The anxiolytic etifoxine protects against convulsant and anxiogenic aspects of the alcohol withdrawal syndrome in mice

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Abstract

Change in the function of γ-aminobutyric acid$_A$ (GABA$_A$) receptors attributable to alterations in receptor subunit composition is one of main molecular mechanisms with those affecting the glutamatergic system which accompany prolonged alcohol (ethanol) intake. These changes explain in part the central nervous system hyperexcitability consequently to ethanol administration cessation. Hyperexcitability associated with ethanol withdrawal is expressed by physical signs, such as tremors, convulsions, and heightened anxiety in animal models as well as in humans. The present work investigated the effects of anxiolytic compound etifoxine on ethanol-withdrawal paradigms in a mouse model. The benzodiazepine diazepam was chosen as reference compound. Ethanol was given to NMRI mice by a liquid diet at 3% for 8 days, then at 4% for 7 days. Under these conditions, ethanol blood level ranged between 0.5 and 2 g/L for a daily ethanol intake varying from 24 to 30 g/kg. These parameters permitted the emergence of ethanol-withdrawal symptoms once ethanol administration was terminated. Etifoxine (12.5–25 mg/kg) and diazepam (1–4 mg/kg) injected intraperitoneally 3 h 30 min after ethanol removal, decreased the severity in handling-induced tremors and convulsions in the period of 4–6 h after withdrawal from chronic ethanol treatment. In addition when administered at 30 and 15 min, respectively, before the light and dark box test, etifoxine (50 mg/kg) and diazepam (1 mg/kg) inhibited enhanced aversive response 8 h after ethanol withdrawal. Etifoxine at 25 and 50 mg/kg doses was without effects on spontaneous locomotor activity and did not exhibit ataxic effects on the rota rod in animals not treated with ethanol. These findings demonstrate that the GABAergic compound etifoxine selectively reduces the physical signs and anxiety-like behavior associated with ethanol withdrawal in a mouse model and may hold promise in the treatment of ethanol-withdrawal syndrome in humans. © 2009 Elsevier Inc. All rights reserved.

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Introduction

The molecular mechanisms by which alcohol (ethanol) produces its pharmacologic actions, including the development of tolerance and withdrawal, are not clearly established. Many neurotransmitter systems and certain membrane ion channels have been implicated in the effects of ethanol, for example, glutamate, noradrenaline, dopamine, serotonin, opiates, and voltage-sensitive calcium channels (Chandler et al., 1998; Chastain, 2006; Finn and Crabbe, 1997; Johnson, 2004; Littleton, 1998; Whittington et al., 1995). However, abundant behavioral, biochemical, and electrophysiological data for decades have established the involvement of γ-aminobutyric acid type A (GABA$_A$) receptors in the actions of ethanol and in alcoholism (Grobin et al., 1998; Harris, 1999; Kumar et al., 2004; Mehta and Ticku, 1988; Olsen

et al., 2007). Several studies have also shown that effects of short- or long-term exposure to ethanol on the mammalian central nervous system are accompanied by enhancement or impairment of inhibitory synaptic transmission at the level of the GABA$_A$ receptors (Grobin et al., 1998; Liang et al., 2006; Littleton, 1998).

Although the precise molecular mechanisms by which prolonged ethanol consumption modifies GABA$_A$ receptors function remain unclear, it has been proposed that they include at least in part alterations in the expression and/or the composition of various constituent subunits of GABA$_A$ receptors in several brain regions. For example, many studies have shown that long-term ethanol administration in rodents results in marked changes in expression of the genes for various GABA$_A$ receptor subunits, including a decrease in the abundance of α_1, α_2, α_3, and α_5 subunit mRNAs and proteins (Devaud et al., 1995; Mhatre et al., 1993; Montpied et al., 1991) and an increase in that of α_4, γ_1, and γ_2S mRNAs (Devaud et al., 1995) as well as in that of β_1, β_2, and β_3 mRNAs and proteins (Mhatre and Ticku, 1994) in the cerebral cortex. Recently, it

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has been shown that chronic exposure of hippocampal neurons to ethanol resulted in a marked increase in the abundance of the δ subunit mRNA and peptide (Biggio et al., 2007; Follesa et al., 2005). Globally, it is proposed that alterations in $GABA_A$ receptors expression in many brain regions may contribute to dysfunction in $GABA_A$ receptors (Biggio et al., 2007; Follesa et al., 2004; Kumar et al., 2004) in the way of a reduction in inhibitory GABAergic transmission (Bayard et al., 2004; Golovko et al., 2002; Grobin et al., 1998; Kang et al., 1998). Consequently, abrupt cessation of ethanol exposure may be responsible for the rebound central nervous system hyperexcitability masked by ethanol (Kokka et al., 1993). Hyperexcitability underlies emergence of ethanol-withdrawal signs as expressed by agitation, tremors, tachycardia, seizures, irritability, sweating, nausea, and anxiety in alcoholic patients (Becker, 2000; Saitz and O'Malley, 1997). Such effects are also apparent in rodent models and among the multiple behaviors assessed during ethanol withdrawal, tremors, or convulsions, and anxiety can be modeled and measured (Becker, 2000; Goldstein, 1972; Kliethermes, 2005). Based on the earlier discussion, it is not surprising that enhancers of the GABAergic transmission, such as benzodiazepines, have been found to reverse ethanol-withdrawal–induced convulsions and anxiety-like behaviors (Costall et al., 1988; File et al., 1992; Jung et al., 2000). Currently, benzodiazepines are the treatment of choice for ethanol-withdrawal disorders in humans (Mayo-Smith, 1997), due to the anti-anxiety and anti-seizure effects of this class of drugs (Gatch and Lal, 1998). However, the large doses typically used lead to sedation and motor impairments coupled with the potential risk of further substance dependence. This has limited their clinical usefulness and has motivated the search for better treatments of ethanol withdrawal.

Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3, 1-benzoxazine hydrochloride, Stresam®), a molecule structurally unrelated to benzodiazepines, has demonstrated anxiolytic and anticonvulsant properties in rodents (Boissier et al., 1972; Kruse and Kuch, 1985; Verleye and Gillardin, 2004). This compound exhibits anxiolytic activity (Nguyen et al., 2006; Servant et al., 1998) in humans without sedative, and myorelaxant and mnesic side effects at anxiolytic concentrations (Micallef et al., 2001). Mechanistically, etifoxine facilitates GABAergic transmission after binding directly on a site near or on the chloride channel coupled to the $GABA_A$ receptor complex (Schlichter et al., 2000; Verleye et al., 1999) and/or indirectly by stimulating the synthesis of neuroactive steroids in the brain (Verleye et al., 2005). The effects of etifoxine on ethanol-withdrawal syndrome or ethanol dependence have never been evaluated, so the purpose of the present study was to investigate the effects of this compound on tremors or convulsions and anxiety-related behavior in a mouse model of ethanol withdrawal. Diazepam, chosen as a positive control, and the effects of both drugs were studied on the responses to gentle handling that measures tremor convulsive behavior and behavior in the light and dark box, which is a well-established index of anxiety-related behavior (Bourin and Hascoet, 2003; Crawley and Goodwin, 1980). The sedative and ataxic effects of etifoxine and diazepam were assessed in naïve animals (i.e., not treated with ethanol) to establish the specificity of the effects observed in the withdrawal studies.

Materials and methods

Animals

Male NMRI mice (25–30 g), 6 weeks old at the time of experiments were purchased from Janvier breeding (France). A 1-week adaptation period preceded the onsets of experiments. They were housed (10/cage: $43 \times 43 \times 19$ cm) at a controlled temperature ($22 \pm 2°C$), with a relative hygrometry of $50 \pm 20\%$ and with a 12:12 h light/dark cycle (lights on at 7:00 a.m.). Food (AO4, Safe, France) and water (tap water) were freely available. All animal procedures involved in this work were in strict compliance with the European Community council directive of November 24, 1986 (86/609/EEC) and the French government guidelines (authorization B60-159-04-January 2005) for the care and handling of laboratory animals.

Drugs

Etifoxine (batches 219 and 350, Biocodex, France) was suspended in 0.9% saline containing 1% Tween 80 (vol/vol), whereas diazepam (Valium®; Roche, France) was dissolved in 0.9% saline. These two compounds or their corresponding vehicle were administered intraperitoneally (i.p.) in a volume of 10 mL/kg of body weight.

Alcohol treatment procedure

During the first time period, all singly housed animals received a liquid diet (40 mL/day at 08:00 a.m.) for 7–10 days ad libitum to habituate them to these sole food and fluid sources. The liquid diet consisted of chocolate milk (Candia, France) supplemented with 5 g/L of minerals (Salt Mixture XIV; ICN, France) and 3 g/L of vitamins mixture (Vitamin Diet Mixture; ICN, France). Mice consumed 900–1100 g/kg/day over this period. There were no differences in the weights of animals at the end of this habituation period: weights ranged between 29 and 31 g. During the second time period, the ethanol administration procedure described by Naassila et al., (1998) with slight modifications was used. Briefly, ethanol-treated mice received a diet containing 3% (vol/vol) ethanol (99%, vol/vol; Sigma, France) for 8 days then a diet containing 4% (vol/vol) ethanol for 7 days. Control mice received the same chocolate diet where ethanol was replaced by an isocaloric amount of dextrose (VWR, France). No extra chow or water was supplied over this period and all animals had unlimited access to the diet. At day 15 at 08:00 a.m., alcohol–chocolate diet was replaced by the non-alcohol diet until use of animals in the different experiments. Separate groups of mice were used for each set of experiments.

Blood ethanol determination

During the alcoholization period, blood samples (300–500 µL) were collected from cardiac puncture in anaesthetized animals with 2.8% isoflurane on days 7 and 14 at 08:00 a.m. and 05:00 p.m. Blood samples were kept refrigerated until assayed for ethanol by a fully automated spectrophotometric system (Roche Diagnostics, France) based on the enzymatic method developed by Bucher and Redetzki (1951). Blood ethanol level measurements were carried out in the Demarquilly Laboratories (Hénin-Beaumont, France).

Measurement of the ethanol-withdrawal syndrome: handling-induced behavior

The severity of the physical signs of withdrawal syndrome was measured by ratings of behavior produced by handling. This method, originated by Goldstein and Pal (1971), and modified by Watson et al. (1997) measures tremor, myoclonic jerks, and clonic convulsive behavior.

