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The Increased Ethanol Preference in Rats Induced by Choice, Darkness, or Drugs is Reduced by Ritanserin

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LIN, N. AND J.I. HUBBARD. *The increased ethanol preference in rats induced by choice, darkness, or drugs is reduced by ritanserin.* BRAIN RES BULL **33**(6) 633–638, 1994.—We tested the hypothesis that ritanserin, a serotonin S_2 antagonist, reduces voluntary and induced forms of ethanol drinking. We gave 10 mg/kg ritanserin IP or SC to groups of rats given either a) a free choice between 3% ethanol and water, or b) kept in the dark for 5 weeks and given a choice between a range of ethanol concentrations (3–25%) and water, or c) implanted with osmotic pumps filled with tetrahydro-β carboline and given a choice between a range of ethanol concentrations and water. In each case, ritanserin signficantly reduced ethanol consumption and ethanol preference for 8–10 days after the last injection.

Ethanol Rat Ritanserin Dark cycle Tetrahydro-β carboline

RITANSERIN is a very potent and long lasting serotonin-S_2 antagonist (8,10). Indeed, chronic treatment with ritanserin brings about downregulation of serotonin-S_2 sites (11). In both man and rats ritanserin treatment reduces the intake of and the preference for 3% alcohol (12,13,16). Panocka and Massi (1992) suggest that ritanserin has its effect on ethanol drinking by disinhibition of the dopamine neurons of the A10 group in the midbrain. We have investigated whether the drug will also affect the increase in ethanol preference produced by either keeping rats on a 24 h dark cycle (6) or by infusing IVT 2,3,4,9-tetrahydro-1h-pyrido[3.4-6]indole [tetrahydro-β carboline, THBC-Sigma Chemical Co. (19)]. Our purpose was twofold. Firstly we tested ritanserin to see if it acts at a motivational stage of ethanol drinking, as suggested by Panocka and Massi (16), as it should then reduce all methods of inducing ethanol drinking. Secondly, we have investigated the increase in ethanol preference brought about by keeping rats on a 24 h dark cycle using a range of ethanol strengths, an aspect of ethanol drinking that has not to our knowledge been previously tested.

METHOD

Animals

Male Sprague–Dawley rats, weighing 250–400 g, were used. They were kept in individual cages at a temperature of 24 ± 2°C and fed an ad lib diet. Unless otherwise noted, they were kept on a 12L:12D cycle in which the dark cycle began at 0900 h and drinking of water and ethanol was measured after 1 h of the dark cycle, i.e, at 1000 h. Rats were given the choice of three tubes, one filled with water, one with the concentration of ethanol being tested on that day (3–30%) by weight/volume), and one empty. The position of the tubes was varied randomly each day to avoid the setting up of a position habit (14). Preference was measured as the ratio of ethanol consumed to total fluid (ethanol and water) consumed.

Drugs

Ritanserin (6-[2-[4-[bis(4-Fluorphenyl)methylene]-1-piperidinyl]-ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one) was obtained from Research Biochemicals Inc. Natick, MA. Tetrahydro-β carboline (2,3,4,9-Tetrahydro-1H-pyrido[3,4-b]indole) (THBC) was obtained from the Sigma Chemical Co., St. Louis, MO.

Drug Administration

Ritanserin was dissolved (10 mg/ml/kg) in a vehicle consisting of 20% propylene glycol, 0.5% acetic acid, and 2 M NaOH to adjust the pH to 5 and injected either SC or IP. THBC was infused IVT from an osmotic pump (Model 2002, mean pumping

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rate, at 37°C, 0.48 ± 0.02 μl/h, Alza Corporation, Palo Alto, CA). Rats were anaesthetized with Equithesin (3 ml/kg), and IVT cannulas were implanted (coordinates P, 0.7, L, 1.6, H, 4.0) and connected to osmotic pumps placed under the skin of the neck. The pumps contained either THBC, 35 nmol/0.48 μl, dissolved in the vehicle, artificial cerebrospinal fluid (15), or the vehicle. These solutions were sterilized by passing them through a membrane filter (Millex-GV-0.22 μm filter unit). At the conclusion of this experiment all rats were deeply anaesthetized with Equithesin IP and the pump and connection with the cannula were exposed and checked for continuity and patency. A 10μl injection of methylene blue was then given through the connecting tube and cannula to fill the CSF with dye. The rats were then perfused intracardially with normal saline followed by formol saline. Their brains were removed and hardened in formalin for 2–3 days. Coronal sections (50 μm) were then cut with a freezing microtome for histological verification of the cannula placements.

