THE EFFECT OF DIAZEPAM ON VOLUNTARY ETHANOL INTAKE IN A RAT MODEL OF ALCOHOLISM

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Abstract — This paper reports the effects of a diazepam treatment on voluntary ethanol intake in rats included in an animal model of alcoholism. In a first dose-seeking experiment, rats had a choice between 10% (w/v) ethanol and water for 24 h each week. Single doses of diazepam between 2 and 20 mg/kg injected i.p. prior to the 24-h choice caused a dose-dependent decrease in voluntary ethanol intake from 3.2 ± 0.4 g/kg/day down to 2.3 ± 0.3 g/kg/day \((P < 0.01)\) after a dose of 20 mg/kg. In a second experiment, psychological dependence was induced by a 1-year intermittent exposure to ethanol (a choice between 10% ethanol and water for 24 h each week, followed by an i.p. injection of 2.0 g/kg of ethanol). After this year, the rats were given a continuous choice between ethanol and water. A 3-week treatment with diazepam (20 mg/kg/day, i.p.) was started in week 68, during which period a choice of 10% (w/v) ethanol was available only on the first and the last days of treatment. On the first day of the diazepam treatment, ethanol intake was decreased from a pre-experimental value of 2.7 ± 0.3 g/kg/day to 1.2 ± 0.1 g/kg/day \((P < 0.001)\). On the last day of the treatment, voluntary intake was higher than before the treatment \(3.8 ± 0.27 \) g/kg/day, \(P < 0.01\). Ethanol intake remained elevated during the week after the end of the diazepam treatment \(P < 0.05\). When single doses of diazepam (20 mg/kg) were re-tested 10 and 19 weeks after the treatment, there was no decrease in ethanol intake, indicating that the initial effect had not been re-established.

INTRODUCTION

Ethanol intake produces a multitude of effects on many transmitter systems in the brain (Liljequist and Tabakoff, 1984; Peoples et al., 1996). The effect of ethanol on the \(\gamma\)-aminobutyric acid (GABA)-ionophore is one of the most extensively studied systems. Earlier experiments have shown that there is an interaction between the acute effects of ethanol and the GABA-ergic system (Liljequist and Tabakoff, 1984; Allan et al., 1991; Szmigielski et al., 1992; Ticku and Mehta, 1995). Furthermore, some rats with a changed acute response to ethanol have changes in their GABA receptors (Korpi et al., 1992a; Uusi-Oukari and Korpi, 1992). In humans with chronic alcoholism, there are also changes in GABA function, suggested by the decreased affinity and density of benzodiazepine (BDZ) receptors in postmortem material (Freund and Ballenger, 1988, 1989; Korpi et al., 1992b).

The effect of an agonist at the GABA\(_A\) receptor is a general depression of signal transduction in the central nervous system. Acute ethanol and endogenous GABA agonists have synergistic effects, so that ethanol enhances this depressing effect (Liljequist and Tabakoff, 1984; Korpi, 1994; Ticku and Mehta, 1995). If ethanol exposure is constant for a period of time, adaptive changes take place to counteract this positive interaction (Szmigielski et al., 1992; Sherif et al., 1994; Katsura et al., 1995; Ticku and Mehta, 1995). When ethanol is withdrawn, a withdrawal syndrome appears, which is due at least partly to this adaptive state. The withdrawal syndrome in humans is effectively treated with diazepam, a drug with agonistic properties at the GABA/BDZ receptor complex (Salloum et al., 1995; Shaw, 1995; Verbanck, 1995). Under these conditions, diazepam has anticonvulsant and anxiolytic effects.

