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The Role of the δ GABA(A) Receptor in Ovarian Cycle-Linked Changes in Hippocampus-Dependent Learning and Memory

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Abstract The δ subunit of the GABA_AR is highly expressed in the dentate gyrus of the hippocampus where it mediates a tonic extrasynaptic inhibitory current that is sensitive to neurosteroids. In female mice, the expression level of the δ subunit within the dentate gyrus is elevated in the diestrous relative to estrous phase of the estrous cycle. Previous work in our lab found that female δ -GABA_AR KO mice showed enhanced hippocampus-dependent trace but normal hippocampus-independent delay fear conditioning. Wild-type females in this study showed a wide range of freezing levels, whereas δ -GABA_AR KO mice expressed only high levels of fear. We hypothesized that the variability in the wild-type mice may have been due to estrous cycle-mediated changes in the expression of the δ -GABA_AR, with low levels of freezing in mice that were in the diestrous phase when dentate gyrus tonic inhibition is high. In the present study we tested this hypothesis by utilizing contextual, delay, and trace fear conditioning protocols in mice that were trained and tested in either the diestrous or estrous phases. Consistent with our hypothesis, we found a significant impairment of hippocampus-dependent learning and memory during diestrus relative to estrus in wild-type mice and this impairment was absent in δ -GABA_AR mice. These findings argue that the δ -GABA_AR plays an important role

in estrous cycle-mediated fluctuations in hippocampus-dependent learning and memory.

Keywords Hippocampus · GABA · Estrus · Fear conditioning · Sex difference

Introduction

Sex differences in brain function and disease have traditionally been viewed as the combined result of the organizational and activational effect of gonadal hormones. However, males and females differ not only in the relative levels of gonadal hormones, they also differ in that females of reproductive age are in constant flux across the ovarian cycle. In humans, menstrual phase has been shown to modulate a variety of cognitive and neurophysiological measures [1–6]. For example, pre-pulse inhibition, a measure correlated with psychiatric disturbances, shows cycle-related changes [7] and in women with catamenial epilepsy, seizure probability and severity is strongly modulated by menstrual phase [5, 8–10]. In rodents, the estrous cycle has been shown to exert similarly profound effects, ranging from changes in synaptic spine density in the hippocampus [11] to amphetamine responsiveness in the striatum [12]. Despite important advances in recent years, the underlying mechanisms of ovarian cycle-linked changes in brain and behavior are still poorly understood. Unfortunately, this lack of understanding has led to the widespread exclusion of female subjects from both basic and clinical research [13–15].

The estrous cycle causes characteristic cyclical fluctuations in sex hormones, which can act directly on hormone receptors or indirectly via their metabolites. The neurosteroid metabolite of progesterone, allopregnanolone, is a

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powerful allosteric modulator of the GABA_A receptor, particularly subtypes containing the δ subunit [16–18]. The δ -containing GABA_A receptors are expressed extra synaptically, primarily in the cerebellum, thalamus and the hippocampus [19], where they mediate a powerful tonic inhibitory current. Exogenously administered allopregnanolone has memory-impairing [20–23] and anxiolytic [24, 25] effects and at high doses it has sedative and anesthetic effects [26, 27]. The expression level of δ -containing GABA_A receptors has been shown to fluctuate across the estrous cycle [28–30]. In the hippocampus, δ expression is highest during late diestrus corresponding to enhanced tonic inhibition in the dentate gyrus and decreased seizure severity [29, 30], whereas in the periaqueductal gray δ expression is enhanced during late diestrus specifically in inhibitory interneurons [31]. This enhanced expression is thought to result from the direct action of neurosteroid metabolites of progesterone on granule cells [5, 30, 32, 33].

The goal of the present study was to test the hypothesis that estrous cycle-mediated fluctuations of the GABA_A δ subunit modulate hippocampus-dependent learning and memory. This hypothesis is based on an intriguing sex difference that was observed in the learning and memory ability of δ -GABA_A KO mice: genetic deletion of the GABA_A δ subunit produced an enhancement of hippocampus-dependent learning and memory specifically in female mice. While female δ -GABA_A KO mice showed enhanced hippocampus-dependent trace tone fear conditioning, male GABA_A KO mice were normal. Both males and females showed normal hippocampus-independent delay tone fear conditioning, suggesting that the enhancement in females was due to a specific enhancement of learning and memory-related plasticity within the hippocampus. An analysis of the distribution of freezing scores showed that wild-type females exhibited a range of freezing to the trace conditioned tone with some animals exhibiting very little freezing, suggesting a failure to learn the tone-shock association. None of the female δ -GABA_A KO mice exhibited low freezing levels suggesting they all successfully learned the tone-shock association. This experiment did not control for phase of the estrous cycle. We therefore hypothesized that the “failures to learn” in wild-type mice could have been from mice that were trained during diestrus when δ subunit expression and tonic inhibition in the dentate gyrus is high. This enhanced inhibition would be absent in δ -GABA_A KO mice and thus explain the absence of “failures to learn”. The present study was therefore designed to test the hypothesis that activity at δ -GABA_A produces an impairment in hippocampus-dependent learning during late diestrus relative to estrus and that this cyclic variation would be mitigated in δ -GABA_A KO mice. We tested hippocampus-dependent

