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Effects of γ-hydroxybutyric acid and flunitrazepam on ethanol intake in male rats

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Abstract

Both γ-hydroxybutyric acid (GHB) and flunitrazepam are often used illicitly in combination with ethanol. Nevertheless, the effects that these and other drugs of abuse have on the reinforcing effects of ethanol remain inconclusive. To test the effects of GHB and flunitrazepam on contingent ethanol intake, twelve male Long–Evans rats were trained to orally consume ethanol using a saccharin-fading procedure. After training, all animals preferentially consumed ethanol instead of water at each of five ethanol concentrations (0–32%) when tested with a two-bottle preference test in the homecage. Animals then received a noncontingent dose of ethanol (0.32, 0.56, 1, and 1.33 g/kg), flunitrazepam (0.032, 0.1, and 0.32 mg/kg), or GHB (100, 180, 320, and 560 mg/kg) prior to each subject's daily access to ethanol (18% v/v). Noncontingent doses of ethanol decreased ethanol intake, however, the subjects consumed enough ethanol to maintain a consistent total ethanol dose in g/kg. Flunitrazepam did not affect ethanol intake at any dose tested, whereas GHB only affected intake at the highest dose (560 mg/kg), a dose that also produced sedation. These data suggest that there are perceptible or qualitative differences between GHB, flunitrazepam, and ethanol in terms of their capacity for modulating oral ethanol intake in outbred rats.

Keywords: Voluntary ethanol intake; Flunitrazepam; γ-Hydroxybutyric acid; $GABA_A$

1. Introduction

Although both γ-hydroxybutyric acid (GHB) and flunitrazepam are often used illicitly in combination with ethanol, the effects that these and other drugs of abuse have on oral intake of ethanol remain inconclusive. For example, some studies in rats have demonstrated no change in ethanol consumption after pretreatment with the benzodiazepine diazepam (Rimondini et al., 2002; Shelton and Balster, 1997). In contrast, other investigators have reported that diazepam can either increase (Schmitt et al., 2002) or decrease (Hedlund and Wahlstrom, 1998) ethanol intake in a dose-dependent manner. Midazolam and the $GABA_A$ agonist THIP have been shown to increase consumption of ethanol (Boyle et al., 1992, 1993; Smith et al., 1992; Soderpalm and Hansen, 1998; Wegelius et al., 1994), whereas chlordiazepoxide increased ethanol intake at low doses (1–2 mg/kg; Petry, 1995) and decreased intake at larger doses (5–20 mg/kg; Petry, 1995; Roehrs et al., 1984; Samson and Grant, 1985), suggesting that the dose of the benzodiazepine may be critical. Interestingly, few, if any, studies have tested the effects of flunitrazepam on oral ethanol intake even though it is frequently used illicitly in combination with ethanol (Dåderman and Lidberg, 1999; Rickert et al., 1999; Simmons and Cupp, 1998).

γ-Hydroxybutyric acid has specific CNS depressant effects similar to the benzodiazepines, and like flunitrazepam, it is

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often abused illicitly in combination with ethanol (Chin et al., 1998; Degenhardt et al., 2002; Miró et al., 2002). Consistent with its reported central nervous system depressant effects, GHB has been proposed as a novel treatment for ethanol withdrawal and dependence. Interest in GHB as a treatment for ethanol dependence increased after it was reported to reduce symptoms associated with ethanol withdrawal in both humans and rats, and to increase the length of abstinence in humans (Fadda et al., 1989; Gallimberti et al., 1989, 1992; Moncini et al., 2000; Poldrugo and Addolorato, 1999). Pretreatment with GHB has also been reported to reduce oral ethanol intake in Sardinian alcohol-preferring (sP) and other alcohol-preferring (e.g., P) lines of rats (Agabio et al., 1998; Fadda et al., 1988; Gessa et al., 2000; June et al., 1995). However, most experiments investigating the effects of GHB on ethanol intake involved only ethanol-preferring rodents. This raises questions regarding the generality of these findings, particularly because inbred alcohol-preferring rats exhibit a higher affinity of [2,3-^{3}H]GHB binding at both high- and low-affinity sites in the cortex (Frau et al., 1995), and a higher sensitivity to the reinforcing effects of both ethanol and GHB compared to alcohol non-preferring rats (Colombo et al., 1998a,b).