Experiment 1: The Effects of Ritanserin on Ethanol Preference in Randomly Selected Rats

The experimental procedure of Panocka and Massi (16) was used, i.e., rats were forced to drink only 3% ethanol for a week and then had only water for a week. They were then tested with the three-bottle technique and rats with a preference of 50% or more were allotted randomly to test (n = 9) and control groups (n = 8) and injected SC with ritanserin or vehicle (1 ml/kg) daily for 10 days.

Experiment 2: The Effect of Ritanserin on the Increase in Ethanol Preference Produced by Exposure to a 24 h Dark Cycle

Sixteen rats were kept on a 24 h dark cycle for 5 weeks. At the beginning of the fourth week they were randomly allotted to test (n = 10) and control groups (n = 6). The concentrations of ethanol tested were 3, 4, 5, 7.5, 10, 15, 20% by weight/volume. It took 1 week to test the response to all these concentrations of ethanol. At the beginning of the fourth week (Fig. 1) the test group were injected once IP with 10 mg/ml/kg of ritanserin, and the control group were injected with a ml/kg of the vehicle.

Experiment 3: The Effects of THBC and Ritanserin on Alcohol Preference

A group of rats (n = 13) were kept on a 12L:12D cycle. The concentrations of ethanol tested, each on 1 day were: 3, 4, 5, 6, 8, 10, 12, 15, 20, 25% by weight/volume. It took 10 days to test the response to all the concentrations of ethanol. Between the pretreatment and treatment periods all rats were implanted with IVT cannulas and connected to Alzet osmotic pumps containing THBC, 47 nM dissolved in artificial cerebrospinal fluid (15). Rats were allowed 4 days to recover and then again given the choice of the 3–25% graded ethanol series or water. At this stage the THBC-treated rats were divided randomly into test (n = 7) and control groups (n = 6). The test group received SC injections of ritanserin 10 mg/ml/kg and the control group injections of the vehicle (ml/kg).

Statistics

The measured data, water, and ethanol consumption, was not normally distributed. Preference is a ratio and cannot, therefore, be used untransformed in an anova (23). Zar (23) recommends transformation to the arcsine. Our data after this conversion were still markedly skew so nonparametric methods were used for all

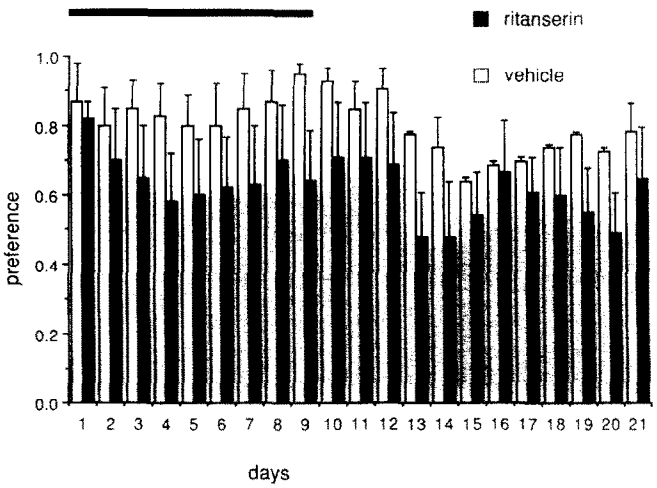

FIG. 1. Effect of ritanserin on preference for 3% ethanol. Histograms show preference defined as ethanol consumption (ml) divided by total consumption (water + ethanol consumption). Filled histograms—preference of a group of rats injected with ritanserin on days 1–10. Open histograms—preference of group of rats injected with vehicle on days 1–10. The preference of the ritanserin-treated group is significantly less than that that of the vehicle-treated group for the period of ritanserin treatment and for the 10 days afterwards.

data. Firstly, Kruskal–Wallace tests were used to examine whether there was a significant difference between the measured data on particular days (Experiment 1) and with particular alcohol treatments (Experiments 2, 3). If the comparison of the measured data from days, or particular alcohol treatnents, were not significantly different, the data from those days or treatments was combined. Mann–Whitney tests were then used with the combined data to compare test and control results.

