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Chronic progesterone treatment augments while dehydroepiandrosterone sulphate prevents tolerance to ethanol anxiolysis and withdrawal anxiety in rats

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Abstract

We have recently shown that the neurosteroid allopregnanolone modulates anxiolytic effect of ethanol. In the present report, we attempted to examine whether neurosteroids progesterone and dehydroepiandrosterone sulphate (DHEAS), which modulate γ-aminobutyric acid ($GABA_A$) receptor function, affects development of tolerance to ethanol anxiolysis and withdrawal anxiety. Rats on ethanol (6% v/v in nutritionally balanced liquid diet) for prolong period (10 days) were injected twice daily either with vehicle, progesterone (a precursor of allopregnanolone, positive $GABA_A$ receptor modulator), finasteride (5α-reductase inhibitor) or DHEAS (negative $GABA_A$ receptor modulator). During this period, rats were acutely challenged periodically with ethanol (2 g/kg, i.p., 8% w/v) and subjected to the elevated plus maze test. For withdrawal studies, similar treatment protocols (except ethanol challenge) were employed and on day 11, rats were subjected to the elevated plus maze test at different time intervals post-ethanol withdrawal. While progesterone significantly advanced the development of tolerance to ethanol anxiolysis and enhanced withdrawal anxiety, DHEAS and finasteride prevented such behavioral alterations. These data highlight the important role played by GABAergic neurosteroids progesterone and DHEAS in the development of tolerance to ethanol anxiolysis and withdrawal anxiety in rats. Moreover, it points to the potential usefulness of specific neurosteroids as targets in the treatment of alcoholism.

Keywords: Neurosteroids; Allopregnanolone; DHEAS; $GABA_A$ receptor; Ethanol tolerance; Withdrawal anxiety

1. Introduction

Prolonged ethanol (alcohol) consumption leads to the development of tolerance and dependence. This induces long lasting changes in the brain neuronal systems leading to adverse behavioral consequences. Considerable evidences suggest that the anxiolytic effect of ethanol may be one of the factors that promote alcohol consumption (Henniger et al., 2002; Langen and Fink, 2004). Anxiety is one of the most prominent subjective effects associated with ethanol dependence (Baldwin et al., 1991; Koob, 2000). Although ethanol has many sites of action in the central nervous system, recently effect on multiple receptors operated ion channels including γ-aminobutyric acid ($GABA_A$) receptors, *N*-methyl-d-aspartate (NMDA) receptors, 5-hydroxytryptamine ($5HT_3$) receptors and voltage sensitive calcium ion (Ca^{2+}) channels (Ollat et al., 1988; Morrow et al., 1990; Rossetti and Carboni, 1995; Devaud et al., 1997; Biggio et al., 2003; Krystal et al., 2003) have been demonstrated. However, it is doubtful whether all these contribute to the ethanol reinforcement. GABAergic adaptations were considered as one of the important basis in the neurobiology of ethanol dependence and withdrawal (Eravci et al., 2000; Alele and Devaud, 2005). A number of preclinical studies have advocated

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the role of neurosteroids in the complex interaction of ethanol with $GABA_A$ receptor system (Morrow et al., 1998; Barbaccia et al., 1999; Vanover et al., 1999; VanDoren et al., 2000; Hirani et al., 2002, 2005; Khisti et al., 2002, 2003). Several effective treatments for anxiety target the primary inhibitory neurotransmitter GABA and modulate the overall effect of GABAergic system.

Neurosteroids can be synthesized de novo in the brain independent of steroidogenic endocrine glands (Corpechot et al., 1981). They interact with targets within the family of ligand-gated ion channels mentioned above and rapidly alter the neuronal excitability (Paul and Purdy, 1992; Lambert et al., 1995). In variety of physiological conditions like anxiety, stress, stages of estrous cycle, pregnancy, aggressive behavior and seizures, pharmacologically relevant concentrations of some neurosteroids including allopregnanolone (3α, 5α-tetrahydroprogesterone) have been detected in brain (Bicikova et al., 1998, 2000; Herbison, 2001; Reddy and Rogawski, 2001). At this concentration, allopregnanolone and related neurosteroids can modulate the expression and function of $GABA_A$ receptor (Follesa et al., 2004; Birzniece et al., 2006). Administration of these neurosteroids exhibits myriad of behavioral effects including anxiolysis, antistress, sedation, analgesia, antiseizure, antidepressant and cognitive impairment in rodents (Zimmerberg and Blaskey, 1998; Khisti and Chopde, 2000; Khisti et al., 2000; Hirani et al., 2002, 2005). Acute ethanol administration differentially modifies cerebral concentrations of neurosteroids, typically increasing allopregnanolone concentration (Morrow et al., 1999; VanDoren et al., 2000) and preferentially reducing dehydroepiandrosterone sulphate (DHEAS) levels (Baulieu and Robel, 1996). The neurosteroid allopregnanolone is a potent endogenous positive allosteric modulator of $GABA_A$ receptor, whereas DHEAS is a negative modulator (Majewska et al., 1990; Majewska, 1992). Pretreatment with finasteride, an inhibitor of enzyme 5α-reductase, which is the rate-limiting enzyme in the biotransformation of progesterone to allopregnanolone, attenuates several effects of ethanol (Morrow et al., 1999; VanDoren et al., 2000) indicating that allopregnanolone can modulate sensitivity to some but not all effects of ethanol. We have demonstrated earlier that allopregnanolone accounts for antidepressant and anxiolytic actions of ethanol (Hirani et al., 2002, 2005). Allopregnanolone has been shown to heighten ethanol reinforcement (Nie and Janak, 2003) and self-administration (Janak et al., 1998). However, Morrow et al. (2001) demonstrated that allopregnanolone administration reduces ethanol consumption in dependent rats.

In contradiction to acute ethanol, rats dependent on ethanol exhibited reduced cerebral levels of allopregnanolone and withdrawal from ethanol normalized cortical levels to control values (Janis et al., 1998). Chronic ethanol exposure influences the orchestration of $GABA_A$ receptor subunits and also its functional properties (Morrow et al., 1990; Mhatre et al., 1993; Devaud et al., 1997). Similar to $GABA_A$ receptor modulators (Holt et al., 1996; Devaud et al., 1997; Mahmoudi et al., 1997; Follesa et al., 2001; Cagetti et al., 2003), repeated administration of neurosteroids or withdrawal can regulate specific α_4 subunit expression of the $GABA_A$ receptor (Smith et al., 1998a,b; Follesa et al., 2001). Such increased α_4 subunit peptide expression and mRNA levels were also observed in cerebral region of the brain in ethanol-dependent and withdrawn animals (Devaud et al., 1995, 1997; Follesa et al., 2002; Cagetti et al., 2003) and this subunit is insensitive to benzodiazepines (Barnard et al., 1998). Recently it has been shown that the variations in gene GABRA2, encoding the α_2 subunit of $GABA_A$ receptor, influence susceptibility to alcohol dependence by modulating the level of neural excitation (Edenberg et al., 2004).

In view of close relationship between the endogenous neurosteroid levels in central nervous system and the behavioral actions of ethanol, we examined whether neurosteroids that cause positive or negative modulation of $GABA_A$ receptors, influence tolerance to anxiolytic effect of ethanol and withdrawal anxiety. Here we report that chronic administration of progesterone, the precursor of allopregnanolone and the positive allosteric modulator of $GABA_A$ receptor promotes the development of tolerance to ethanol anxiolysis and withdrawal anxiety. In contrast, DHEAS, the negative $GABA_A$ receptor modulator or finasteride, an inhibitor for conversion of progesterone to allopregnanolone, prevents these effects.

2. Materials and methods

2.1. Subjects

Subjects were Sprague–Dawley rats, born and reared in the animal house of Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University from a stock originally purchased from National Institute of Nutrition, Hyderabad, India. Young healthy male rats (220–250 g body weight and 80–90 days old) were group housed (five per cage) in opaque polypropylene cages (640×410×250 mm) and maintained at 24±1 °C under 12:12 h light/dark cycle (lights on 0700–1900 h) with rodent chow (Lipton, India) and water ad libitum. Animals were brought to the experimental room and were housed individually 12 h prior to treatments to minimize nonspecific stress-induced steroid changes. The experimental procedures were in strict accordance with the guidelines approved by the Institutional Experimental Animal Ethical Committee of Rashtrasant Tukadoji Maharaj Nagpur University, Department of Pharmaceutical Sciences, Nagpur, India. Animals were naïve to drug treatment and experimentation at the beginning of all studies. Each experimental group had a separate set of 6–7 animals.

2.2. Drugs

The drugs used were DHEAS, progesterone (RBI, USA), finasteride (Dr. Reddy's Laboratories, Hyderabad, India) and absolute alcohol (MSSIDC, Mumbai, India). Finasteride, DHEAS and progesterone were dissolved in 2-hydroxypropyl-β-cyclodextrin (45% w/v) solution and diluted with 0.9% saline. All drugs were injected intraperitoneally (i.p.), with the exception of finasteride that was given by the subcutaneous (s.c.) route. Ethanol was diluted with 0.9% saline to a concentration of 8% w/v for i.p. injection or to 6% v/v in a liquid diet for oral chronic consumption.

2.3. Elevated plus maze test

This test exploits the conflict behavior between exploration of a novel area and aversion to open areas and heights. The plus maze was made up of Plexiglas painted black. The plus maze consisted of opposite facing two open (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) connected by a central platform (10 × 10 cm). The whole maze was raised 60 cm above the floor. Rats were tested on the plus maze in a room with low, indirect incandescent lighting (100-W lamp, fixed 2 m above the maze floor) and very low noise levels. On the day of testing, rats were placed singly at the center of the maze, head facing an open arm and allowed to explore for 5 min. The number of entries into and time spent in each arm was recorded by an observer blind to the treatment. An entry was registered only when all four paws of the animal were placed on the arm. The maze was wiped clean with damp cotton and dried after testing each rat. Anxiogenic or anxiolytic effects were assessed based on the frequency of open arms entries as well as time spent in the open arms (Pellow et al., 1985). Decrease in time spent on the open arms and a low frequency of open arms entries relative to control animals was considered as an increase in anxious behavior. Separate groups of animals were used for each treatment and each subject tested was given a single 5 min trial. All animals were tested between 0900–1400 h to minimize circadian influences.