The mice were gently picked up by the tail for 30 s, 30 cm under an anglepoise lamp with a 60-W bulb. The animal was gently rotated and its ensuing behavior rated on a scale from 0 to 5 according to the following criteria: 0 corresponded to no reaction; 1 corresponded to a mild tremor on lifting and turning; 2 corresponded to a continuous severe tremor on lifting and turning; 3 corresponded to a clonic forelimb extensor spasm on lifting; 4 corresponded to the previous signs that continued after placing mouse on cage top; 5 corresponded to spontaneous myoclonic activity followed by the previous sequences. Ratings of handling-induced behavior were assessed by the same experimenter unaware of the treatment status, on the same mice, before alcoholization (basal conditions), 24 h before ethanol withdrawal and every hour from 1 to 7 h after withdrawal from ethanol. The selection of these post-withdrawal time points was based on previous studies that showed that withdrawal from this ethanol treatment schedule produces maximal behavioral signs between 3 and 7 h post-ethanol exposure. Ratings carried out before the experiment and during alcoholization confirmed the lack of spontaneous convulsive behavior in mice. Etifoxine (6.25–50 mg/kg) and diazepam (1 and 4 mg/kg) were administered 3 h 30 min after the alcoholized diet removal. Control animals received an equivalent volume of corresponding vehicle. The delay of 3 h 30 min determined in pilot studies, corresponded to the time where the signs of withdrawal (at least score ≥ 2) were present in all animals.

Anxiety-related behavior: the light and dark box test

Anxiety-related behavior was tested 8 h after ethanol removal. This time corresponded to the optimum time for measurement of such behavior following withdrawal from this ethanol treatment schedule. It was chosen on the basis of literature data (Barnes et al., 1990; Costall et al., 1990; Watson and Little, 1994) and from preliminary studies. At this time point, the intensity of physical signs is such that it did not elicit disruptive effects on animal behavior in the apparatus described later.

The light and dark paradigm was according to the design by Crawley and Goodwin (1980) with slight modifications. This test makes use of rodents' natural aversion to bright areas compared to darker ones. In the two-compartment light and dark box, rodents prefer the smaller dark area and hesitate to enter the brightly lit, open area. The apparatus (OSYS, Orga systems; France) is a Perspex rectangular box (46 × 27 × 30 cm), divided into a small area (18 × 27 cm) and a large area (27 × 27 cm) with an opening door (7.5 × 7.5 cm) located in the center of the partition at floor level. The close-topped small compartment is painted black and illuminated by a dim red light 60 W (4 lux), whereas the open-topped large compartment is painted white and brightly illuminated with a 60 W (400 lux) light source. The compartments are equipped with infrared beam sensors enabling the detection of locomotion in each zone, latency of the first crossing from one compartment to the other and shuttle crossings between both compartments. The test was conducted in a sound-attenuated room, under a light intensity of 400–500 lux. Mice were placed individually in the middle of the light area facing the opening. A 5-min test was given during which the latency to enter the brightly lit area with all four paws, the number of crossings in the white compartment, and the number of transitions between the two compartments were recorded. The floor of each box was cleaned with 10% ethanol between sessions. Etifoxine (6.25–50 mg/kg) and diazepam (1 mg/kg) were administered i.p. 30 and 15 min, respectively, before the test. Control animals received an equivalent volume of corresponding vehicle.

Effects on spontaneous locomotor activity

Possible sedative effects of etifoxine were studied in naïve animals (i.e., not treated with ethanol) by measuring the spontaneous locomotor activity in a separate set of experiments. The motor activity cages (265 × 160 × 140 mm height) were made of clear plastic, and contained a minimum amount of sawdust. Locomotor activity whose reduction expressed a sedative effect, was measured by the interruption of infra-red beams. When one of these beams was broken, a counter in the control unit (OptaVarimex, Colombus, USA) was incremented. The sensitivity of this unit was set so that walking (ambulatory activity) and rearing (vertical activity) were measured. The total beam breaks corresponding to the locomotor activity was measured for 10 min at 15, 30, 60, and 120 min after compound injection.

Effects in the rota rod test

Possible ataxic effects of etifoxine were studied in naïve animals (i.e., not treated with ethanol) using the rota rod assay. Animals were trained before the drug treatment to remain on the rod (3 cm diameter) rotating (Ugo-Basile

7600-Apelex, France) at 16 rpm for at least 120 seconds. Several trials over two consecutive days were necessary to reach this performance criterion and mice that did not do so were excluded from the study (10% of animals). In the drug evaluation session, rota rod performance time was measured three times with a cut-off time of 120 seconds, 15, 30, 60, and 120 min after compound injection, and the mean was adopted as the performance time for each animal. Separate groups of mice were used for each set of measurements. An early fall of the animal from the bar expressed an ataxic effect or a disturbance of motor coordination.

Expression of results and statistical analysis

Statistical tests were run using SigmaStat V3.5 (SPSS Inc., Chicago, IL, USA). Data are presented as means ± standard error of means. The results were analyzed with the one- or two-way analysis of variance (ANOVA) or with the nonparametric Kruskal—Wallis procedure if the two conditions, normality of the data distribution and equality of variances, were not fulfilled. The Student—Newman—Keuls test or Dunnett's test was used for post hoc comparisons following ANOVA, whereas Dunn's test was applied for post hoc comparisons following the nonparametric Kruskal—Wallis procedure. The ratings of handling-induced responses were compared by two-way ANOVA (treatment and time as factors) to repeated measures on the factor time followed post hoc by the Student—Newman—Keuls test to locate the differences between the groups. The effects of the drugs on ethanol-withdrawal signs were also expressed as area under the curve to evaluate the overall effectiveness of the compounds between 3 and 7 h after ethanol removal (trapezoidal rule using the procedure 25 of the software PCS/PHARM; V4.2; USA). These results were then compared by one-way ANOVA or nonparametric Kruskall—Wallis procedure when appropriate, followed by post hoc Dunnett's or Dunn's test, respectively, with the alcoholized controls as reference group.