RESULTS

Experiment 1: The Effects of Ritanserin on Ethanol Preference in Randomly Selected Rats

Before the injection of ritanserin, control and test groups drank volumes of ethanol and water that were not significantly different (ethanol $Z = -1.33$, $p = 0.18$; water, $Z = -1.01$, $p = 0.31$). The control group continued for the 20 days of the experiment to drink volumes of ethanol (mean 23.47 ± 0.68 ml/day) and water (mean 5.92 ± 0.63 ml/day) which were not significantly different (ethanol, $H = 10.48$, $p = 0.94$; water, $H = 13.63$, $p = 0.83$). When the test and control groups were compared over the 20 days of the experiment (Fig. 1) it was found that, after the injection of ritanserin, the test group drank significantly smaller volumes of ethanol and significantly greater volumes of water than the control group and, thus, had a significantly smaller preference for ethanol (ethanol: $Z = -4.63$, $p < 0.0001$; water: $Z = -5.37$, $p < 0.0001$; preference: $Z = -5.38$, $p < 0.0001$). For the 10 days of ritanserin injection the total fluid (ethanol + water) drunk/day by the test group was significantly more than the total drunk by the control group ($Z = -2.64$, $p = 0.008$), but in the following 10 days, the total volumes drunk/day by the test and control groups were not significantly different ($Z = -0.8$, $p = 0.43$).

Experiment 2: The Effect of Ritanserin on the Increase in Ethanol Preference Produced by Exposure to a 24 h Dark Cycle

The total volume of fluid (ethanol and water) drunk each day on the 12L:12D cycle was not significantly different over the

