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# Mice performance on the staircase test following acute ethanol administration

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**Abstract**

This study examined the effect of acute ethanol administration as compared to diazepam on the number of rearing events and the number of steps ascended in the mouse staircase test, an animal model sensitive to benzodiazepines. Acute ethanol administration, similar to acute diazepam administration, reduces rearing (at doses that do not reduce climbing) in the staircase test. This effect of acute ethanol administration is insensitive to the benzodiazepine antagonist flumazenil and is not consistently counteracted by the partial inverse agonist Ro15-4513. It seems that the mouse staircase test is an efficient paradigm for studying agents active at the gamma-aminobutyric acid ($GABA_A$) receptor complex, including ethanol. 

*Keywords:* Staircase test; Benzodiazepine; Diazepam; Ethanol; Gamma-aminobutyric acid (GABA) receptor; Flumazenil

## 1. Introduction

Pharmacologically, ethanol displays a similar anxiolytic, anticonvulsant, and muscle relaxant effect to benzodiazepines and barbiturates. It has been found to modulate gamma-aminobutyric acid ($GABA_A$) receptor complex activity in an allosteric fashion by potentiating the GABA-induced opening of the chloride ion channels (Suzdak et al., 1986a). Some authors have suggested that ethanol interacts with a number of ligand-gated ion channels, and that its agonistic activity at the $GABA_A$ receptor is linked to alcohol reinforcement (Dietrich et al., 1989; Tabakoff and Hoffman, 1996). The ethanol activity at the $GABA_A$ complex and its behavioral effects can be antagonized by the partial inverse benzodiazepine receptor agonist imidazobenzodiazepine (Ro15-4513), which has proconflict (Bonetti et al., 1988; Glowa et al., 1989; Suzdak et al., 1986b, 1988) and proconvulsant properties (Corda et al., 1989; Nutt and Lister, 1987).

The mouse staircase paradigm is a relatively simple and efficient procedure for screening anxiolytic agents. It combines step-climbing, which serves as an index of exploratory and locomotor activity, and rearing, which serves as an index of anxiety (Emmanouil and Quock, 1990; Simiand et al., 1984; Steru et al., 1987). The model can determine the impact of psychotropic agents on rearing and climbing separately and can detect behavioral effects of agents active at the $GABA_A$ receptor (Simiand et al., 1984). The finding that rearing (“anxiety”) in mice is sensitive to the benzodiazepines (e.g., chlordiazepoxide, diazepam, alprazolam, midazolam) at doses that do not suppress climbing indicates that this activity may reflect both central anxiolytic effect as well as the muscle relaxant effect of these and other GABA-potentiating agents. The GABA-active neurosteroids [3$\alpha$,5$\alpha$-tetrahydrodeoxycorticosterone ($\alpha$-THDOC) and alphaxolone], as well as the barbiturate phenobarbital (Pick et al., 1996, 1997) and ethanol (Belzung et al., 1988; Pollard and Howard, 1986), alter staircase behavior patterns in a manner similar to that of benzodiazepine agonists. Furthermore, the behavioral effects of ethanol in the staircase test were reversed by the benzodiazepine partial inverse agonist Ro15-4513 (Belzung et al., 1988). These series of experiments (Belzung et al., 1988; Emmanouil and Quock, 1990; Simiand et al., 1984; Steru et al., 1987) suggested that the

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staircase test is a reliable tool for the assessment of central GABA-mediated behaviors. However, such specificity of rearing activity for anxiolytic agents has not been demonstrated in other studies for alprazolam, although it reduced rearings more than steps climbed (Pollard and Howard, 1986). It is noteworthy that a similar suppression of both rearing and climbing behavior has been induced by other non-benzodiazepine compounds, such as neuroleptics, tricyclic antidepressants, and buspirone, whereas a dissociation of the two behaviors (i.e., suppression of rearing but not climbing) occurred only in the presence of agents with agonistic activity at the $GABA_A$/benzodiazepine receptor/chloride ion channel complex (Simiand et al., 1984).