Previous studies of the effect of GABA agonists on voluntary ethanol intake in laboratory animals have been inconclusive, as both decreases and increases (Chan, 1984; Smith et al., 1992) as well as no effect (Beaman et al., 1984; Daoust et al., 1987; Korpi, 1994) have been found. Furthermore,
one study reported a decrease in intact rats, while no effect was seen in physically dependent rats (Ferko et al., 1979). With regard to the BDZ component of the GABA-ionophore complex, BDZ partial inverse agonists (McBride et al., 1988; Balakleevsky et al., 1990; June et al., 1992; Rassnick et al., 1992; Korpi, 1994) and antagonists (Korpi, 1994) have been shown to decrease voluntary ethanol intake. However, a major concern when interpreting results from these animal studies is how the animals have been exposed to ethanol. A number of experimental techniques to increase the ethanol intake of the rats have been used (Carroll et al., 1990; Sinclair et al., 1992; Stewart and Grupp, 1992), but few of these resemble the exposure pattern in human alcoholics. Furthermore, in many experimental studies, only single doses of drugs acting at the GABA/BDZ receptor complex have been tested. This is in contrast to the clinical situation. Diazepam, when used as a treatment during ethanol withdrawal in humans, can be given for at least half a week (Salloum et al., 1995; Shaw, 1995). Longer exposures are also seen in alcoholics, since many general practitioners fail to record alcohol abuse when prescribing benzodiazepines (Graham et al., 1992), and many alcoholics use benzodiazepines, with or without prescription (Woods et al., 1992).

In our laboratory, we have developed a model of psychological dependence in the rat. This model uses an empirical definition of psychological dependence, i.e. that the individual rats, when given a choice between ethanol and water, take the same daily dose of ethanol (in g/kg) independently of the ethanol concentration offered, within the range of 5 to 25% (Wahlström, 1987). This state of psychological dependence is induced during a 1-year treatment period. This stability of the ethanol intake is not seen in rats recently introduced to ethanol (Myers and Oblinger, 1977). The stability of the ethanol intake makes it possible to treat these psychologically dependent rats with a drug for different periods of time with or without access to ethanol. This is intended to resemble the clinical in-patient or out-patient situation when a drug is given to, or taken by, human alcoholics.

Since diazepam is given to alcoholics during withdrawal, and also used by alcoholics both with and without prescription, and since the effect of diazepam on ethanol intake is unclear, despite a possible common mechanism of action, we decided to test the effect of a diazepam treatment on voluntary ethanol intake in rats participating in our rat model of alcoholism.

Parts of this paper were previously presented at the XXIVth Annual Nordic Meeting on Biological Alcohol Research (BAR), Imatra, Finland, 24–26 May 1996 and have been published as an abstract (Hedlund and Wahlström, 1996).

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats were purchased from the Möllegaards Breeding Centre Ltd, Li Skensved, Denmark (Mol:SPRD Han). They were housed individually for 10 days before the start of experiments. Each cage was equipped with two drinking bottles. One of the bottles always contained tap water; the other contained either water or an ethanol solution (5, 10 or 20%, w/v). Food (commercial rat pellets, R34, Lactamin, Stockholm, Sweden) consisting of 16.5% raw protein, 4.0% raw fat, and 58.0% NFE (carbohydrates), 1255 kJ/100 g, was available ad libitum. The room where the rats were housed had a reversed light/dark schedule (lights on 19:00–07:00) and a room temperature around 23°C. All handling of the rats was done between 07:00 and 09:00. The age of the animals was determined from the age/weight curve (supplied by the breeder) and was approximately 10 weeks at the start of the first experiment, and 5 weeks at the start of the second experiment, with a chronic ethanol exposure.

Chemicals

Ethanol (AB Svensk Sprit, Sweden) as a drinking fluid was mixed with tap water to give concentrations of 5, 10, and 20% (w/v). Ethanol for injection was dissolved in 0.9% (w/v) NaCl to give a concentration of 10% (w/v) and administered i.p. at a dose of 2.0 g/kg. Diazepam (Apoteksbolaget, Sweden) was suspended in 5% gum arabic (Apoteksbolaget, Sweden) prior to injection (Carlsson and Wahlström, 1993) and injected i.p. Control animals were given the vehicle in which the active drug was given.