2.4. Chronic ethanol administration

Ethanol was administered to rats for prolonged period as described previously with some modifications (Miller et al., 1980; Lal et al., 1988; Jung et al., 2000; Hirani et al., 2002; Kokare et al., 2006). Briefly, rats were assigned to different treatment groups and housed individually in polypropylene cages. Initially they received nutritionally balanced liquid diet (Dextrose diet, ICN Biochemicals) for two days to allow adaptation to novel food. Water was available ad libitum. From third day onwards, ethanol was added to the liquid diet of some groups (final concentration 6% v/v, ethanol-fed), while isocaloric amount of ethanol was substituted with dextrose in the liquid diet of remaining groups (pair-fed control) and had free access to it for 1, 3, 5, 7 or 10 days. Fresh aliquot (100 ml/rat) of ethanol containing liquid diet was introduced in the respective cages each morning at 0800 h. Diet of pair-fed groups was unchanged but was restricted so that consumption matched the mean amount of the ethanol-containing diet consumed. Rats having access to ethanol diet for 10 days were discontinued from ethanol on 11th day morning (0800 h) and received dextrose containing diet until the termination of the experiment (ethanol withdrawal).

The dietary consumption and body weight of each animal was monitored daily (0800 h) for all the groups. The average daily ethanol consumption was found to be 10.93 ± 0.32 g/kg. Body weights of ethanol-fed rats were not different in comparison to pair-feds during initial drinking phase or throughout the course of the experiment. The animals were subjected for 5 min to elevated plus maze test at different time intervals, but individual animal was tested only once to avoid 'one trial tolerance' to drug effect (Bertoglio and Carobrez, 2002).

2.5. Tolerance to ethanol anxiolysis

These experiments were designed to examine the role of neurosteroids in the development of tolerance to anxiolytic effect of ethanol. Animals were randomly assigned to different treatment groups. During chronic ethanol studies, ethanol-fed as well as pair-fed rats were injected twice daily (1000 h and 2200 h) either with vehicle (5 ml/kg, i.p. or 1 ml/kg, s.c.), progesterone (5 mg/kg, i.p.), DHEAS (5 mg/kg, i.p.) or finasteride (25 mg/kg, s.c.) for 10 days. On days 1, 3, 5, 7 or 10 (0900 h), some of these rats were challenged with ethanol (2 g/kg, i.p., 8% w/v). Thirty minutes thereafter, individual rat was subjected to elevated plus maze test for 5 min session. Separate groups of animals were employed for different experimental days and the group once challenged with ethanol and tested for anxiety parameters, was discontinued from further studies. The doses of neurosteroids or their modulators used were based on preliminary experiments and previous reports in literature (VanDoren et al., 2000; Khisti et al., 2002; Reddy, 2003; Ren et al., 2004) such that they effectively modulate endogenous neurosteroid levels without causing motor dysfunction or affecting anxiety parameters.

2.6. Withdrawal anxiety

For this study, separate groups of animals were used. During 10 days chronic ethanol consumption, the ethanol-fed as well as pair-fed groups were injected with progesterone, DHEAS, finasteride or vehicle twice daily as per the protocol described above. However, they were not challenged with i.p. ethanol during 10 days program. On day 11 mornings (0800 h), ethanol liquid diet was discontinued and replaced with control dextrose containing liquid diet. At 1, 4, 8, 12 or 18 h post-withdrawal, ethanol naïve (pair-fed groups) as well as ethanol-dependent rats were subjected to elevated plus maze test. The time spent in open arms and entries into open and closed arms were recorded for 5 min.

2.7. Data analysis

The results are presented as means ± S.E.M. and analyzed by Graphpad Prism 3.02. Data obtained from chronic ethanol treatment and combination treatments of neurosteroid modulators with ethanol were analyzed by two-way analysis of variance (ANOVA). Post-hoc comparisons were performed using Bonferroni test. Value of $P < 0.05$ was considered significant.

3. Results

3.1. Tolerance to ethanol-induced anxiolysis

As shown in Fig. 1, significant anxiolytic-like effect was observed in pair-fed groups challenged with ethanol (2 g/kg, i.p.,

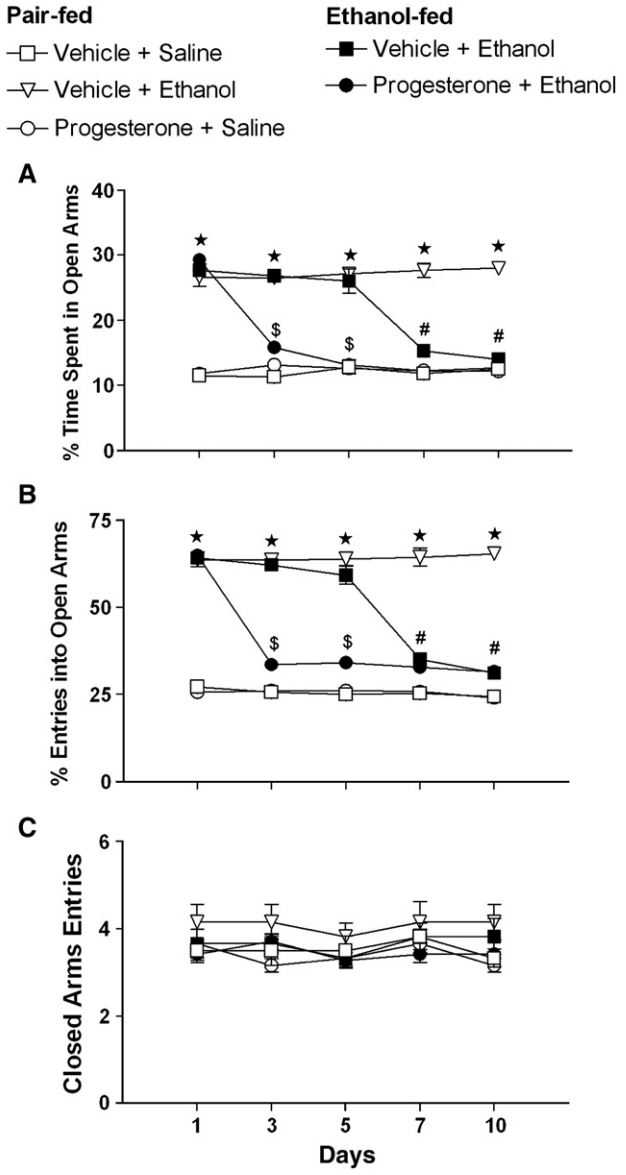

Fig. 1. Effect of daily administration of progesterone on the development of tolerance to ethanol-induced anxiolysis showing (A) % time spent in open arms, (B) % entries into open arms and (C) number of closed arms entries in elevated plus maze test. Several groups of rat consuming control liquid diet (pair-fed groups) or ethanol (6% v/v) containing liquid diet (ethanol-fed groups) were treated (twice daily 1000 and 2200 h) with vehicle (5 ml/kg, i.p.) or progesterone (5 mg/kg, i.p.) for 10 days. Rats were challenged i.p. on days 1, 3, 5, 7 or 10 with ethanol (2 g/kg, 8% w/v) or saline (5 ml/kg, i.p.) and 30 min thereafter individual rat was placed on the central platform to explore the elevated plus maze for 5 min. Each bar represents means±S.E.M. of data from 6–7 rats per group. *$P<0.05$ vs. vehicle+saline (pair-fed group); $^{\#}P<0.05$ vs. vehicle+ethanol (pair-fed group); $^{\$}P<0.05$ vs. vehicle+ethanol (ethanol-fed group) (two-way ANOVA followed by Bonferroni test).

8% w/v) on days 1, 3, 5, 7 and 10 of liquid diet consumption as compared to pair-fed rats with saline treatment (5 ml/kg, i.p.). In particular, two-way ANOVA indicated significant effect of factor 'treatment' [% time spent into open arms F (1, 50)= 696.2, $P<0.0001$) and % entries into open arms F (1, 50)= 1544, $P<0.0001$] but not of factor 'day' ($P>0.05$) and interaction between factors 'treatment × day' ($P>0.05$) on open arms indices. However, on days 7 and 10 in ethanol-fed rats (6% v/v), significant reduction in % time spent into open arms [factor 'treatment' F (1, 50)=59.81, $P<0.0001$, factor 'day' F (4, 50)=16.32, $P<0.0001$ and interaction 'treatment × day' F (4, 50)=23.34, $P<0.0001$] and % entries into open arms [factor 'treatment' F (1, 50)=154.1, $P<0.0001$, factor 'day' F (4, 50)=37.54, $P<0.0001$ and interaction 'treatment × day' F (4, 50)=44.48, $P<0.0001$] was observed as compared to pair-fed rats with ethanol challenge (2 g/kg, i.p., 8% w/v) indicating tolerance to ethanol's anxiolytic action. There was no significant change in closed arms entries during these treatment schedules ($P>0.05$).

3.2. Chronic progesterone administration facilitated development of tolerance to ethanol anxiolysis

As depicted in Fig. 1, daily progesterone administration (5 mg/kg, i.p.; 1000 and 2200 h) to ethanol-fed groups (6% v/v) resulted in early development of tolerance to antianxiety action of ethanol (2 g/kg, i.p., 8% w/v). Significant reduction in % time spent [factor 'treatment' F (1, 55)=105.2, $P<0.0001$, factor 'day' F (4, 55)=114.7, $P<0.0001$ and interaction 'treatment × day' F (4, 55)=29.53, $P<0.0001$] and % entries [factor 'treatment' F (1, 55)=217.4, $P<0.0001$, factor 'day' F (4, 55)= 252.5, $P<0.0001$ and interaction 'treatment × day' F (4, 55)= 76.71, $P<0.0001$] into open arms was observed on days 3 and 5 in daily progesterone treated ethanol-fed groups as compared to respective vehicle treated ethanol-fed groups. Progesterone treatment at the dose used here per se did not alter open arms activity ($P>0.05$). However, there was no significant change in entries into closed arms ($P>0.05$) at any of the treatment days.