The present study was designed in an attempt to demonstrate that the staircase paradigm is sensitive to GABA-related behaviors, including ethanol, and not just benzodiazepine-induced behaviors, as claimed in the literature. To this end, we used the staircase test to reevaluate the in vivo responsivity of the $GABA_A$ receptor complex to acute administration of ethanol, using the benzodiazepine agonist diazepam as reference drug. In addition, in order to clarify the relationship between behavior and GABAergic activity, we assessed the interactions between ethanol and the benzodiazepine antagonist flumazenil (Ro15-1788) as well as the partial inverse agonist Ro15-4513 in the staircase test.

## 2. Method

### 2.1. Animals

Male ICR mice (25–35 g) were purchased from the Levinstein colony (Yokneam, Israel). The animals were housed in groups of 20 under standard conditions and maintained on a 12-h light: 12-h dark cycle. They were randomly divided into groups of five in plastic cages and transferred to the laboratory at least 1 day before the start of the test. Each animal was used only once because lack of familiarity with the staircase was required in order to evoke anxiety.

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Sackler Faculty of Medicine at Tel Aviv University (No. 11-96-014), and complied with the guidelines for animal experimentation of the National Institute of Health, Bethesda, MD, USA [DHEW Publication (NIH) 85-23, revised, 1995].

### 2.2. Drugs

Flumazenil (Ro15-1788) and Ro15-4513 were a generous gift from Hoffman-LaRoche (Nutley, NJ). Diazepam was a gift from Teva (Jerusalem, Israel). All other compounds were purchased from commercial sources.

Flumazenil was dissolved in saline with a drop of Tween 80, and diazepam was dissolved in 30% dimethyl sulfoxide and 70% saline with a drop of Tween 80. Ro15-4513 was suspended in normal saline to which a drop of Tween 80 was added. The drugs were injected in a constant volume of 1 ml/100 g body weight.

### 2.3. Behavioral testing

The staircase was built from polyvinyl chloride according to Simiand et al. (1984). It consisted of five identical steps, $2.5 \times 10 \times 7.5$ cm each. The height of the walls was constant (12.5 cm above the stairs) along the entire length of the staircase. Each mouse was placed individually on the floor of the staircase with its back to the staircase. A step was considered climbed only if the mouse placed all four paws on it. Rearing was recorded when the mouse rose on its hind legs either on the step or against the wall to sniff the air. The number of steps descended was not counted. At the end of 3 min, the mouse was removed, and the staircase was cleaned with an alcohol sponge to eliminate any residual odors. At least 10 min elapsed between sponging and the testing of the next mouse. All experiments were performed between 10:00 a.m. and 3:00 p.m.

### 2.4. Procedure

Mice were offered Purina rodent chow and water ad libitum. Ethanol (1, 2, 3, and 5 g/kg, ip), flumazenil (10 mg/kg, sc), and Ro14-4513 (5 mg/kg, sc) were administered 30 min prior to testing. Control animals were treated with saline. Previous results of acute diazepam (4, 8, and 16 mg/kg, ip) administration with and without flumazenil coadministration were used as a reference (Pick et al., 1996).

### 2.5. Statistical analysis

The effects of the different drug treatments on the number of rearing events and the number of stairs ascended was evaluated by one-way analysis of variance (ANOVA) followed by the Dunnet post-hoc test. The two-tailed unpaired Student's $t$ test was used as appropriate. Results are expressed as mean ± S.E.M.

## 3. Results

### 3.1. Acute ethanol

Figs. 1 and 2 show the effect of four doses (1,2,3, and 5 g/kg) of ethanol alone and in combination with Ro15-4513 (5 mg/kg) on the outcome of the staircase test. The control values ($n = 20$) for steps ascended and rearings (mean ± S.E.M.) were 21.2 ± 1.1 and 25.9 ± 1.0, respectively. Ethanol administration was not associated with behavioral reaction to pain (as observed by squealing, writhing, running, or immobility) either immediately after injection or at the time of testing.

