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Interactions of Three Benzodiazepine Receptor Inverse Agonists with Ethanol in a Plus-Maze Test of Anxiety

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LISTER, R. G. *Interactions of three benzodiazepine receptor inverse agonists with ethanol in a plus-maze test of anxiety.* PHARMACOL BIOCHEM BEHAV 30(3) 701–706, 1988.—The effects of RO 15-4513, RO 15-3505 and FG 7142 on the anxiolytic properties of ethanol in mice were investigated using the plus-maze test of anxiety. Before being tested on the plus-maze, the mice were tested in a holeboard apparatus. All three inverse agonists attenuated the reduction in exploration caused by ethanol in the holeboard test. In the plus-maze, only RO 15-4513 and FG 7142, which possess anxiogenic properties when administered alone, attenuated ethanol's anxiolytic effect. RO 15-3505, which alone had no effect on anxiety, failed to significantly reduce ethanol's anxiolytic effect. Neither RO 15-4513 nor FG 7142 reduced the increase in the total number of arm entries caused by ethanol. These data indicate that the interaction between ethanol and benzodiazepine receptor ligands depends both on the intrinsic properties of the ligands and the behavior under investigation.

Alcohol Benzodiazepine receptor Motor activity Exploration Anxiety Mouse RO 15-4513 FG 7142 RO 15-3505

THERE have been a number of reports that RO 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a] [1,4] benzodiazepine-3-carboxylate) a benzodiazepine receptor partial inverse agonist [1, 19, 26], can reverse at least partially some of the behavioral effects of ethanol [2, 3, 10, 12, 13, 18, 20, 24, 25, 27]. In some paradigms other benzodiazepine receptor inverse agonists (e.g., FG 7142) are also capable of antagonising ethanol's effects [3, 11, 13]. In other tests [24], in particular one in which an observer rates ethanol-induced intoxication in rats, it has been suggested that RO 15-4513 differs from other benzodiazepine receptor inverse agonists in its ability to reverse ethanol's effects [27].

The aim of the present study was to examine the interactions of three different inverse agonists with ethanol in the plus-maze test of anxiety [8, 14, 22]. This test is sensitive to the anxiolytic effect of alcohol, and the anxiogenic effect of benzodiazepine receptor inverse agonists [14,22]. The three inverse agonists used are RO 15-4513 (mentioned above), FG 7142 a β-carboline derivative which is anxiogenic both in animals and humans [4,21], and RO 15-3505, a structural analogue of RO 15-4513 with a high affinity for benzodiazepine receptors that has been used to antagonise the effects of benzodiazepines in humans [6,7]. It possesses weak inverse agonist properties [23]. The doses used were ones we have employed in previous studies with these agents. Immediately before testing animals on the plus-maze, they received a 5-min exposure to a holeboard apparatus. This procedure has been used in other studies with the plus-maze [14,22]. The holeboard test assesses an animal's exploratory behavior (head-dipping) independently of locomotor activity [5].

METHOD

RO 15-4513, FG 7142 and RO 15-3505 were suspended in distilled water to which a drop of Tween 20/10 ml had been added. These drugs were administered intraperitoneally (IP) in a volume of 10 ml/kg. Ethanol was prepared as a 20% (w/v) solution in distilled water. All injections were made IP using an injection volume of 10 ml/kg.

Male NIH Swiss mice, Weighing approximately 24 g, were housed in groups of 10, maintained on a 12 hr light:12 hr dark cycle and allowed ad lib access to food and water.

Apparatus

The holeboard apparatus was made of Plexiglas (40× 40×30 cm) and had four holes 3 cm in diameter equally spaced in the floor. Infra-red photocells in the walls of the box and directly beneath each hole provided automated measures of locomotor activity, of the number of exploratory head-dips made and the duration of head-dipping.

The plus-maze was also made of Plexiglas and consisted of two open (30×5 cm) and two enclosed (30×5×15 cm) arms. The arms extended from a central platform 5×5 cm. The open arms, the central platform, and the floor of the closed arms were made of black Plexiglas, and the sides of the closed arms were made of clear Plexiglas. The apparatus was mounted on a Plexiglas base raising it 38.5 cm above the

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floor. The test consisted of placing a mouse in the center of the apparatus and allowing it to freely explore for 5 min. The number of entries made on to the open and closed arms and the time spent on each type of arm were recorded using an event recorder interfaced with a micro PDP-11. Three measures were obtained from the test: the total number of arm entries; the percentage of arm entries made on to the open arms; the time spent on the open arms expressed as a percentage of the time spent on both the open and closed arms. The last two measures are used as indices of anxiety. They increase following the administration of anxiolytics and decrease following the administration of anxiogenic drugs [8, 14, 22]. Both the holeboard and plus-maze tests were performed under low lighting conditions.