The light and dark box results were compared using the nonparametric Kruskall—Wallis procedure followed by post hoc Dunn's test to locate the differences between the groups. One-way ANOVA or Kruskall—Wallis procedure was used for comparisons at each interval of the locomotor activity counts and staying time on the rota rod followed by post hoc Dunnett's or Dunn's test, respectively, with vehicle group as reference group. Other variables (body weight and food intake) were compared with Student's t test for unpaired data. Significance was set at $P < .05$.

Results

Alcohol treatment period

During the period of alcohol treatment, no obvious signs of ethanol intoxication (sedation, ataxia) were observed. At the end of alcohol exposure, the body weight of the non—alcohol-treated animals was significantly higher than that of alcoholized animals: 33.6 ± 0.3 vs 29.1 ± 0.4 g, respectively, (Student's t test; $P < .001$). This difference in body weight is explained by a temporary (for 1—2 days) decrease in body weight when ethanol was introduced in the diet. Despite an increase in body weight in these same animals during the ethanol exposure intermediate period, the body weight difference between the two groups of animals remained steady up to the term of ethanol ingestion period. Alcohol intake varied slightly in all experiments and was 28.9 ± 0.7 g/kg/day.

Blood ethanol levels after chronic ethanol administration

At days 7 and 14 ($n = 3$—4 mice) for an average ethanol intake of 24—30 g/kg/day, the corresponding blood ethanol level at 8:00 a.m. was 2.05 ± 0.04 g/L, whereas the levels varied from 0.5 to 1.1 g/L at 0500 p.m. These blood ethanol levels at 5:00 p.m. were coupled to a diurnal food intake ranging from 3 to 4 g/kg.

Handling response

As shown in Figures 1 and 2, the ratings of behavior in response to handling showed the expected increase following withdrawal from ethanol. The score in alcoholized animals increased from 0 to 2.5 between 4 and 6 h after ethanol removal then decreased, whereas the score in non-alcoholized animals remained close to zero over this same period (Figs. 1A and 2). Two-way repeated measures ANOVA revealed a significant interaction (treatment × time) for etifoxine (F [61,488] = 6.08; $P < .001$) on the handling scores. When administered 3.3 h after withdrawal, etifoxine, at 12.5 and 25 mg/kg, significantly reduced the increase in score compared to the alcoholized controls over the same period (Figs. 1 and 2). The effect of etifoxine at these same doses showed a marked reduction in handling scores at around 4 h (Fig. 2). It was noticed that 50 mg/kg of etifoxine dose had no effect and consequently the dose—response relationship exhibited a U-shape curve (Fig. 2). In the positive control (diazepam) group, the ethanol-withdrawal group administered with diazepam at 1 and 4 mg/kg showed a significant reduction in scores on handling (Figs. 1B and 2). Up to the highest doses tested, etifoxine and diazepam did not significantly alter the scores in non-alcoholized animals (results not shown).