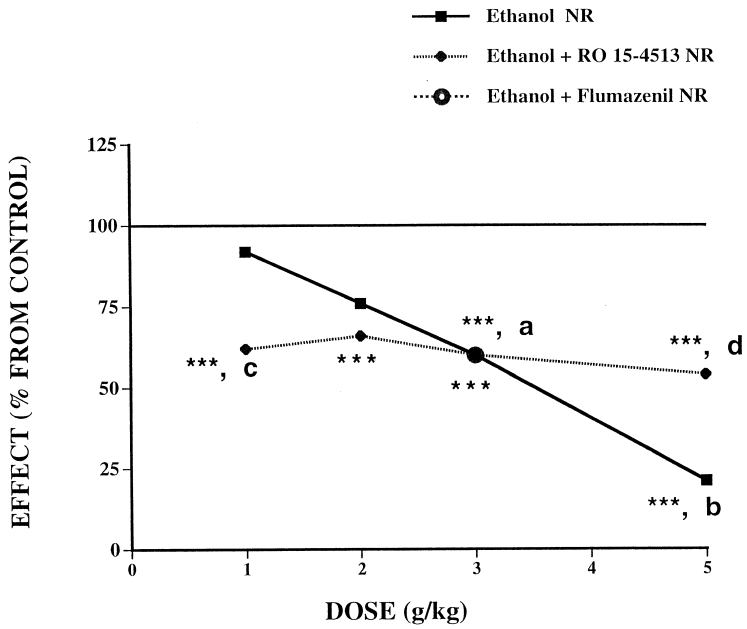


Fig. 1. Effect of acute ethanol administration on the number of rearing events (NR) in a 3-min period. Ethanol (ip), flumazenil (10 mg/kg, sc), and Ro 15-4513 (5 mg/kg, sc) were given 30 min before starting the test. Each experimental group contained at least 10 mice. Results are expressed as percentage of control group. * * * $P<.001$ vs. controls. (a) $P<.01$ vs. 1 g/kg ethanol; (b) $P<.001$ vs. 1, 2, 3 g/kg ethanol; (c) $P<.05$ vs. 1 g/kg ethanol; (d) $P<.01$ vs. 5 g/kg ethanol.

### 3.2. Rearing

The number of rearing events in the mice treated with 1 g/kg ethanol did not differ significantly from that of the control animals. However, an increase in the dose of ethanol to 2, 3, and 5 g/kg induced a significant dose-dependent decrease ($P<.001$ and $P<.001$ vs. control, respectively) in the number of rearing events compared to control animals [$F(8,119)=18.55$; $P<.0001$]. The effect of 3 g/kg ethanol was not altered by the addition of flumazenil (10 mg/kg) [3 g/kg ethanol ($n=12$) vs. 3 g/kg ethanol + flumazenil ($n=10$): $59.5\pm9.3$ vs. $59.8\pm7.7$; $t=0.024$, $df=20$, NS)]. Flumazenil (10 mg/kg) by itself did not affect rearing behavior (unpublished data). The administration of Ro15-4513 (5 mg/kg) alone was associated with a significant decrease in the number of rearing events compared to controls [Ro15-4513 ($n=10$) vs. control ($n=20$): $70.3\pm7.3$ vs. $100.0\pm3.9$; $t=3.95$, $df=28$, $P<.001$]. The addition of Ro15-4513 altered the effect of 1 and 5 g/kg ethanol ($P<.05$ and $P<.01$, respectively), but not that of 2 and 3 g/kg, resulting in the abolishment of the dose-dependent ethanol effects (Fig. 1). This unexpected and inconsistent interaction of Ro15-4513 with ethanol may be attributable to the partial inverse agonistic activity of Ro15-4513 at the benzodiazepine receptor.