3.3. Chronic DHEAS or finasteride administration prevented development of tolerance to ethanol-induced anxiolytic effect

Daily DHEAS injections (5 mg/kg, i.p., 1000 and 2200 h) to ethanol-fed rats (6% v/v) prevented tolerance to ethanol (2 g/kg, i. p., 8% w/v)-induced anxiolysis. Two-way ANOVA followed by Bonferroni test revealed significant increase in % time spent [factor 'treatment' F (1, 50)=59.72, $P<0.0001$, factor 'day' F (4, 50)= 32.30, $P<0.0001$ and interaction 'treatment × day' F (4, 50)= 12.40, $P<0.0001$] and % entries into open arms [factor 'treatment' F (1, 50)=352.7, $P<0.0001$, factor 'day' F (4, 50)=110.2, $P<0.0001$ and interaction 'treatment × day' F (4, 50)=144.1, $P<0.0001$] with acute ethanol challenge (2 g/kg, i.p.) in DHEAS injected ethanol-fed rats as compared to ethanol-fed rats on days 7 and 10 (Fig. 2). Similarly, as shown in Fig. 3, daily treatment with finasteride (25 mg/kg, s.c.; 1000 and 2200 h) to ethanol consuming rats altered open arms activity, as significant increase in % time spent [factor 'treatment' F (1, 50)= 51.13, $P<0.0001$, factor 'day' F (4, 50)=36.19, $P<0.0001$ and interaction 'treatment × day' F (4, 50)=16.11, $P<0.0001$] and % entries into open arms [factor 'treatment' F (1, 50)= 159.2, $P<0.0001$, factor 'day' F (4, 50)=103.8, $P<0.0001$ and interaction 'treatment × day' F (4, 50)=57.29, $P<0.0001$] was

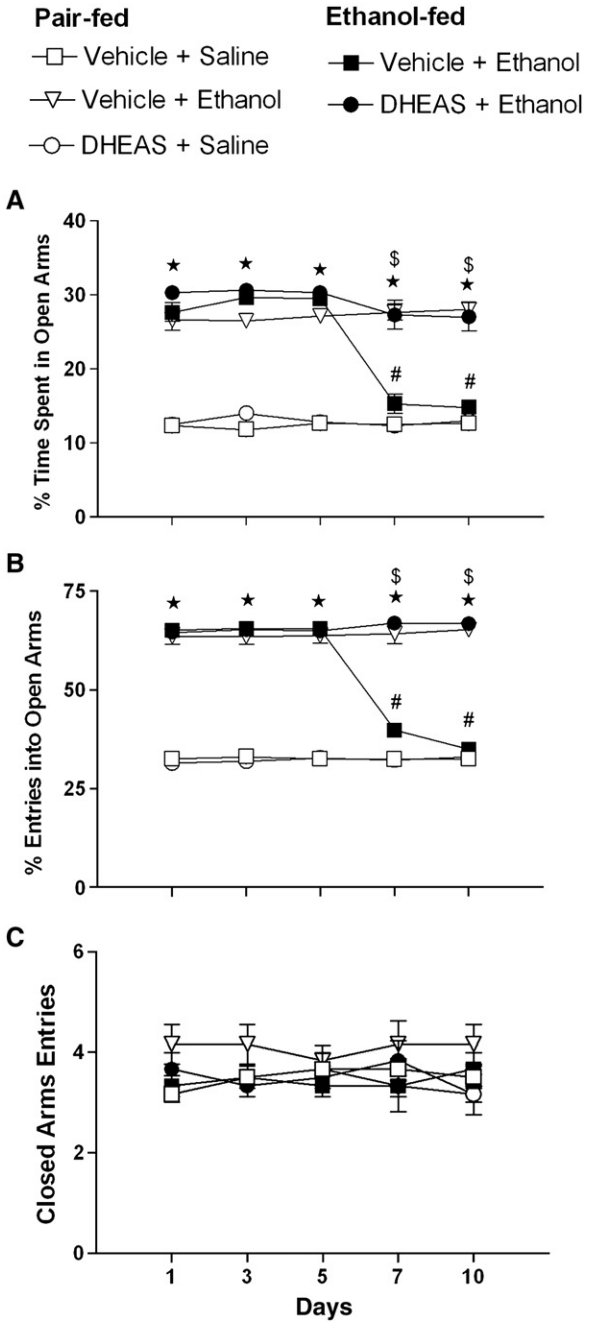

Fig. 2. Effect of daily administration of DHEAS on the development of tolerance to ethanol-induced anxiolysis showing (A) % time spent in open arms (B) % open arms entries and (C) number of closed arms entries. Different groups of rats were treated either with DHEAS (5 mg/kg, i.p.) or vehicle control (5 ml/kg, i.p.) (twice daily 1000 and 2200 h) consuming ethanol (6% v/v) containing liquid diet (ethanol-fed groups) or control liquid diet (pair-fed groups) for 10 days. Rats were challenged on days 1, 3, 5, 7 or 10 with ethanol (2 g/kg, i.p.) and 30 min thereafter, individual rat was placed on the central platform to explore the elevated plus maze for 5 min. Each bar represents means ± S.E.M. of data from 6 rats per group. $^{*}P<0.05$ vs. vehicle + saline (pair-fed group); $^{\#}P<0.05$ vs. vehicle + ethanol (pair-fed group); $^{\$}P<0.05$ vs. vehicle + ethanol (ethanol-fed group) (two-way ANOVA followed by Bonferroni test).

observed when compared to experimental days 7 and 10 ethanol-fed rats. DHEAS or finasteride daily administration at the doses used here per se did not significantly alter open arms indices in elevated plus maze test ($P>0.05$). Moreover, there was no significant effect of these treatments on number of closed arms entries ($P>0.05$).

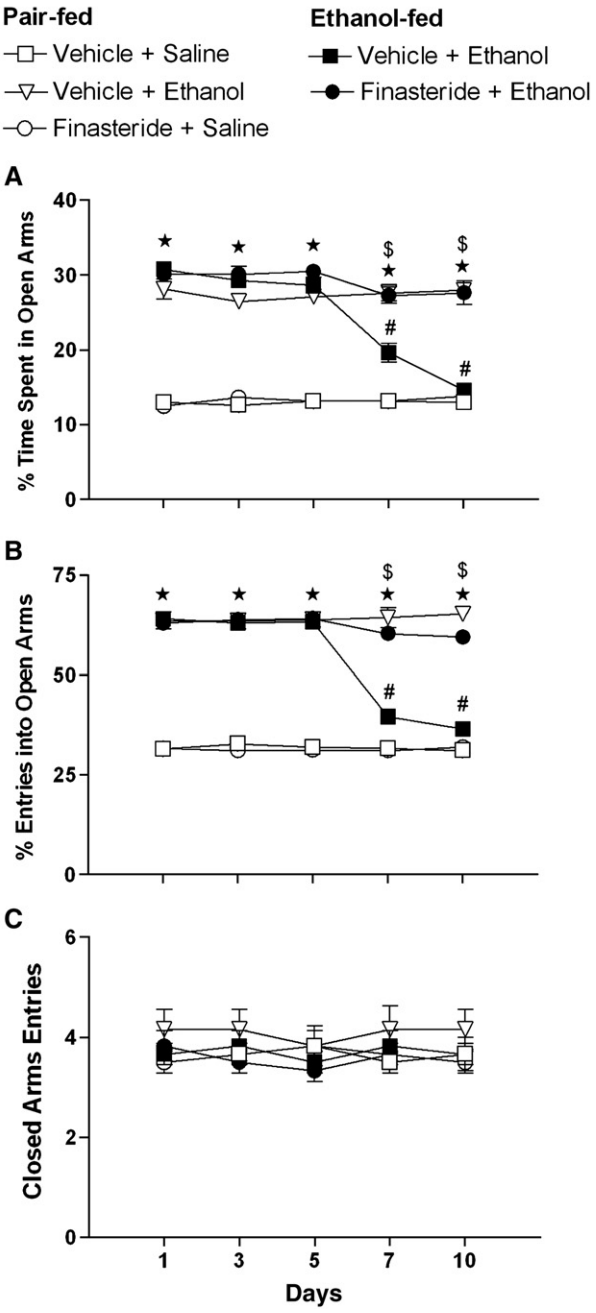

Fig. 3. Effect of daily administration of finasteride on the development of tolerance to ethanol-induced anxiolysis showing (A) % time spent in open arms (B) % open arms entries and (C) number of closed arms entries. Different groups of rats were treated either with finasteride (25 mg/kg, s.c.) or vehicle control (5 ml/kg, i.p.) (twice daily 1000 and 2200 h) consuming ethanol (6% v/v) containing liquid diet (ethanol-fed groups) or control liquid diet (pair-fed groups) for 10 days. Rats were challenged on days 1, 3, 5, 7 or 10 with ethanol (2 g/kg, i.p.) and 30 min thereafter individual rat was placed on the central platform to explore the elevated plus maze for 5 min. Each bar represents means ± S.E.M. of data from 6 rats per group. $^{*}P<0.05$ vs. vehicle + saline (pair-fed group); $^{\#}P<0.05$ vs. vehicle + ethanol (pair-fed group); $^{\$}P<0.05$ vs. vehicle + ethanol (ethanol-fed group) (two-way ANOVA followed by Bonferroni test).