Experimental design

The main experiment involved treatment with diazepam in psychologically dependent rats
DIAZEPAM AND ALCOHOL CONSUMPTION

Diazepam injections (DZ):

- Treatment period to induce psychological dependence. 54 weeks. Voluntary choice (10% ethanol/water) restricted to 24 h each week followed by 2.0 g/kg of ethanol i.p.
- Evaluation period with a continuous choice between ethanol and water, no ethanol injections. Basic ethanol concentration 10%, unless otherwise indicated by 5 or 20%.
- Drug treatment. Diazepam 20 mg/kg/day i.p. for 3 weeks. Ethanol withdrawn except for the first and the last day. One control group also had an interrupted choice, the other control group a continuous choice between 10% ethanol and water.

Fig. 1. The model used in experiment 2.

DZ = diazepam injection at a dose of 20 mg/kg/day. Diazepam was given as a treatment during the period marked DT and later re-tested twice as a single dose.

(experiment 2). A dose-seeking experiment (experiment 1) was performed prior to this experiment. In experiment 1, doses of diazepam in the range of 2–40 mg/kg were tested on voluntary ethanol intake. From this experiment, 20 mg/kg was chosen as an effective dose for the treatment of psychologically dependent rats.

Experiment 1. Twenty-six rats were randomly assigned to two groups, in which five doses of diazepam were tested in a cross-over design. Both groups had, during all weeks of the treatment period, two bottles of water available for the first 6 days. On the seventh day, a bottle of 10% (w/v) ethanol was inserted for 24 h instead of the bottle from which the rats had consumed most water during the previous 6 days. In week 3, after 2 weeks with this 24-h/week exposure pattern, one group was injected with the first dose of diazepam and the other group with the vehicle prior to the 24-h choice. In week 4, the injections were reversed, but still given prior to the 24-h choice. The following week no injections were given. This 3-week pattern (weeks 3–5) was repeated throughout the experiment, with diazepam injections every third week. Five doses were tested: 2, 4, 10, 20, and 40 mg/kg. The 40 mg/kg dose made the rats heavily sedated. This sedation made some rats stop drinking during the whole 24-h period, consuming only a few millilitres of fluid, whereas other rats almost doubled their total fluid intake. Because of this, it was decided to exclude the 40 mg/kg dose.

Experiment 2. Thirty-six rats were randomly assigned to three groups, a diazepam-treated group, and two control groups. All three groups were treated identically, except during the time when diazepam was given. The experimental design used in this experiment is illustrated in Fig. 1, and has also been described in detail in earlier publications (Wahlström, 1983, 1987, 1994). It consisted of two periods. First a 1-year treatment period during which psychological dependence was induced (see below). This year was followed by an evaluation period where our empirical criterion of psychological dependence was evaluated, and the drug treatment was given (see below).

Our model is founded on three prerequisites involved in human alcoholism. (1) Intoxication once a week: a pattern seen in Nordic countries (Simpura, 1987) and in Wales (Bennett et al., 1991), where ‘Saturday night’ intoxication is a common feature, prior to the development of alcoholism. In the model, this is mainly accomplished by ethanol injections once a week. (2) Chronic treatment: since it takes a long period of...
exposure to ethanol before psychological dependence develops in humans. In the model, the rats are treated with ethanol injections for a year, and then have a continuous choice between ethanol and water for 3 months prior to drug treatment. (3) Voluntary oral intake: since in humans, alcohol is taken orally with a choice of other drinking fluids always available. In our model, one of the bottles always contains tap water. During the evaluation period, rats treated according to these prerequisites take the same dose of ethanol in a voluntary choice situation independent of the concentration of the offered ethanol solution in the range 5 to 25% (Wahlström, 1987). This is not the case with other rats, e.g. rats recently introduced to ethanol (Myers and Oblinger, 1977), or rats which during the treatment period have received saline, instead of ethanol, injections together with a continuous choice between 10% ethanol and water (Wahlström, 1987). Thus, this treatment causes the rats to seek a certain dose of ethanol, indicating a defined need (craving) for the drug.