Light and dark box paradigm

As illustrated in Fig. 3, 8 h after cessation of ethanol treatment, mice showed an increased latency in moving from the dark to the light compartment (Fig. 3A), a decrease in crossings in the light area (Fig. 3B) and in number of transitions between the compartments (Fig. 3C) compared to non-alcoholized animals. The administration of etifoxine (6.25—50 mg/kg) reversed the profile of changes caused by withdrawal ethanol (H [5] ranged from 13.9 to 15.4; with $P \leq .016$) with
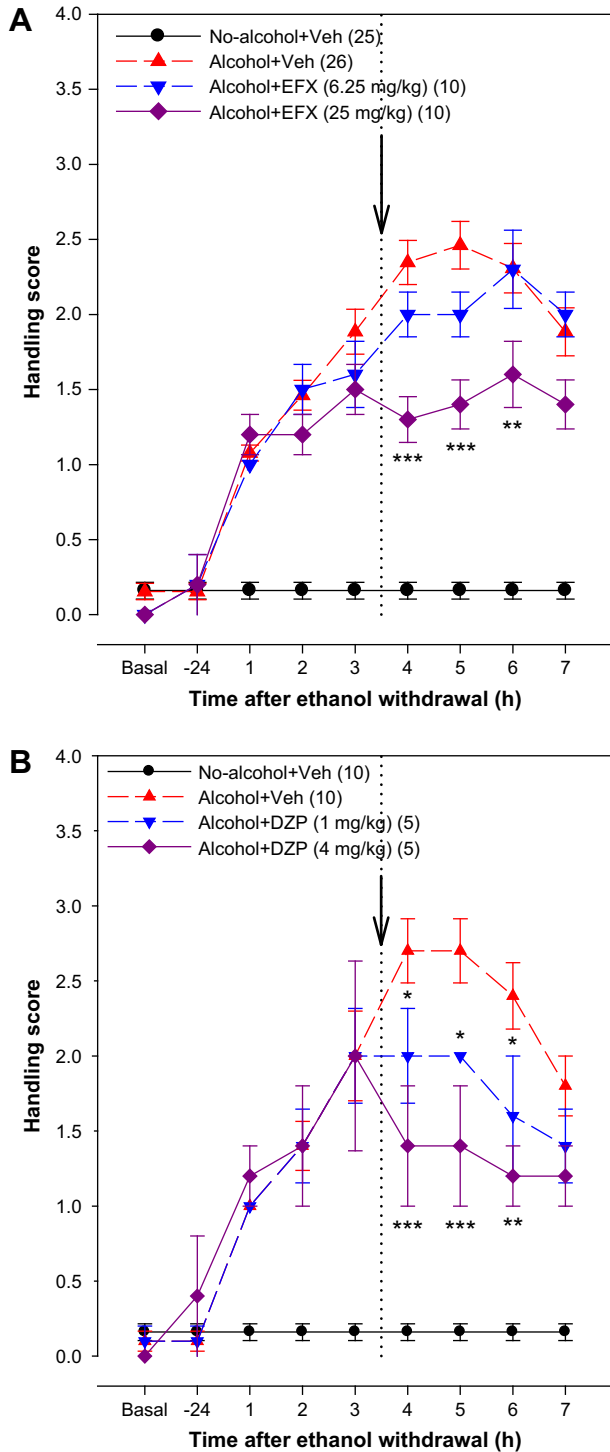
Fig. 1. Effects of etifoxine (EFX) (A) and diazepam (DZP) (B) or their respective vehicle (Veh) administered 3 h 30 min after ethanol removal (shown by *arrow*) on tremors and convulsions score. Values are means ± standard error of means (number of animals used in brackets). *$P < .05$; **$P < .01$; ***$P < .001$ versus alcohol + vehicle (two-way analysis of variance with repeated measures and Student–Newman–Keuls test). For figure clarity, all the doses of EFX are not shown.
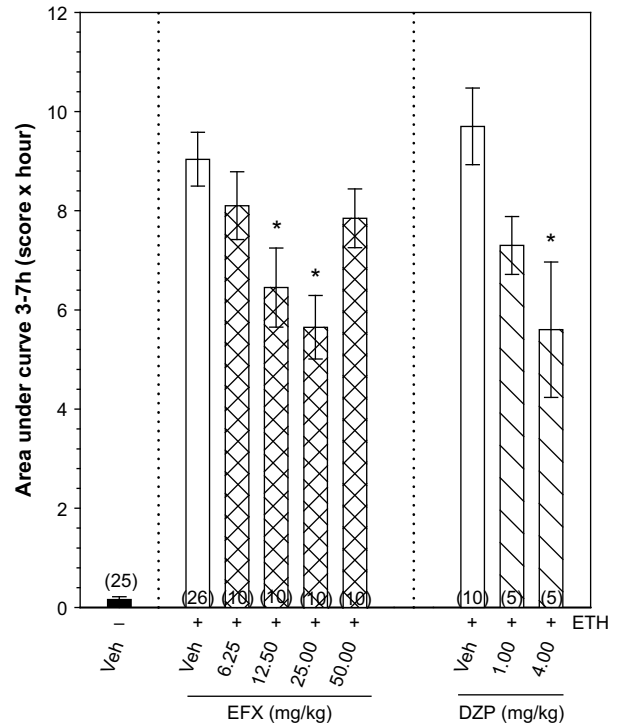

Fig. 2. Effects of etifoxine (EFX) and diazepam (DZP) or their respective vehicle (Veh) against ethanol (ETH) withdrawal signs expressed as areas under the curve for ratings of the responses to handling between 3 and 7 h after ethanol removal. Values are means ± standard error of means. The number of animals appears in brackets. *$P < .05$ vs alcohol + vehicle (one-way analysis of variance and Dunnett's test for EFX and Kruskall–Wallis procedure followed by Dunn's test for DZP).

Spontaneous locomotor activity

The effects of etifoxine and diazepam on spontaneous locomotor activity in naïve animals are illustrated in Figure 4. Etifoxine at 25 and 50 mg/kg doses was devoid of any significant effects on locomotor activity compared to vehicle-treated animals. Similarly, diazepam at the 1 mg/kg dose did not alter locomotor activity up to 120 min after administration, whereas at the 4 mg/kg dose, this compound produced a marked decrease in the spontaneous locomotor activity with a significant effect at 15, 30, and 60 min after administration.

Rota rod test

Etifoxine at 25 and 50 mg/kg doses did not affect motor coordination assessed with rota rod performance time up to 2 h after administration (Fig. 5). Diazepam at the dose of 1 mg/kg did not have significant effects on the performance of animals. In contrast, at 4 mg/kg dose, diazepam significantly lowered the duration the mice were able to stay on the rotating rod compared to vehicle-treated animals up to 60 min after administration (Fig. 5).