context and trace fear conditioning and hippocampus-independent delay fear conditioning in animals that are trained and tested in either estrus or diestrus. In addition, in order to test the specific hypothesis of δ -GABA_A involvement, hippocampus-dependent contextual fear was assessed across estrus in δ -GABA_A KO mice.

Methods

Animals

Female C57Bl/6 mice ranging in age from 4 to 6 months were purchased from Taconic Farms and housed in UCLA’s Psychology Department vivarium on a 12 h light/12 h dark cycle with all experiments performed during the light phase. For the study in δ -GABA_A KO mice heterozygous breeding pairs of δ HET (+/–) were used to produce δ KO (–/–), δ HET (+/–) and δ WT (+/+) mice. These mice were originally generated on the 129 background, but were back-crossed for >10 generations with C57Bl/6 mice.

Estrous Cycle Monitoring

The estrous cycle was determined by cellular profile analysis (Giemsa staining, Fischer Diagnostics) in vaginal smears and by measuring electrical resistance of the vaginal mucosa (Estrous cycle monitor EC40), as outlined in Fig. 1. Estrus was defined as the day after the proestrus peak of vaginal impedance accompanied by large numbers of cornified epithelial cells. Diestrus was defined as the second low day of vaginal impedance after estrus characterized by very few cells, mucous streaks and occasional nucleated parabasal cells [29]. Mice were followed through one to two cycles prior to behavioral procedures. This was done to ensure that only mice with normal estrous cycles were included and to exclude females in which pseudopregnancy was induced by the monitoring procedure. Any mice that failed to show normal estrous cycles were excluded (Fig. 1).

Fear Conditioning Procedures

For all procedures training took place in either estrus or diestrus and testing took place when the animals again entered the same phase. Trace and delay fear conditioning were conducted according to a previously established protocol [34, 35]. Training took place in four identical chambers (Training context) (28 × 21 × 21 cm; Lafayette Instrument Co.) in a brightly lit room. The floor of each chamber consisted of a shock grid wired to a shock generator and scrambler (Med-Associates Inc.) to deliver foot

Fig. 1 Experimental design and criteria for determining estrus versus diestrus. Mice were trained in either estrus or diestrus and then tested when back in the same phase. Vaginal impedance was monitored every day and combined with vaginal cytology to determine estrous phase

shock. On the training day mice underwent either trace or delay fear conditioning. For both procedures, mice were placed in the training context and left to explore for 3 min before tone onset (20 s, 75 dB, 2,800 Hz). For delay conditioning, termination of tone was contiguous with foot shock (2 s, 0.5 mA). For trace conditioning, tone and foot shock were separated by a 20-s trace interval (See Fig. 3b for a diagram). Both groups received five tone-shock trials. In order to keep the overall session length the same, the delay-conditioned group had a 220-s interval between shock termination and the next tone onset whereas the trace-conditioned group had 200-s interval. After the last shock, animals were left for 2 min and then returned to their home cages. When the animals were back in the same estrous cycle phase as training they were placed in a separate room with four novel and structurally distinct chambers (Test context) for the tone test. The tone was delivered in the same way as in the training, but with shock omitted.

For contextual fear conditioning, on the training day mice were placed in the training context and left to explore for 3 min before the first shock onset (2 s, 0.5 mA). A total of 5 shocks were presented with a 220 s inter-trial interval. After the last shock, animals were left for 2 min and then returned to their home cages. When the animals were back in the same estrous cycle phase as training they were placed back in the training context for an 8 min context test. Context and Tone were not tested in the same mice because of logistical issues in keeping training and testing

at the same phase of the cycle.

An observer blind to the genotype of the animal scored the presence or absence of freezing as a measure of conditional fear. In addition we utilized MedAssociates automated scoring as described previously [36]. For the tone test, freezing scores were grouped into 3 different bins: baseline (BL), tone, and post-tone interval (the 20 s immediately following tone termination). For the BL bin, freezing was scored every 8 s. For both tone and trace bins, freezing was scored every 2 s. Freezing data from the five tone presentations were averaged together. For the context test freezing was scored every 8 s. All freezing scores were transformed into percent freezing by dividing the number of times the animals were observed to be freezing by the total number of observations and multiplying by 100 ($\% \text{ Freezing} = \text{Freeze}_{\text{TOT}} / \text{Observations}_{\text{TOT}} \times 100$).