Treatment period. During the treatment period (weeks 1–54), the weekly ethanol exposure consists of two components: (1) a voluntary choice between 10% (w/v) ethanol and water for 24 h, with ethanol in the bottle from which the rat had consumed most water during the previous week; (2) an ethanol injection (2.0 g/kg i.p.) given immediately after the end of the 24-h voluntary choice. The injection is given to ensure a defined intoxication once a week. At the beginning of the treatment period, this injection causes an inhibition of voluntary ethanol intake in the 24-h period of the following week. This reduction of ethanol intake subsides to a minimum after 5 to 6 weeks. If the treatment is continued, this inhibition of ethanol intake gradually disappears. In a basic experiment, the treatment is pursued for a year. At this stage, inhibition had disappeared when compared to saline-injected controls included in earlier experiments (Wahlström, 1994). In all rats participating in experiment 2, ethanol intake (in g/kg/day) was, in the first week: 7.10 ± 0.34; in week 5: 2.37 ± 0.23; and, in week 54: 4.39 ± 0.23.

Evaluation period. During the evaluation period our empirical criteria of psychological dependence are tested. This is done by changing the basic ethanol concentration (10%), usually to 20%, over 3 weeks. After this test the mean intake of the different concentrations is compared for the individual rats, by regression analysis. The statistical criteria are that there have to be significant correlations between intakes of the different concentrations, the slope of the regression line must not deviate from 1.0, and the regression line must not deviate from origin. Thus, all rats must have the same intake in g/kg/day with both concentrations. After this ‘test’, the drug can be given. After drug treatment, a second test of psychological dependence is performed by changing the concentration of the ethanol solution for 3 weeks, to see if the psychological dependence of the rats has been altered by the drug treatment.

During the evaluation period, which was started in week 55, all ethanol exposures were voluntary and given as a continuous oral choice between ethanol and water, with random placement of the ethanol bottle each week. No ethanol injections were given. The basic ethanol concentration was 10% (w/v). In the period prior to drug treatment in this experiment, the ethanol concentration was changed to 20% for 3 weeks (weeks 61, 62, and 63) to test the psychological dependence of the rats. All rats in the three groups of the present experiment fulfilled our criteria of psychological dependence in this test.

The diazepam treatment in experiment 2 was given in weeks 68, 69, and 70, during the evaluation period. The rats randomized to drug treatment were given diazepam (20 mg/kg/day) for 23 days. Ethanol was withdrawn, except for the first and the last days of the treatment. This is the diazepam interrupted choice (DIC) group. Control rats were given the vehicle in which diazepam was suspended in the same volume. One of the control groups had ethanol withdrawn, as the diazepam-treated group, during the same 3-week period. This is the control interrupted choice (CIC) group. The other control group had a continuous choice between 10% (w/v) ethanol and water. This is the control continuous choice (CCC) group.

Four weeks after diazepam treatment, the ethanol concentration was again changed to 20% (weeks 75, 76, and 77), and the psychological dependence of the rats was re-tested as described above. To evaluate any long-term effects of the diazepam treatment, the DIC group was given single doses of 20 mg/kg of diazepam 10 (week 80) and 19 (week 89) weeks after the start of the initial treatment. Control rats were given vehicle in a corresponding volume. A 3-week test with 5%


(w/v) ethanol was also performed between these two re-tests (week 84, 85, and 86).

Twenty rats (including seven in the DIC group) had permanent registration of licks at the ethanol bottle. They had a difference in voltage between the ethanol bottle and the floor of the cage, which made a weak current pass when the rat licked at the ethanol bottle. After amplification, licks were recorded on an event recorder. Thus, records were obtained at all occasions when diazepam was given prior to a choice of ethanol, as well as in week 91. In week 91, blood samples were taken from these 20 rats 18–42 min after a period of ethanol drinking and analysed by gas chromatography (Wahlström, 1987), to measure blood-ethanol concentrations.

Statistical methods

Conventional statistical methods were used. Differences between two groups were tested with Student’s t-test, ANOVAs, or the Mann-Whitney U-test. A P < 0.05 was used as the basic level of significance, NS denotes not significant (P > 0.05), n denotes numbers of observations, error bars denote 1 standard error of the mean (SEM). Correlation (r) and regression (b) coefficients were tested against 0. If the regression coefficient differed from 0, it was also tested against 1.0. In this case, NSD1 denotes that the slope of the regression line was not significantly different from 1.0.

