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Similar effects of ethanol and flumazenil on acquisition of a shuttle-box avoidance response during withdrawal from chronic ethanol treatment

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1 Acquisition of a two-way shuttle-box avoidance response is facilitated by ethanol. This facilitated acquisition of an avoidance response to ethanol was attenuated during withdrawal from chronic-ethanol diet intake (i.e. tolerance developed by ethanol). The deficit in the avoidance task after chronic ethanol treatment could be overcome by increasing the dose of ethanol.

2 Flumazenil, a benzodiazepine antagonist, also facilitated acquisition of the avoidance response in control rats. This response to flumazenil was significantly reduced during withdrawal from chronic-ethanol treatment. This reduced avoidance responding during withdrawal also could be overcome by increasing the dose of flumazenil.

3 The benzodiazepine-inverse agonist, RO 15-4513, produced a deficit in avoidance responding that was antagonized by both ethanol and flumazenil in a dose-related manner.

4 To determine whether flumazenil has the properties of a benzodiazepine agonist, it was established that, unlike the benzodiazepine chlordiazepoxide, flumazenil did not enhance the ethanol-induced deficit in the aerial righting reflex. Additionally, flumazenil blocked the action of chlordiazepoxide in this procedure, consistent with the benzodiazepine antagonist action of flumazenil.

5 Data collected are consistent with the hypothesis that an endogenous substance with the properties of a benzodiazepine-inverse agonist antagonizes the anticonflict actions of acutely administered ethanol during withdrawal from chronic-ethanol exposure.

Keywords: Flumazenil; ethanol; chronic ethanol treatment; shuttle-box avoidance; avoidance conflict responding; aerial righting reflex; anxiogenic; anxiolytic; RO 15-4513; ethanol tolerance

Introduction

In 1980, our laboratory compared the effects of ethanol and benzodiazepines on performance in various behavioural tasks and concluded they may share a common mechanism of action (Frye *et al.*, 1980; Vogel *et al.*, 1980). However, ethanol does not compete with a benzodiazepine for receptor binding (Frye *et al.*, 1980; Breese *et al.*, 1983). Several early studies have indicated that ethanol, like benzodiazepines, has an anticonflict action (Conger, 1956; Barry *et al.*, 1963; Mansfield *et al.*, 1977; Vogel *et al.*, 1980). More recently, our laboratory (Frye *et al.*, 1981; Criswell & Breese, 1989) demonstrated that chlordiazepoxide and ethanol facilitated acquisition of a shuttle-box avoidance response, a finding consistent with other studies demonstrating that the two-way avoidance task may have a conflict component (Gray, 1977; Fernandez-Teruel *et al.*, 1991a,b). During withdrawal from chronic-ethanol treatment, acquisition of the avoidence response was not facilitated by either ethanol or chlordiazepoxide, suggestive that there was cross-tolerance between these drugs (Criswell & Breese, 1989). This latter observation provided additional support for the position that ethanol has actions similar to those of the benzodiazepines but the basis of the tolerance to the action of chlordiazepoxide and ethanol in the avoidance task following withdrawal from chronic-ethanol treatment was unknown.

File *et al.* (1989) reported that flumazenil, a benzodiazepine antagonist, reversed the deficit to ethanol seen in a conflict procedure in rats withdrawn from chronic ethanol treatment. Furthermore, in a social interaction test, flumazenil alone reversed the behavioural response associated with withdrawal from chronic-ethanol or from chronic-benzodiazepine administration (Baldwin & File, 1987; File *et al.*, 1989; File & Hitchcott, 1990). The effect of flumazenil in these conflict tasks during withdrawal from ethanol or a benzodiazepine (File & Hitchcott, 1990) could be explained if flumazenil had a benzodiazepine agonist action or if it was antagonizing the effect of an endogenous compound with benzodiazepine-inverse agonist activity (File *et al.*, 1989; File & Hitchcott, 1990).

It is not known whether the reduced responding to ethanol in the avoidance task observed during withdrawal from chronic-ethanol treatment would be attenuated by flumazenil, as has been demonstrated in another conflict task (File *et al.*, 1989). For this reason, the present investigation examined (1) the effect of the benzodiazepine antagonist, flumazenil, alone and on the action of an acute dose of ethanol in rats performing the avoidance procedure, (2) the effect of varying doses of flumazenil and ethanol on shuttle-box avoidance responses following withdrawal from chronic ethanol treatment; (3) whether a benzodiazepine-inverse agonist would produce a deficit in the acquisition of the avoidance response; (4) the action of ethanol and flumazenil on the deficit produced by a benzodiazepine-inverse agonist in the avoidance task, and (5) the possibility that the anticonflict action of flumazenil was due to a benzodiazepine agonist-like action of this drug. From these data, it could be assessed whether findings were consistent with the hypothesis of File *et al.* (1989) that an endogenous compound with benzodiazepine-inverse agonist properties is responsible for the deficit in the anticonflict action of ethanol during withdrawal from chronic-ethanol treatment (Criswell & Breese, 1989; 1990).

Some of these data were presented in abstract form at the annual Research Society on Alcoholism meeting (Criswell & Breese, 1990).

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Methods

Animals

Male Sprague-Dawley rats weighing 250 to 275 g were obtained from Charles River Laboratories (Raleigh, NC, U.S.A.). The animals were maintained in our animal facilities until they reached 350 to 550 g. Rats were in the animal facility for at least two weeks before being used. The rats were housed individually with a 12 h light-dark cycle and allowed free access to food and water or a liquid diet as described below.