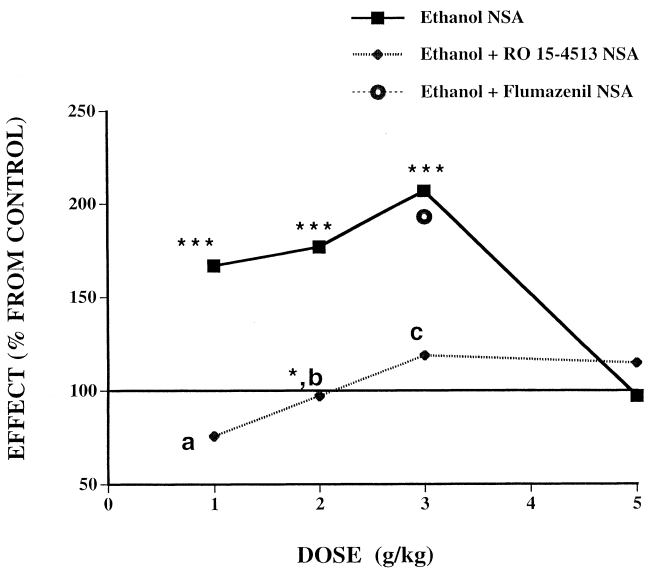


Fig. 2. Effect of acute ethanol administration on the number of steps ascended (NSA) in a 3-min period. Each experimental group contained at least 10 mice. Results are expressed as percentage of control group. * $P<.05$ vs. controls, * * * $P<.001$ vs. controls. (a) $P<.001$ vs. 1 g/kg ethanol; (b) $P<.001$ vs. 2 g/kg ethanol; (c) $P<.001$ vs. 3 g/kg ethanol.

### 3.3. Climbing

The administration of 1, 2, and 3 g/kg ethanol led to a significant increase in the number of steps ascended compared to controls ($P<.001$ for all), but no effect was obtained at 5 g/kg [$F(8,119)=21.59$, $P<.0001$] (Fig. 1). Like for the number of rearing events, the addition of flumazenil (10 mg/kg) did not affect the impact of 3 g/kg ethanol on the number of stairs climbed [3 g/kg ethanol vs. 3 g/kg ethanol + flumazenil ($n=10$): $t=0.67$, $df=20$, NS]. Flumazenil (10 mg/kg) by itself did not affect rearing behavior (unpublished data). The administration of Ro15-4513 alone decreased the number of stairs ascended com-

pared to controls (Ro14-4513 vs. controls: $t = 2.64$, $df = 28$, $P < .05$). The addition of Ro15-4513 to 1, 2, and 3 g/kg ethanol abolished the effect achieved by ethanol at these doses ($P < .001$ vs. ethanol) (Fig. 2). As for rearing, this interaction with ethanol may be ascribed to the benzodiazepine partial inverse agonistic activity of Ro15-4513.

### 3.4. Acute diazepam

The effect of acute diazepam administration was previously described by us (Pick et al., 1996). Briefly, three doses of diazepam (4, 8, and 16 mg/kg) alone and in combination with flumazenil (10 mg/kg) were assessed. Exposure to diazepam caused a reduction in rearing activity, and this reduction was prevented by the addition of flumazenil. By contrast, the number of stairs ascended was not suppressed by diazepam. Indeed, at low doses, diazepam induced an increase in the number of stairs ascended, while the addition of flumazenil did not consistently antagonize this effect (for more details, see Suzdak et al., 1986b).

## 4. Discussion

The present study using the mouse staircase test shows that acute exposure to ethanol is associated with a dose-dependent reduction in rearing activity. The reduction in rearing induced by ethanol was not prevented by the addition of the benzodiazepine antagonist flumazenil, and was inconsistently counteracted (effect at 1 and 5 g/kg ethanol, but not at 2 and 3 g/kg ethanol) by the benzodiazepine partial inverse agonist Ro15-4513. However, it should be noted that only one dose of flumazenil and Ro15-4513 was given in this study.

Acute ethanol administration did not suppress the number of stairs ascended at low doses even induced an increase. The addition of flumazenil did not antagonize the effect of 3 g/kg ethanol, whereas the addition of Ro15-4513 did.