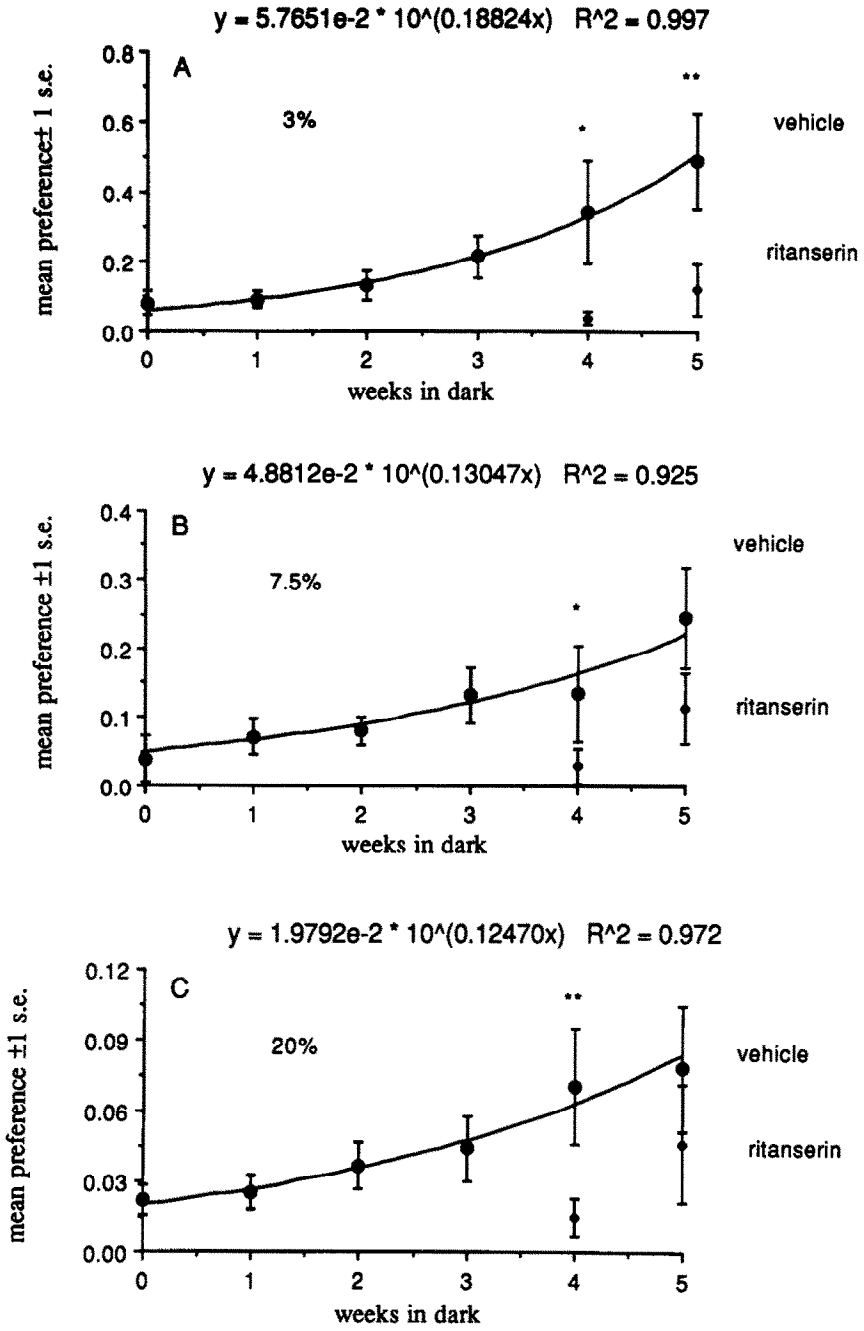

FIG. 2. The exponential increase in preference for ethanol in rats living in complete darkness and the effect of a single IP ritanserin injection at the beginning of week 4. Each strength of ethanol was tested once a week and the mean preference is shown ± 1 SE. (A) Shows the preference for 3% ethanol tested on the first day of each week. (B) Shows the preference for 7.5% ethanol tested on the fourth day of every week. (C) Shows the preference for 20% ethanol tested on the seventh day of every week The exponential curves were computer fitted to the points obtained in the dark according to the equation above each graph. The group given ritanserin had a significantly reduced preference for all grades of ethanol in week 4 and for 3% ethanol in week 5 when compared with the vehicle treated group (*$p = 0.05$, **$p = 0.01$).

period. The mean volume ± 1 SE was 30.49 ± 0.27 ml. Immediately when changed to complete darkness the rats began to drink more fluid; the mean for the first 2 weeks was 37.43 ± 0.43 ml. This increase was highly significant (comparison of 2 weeks on 12L:12D and 2 weeks in complete dark, $Z = -2.73$, $p = 0.0001$). A similar increase in total fluid drunk in the dark cycle was reported by Burke and Cramer, 1974). There was no significant difference in the total daily fluid intake for the rest of

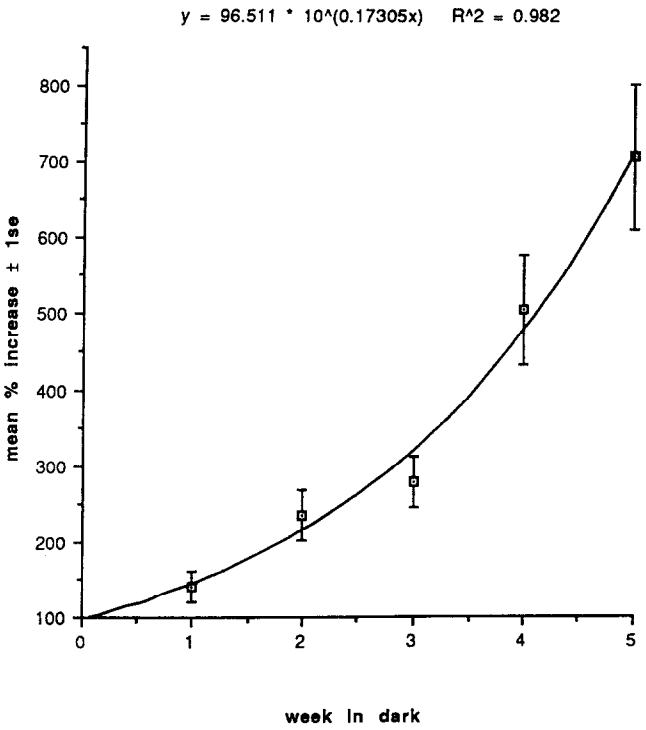

FIG. 3. To make this figure, the increases in preference shown in Fig. 2 were expressed as a percentage of the appropriate predark mean preference. The percentages for each week for all strengths of ethanol were compared and found not to be significantly different. In this figure the mean percentage ± 1 SE for each week is shown together with a computer-fitted exponential curve, the equation for which is shown above the graph.

the experiment except on the day after the intraperitoneal injection of ritanserin when here was a significant drop in the total fluid consumption of the test group.

