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Effects of a low dose of ethanol in an animal model of premenstrual anxiety

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Abstract

Low (1 mM), but not 10 mM, concentrations of ethanol selectively potentiate current gated by $\alpha_4\beta_2\delta$ subunit combinations of the gamma-aminobutyric acid type A (GABA_A) receptor, a subtype increased in hippocampus after withdrawal from progesterone in a rodent model of premenstrual anxiety. In the current study, we tested the hypothesis that the anxiolytic effect of ethanol would exhibit a similar dose-response effect by using the acoustic startle response (ASR) and elevated plus-maze as behavioral models. To this end, adult, female rats were tested (1) 24 h after removal of a progesterone-filled capsule implanted subcutaneously for 21 days (progesterone withdrawal) or (2) on the day of diestrus, a low hormone state. Low doses of ethanol (0.2–0.4 mg/kg) produced a significant 60%–70% decrease in the ASR only in animals undergoing progesterone withdrawal. However, higher doses of ethanol (0.8–1.2 g/kg) were ineffective in these animals, resulting in an “inverted U” ethanol dose effect similar to that observed at recombinant $\alpha_4\beta_2\delta$ subunit combinations of the GABA_A receptor. Consistent with these findings, significant 70% attenuation of the ASR was also achieved after progesterone withdrawal with 3 mg/kg of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), a GABA_A receptor partial agonist with greater potency at $\alpha_4\beta_2\delta$ receptors than at other known isoforms. In contrast, this partial agonist was not anxiolytic in control animals. These results support the suggestion that very low doses of ethanol are anxiolytic in a model of premenstrual anxiety, whereas higher, potentially sedative, doses are without effect. The results may be relevant for altered ethanol sensitivity during premenstrual syndrome, when increased ethanol consumption has been reported.

Keywords

Ethanol; Progesterone; Allopregnanolone; Withdrawal; Acoustic startle response; Elevated plus maze; Hippocampus; Alpha-4; Delta; GABA_A receptor; GABA; Premenstrual syndrome; PMS; Steroid

1. Introduction

Ethanol is a widely used drug with dose-dependent behavioral effects, reported to be anxiolytic at low doses and sedative or anesthetic at higher doses, similar to most known

positive modulators of the gamma-aminobutyric acid type A (GABA_A) receptor. This receptor has been shown to play a central role in anxiolytic drug actions (Low et al., 2000). Through work from our laboratory (Sundström-Poromaa et al., 2002), a novel subunit combination of the GABA_A receptor, $\alpha_4\beta_2\delta$, has been identified. It is potentiated by very low concentrations of ethanol (1–3 mM), assessed by using two electrode voltage clamp procedures and heterologous expression in oocytes. However, higher concentrations of ethanol (>10 mM) were ineffective in this regard, resulting in an “inverted U” concentration-response relation. Because other subtypes were unresponsive to this low concentration of ethanol, including $\alpha_4\beta_2\gamma_2$ subunit isoforms of the GABA_A receptor, this finding supports the suggestion that $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor possess a unique sensitivity to ethanol (Sundström-Poromaa et al., 2002; Wallner et al., 2003).

Ethanol is known to exert behavioral (Jones et al., 2000) and electrophysiologic (Roberto et al., 2003) effects at relatively low concentrations. Low concentrations of ethanol can exert both postsynaptic and presynaptic effects in hippocampus and amygdala (Carta et al., 2003; Roberto et al., 2003; Wan et al., 1996). This presynaptic effect of ethanol is enhanced when GABA_B receptors are blocked (Wan et al., 1996), supporting the suggestion of an effect of the drug on transmitter release, whereas effects on GABAergic transmission in amygdala are mediated through corticotropin-releasing factor type 1 (CRF₁) receptors (Nie et al., 2004). Findings from behavioral studies indicate that GABA_A receptor antagonists/negative modulators can decrease ethanol consumption (Hyytiä & Koob, 1995; Koob et al., 1988), and ethanol can substitute for other GABA-modulatory drugs in drug-discrimination tasks (Engel et al., 2001). Despite this evidence at native receptors, ethanol modulation of most recombinant GABA_A receptor subtypes (Harris et al., 1997; Mihic et al., 1997), *N*-methyl-D-aspartate (NMDA) receptors (Lovinger et al., 1989), and glycine receptors (Mihic et al., 1997) requires comparatively high concentrations of the drug. The anesthetic effect of ethanol requires identified residues on the M2 region of the GABA_A receptor (Mihic et al., 1997). In contrast, the only recombinant receptor thus far identified as responsive to low concentrations of ethanol is the $\alpha_4\beta\delta$ subunit combination of the GABA_A receptor.