Procedure

In Experiment 1, 70 mice were divided into two equal groups receiving either ethanol (2 g/kg) or the vehicle. Twenty-three minutes later approximately one-third of the animals in each group received RO 15-4513 (1.5 mg/kg), one third received RO 15-4513 (3.0 mg/kg) and the remaining mice received the vehicle. Each mouse was placed in the holeboard for a 5-min test immediately after the injection of RO 15-4513 (or vehicle). At the end of the holeboard test, each mouse was tested for 5 min on the plus-maze. In this and all subsequent experiments testing was carried out in an order randomised for drug treatment between 08:00 and 13:30.

In Experiment 2, 59 mice were divided into 2 approximately equal groups receiving either ethanol (2 g/kg) or its vehicle. Twenty-three minutes later approximately half the animals in each group received RO 15-3505 (1.5 mg/kg) and the rest received the vehicle. Each mouse was placed in the holeboard for a 5-min test immediately after the second injection. At the end of the holeboard test, each mouse was tested for 5 min on the plus-maze.

In Experiment 3, 59 mice were divided into 2 approximately equal groups receiving ethanol (2 g/kg) or its vehicle. Eight minutes later, approximately one-third of the animals in each group received FG 7142 (20 mg/kg), one-third received FG 7142 (40 mg/kg) and the remaining animals received the vehicle. Fifteen minutes later each mouse was tested individually in the holeboard for 5 minutes and then tested on the plus-maze immediately after that.

In order to replicate the results of Experiment 1 and to further investigate the intrinsic effects of RO 15-4513 in the plus-maze, a final experiment was performed. Forty mice were divided into two groups receiving ethanol (2 g/kg) or the vehicle. Twenty-three minutes later half the mice in each group received RO 15-4513 (3.0 mg/kg) and the rest received the vehicle. The mice were tested for 5 min in the holeboard immediately after the second injection. After removal from the holeboard each mouse was tested on the plus-maze.

In all experiments data were analysed using analysis of variance. Simple main effects were evaluated as described in [28].

RESULTS

For clarity, the results of the holeboard and plus-maze experiments are reported separately.

The Plus-Maze Test

Since the pattern of results in all experiments was similar regardless of which index of anxiety was used (either the proportion of entries made onto the open arms, or the proportion of time spent on the open arms), for convenience, only the data using the latter measure are presented here.