Shuttle-box avoidance task

The functional response measured in this investigation was the rate of acquisition of a two-way shuttle-box avoidance procedure, with a tone as the warning stimulus (Criswell & Breese, 1989). For the experimental procedure, each rat was allowed a 5 min adaptation period prior to avoidance training after being placed in a shuttle-box (Lehigh Valley Electronics). Following this adaptation period, the onset of a tone signalled the beginning of a 10 s avoidance period during which the animals could shuttle to the opposite side of the cage to avoid a 0.7 m footshock. Failure to respond within the 10 s period resulted in shock onset. Footshock was terminated when the animals moved to the far side of the cage (escaped) or at the end of a 5 s period. Avoidance of the shock or shock termination initiated a 30 s intertrial interval. Data were collected for 100 trials with the number of avoidance responses, escapes from shock, crosses during the intertrial interval, and response omissions (failure to escape within 5 s) recorded at the end of each 25 trial block for analysis. During the first few training trials, some rats made responses during the avoidance period before having received their first shock; therefore, data were analysed only for the last 75 trials out of the 100 trial session. Since the avoidance task does not require food and water deprivation, the behavioural consequences of chronic exposure to ethanol in a liquid diet can be assessed (Criswell & Breese, 1989).

In the latter portions of this investigation, we encountered a shift in the number of untreated rats exhibiting low rates of avoidance responding during acquisition. Rech (1966) and Fernandez-Teruel *et al.* (1991a,b) have described such differences within populations of rats. This has been described as a 'batch' effect by File & Hitchcott (1990). Therefore, we initiated a screening programme as new batches of rats ($n = 36$) were acquired from the supplier, whereby 4 to 6 animals were tested without drug prior to being assigned to an experiment. Only batches of rats from which this screen produced a mean of 30 or fewer avoidance responses during the last 75 trials were used to evalute the actions of ethanol and flumazenil. A possible 'floor' effect was avoided with treatments which were expected to produce a negative action on avoidance responding by choosing batches of rats with a mean of 45 avoidance responses or greater during acquisition. Rats with such high response scores were used to examine the action of a benzodiazepine inverse agonist, RO-15-4513, in the avoidance task and to evaluate performance in the avoidance task of a group withdrawn from chronic ethanol administration. The rats given saline or liquid diet with each experiment provided a cross-validation of our original measure of acquisition of the task (i.e. screen) for appointment to the appropriate experimental protocol.

Aerial righting

The impairment of aerial righting induced by ethanol in the presence or absence of chlordiazepoxide or flumazenil was measured by the modified procedure of Leitch *et al.* (1977) described by Frye & Breese (1982). The animals were held upside down by grasping the back of the neck and tail and dropped onto a soft surface (i.e. a foam rubber pad). Landing on all four feet on two consecutive releases was considered a successful trial. The distance from the back of the rat to the foam rubber pad was the measured height. A control rat is able to demonstrate aerial righting at a height of approximately 5 cm. Rats treated with ethanol or chlordiazepoxide were not released from heights above 50 cm.

Drugs and treatments

Ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY, U.S.A.) was administered i.p. as a 10% (weight-volume) solution in saline. This concentration minimizes the irritating effects of the ethanol. Chlordiazepoxide (Hoffman LaRoche, Nutley, NJ, U.S.A.) was dissolved in saline and administered i.p. (1 ml kg^{-1} volume). Ethanol was administered chronically by making a nutritionally complete liquid diet containing ethanol available to the rats as previously described (Frye *et al.*, 1981). Control rats received liquid diet with the ethanol replaced by an equicaloric amount of dextrose. For the experiments involving 12-day exposure to chronic ethanol, all animals were habituated to the control liquid diet for 2 days. On day 3, a diet containing 5% ethanol was substituted for the control liquid diet in the experimental group. After 2 days, the concentration of ethanol was increased to 7.5% and 2 days later to 10% where it remained for 8 days. Control animals were limited to 70 ml day^{-1} of liquid diet. Animals drinking the ethanol-liquid diet ingested 11.6 ± 0.7 g kg^{-1} day^{-1}. This diet regimen results in similar weight gains for the two groups (Frye *et al.*, 1981). Whereas the chronic-ethanol treated rats exhibit tremors and irritability to handling, they did not have spontaneous seizures or seizures to the sound exposure in the avoidance task.

Following chronic exposure of the rats to control liquid diet or ethanol-containing liquid diet, the diet was removed and replaced by lab-chow. After 7–9 h of withdrawal from the ethanol-containing or control-liquid diets, treated and control rats were given saline or various doses of ethanol, i.p. This protocol demonstrated tolerance to acute action of ethanol in the avoidance task in the rats withdrawn from chronic treatment (Criswell & Breese, 1989). Additional rats received saline or various doses of flumazenil (RO 15-1788; Hoffmann-La Roche Pharmaceutical Co., Nutley, NJ. U.S.A.), either alone or 5 min prior to ethanol. In other experiments to evaluate the agonist properties of flumazenil, either flumazenil or chlordiazepoxide (Hoffmann-La Roche Pharmaceutical Co.) were given alone or in combination with ethanol and the effect of these drug treatments on aerial righting was evaluated. The benzodiazepine-inverse agonist RO-15-4513 (ethyl 8-azido-5,6-dihydro-5 methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; Hoffmann-La Roche Pharmaceutical Co.) was suspended in 0.25% carboxylmethylcellulose and injected i.p.