The $\alpha_4\beta\delta$ subunits of the GABA_A receptor have very low expression in most areas of the CNS (Peng et al., 2002; Wisden et al., 1992). However, this subunit isoform is markedly increased in the rodent model of premenstrual syndrome (PMS) used in our laboratory (Sundström-Poromaa et al., 2002). For this model, intact, cycling female rats are administered progesterone for 3 weeks with the use of a paradigm that results in physiologic, proestrous-like levels of progesterone and its GABA-modulatory metabolite 3 α -OH-5 α -pregnan-20-one (3 α ,5 α -THP) in the circulation (Moran & Smith, 1998) and temporarily halts the cycle in this high progestin state. Animals are subsequently tested 24 h after discontinuation of the steroid during a “withdrawal” syndrome of increased anxiety and behavioral reactivity produced by declining levels of 3 α ,5 α -THP (Smith et al., 1998a, 1998b). As such, the hormonal milieu replicates that of the late luteal phase, when levels of both progesterone and 3 α ,5 α -THP abruptly decrease, and women with PMS report increased emotional reactivity (McLeod et al., 1994; Sundström et al., 1997).

The $\alpha_4\beta\delta$ subunit combination of the GABA_A receptor also exhibits a distinctive response to GABA agonists, producing a greater maximum current in response to 4,5,6, 7-

tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP or gaboxadol), a GABA partial agonist, compared with the maximum current gated by GABA (Brown et al., 2002), supporting the suggestion that 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol is a full agonist at $\alpha_4\beta\delta$ subunit combinations of the GABA_A receptor. A similar increase in the ratio of maximum 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol response to maximum GABA response was noted in isolated hippocampal neurons after progesterone withdrawal (Gulinello et al., 2003a; Sundström-Poromaa et al., 2002), consistent with increased expression of $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor at this time. Co-expression of α_4 and δ subunits of the GABA_A receptor was also determined directly in hippocampus by using co-immunoprecipitation techniques (Sundström-Poromaa et al., 2002).

Under these described conditions of increased expression of $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor after progesterone withdrawal, GABA-gated current recorded from isolated hippocampal neurons was also potentiated by concentrations of ethanol ranging from 1 to 3 mM, but not by higher (>10 mM) concentrations of ethanol (Sundström-Poromaa et al., 2002). Suppression of α_4 expression prevented the response to 1–3 mM ethanol after progesterone withdrawal, further supporting the suggestion that $\alpha_4\beta\delta$ sub-unit isoforms of the GABA_A receptor possess a unique sensitivity to ethanol. It is interesting that ethanol withdrawal also increases expression of the α_4 subunit of the GABA_A receptor (Devaud et al., 1997; Mahmoudi et al., 1997), but it decreases expression of the δ subunit of the GABA_A receptor (Cagetti et al., 2003), an effect correlated with a decrease in response to ethanol.

The purpose of the current study was to characterize further the dose-dependent effects of ethanol on the acoustic startle response (ASR) and the elevated plus-maze to test the hypothesis that only very low, but not higher, doses of this drug are effective during hormone withdrawal when expression of $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor increase. We also compared ethanol effects on the ASR with those produced by 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol as a GABA agonist partially selective for $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor (Brown et al., 2002). The results obtained after progesterone withdrawal may be relevant for the increased sensitivity of women with PMS to very low doses of ethanol (Sundström et al., 1998).