The purpose of this study was to examine the effects of GHB and flunitrazepam on ethanol intake using an established method of oral ethanol intake. To meet these objectives, male Long–Evans hooded rats were trained to consume ethanol using a saccharin-fading procedure (Porrino et al., 1998; Samson, 1986), and after voluntary intake was established, GHB and flunitrazepam were administered intraperitoneally (i.p.) prior to daily drinking sessions. Ethanol was also administered i.p. prior to ethanol drinking sessions to serve as a control.

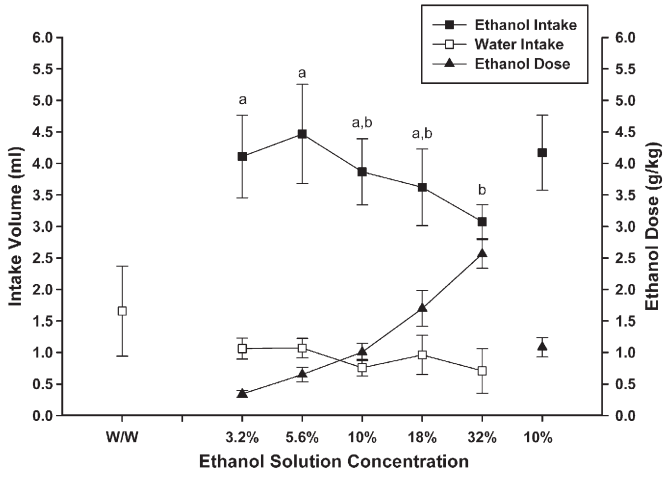

Fig. 1. Intake of water or ethanol during a two-bottle preference test ($n=12$). Filled squares represent the volume (ml) of ethanol consumed (left axis), unfilled squares represent the volume of water consumed (left axis), and filled triangles represent the dose of ethanol consumed in g/kg (right axis). Values are mean±SEM. Letters indicate significant difference between ethanol concentrations (Tukey's HSD test, $p<0.05$). W/W represents the mean intake of water consumed from two tubes of water and is presented as a control. Data points depicted after the concentration–effect curve represent the redetermination of the mean intake level for 10% ethanol alone after completion of the two-bottle preference tests.

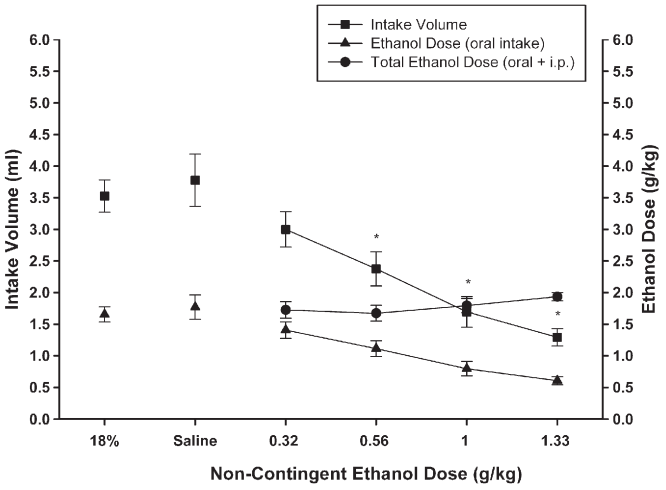

Fig. 2. Effect of noncontingent i.p. ethanol injections on ethanol intake in 12 subjects during a 30-min drinking session. Squares represent the volume of ethanol consumed (left axis), triangles represent the dose of ethanol consumed (right axis), and circles represent the total ethanol dose in g/kg (oral+i.p.; right axis). Values are mean±SEM. Asterisks indicate significant differences from control (Dunnett's test, $p<0.05$). Data points at 18% indicate the mean intake and mean dose for this concentration of ethanol prior to the drug interaction tests and are plotted as a reference point.