Statistics

Total number of avoidance responses, response omissions, intertrial interval crosses during the last 75 trials of a 100 trial session (see avoidance task) and aerial righting distance were analysed by ANOVA (Winer, 1962). Individual means were compared to controls using the Dunnett test (Winer, 1962).

Results

Effects of flumazenil on the response to acutely administered ethanol in the avoidance task

In agreement with previous work (Frye & Breese, 1981; Criswell & Breese, 1989), an acute dose of ethanol caused an increase in avoidance responding in rats treated with control-liquid diet (Figure 1). Flumazenil pretreatment did not block

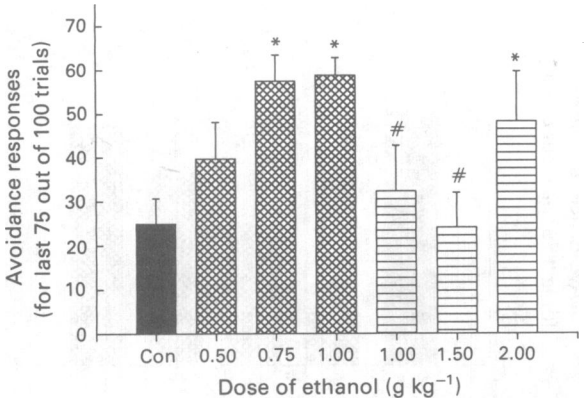

Figure 1 Dose-response effects of ethanol on acquisition of a two-way shuttle-box avoidance response following withdrawal from control or ethanol-liquid diet. The solid column (■) refers to a control (Con) group which was formed from rats that received ethanol-liquid diet or control-liquid diet, after it was established that there was no difference in the responses for these groups ($P>0.05$); (▩) refer to groups of rats that received various doses of acutely administered ethanol (0.5, 0.75, 1.0 g kg^{-1}) during withdrawal from control-liquid diet; (▤) refer to groups of rats that were tested with various doses of ethanol (1.0, 1.5, 2.0 g kg^{-1}) during withdrawal from ethanol-containing liquid diet administered for 12 days. Ethanol was administered 10 min prior to behavioural testing. There were no differences in the number of response omissions or intertrial crosses between groups ($P>0.1$). The mean ± s.e.mean of 5 to 10 rats are shown for each dose of ethanol. *$P<0.05$ when compared to saline control. †$P<0.05$ when compared to the response to 1 g kg^{-1} of ethanol in the CLD group.

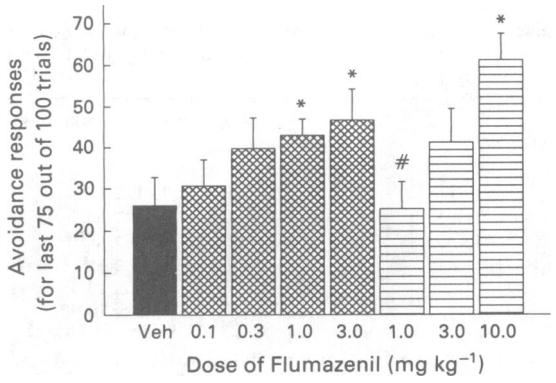

Figure 2 Effect of increasing doses of flumazenil on acquisition of an avoidance task in control rats and in rats following withdrawal from 12 days on an ethanol-liquid diet. Responses to vehicle in rats that received either control diet or ethanol-liquid diet were not significantly different ($P>0.05$) and were combined (Veh). This group is designated (■). Other rats that received 12 days of a control-liquid diet were given various doses of flumazenil (Flu) and are referred to by (▩). Rats treated for 12 days on an ethanol-liquid diet and then withdrawn are designated by (▤). Each dose of flumazenil or vehicle was administered i.p. 5 min prior to testing. n for all groups was from 8 to 13, except for the 1 mg kg^{-1} CLD group ($n=28$) and the 10 mg kg^{-1} ELD group ($n=6$). There were no differences in response omissions between groups ($P>0.05$) and only the 3 mg kg^{-1} group in the untreated group showed a greater number of intertrial crosses than did controls ($P<0.05$). Each value and bar represent the mean ± s.e.mean. *$P<0.05$ when compared to the Veh group. †$P<0.05$ when compared to the CLD-Flu 1.0 mg kg^{-1} group.

Table 1 Effect of flumazenil on the ethanol-induced facilitation of the acquisition of an avoidance task in control rats*

Treatment	*Drug doses* (mg kg^{-1})	*Avoidance responses* (n/75 trials)	n
Vehicle	–	31 ± 6	12
Flumazenil	3	47 ± 7*	11
Ethanol	1	56 ± 5*	11
Ethanol + flumazenil	1 + 3	59 ± 5*	11

*Flumazenil (Flu; 3 mg kg^{-1}) was administered i.p 5 min prior to testing. Ethanol (EtOH; 1 g kg^{-1}) was administered 10 min prior to testing. No antagonism of the response to ethanol was observed when the two drugs were co-administered (Et + Flu; $P>0.1$). There were no differences in response omissions between the groups ($P>0.05$) and only the Et + Flu group showed a greater number of intertrial crosses than the control group ($P<0.05$). n = number. *$P<0.05$ when compared to vehicle control.

the facilitated avoidance responding produced by ethanol (Table 1), indicating that blockade of benzodiazepine receptors does not affect the ethanol-induced increase in avoidance responding. However, flumazenil itself produced a significant increase in avoidance responding (Table 1; Figure 2), demonstrating that flumazenil induces the same behavioural action in this task as ethanol and chlordiazepoxide (Criswell & Breese, 1989). The dose-response effect of flumazenil in the avoidance task was shallow (Figure 2).