2. Materials and methods

2.1. Animals

Adult, female, Long–Evans rats, each weighing between 200 and 250 g at the time of testing, were used for all studies. Animals were housed in pairs under controlled conditions (14-h light/10-h dark cycle; room temperature maintained at 24°C) with free access to food and water. Progesterone (Sigma Chemical Co., St. Louis, MO) (crystal-line)-filled capsules of silicone tubing (Nalgene Co., Rochester, NY; 1/16 in × 1/8 in O.D., 10 mm per 100 grams of body weight) were implanted subcutaneously in the lower aspect of the back after induction of halothane anesthesia (3%–6% in oxygen, 1 l/min) in the experimental animals. This paradigm has been shown to result in hippocampal and circulating levels of 3 α ,5 α -THP and circulating levels of progesterone in the physiologic range (Moran & Smith, 1998), and it halts estrous cyclicity until 8 h after removal of the capsule. Capsules were removed under

halothane anesthesia after a 21 -day progesterone administration period, and animals were tested 24 h after the last injection (progesterone withdrawal). Control animals were tested on the day of diestrus (diestrous control animals), a low hormone state, as determined by vaginal lavage. This stage results in α_4 levels similar to those recorded with chronic progesterone treatment (Smith et al., 1998a). In addition, separate control animals (sham control animals) were implanted with empty capsules and tested with selected high or low doses of ethanol in the ASR paradigm or elevated plus-maze 24 h after implant removal. All animals were tested between 0730 and 1100. Euthanasia was either by decapitation (electrophysiology experiments) or carbon dioxide inhalation (behavioral experiments) after the indicated protocols. Both care and use of, as well as all procedures involving, animals were approved by the SUNY Downstate Medical Center Institutional Animal Care and Use Committee. In addition, all procedures were performed in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

2.2. Patch clamp electrophysiology

Pyramidal neurons were acutely dissociated from CA1 hippocampus and GABA-gated Cl^- currents analyzed by using whole cell patch clamp procedures, as previously described (Sundström-Poromaa et al., 2002). Briefly, tissue was digested at 32°C for 50–60 min under 100% O_2 in piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES)-buffered saline, containing sodium chloride [(NaCl) 120 mM], potassium chloride [(KCl) 5 mM], calcium chloride [(CaCl₂) 1 mM], magnesium chloride [(MgCl₂) 1 mM], D -glucose (25 mM), and PIPES 20 and trypsin (type XI; Sigma Chemical Co.) (0.8 mg/ml, pH 7.0). After a 1-h enzyme-free incubation at room temperature, tissue was dissociated by trituration in 1 ml of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Dulbecco's modified Eagle's medium (DMEM), which was replaced by recording medium after transfer to the recording chamber. The GABA-gated currents were recorded at room temperature (20°C–25°C) at a holding potential of –50 mV in a bath, containing NaCl (120 mM), cesium chloride [(CsCl) 5 mM], CaCl₂ (2 mM), MgCl₂ (1 mM), tetraethylammonium chloride [(TEA Cl) 15 mM], 4-aminopyridine (5 mM), HEPES (10 mM), and glucose (25 mM) (pH 7.4, 320 mOsm/kg H_2O). The pipette solution contained *N*-methyl- D -glucamine chloride (120 mM), cesium 4,1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetate [(Cs₄BAPTA) 5 mM], and magnesium (Mg)-ATP (5 mM). The ATP regeneration system Tris phosphocreatine (20 mM) and creatine kinase were added to minimize GABA rundown. All drugs were obtained from Sigma Chemical Co. except for BAPTA, which was obtained from Calbiochem (San, Diego, CA).

For the ethanol administration studies, drug delivery was accomplished with a superfusion system positioned within 50–100 μM of the cell, which yielded approximately 40- to 100-ms exposure times. Ethanol (3 or 20 mM) was applied concomitant with GABA ($\text{EC}_{20} = 10 \mu\text{M}$). Peak currents were calculated for all drug concentrations as relative to the current gated by 10 μM GABA alone by using the equation

$$([\text{peak current: GABA + ethanol}] - [\text{peak current: GABA alone}]) / [\text{peak current: GABA alone}].$$

Currents were recorded by using an Axopatch 1D amplifier (Axon Instruments, Union City, CA) filtered at 2 kHz (four-pole Bessel filter) and detected at 500 Hz (pClamp 5.1).