2. Materials and methods

2.1. Animals

Twenty-three Long–Evans hooded rats were maintained at 90% of their free-feeding weight on a diet of standard rat chow (Rodent Diet 5001, PMI Inc., St. Louis, MO, USA). Of these, 12 subjects were used for the drug interaction studies, whereas 11 were used for establishing mean blood alcohol levels (BAL) for each ethanol concentration tested. Water was provided ad libitum in the subject's homecage except during the experimental sessions. All animals were housed individually in plastic rodent cages containing hardwood chip bedding. The colony room was maintained at 21±2 °C with 50±10% relative humidity on a 14L:10D light/dark cycle (lights on 0600 h; lights off 2000 h). Training and experimental sessions were conducted daily during the light cycle between 1200 h and 1430 h. Animals used in these studies were maintained in accordance with the Institutional Animal Care and Use Committee, Louisiana State University Health Sciences Center, and in compliance with the guidelines of the National Institute of Health Guide for Care and Use of Laboratory Animal Resources (Publication No. 85-23, revised 1996).

2.2. Training procedure

Subjects were trained to consume ethanol orally using a modified fading procedure (Porrino et al., 1998; Samson, 1986). Prior to each daily training session, animals were weighed and then returned to their homecage. Water bottles were removed

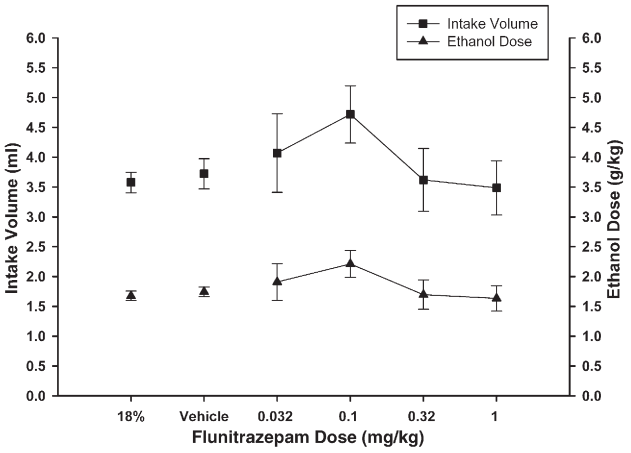

Fig. 3. Effect of noncontingent i.p. flunitrazepam injections on ethanol intake in 6 subjects during a 30-min drinking session. Squares represent volume of ethanol consumed (left axis) and triangles represent the dose of ethanol in g/kg (right axis). For additional details, see legend for Fig. 2.

and replaced with 50-ml plastic centrifuge tubes fitted with a rubber stopper and metal sipper tube containing a saccharin/ethanol solution. Rats were allowed 30 min of free access to the saccharin/ethanol solution. At the end of the 30-min session, the drinking tubes were removed, water bottles were replaced, and the volume of the solution consumed was measured. The dose of ethanol was calculated as g/kg body weight based on the volume of solution consumed and the mean body weight. All solutions were prepared fresh daily.

Rats initially received a 0.2% (w/v) saccharin sodium solution that was gradually replaced with 10% ethanol (v/v) over subsequent training sessions (i.e., as the saccharin concentration was reduced from 0.2% to 0%, the ethanol concentration was increased from 0% to 10%). The solutions were presented in the following order: 0.2% saccharin/0% ethanol, 0.15% saccharin/0.5% ethanol, 0.125% saccharin/1% ethanol, 0.1% saccharin/2% ethanol, 0.05% saccharin/5% ethanol, 0.01% saccharin/8% ethanol, and 0% saccharin/10% ethanol. Each saccharin/ethanol solution was presented daily until either a stable level of intake was observed (±20% of the mean for 3 consecutive days) or a maximum of 8 days had elapsed. Average length of time to completion of training was 60–90 sessions.