Effect of flumazenil and ethanol in rats withdrawn from chronic ethanol treatment

Chronic-ethanol treatment for 12 days antagonized the acute action of ethanol in the avoidance task for doses of ethanol up to 1.5 g kg^{-1} (Figure 1). A higher dose of ethanol (2 g kg^{-1}) was able to overcome the reduced responses observed for lower doses of ethanol in the avoidance task during withdrawal from chronic-ethanol treatment (Figure 1). Additionally, Figure 2 shows that 12 days on the chronic ethanol-liquid diet also reduced the usual facilitation of avoidance responding by 1 mg kg^{-1} of flumazenil. As observed when the dose of ethanol was increased, this reduced effectiveness of flumazenil during withdrawal was overcome if the dose of flumazenil was increased to 10 mg kg^{-1} (Figure 2).

Effect of the benzodiazepine-inverse agonist R0-15-4513 on avoidance responding and reversal by flumazenil and ethanol

Various doses of R0-15-4513, a benzodiazepine-inverse agonist (Mereu *et al.*, 1987; Bonetti *et al.*, 1989; Mehta & Ticku, 1989) were administered to groups of rats chosen for their rapid acquisition of this avoidance response (see Methods). In these rats, R0 15-4513 decreased acquisition of the avoidance response in a dose-related fashion (Figure 3). Conversely, the deficit in avoidance responding produced by RO 15-4513 could be overcome in a dose-related fashion by flumazenil (Table 2). As expected, ethanol also antagonized the reduced avoidance responding produced by RO-15-4513 (Figure 4). Due to a probable 'ceiling effect', ethanol and flumazenil alone had no significant action on avoidance responding in these rats ($P>0.1$; data not shown).

However, we did establish that RO 15-4513 induced a significant deficit in rats with intermediate levels of responding (data not shown), indicating that this action of RO 15-4513 is not dependent upon the performance level of the animals.

The effect of ethanol withdrawal on acquisition of avoidance responding also was examined in a group of rats selected for rapid acquisition of the avoidance response. A significant reduction in responding was observed in these rats during withdrawal from 12 days of chronic-ethanol exposure (chronic ethanol-liquid diet = 41.0 ± 8.5 avoidance response/last 75 trials vs control-liquid diet = 63.6 ± 2.8 avoidance responses/last 75 trials; $P<0.05$). Thus, a behavioural response like that for RO-15-4513 was observed during acute withdrawal from the chronic-ethanol treatment in these rats that had high avoidance response scores during acquisition.

Table 2 Effect of flumazenil (Flu) on the deficit in acquisition of an avoidance task produced by RO-15-4513 (RO) in control rats*

Treatments	*Drug doses*	*Avoidance responses* (*n*/75 trials)	n
Veh	0	41.1 ± 4.1	17
Flumazenil (Flu)	1	48.1 ± 5.6	8
RO-15-4513 (RO)	1	16.3 ± 5.9†	7
Flu + RO	0.3 + 1	11.2 ± 4.1	4
Flu + RO	0.6 + 1	32.0 ± 13.7	5
Flu + RO	1.0 + 1	43.3 ± 10.0*	6

*Rats were taken from groups shown to aquire a two-way shuttle box avoidance response (see Methods). Various doses of flumazenil (Flu, 0.3, 0.6 and 1 mg kg^{-1}) were administered i.p. just prior to rats receiving 1 mg kg^{-1} of RO-15-4513 (RO) which was 5 min before testing. The dose of ethanol is 1 g kg^{-1}. There were no differences in response omissions or intertrial crosses between the group ($P<0.1$). '0' under drug doses indicates that no drug was administered. See Figure 3 for deficit in responding during acquisition of the avoidance response for RO-15-4513. n = number.
†$P<0.05$ when compared to vehicle.
*$P<0.05$ when compared to RO-15-4513.

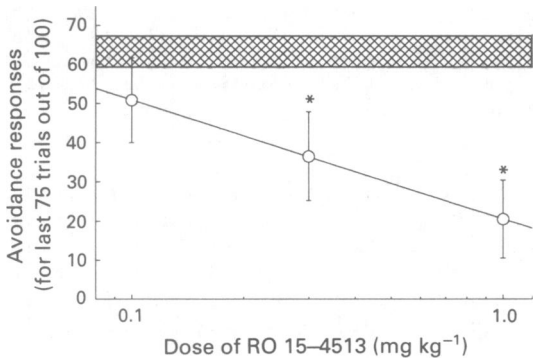

Figure 3 Dose response curve for the effect of RO 15-4513 on acquisition of an avoidance task. Naive rats were selected from a group which had been shown to make a high number of avoidance responses (see Methods). (○) Is the dose-response curve for RO 15-4513; points show mean ± s.e.mean for 6 rats. The vehicle response in the avoidance task is illustrated by the cross-hatched bar above the curve (vehicle response ± s.e.mean). Vehicle or RO 15-4513 were injected i.p. 5 min prior to testing. There were no differences in the number of response omissions or intertrial crosses between groups ($P>0.05$). *$P<0.05$ when compared to vehicle treatment.