2.3. Acoustic startle response

Animals were tested in a stabilimeter device (SR-Lab Startle Response System; San Diego Instruments, Inc., San Diego, CA) that permits vertical movement of a Plexiglas platform contained within a lit, well-ventilated, sound-attenuating chamber (Gulinello et al., 2003b). The amplitude of movement is detected by a piezoelectric device with a voltage output proportional to platform displacement. The analog output of the accelerometer is subsequently amplified and digitized before computer analysis. Five minutes after intra-peritoneal administration of 10% ethanol (0.05–1.6 g/kg) or saline vehicle (1.5 ml volume) or 25 min after 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol injection (3 mg/kg, i.p.), animals were placed in the chamber for a 5-min acclimation period. The drug latencies were previously established in this laboratory (Gulinello et al., 2003a; Sundström-Poromaa et al., 2002), as well as in the laboratory of other investigators (Jones et al., 2000), as optimal for the anxiolytic effects of these two drugs, which have distinct pharmacokinetics. Four acoustic stimuli (116 dB, 40 ms duration) were subsequently presented at random intervals once every 10–15 s by means of a white-noise generator through speakers positioned on either side of the cage. The 40-ms, 116-dB acoustic stimulus has been demonstrated in the paradigm used in our laboratory (Gulinello et al., 2003b) as the minimum effective stimulus to produce a robust startle response in all animals. For each acoustic stimulus, the amplitude and integrated response (amplitude and duration) were determined and normalized to the weight of the animal. Values for the integrated response after administration of each dose of ethanol or 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol were averaged and expressed as a fraction of the saline control average for the indicated treatment (progesterone withdrawal versus control) group to minimize variability between groups. This paradigm has been used to demonstrate the anxiolytic effects of low doses of ethanol in alcohol-preferring (P) rats (Jones et al., 2000). It is also routinely used to assess behavioral excitability after withdrawal from benzodiazepines and ethanol (van Erp & Miczek, 2001).

2.4. Elevated plus-maze

The elevated plus-maze consists of four 10 × 50-cm arms at 90° angles, with two arms enclosed by 40-cm walls, and two open arms, all elevated 50 cm above the floor. The open arms are also partially bordered by small rails extending to the proximal half of the arm, and the floor of the maze is marked with grid lines every 25 cm. After a 30- to 45-min acclimation period, each animal was placed in the center of the maze 10 min after ethanol administration, and exploratory activity was recorded for 10 min. In some cases, animals were tested 10 min after rapid intraperitoneal insertion and removal of a needle coated with dimethylsulfoxide (DMSO) to replicate the aversive qualities of an ethanol injection. Both number of entries and duration of time spent in the open and closed arms were tabulated, as were the total arm entries, a measure of general activity level. To be considered an open arm entry, the animal had to cross the line of the open platform with all four paws. An increase

in open arm entries or time spent in the open arm versus the total time is considered to be a measure of decreased anxiety (Gulinello et al., 2003a).

2.5. Statistical analyses

Average values for ethanol-induced changes in peak GABA-gated current and the ASR were evaluated across drug-treatment groups by one way analysis of variance (ANOVA) with a post hoc Tukey test. Data obtained from the elevated plus-maze were analyzed in an ANOVA followed by post hoc *t* tests [Fisher protected least significant difference (PLSD)]. A *P* value of <.05 was used as an indication of statistical significance.

3. Results

3.1. Ethanol modulation of GABA-gated current

After progesterone withdrawal, GABA-gated responses of acutely isolated CA1 hippocampal pyramidal cells were potentiated by a mean $75\% \pm 10\%$ ($P < .01$) with a 3 mM concentration of ethanol (Fig. 1). In contrast, a higher (20 mM) concentration of the drug was ineffective in potentiating this parameter, whereas neither concentration (3 or 20 mM) produced an effect on GABA responses in cells obtained from control animals. These results extend findings described previously (Sundström-Poromaa et al., 2002) and support the suggestion that progesterone withdrawal produces a state in which GABA_A receptors are more responsive to very low, but not higher, concentrations of ethanol.

