neuronal excitability is reflected by diminished seizure susceptibility and anxiety. Eliminating cycling of $\delta GABA_A$Rs by antisense RNA treatment or gene knockout prevents the lowering of excitability during diestrus. Our findings are consistent with possible deficiencies in regulatory mechanisms controlling normal cycling of $\delta GABA_A$Rs in individuals with catamenial epilepsy or PMDD.

Fluctuations in neuronal excitability and anxiety levels during the ovarian cycle[1,2] have been attributed to cyclic changes in endogenous neurosteroid levels[1–3]. Albeit indirectly, previous reports have suggested that changes occur in $GABA_A$Rs during the ovarian cycle[4–6], but these studies relied on agonist binding and benzodiazepine sensitivity, which do not distinguish between specific subunit combinations of these receptors. The brain contains dozens of $GABA_A$R subtypes assembled from different subunits that define their kinetics and pharmacology[7,8]. Of the many $GABA_A$R assemblies, only a few are sensitive to physiological concentrations of neurosteroids, limiting the number of subtypes that are ideally suited to mediate changes in neuronal excitability during the ovarian cycle. The $\delta GABA_A$Rs, when expressed in expression systems, have a high sensitivity to neurosteroids[9–11] and the tonic inhibition mediated by these receptors is a preferred target of neurosteroid action[12]. The same receptors may also be involved in anxiety, because they are highly ethanol sensitive[13,14] and anxiolytic concentrations of ethanol preferentially enhance tonic inhibition mediated by $\delta GABA_A$Rs[15].

The control of neuronal excitability by tonic inhibition involves the generation of a steady conductance that is pivotal in reducing the gain of neuronal input-output functions[16]. Tonic inhibition is 'on' most of the time, as it is activated by the ambient low levels of GABA present in the extracellular space[16]. Enhancing tonic inhibition by physiological concentrations of neurosteroids is an effective means of reducing neuronal excitability[12]. In contrast, phasic (synaptic) inhibition is intermittently activated at GABAergic synapses whenever presynaptic terminals release transmitter[16]. Because phasic inhibition is mediated by γ subunit–containing $GABA_A$Rs, it is not particularly sensitive to physiological levels of neurosteroids[12] or to anxiolytic ethanol concentrations[15].

The high sensitivity of $\delta GABA_A$Rs and the tonic inhibition mediated by these receptors in response to neurosteroids[12] and to anxiolytic concentrations of ethanol[15] suggests that this type of inhibition may be crucial in the alterations of neuronal excitability and anxiety associated with changes in neurosteroid levels over the ovarian cycle. Little is known about possible alterations in specific neurosteroid-sensitive $GABA_A$R subunits during the ovarian cycle, but changes in seizure threshold consistent with altered neuronal excitability have been associated with various stages of the cycle[17,18]. This may be relevant to the human condition of catamenial epilepsy, in which 75% of epileptic women report seizure exacerbation related to specific stages of their menstrual cycle[2]. The three different forms of catamenial epilepsy—seizures arising at the time of ovulation, seizures arising immediately before and during menstruation and seizures arising in patients with inadequate luteal-phase cycles—all occur when progesterone levels are low[2]. The anxiety and dysphoric disorder PMDD follows a similar pattern of symptom manifestation, occurring before and/or during menstruation[1].

Here we examined changes in tonic inhibition, seizure susceptibility and anxiety along with the expression pattern of the highly neurosteroid-sensitive $\delta GABA_A$Rs over the estrous cycle in mice. We show that specific $GABA_A$R subunits are periodically reorganized during the

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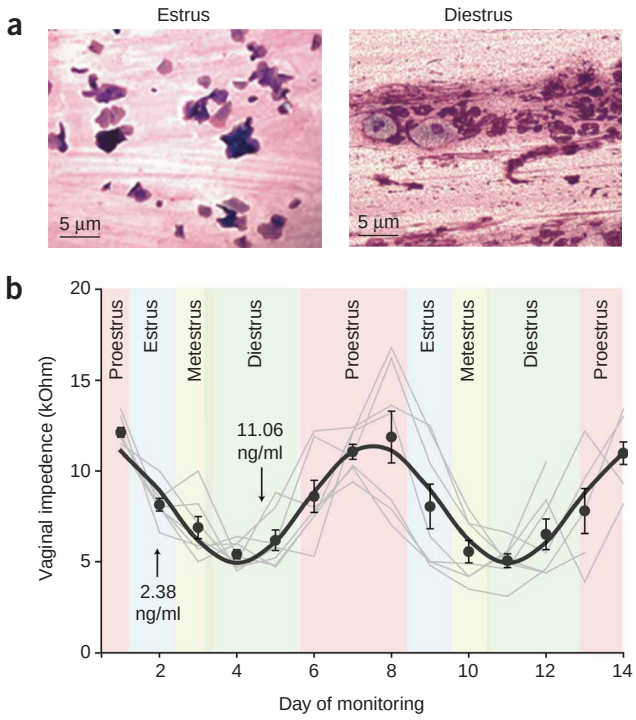

Figure 1 Stages of the estrous cycle in C57/Bl6 mice. (**a**) In Giemsa stains, the cellular profile of smears obtained during estrus is characterized by the presence of irregularly shaped cornified cells, whereas the hallmark of diestrus is the presence of leukocytes and a sparse number of parabasal cells. (**b**) The graph shows the average (± s.e.m.) values of the vaginal mucosa electrical resistance in eight mice (gray lines) over two complete cycles. The fit is a sinusoid (thick black line) with the period 7.0 ± 0.35 d. The phases of the cycle are indicated in relationship to the sinusoid, and arrows show the measured levels of progesterone at the two stages relevant to our experiments.

cycle, resulting in changes in tonic inhibition and neuronal excitability. These changes may be responsible for the observed cyclic alterations in seizure susceptibility and anxiety during the ovarian cycle.

RESULTS

Determination of the estrous cycle in mice

Different stages of the estrous cycle were accurately determined by analyzing cellular profiles of vaginal smears and by measuring vaginal impedance (**Fig. 1**). The average cycle length in C57/Bl6 mice was 7.0 ± 0.35 d (**Fig. 1**). Plasma progesterone concentrations determined by immunoassay were correlated to the cycle stage. Circulating progesterone levels were significantly higher at late diestrus (11.06 ± 0.76 ng ml^{-1}) than in estrus (2.38 ± 0.29 ng ml^{-1}; **Fig. 1**; $n = 10$; $P < 0.05$). All estrous cycle–related experiments described were carried out in mice with well-established ovarian cycles. Two stages of the estrous cycle were chosen for comparison: the estrus phase, when estrogen levels are high and progesterone levels are low during the day, and the late diestrus phase, when progesterone levels peak and estrogen levels are low[19].