Open Field Procedure

The open field procedure was based on a previously developed procedure [37]. Animals were placed in an open field (30 × 30 cm; Couborn, Inc.) for 16 min. During the first 8 min the lights in the experimental room were turned off. During the second 8 min the lights were turned on. Locomotor activity was analyzed using a series of infrared beams arranged around the perimeter of the open field apparatus. This procedure was designed to assess locomotor habituation under conditions of both light and dark and the activity response to stimulus change [37].

Results

Delay, Trace and Contextual Fear Across Estrus in C57Bl/6 Mice

Our results indicate that estrous cycle phase specifically modulates hippocampus-dependent fear conditioning, with an impairment in diestrus relative to estrus. As shown in Fig. 2, contextual fear was significantly attenuated in diestrus relative to estrus ($F_{(1,14)} = 5.954$, $p = 0.031$, Fig. 2). For conditioning involving a discrete tone stimulus, post-tone freezing was significantly attenuated in diestrus relative to estrus regardless of whether the animals were trained with a delay or trace conditioning protocol ($F_{(1,40)} = 8.786$, $p = 0.005$, Fig. 3a, right). Hippocampal lesions produce a similar reduction in post-tone freezing [38]. Baseline freezing prior to tone presentation did not differ ($F_{(1,40)} = 0.002$, $p = 0.857$, Fig. 3a, left) indicating similar levels of generalization between the training context and the tone test context and arguing the fear expression per se is not different. Freezing in response to the delay conditioned tone ($F_{(1,19)} = 0.018$, $p = 0.893$, Fig. 3a, middle) also did not differ indicating that estrous cycle-mediated differences do not affect this hippocampus-independent form of conditioning. Freezing to the trace-conditioned tone did not differ ($F_{(1,21)} = 0.841$, $p = 0.371$, Fig. 3a, middle) suggesting that this form of hippocampus-dependent learning and memory is less reliably affected by the estrous cycle. Delay conditioning produced overall higher levels of freezing during the tone and post-tone interval, as expected ($F_{(1,40)} = 23.801$, $p < 0.001$ and $F_{(1,40)} = 10.078$, $p = 0.003$, respectively, Fig. 3a middle). Shock reactivity, as determined by the MedAssociates automated measurement of activity during the shock presentations, also did not differ (Diestrus (mean $\pm$ SD):

Fig. 2 Female mice in diestrus show impaired contextual fear relative to estrous mice. Percent freezing was reduced in diestrus relative to estrus during the context test

Fig. 3 Freezing during the tone test in mice that were trained and tested in estrus versus diestrus. **a** Female mice trained and tested in diestrus showed reduced levels of freezing during the post-tone interval after both trace and delay fear conditioning ($p < 0.01$). **b** Diagram illustrating the temporal relationship between tone and shock in trace versus delay fear conditioning

1,058.89 $\pm$ 238.8 arbitrary units; Estrus: 963.65 $\pm$ 187.64 arbitrary units; $F_{(1,19)} = 0.950$, $p = 0.343$).

Open Field Activity Across Estrus

Locomotor activity was similar between estrus and diestrus (Fig. 4). There was an overall habituation of exploratory

Fig. 4 Open field activity did not differ between estrus and diestrus. Assessment of locomotor activity across a 16 min session. The light in the experimental room was turned on halfway through the session in order to assess light-induced locomotor suppression as well as responsiveness to stimulus change (i.e., the activity response). There were no significant differences in locomotor activity in either lighting condition or in the activity response

activity across the 16 min in the open field as indicated by a significant repeated measures effect ($F_{(15,225)} = 19.8$, $p < 0.001$, Fig. 4), but there was no main effect of estrous phase ($F_{(1,15)} = 0.070$, $p = 0.795$, Fig. 4) or repeated measures interaction ($F_{(15,225)} = 0.587$, $p = 0.883$, Fig. 4). The activity response to the change in lighting conditions also did not differ ($F_{(1,17)} = 0.435$, $p = 0.520$, Fig. 4). These findings suggest that the reduced freezing during diestrus is not a result of hyperactivity interfering with expression of freezing.