RESULTS

Effects of diazepam on body weight, total fluid intake and ethanol drinking bouts

Total fluid intake was not significantly affected by any of the doses of diazepam tested in any of the present experiments. The single doses of diazepam did not affect the weight of the rats in any of the experiments. However, during the 3-week period of treatment with diazepam in experiment 2, the weights of the rats dropped from the pre-treatment value of 507 ± 8 g to 497 ± 8 g on the last day of the treatment, a 2% decrease. Although this decrease was significant (P < 0.05), the biological significance of this slight weight reduction is uncertain. The effect of diazepam on the number of ethanol drinking bouts and total fluid intake for experiment 2 are given in Table 1 both for the first day of the treatment (day 1) and the last day of the treatment (day 23), as well as the two single dose re-tests (re-test 1 and re-test 2). On the first day of the diazepam treatment sedation was recorded during the first hour after the diazepam injection. During the first 24-h period of treatment, there was no decrease in bouts of drinking at the ethanol bottle compared to the controls. The total fluid intake was also

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Table 1. The effect of diazepam on number of ethanol drinking bouts and total fluid intake

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>Dark period (no. of ethanol drinking bouts)</th>
<th>Light period</th>
<th>Total fluid intake (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-3</td>
<td>3-6</td>
<td>6-11</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>5.11 ± 1.86</td>
<td>3.00 ± 0.90</td>
<td>1.78 ± 0.78</td>
</tr>
<tr>
<td>Control</td>
<td>2.00 ± 0.46</td>
<td>0.75 ± 0.25</td>
<td>0.62 ± 0.26</td>
</tr>
<tr>
<td>Day 23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>6.88 ± 1.30**</td>
<td>5.25 ± 1.50*</td>
<td>2.50 ± 0.63</td>
</tr>
<tr>
<td>Control</td>
<td>2.62 ± 0.38</td>
<td>1.50 ± 0.38</td>
<td>1.50 ± 0.65</td>
</tr>
<tr>
<td>Re-test 1</td>
<td>No data</td>
<td>10.25 ± 3.07**</td>
<td>14.62 ± 6.03*</td>
</tr>
<tr>
<td>Diazepam</td>
<td>10.50 ± 2.49**</td>
<td>5.62 ± 1.55**</td>
<td>6.75 ± 2.58**</td>
</tr>
<tr>
<td>Control</td>
<td>1.75 ± 0.53</td>
<td>1.00 ± 0.38</td>
<td>0.62 ± 0.26</td>
</tr>
</tbody>
</table>

Diazepam was given as a single daily i.p. injection of 20 mg/kg. Day 1 is the first day of the 3-week treatment. Day 23 is the last day of the 3-week treatment. Re-test 1 and 2 are the two single dose re-tests. There were eight to 10 rats in each group. Lights were off during the first 11 h after the injection, and on during the subsequent 12 h. Differences between the two groups were tested with the Mann–Whitney U-test (2-tailed). *P < 0.05, **P < 0.01, when comparing diazepam to controls. Values are given as means ± 1 SEM.
Experiment 1: Diazepam dose-response effects on alcohol consumption in the limited access design

The results of experiment 1 are given as a dose–response curve in Fig. 2, showing the effect on ethanol intake during the 24-h choice period following diazepam injection. In this experiment, the rats had limited access to ethanol (24 h/week for 3–16 weeks prior to the different doses of diazepam). A repeated measures ANOVA showed that diazepam significantly affected ethanol intake (P < 0.01). Due to four planned comparisons, reduction of the α-level was performed. There were significant decreases in ethanol intake after 2 mg/kg (P < 0.05) and 20 mg/kg (P < 0.01) of diazepam. The curve seemed to plateau through 2, 4, and 10 mg/kg, with the effects of 4 and 10 mg/kg being not significant. From these results,
20 mg/kg was the dose chosen for treatment of psychologically dependent rats.