3.2. Effects of ethanol on the acoustic startle response

To compare the dose dependence of ethanol effects in a behavioral paradigm, ethanol was tested across a dose range (0.05–1.6 g/kg, i.p.) for its effect in modulating the ASR, a measure of behavioral excitability, which is increased after progesterone withdrawal (Gulinello et al., 2003b). To this end, animals were injected with ethanol or saline vehicle 5 min before placement in an acoustically isolated startle chamber. After an additional 5-min acclimation period, the startle responses to four 116-dB acoustic stimuli were averaged and adjusted for the weight of the animal. Both average peak and integrated startle responses were significantly ($P < .05$) attenuated by about 70% with very low (0.2–0.4 g/kg) doses of ethanol after progesterone withdrawal, an effect not observed in diestrous control animals or in sham control animals (Fig. 2A and 2B). Higher doses (0.8–1.2 g/kg) of the drug, however, were ineffective in altering the ASR in progesterone-withdrawn animals, but they significantly decreased the ASR in both control groups ($P < .05$). The highest dose tested (1.6 g/kg) decreased the ASR in both progesterone-withdrawn and diestrous control animals to a similar degree, whereas the lowest dose tested (0.05 g/kg) was equally ineffective in these two groups. These results support the suggestion that the dose dependence of the anxiolytic effects of ethanol after progesterone withdrawal is similar to that reported from previous work in our laboratory for the physiologic effects of this drug on GABA-gated current (Sundström-Poromaa et al., 2002), with very low doses being highly effective, but higher doses being ineffective, in reducing the ASR.

Capsule implantation was not responsible for the altered response to ethanol, as both diestrous (unimplanted) control animals and sham (implanted with an empty capsule)

control animals reacted in a similar manner, with 0.8 g/kg of ethanol being effective, but 0.2 g/kg of ethanol being ineffective, in decreasing the peak ASR (Fig. 2A and 2B). The two control groups also exhibited similar levels of anxiety, assessed by nearly identical mean ASR scores. In contrast, the progesterone-withdrawn animals had significantly higher ($P < .01$) baseline ASR scores after saline injection compared with findings for the control groups (integrated ASR, $1,165 \pm 120$, progesterone-withdrawn animals vs. 485.4 ± 114 , diestrous control animals, 433.5 ± 56 , sham control animals).

3.3. Effects of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol on acoustic startle response

Because it has been demonstrated through work in our laboratory that $\alpha_4\beta\delta$ subunit combinations of the GABA_A receptor exhibit a unique sensitivity to very low concentrations of ethanol, as well as that expression of this GABA_A receptor isoform is increased after progesterone withdrawal (Sundström-Poromaa et al., 2002), we tested the effects of a GABA partial agonist, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, which has greater potency at $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor than at the predominant native GABA_A receptor isoforms expressed in CA1 hippocampus (Brown et al., 2002). In this case, either 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol or vehicle was administered 30 min before ASR testing. A 3-mg/kg intraperitoneal dose of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol ($P < .05$) attenuated the ASR by 70% in progesterone-withdrawn animals, but it exerted the opposite effect in control animals, increasing the ASR by about 40% ($P < .05$; Fig. 2). These results are consistent with the observation that $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor are increased after progesterone withdrawal, and they support the suggestion that activation of this receptor subunit combination produces an effect similar to that of a low dose of ethanol.

3.4. Effects of ethanol on elevated plus-maze

It has been demonstrated through work in our laboratory that the anxiety state triggered by progesterone withdrawal results in a decreased number of open arm entries as well as time spent in the open arm of the elevated plus-maze compared with findings for sham control animals (Gulinello et al., 2003a). With the use of this paradigm, a low dose (0.5 g/kg) of ethanol significantly ($P < .05$) increased the time in the open arm more than 20-fold and also increased the percentage of open arm entries by sevenfold compared with findings for vehicle treatment in progesterone-withdrawn animals (Fig. 3) (These values are threefold and 50% greater than those observed for control animals, respectively, after ethanol administration.) In contrast, this dose of the drug produced no significant effect on these parameters tested in control animals. Locomotor activity was unaffected by this dose of ethanol in the study, as assessed by the total number of arm entries, which was not significantly altered in either group of animals tested (Fig. 3). Therefore, these results support the suggestion that, consistent with the results from the ASR study, a low dose of ethanol, which is ineffective in reducing anxiety in control animals, has significant anxiolytic effects after progesterone withdrawal.