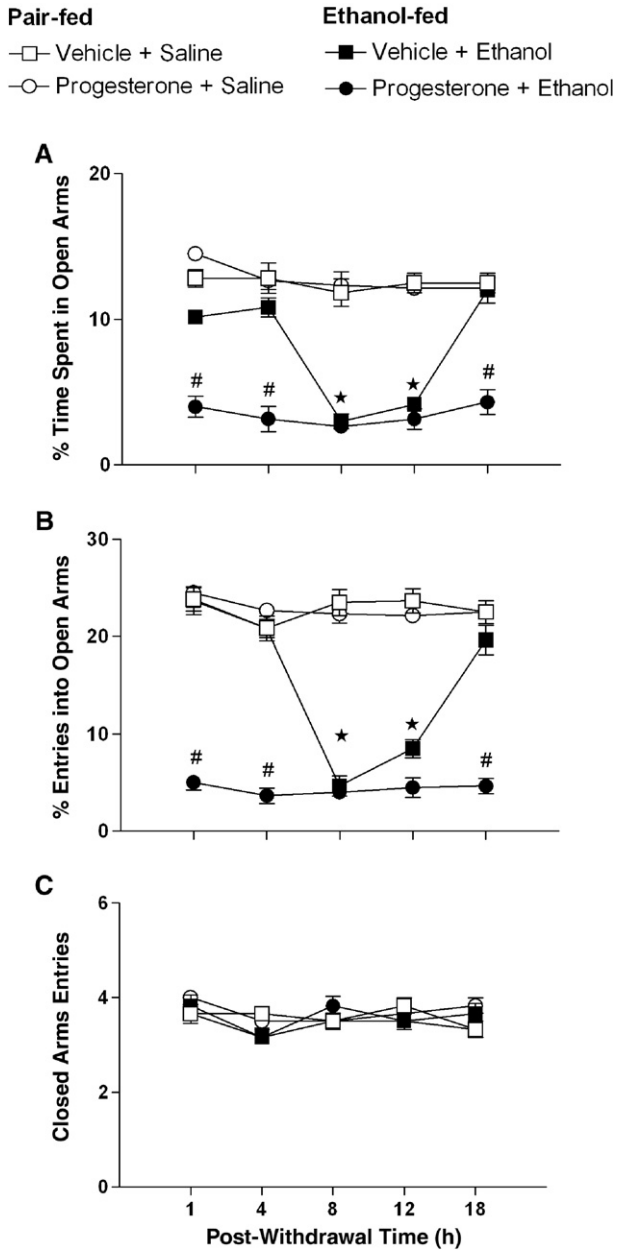

Fig. 4. Concomitant progesterone administration precipitates early ethanol withdrawal-induced anxiety in elevated plus maze test showing (A) % time spent in open arms (B) % open arms entries and (C) number of closed arms entries. Several groups of rats were treated with progesterone (5 mg/kg, i.p.) (twice daily 1000 and 2200 h) consuming ethanol (6% v/v) containing liquid diet (ethanol-fed groups) or control liquid diet (pair-fed groups) for 10 days. On day 11 (0800 h), ethanol was replaced with control liquid diet aliquot. Separate groups of withdrawn rats naïve to plus maze were subjected to elevated plus maze for 5 min at 1, 4, 8, 12 or 18 h post-withdrawal. Each point represents means±S.E.M. of data from 6 rats per group. *$P<0.05$ vs. vehicle+saline (pair-fed group); $^{\#}P<0.05$ vs. vehicle+ethanol (ethanol-fed group) (two-way ANOVA followed by Bonferroni test).

3.4. Early induction of ethanol withdrawal anxiety in progesterone treated rats

As shown in Fig. 4, significant anxiety was observed by 8 h and 12 h of ethanol (6% v/v) withdrawal as evident from significant reduction in % time spent [factor 'treatment' $F(1, 50)=97.11$, $P<0.0001$, factor 'hours' $F(4, 50)=19.23$, $P<0.0001$ and interaction 'treatment×hours' $F(4, 50)=14.37$, $P<0.0001$] and % entries into open arms [factor 'treatment' $F(1, 50)=91.84$, $P<0.0001$, factor 'hours' $F(4, 50)=21.05$, $P<0.0001$ and interaction 'treatment×hours' $F(4, 50)=26.73$, $P<0.0001$] in

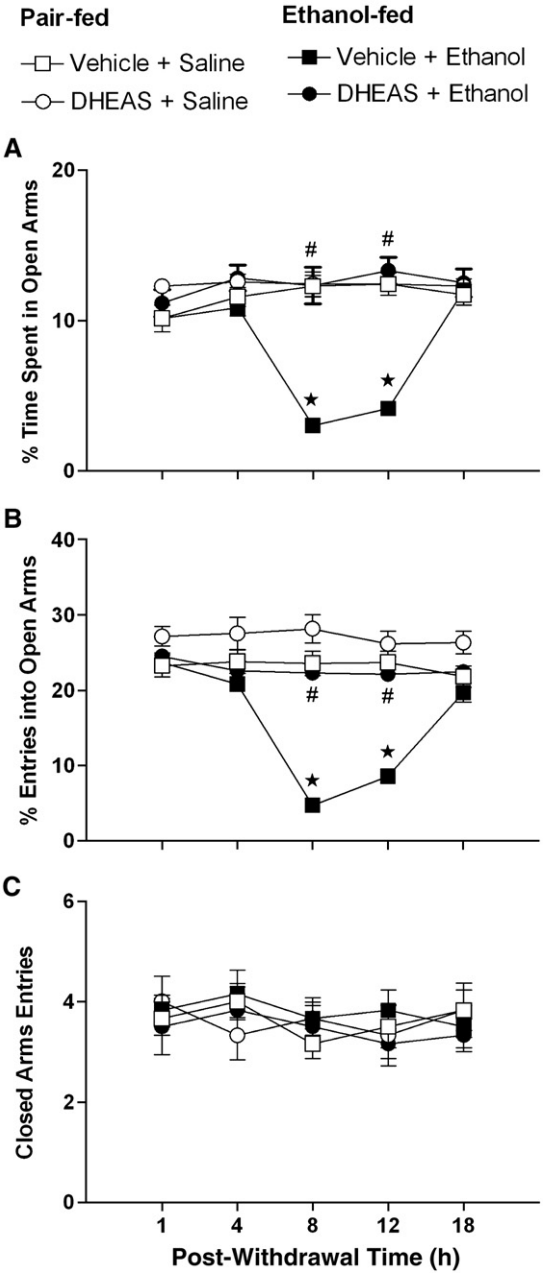

Fig. 5. Concomitant DHEAS administration prevents ethanol withdrawal-induced anxiety in elevated plus maze test showing (A) % time in open arms (B) % open arms entries and (C) number of closed arms entries. Several groups of rats were treated with DHEAS (5 mg/kg, i.p.) or vehicle control (5 ml/kg, i.p.) (twice daily 1000 and 2200 h) consuming ethanol (6% v/v) containing liquid diet (ethanol-fed groups) or control liquid diet (pair-fed groups) for 10 days. On day 11 (0800 h), ethanol was replaced with liquid diet aliquot. Separate groups of withdrawn rats that were naïve to plus maze, were subjected to elevated plus maze test for 5 min at 1, 4, 8, 12 or 18 h after withdrawal. Each point represents means±S.E.M. of data from 6–7 rats per group. *$P<0.05$ vs. vehicle+saline (pair-fed group); $^{\#}P<0.05$ vs. vehicle+ethanol (ethanol-fed group) (two-way ANOVA followed by Bonferroni test).

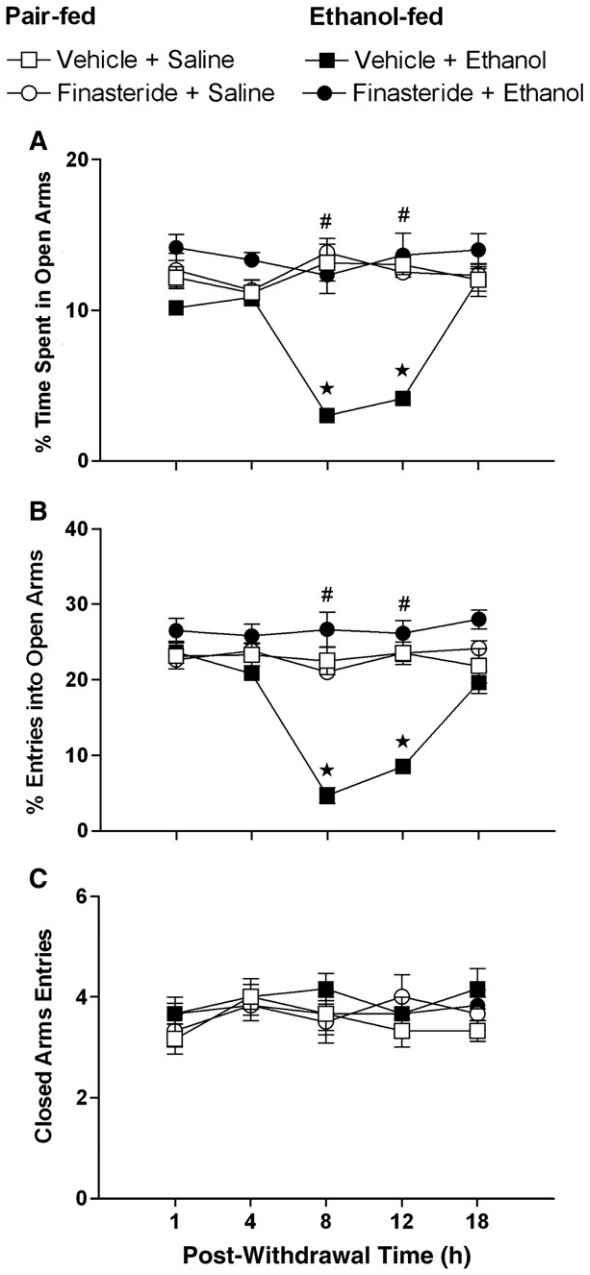

Fig. 6. Concomitant finasteride administration prevents ethanol withdrawal-induced anxiety in elevated plus maze test showing (A) % time in open arms (B) % open arms entries and (C) number of closed arms entries. Several groups of rats were treated with finasteride (25 mg/kg, s.c.) or vehicle control (5 ml/kg, i.p.) (twice daily 1000 and 2200 h) consuming ethanol (6% v/v) containing liquid diet (ethanol-fed groups) or control liquid diet (pair-fed groups) for 10 days. On day 11 (0800 h), ethanol was replaced with liquid diet aliquot. Separate groups of withdrawn rats that were naïve to plus maze, were subjected to elevated plus maze test for 5 min at 1, 4, 8, 12 or 18 h after withdrawal. Each point represents means ± S.E.M. of data from 6–7 rats per group. *$P<0.05$ vs. vehicle + saline (pair-fed group); $^{\#}P<0.05$ vs. vehicle + ethanol (ethanol-fed group) (two-way ANOVA followed by Bonferroni test).

comparison to pair-fed groups. On the other hand, repeated progesterone treated rats (5 mg/kg, i.p. twice daily for 10 days) with chronic ethanol (6% v/v) intake experienced persistent withdrawal anxiety as evident from significant reduction in % time spent in open arms [factor 'treatment' F (1, 50) = 113.9, $P<0.0001$, factor 'hours' F (4, 50) = 24.08, $P<0.0001$ and interaction 'treatment × hours' F (4, 50) = 14.30, $P<0.0001$] and % entries into open arms [factor 'treatment' F (1, 50) = 298.2, $P<0.0001$, factor 'hours' F (4, 50) = 35.26, $P<0.0001$ and interaction 'treatment × hours' F (4, 50) = 32.52, $P<0.0001$] at 1, 4 and 18 h post-withdrawal as compared to ethanol-fed rats. Further, progesterone withdrawal per se did not significantly alter exploration of elevated plus maze as compared to pair-fed rats ($P>0.05$). Moreover, there was no significant effect of any of these treatments on number of closed arms entries as compared to pair-fed controls ($P>0.05$).