Altered $GABA_AR$ subunit expression during the ovarian cycle

To determine the effects of the ovarian cycle on $GABA_AR$ subunit composition, we carried out western blot analysis on membrane fractions isolated from the hippocampi of mice in estrus and late diestrus. We measured the abundance of three $GABA_AR$ subunits, α4, γ2 and δ, directly in samples containing the same amount of total protein to ensure consistent comparisons across the ovarian cycle. Hippocampal membrane fractions expressed significantly different levels of $GABA_AR$ subunits in the estrus as compared to the late diestrus phase. The optical density of δ$GABA_AR$ blots decreased from 0.53 ± 0.01 OD per 100 µg of total protein during late diestrus to 0.37 ± 0.04 OD per 100 µg at estrus (**Fig. 2**; $n = 6$; $P < 0.05$). $GABA_AR$ γ2 subunit expression showed a pattern complementary to that for δ subunits. The optical density of $GABA_AR$ γ2 subunits was 0.34 ± 0.01 OD per 100 µg of total protein during late diestrus and increased to 0.51 ± 0.02 OD per 100 µg of total protein during estrus (**Fig. 2**; $n = 6$; $P < 0.05$). These results demonstrate dynamic alterations in $GABA_AR$ subunit composition over the estrous cycle, whereby elevated progesterone levels correlated with increased δ$GABA_AR$ expression and decreased γ2$GABA_AR$ expression. In accordance with suggested rules of $GABA_AR$ partnership[20,21], α4 subunits coassemble with δ subunits[21,22] or potentially with γ2 subunits[21,23]. Moreover, loss of one subunit may result in an increased assembly of the α4 subunits with another partner[23]. Therefore, it is not surprising that alterations in $GABA_AR$ δ and γ2 subunit expression over the estrous cycle were not paralleled by changes in the expression of α4 subunits. Expression of $GABA_AR$ α4 subunit was 0.54 ± 0.01 OD per 100 µg for mice in estrus and 0.55 ± 0.01 OD per 100 µg for those in late diestrus (**Fig. 2**; $n = 6$; $P > 0.05$).

Altered $GABA_AR$-mediated tonic inhibition during the cycle

Functional changes in GABAergic inhibition over the cycle were assessed by recording tonic and phasic inhibitory currents in dentate gyrus granule cells (DGGCs) and CA1 pyramidal cells, two cell types in which tonic inhibition is mediated by distinct $GABA_ARs$[12,24]. We measured a twofold higher tonic conductance in DGGCs from mice in late diestrus as compared to estrus (**Fig. 3**). The average tonic conductance normalized to whole-cell capacitance was 29.8 ± 5.5 pS pF^{-1} in mice in estrus and 57.6 ± 10.2 pS pF^{-1} during late diestrus (**Fig. 3**; $n = 18$ cells; $n = 6$ mice; $P < 0.05$). However, there was no significant difference in the tonic conductance recorded in CA1 pyramidal cells (estrus: 38.1 ± 8.5 pS pF^{-1}; diestrus: 29.9 ± 2.7 pS pF^{-1}; **Fig. 3**; $n = 10$ cells; $n = 5$ mice; $P = 0.38$). The absence of changes in the CA1 region, where δ$GABA_AR$ expression is low[22,25] and these subunits do not contribute to the tonic current in this region[12,24], is consistent with a selective regulation of δ subunits during the ovarian cycle. The idea that other $GABA_AR$ subunits are not substantially altered during the cycle is also supported by the lack of significant

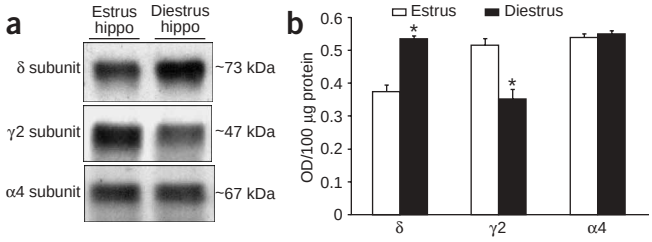

Figure 2 Alterations in abundance of $GABA_AR$ subunits over the estrous cycle. (**a**) Representative immunoblots of total membrane protein isolated from the hippocampi of mice at estrus or late diestrus. (**b**) δ$GABA_AR$ levels in membrane fractions are expressed as OD per 100 µg of total protein. Membrane δ$GABA_ARs$ were more abundant, but γ2 subunit expression was lower, during late diestrus than during estrus. There were no differences in α4 subunit levels between mice in estrus and in late diestrus. *$P < 0.05$, unpaired *t*-test, compared to estrus ($n = 5$–7 for each experimental group).

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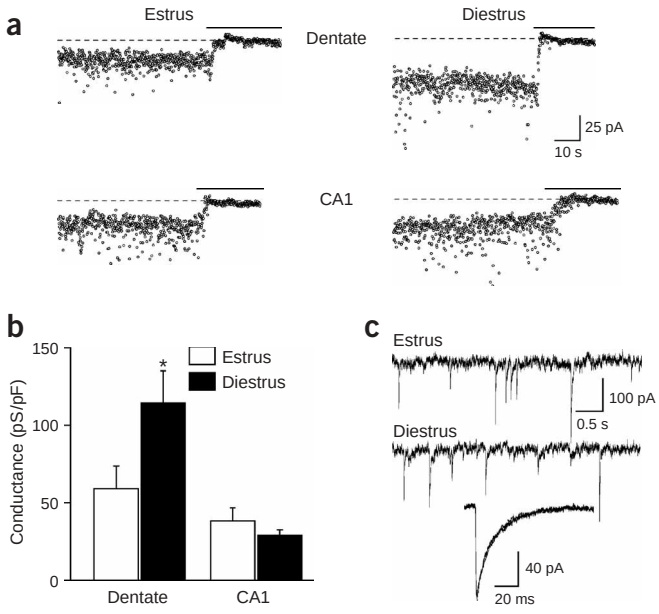