An additional study was conducted to test for the possibility that the stress produced by an ethanol injection was different in control than in progesterone-withdrawn animals. To this end, a separate group of animals was tested on the elevated plus-maze after rapid insertion

of a DMSO-coated needle to simulate the irritation produced by ethanol injection. There was no significant change in open arm entries in either group: This stimulus decreased open arm entries by $10\% \pm 4\%$ for the control group and by $8\% \pm 3.6\%$ for the progesterone-withdrawn group. Closed arm entries were decreased by $31\% \pm 11.5\%$ for the control animals, but increased $30\% \pm 14\%$ for the progesterone-withdrawn animals, changes that were not significant. In addition, the stimulus produced no significant change in locomotor activity, increasing total arm entries by $18\% \pm 9\%$ (control group) and $28\% \pm 12\%$ (progesterone-withdrawn group).

4. Discussion

Results from the current study demonstrate that very low doses of ethanol attenuate the ASR and decrease anxiety in the elevated plus-maze during a progesterone withdrawal model of premenstrual anxiety in the rodent. This anxiolytic action of ethanol was seen only for very low, but not higher, doses, supporting the suggestion of an inverted U dose-response relation. These results may have relevance for PMS, in which an increased response to a low dose of ethanol has been reported (Sundström et al., 1998).

The inverted U ethanol dose-response relation observed in the current study is characteristic of $\alpha_4\beta\delta$ subunit combinations of the GABA_A receptor (Sundström-Poromaa et al., 2002), which are increased after progesterone withdrawal, but it is not characteristic of other receptors or mechanisms, such as phosphorylation state (Choi et al., 2002) and neurosteroid release (VanDoren et al., 2000). A similar concentration-response relation was observed in isolated neurons after progesterone withdrawal (Sundström-Poromaa et al., 2002), an effect that depends on expression of α_4 subunits of the GABA_A receptor. Taken together, these findings support the notion that the $\alpha_4\beta\delta$ subunit isoform of the GABA_A receptor is likely to mediate the concentration-dependent effect of ethanol in the current study. The $\alpha_4\beta\delta$ subunit isoforms of the GABA_A receptor are localized perisynaptically (Wei et al., 2003), where they produce a tonic current (Brickley et al., 2001) under conditions of transmitter spillover (Wei et al., 2003), which can be increased by GABA modulators such as neurosteroids (Stell et al., 2003). Potentiation of this current by a low dose of ethanol after progesterone withdrawal, when these receptors increase, would act as a resistive shunt to limit neuronal excitability, and it may produce the anxiolytic effects we have reported in this article.

Other investigators have suggested that there is a link between δ or α_4 GABA_A receptor subunit expression and ethanol effects. Transgenic mice lacking expression of the δ subunit have reduced responsiveness to ethanol, as well as reduced consumption of the drug (Mihalek et al., 2001). In addition, the anxiolytic effects of ethanol are greater on proestrus (Brot et al., 1995), when hormone levels are high and α_4 expression is increased (unpublished observations, F.-C. Hsu, Y. Ruderman, and S. S. Smith, 2000).

Biphasic effects of ethanol have often been reported in the literature [see Aguayo et al. (2002) for review]. The most parsimonious explanation for the inverted U effect of ethanol reported in this article is that ethanol is acting at two sites on $\alpha_4\beta\delta$ subunit combinations of the GABA_A receptor: a high affinity potentiating site and a low affinity inhibitory site. Inhibitory effects of ethanol have been identified on other atypical GABA receptors, such as

the homomeric rho 1 receptor (Mihic & Harris, 1996), where mutations of the TM2-TM3 region abolish the effect (Mihic et al., 1997), supporting the suggestion of a structural mechanism for such a hypothesis. In contrast, this biphasic effect of ethanol may be due to effects of higher doses of ethanol (10 mM) directed at other receptors. Alternatively, inhibition of interneuron activity by higher doses of ethanol (Carta et al., 2003) could also produce a biphasic effect.