3.5. Reduced ethanol withdrawal anxiety in DHEAS or finasteride treated rats

Two-way ANOVA followed by Bonferroni test revealed significant attenuation of ethanol withdrawal-induced anxiety in DHEAS treated rats as statistically significant increase in % time spent [factor 'treatment' F (1, 55) = 87.11, $P<0.0001$, factor 'hours' F (4, 55) = 14.07, $P<0.0001$ and interaction 'treatment × hours' F (4, 55) = 17.91, $P<0.0001$] and % entries into open arms [factor 'treatment' F (1, 55) = 175.2, $P<0.0001$, factor 'hours' F (4, 55) = 53.10, $P<0.0001$ and interaction 'treatment × hours' F (4, 55) = 39.07, $P<0.0001$] was evident at 8 h and 12 h post-withdrawal when compared with DHEAS naïve ethanol-fed rats (Fig. 5). Similar results were obtained with repeated finasteride and ethanol co-treatment as significant increase in % time spent [factor 'treatment' F (1, 55) = 115.2, $P<0.0001$, factor 'hours' F (4, 55) = 16.75, $P<0.0001$ and interaction 'treatment × hours' F (4, 55) = 10.47, $P<0.0001$] and % entries into open arms [factor 'treatment' F (1, 55) = 149.1, $P<0.0001$, factor 'hours' F (4, 55) = 17.23, $P<0.0001$ and interaction 'treatment × hours' F (4, 55) = 16.43, $P<0.0001$] was observed at 8 h and 12 h post-withdrawal when compared with finasteride naïve ethanol-fed rats (Fig. 6). Additionally, DHEAS or finasteride withdrawal per se did not significantly alter exploration of elevated plus maze as compared to pair-fed rats ($P>0.05$). However, there was no significant change in entries into closed arms at any point of withdrawal interval ($P>0.05$) (Figs. 5 and 6).

4. Discussion

Present study provides evidence for the differential role of GABAergic neurosteroids in development of tolerance to anxiolytic effect of ethanol and withdrawal anxiety. Prolonged ethanol consumption led to the development of tolerance to acute ethanol (2 g/kg, i.p., 8% w/v) anxiolysis as compared to pair-fed liquid diet group. This effect was further augmented by chronic concomitant treatment with the sub-effective doses of progesterone (5 mg/kg, i.p.), the precursor of allopregnanolone (Paul and Purdy, 1992; Rupprecht et al., 1993; Prasad et al., 1994; Lambert et al., 1995). Conversely, neurosteroid DHEAS (5 mg/kg, i.p.), the negative allosteric modulator of $GABA_A$ receptor (Majewska et al., 1990) or allopregnanolone biosynthesis blocker, finasteride (25 mg/kg, s.c.) (Morrow et al., 1999; VanDoren et al., 2000) attenuated the development of tolerance

to ethanol anxiolysis. At the doses employed herein, these drugs given for 10 days did not significantly alter elevated plus maze behavior in pair-fed control groups per se. Indeed, it is difficult to perform this kind of study in group-housed rats and hence isolation of subjects is widely practiced for ethanol tolerance studies. However, we observed no significant differences in the basal anxiety parameters of pair-fed control rats isolated for either days 1, 3, 5, 7 or 10 ruling out the impact of social isolation for the outcome of the present study. Moreover, as reported earlier (Dong et al., 2001, Guidotti et al., 2001, Hirani et al., 2005), social isolation for 4–6 weeks, but not 2 weeks (as in present study), is required to induce anxiety-like behavior. Interestingly, previous studies have shown that social isolation of rodents for 6–10 weeks and not for 2 weeks decreases cerebro-cortical content of allopregnanolone (Matsumoto et al., 1999; Dong et al., 2001).

It is believed that GABAergic inhibitory neurotransmission is reduced as opposed to the increase in glutamatergic excitatory transmissions after prolong ethanol consumption (Morrow, 1995; Tabakoff and Hoffman, 1996). Some deleterious effects of ethanol, like tolerance and withdrawal symptoms, are also thought to be mediated, at least in part via glutamate NMDA receptors (Kumari and Ticku, 2000). However, there are considerable evidences that acute ethanol administration enhances the $GABA_A$ receptor mediated chloride channel opening (Suzdak et al., 1986; Allan and Harris, 1987), while chronic ethanol consumption reduces sensitivity of $GABA_A$ receptor, which in turn alters pharmacological and behavioral sensitivity characteristic of ethanol tolerance and dependence (Morrow et al., 1988; Samson and Harris, 1992; Follesa et al., 2006). It is noteworthy that endogenous neurosteroids are thought to be implicated in certain effects of ethanol on $GABA_A$ receptors (Randall et al., 1995; Khisti et al., 2002, 2003; Sanna et al., 2004; Hirani et al., 2002, 2005). Likewise, acute allopregnanolone enhances the GABA-induced opening of $GABA_A$ receptor associated chloride flux in the rat brain synaptoneurosomes at nanomolar concentrations (Morrow et al., 1987), while its chronic treatment downregulates $GABA_A$ receptor functioning (Gulinello et al., 2002) and produces anxiogenic effect (Gulinello et al., 2001, 2002).

In vitro studies revealed that chronic treatment with $GABA_A$ receptor agonistic neurosteroid allopregnanolone resulted in reduced efficacy of benzodiazepines, GABA and neurosteroids themselves at the $GABA_A$ receptor complex in mammalian cortical neurons (Yu and Ticku, 1995). Allopregnanolone decreased the *E*-max value of benzodiazepine agonists to potentiate, and of inverse agonists to inhibit, GABA-induced [$^{36}Cl^-$] influx. Such decreased efficacy of GABA and allopregnanolone at the $GABA_A$ receptor complex was reversed by concomitant chronic exposure of cortical neurons to competitive $GABA_A$ receptor antagonist, R5135 (3α-hydroxy-16-imino-5-β-17-androstan-11-one) (Yu and Ticku, 1995). Tolerance to ethanol-induced anxiolysis following its chronic consumption, and further earlier manifestation of this effect by prolonged progesterone treatment, may result from compensatory decrease in $GABA_A$ receptor mediated neurotransmission. Further, withdrawal symptoms perhaps are associated from unmasking this decrease in $GABA_A$ receptor function or receptor trafficking (Grobin et al., 1998). Moreover, progesterone is lipophilic and readily crosses the blood brain barrier. It is known that peripheral sources of precursor steroids are required for the increases in brain allopregnanolone levels following ethanol administration (Khisti et al., 2003; O'Dell et al., 2004).

Ethanol dependence arising from chronic ethanol exposure is associated with adaptation of $GABA_A$ receptor and/or its subunit gene expression (Petrie et al., 2001; Sanna et al., 2003; Smith and Gong, 2004; Marutha Ravindran and Ticku, 2006). Chronic treatment with progesterone resulted in similar changes in the gene expression and function of $GABA_A$ receptor assembly as that of ethanol in rat cerebellar granule cells in culture (Biggio et al., 2003). Such prolong treatments have been shown to decrease the expression of α_1 and α_2 subunits of $GABA_A$ receptor and a parallel increase in other subunits including α_4 subunit at mRNA and protein levels in various brain regions (Grobin et al., 2000). Such changes might be responsible for tolerance and withdrawal reactions associated with ethanol alone or in presence of $GABA_A$ receptor agonistic neurosteroids. The α_4 subunits of $GABA_A$ receptors are insensitive to the classical benzodiazepines (Cagetti et al., 2003) and are positively modulated by competitive benzodiazepine antagonists and inverse agonists (Smith et al., 1998b). The coupling between inverse agonist benzodiazepine sites or its agonist sites and the Cl^- channel is reported to be increased or decreased respectively following chronic ethanol exposure (Buck and Harris, 1990). $GABA_A$ receptor agonists or positive modulators are known to increase both acquisition of drinking behavior (Smith et al., 1992; Petry, 1997) and volume of ethanol consumed (Pohorecky and Brick, 1988; Boyle et al., 1993). Additionally, $GABA_A$ receptor agonist neurosteroid allopregnanolone has been shown to increase ethanol intake in nondependent rats (Janak et al., 1998) and mice (Sinnott et al., 2002).

In contrast to this, the negative modulator of $GABA_A$ receptor, DHEAS prevented the development of tolerance to ethanol-induced anxiolysis and also withdrawal anxiety. In fact some workers have proposed that the persistent ethanol exposure affects receptor density (Ticku and Burch, 1980), post-translational modifications (Kumar et al., 2002) and subunit expression (Mhatre et al., 1993; Devaud et al., 1995, 1997; Follesa et al., 2003; Sanna et al., 2003) in the development of tolerance and dependence. Paradoxically, in spite of being a negative $GABA_A$ receptor modulator or positive modulator of NMDA receptor, DHEAS at low doses has anxiolytic effect (Melchior and Ritzmann, 1994).

Withdrawal from chronic ethanol consumption resulted in a decrease in percent time and entries into open arms in elevated plus maze test indicating anxiety-like effect (Kokare et al., 2006). The maximum withdrawal anxiety was evident at 8 h and disappeared by 18 h. This 6–8 h post-ethanol withdrawal duration characterized the peak behavioral withdrawal symptoms (Morrow et al., 1992; Devaud et al., 1997). Interestingly during chronic ethanol consumption, co-administration of progesterone enhanced while DHEAS prevented the development of ethanol withdrawal-induced anxiety. Similarly, finasteride that inhibits the formation

of allopregnanolone from progesterone (VanDoren et al., 2000; Dazzi et al., 2002) also prevented the withdrawal anxiety. Finasteride treatment during chronic ethanol consumption significantly decreased blood ethanol concentration by altering pharmacokinetics of ethanol (Finn et al., 2004; Gorin et al., 2005) and also reduced the allopregnanolone levels in blood or brain (Matsumoto et al., 1999; Pinna et al., 2000; Gorin et al., 2005). This perhaps leads to decrease in GABAergic adaptive responses involved in the development of tolerance (Gorin et al., 2005). Although there are no direct preclinical evidences affecting the pharmacokinetics of ethanol by progesterone or DHEAS (Crippens et al., 1999; Robinson et al., 2002), some clinical studies demonstrated that progesterone could enhance ethanol elimination at low blood concentrations (<0.025%) (King and Hunter, 2005). On the other hand, Mumenthaler et al. (1999) observed no such alterations in ethanol pharmacokinetics with varying progesterone levels. However, a possibility of altered ethanol pharmacokinetic by these neurosteroids cannot be excluded. It is also possible that finasteride in addition to inhibiting the allopregnanolone biosynthesis, may affect other hormonal systems or pathways including testosterone and deoxycorticosterone (Gorin et al., 2005). Finasteride treatment has been reported to prevent changes in mRNA expression of $GABA_A$ receptor α_4 subunit elicited by chronic progesterone treatment (Biggio et al., 2003). Chronic ethanol treatment leading to reduction in $GABA_A$ receptor sensitivity to allopregnanolone (Sanna et al., 1993; Faingold et al., 1998; Chandler et al., 1998) may heighten sensitivity to the negative allosteric action of DHEAS at $GABA_A$ receptors in ethanol dependent animals. Such altered sensitivity of $GABA_A$ receptors for endogenous neurosteroids may serve here as the prevalent factor for differential modulation of tolerance to ethanol anxiolysis and withdrawal anxiety.