Figure 3 Tonic conductance is elevated during late diestrus in dentate gyrus granule cells. Whole-cell patch clamp recordings were performed on dentate gyrus granule cells (Dentate) and CA1 pyramidal cells (CA1) with the investigator blinded as to the origin of the slices. (**a**) Representative recordings from mice in estrus and in late diestrus indicate that tonic conductance in DGGC cells is higher in late diestrus, but show no estrous cycle–dependent changes in tonic currents in CA1 pyramidal cells. Dashed lines indicate the basal current in the presence of saturating concentrations of the $GABA_AR$ antagonist SR-95531 perfused during the time indicated by the horizontal black bars. (**b**) Bar graphs of the average tonic conductance (normalized for cell capacitance) values in DGGCs show that this is significant higher in late diestrus than in estrus, whereas no difference was detected in CA1 pyramidal cells ($^*P < 0.05$, *t*-test, compared to estrus; $n = 18$ cells, 6 mice). (**c**) Recordings of sIPSCs (phasic currents) show no changes in frequency, peak amplitude or τ_w over the estrous cycle. Two superimposed averaged sIPSCs, each recorded in a DGGC at a different stage of the estrous cycle, show no changes in the kinetics of the spontaneous events.

changes in the kinetics or frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) observed in either DGGCs or CA1 pyramidal cells over the cycle (**Fig. 3**). In DGGCs sIPSCs, frequency was 2.48 $\pm$ 0.69 Hz in estrus and 1.69 $\pm$ 0.48 Hz in late diestrus (**Fig. 3**; $n = 18$ cells; $n = 6$ mice; $P = 0.93$). The peak amplitude of sIPSCs recorded in diestrus was 114.5 $\pm$ 20.5 pA as compared to 90.6 $\pm$ 13.4 pA in estrus. The weighted decay time constant (τ_w) in diestrus was 15.1 $\pm$ 0.91 ms as compared to 16.0 $\pm$ 0.82 ms in estrus (**Fig. 3**). Neither of these differences was statistically significant ($P = 0.33$ and $P = 0.44$, respectively, two-sample paired *t*-test).

Altered seizure susceptibility during the ovarian cycle

To investigate the effects of $GABA_AR$ alterations and the resulting changes in tonic GABAergic inhibition on neuronal excitability, we measured seizure susceptibility in response to an intraperitoneal (i.p.) injection of 15 mg kg^{-1} kainic acid in mice in estrus and diestrus. Consistent with the upregulation $\delta GABA_ARs$ during late diestrus and the consequent increase in tonic inhibition, we observed a decrease in seizure susceptibility at late diestrus. The latency to seizure onset, measured as the elapsed time to the first seizure after the mice were injected with kainic acid, was nearly twofold longer in diestrus than in estrus (**Fig. 4**, **Table 1**; $n = 9$; $P < 0.05$; see also next section). Mice in estrus spent significantly more cumulative time seizing during 120-min recording sessions than did mice in diestrus. Mice in estrus had cumulative seizure times of 79 $\pm$ 1.5% of 120 min (**Fig. 4**, **Table 1**; see also next section), whereas those in late diestrus underwent seizures during only 45.2 $\pm$ 6.8% of the total observation time (**Fig. 4**, **Table 1**; $n = 9$; $P < 0.05$; see also next section). The average duration of the individual electrographic events was longer in estrus (9.4 $\pm$ 1.7 min) than in late diestrus (1.7 $\pm$ 0.38 min; also see **Fig. 6**). These results are consistent with the idea that alterations in tonic GABAergic inhibition over the estrous cycle result in alternating periods of increased seizure susceptibility (during estrus) and periods of seizure resistance (during diestrus).

To confirm the greater $\delta GABA_AR$ expression during diestrus as compared to estrus by pharmacologic means, we examined the effects of a non-sedative dose[26,27] of 4,5,6,7-tetrahydroisothiazolo-[5,4-*c*]pyridin-3-ol (THIP or gaboxadol) on seizure susceptibility at the two stages of the estrous cycle. In a separate set of experiments (**Supplementary Fig. 1**) using wild-type and $\delta GABA_AR$-null mutant (*Gabrd*$^{-/-}$) mice, 5 μM THIP produced a much larger tonic current in wild-type DGGCs than did 5 μM GABA, consistent with its higher efficacy at

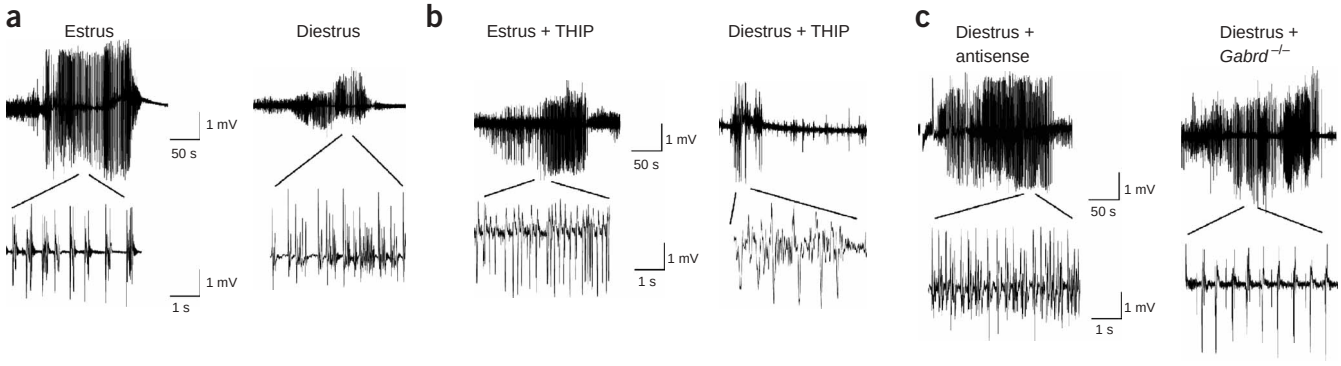

Figure 4 Electrographic seizures vary with stages of the estrous cycle and are affected by alterations in $\delta GABA_ARs$. Seizures in response to a single i.p. injection of 15 mg kg^{-1} kainic acid were recorded in female mice by hippocampal EEG (shown here band-pass filtered at 0.1–250 Hz). (**a**) More severe seizure episodes are seen in the representative traces from mice in estrus as compared to mice in diestrus. (**b**) The $\delta GABA_AR$-specific agonist THIP, injected i.p. at a dose of 10 mg kg^{-1} 30 min before kainic acid injection, attenuates seizures more effectively during late diestrus than during estrus. (**c**) The involvement of $\delta GABA_AR$ is shown by the exacerbation of the kainic acid–induced seizures during diestrus in $\delta GABA_AR$ antisense mRNA–treated mice (left) and in *Gabrd*$^{-/-}$ mice (right), as evidenced by comparison of these EEG traces to that from a control mouse during diestrus (**a**).