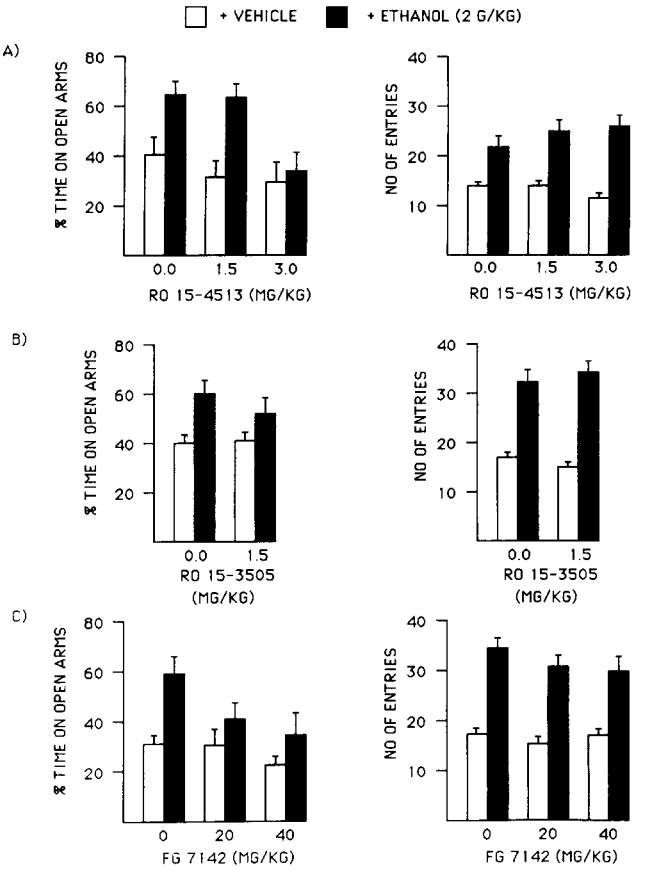

FIG. 1. The time spent on the open arms of a plus-maze expressed as a percentage of the time spent on both open and closed arms (left) and the total number of arm entries (right) in a 5 min test (A) 30 min after treatment with ethanol (0 or 2 g/kg) and 7 min after treatment with RO 15-4513 (0, 1.5 or 3 mg/kg); (B) 30 min after treatment with ethanol (0 or 2 g/kg) and 7 min after treatment with RO 15-3505 (0 or 1.5 mg/kg); and (C) 30 min after treatment with ethanol (0 or 2 g/kg) and 22 min after treatment with FG 7142 (0, 20 or 40 mg/kg). Scores are means±SEM, n=9–15 per group.

The results of Experiment 1 are shown in Fig. 1A. There were significant main effects of ethanol, $F(1,64)=13.2$, $p<0.001$, and of RO 15-4513, $F(2,64)=5.1$, $p<0.01$, on the percentage of time spent on the open arms of the plus-maze. Ethanol increased this measure reflecting its anxiolytic properties, and RO 15-4513 reduced this measure. The reduction in the proportion of time spent on the open arms seen in animals receiving RO 15-4513 alone failed to reach significance, buth RO 15-4513 significantly attenuated the increase in this measure caused by ethanol ($p<0.01$). Ethanol also increased the total number of arm entries, $F(1,64)=59.3$, $p<0.0001$. RO 15-4513 did not alter this measure either alone, or in combination with ethanol.

In Experiment 2, ethanol again significantly increased the percentage of time spent on the open arms, $F(1,55=10.2$, $p<0.005$. RO 15-3505 alone failed to alter this measure. Although the mean of animals receiving the drug combination was below that of animals that received ethanol alone, this difference did not reach significance. Ethanol also increased

the total number of arm entries, $F(1,55)=86.0, p<0.0001$. RO 15-3505 showed no indication of reversing this effect (see Fig. 1B).

In Experiment 3, there were significant main effects of ethanol, $F(1,53)=10.4$, $p<0.005$, and of FG 7142, $F(2,53)=3.3$, $p<0.05$, in the analysis of the percentage of time spent on the open arms. In Fig. 1C it can be seen that ethanol alone increased this measure ($p<0.01$) and both doses of FG 7142 reversed this effect ($p<0.01$). The reduction in the proportion of time animals treated with FG 7142 alone spent on the open arms failed to reach significance, although FG 7142 did decrease the proportion of arm entries made on to the open arms ($p<0.05$). Ethanol increased the total number of arm entries, $F(1,53)=86.4$, $p<0.0001$. FG 7142 did not alter this measure.

In the final experiment there were main effects of ethanol, $F(1,36)=48.1$, $p<0.0001$, and RO 15-4513, $F(1,36)=9.1$, $p<0.005$, in the analysis of the percentage of time spent on the open arms, ethanol increasing and RO 15-4513 decreasing this index of anxiety (see Fig. 4A). Between group comparisons showed that animals that received RO 15-4513 alone spent a lesser proportion of their time on the open arms than vehicle-treated animals ($p<0.05$). Ethanol increased, $F(1,36)=78.7$, $p<0.0001$, but RO 15-4513 did not alter the total number of arm entries.

The Holeboard Test

Exploration. There were significant RO 15-4513 × ethanol interactions in the analysis of both the number, $F(2,64=18.4$, $p<0.0001$, and duration, $F(2,64)=19.2$, $p<0.0001$, of exploratory head-dips. In Fig. 2A, it can be seen that ethanol and both doses of RO 15-4513 alone reduced the number of head-dips ($p<0.01$), but animals that received the drug combinations made more head-dips than those that received either drug alone ($p<0.01$). A similar pattern was observed with the duration of head-dipping, both drugs alone reducing this measure ($p<0.01$), but the combination of RO 15-4513 with ethanol tending to produce more head-dipping than either drug alone. This difference however only reached significance ($p<0.01$) for the lower dose of RO 15-4513.

In Experiment 2 there was a significant departure from homogeneity of variance for the measure of the duration of head-dipping ($p<0.01$, Hartley's test). These data were, therefore, subjected to a log transformation prior to analysis. Ethanol alone significantly reduced the number of head-dips ($p<0.01$) and this effect was partially reversed by RO 15-3505 ($p<0.01$), which alone did not significantly alter this measure, see Fig. 2B. A similar pattern of results was observed with the duration of head-dipping, ethanol alone reducing this measure ($p<0.01$) and RO 15-3505 alone exerting no effect, but attenuating the reduction caused by ethanol ($p<0.05$).