The results obtained following acute ethanol are similar to those reported previously in the mouse staircase test with a single-dose administration of benzodiazepine agonists (Emmanouil and Quock, 1990; Pick et al., 1996, 1997; Simiand et al., 1984), namely, a dose-dependent inhibitory effect on rearing behavior, accompanied by a stimulatory effect, at low doses, on step-climbing. The role of the $GABA_A$/benzodiazepine receptor complex in rearing behavior is supported by the observation that the administration of the benzodiazepine inverse agonist β-carboline norelegnine (anxiogenic agent) was associated with a greater number of rearing events with no effect on the number of stairs climbed (Emmanouil and Quock, 1990). The increase in rearing events was blocked by the benzodiazepine antagonist flumazenil, which was inactive when given alone. It is noteworthy that suppression of both rearing and climbing behavior has been induced by non-benzodiazepine psychotropic agents, such as antipsychotics, tricyclic antidepressants, and buspirone, but a dissociation of the two behaviors (i.e., suppression of rearing but not climbing) occurred only in the presence of agents with agonistic activity at the $GABA_A$/benzodiazepine receptor/chloride ion channel complex (Simiand et al., 1984), including ethanol (Belzung et al., 1988; Pollard and Howard, 1986). However, such specificity of rearing activity for agents active at the benzodiazepine receptor has not been demonstrated in all studies (Belzung et al., 1988; Pollard and Howard, 1986). If rearing behavior is indeed an indicator of anxiety, the administration of benzodiazepine inverse agonists should provoke an increase at doses that either reduce or have no effect on climbing behavior. However, the partial benzodiazepine inverse agonist *N*-methyl-β-carboline-3-carboxamide (FG 7142) and the non-β-carboline $GABA_A$ inhibitor pentylenetetrazol did not affect either rearing or stairs ascending behaviors (Pollard and Howard, 1986). The partial inverse agonist of the benzodiazepine receptor Ro15-4513 given alone at a dose of 2 mg/kg also did not affect mice behavior in the staircase test. Moreover, the inverse agonist methyl-β-carboline-3-carboxylate (β-CCM) induced a reduction in both rearing and step ascending behavior (Belzung et al., 1988). A similar suppressive effect on rearing and climbing was obtained also in our study with a high dose of Ro15-4513 (5 mg/kg) when given alone. Belzung et al. (1988) demonstrated that the increase in ascending behavior induced by ethanol administration (1 g/kg) could be reversed by coadministration of Ro15-4513, at a dose of 2 mg/kg, a phenomenon similar to that obtained in the present study. The capacity of Ro15-4513 to reverse the anxiolytic effects of ethanol as assessed in the staircase test may be related to its partial inverse agonistic activity at the $GABA_A$ receptor.

It is of note that the rearing-suppression effect of the benzodiazepine agonists and the GABA-active neurosteroid 3α,5α-tetrahydrodeoxycorticosterone was blocked by the benzodiazepine antagonist flumazenil (Pick et al., 1996), whereas the similar effect achieved by acute ethanol (3 g/kg) administration in the present study was insensitive to flumazenil. This lack of sensitivity to flumazenil was also observed with phenobarbital and the pregnane-related GABA-active steroid alphaxalone (Pick et al., 1996, 1997), and may therefore indicate that ethanol, phenobarbital, and alphaxalone do not act via the benzodiazepine recognition site at the $GABA_A$ receptor complex, but rather at distinct sites on the complex or via allosteric modulation of the GABA-gated chloride ion conductance. Furthermore, using biochemical, electrophysiological, and behavioral methods, it was demonstrated that several specific actions of acute ethanol administration are mediated by the neurosteroid 3α-5α-pregnan-20-one (3α,5α-THP; allopregnanolone) (VanDoren et al., 2000). Studies with specific GABA antagonists may further clarify the interaction of ethanol with the $GABA_A$ receptor.

In conclusion, the present study demonstrates that acute ethanol administration reduces rearing (at doses that do not reduce climbing) in the staircase test. This effect is insensitive to the benzodiazepine antagonist flumazenil and is not consistently counteracted by the partial inverse agonist Ro15-4513. These data indicate that the mouse staircase test is an efficient paradigm for studying agents active at the $GABA_A$ receptor complex, including ethanol. It remains unclear, however, whether the behavioral effects obtained by the GABA-potentiating effects of ethanol administration are due to its anxiolytic activity or its muscle relaxant activity.

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