Discussion

The present study set out to investigate the effects of etifoxine in ethanol-dependent mice using diazepam as

a significant effect at 50 mg/kg dose. The behavior changes induced by ethanol withdrawal were also significantly attenuated in presence of diazepam at the 1 mg/kg dose (Fig. 3).
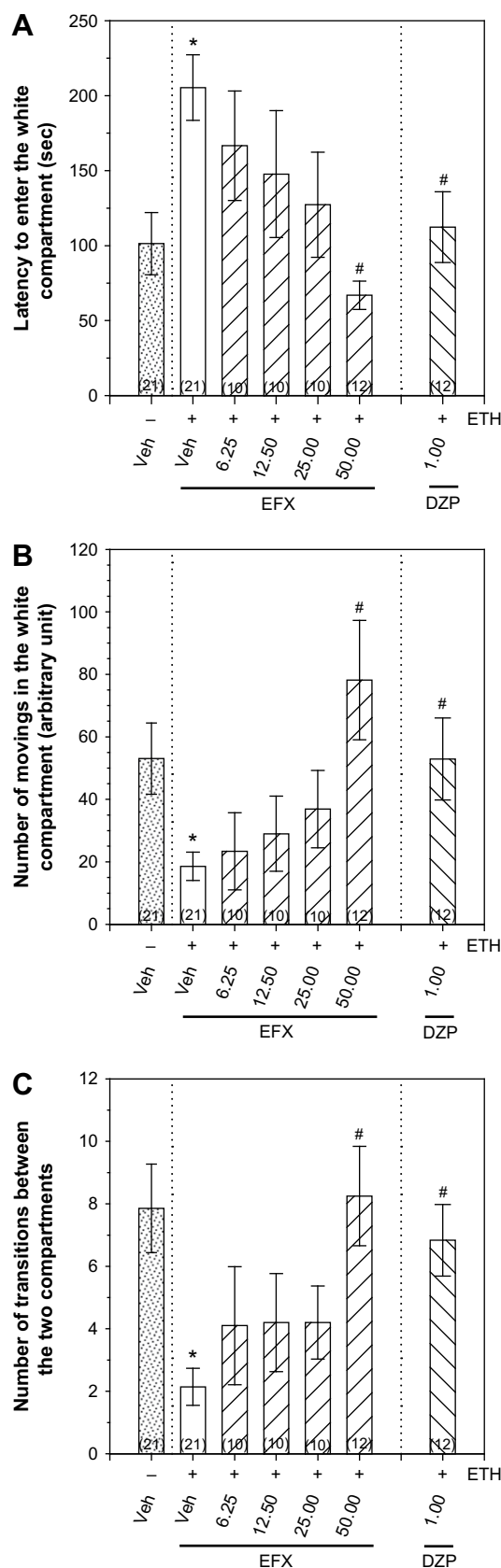
Fig. 3. Effects of etifoxine (EFX, mg/kg) and diazepam (DZP, mg/kg) or their respective vehicle (Veh) on the latency to enter the white compartment (A), the number of moving in the white compartment (B) and the

a control. Measures made in this model were consistent with literature data (Goldstein and Pal, 1971; Naassila et al., 1998; Watson and Little, 2002) in that a daily ethanol consumption ranging from 24 to 30 g/kg yielding ethanol blood level close to 2 g/L (43 mM) produced the emergence of symptoms such as hyperexcitability and heightened anxiety due to ethanol treatment cessation in mice. Brain ethanol levels were not measured in the present study because the purpose of blood alcohol measurements was simply to ascertain that ethanol consumption yielded blood concentrations required to produce intoxication whose consequences were observed after the cessation of ethanol exposure in the current experiment. Also, because brain to blood alcohol partition ratios measured in mice (Smolen and Smolen, 1989) are larger than unity, the blood concentrations measured in this study would produce brain concentrations larger than the range of 5–15 mM associated with withdrawal symptoms (Harris et al., 1998). However, in the present study, animals were forced to consume ethanol with the diet and this procedure is different from ethanol consumption in human alcoholics. Nevertheless, this pattern of ethanol exposure allowed a control of the consumed ethanol quantities and maintenance of high blood ethanol concentrations for a longer duration, resulting in the development of withdrawal signs once alcohol administration was terminated. It is noteworthy that the alcohol-treated animals temporarily consumed less food than the non–alcohol-treated animals with a subsequent reduction in body weight when alcohol was introduced in the diet. It is likely that the taste of alcohol, described to be aversive to rodents (Nachman et al., 1971), was not fully masked by chocolate and consequently led to this transient reduction in food intake.

The protective effect of etifoxine at 12.5 and 25 mg/kg doses on ethanol-withdrawal hyperexcitability manifested by tremors and clonic convulsions is shared with other GABA$_A$–related compounds, such as diazepam, as shown in the present study. As expected, such drugs that enhance the inhibitory GABAergic transmission (Schlichter et al., 2000; Sieghart, 2006) counterbalance the hyperexcitability induced by ethanol treatment cessation. In addition, it has been reported that etifoxine exhibited anticonvulsant properties against seizures induced by blockade of GABAergic system in mice (Kruse and Kuch, 1985; Verleye et al., 1999). Unlike diazepam at the 4 mg/kg dose, etifoxine is devoid of sedative and ataxic effects at the effective doses of 25 and 50 mg/kg. Indeed, such motor sedative and ataxic effects might interfere with or be related to the anti-tremor