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Table 1 Efficacy of THIP in attenuating seizure susceptibility is altered over the estrous cycle

	Seizure duration (min)	% time seizing	Latency to seizure onset (min)	*n*
Diestrus	1.7 ± 0.4	45.2 ± 6.8	3.2 ± 0.7	4
Estrus	9.4 ± 1.7*	81.7 ± 2.7*	1.3 ± 0.1*	5
Diestrus + THIP	3.1 ± 0.7	22.6 ± 1.1	27.4 ± 4.6	5
Estrus + THIP	6.9 ± 0.3*	73.5 ± 1.5*	2.0 ± 0.6*	5

Seizure susceptibility in response to 15 mg kg^{-1} i.p. kainic acid was analyzed in wild-type mice in estrus and diestrus. The three seizure parameters were measured in mice in estrus and diestrus with or without administration of 10 mg kg^{-1} THIP 30 min before kainic acid. * denotes statistically significant difference ($P < 0.05$) compared to diestrus.

$\delta GABA_ARs$[10]. In contrast, there were no differences between THIP and GABA in activating a residual tonic current in *Gabrd*$^{-/-}$ mice, and this low concentration of THIP had no effect on the properties of sIPSCs (**Supplementary Fig. 1**). Our findings on seizure susceptibility demonstrate that, as compared to estrus, THIP was more effective in attenuating seizure activity during late diestrus (**Table 1**) when there are more $\delta GABA_AR$s. THIP increased the latency to seizure onset by only 1.5-fold (1.3 ± 0.1 to 2.0 ± 0.6 min) in estrus but by more than 6-fold (3.2 ± 0.7 to 21.6 ± 6.3 min) in late diestrus (**Fig. 4**, **Table 1**; $n = 9$; $P < 0.05$). THIP was also less effective during estrus in decreasing the fraction of time seizing, causing a decrease from 79 ± 1.5% to 73.5 ± 1.5% in estrus as compared to a reduction from 45.2 ± 6.8% to 22.6 ± 1.1% in late diestrus (**Fig. 4**, **Table 1**; $n = 10$; $P < 0.05$). These results demonstrate that the $\delta GABA_AR$ agonist THIP[10] is more effective during late diestrus, when $\delta GABA_AR$ expression is high. Apparently, doses of THIP that do not cause sedation are most effective as anticonvulsants when there is an increased $\delta GABA_AR$ expression during late diestrus, presumably because the drug augments the tonic conductance mediated by these receptors and thus further decreases seizure susceptibility.

Reduction of $\delta GABA_AR$s prevents ovarian cycle–related changes

To ascertain whether the changes seen in tonic inhibition and excitability over the estrous cycle result from specific alterations in $\delta GABA_AR$s, we investigated cycle-linked changes in seizure susceptibility and tonic inhibition in *Gabrd*$^{-/-}$ mice[28] and in wild-type mice treated with $\delta GABA_AR$ antisense oligonucleotides. To demonstrate knockdown of $\delta GABA_AR$s at diestrus after intracerebroventricular administration of $\delta GABA_AR$ antisense mRNA, we compared $\delta GABA_AR$ expression in hippocampal membranes of mice treated with $\delta GABA_AR$ missense mRNA or with $\delta GABA_AR$ antisense mRNA. Mice were treated with either 5 nmol $\delta GABA_AR$ missense mRNA or 5 nmol $\delta GABA_AR$ antisense mRNA at estrus, and analysis was performed approximately 2–3 d later, when the mice reached the late diestrus phase. Antisense treatment significantly decreased $\delta GABA_AR$ expression at this phase, from 0.53 ± 0.01 OD per 100 μg to 0.34 ± 0.01 OD per 100 μg, a level comparable to the expression during estrus (**Fig. 5**; $n = 7$; $P < 0.05$). However, for mice in late diestrus, there was no significant difference in $\delta GABA_AR$ expression between negative control RNA– (0.53 ± 0.01 OD per 100 μg) and $\delta GABA_AR$ missense mRNA–treated mice (0.54 ± 0.01 OD per 100 μg) (**Fig. 5**; $n = 4$). In $\delta GABA_AR$ antisense mRNA–treated mice at late diestrus, the optical density of γ2 subunits was 0.37 ± 0.02 OD per 100 μg, which is not significantly different from the 0.39 ± 0.01 OD per 100 μg in $\delta GABA_AR$ missense mRNA–treated mice ($P = 0.13$) or the 0.34 ± 0.01 OD per 100 μg in control mice (**Fig. 5**; $n = 7$; $P = 0.23$).

We carried out whole-cell patch-clamp recordings performed on DGGCs in hippocampal slices prepared from mice in late diestrus that had been treated at estrus with 5 nmol negative control RNA or 5 nmol $\delta GABA_AR$ antisense mRNA, and thereby confirmed that tonic GABAergic inhibition was attenuated after $\delta GABA_AR$ antisense mRNA administration. Antisense treatment decreased the tonic conductance in DGGCs: in mice in late diestrus, the conductance was 89.9 ± 18.4 pS pF^{-1} in those treated with negative control RNA and 22.0 ± 6.4 pS pF^{-1} in those treated with antisense mRNA (**Fig. 5**; $n = 7$; $P < 0.05$). Thus, preventing upregulation of $\delta GABA_AR$s that normally occurs at late diestrus blocked the enhancement of tonic GABAergic inhibition. We did not observe significant changes in the kinetics or frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) of DGGCs in mice treated with negative control or $\delta GABA_AR$ antisense mRNA. In DGGCs, the frequency of sIPSCs was 0.78 ± 0.35 Hz for negative control mRNA–treated mice and 0.63 ± 0.11 Hz for antisense mRNA–treated mice ($P = 0.37$). The peak amplitude of sIPSCs recorded from mice treated with $\delta GABA_AR$ antisense mRNA was 102.9 ± 14.1 pA, as compared to 93.1 ± 15.4 pA in mice given negative control mRNA. The τ_w was 10.0 ± 0.61 ms, as compared to 11.1 ± 0.67 ms in mice given negative control mRNA. Neither of these differences was statistically significant ($P = 0.64$ and $P = 0.25$, respectively, two-sample paired *t*-test).