Interactions of flumazenil with ethanol and chlordiazepoxide on aerial righting

The possibility that flumazenil possessed benzodiazepine agonist properties was tested by comparing the action of the benzodiazepine, chlordiazepoxide, to increase ethanol sedation to any change in sedation produced by flumazenil when combined with ethanol. In accordance with previous studies (Barthalmus *et al.*, 1978; Okamoto *et al.*, 1985), chlordiazepoxide enhanced the ethanol-induced deficit in aerial righting (Figure 5). Subsequently, it was reasoned that if flumazenil had a benzodiazepine agonist action, flumazenil, like chlordiazepoxide, would enhance the deficit in aerial righting produced by ethanol. The effects of flumazenil on the deficit in aerial righting produced by ethanol and chlordiazepoxide are shown in Figure 6. Flumazenil had no action in this task either to block or augment the action of ethanol (Figure 6). On the other hand, flumazenil (3 mg kg^{-1}) blocked the aerial righting deficit produced by 10 mg kg^{-1} of chlordiazepoxide (Figure 7). Furthermore, flumazenil (3 mg kg^{-1}) blocked the

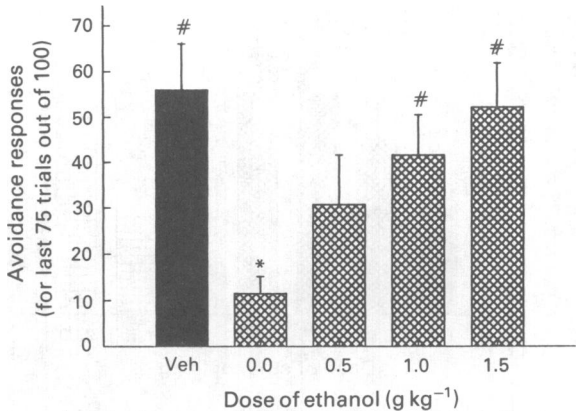

Figure 4 Reversal by ethanol of the effect of RO 15-4513 on performance in the shuttle-box avoidance task. The control group (no drug) which received only vehicle (Veh) 5 min prior to testing is designated by (■). Various doses of ethanol, (▩) were administered 5 min before 1 mg kg^{-1} of RO 15-4513 to a group of rats that readily acquired the avoidance response (see Methods). Testing began 5 min after administration of RO 15-4513 (1.0 mg kg^{-1}). The group designated as the 0.0 dose of ethanol in the column with cross-hatches received vehicle plus RO-15-4513. There were no differences in the number of response omissions or intertrial crosses between the drug-treated and control groups ($P>0.05$). The mean ± s.e.mean of 4–6 rats are shown for each dose. Ethanol (1 g kg^{-1}) alone did not produce a significant change from vehicle (see text). *$P<0.05$ when compared to vehicle alone (Veh). †$P<0.05$ when compared to RO-15-4513 alone designated to the 0.0 dose.

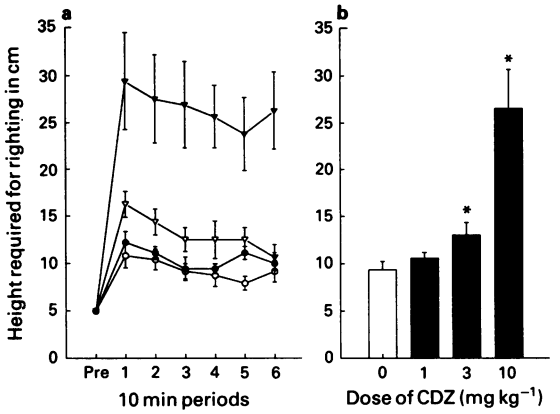

Figure 5 Dose-response relationship for enhancement by chlordiazepoxide (CDZ) of the ethanol-induced aerial-righting deficit. (a) Shows the mean ± s.e.mean of height required for righting for each 10 min period: (○) group that received ethanol alone; (●) group that received ethanol and 1 mg kg^{-1} of CDZ; (▽) group that received ethanol plus 3 mg kg^{-1} of CDZ; (▼) group that received ethanol plus 10 mg kg^{-1} of CDZ. Chlordiazepoxide (1, 3 or 10 mg kg^{-1}) was administered 5 min prior to ethanol (1.5 g kg^{-1}) administration. Aerial righting was assessed every 10 min for 1 h. Rats given only saline have scores of approximately 5 cm (Frye & Breese, 1982), as designated by Pre in (a). The columns in (b) depict the mean ± s.e.mean score for aerial righting scores for the rats in (a) for the total time period (1 h): (□) group that received only ethanol; (■) are the three doses of CDZ (1, 3 and 10 mg kg^{-1}). The score for aerial righting observed after 10 mg kg^{-1} of chlordiazepoxide (CDZ) alone is presented in Figure 6. Each group consisted of 8 to 12 rats. *$P<0.05$ when compared to ethanol alone.

increased deficit in aerial righting produced by chlordiazepoxide when administered to rats given ethanol (Figure 7). Thus, flumazenil continued to act as a benzodiazepine antagonist when given with only chlordiazepoxide as well as when chlordiazepoxide and ethanol were combined. Consequently, these results provide evidence that flumazenil is not acting as a benzodiazepine agonist at the doses used in the avoidance task but acts only as a benzodiazepine antagonist.