It cannot be excluded that neurosteroids in addition to allosteric interaction and alterations in subunit composition of $GABA_A$ receptors may affect other intracellular transduction cascades including protein kinase C isomers (Hodge et al., 1999, 2002). Recent reports suggested that neurosteroid modulation of $GABA_A$ receptors depends on endogenous balance between Ca^{2+}-dependent phosphatase and protein kinase C activity (Brussaard and Koksma, 2003; Koksma et al., 2003). Shifting this balance towards high levels of protein kinase C and low levels of phosphatase activity results in insensitivity of $GABA_A$ receptors to allopregnanolone and vice versa (Brussaard and Koksma, 2003). Further, altered sensitivity of $GABA_A$ receptors to its allosteric modulators was observed in transgenic mice lacking protein kinase C_γ isomer (Harris et al., 1995) or protein kinase C_ε isomer (Hodge et al., 1999, 2002). It is plausible that neurosteroids, apart from allosteric interaction with $GABA_A$ receptors, may have alternative mechanism, translocating specific protein kinase C isomer towards membrane, which in turn may affect $GABA_A$ receptor sensitivity (Brussaard and Koksma, 2003). It is also unlikely that the alterations in anxiety levels during chronic administration evident here are mere functions of the allopregnanolone and DHEAS concentration. These neurosteroids in the pair-fed rats per se did not affect the behavioral indices in elevated plus maze test. If endogenous levels of neurosteroids were the only major factor regulating anxiety, one would expect progesterone-injected rats to be significantly less anxious than controls, and DHEAS or finasteride treated rats to be more anxiogenic. However, it was not the case with present study. Recently, Gorin et al. (2005) reported that pretreatment with allopregnanolone biosynthesis inhibitor, finasteride reduced ethanol withdrawal severity, measured by handling-induced seizures, and anxiety-related behavior in the withdrawal seizure prone selected line of mice.

In summary, we report that co-administration of progesterone during chronic ethanol consumption facilitates tolerance to anxiolytic action of ethanol and its withdrawal-induced anxiety. On the other hand, neurosteroid DHEAS, the negative modulator of $GABA_A$ receptor or finasteride treatment that inhibits allopregnanolone synthesis prevents such behavioral effects of ethanol. Further studies are needed to delineate the role of neurosteroids in ethanol dependent adaptations to $GABA_A$ receptors.

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References

Allan, A.M., Harris, R.A., 1987. Acute and chronic ethanol treatments alter GABA receptor-operated chloride channels. Pharmacol. Biochem. Behav. 27, 665–670.

Alele, P.E., Devaud, L.L., 2005. Differential adaptations in GABAergic and glutamatergic systems during ethanol withdrawal in male and female rats. Alcohol., Clin. Exp. Res. 29, 1027–1034.

Baldwin, H.A., Rassnick, S., Rivier, J., Koob, G.F., Britton, K.T., 1991. CRF antagonist reverses the "anxiogenic" response to ethanol withdrawal in the rat. Psychopharmacology 103, 227–232.

Barbaccia, M.L., Affricano, D., Trabucchi, M., Purdy, R.H., Colombo, G., Agabio, R., Gessa, G.L., 1999. Ethanol markedly increases "GABAergic" neurosteroids in alcohol-preferring rats. Eur. J. Pharmacol. 384, R1–R2.

Barnard, E.A., Skolnick, P., Olsen, R.W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A.N., Langer, S.Z., 1998. International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacol. Rev. 50, 291–313.

Baulieu, E.E., Robel, P., 1996. Dehydroepiandrosterone and dehydroepiandrosterone sulfate as neuroactive neurosteroids. J. Endocrinol. 150, S221–S239.

Bertoglio, L.J., Carobrez, A.P., 2002. Prior maze experience required to alter midazolam effects in rats submitted to the elevated plus-maze. Pharmacol. Biochem. Behav. 72, 449–455.

Bicikova, M., Putz, Z., Hill, M., Hampl, R., Diebbelt, L., Tallova, J., Starka, L., 1998. Serum levels of neurosteroid allopregnanolone in patients with premenstrual syndrome and patients after thyroidectomy. Endocr. Regul. 32, 87–92.

Bicikova, M., Tallova, J., Hill, M., Krausova, Z., Hampl, R., 2000. Serum concentrations of some neuroactive steroids in women suffering from mixed anxiety-depressive disorder. Neurochem. Res. 25, 1623–1627.

Biggio, G., Dazzi, L., Biggio, F., Mancuso, L., Talani, G., Busonero, F., Mostallino, M.C., Sanna, E., Follesa, P., 2003. Molecular mechanisms of tolerance to and withdrawal of GABA(A) receptor modulators. Eur. Neuropsychopharmacol. 13, 411–423.

Birzniece, V., Türkmen, S., Lindblad, C., Zhu, D., Johansson, I.M., Bäckström, T., Wahlström, G., 2006. GABA(A) receptor changes in acute allopregnanolone tolerance. Eur. J. Pharmacol. 535, 125–134.

Boyle, A.E., Segal, R., Smith, B.R., Amit, Z., 1993. Bidirectional effects of GABAergic agonists and antagonists on maintenance of voluntary ethanol intake in rats. Pharmacol. Biochem. Behav. 46, 179–182.

Brussaard, A.B., Koksma, J.-J., 2003. Conditional regulation of neurosteroid sensitivity of GABAA receptors. Ann. N. Y. Acad. Sci. 1007, 29–36.

Buck, K.J., Harris, R.A., 1990. Benzodiazepine agonist and inverse agonist actions on GABAA receptor-operated chloride channels. II. Chronic effects of ethanol. J. Pharmacol. Exp. Ther. 253, 713–719.

Cagetti, E., Liang, J., Spigelman, I., Olsen, R.W., 2003. Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of GABAA receptors. Mol. Pharmacol. 63, 53–64.

Chandler, L.J., Harris, R.A., Crews, F.T., 1998. Ethanol tolerance and synaptic plasticity. Trends Pharmacol. Sci. 19, 491–495.

Corpechot, C., Robel, P., Axelson, M., Sjovall, J., Baulieu, E.E., 1981. Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc. Natl. Acad. Sci. U. S. A. 78, 4704–4707.

Crippens, D., White, M.L., George, M.A., Jaworski, J.N., Brunner, L.J., Lancaster, F.E., Gonzales, R.A., 1999. Gender differences in blood levels, but not brain levels, of ethanol in rats. Alcohol., Clin. Exp. Res. 23, 414–420.

Dazzi, L., Serra, M., Seu, E., Cherchi, G., Pisu, M.G., Purdy, R.H., Biggio, G., 2002. Progesterone enhances ethanol-induced modulation of mesocortical dopamine neurons: antagonism by finasteride. J. Neurochem. 83, 1103–1109.

Devaud, L.L., Smith, F.D., Grayson, D.R., Morrow, A.L., 1995. Chronic ethanol consumption differentially alters the expression of γ-aminobutyric acidA receptor subunit mRNAs in rat cerebral cortex: competitive, quantitative reverse transcriptase-polymerase chain reaction analysis. Mol. Pharmacol. 48, 861–868.

Devaud, L.L., Fritschy, J.M., Sieghart, W., Morrow, A.L., 1997. Bidirectional alterations of GABA(A) receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. J. Neurochem. 69, 126–130.

Dong, E., Matsumoto, K., Uzunova, V., Sugaya, I., Takahata, H., Nomura, H., Watanabe, H., Costa, E., Guidotti, A., 2001. Brain 5α-dihydroprogesterone and allopregnanolone synthesis in a mouse model of protracted social isolation. Proc. Natl. Acad. Sci. U. S. A. 98, 2849–2854.

Edenberg, H.J., Dick, D.M., Xuei, X., Tian, H., Almasy, L., Bauer, L.O., Crowe, R.R., Goate, A., Hesselbrock, V., Jones, K., Kwon, J., Li, T.K., Nurnberger Jr., J.I., O'Connor, S.J., Reich, T., Rice, J., Schuckit, M.A., Porjesz, B., Foroud, T., Begleiter, H., 2004. Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations. Am. J. Hum. Genet. 74, 705–714.

Eravci, M., Schulz, O., Grospietsch, T., Pinna, G., Brodel, O., Meinhold, H., Baumgartner, A., 2000. Gene expression of receptors and enzymes involved in GABAergic and glutamatergic neurotransmission in the CNS of rats behaviourally dependent on ethanol. Br. J. Pharmacol. 131, 423–432.

Faingold, C.L., N'Gouemo, P., Riaz, A., 1998. Ethanol and neurotransmitter interactions—from molecular to integrative effects. Prog. Neurobiol. 55, 509–535.

Finn, D.A., Long, S.L., Tanchuck, M.A., Crabbe, J.C., 2004. Interaction of chronic ethanol exposure and finasteride: sex and strain differences. Pharmacol. Biochem. Behav. 78, 435–443.

Follesa, P., Concas, A., Porcu, P., Sanna, E., Serra, M., Mostallino, M.C., Purdy, R.H., Biggio, G., 2001. Role of allopregnanolone in regulation of GABA(A) receptor plasticity during long-term exposure to and withdrawal from progesterone. Brain Res. Rev. 37, 81–90.