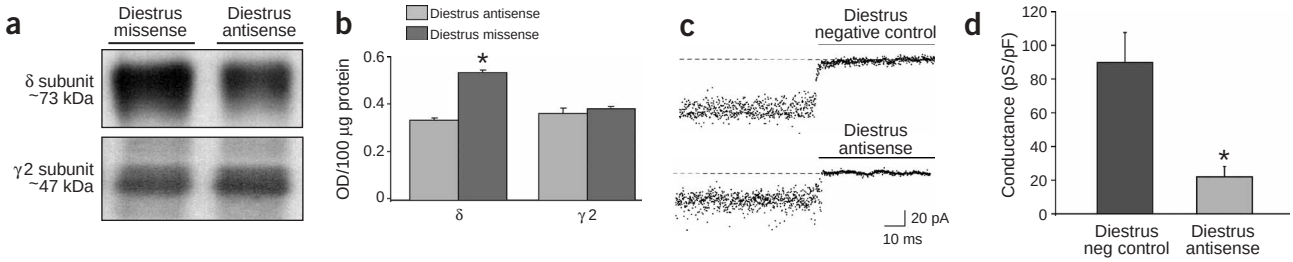

Figure 5 Decreased $\delta GABA_AR$ expression and tonic GABAergic inhibition after $\delta GABA_AR$ antisense mRNA treatment. (**a**) Treatment with $\delta GABA_AR$ antisense mRNA results in lower membrane $\delta GABA_AR$ expression, measured by western blotting, as compared to that in mice treated with $\delta GABA_AR$ missense mRNA. Representative immunoblots from $\delta GABA_AR$ missense– or antisense mRNA–treated diestrus-phase mice demonstrate decreased $\delta GABA_AR$ expression after antisense treatment. (**b**) Densitometric quantification of changes in $\delta GABA_AR$ expression in mice in diestrus after $\delta GABA_AR$ antisense or missense treatment, as compared to control mice in estrus and diestrus (*$P < 0.05$, ANOVA; $n = 4$–6 for each experimental group). (**c**) Representative whole-cell recordings of tonic currents from DGGCs of mice treated with negative control mRNA and $\delta GABA_AR$ antisense mRNA. Dashed lines indicate basal current (as in **Fig. 3**). (**d**) Tonic GABAergic inhibition is significantly lower in DGGCs of mice in late diestrus 2 d after intraventricular administration of $\delta GABA_AR$ antisense mRNA as compared to those treated with negative control RNA (*$P < 0.05$, *t*-test, $n = 17$ cells from seven mice for each experimental group).

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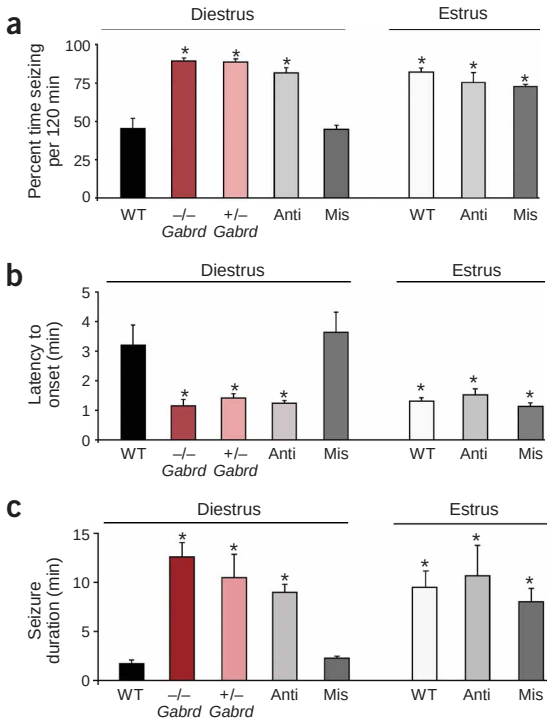

Figure 6 Characteristics of kainic acid–induced seizures as a function of δGABA$_A$R expression during the estrous cycle and after experimental manipulation of these subunits. (**a**) The cumulative percent seizure time per 120 min after kainic acid administration was lower in diestrus than in estrus for wild-type mice, but not *Gabrd*$^{-/-}$ or heterozygous *Gabrd*$^{+/-}$ mice or mice treated with δGABA$_A$R antisense mRNA. Mice treated with missense mRNA showed characteristics similar to those of late diestrus controls. Estrus wild-type mice were similar to estrus missense mRNA– or antisense mRNA–treated mice ($n = 3$–6 mice; $^*P < 0.05$ from unlabeled groups; post-ANOVA multiple comparisons). (**b**) The latency to seizure onset was longer in diestrus than estrus. However, in late diestrus *Gabrd*$^{-/-}$ or *Gabrd*$^{+/-}$ mice, or mice treated with δGABA$_A$R antisense mRNA, had shorter latencies than controls. During late diestrus, missense mRNA–treated mice were similar to late diestrus wild-type controls. In the estrus phase, wild-type, missense mRNA–treated and antisense mRNA–treated mice showed similar latencies to onset. ($n = 3$–6 mice; $^*P < 0.05$ as in **a**). (**c**) The average duration of single electrographic seizures was shorter in diestrus than in estrus. *Gabrd*$^{-/-}$, *Gabrd*$^{+/-}$ and δGABA$_A$R antisense mRNA–treated mice had longer seizure durations than diestrus controls. In diestrus, missense mRNA–treated mice were similar wild-type controls. In estrus, wild-type control, missense mRNA– and antisense mRNA–treated mice had similar seizure durations ($n = 3$–6 mice; $^*P < 0.05$ as in **a**).