In Experiment 3, there was a departure from homogeneity of variance in the duration of head-dipping scores ($p<0.01$). These were subjected to a log transformation prior to analysis. There was a significant ethanol × FG 7142 interaction in the analysis of both the number, $F(2,53)=4.0, p<0.03$, and duration, $F(2,53)=5.6, p<0.01$, of head-dips. In Fig. 2C it can be seen that ethanol alone failed to reduce the number of head-dips, although it did reduce the time spent head-dipping ($p<0.01$). FG 7142 (20 mg/kg) reduced the number and duration of head-dips ($p<0.05$) and this effect was reversed by ethanol ($p<0.05$). Mice that received ethanol in combination with FG 7142 (40 mg/kg) made significantly more head-dips

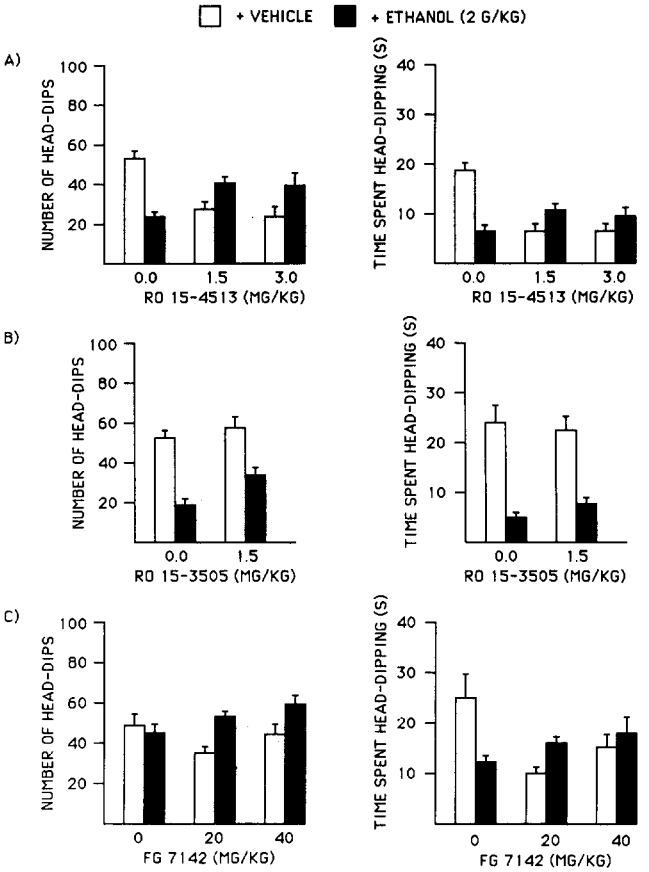

FIG. 2. The number of head-dips (left) and the time spent head-dipping (right) in a 5-min holeboard test (A) 23 min after treatment with ethanol (0 or 2 g/kg) and immediately after treatment with RO 15-4513 (0, 1.5 or 3 mg/kg); (B) 23 min after treatment with ethanol (0 or 2 g/kg) and immediately after treatment with RO 15-3505 (0 or 1.5 mg/kg); and (C) 23 min after treatment with ethanol (0 or 2 g/kg) and 15 min after treatment with FG 7142 (0, 20 or 40 mg/kg). Scores are means±SEM, n=9–15 per group.

than those that received either drug alone ($p<0.05$).

In the final experiment there was again an interaction between RO 15-4513 and ethanol in the analysis of both the number, $F(1,36)=21.7$, $p<0.001$, and duration, $F(1,36)=33.1$, $p<0.0001$, of head-dips. In Fig. 4B it can be seen that while both RO 15-4513 and ethanol alone decreased both measures ($p<0.01$), mice that received the drug combination made more head-dips and spent longer head-dipping than those that received either drug alone.

Locomotion. In Experiment 1 ethanol significantly increased motor activity, $F(1,64)=41.6, p<0.0001$. RO 15-4513 alone failed to alter this measure in animals pretreated with either ethanol or the vehicle (see Fig. 3A).

In Experiment 2, ethanol again increased motor activity, $F(1,55)=93.5$, $p<0.0001$. RO 15-3505 failed to alter this measure in either vehicle- or ethanol-treated mice (see Fig. 3B).