Follesa, P., Porcu, P., Sogliano, C., Cinus, M., Biggio, F., Mancuso, L., Mostallino, M.C., Paoletti, A.M., Purdy, R.H., Biggio, G., Concas, A., 2002. Changes in GABAA receptor gamma2 subunit gene expression induced by long-term administration of oral contraceptives in rats. Neuropharmacology 42, 325–336.

Follesa, P., Mancuso, L., Biggio, F., Mostallino, M.C., Manca, A., Mascia, M.P., Busonero, F., Talani, G., Sanna, E., Biggio, G., 2003. Gamma-hydroxybutyric acid and diazepam antagonize a rapid increase in GABA(A) recptors alpha(4) subunit mRNA abundence induced by ethanol withdrawal in cerebellar granule cells. Mol. Pharmacol. 63, 896–907.

Follesa, P., Biggio, F., Mancuso, L., Cabras, S., Caria, S., Gorini, G., Manca, A., Orru, A., Biggio, G., 2004. Ethanol withdrawal-induced up-regulation of the alpha2 subunit of the GABAA receptor and its prevention by diazepam or gamma-hydroxybutyric acid. Mol. Brain Res. 120, 130–137.

Follesa, P., Biggio, F., Talani, G., Murru, L., Serra, M., Sanna, E., Biggio, G., 2006. Neurosteroids, GABAA receptors, and ethanol dependence. Psychopharmacology 186, 267–280.

Gorin, R.E., Crabbe, J.C., Tanchuck, M.A., Long, S.L., Finn, D.A., 2005. Effects of finasteride on chronic and acute ethanol withdrawal severity in the WSP and WSR selected lines. Alcohol., Clin. Exp. Res. 29, 939–948.

Grobin, A.C., Matthews, D.B., Devaud, L.L., Morrow, A.L., 1998. The role of GABA(A) receptors in the acute and chronic effects of ethanol. Psychopharmacology 139, 2–19.

Grobin, A.C., Papadeas, S.T., Morrow, A.L., 2000. Regional variations in the effects of chronic ethanol administration on GABA(A) receptor expression: potential mechanisms. Neurochem. Int. 37, 453–461.

Guidotti, A., Dong, E., Matsumoto, K., Pinna, G., Rasmusson, A.M., Costa, E., 2001. The socially-isolated mouse: a model to study the putative role of allopregnanolone and 5α-dihydroprogesterone in psychiatric disorders. Brain Res. Rev. 37, 110–115.

Gulinello, M., Gong, Q.H., Li, X., Smith, S.S., 2001. Short-term exposure to a neuroactive steroid increases alpha4 GABA(A) receptor subunit levels in association with increased anxiety in the female rat. Brain Res. 910, 55–66.

Gulinello, M., Gong, Q.H., Smith, S.S., 2002. Progesterone withdrawal increases the alpha4 subunit of the GABA(A) receptor in male rats in association with anxiety and altered pharmacology — a comparison with female rats. Neuropharmacology 43, 701–714.

Harris, R.A., McQuilkin, S.J., Paylor, R., Abeliovich, A., Tonegawa, S., Wehner, J.M., 1995. Mutant mice lacking the gamma isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of gamma-aminobutyrate type A receptors. Proc. Natl. Acad. Sci. U. S. A. 92, 3658–3662.

Henniger, M.S., Spanagel, R., Wigger, A., Landgraf, R., Holter, S.M., 2002. Alcohol self-administration in two rat lines selectively bred for extremes in anxiety-related behavior. Neuropsychopharmacology 26, 729–736.

Herbison, A.E., 2001. Physiological roles for the neurosteroid allopregnanolone in the modulation of brain function during pregnancy and parturition. Prog. Brain Res. 133, 39–47.

Hirani, K., Khisti, R.T., Chopde, C.T., 2002. Behavioral action of ethanol in Porsolt's forced swim test: modulation by 3 alpha-hydroxy-5 alpha-pregnan-20-one. Neuropharmacology 43, 1339–1350.

Hirani, K., Sharma, A.N., Jain, N.S., Ugale, R.R., Chopde, C.T., 2005. Evaluation of GABAergic neuroactive steroid 3alpha-hydroxy-5 alpha-pregnane-20-one as a neurobiological substrate for the anti-anxiety effect of ethanol in rats. Psychopharmacology 180, 267–278.

Hodge, C.W., Mehmert, K.K., Kelley, S.P., McMahon, T., Haywood, A., Olive, M.F., Wang, D., Sanchez-Perez, A.M., Messing, R.O., 1999. Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. Nat. Neurosci. 2, 997–1002.

Hodge, C.W., Raber, J., McMahon, T., Walter, H., Sanchez-Perez, A.M., Olive, M.F., Mehmert, K., Morrow, A.L., Messing, R.O., 2002. Decreased anxiety-like behavior, reduced stress hormones, and neurosteroid supersensitivity in mice lacking protein kinase Cepsilon. J. Clin. Invest. 110, 1003–1010.

Holt, R.A., Bateson, A.N., Martin, I.L., 1996. Chronic treatment with diazepam or abecarnil differently affects the expression of GABAA receptor subunit mRNAs in the rat cortex. Neuropharmacology 35, 1457–1463.

Janak, P.H., Redfern, J.E., Samson, H.H., 1998. The reinforcing effects of ethanol are altered by the endogenous neurosteroid, allopregnanolone. Alcohol., Clin. Exp. Res. 22, 1106–1112.

Janis, G.C., Devaud, L.L., Mitsuyama, H., Morrow, A.L., 1998. Effects of chronic ethanol consumption and withdrawal on the neuroactive steroid 3alpha-hydroxy-5alpha-pregnan-20-one in male and female rats. Alcohol., Clin. Exp. Res. 22, 2055–2061.

Jung, M.E., Wallis, C.J., Gatch, M.B., Lal, H., 2000. Abecarnil and alprazolam reverse anxiety-like behaviors induced by ethanol withdrawal. Alcohol 21, 161–168.

Khisti, R.T., Chopde, C.T., 2000. Serotonergic agents modulate antidepressant-like effect of the neurosteroid 3alpha-hydroxy-5alpha-pregnan-20-one in mice. Brain Res. 856, 291–300.

Khisti, R.T., Chopde, C.T., Jain, S.P., 2000. Antidepressant-like effect of neurosteroid 3alpha-hydroxy-5 alpha-pregnan-20-one in mice forced swim test. Pharmacol. Biochem. Behav. 67, 137–143.

Khisti, R.T., Penland, S.N., VanDoren, M.J., Grobin, A.C., Morrow, A.L., 2002. GABAergic neurosteroid modulation of ethanol actions. World J. Biol. Psychiatry 3, 87–95.

Khisti, R.T., VanDoren, M.J., O'Buckley, T., Morrow, A.L., 2003. Neuroactive steroid 3 alpha-hydroxy-5 alpha-pregnan-20-one modulates ethanol-induced loss of righting reflex in rats. Brain Res. 980, 255–265.

King, A.R., Hunter, P.J., 2005. Alcohol elimination at low blood concentrations among women taking combined oral contraceptives. J. Stud. Alcohol 66, 738–744.

Kokare, D.M., Chopde, C.T., Subhedar, N.K., 2006. Participation of alpha-melanocyte stimulating hormone in ethanol-induced anxiolysis and withdrawal anxiety in rats. Neuropharmacology 51, 336–345.

Koksma, J.-J., Kesteren, R.E., Rosahl, T.W., Zwart, R., Smit, A.B., Luddens, H., Brussaard, A.B., 2003. Oxytocin regulates neurosteroid modulation of GABA(A) receptors in supraoptic nucleus around parturition. J. Neurosci. 23, 788–797.

Koob, G.F., 2000. Neurobiology of addiction. Toward the development of new therapies. Ann. N. Y. Acad. Sci. 909, 170–185.

Krystal, J.H., Petrakis, I.L., Krupitsky, E., Schutz, C., Trevisan, L., D'Souza, D.C., 2003. NMDA receptor antagonism and the ethanol intoxication signal: from alcoholism risk to pharmacotherapy. Anal. N. Y. Acad. Sci. 1003, 176–184.

Kumar, S., Sieghart, W., Morrow, A.L., 2002. Association of protein kinase C with GABA(A) receptors containing alpha1 and alpha4 subunits in the cerebral cortex: selective effects of chronic ethanol consumption. J. Neurochem. 82, 110–117.

Kumari, M., Ticku, M.K., 2000. Regulation of NMDA receptors by ethanol. Prog. Drug Res. 54, 152–189.

Lal, H., Harris, C.M., Benjamin, D., Springfield, A.C., Bhadra, S., Emmett-Oglesby, M.W., 1988. Characterization of a pentylenetetrazol-like interoceptive stimulus produced by ethanol withdrawal. J. Pharmacol. Exp. Ther. 247, 508–518.

Lambert, J.J., Belelli, D., Hill-Venning, C., Peters, J.A., 1995. Neurosteroids and GABAA receptor function. Trends Pharmacol. Sci. 16, 295–303.

Langen, B., Fink, H., 2004. Anxiety as a predictor of alcohol preference in rats? Prog. Neuropsychopharmacol. Biol. Psychiatry 28, 961–968.

Mahmoudi, M., Kang, M.H., Tillakaratne, N., Tobin, A.J., Olsen, R.W., 1997. Chronic intermittent ethanol treatment in rats increases GABA(A) receptor alpha4-subunit expression: possible relevance to alcohol dependence. J. Neurochem. 68, 2485–2492.

Majewska, M.D., 1992. Neurosteroids: endogenous bimodal modulators of the GABAA receptor. Mechanism of action and physiological significance. Prog. Neurobiol. 38, 379–395.

Majewska, M.D., Demirgoren, S., Spivak, C., London, E., 1990. The neurosteroid dehydroepiandrosterone sulfate is an antagonist of the GABAA receptor. Brain Res. 526, 143–146.

Marutha Ravindran, C.R., Ticku, M.K., 2006. Tyrosine kinase phosphorylation of GABAA receptor subunits following chronic ethanol exposure of cultured cortical neurons of mice. Brain Res. 1086, 35–41.

Matsumoto, K., Uzunova, V., Pinna, G., Taki, K., Uzunov, D.P., Watanabe, H., Mienville, J.M., Guidotti, A., Costa, E., 1999. Permissive role of brain allopregnanolone content in the regulation of pentobarbital-induced righting reflex loss. Neuropharmacology 38, 955–963.

Melchior, C.L., Ritzmann, R.F., 1994. Dehydroepiandrosterone is an anxiolytic in mice on the plus maze. Pharmacol. Biochem. Behav. 47, 437–441.