To determine whether the decreased seizure susceptibility observed during diestrus was a direct result of δGABA$_A$R upregulation, we investigated seizure susceptibility during diestrus in *Gabrd*$^{-/-}$ mice and in mice treated with δGABA$_A$R antisense mRNA. Knockdown of δGABA$_A$R expression in mice in diestrus after antisense treatment resulted in an increase in seizure susceptibility to a level comparable to that observed during estrus (**Fig. 6**). In response to i.p. administration of 15 mg kg^{-1} kainic acid, mice in late diestrus pretreated with δGABA$_A$R antisense mRNA spent a significantly larger fraction of time seizing (81.4 ± 3.5%) than those pretreated with δGABA$_A$R missense mRNA (44.7 ± 2.7%) (**Fig. 6**, $n = 4$; $P < 0.05$). Antisense treatment also decreased the latency to seizure onset in late diestrus, from 3.6 ± 0.7 min in missense mRNA–treated mice to 1.2 ± 0.1 min in δGABA$_A$R antisense mRNA–treated mice (**Fig. 6**, $n = 4$; $P < 0.05$). We measured seizure susceptibility in mice in diestrus only in the total absence of δGABA$_A$Rs. Estrus-phase mice of this seizure-prone phenotype were not analyzed owing to their already elevated seizure susceptibility. In the diestrus phase, homozygous *Gabrd*$^{-/-}$ and heterozygous *Gabrd*$^{+/-}$ mice spent 89.2 ± 2.2% and 88.6 ± 2.1% of the time undergoing seizures, respectively, as compared to 45.2 ± 6.8% for wild-type mice (**Fig. 6**; $n = 3$–5; $P < 0.05$). In diestrus, the latency to seizure onset was 3.2 ± 0.7 min in wild-type mice but only 1.2 ± 0.2 min in *Gabrd*$^{-/-}$ mice and to 1.4 ± 0.2 min in *Gabrd*$^{+/-}$ mice (**Fig. 6**; $n = 3$–5; $P < 0.05$). Our findings seem to indicate that the lower δGABA$_A$R expression during estrus underlies the increased seizure susceptibility in wild-type mice during estrus, as it does the susceptibility in *Gabrd*$^{-/-}$ mice and in δGABA$_A$R antisense mRNA–treated mice during diestrus. A similar antisense or missense mRNA treatment did not affect seizure susceptibility in mice at the estrus stage of the cycle (**Fig. 6**). For mice in estrus, the fraction of time spent seizing per recording session did not differ between those treated with antisense mRNA (76.5 ± 6.4%) and those treated with missense mRNA (73.8 ± 1.52%). The latency to onset of electrographic seizures and the average duration of individual seizure episodes also did not differ between the two groups (latency 1.5 ± 0.19 min, duration 10.7 ± 3.1 min for antisense mRNA–treated mice; latency 1.1 ± 0.01 min, duration 8.0 ± 1.4 min for missense mRNA–treated mice).

Altered levels of anxiety during the ovarian cycle

In light of the specific effects of anxiolytic ethanol concentrations on δGABA$_A$Rs[13–15] and on the tonic inhibition mediated by these receptors[15], together with the changes in mood and anxiety during PMDD[1] and associated changes in the effects of ethanol[29,30], we wanted to examine the levels of anxiety in mice at different stages of the ovarian cycle. We used an elevated plus-maze to measure anxiety levels[31] in female mice with well-established ovarian cycles. Mice in late diestrus, a time when the δGABA$_A$Rs are upregulated, showed decreased anxiety. Individual mice tested throughout the course of their cycles spent more time in the open arms of the maze during diestrus than during estrus (**Table 2**). In contrast, mice in estrus spent more time in the closed arms than mice in diestrus (**Table 2**). Mice in diestrus spent significantly more time in the center of the maze (diestrus: 9.26 ± 1.83%; estrus: 4.91 ± 0.57%; $n = 6$; $P < 0.05$) and made significantly more total arm entries (diestrus: 37.47 ± 3.64; estrus: 25.00 ± 2.98; $n = 6$; $P < 0.05$). Although the number of total entries was significantly higher during late diestrus, analysis of covariance in all experimental groups (two groups of females and two groups of males) did not show any significant relationship ($P = 0.37$) between the number of total entries and the fraction of entries into open or closed arms. Male mice tested daily on the elevated plus-maze over a period of 15 d did not show fluctuations in anxiety levels. The average fraction of time spent by male mice in the open arms during three successive test periods

Table 2 Effect of the estrous cycle on anxiety evaluated using the elevated plus-maze

	Open arms		Closed arms	
	Entries (% total)	Time (% total)	Entries (% total)	Time (% total)
Estrus	5.68 ± 1.11*	1.36 ± 1.05*	44.53 ± 1.06*	93.71 ± 0.69*
Diestrus	9.69 ± 2.29	3.80 ± 0.32	40.08 ± 1.04	86.90 ± 1.68

*denotes significant difference ($P < 0.05$) between mice in estrus and in diestrus (paired *t*-test; $n = 6$).

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separated by 7 d was 3.67 ± 1.09% ($n = 9$). This was not significantly different from the values obtained for the same male mice tested during the middle of the two 7-d periods to resemble the tests done in females (4.41 ± 1.25%; $n = 9$; $P = 0.67$, paired t-test). Our data are consistent with fluctuating anxiety levels in female mice related to the stages of the estrous cycle. Mice in diestrus, when levels of δGABA$_A$Rs are high, have lower anxiety levels than mice in estrus. The attenuated tonic inhibition during estrus resulting from decreased δGABA$_A$R expression corresponds to a stage of enhanced anxiety during this phase of cycle.

DISCUSSION

We have identified specific GABA$_A$R subunits that undergo fluctuations over the estrous cycle, resulting in changes in tonic inhibition paralleled by commensurate alterations in seizure susceptibility and anxiety levels. During late diestrus, when circulating levels of progesterone are high, enhanced expression of δGABA$_A$Rs and a consequent increase in tonic inhibition are accompanied by decreased seizure susceptibility and lowered anxiety. Until now, estrous cycle–related alterations in excitability have been attributed to direct effects of neurosteroids on GABA$_A$Rs. Our study is the first to focus on cellular and molecular events underlying changes in seizure susceptibility and anxiety over the course of the cycle, demonstrating cycle-dependent changes in the expression of GABA$_A$R δ and γ2 subunits. In the future, the precise mechanisms responsible for cycle-related alterations of GABA$_A$R subunits will need to be determined, as there may be links between the subunit changes and fluctuations in hormone levels.