The effect of very low doses of ethanol in attenuating the ASR in progesterone-withdrawn animals was paralleled by a similar effect of 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, a GABA partial agonist with greater potency at $\alpha_4\beta_2\delta$ subunit isoforms of the GABA_A receptor than at other isoforms (Brown et al., 2002; Sundström-Poromaa et al., 2002). Thus, these results also support the idea that anxiolytic effects of a low dose of ethanol target $\alpha_4\beta_2\delta$ subunit combinations of the GABA_A receptor. In contrast, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol and low doses of ethanol exerted potentiating effects on the ASR in control animals. This effect may be due to disinhibition of limbic interneurons, which express δ -containing GABA_A receptors (Peng et al., 2002). Low concentrations of ethanol, close to the range reported in this article, exert excitatory effects in hippocampus by inhibiting kainate receptor activation of GABAergic interneurons (Carta et al., 2003). Excitatory effects of a low dose of ethanol have been reported in other areas such as cortex (Chapin et al., 1986).

Indirect mechanisms related to the stress of injection are unlikely to be implicated in the current study because the aversive stimulus tested did not produce different effects in control and progesterone-withdrawn animals. Results of earlier work from our laboratory (Gallo & Smith, 1993) showed that a stronger aversive stimulus (electrified prod) triggered a greater reaction in animals withdrawing from progesterone than it did in control animals. Thus, on the basis of these findings, the stress produced by an ethanol injection should have increased peak ASR in the progesterone-withdrawn animals, rather than in the control animals. When these findings are considered together, the excitatory effect of a low dose of ethanol in control animals is more likely due to the pharmacological effects of ethanol in CNS circuits, rather than to a stress response.

The progesterone withdrawal paradigm used in our laboratory may serve as a rodent model of PMS. It is estimated that up to 70% of the female population exhibits mild to moderate dysphoria, including anxiety and irritability, during the late luteal phase when circulating levels of progesterone and its GABA-modulatory metabolite 3 α ,5 α -THP decline (Rapkin et al., 1997). Decreases in circulating 3 α ,5 α -THP levels have been correlated with adverse mood (Rapkin et al., 1997), supporting the notion that the decline in this steroid level precipitates PMS symptoms. Similarly, in the progesterone withdrawal paradigm, anxiety and behavioral reactivity occur in correlation with declining levels of 3 α ,5 α -THP (Gulinello et al., 2003a, 2003b; Smith et al., 1998a, 1998b). Systemic administration of progesterone is a physiologic means to increase CNS levels of 3 α ,5 α -THP, as this conversion occurs in the CNS (Corpechot et al., 1993). However, results of early work from our laboratory established that withdrawal effects of progesterone were not observed if 3 α ,5 α -THP formation was prevented (Smith et al., 1998a) and could be replicated by a direct withdrawal from 3 α ,5 α -THP in the presence of high progesterone levels (Smith et al., 1998b). Thus, the

hormonal and behavioral changes that are produced by progesterone withdrawal are characteristic of PMS.

Many of the pharmacological changes that occur are also common to both progesterone withdrawal and PMS. The increase in expression of the α_4 subunit of the GABA_A receptor produced by steroid withdrawal resulted in a relative insensitivity to the anxiolytic and GABA-modulatory effects of a benzodiazepine (Smith et al., 1998a), consistent with the characterization of GABA_A receptors containing this subunit as benzodiazepine-insensitive (Wisden et al., 1992). A corresponding benzodiazepine insensitivity has also been reported in women with PMS (Sundström et al., 1997). In addition, both progesterone withdrawal and PMS produce a reduced sensitivity to 3 α ,5 β -THP (Smith et al., 1998a; Sundström et al., 1998). Results from the current study demonstrate a unique sensitivity of female rats to very low doses of ethanol, which reduce behavioral reactivity. The effective doses are equivalent to those that were shown to produce changes in saccade velocity and relaxation in women with PMS (Sundström et al., 1998), but that were ineffective in control subjects. Thus, results from the current study support the notion that sensitivity to very low doses of ethanol is an additional feature common to both progesterone withdrawal and PMS.