Context Fear Across Estrus in δ GABA_AR KO Mice

Examining contextual fear across estrus in δ KO mice (Fig. 5) showed that while their WT littermates did show an impairment during diestrus, δ KO mice did not (Genotype $\times$ Phase interaction: $F_{(1,26)} = 5.534$, $p = 0.028$; effect of phase in WT: $F_{(1,12)} = 12.99$, $p = 0.005$; effect of phase in KO: $F_{(1,14)} = 0.030$, $p = 0.865$). Overall, this led to a significant enhancement of context fear in the δ KO mice (Main effect of genotype: $F_{(1,26)} = 4.359$, $p = 0.049$). These results confirm our hypothesis that it is the lack of impairment during diestrus, when δ -GABA_AR-mediated, steroid-enhance able tonic inhibition in the dentate gyrus is high, that underlies the sex-specific enhancement of hippocampus-dependent learning and memory in δ -GABA_AR KO mice.

Fig. 5 δ -GABA_AR KO mice fail to show the impairment in contextual fear during diestrus. Percent freezing during the context test for WT and δ -GABA_AR KO trained and tested in either estrus or diestrus. WT mice displayed a deficit in freezing in diestrus relative to estrus mice (*, $p = 0.005$), whereas δ -GABA_AR KO did not. This lack of a deficit underlies the overall enhancement in δ -GABA_AR KO relative to WT mice

Discussion

These findings provide strong evidence that hippocampus-dependent learning and memory, as assessed by widely used fear conditioning tasks, fluctuates across the estrous cycle in mice, with impairment in diestrus relative to estrus. The absence of this impairment during diestrus appears to underlie the sex-specific enhancement of hippocampus-dependent learning and memory in δ -GABA_AR KO mice (Fig. 5). This is consistent with our hypothesis that the diestrus impairment in wild-type mice may be due to increased δ -GABA_AR-mediated, neurosteroid-enhanceable tonic inhibition in the dentate gyrus [29, 30, 35]. This elevated expression results in increased tonic inhibitory tone, specifically in the dentate gyrus [29, 30] and pharmacological agents that increase tonic inhibition, such as ethanol and allopregnanolone, are known to have memory-impairing effects [21].

Our findings are in general agreement with previous fear conditioning and novel object recognition studies [39, 40]. Markus et al. 1997 used a similar methodology, e.g., the rats were trained and subsequently tested in the same phase, and found enhanced contextual fear, but equivalent delay tone fear, in estrus relative to proestrus. Walf et al. [40] and Van Goethem et al. [41] found a relative enhancement of novel object recognition during estrus which, in combination with the present findings, argues for a general enhancement in hippocampus-dependent learning and memory during this phase. Our studies add to this by showing that the δ -GABA_AR makes significant contributions to these cyclical changes in hippocampal function, potentially via allopregnanolone-mediated modulation of tonic inhibition within dentate granule cells. Allopregnanolone is increased during diestrus as well as by stressful experiences [29, 30], which argues that stressful learning experiences, such as fear conditioning, may be particularly sensitive to disruption during this phase. As we trained and tested in the same phase, however, future studies will be required to determine whether these changes are mediated by alterations in acquisition, consolidation or retrieval mechanisms.

Several studies using avoidance paradigms have found precisely the opposite results to ours [42–45], e.g., impaired active avoidance in estrus relative to diestrus. Although, on the surface, these findings may seem contradictory, in active avoidance tasks, freezing and the avoidance response are in competition [46]. Thus, enhanced context freezing during estrus as observed in the present study could result in an impairment in an active avoidance situation [47].

Our findings are inconsistent with the estrous cycle-mediated fluctuations in δ -GABA_AR expression in the PAG that has been observed in rats [31, 48]. In that study

δ -GABA_AR expression was increased, specifically in inhibitory interneurons, during diestrus relative to estrus. They hypothesized that this should produce disinhibition of the defensive motivational system that is orchestrated by the PAG and result in increased panic-like behavior [49]. The decrease in freezing we observed during diestrus is therefore inconsistent with their hypothesis. This could be due to species differences; however, as their findings are in rats and the present study is in mice. Rats and mice potentially differ in hormonal changes across the estrous cycle as well as the cognitive impact of exogenous neurosteroid administration [21, 29, 41].

Overall, these findings have important implications for our understanding of ovarian-cycle linked changes in cognitive function, but also for the field of mouse transgenic as a whole. While this study confirms the underlying assumption that the estrous cycle does confer variability, which is the usual justification for exclusion of female subjects [15], it also helps to characterize this variability and indicates that it should not be ignored. These results clearly indicate that inclusion of female subjects and careful monitoring of the estrous cycle can reveal phenotypes in transgenic mice that would otherwise be missed [50]. We do acknowledge, however, that monitoring the estrous cycle is time-consuming, labor-intensive and requires extensive handling of the animal. We therefore argue that, at the very least, initial studies in transgenic mice should be conducted with both males and females and follow-up studies should be conducted when the data suggest potential estrous cycle-mediated effects.

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