Estrous cycle related changes in GABA$_A$Rs

Our findings are consistent with previous studies showing alterations in muscimol binding and changes in neurosteroid and benzodiazepine sensitivities over the cycle[4–6,32]. Increased sensitivity to neurosteroids[32] and decreased efficacy of benzodiazepines[4–6] have been reported at diestrus, consistent with our findings of increased neurosteroid-sensitive[10,11] δ and decreased benzodiazepine-sensitive, neurosteroid-insensitive[10,11] γ2 subunit expression at diestrus. Previous studies have shown that withdrawal of female rats from 3-week-long exogenous progesterone administration results in marked alterations in GABA$_A$Rs and phasic inhibition in the CA1 region, including increased α4 and δ subunit expression and decreased benzodiazepine sensitivity[33,34]. The increased δGABA$_A$R expression in our study occurs at a time when progesterone levels are relatively high during diestrus. Therefore, it seems unlikely that progesterone withdrawal might be responsible for the changes we observed. We did not find any changes in α4 subunit expression, but our data demonstrate tightly regulated expression of GABA$_A$R δ and γ2 subunits over the course of the estrous cycle, resulting in specific changes in tonic inhibition, seizure susceptibility and anxiety levels. It seems unlikely that additional hippocampal GABA$_A$R subunits undergo substantial alterations during the cycle, as there were no alterations of the phasic inhibition (sIPSCs) in DGGCs or CA1 pyramidal cells or in the tonic inhibition in the CA1. The specificity of the effects of δGABA$_A$R antisense mRNA also diminish the likelihood that substantial ovarian cycle–related changes occur in other GABA$_A$R subunits.

The complementary regulation of γ2 and δ subunit expression during the estrous cycle is reminiscent of the upregulation of γ2 subunits in *Gabrd*$^{-/-}$ mice[23]. Because γ2 subunits are found mostly at synapses[35], we also expected to see changes in the phasic (synaptic) component of inhibition over the estrous cycle. As phasic inhibition remained constant, γ2 subunits may have replaced δ subunits at estrus solely at extrasynaptic sites where the continuous presence of GABA prevented the γ2GABA$_A$Rs from generating tonic currents owing to desensitization[10,11]. Alternatively, an increase in γ2 subunits at dendritic synapses where spontaneous GABA release is extremely low[36] may have gone undetected because we recorded sIPSCs of predominantly somatic origin. The abundance of these two subunits may be independently regulated by a common ovarian cycle–related mechanism: when the increase in δ subunits during late diestrus was stalled by antisense mRNA, γ2 subunit levels still decreased.

Tonic inhibition during the estrous cycle

In many neurons in which a tonic current is activated by the GABA levels present in the extracellular space[8,16], the overall charge carried by the activation of tonically active GABA$_A$Rs can be more than three times larger than that produced by phasic inhibition[8]. Experimental and theoretical studies indicate that a continuously active inhibitory conductance significantly affects excitability and gain control both *in vitro* and *in vivo*[16].

Selectively increasing the tonic inhibition of DGGCs by about twofold with neurosteroids results in an overall reduction in the neuronal excitability of the dentate gyrus[12]. The similar twofold larger tonic inhibition in diestrus as compared to estrus should substantially reduce the throughput of the dentate gyrus during late diestrus. This decreased throughput could be further diminished at diestrus by the potentiating actions of progesterone-derived neurosteroids on the elevated number of δGABA$_A$Rs. It is unlikely that elevated neurosteroid levels could have persisted in acute hippocampal slices to account for the enhanced tonic currents found in late diestrus. A recent study investigating the relationship between ethanol and neurosteroid effects[37] has shown that inhibiting neurosteroid synthesis in acute brain slices had no bearing on neurosteroid-dependent effects.

The magnitude of the tonic current at late diestrus is comparable to that found in male mice[12]. This finding also matches well with the similar anxiety levels of male mice and females at late diestrus. As the dentate gyrus is not the only brain area involved in anxiety, future studies will need to focus on ovarian cycle–related δGABA$_A$R expression and tonic inhibition in other brain areas enriched in δGABA$_A$Rs.

Seizure susceptibility during the estrous cycle

Exogenous progesterone administration has anticonvulsant effects in women with catamenial epilepsy as well as in experimental models of epilepsy[38–40]. Likewise, progesterone withdrawal or inhibition of progesterone metabolism exacerbates seizure susceptibility[41,42]. Yet few studies have focused on the effect of endogenous alterations in neurosteroid levels on seizure susceptibility. Our study clearly shows changes in seizure susceptibility over the estrous cycle: seizure susceptibility is decreased during late diestrus, when progesterone levels are elevated and δGABA$_A$Rs are increased resulting in enhanced tonic inhibition.

In the mouse pilocarpine model of temporal-lobe epilepsy, δGABA$_A$R expression in DGGCs becomes progressively reduced[43]. In addition, loss-of-function mutations in δGABA$_A$Rs have been identified in patients with generalized epilepsy with febrile seizures plus[44]. Thus, δGABA$_A$Rs may be an important therapeutic target for the treatment of epilepsy. Our studies show a clear attenuation in seizure activity by the GABA$_A$R agonist THIP, which at low concentrations is a preferred agonist at δGABA$_A$Rs[10]. This drug is presently undergoing clinical trials as a deep sleep–inducing hypnotic[45], but lower doses of THIP may offer a safe and effective treatment for patients with various forms of epilepsy. Other potential treatments may include the GABA uptake inhibitor tiagabine (Gabitril), as inhibition of GABA uptake specifically enhances tonic inhibition[46]. However, as shown by the lack of an effect for THIP on seizure susceptibility

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during estrus, such drugs may be ineffective if an impaired upregulation of δGABA$_A$Rs during the luteal phase is responsible for the increased seizure frequency.

Anxiety during the estrous cycle

Neurosteroid modulation of GABA$_A$Rs is thought to have a role in depression, anxiety, and mood disorders associated with PMDD[1]. Patients with PMDD have a much higher incidence of catamenial epilepsy than the general population[47], and show increased luteal-phase excitability[2,48], suggesting shared pathogenesis. Women with PMDD lack the enhancement of GABAergic inhibition normally present during the luteal phase[32,48] when progesterone-derived neurosteroid levels are high. Thus, a failure in neurosteroid-sensitive inhibition may underlie both dysphoria and increased seizure susceptibility. According to our findings, catamenial epilepsy and PMDD may both result from a disturbance in the normal hormone-dependent potentiation of δGABA$_A$R-mediated inhibition during the menstrual cycle. These patients may benefit from treatments targeting the enhancement of neurosteroid-sensitive tonic GABAergic inhibition. Consistent with this hypothesis, women with PMDD have decreased sensitivity to ethanol[29], a drug that selectively enhances tonic GABAergic inhibition mediated by δGABA$_A$Rs[15]. Consequently, women with PMDD may have a deficit in δGABA$_A$R-mediated tonic inhibition during the luteal phase, perhaps secondary to an impaired expression of the δGABA$_A$Rs sensitive to neurosteroids and ethanol.