2.3. Ethanol preference test

After a stable baseline of ethanol drinking was established with a 10% ethanol solution, the effects of varying concentrations of ethanol (all in the absence of saccharin) were examined using the standard two-bottle preference test. During these tests, subjects were allowed simultaneous access to two drinking tubes daily for 30 min in their homecage. One drinking tube contained deionized water (vehicle) and the other contained varying concentrations of ethanol (0, 3.2, 5.6, 10, 18, or 32% v/v) presented in a mixed order. To avoid development of a positional bias, the positions of the drinking tubes were reversed each day. Immediately following the 30-min session, drinking tubes were removed, the water bottles were replaced, and the volume of water and ethanol consumed was measured. Each concentration of ethanol was presented daily until either a stable level of intake was observed (±20% of the mean for 3 consecutive days) or a maximum of 8 days had elapsed. After each change in the ethanol concentration, the subjects were always returned to the 10% ethanol concentration until the specified criterion was met. Daily ethanol intake (g/kg body weight) was calculated from the volume of the ethanol solution consumed and the mean body weight. Ethanol preference was calculated as the percent ratio between ethanol consumption and total fluid (ethanol plus water) intake (i.e., ethanol intake / total fluid intake × 100).

2.4. Drug interaction tests

For each drug interaction test, only a single bottle was available during the 30-min session. The concentration of the ethanol solution was maintained at 18% (v/v), and each animal received a single i.p. dose of ethanol (0.32, 0.56, 1, and 1.33 g/kg), GHB (100, 180, 320, and 560 mg/kg), or flunitrazepam (0.032, 0.1, 0.32, and 1 mg/kg) prior to their daily 30-min session. An 18% ethanol solution was used for the drug interaction studies because the average ethanol intake decreased over time with the 10% ethanol solution. Rats received a particular noncontingent dose daily until one of the criteria was met, i.e., either a stable level of intake was observed (±20% of the mean for 3 consecutive days) or a maximum of 8 days had elapsed. After the testing of each dose, the subjects were always returned to the 18% ethanol concentration for the specified criterion. Both ethanol and flunitrazepam were administered 15 min prior to the start of the session, whereas GHB was administered 30 min prior to the start of the session. These pretreatment times were based on preliminary data and on the duration of action of these drugs as shown in male rats responding under a fixed-ratio schedule of food presentation (Carter et al., 2004). γ-Hydroxybutyric acid was dissolved in sterile saline, and flunitrazepam was dissolved in a vehicle consisting of 70% saline, 20% emulphor, and 10% ethanol. For

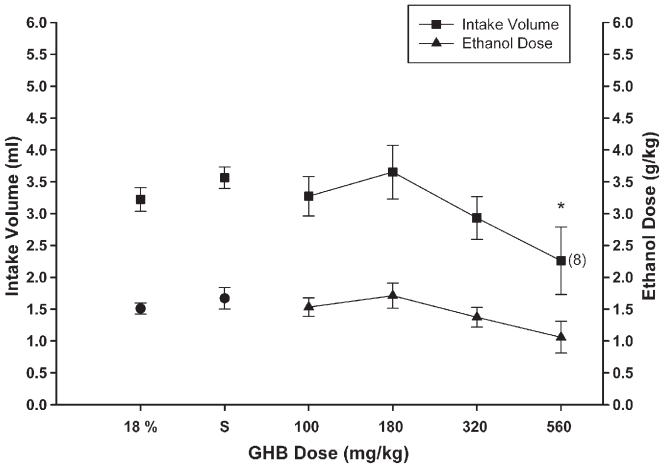

Fig. 4. Effect of noncontingent i.p. GHB injections on ethanol intake in 11 subjects during a 30-min drinking session. Squares represent the volume of ethanol consumed (left axis) and triangles represent the dose of ethanol consumed in g/kg (right axis). The number in parenthesis indicates the number of animals when less than 11. For additional details, see legend for Fig. 2.