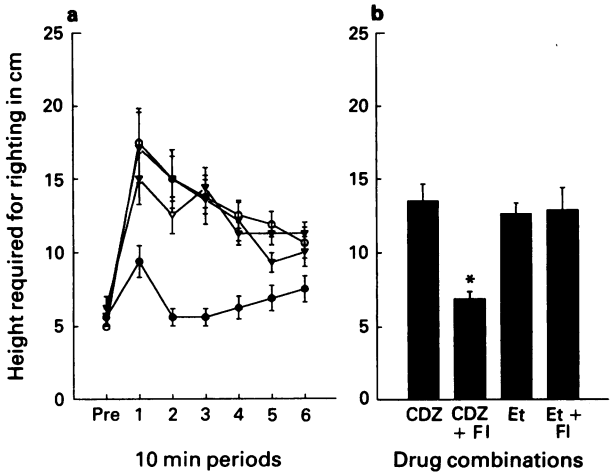

Figure 6 Effect of flumazenil on the aerial-righting deficit induced by chlordiazepoxide or ethanol. (a) Shows the mean ± s.e.mean aerial-righting score for 4 groups of 8 rats tested every 10 min for 1 h. Flumazenil (Fl, 3 mg kg^{-1}) was administered 5 min prior to either chlordiazepoxide (CDZ; 10 mg kg^{-1}) or ethanol (Et, 1.5 g kg^{-1}). Control data (Pre) were obtained immediately prior to drug administration; and (○) group that received CDZ; (▽) refers to the group that received only ethanol; (●) group that received CDZ plus flumazenil, and (▼) group that received ethanol and flumazenil. (b) The columns in this panel shown the mean ± s.e.mean aerial righting score for the accumulated 1 h period for each of the drug combinations in (a). These groups include CDZ, Et, CDZ + Fl, and Et + Fl as noted under the columns. Flumazenil alone did not affect aerial righting in untreated rats ($P > 0.1$). *$P < 0.05$ when compared to the chlordiazepoxide-treated group.

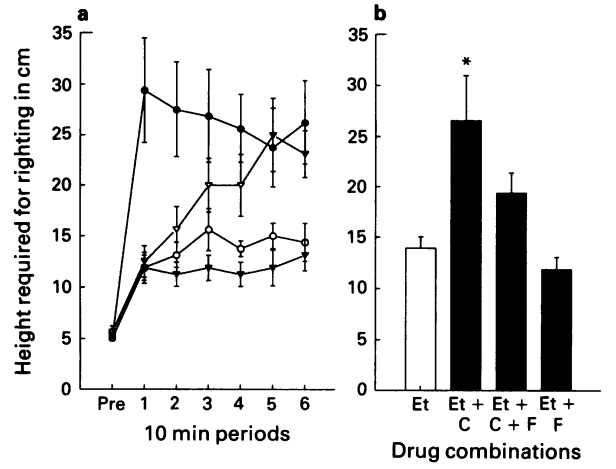

Figure 7 Effect of flumazenil on the chlordiazepoxide enhancement of ethanol-induced aerial-righting deficit. (a) Shows the mean ± s.e.mean aerial righting scores every 10 min for rats given ethanol (Et, 1.5 g kg^{-1}) 5 min after the following treatments were administered: saline (○, ethanol alone); ethanol plus 10 mg kg^{-1} chlordiazepoxide (●), ethanol plus 3 mg kg^{-1} of flumazenil (▼) or ethanol with both flumazenil and chlordiazepoxide (▽). Flumazenil blocked the enhancement of the ethanol-induced aerial-righting deficit by CDZ for the first 20 min ($P < 0.05$), but did not block the action of ethanol alone. (b) The columns show mean ± s.e.mean aerial righting score for the accumulated 1 h test period for each group in (a); (□) is for ethanol (Et) alone. The ■ are for the following groups: ethanol plus CDZ (Et + C); ethanol plus flumazenil (Et + F), and ethanol plus CDZ plus flumazenil (Et + C + F). Flumazenil alone did not affect aerial righting in untreated rats ($P > 0.1$). Eight rats were tested in each group. *$P < 0.05$ when compared to ethanol alone.

Discussion

Ethanol can facilitate acquisition of a two-way shuttle-box avoidance response (Frye & Breese, 1981; Criswell & Breese, 1989). This facilitatory effect of ethanol on avoidance responding has previously been cited as an indication that ethanol and chlordiazepoxide exhibit a type of anticonflict action in this task (Gray, 1977; Criswell & Breese, 1989; Fernandez-Teruel *et al.*, 1991a,b), similar to that seen in the acute lick suppression paradigm where shock suppressed licking is enhanced by these drugs (Vogel *et al.*, 1971; 1980). While motor stimulants such as (+)-amphetamine can facilitate avoidance responding (Rech *et al.*, 1966), the effect of ethanol in the avoidance task cannot be attributed to enhanced locomotion, because no dose of ethanol causes an increased locomotion in our Sprague-Dawley rats (unpublished data; Frye & Breese, 1981; Criswell & Breese, 1989). In the present work, tolerance to the action of ethanol in facilitating avoidance responding was observed during withdrawal from 12 days of chronic-ethanol treatment (i.e. liquid diet containing ethanol), replicating our previous findings (Criswell & Breese, 1989). In an earlier study, tolerance to ethanol during withdrawal from chronic ethanol treatment was accompanied by cross-tolerance to the action of a benzodiazepine in this task (Criswell & Breese, 1989). Tolerance to the anticonflict action of ethanol during withdrawal from chronic ethanol treatment also has been reported by File *et al.* (1989). Furthermore, an anxiogenic response has been observed in these behavioural tasks during withdrawal from chronic administration of both ethanol and benzodiazepines (Baldwin & File, 1987; File *et al.*, 1989; File & Hitchcott, 1990; Baldwin *et al.*, 1991). This series of studies suggested that there was a link between the tolerance to the anticonflict action of ethanol during withdrawal from chronic-ethanol treatment and benzodiazepine receptor mechanisms.