Our findings provide new insight into ovarian cycle–related changes in neuronal excitability. These estrous cycle–related changes in GABAergic inhibition may underlie the increased seizure susceptibility and anxiety in catamenial epilepsy and PMDD, respectively. The molecular mechanisms underlying the cyclic regulation of GABA$_A$R subunits require further elucidation and are expected to uncover new potential therapeutic targets.

METHODS

Estrous cycle detection. The estrous cycle of adult C57/Bl6 mice (10–14 weeks of age) (Harlan) was determined by cellular profile analysis (Giemsa staining, Fisher Diagnostics) in vaginal smears and by measuring the electrical resistance of the vaginal mucosa (Estrus cycle monitor EC40, Fine Science Tools). Only mice showing regular estrous cycles were used (**Fig. 1**).

Progesterone level determination. Trunk blood was collected from wild-type mice in estrus and late diestrus. Duplicate 25-μl plasma samples were measured using a spectrophotometer (at 450 nm) against a standard curve according to the manufacturer's specification (Progesterone-HRP enzyme immunoassay test kit, BioCheck).

Whole-cell recordings. Whole-cell recordings were performed at 21–23 °C on visually identified DGGCs and CA1 pyramidal cells ($V_h = -60$ mV) in coronal hippocampal slices 350 μm thick as previously described[12,15] (**Supplementary Methods**). Measurements and data analysis were performed as previously described[12,46] (**Supplementary Methods**). The experimenter was blinded to the stage of the estrous cycle throughout the recordings and analyses.

Western blot analysis. Mice were anesthetized with halothane and killed by cervical dislocation. Hippocampi were homogenized in a buffer containing 50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA and 0.5 mM dithiothreitol along with protease inhibitors (Complete Mini, Roche). The homogenate was centrifuged at 100,000*g* for 30 min at 4° C; the pellet was resuspended in homogenization buffer containing 1% Triton X-100, incubated on ice for 1 h and centrifuged at 100,000*g* for 30 min at 4 °C. The supernatant was then collected as the membrane fraction.

Protein concentrations were determined using the DC Protein Assay (Bio-Rad). Protein (100 μg) was loaded onto a 12% SDS-polyacrylamide gel, subjected to electrophoresis and transferred to a pure nitrocellulose membrane (Amersham). The membrane was blocked in 10% nonfat milk and probed with polyclonal antibodies specific for α4 (1:5,000) or δ (1:5,000), both gifts from W. Sieghart, or γ2 (1:10,000) from Novus. The blots were incubated with peroxidase-labeled anti–rabbit IgG (1:2,000, Vector Laboratories) and immunoreactive proteins were visualized using enhanced chemiluminescence (Amersham). Optical density was determined using the NIH Image J software. Pixel intensities were converted to optical density (OD) using the calibration curve of the software, and background-subtracted values were expressed as OD per 100 μg total protein.

Electroencephalogram (EEG) recordings. Age-matched adult C57/Bl6 mice (Harlan) were anesthetized with 100 mg kg^{-1} ketamine, 5.2 mg kg^{-1} xylazine and 1.0 mg kg^{-1} acepromazine according to a protocol approved by the UCLA Chancellor's Animal Research Committee. A hippocampal depth electrode (Plastics One) was placed 2.2 mm posterior to bregma and 1.7 mm lateral to the midline at a depth of 2.0 mm. The electrode was fixed to the skull using dental cement and the mouse was allowed to recover for 48 h. EEG recordings were started 10 min before an i.p. injection of 15 mg kg^{-1} kainic acid (Sigma) and continued for more than 120 min. Mice treated with 10 mg kg^{-1} THIP (Sigma) were injected i.p. 30 min before kainic acid treatment. Recordings were band-pass filtered between 0.1 and 200 Hz (8-pole Bessel, Frequency Devices) and were sampled at 1 kHz using an in-house Labview-based (National Instruments) software. Electrographic seizure events were defined as changes in the amplitude and frequency of the EEG activity, and their duration was calculated by the software. Measures of seizure susceptibility were the seizure latency, the cumulative time seizing expressed as a fraction (%) of the total recording time, and the average duration of individual electrographic events. Seizure latency was defined as the time elapsed from the injection of kainic acid to the start of the first electrographic seizure. The fraction of total time spent in seizures (% time seizing) was calculated as the cumulative time of all seizure activity during a 120-min recording period divided by 120 min. The durations of individual electrographic events were measured from the start of the repetitive EEG pattern until return to baseline. The average time of these events observed over 120 min was calculated to obtain the average seizure duration. The number of electrographic events over the 120-min period can be calculated by dividing the (% time seizing × 120 min) by the average seizure duration.

Antisense generation and administration. Antisense oligodeoxynucleotides (ODNs) were designed complementary to the mouse δGABA$_A$R mRNA. The 20-mer ODN, CGT TTG TAC CTT ATG TGG TA, does not show any significant homology with any other sequence identified in GenBank. A 20-mer ODN missense mRNA was also constructed as AT GGT GTA TTC CAT GTT TGC, and did not show any significant homology with any other sequence identified in GenBank. A 20-mer negative control RNA with no significant sequence similarity to mouse, rat or human gene sequences (Silencer Negative Control siRNA, Ambion) was used as a negative control in the whole-cell tonic recording experiments. ODNs (5 nmol) were injected in a 5 μl volume of sterile saline into the lateral ventricle at coordinates: 0.6 mm posterior to bregma and 1.1 mm lateral to the midline at a depth of 2.0 mm. The mice were injected with ODNs at estrus and allowed to recover for 48 h until the diestrus phase.

Behavioral anxiety test. Mice were tested for 10 min on an elevated (57 cm) plus-maze every day for 14 d during the light period (13:00–6:00 h). At the beginning of each test, each mouse was placed in the central platform facing an open arm. The activity of the mice was evaluated based on the number of entries into the closed arms and open arms and the percentage of time spent in the open versus closed arms. After blind testing and analysis, vaginal smears and impedance monitoring were performed to correlate the behavior to the stages of the estrous cycle.

All data are presented as mean ± s.e.m. Significance was considered to be $P < 0.05$, as determined by paired and unpaired *t*-tests and by ANOVA, as indicated. Further details of methods are available online in the **Supplementary Methods**.

Accession code. GenBank: δGABA$_A$R mRNA, LocusID 14403.

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Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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