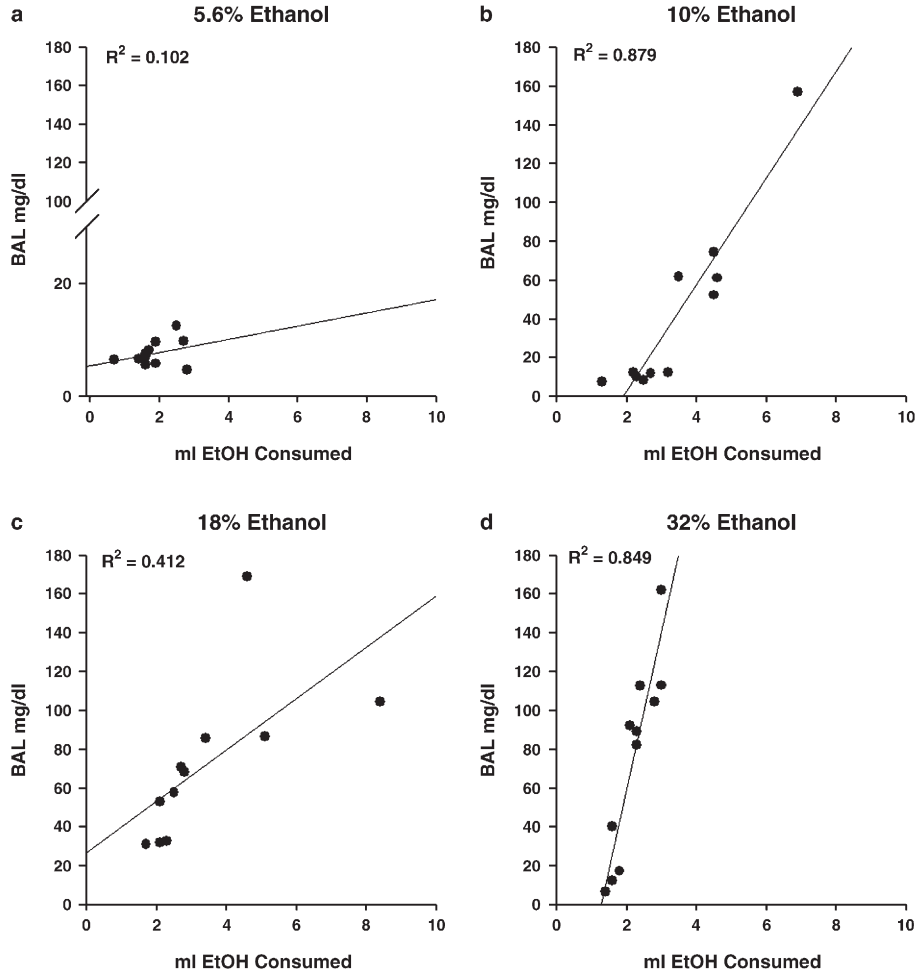

Fig. 5. Ethanol intake (ml) and corresponding blood ethanol levels (mg/dl) for four ethanol solutions: a) 5.6%, b) 10%, c) 18%, and d) 32%. Each filled circle represents data from 11 individual subjects. Blood samples were obtained immediately after a 30-min two-bottle preference test.

i.p. injections, ethanol (100%) was always diluted to a 20% solution with saline, and the injection volume varied accordingly for each dose; saline (control) injections matched these various volumes. For flunitrazepam and GHB, the volume for both control (saline or flunitrazepam vehicle) and drug injections was 0.1 ml/100 g body weight.

2.5. Blood collection and blood alcohol level determination

To assess mean BALs, 11 male Long–Evans rats were trained to consume ethanol orally using the above procedure. Blood samples were collected by saphenous venepuncture immediately after a 30-min two-bottle preference test. Samples across four ethanol concentrations were taken the day each subject met criteria. Serum was isolated and stored at −80 °C until blood alcohol levels (mg/dl) were quantified in duplicate using the MicroStat GM7 Analyzer (Analox Instruments, Inc., Lunenburg, MA). The intra-assay coefficient of variation was 2.5%.