number of transitions between the two compartments (C) 8 h after ethanol (ETH) removal in mice. Values are means ± standard error of means. The number of animals used appears in brackets. *P < .05 versus non-alcoholized animals + vehicle; #P < .05 versus alcoholized animals + vehicle (Kruskall–Wallis procedure and Dunn's test for EFX and DZP). The lack of statistically significant differences in the non-alcoholized animals and the alcoholized animals treated with vehicle between the experiments with etifoxine and with diazepam allow the pool of data.
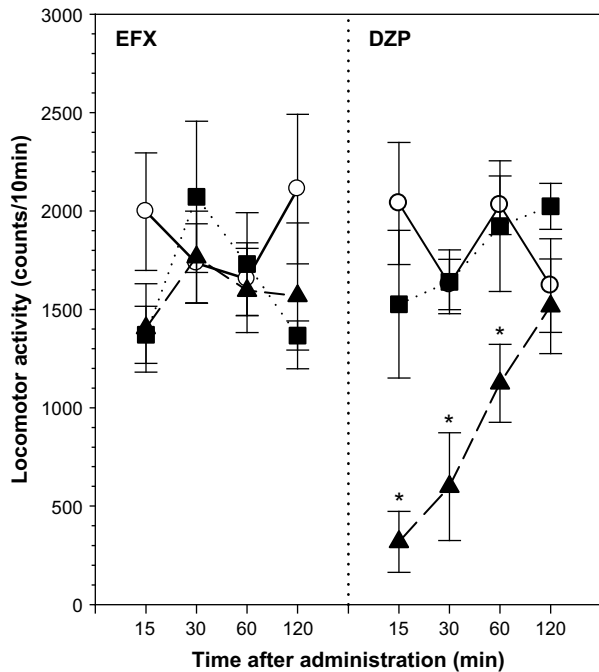
Fig. 4. Effects of etifoxine (EFX) at 25 (*black square*) and 50 mg/kg (*black triangle*) and diazepam (DZP) at 1 (*black square*) and 4 mg/kg (*black triangle*) or their respective vehicle (*white circle*) on the locomotor activity in naïve mice (non-alcoholized mice). Values are means ± standard error of means. The number of animals used per group = 10 for vehicle-treated animals and eight for drug-treated animals. *P < .05 versus vehicle-treated animals (one-way analysis of variance or Kruskall–Wallis procedure and Dunnett's or Dunn's test, respectively, at each time point for EFX and DZP).
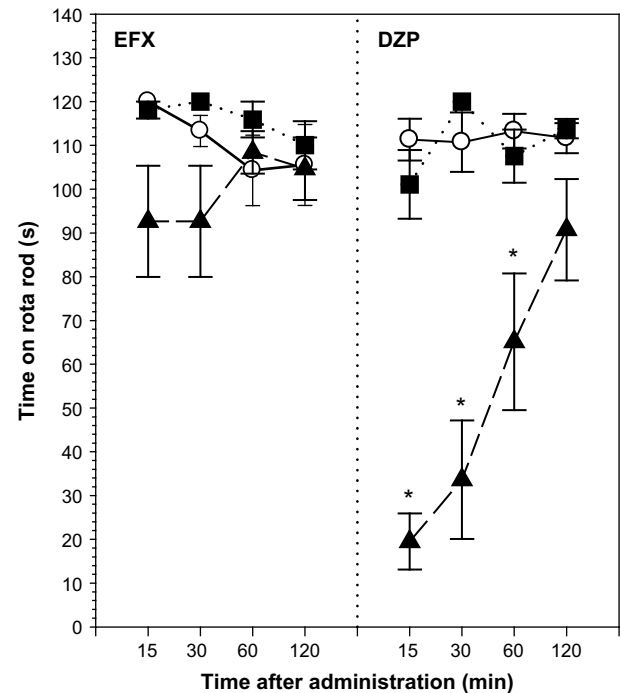
Fig. 5. Effects of etifoxine (EFX) at 25 (*black square*) and 50 mg/kg (*black triangle*) and diazepam (DZP) at 1 (*black square*) and 4 mg/kg (*black triangle*) or their respective vehicle (*white circle*) on the rota rod performance in naïve mice (non-alcoholized mice). Values are means ± standard error of means. The number of animals used per group = 10 for vehicle-treated animals and eight for drug-treated animals. *P < .05 versus vehicle-treated animals (Kruskall–Wallis procedure and Dunn's test at each time point for EFX and DZP).

and anti-seizure activities, pointing out the non-specificity of these latter properties. About the activity profile, etifoxine was ineffective on the physical signs at the lower dose tested (e.g., 6.25 mg/kg) and at the higher dose tested (e.g., 50 mg/kg) but effective at 12.5 and 25 mg/kg. Consequently, the dose–response curve had a U-shape, suggesting that the maximum anti-tremor/anti-seizure activity was observed within a certain critical dose range.

The anxiolytic activities of etifoxine and diazepam were seen clearly at the 50 and 1 mg/kg dose, respectively, in mice undergoing ethanol withdrawal. Indeed, these two compounds inhibited the anxiogenic-like effects of ethanol withdrawal in the light and dark test. As shown by other authors, withdrawal from chronic ethanol caused changes in behavior in the way of exacerbation in aversive response in this test (Barnes et al., 1990; Costall et al., 1990; Timpl et al., 1998; Wallis et al., 1995). Thus, 8 h after the cessation of ethanol administration in the present study, heightened anxiety was evidenced by a reduced exploration of the white compartment and by an increased latency to enter the light area with a decrease in number of crossings in the same area and in the number of transitions. However, it has been proposed that the number of transitions between the two compartments is considered as an index of locomotor and exploratory behavior (Crawley and Goodwin, 1980; Kliethermes, 2005; Wallis et al., 1995; for review see