In Experiment 3 ethanol increased locomotor activity, $F(2,54)=117.1$, $p<0.0001$. FG 7142 was without effect (see Fig. 3C).

In the final experiment ethanol again increased locomotor activity, $F(1,36)=73.5$, $p<0.0001$. RO 15-4513 failed to alter locomotion (see Fig. 4C).

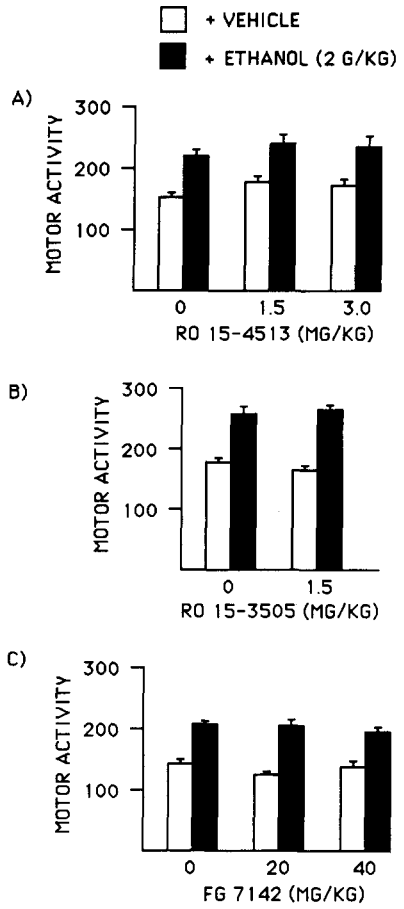

FIG. 3. The locomotor activity scores of mice in a 5-min holeboard test (A) 23 min after treatment with ethanol (0 or 2 g/kg) and immediately after treatment with RO 15-4513 (0, 1.5 or 3 mg/kg); (B) 23 min after treatment with ethanol (0 or 2 g/kg) and immediately after treatment with RO 15-3505 (0 or 1.5 mg/kg); and (C) 23 min after treatment with ethanol (0 or 2 g/kg) and 15 min after treatment with FG 7142 (0,20 or 40 mg/kg). Scores are means±SEM, n=9–15 per group.

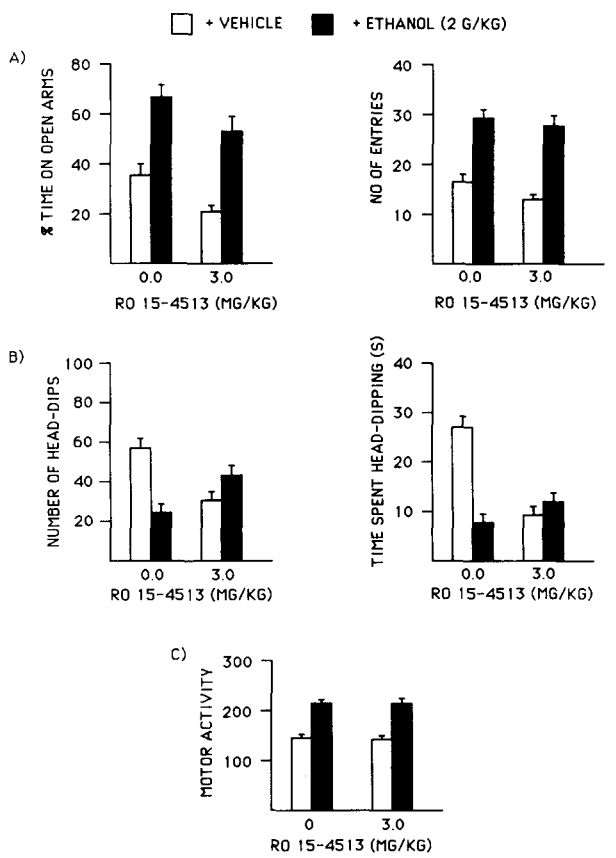

FIG. 4. (A) The time spent on the open arms of a plus-maze expressed as a percentage of the time spent on both open and closed arms (left) and the total number of arm entries (right) in a 5-min plus-maze test 30 min after treatment with ethanol (0 or 2 g/kg) and 7 min after treatment with RO 15-4513 (0 or 3 mg/kg); (B) The number of head-dips (left) and the time spent head dipping (right) in a 5 min test in a holeboard 23 min after treatment with ethanol (0 or 2 g/kg) and immediately after treatment with RO 15-4513 (0 or 3 mg/kg); (C) The locomotor activity in a 5-min test in a holeboard 23 min after treatment with ethanol (0 or 2 g/kg) and immediately after treatment with RO 15-4513 (0 or 3 mg/kg). Scores are means± SEM, n=10 per group.