2.6. Data analysis

For the ethanol preference test, a two-way repeated measures ANOVA (ethanol concentration × type of solution) was used to determine if significant differences occurred between ethanol and water intake at each concentration of ethanol. When significant main effects were detected, post-hoc Tukey's HSD tests were used to identify significant differences between ethanol and water intake at each ethanol concentration. The mean data for each animal were also grouped and analyzed for an effect of treatment at each dose of drug (ethanol, flunitrazepam, and GHB) using one-way repeated measures ANOVA (SigmaStat Statistical Software, SYSTAT Software, Inc. Point Richmond, CA, USA). When a significant effect of treatment was detected, post-hoc Dunnett's tests were used to compare each dose with the respective control condition. Correlations between intake of the four ethanol concentrations and BALs were analyzed using a simple linear regression, while a one-way repeated measures ANOVA was used to determine whether mean BALs differed across each of these four concentrations. Statistical significance was accepted at α level ≤ 0.05 for all statistical tests.

3. Results

Both ethanol and water intake during the two-bottle preference tests are plotted in Fig. 1. Ethanol was preferentially

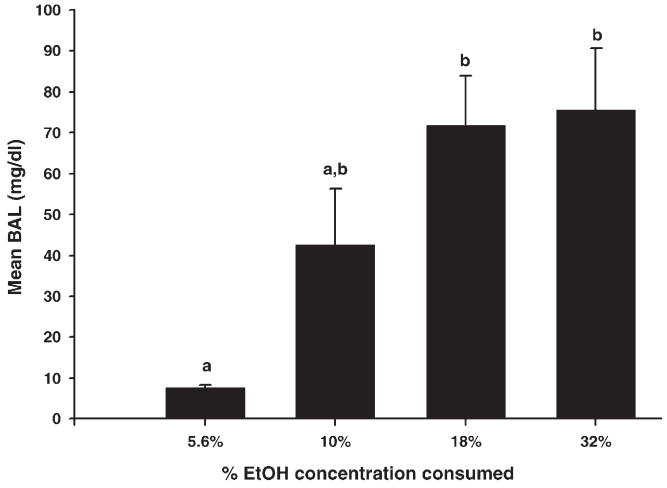

Fig. 6. Mean blood ethanol levels (mg/dl) for four ethanol solutions: 5.6%, 10%, 18%, and 32%. Values are mean±SEM (n=11). Letters indicate significant differences between the four ethanol solutions (Tukey's HSD test, $p<0.05$). Blood samples were obtained immediately after a 30-min two-bottle preference test.

consumed instead of water at each of the five ethanol concentrations (3.2, 5.6, 10, 18, and 32%), with preference ratios for ethanol ranging from 79.5% to 83.6%. A two-way repeated measures ANOVA revealed a significant main effect of ethanol concentration [$F(4, 119)=2.904$, $p<0.032$] and type of solution [$F(1, 119)=50.966$, $p<0.001$]; the interaction between the two main factors was not significant. Post-hoc analysis revealed that consumption of the 3.2% and 5.6% ethanol concentrations was both significantly higher than that for the 32% ethanol concentration (Tukey's, $p<0.05$). No significant group differences in water consumption across the five ethanol concentrations were revealed. The total ethanol dosage consumed increased from a mean of 0.34 g/kg to a mean of 2.6 g/kg as the concentration of ethanol increased from 3.2 to 32%. After completion of the two-bottle preference tests, intake for 10% ethanol was redetermined in the absence of water and intake levels of 10% ethanol were comparable to those observed during the two-bottle preference tests.

The effects of a noncontingent ethanol pretreatment on ethanol intake are shown in Fig. 2. Ethanol pretreatment significantly decreased oral consumption of an 18% ethanol solution in a dose-dependent manner for all subjects [$F(11, 59)=20.687$, $p<0.001$]. Pretreatment with ethanol doses of 0.56, 1 and 1.33 g/kg significantly reduced the volume of ethanol consumed relative to that consumed after pretreatment with saline (Dunnett's, $p<0.05$). No change in ethanol intake was observed following a noncontingent dose of 0.32 g/kg. The total dose of ethanol in g/kg remained consistent after each ethanol pretreatment dose, thereby producing a constant total ethanol dose (see filled circles).