Mhatre, M.C., Pena, G., Sieghart, W., Ticku, M.K., 1993. Antibodies specific for GABAA receptor alpha subunits reveal that chronic alcohol treatment down-regulates alpha-subunit expression in rat brain regions. J. Neurochem. 61, 1620–1625.

Miller, S.S., Goldman, M.E., Erickson, C.K., Shorey, R.L., 1980. Induction of physical dependence on and tolerance to ethanol in rats fed a new nutritionally complete and balanced liquid diet. Psychopharmacology 68, 55–59.

Morrow, A.L., 1995. Regulation of GABAA receptor function and gene expression in the central nervous system. Int. Rev. Neurobiol. 38, 1–41.

Morrow, A.L., Suzdak, P.D., Paul, S.M., 1987. Steroid hormone metabolites potentiate GABA receptor-mediated chloride ion flux with nanomolar potency. Eur. J. Pharmacol. 142, 483–485.

Morrow, A.L., Suzdak, P.D., Karanian, J.W., Paul, S.M., 1988. Chronic ethanol administration alters gamma-aminobutyric acid, pentobarbital and ethanol-mediated $36Cl^-$ uptake in cerebral cortical synaptoneurosomes. J. Pharmacol. Exp. Ther. 246, 158–164.

Morrow, A.L., Montpied, P., Lingford-Huges, A., Paul, S.M., 1990. Chronic ethanol and pentobarbital administration in the rat: effects on GABA(A) receptor function and expression in brain. Alcohol 7, 237–244.

Morrow, A.L., Herbert, J.S., Montpied, P., 1992. Differential effects of chronic ethanol administration on $GABA_A$ receptor $\alpha 1$ and $\alpha 6$ subunit mRNA levels in rat cerebellum. J. Mol. Cell. Neurosci. 3, 251–258.

Morrow, A.L., VanDoren, M.J., Devaud, L.L., 1998. Effects of progesterone or neuroactive steroid? Nature 395, 652–653.

Morrow, A.L., Janis, G.C., VanDoren, M.J., Matthews, D.B., Samson, H.H., Janak, P.H., Grant, K.A., 1999. Neurosteroids mediate pharmacological effects of ethanol: a new mechanism of ethanol action? Alcohol., Clin. Exp. Res. 23, 1933–1940.

Morrow, A.L., VanDoren, M.J., Penland, S.N., Matthews, D.B., 2001. The role of GABAergic neuroactive steroids in ethanol action, tolerance and dependence. Brain Res. Rev. 37, 98–109.

Mumenthaler, M.S., Taylor, J.L., O'Hara, R., Fisch, H.U., Yesavage, J.A., 1999. Effects of menstrual cycle and female sex steroids on ethanol pharmacokinetics. Alcohol., Clin. Exp. Res. 23, 250–255.

Nie, H., Janak, P.H., 2003. Comparison of reinstatement of ethanol-and dextrose-seeking by conditioned stimuli and priming injections of allopregnanolone after extinction in rats. Psychopharmacology 168, 222–228.

O'Dell, L.E., Alomary, A.A., Vallee, M., Koob, G.F., Fitzgerald, R.L., Purdy, R.H., 2004. Ethanol-induced increases in neuroactive steroids in the rat brain and plasma are absent in adrenalectomized and gonadectomized rats. Eur. J. Pharmacol. 484, 241–247.

Ollat, H., Parvez, H., Parvez, S., 1988. Alcohol and central neurotransmission. Neurochem. Int. 13, 275–300.

Paul, S.M., Purdy, R.H., 1992. Neuroactive steroids. FASEB J. 6, 2311–2322.

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods 14, 149–167.

Petrie, J., Sapp, D.W., Tyndale, R.F., Park, M.K., Fanselow, M., Olsen, R.W., 2001. Altered gaba receptor subunit and splice variant expression in rats treated with chronic intermittent ethanol. Alcohol., Clin. Exp. Res. 25, 819–828.

Petry, N.M., 1997. Benzodiazepine-GABA modulation of concurrent ethanol and dextrose reinforcement in the rat. Exp. Clin. Psychopharmacol. 5, 183–194.

Pinna, G., Uzunova, V., Matsumoto, K., Puia, G., Mienville, J.M., Costa, E., Guidotti, A., 2000. Brain allopregnanolone regulates the potency of the GABA(A) receptor agonist muscimol. Neuropharmacology 39, 440–448.

Pohorecky, L.A., Brick, J., 1988. Pharmacology of ethanol. Pharmacol. Ther. 36, 335–427.

Prasad, V.V., Vegesna, S.R., Welch, M., Lieberman, S., 1994. Precursors of neurosteroids. Proc. Natl. Acad. Sci. U. S. A. 91, 3220–3223.

Randall, R.D., Lee, S.Y., Meyer, J.H., Wittenberg, G.F., Gruol, D.L., 1995. Acute alcohol blocks neurosteroid modulation of synaptic transmission and long-term potentiation in the rat hippocampal slice. Brain Res. 701, 238–248.

Reddy, D.S., 2003. Pharmacology of endogenous neuroactive steroids. Crit. Rev. Neurobiol. 15, 197–234.

Reddy, D.S., Rogawski, M.A., 2001. Enhanced anticonvulsant activity of neuroactive steroids in a rat model of catamenial epilepsy. Epilepsia 42, 337–344.

Ren, X., Noda, Y., Mamiya, T., Nagai, T., Nabeshima, T., 2004. A neuroactive steroid, dehydroepiandrosterone sulfate, prevents the development of morphine dependence and tolerance via c-fos expression linked to the extracellular signal-regulated protein kinase. Behav. Brain Res. 152, 243–250.

Robinson, D.L., Brunner, L.J., Gonzales, R.A., 2002. Effect of gender and estrous cycle on the pharmacokinetics of ethanol in the rat brain. Alcohol., Clin. Exp. Res. 26, 165–172.

Rossetti, Z.L., Carboni, S., 1995. Ethanol withdrawal is associated with increased extracellular glutamate in the rat striatum. Eur. J. Pharmacol. 283, 177–183.

Rupprecht, R., Reul, J.M., Trapp, T., van Steensel, B., Wetzel, C., Damm, K., Zieglgansberger, W., Holsboer, F., 1993. Progesterone receptor-mediated effects of neuroactive steroids. Neuron 11, 523–530.

Samson, H.H., Harris, R.A., 1992. Neurobiology of alcohol abuse. Trends Pharmacol. Sci. 13, 206–211.

Sanna, E., Serra, M., Cossu, A., Colombo, G., Follesa, P., Cuccheddu, T., Concas, A., Biggio, G., 1993. Chronic ethanol intoxication induces differential effects on GABAA and NMDA receptor function in the rat brain. Alcohol., Clin. Exp. Res. 17, 115–123.

Sanna, E., Mostallino, M.C., Busonero, F., Talani, G., Tranquilli, S., Mameli, M., Spiga, S., Follesa, P., Biggio, G., 2003. Changes in GABA(A) receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal. J. Neurosci. 23, 11711–11724.

Sanna, E., Talani, G., Busonero, F., Pisu, M.G., Purdy, R.H., Serra, M., Biggio, G., 2004. Brain steroidogenesis mediates ethanol modulation of GABAA receptor activity in rat hippocampus. J. Neurosci. 24, 6521–6530.

Sinnott, R.S., Phillips, T.J., Finn, D.A., 2002. Alteration of voluntary ethanol and saccharin consumption by the neurosteroid allopregnanolone in mice. Psychopharmacology 162, 438–447.

Smith, S.S., Gong, Q.H., 2004. Ethanol administration rapidly reverses alpha4 GABAA receptor subunit upregulation following steroid exposure. Neuropharmacology 47, 9–16.

Smith, B.R., Robidoux, J., Amit, Z., 1992. GABAergic involvement in the acquisition of voluntary ethanol intake in laboratory rats. Alcohol 27, 227–231.

Smith, S.S., Gong, Q.H., Hsu, F.C., Markowitz, R.S., ffrench-Mullen, J.M., Li, X., 1998a. GABA(A) receptor alpha4 subunit suppression prevents withdrawal properties of an endogenous steroid. Nature 392, 926–930.

Smith, S.S., Gong, Q.H., Li, X., Moran, M.H., Bitran, D., Frye, C.A., Hsu, F.C., 1998b. Withdrawal from 3alpha-OH-5alpha-pregnan-20-One using a pseudopregnancy model alters the kinetics of hippocampal GABAA-gated current and increases the GABAA receptor alpha4 subunit in association with increased anxiety. J. Neurosci. 18, 5275–5284.

Suzdak, P.D., Schwartz, R.D., Skolnick, P., Paul, S.M., 1986. Ethanol stimulates gamma-aminobutyric acid receptor-mediated chloride transport in rat brain synaptoneurosomes. Proc. Natl. Acad. Sci. U. S. A. 83, 4071–4075.

Ticku, M.K., Burch, T., 1980. Alterations in gamma-aminobutyric acid receptor sensitivity following acute and chronic ethanol treatments. J. Neurochem. 34, 417–423.

Tabakoff, B., Hoffman, P.L., 1996. Alcohol addiction: an enigma among us. Neuron 16, 909–912.

VanDoren, M.J., Matthews, D.B., Janis, G.C., Grobin, A.C., Devaud, L.L., Morrow, A.L., 2000. Neuroactive steroid 3alpha-hydroxy-5alpha-pregnan-20-one modulates electrophysiological and behavioral actions of ethanol. J. Neurosci. 20, 1982–1989.

Vanover, K.E., Suruki, M., Robledo, S., Huber, M., Wieland, S., Lan, N.C., Gee, K.W., Wood, P.L., Carter, R.B., 1999. Positive allosteric modulators of the GABA(A) receptor: differential interaction of benzodiazepines and neuroactive steroids with ethanol. Psychopharmacology 141, 77–82.

Yu, R., Ticku, M.K., 1995. Chronic neurosteroid treatment decreases the efficacy of benzodiazepine ligands and neurosteroids at the gamma-aminobutyric acidA receptor complex in mammalian cortical neurons. J. Pharmacol. Exp. Ther. 275, 784–789.

Zimmerberg, B., Blaskey, L.G., 1998. Prenatal stress effects are partially ameliorated by prenatal administration of the neurosteroid allopregnanolone. Pharmacol. Biochem. Behav. 59, 819–827.