DISCUSSION

The various interactions between ethanol and the three inverse agonists reported above and discussed below appear to have a pharmacodynamic rather than a pharmacokinetic basis since none of the drugs alters blood alcohol concentrations at the time of testing [13,15].

In all experiments ethanol increased the proportion of time mice spent on the open arms of the plus-maze, reflecting its anxiolytic action. The benzodiazepine receptor partial inverse agonists RO 15-4513 and both FG 7142 reversed this effect. Ethanol also consistently increased the total number of arm entries. Interestingly, none of the inverse agonists tested reduced this effect, providing further evidence for the dissociation of the anxiolytic from the stimulant effect of ethanol. The reversal of the anxiolytic effect of ethanol should be considered in relation to the intrinsic effects of inverse agonists on anxiety. FG 7142 has been shown to possess anxiogenic properties in many animal paradigms (including the plus-maze [14,22]), and also in humans [4]. In the present study the reduction in the proportion of time spent on the open arms by FG 7142 alone just failed to reach significance, although FG 7142 did decrease the proportion of entries made on to the open arms. In both Experiments 1 and 4, RO 15-4513 alone reduced the proportion of time spent on the open arms, although this effect only reached significance in the final experiment. This is consistent with the results of other studies showing this compound to be anxiogenic [9]. It seems likely, therefore, that in the plus-maze paradigm the intrinsic anxiogenic activity of the inverse agonists underlies their ability to reverse ethanol's anxiolytic effect. This interpretation is also consistent with results from a conflict model [3] and also parallels results obtained using a seizure threshold paradigm [20]. The inverse agonist properties of RO 15-3505 do not appear to be as marked as those of RO 15-4513 [16]. At high doses it has been found to possess a slight anxiogenic action in the plus-maze and social interaction tests of anxiety in rats (File, personal communication). In the present study, RO 15-3505 alone showed no indication of reducing either index of anxiety, and the attenuation of ethanol's anxiolytic effect was not significant. Together, these results strongly suggest that

for a benzodiazepine receptor ligand to antagonise ethanol's anxiolytic effect it must possess anxiogenic activity when administered alone.

The holeboard study differed in two respects from the studies published previously [12, 13, 15]. Firstly, the imidazodiazepines were given immediately before animals were tested in the holeboard, as opposed to 8 min before the holeboard test. (This was done to optimise drug concentrations for the plus-maze test.) Secondly, the holeboard test lasted 5 rather than rather than 8 minutes. Despite these procedural differences the overall pattern of results resembled those of the previous studies [12,15]. That is, RO 15-4513 and RO 15-3505 both attenuated the reductions in exploration caused by ethanol. These drugs appear, therefore, to have a very rapid onset of action. The short duration of action of RO 15-4513 has already been noted [17]. The interaction of FG 7142 with ethanol also resembled that found in a previous study in which a longer interval was used between drug administration and testing [13].

In some respects the data from the holeboard test contrast with those from the plus-maze. Firstly, in the plus-maze the effect of ethanol was opposite in direction to that of RO 15-4513 and FG 7142, and the effects of these inverse agonists appeared to subtract from those of ethanol. In the holeboard test the effects of RO 15-4513 and FG 7142 on exploration were in the same direction as that of ethanol (i.e., a reduction was observed), and yet a mutual antagonism was found when the inverse agonists and ethanol were combined. Secondly RO 15-3505 was able to attenuate ethanol's effects in the holeboard but not the plus-maze. This selectivity may result from a difference in sensitivity between the two tests, or from the fact that the two tests are measuring different behaviors [14], and ethanol's effects on these behaviors may be mediated by different neurobiological mechanisms. Experiments are currently in progress to determine whether RO 15-3505 antagonises ethanol's effects in other paradigms.

In conclusion, the ability of RO 15-4513 and FG 7142 to antagonise the anxiolytic effect of ethanol appeared to be related to their intrinsic anxiogenic effects—the anxiogenic action of each inverse agonist merely subtracting from the anxiolytic effect of ethanol. In the holeboard all three inverse agonists partially reversed ethanol's effects on exploratory head-dipping. These data indicate that the nature of the interaction between ethanol and benzodiazepine receptor ligands depends both on the intrinsic properties of the ligands and the behavioral measure being investigated.

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