As shown in Fig. 3, levels of oral ethanol intake did not significantly differ after pretreatment with 0.032, 0.1, 0.32, or 1 mg/kg of flunitrazepam when compared to control data [$F(5, 29)=2.255$, $p=0.099$]. Interestingly, pretreatment with flunitrazepam had no effect on ethanol consumption even at the highest dose, a dose that had sedative effects. At the doses tested, flunitrazepam also did not alter the dose of ethanol in g/kg body weight.

γ-Hydroxybutyric acid decreased the consumption of an 18% ethanol solution significantly as depicted in Fig. 4 [$F(10, 51)=2.685$, $p=0.046$]. Post-hoc analysis indicated that this reduction in ethanol intake was limited to the highest dose of GHB tested (560 mg/kg), a dose that heavily sedated 3 out of 11 animals. When compared to control data, administration of 100, 180, and 320 mg/kg of GHB prior to the daily ethanol drinking sessions did not alter ethanol consumption significantly. The dose of ethanol in g/kg was also not altered at any dose.

Fig. 5 shows the distribution of individual BALs following 30-min drinking sessions in which rats consumed ethanol solutions of 5.6%, 10%, 18%, or 32%. The volumes consumed by the group of subjects participating in the drug interaction studies and those strictly for BALs were comparable, except at the 5.6% ethanol concentration. A significant positive correlation between BALs and the volume consumed was found for the 10% [$R^2=0.879$, $p<0.001$], 18% [$R^2=0.412$, $p=0.033$], and 32% [$R^2=0.849$, $p<0.001$] ethanol solutions but not for the 5.6% ethanol solution [$R^2=0.102$, $p=0.339$]. Mean BALs for each of the four ethanol solutions is shown in Fig. 6. In general, increasing the concentration of ethanol resulted in a linear increase in BALs across all four ethanol concentrations [$F(10, 43)=7.500$, $p<0.001$]. Post-hoc analysis revealed that the BALs of subjects consuming the 5.6% ethanol solution were statistically lower than the BALs of subjects consuming the 18% and 32% ethanol solutions, but did not differ from those consuming the 10% ethanol solution.

4. Discussion

Contingent ethanol intake was successfully established in a group of outbred male Long–Evans hooded rats using a saccharin-fading procedure. Upon completion of training, subjects reliably consumed ethanol in volumes that have been shown to produce pharmacologically detectable BALs (Linseman, 1987; Roberts et al., 1999). More specifically, blood alcohol levels were positively correlated with the amount of ethanol orally consumed and the mean BAL was found to increase significantly with increasing ethanol concentrations. Rats also maintained a clear preference for ethanol over water at ethanol concentrations ranging from 3.2% to 32% as demonstrated with the two-bottle preference test, indicating that drinking was contingent on the presentation of ethanol.

Noncontingent administration of ethanol prior to the drinking session decreased the oral consumption of ethanol in a dose-dependent manner. Thus, the dose of ethanol in g/kg body weight that was consumed decreased as the noncontingently administered dose of ethanol increased; however, the total ethanol dose was relatively stable across each ethanol dose. These findings suggest that the subjects titrated the amount of ethanol they drank and that a noncontingent dose of ethanol may produce effects similar to those of voluntary ethanol intake, as subjects consumed less ethanol in the presence of an existing dose. This conclusion is supported by similar data from two operant studies, although total ethanol dose was not calculated

in these studies. For instance, Shelton and Balster (1997) showed that pretreatment of 180–1560 mg/kg of ethanol dose-dependently decreased responding maintained by ethanol in male Long–Evans hooded rats, and Petry (1995) found that male Wistar rats responded less for ethanol after pretreatment with 0.5 and 1 g/kg of ethanol.