Bourin and Hascoet, 2003), leading to confusion in interpretation between effects related to anxiety-like state and those related to general locomotor activity. Based on the basic premise of this test, which remains the conflict between the natural tendency to explore a novel environment and the aversive nature of a large, bright, open area, it is hypothesized that etifoxine and diazepam suppressed the inhibition of the exploratory behavior by reducing the anxiety level beforehand enhanced by ethanol withdrawal. In other words, the mice treated with etifoxine or diazepam showed reduced aversive behavior for the light area. In addition, the absence of effects of etifoxine at the effective dose of 50 mg/kg on locomotor activity in naïve animals excluded the hypothesis of non-specific stimulating effects underlying the behavior changes in the light and dark box. Similarly, the same assumption can be applied to diazepam (1 mg/kg), which is devoid of any effect on the spontaneous locomotor activity up to 30 min after administration.

In rodents, increased anxiety-like behavior during withdrawal is likely a reflection of the direct effects of ethanol exposure on neuronal functioning affecting particularly the GABAergic transmission (Grobin et al., 1998; Liang et al., 2006; Littleton, 1998). It is known that the GABAergic system plays an important role in the control of anxiety, and dysfunction of GABA_A receptors in some key brain structures might underlie anxious states (see review of

Millan, 2003). As indicated in the Introduction, the physical signs and increased anxiety during ethanol withdrawal might be attributable to differential alterations in GABA$_A$ receptors subunits function and expression (Grobin et al., 1998; Kumar et al., 2004). Recently, it has been reported that the enhancement of GABAergic transmission by etifoxine could be linked to its selectivity to GABA$_A$ receptor β$_2$/β$_3$ subunits (Hamon et al., 2003). Hence, it is suggested that etifoxine could modulate adaptive changes in GABA$_A$ receptors subunits and thus elicit the alleviation in ethanol-withdrawal symptoms.

Classical benzodiazepines exemplified by diazepam possess a relatively narrow window between doses that produce anticonvulsant or anxiolytic effects and those that cause sedation or motor-impairing effects in animal models and humans (Haefely et al., 1990; Mirza et al., 2008). The profile of these compounds is probably related to the non-selectivity of the binding at GABA$_A$ receptor subunits (Korpi et al., 2002; Sieghart, 2006). Pharmacological studies, recently combined with the use of genetically engineered mice, have led to a general consensus that GABA$_A$ receptors containing an α1 subunit mediate diazepam loco-motor depressant and sedative actions, whereas GABA$_A$ receptors containing an α2 subunit are involved in diaz-epam-induced anxiolysis (Rudolph et al., 2001). Considering the complex interactions between ethanol and neuroactive steroids at GABA$_A$ receptors (Biggio et al., 2007; Follesa et al., 2006; Morrow et al., 2006), it is not excluded that alleviation in ethanol-withdrawal disorders can also be mediated by neuroactive steroids whose biosynthesis is increased by etifoxine (Verleye et al., 2005). Indeed, these neuroactive steroids are described as potent regulators of GABA$_A$ receptor function with anticonvulsant and anxiolytic properties (Longone et al., 2008; Majewska, 1992). It is noteworthy that ethanol does not have a specific neuronal target and chronic ethanol exposure results not only in reducing GABA$_A$ function but also in increasing sensitization of glutamate receptors as well as enhancing activity of voltage-sensitive calcium channels (Littleton, 1998). It has been shown that the monoaminergic systems activity, for example, the dopaminergic system is also markedly reduced in ethanol-withdrawal syndrome (Diana et al., 1993). There is no evidence that this syndrome was caused only by decreases in GABA transmission. Indeed, the increases in GABA transmission produced by etifoxine and diazepam can balance out changes in other neurotransmitter systems or indirectly correct the dysfunction of these same neurotransmitter systems induced by the withdrawal. This latter assumption rests on the existence of interactions between the monoaminergic systems and GABA$_A$ receptors contributing to the complexity of the effects of ethanol in the brain (Millan, 2003; Stanford, 1995).

The effectiveness of etifoxine in decreasing tremors, clonic seizures, and heightened anxiety associated with withdrawal from chronic ethanol exposure in mice suggest that this compound might possess some clinical utility in the management of the ethanol-withdrawal syndrome. The fact that this compound, unlike benzodiazepines, is devoid of sedative effects at relevant pharmacological doses in the present work and in humans (Micallef et al., 2001), suggests that it should be further evaluated in other animal models of ethanol withdrawal (e.g., repeated withdrawal experiences, Kokka et al., 1993; Overstreet et al., 2002) with an assessment of its efficiency on ethanol intake and tolerance. In addition, it will be useful in future studies to measure alcohol-withdrawal signs during longer periods (time > 10 h to several days after ethanol cessation) where physical signs and anxiety-like behavior can be still detectable (Kliethermes, 2005) to ascertain that etifoxine reduced rather than postponed alcohol-withdrawal signs. At present, it can only be concluded that etifoxine was active in the acute phase of withdrawal.

In conclusion, etifoxine demonstrated protective effects in mice against the severity of tremors and convulsions and the increase in anxiety-related behavior elicited by withdrawal from chronic ethanol administration. It is proposed that enhancement in GABAergic inhibition underlies the effects of this drug.

Acknowledgments

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