Figure 2 shows the effect of the dark cycle on preference for 3, 7.5, and 20% ethanol. In each case there was an exponential increase in preference. The curves were fitted to the points obtained in the dark cycle, intermediate concentrations had similar effects. Figure 2 also shows the effects of ritanserin in the fourth and fifth weeks. Consumption of and preference for ethanol of the test group was much reduced when compared with the control groups receiving only vehicle. The stars indicate a significant difference between ritanserin- and vehicle-treated groups. In the fourth week the preference for ethanol of all strengths was significantly depressed by ritanserin. The figure shows data from consumption of 3, 7.5, and 20% ethanol representing the first, fourth, and seventh days after ritanserin. A significant reduction was also detected on the eighth day after the injection. This is depicted in the fifth week for 3%. Preference for 4% ethanol on the ninth day just failed to be significantly depressed, but on the tenth day, preference for 5% ethanol was signficantly depressed. On the eleventh day, shown in the graph as 7.5% (fifth week) and at later times, e.g., 20% (14th day, week 5) there were no significant differences between the means of the drug- and vehicle-treated groups though the means of the ritanserin-treated groups were in all cases smaller than the means of the vehicle-treated groups, suggesting that, with larger samples, we may have been able to detect a significant difference for a longer time.

When the exponential curves of ethanol preference in Fig. 2 are examined carefully it can be seen that in each case if one compares preference in the predark situation with the preference at weeks 1, 2, 3, 4, and 5 there is a similar increase. That is, preference increases by about the same proportion with all grades of ethanol. We compared the increase for each grade of ethanol for each week using all our data and not just the three strengths shown in Fig. 2. There was no significant difference between the increases in preference for the different strengths of ethanol in any particular week. We, therefore, combined the results for each week to make Fig. 3, the data of which, again, is well fitted by an exponential curve. These results imply that the effect of the dark cycle is to exponentially increase the volume of ethanol drunk at each concentration by the same proportion.

Experiment 3: The Effects of THBC and Ritanserin on Alcohol Preference

Over the initial 10 days the rats (n = 13) showed a significant difference in their consumption of ethanol offered at different concentrations (Fig. 4A). Consumption, as the figure shows, was significantly greater on days when 3, 4, or 5% ethanol was offered than on days on which any greater concentration (6–25%) was offered (p = 0.045), and there was no significant difference between the volume consumed on days when ethanol of higher strengths was offered (p = 0.06).

Over the 10 day period during which the rats were infused IVT with THBC their ethanol consumption increased markedly (Fig. 4, centre section). The significant increase was in the drinking of ethanol at concentrations of 6% and above when compared with the consumption of these strengths in the pre-THBC period (p = 0.004). The changes in consumption of 3–5% ethanol when similarly compared were not signficant (p = 0.09). In the third

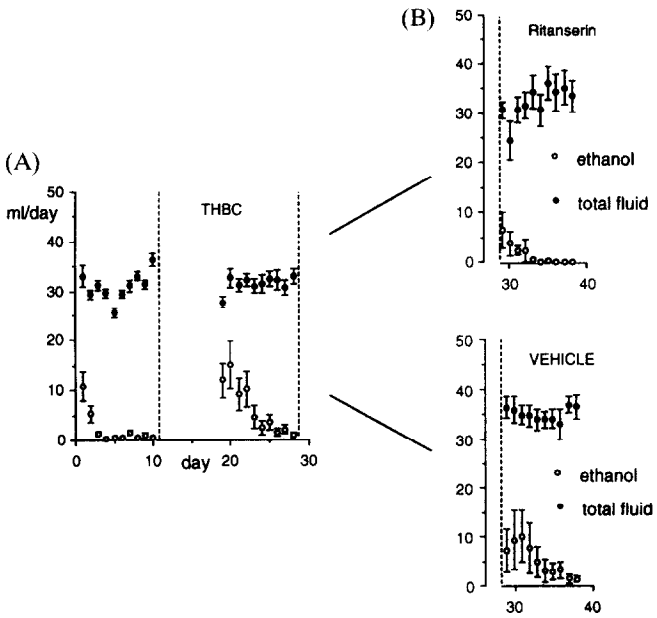

FIG. 4. The effects of tetrahydro β carboline (THBC) and ritanserin on consumption of a range of ethanol strengths (3–25%) taking 10 days for full presentation. (A) Shows the pretreatment results from a group of rats tested sequentially with THBC (B) and then either ritanserin or the vehicle for ritanserin. Closed circles show mean ± 1 SE daily total fluid intake (ml), open circles show mean ± 1 SE daily ethanol intake (ml).