Few, if any, studies have tested the effects of flunitrazepam on ethanol intake even though it, like other benzodiazepines, is used illicitly in combination with ethanol. Unlike pretreatment with ethanol, pretreatment with flunitrazepam had no effect on ethanol consumption even at the highest dose, a dose that also had sedative effects. This finding is in contrast to several studies showing that other benzodiazepines can decrease self-administration of ethanol (Hedlund and Wahlstrom, 1998; Roehrs et al., 1984; Shelton and Balster, 1997). In a study with findings most similar to those of the present study, Shelton and Balster (1997) found that the positive $GABA_A$ modulators diazepam and pentobarbital decreased both ethanol and saccharin self-administration similarly at the highest doses tested (i.e., 5.6 and 20 mg/kg, respectively) in rats responding under a multiple fixed-ratio schedule. However, a direct comparison of the present findings with those in previous studies is difficult to make, due to different methodologies and conditions used for ethanol intake. For instance, ethanol was either available for longer periods of time (Hedlund and Wahlstrom, 1998) or different feeding schedules were associated with ethanol availability (Roehrs et al., 1984) in those previous studies.

Unlike the present study, some studies using outbred rats have reported increases in ethanol intake following administration of a benzodiazepine (Schmitt et al., 2002; Soderpalm and Hansen, 1998) or a $GABA_A$ agonist such as THIP (Boyle et al., 1992, 1993). For example, pretreatment with 5 mg/kg of midazolam increased the average intake of 6% ethanol for male Wistar rats when it was available for 2 h in the homecages (Soderpalm and Hansen, 1998), and 16 mg/kg of THIP increased the intake of 9% ethanol and maintained a preference for ethanol over water in male Long–Evans rats during 5 days of testing (Boyle et al., 1992, 1993). These previous findings, although they are opposite to those found in the present study, suggest that benzodiazepines and other positive modulators of the $GABA_A$ receptor complex might not be appropriate substitution therapies for treating ethanol abuse as intake levels of ethanol only increased in their presence. Part of the difficulty in interpreting the effects of the positive modulators on ethanol self-administration is likely related to the fact that the dose–effect curve for the reinforcing effects of ethanol may be an inverted U-shaped curve. As a consequence, the interactive effects of a single dose combination could be represented as either an increase or a decrease in drinking depending on the dose of ethanol.

Several previous studies using alcohol-preferring rats (sP and P lines) indicate that GHB (50–400 mg/kg) as well as γ-butyrolactone (200 mg/kg) and 1,4 butanediol (100–300 mg/kg) can reduce oral consumption of ethanol in these rats, which are selectively bred for high ethanol preference and consumption (Agabio et al., 1998; Colombo et al., 1990; Fadda et al., 1983, 1988; Gessa et al., 2000; June et al., 1995). In the present study, however, oral ethanol intake was not affected by pretreatment with GHB (100–560 mg/kg). Although the highest dose of GHB did decrease ethanol intake, it was associated with considerable sedation in several animals. One explanation for the absence of an effect with GHB in the present study could be that outbred rats are less sensitive to the effects of GHB. For example, compared with non-preferring rodent lines, selectively bred sP rats have been reported to have a higher sensitivity to the reinforcing (Colombo et al., 1998b) and sedative effects of GHB (Colombo et al., 1998a) compared to alcohol non-preferring (sNP) rats. Furthermore, variations in $GABA_A$ binding have been reported between preferring and non-preferring rodents (Wong et al., 1996), and binding of [2,3-^{3}H]GHB to both the putative low- and high-affinity binding sites is greater in ethanol-preferring rats than in ethanol non-preferring rats (Frau et al., 1995).

In summary, GHB and flunitrazepam failed to significantly alter ethanol intake, indicating that there are perceptible or qualitative differences between these drugs and ethanol in terms of their capacity for modulating oral ethanol intake. Moreover, this was the case even though at least one of these drugs, flunitrazepam, can positively modulate the $GABA_A$ receptor, something that ethanol is also known to do. These results would support the notion that ethanol's reinforcing effects may not be solely mediated by the $GABA_A$ receptor complex (DeBeun et al., 1996; Grant and Colombo, 1993; Green-Jordan and Grant, 2000; Maurell et al., 1997; Shippenberg and Altshuler, 1985; Spanagel, 1996), and are not similar to the effects of GHB, which can be mediated by either GHB receptors or $GABA_B$ receptors (Lobina et al., 1999; Carter et al., 2003, 2004).

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