Flumazenil, a benzodiazepine antagonist, has been reported to reduce the anxiogenic response associated with withdrawal from chronic benzodiazepine or ethanol administration (Baldwin & File, 1987; File *et al.*, 1989; File & Hitchcott, 1990), as does ethanol (Figure 2). Nevertheless, unlike a benzodiazepine or ethanol (Frye *et al.*, 1980; 1983), flumazenil does not prevent the majority of symptoms associated with the ethanol-withdrawal syndrome (Little *et al.*, 1985; Adinoff *et al.*, 1986; Chan *et al.*, 1991). Furthermore, flumazenil did not reduce the acute action of ethanol in the avoidance task in control rats, indicating that this action of ethanol was not due to a direct action on benzodiazepine receptors. However, this investigation demonstrated that flumazenil facilitated acquisition of the avoidance response in control rats. This behavioural response exhibited by flumazenil resembled that seen after administration of ethanol or a benzodiazepine (Criswell & Breese, 1989).

Based on results obtained with flumazenil, File *et al.* (1989) proposed the hypothesis that tolerance to the anticonflict action of ethanol during withdrawal from chronic-ethanol treatment was related to an increase in an endogenous substance acting as a benzodiazepine-inverse agonist. Therefore, one purpose of the present investigation was to determine if flumazenil would affect tolerance to the anticonflict action of ethanol in the avoidance task during withdrawal, as had been noted in another conflict task (File *et al.*, 1989). During withdrawal from 12 days of chronic-ethanol treatment, the facilitated responding in the avoidance task following 1 mg kg^{-1} of flumazenil was reduced to levels observed in rats given vehicle (i.e. tolerance like that observed to ethanol and chlordiazepoxide after chronic ethanol exposure). Administration of a higher dose of flumazenil during withdrawal from chronic ethanol treatment reinstated responding to a level similar to that observed when a lower dose of flumazenil was administered to rats receiving control liquid diet. As noted for flumazenil, increasing the dose of ethanol also overcame the tolerance observed following withdrawal from chronic ethanol treatment, providing additional evidence for similarities between ethanol and flumazenil in the avoidance task. While metabolic factors could contribute to the decreased response to ethanol after chronic-ethanol treatment (e.g. increased ethanol metabolism), a similar ex-

planation for the results with flumazenil seems unlikely. Two additional explanations for these data would be that flumazenil was antagonizing the action of an endogenous substance acting as a benzodiazepine-inverse agonist (i.e. as suggested by File *et al.*, 1989) or that flumazenil was acting as a benzodiazepine agonist to produce an anticonflict action in the avoidance task.

With the logic that an endogenous benzodiazepine inverse agonist might be present during withdrawal, it was reasoned that, if withdrawal from chronic ethanol was increasing the presence of an endogenous benzodiazepine-inverse agonist, then administration of a benzodiazepine-inverse agonist like RO-15-4513 (Mereu *et al.*, 1987), should produce a deficit in responding in the avoidance task and this deficit should be antagonized by flumazenil. In rats chosen for their rapid acquisition of avoidance responding (see Methods), a dose-related deficit in acquisition of the avoidance response was noted after RO-15-4513 administration. This finding is consistent with other reports that benzodiazepine-inverse agonists produce deficits in behavioural tasks with a conflict component (Corda *et al.*, 1983; File & Pellow, 1984; Fernandez-Teruel *et al.*, 1991,a,b; Takada *et al.*, 1992). In earlier work, File & Lister (1983) reported that the anxiogenic response to a β-carboline in the social interaction test is blocked by flumazenil, just as it antagonizes the anticonflict action of a benzodiazepine (Barrett *et al.*, 1985). Ethanol as well as flumazenil effectively reversed the deficit in the avoidance task produced by RO 15-4513. This demonstration that doses of ethanol or flumazenil could antagonize RO-15-4513 in the avoidance task would predict that doses of both ethanol and flumazenil could be found that would produce an effect in tolerant animals like that seen in control rats. This in fact was the case. Since RO-15-4513 acts directly on $GABA_A$ receptors, ethanol could be enhancing γ-aminobutyric acid (GABA) responsiveness or acting through a parallel neural system to overcome the action of RO-15-4513 to reduce responding in the avoidance task. However, flumazenil reversal of both the effect of RO-15-4513 and the tolerance to the anticonflict action of ethanol observed in the avoidance task, seemed most likely to be due to a direct action on benzodiazepine receptors, an interpretation consistent with the hypothesis proposed by File *et al.* (1989). Nonetheless, we next examined the alternative possibility that flumazenil possessed benzodiazepine agonist activity to produce behavioural changes in the avoidance task (i.e. was having an agonist action on benzodiazepine receptors).