Increased responsiveness to a low dose of ethanol has also been demonstrated in the P rat, a genetic strain that uniquely self-administers ethanol. Low doses of ethanol, in the same range as used in the current study, have been reported to reduce the ASR for these animals (Jones et al., 2000), whereas higher doses were ineffective. In addition, people at risk for the development of alcoholism also exhibit increased sensitivity to low blood ethanol levels, but they are less responsive to higher levels compared with findings for normal control subjects (Cohen et al., 1993), similar to the inverted U dose-response pattern observed after progesterone withdrawal.

In conclusion, results from the current study support the suggestion that, in a rodent model of premenstrual anxiety, very low doses of ethanol, equivalent to 1 to 2 oz of wine (Kalant et al., 1975), are anxiolytic. This effect is consistent with the increase in expression of $\alpha_4\beta\delta$ subunits of the GABA_A receptor observed at this time, supporting the idea of these receptors as a likely target for ethanol effects (Sundström-Poromaa et al., 2002). These findings may be relevant for PMS, when increased consumption of ethanol has been reported.

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Fig. 1.

A low concentration of ethanol potentiates gamma-aminobutyric acid (GABA)-gated current after progesterone withdrawal. The effects of ethanol on GABA (EC_{20})-gated current are depicted for two concentrations (3 or 20 mM) relative to current gated by GABA alone after progesterone withdrawal (P Wd) or control conditions. At 3 mM ethanol, GABA-gated current, recorded from pyramidal cells acutely dissociated from CA1 hippocampus after a 24-h period of progesterone withdrawal, was potentiated by 75%. Current was recorded at a -50 mV holding potential by using whole cell patch clamp techniques. In contrast, a higher concentration of ethanol (20 mM) was ineffective in potentiating GABA-evoked current. Neither concentration (3 or 20 mM) was effective when tested on cells from control animals. ($n = 10$ – 15 cells recorded from 5 or 6 rats per group; $*P < .01$ vs. GABA alone.)

Fig. 2.

A low dose of ethanol decreases the peak acoustic startle response after progesterone withdrawal. A. The maximum amplitude of the acoustic startle response [(peak ASR) y axis] is calculated after intraperitoneal injection of either saline vehicle or increasing doses of ethanol or 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) to animals after progesterone withdrawal (P Wd) or in diestrus (Con). In progesterone-withdrawn animals, ethanol, at a dose of 0.2 or 0.4 g/kg, significantly reduced the ASR compared with saline control values, whereas higher doses were ineffective until the 1.6-g/kg dose. In contrast,

these low doses were ineffective in control animals, whereas the threshold dose for this effect was 0.8 g/kg. 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, which has increased potency at $\alpha_4\beta\delta$ subunit combinations of the GABA_A receptor, produced a similar decrease in ASR only in progesterone-withdrawn animals. Results are expressed relative to the saline control animals for each group. B. Comparison of the effects of ethanol in sham control animals, where 0.8 g/kg, but not 0.2 g/kg, of ethanol (ETOH) was effective in decreasing the peak ASR. Results are expressed relative to the diestrous control animals. Panels A and B: $n = 5-8$ animals per group; $*P < .05$ vs. the respective control.

Fig. 3.

Alow dose of ethanol increases time in the open arm of the elevated plus-maze after progesterone withdrawal. Both time in the open arm (top panel) and percentage (%) of open arm entries (middle panel) were significantly increased by a 0.5-g/kg dose of ethanol in animals undergoing progesterone withdrawal (P Wd). This dose was ineffective in control animals (Con). In contrast, this dose of ethanol did not alter the total arm entries (bottom panel), a measure of general locomotor activity, in either group of animals tested. ($n = 7-10$ animals per group; $*P < .0001$ vs. Con; $**P < .0001$ vs. vehicle.) [Note: A needle stick

(precoated with dimethylsulfoxide) did not significantly alter time in the open arm in either Con or P Wd animals ($n = 5$ animals per group).]