10 day period the control group compared with the pre-THBC period continued to drink signficantly more of the higher strengths of ethanol ($p = 0.0001$) while maintaining their consumption of the 3–5% ethanol at the pre-THBC level ($p = 0.43$).

Finally, after the test group was injected with ritanserin (Fig. 4, right section), they drank significantly less ethanol than the control group over the 10 days ($Z = -4.022$, $p < 0.0001$) but the same quantity of water ($Z = -0.308$, $p = 0.758$) so that their preference for ethanol fell significantly below the preference of the control group ($Z = -3.876$, $p = 0.0001$).

DISCUSSION

Figure 1 shows that ritanserin significantly suppresses preference for 3% alcohol in Sprague–Dawley rats as previously reported in Wistar rats (16) and men (13). In addition, the increase in alcoholic preference caused by keeping rats in a complete dark cycle (Fig. 2), and the increase in preference caused by THBC (Fig. 4) were also significantly reduced by ritanserin. The effect of the drug was behaviourly selective as there was no signficant reduction of total fluid intake. The effects on alcohol preference were very long lasting. After daily doses of ritanserin for 10 days (Fig. 1) a significant effect was detected for a further 10 days. The similar long-lasting effect of a single dose of ritanserin was shown in the animals in the dark and those treated with THBC. In both cases (Figs. 2, 4), a significant depression of alcohol preference was detected up to 8–10 days later.

The effect of a dark cycle on ethanol consumption over 5 weeks in our experiments (Figs. 2, 3) was greater than previously reported (2). We attribute this to the strictness of our dark regime. Some previous investigators have put on the lights for a brief period to make necessary observations of fluid intake (2,6). In a pilot experiment using this technique we found the increase in ethanol intake over 5 weeks was only about 260% of the initial predark ethanol intake, comparable to that reported by Blum et al. (3). We then changed to the use of a low intensity red light (4) for making the necessary observations and thereafter observed the progressive changes shown in the present experiments. The effects of a continued dark cycle on intake of a range of ethanol strengths has not previously been reported. We found an exponential increase in the consumption of all strengths from 3–20% (Fig. 3). The absolute magnitude of the effect awaits further investigation.

The effects of THBC observed in the present experiments (Fig. 4) were similar to those reported by Airaksinen et al. (1) who also used an Alzet pump to apply the drug and an even bigger range of strengths of ethanol to test its effects. They found, as we did, that the greatest effect of the drug was on the consumption of higher concentrations of ethanol (9–20%). However, their design was such that higher concentrations were offered when the drug was most effective. We found, as Fig. 4 shows, an effect with all concentrations of 6% and above.

Ritanserin has been found to reduce intake of, and preference for, other drugs of abuse, notably cocaine and fentanyl (12). There is considerable evidence that the rewarding effects of cocaine depend on dopamine release in the nucleus accumbens (9,22). It seems probable that the only neural system coactivated by these drugs and by the three methods of increasing alcohol preference that we have employed is the A10 group of dopaminergic neurons with cell bodies in the ventral tegmentum and axons passing through the median forebrain bundle to terminate in the nucleus accumbens and prefrontal cortex. Activation of this system is strongly implicated in the rewarding effects of drugs of abuse (3,9,21). Ritanserin is known to activate the A10 dopaminergic neurons by blocking a serotoninergic inhibition of their activity exerted by neurons in the prefrontal cortex (18,20). There is supporting evidence linking the reduction of 3% ethanol preference by ritanserin to this system, for this reduction is blocked by previous depletion of endogenous serotonin (16). In conscious, but not anaesthetised rats, the A10 group of dopamine-containing cells are also known to be excited by IV ethanol in a dose-related fashion (7). There is a marked decrease of neuronal firing here during the ethanol withdrawal syndrome in rats (5) and ethanol abstinence produces a marked inhibition of mesolimbic dopamine release (17). As ritanserin reduces ethanol intake and preference for ethanol we suggest that activation of this dopaminergic system by ritanserin produces a long-lasting occlusion of the effects of ethanol and other drugs of abuse on the same system.

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