The possible agonist activity of flumazenil at the benzodiazepine receptor was supported by *in vitro* data in which high concentrations of flumazenil had been reported to possess a benzodiazepine-like agonist action (Skerritt & MacDonald, 1983; Mehta & Ticku, 1989). However, under most *in vivo* conditions, flumazenil has not been reported to exhibit the pharmacological properties of a benzodiazepine agonist (Crawley *et al.*, 1984; Barrett *et al.*, 1985; Koob *et al.*, 1986; Thomas *et al.*, 1990), including a discrimination task for a benzodiazepine (Pugh, *et al.*, 1992). Such a lack of behavioural action of flumazenil was observed in the present investigation when, unlike chlordiazepoxide, it did not affect the sedation induced by ethanol (i.e. did not affect the deficit in aerial righting induced by ethanol; Figure 6). Further, flumazenil not only blocked the deficit produced by chlordiazepoxide on aerial righting, but also antagonized the facilitation of the ethanol-induced aerial righting deficit produced by chlordiazepoxide. Thus, the present evaluation of flumazenil in combination with ethanol and chlordiazepoxide provided convincing evidence that, at doses used in the avoidance task, flumazenil possesses no discernible benzodiazepine agonist action, but rather is an effective benzodiazepine antagonist. Of course, this absence of an effect of flumazenil on aerial righting contrasts with the action of flumazenil to facilitate responding in the avoidance task. File & Hitchott (1990) as well as Fernandez-Teurel *et al.* (1991a,b) have implied that the circumstance under which behavioural measures are taken determine whether flumazenil will affect behaviour. Our present data are consistent with this view. Thus, the data collected in the avoidance task and the lack of evidence that flumazenil acts as a benzodiazepine agonist are in support of the view that an endogenous benzodiazepine-inverse agonist could be involved both in the general poor learning by rats in this behavioural paradigm (Fernandez-Teurel *et al.*, 1991a,b), as well as in the reduced anti-conflict action (i.e. tolerance) to ethanol and flumazenil in the avoidance task during withdrawal from chronic ethanol treatment (File *et al.*, 1989; File & Hitchcott, 1990).

Schatzkik *et al.* (1989) has provided evidence for an increased sensitivity of receptors for inverse agonists and decreased sensitivity of benzodiazepines following withdrawal from chronic lorazepam treatment. Similarly, Buck & Harris (1990) provided evidence that chronic ethanol administration densensitizes benzodiazepine receptors to agonist action while simultaneously increasing their sensitivity to inverse agonists. This change in benzodiazepine receptor function would be expected to produce behavioural changes similar to those observed in the present study, if a benzodiazepine-inverse agonist were to be present in brain to influence these sensitized receptors. However, in the latter study, the increase in sensitivity to a benzodiazepine-inverse agonist was observed after acute as well as chronic ethanol administration, suggesting that this change in receptor sensitivity is associated with acute ethanol tachyphylaxis. Tolerance to the effect of ethanol on shuttle-box avoidance acquisition is not observed until rats have been treated with ethanol for at least seven days (Criswell & Breese, 1989).

There have been nunerous attempts to isolate substances from brain that act on the benzodiazepine receptor (Braestrup *et al.*, 1980; Guidotti *et al.*, 1983; Peña *et al.*, 1986). It seem relatively clear that endogenous benzodiazepines exist in brain (Sangameswaran *et al.*, 1986; Wolfman *et al.*, 1991). Likewise, there are endogenous compounds which are purported to have properties of a benzodiazepine-inverse agonist (Guidotti *et al.*, 1983; Peña *et al.*, 1986; Novas *et al.*, 1988). Assuming that these endogenous compounds have an action in the brain, the absence of an effect of flumazenil in the aerial righting reflex would relate to a physiological state where little endogenous benzodiazepine agonist or inverse agonist were present in brain. Conversely, when rats are performing the avoidance task during withdrawal from chronic ethanol (i.e. aversive situation), the content of the endogenous benzodiazepine-inverse agonist in brain would be expected to increase to a greater degree than would be observed under normal conditions. As noted earlier, others have suggested that the behaviour conditions dictate whether flumazenil has an effect in a behavioural task (File & Hitchcott, 1990; Fernandez-Teurel *et al.*, 1991a,b). In any case, the similar actions of ethanol and flumazenil, in the absence of any evidence for a benzodiazepine agonist like action of flumazenil, provide support for the hypothesis that an endogenous benzodiazepine-inverse agonist is responsible for the reduced effectiveness of flumazenil and ethanol in the avoidance task following withdrawal from chronic-ethanol treatment (File *et al.*, 1989). The endogenous peptide, diazepam binding inhibitor (DBI), which was isolated from mammalian brain (Guidotti *et al.*, 1983), acts as a benzodiazepine inverse agonist (Ferrero *et al.*, 1984; 1986). This endogenous peptide is reportedly low during normal conditions, but is increased by stressful and anxiety producing situations (Ferrero *et al.*, 1988; Ferrarese *et al.*, 1991a,b). Consequently, defining the potential role of this endogenous peptide in the behavioural change in conflict behaviour in the avoidance task during withdrawal from chronic ethanol treatment would be a logical extension of our present investigation.

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