**Experiment 2: Effects of diazepam on ethanol consumption by psychologically dependent rats**

The results of experiment 2 are given in Fig. 3. On the first day of the treatment period with daily injections of 20 mg/kg/day of diazepam (67 weeks after the start of the experiment), all rats in the DIC group reduced their voluntary ethanol intake, compared to the mean intake of the previous week. The decrease was from 2.7 ± 0.3 g/kg/day to 1.2 ± 0.1 g/kg/day. This decrease (56%) was significant (P < 0.001). On the last day of the diazepam treatment period, when the DIC group was again given a choice between 10% (w/v) ethanol and water, ethanol intake was slightly above the level of both control groups. The intake was, compared to the pre-treatment level, increased from 2.7 ± 0.3 g/kg/day to 3.8 ± 0.3 g/kg/day. This increase (40%) was significant (P < 0.01). No similar increase was seen in either of the control groups. Thus, a tolerance to the effect of diazepam on voluntary ethanol intake had developed during the 3-week period of diazepam injections.

To evaluate the stability of ethanol intake, the mean intake of the 3 weeks prior to the diazepam treatment was compared to that in the 2 weeks after treatment. In the first week after the treatment, the DIC group had a higher intake than in the 3 weeks before the treatment (3.23 ± 0.21 vs 2.66 ± 0.20, n = 10, P < 0.05). There was also no correlation between the ethanol intake in these two periods (r = 0.61, n = 10, NS). This indicates that a new source of variance determining ethanol intake had been introduced by the diazepam treatment. When comparing ethanol intake in the first week after treatment, to intake of 3 weeks before the treatment period, both control groups showed significant correlations (CCC: r = 0.80, n = 9, P < 0.01, CIC: r = 0.71, n = 10, P < 0.05). However, the CIC group had a regression line that differed significantly from 1.0 (P < 0.01) and a cut-off point of the regression line with a value on the ordinate that differed significantly from 0.0 (P < 0.01), indicating a changed pattern of intake.

The relationship between voluntary ethanol intake in the second week after, and in the 3 weeks prior to, the diazepam treatment is given in Fig. 4. The DIC group (Fig. 4 A) and the CCC group (Fig. 4 B) had the same voluntary ethanol intake in these two periods, whereas the CIC group (Fig. 4 C), as in the first week, again had a regression line that significantly differed from 1.0 (P < 0.001) and a cut-off point of the regression line with a value on the ordinate that significantly differed from 0.0 (P < 0.001). This indicates that the change in the voluntary ethanol intake pattern of the CIC group remained. No corresponding change was seen either in the DIC group, which also had a 3-week interruption in their choice, or in the CCC group.

No convulsions were observed in the rats in this experiment after the end of the diazepam treatment, but the rats were not kept under constant conditions.
surveillance, as this could have interfered with their drinking behaviour.

Tests of psychological dependence

Tests of psychological dependence with 20% (w/v) ethanol were performed before (weeks 61–63) and after (weeks 75–77) the 3-week period of treatment with diazepam. In the test before the diazepam treatment, there were significant correlations in all three groups between voluntary intake of 10% ethanol and the voluntary intake of 20% ethanol, and the regressions did not significantly deviate from 1.0 (Fig. 5 A, B, C). At the re-test after the treatment, the same criteria of psychological dependence were found (Fig. 5 D, E, F). Thus, the psychological dependence on ethanol had not been changed either by the interruption in the access to ethanol (CIC group), or by the combination of an interruption and treatment with diazepam (DIC group).

Diazepam (20 mg/kg) was re-tested in single doses 10 and 19 weeks after the initial 3-week treatment (Fig. 3) in the DIC group. No effects on voluntary ethanol intake were seen after these diazepam injections. Thus, the initial effect seen in the first day of the 3-week diazepam treatment, that had disappeared on the last day of the treatment, had not been re-established. After the diazepam injections, there was no decrease in bouts of drinking at the ethanol bottle compared to the controls (Table 1, re-test 1 and re-test 2). Instead, the diazepam injections increased the number of drinking bouts. The total fluid intake was uninfluenced at both re-tests (Table 1, re-test 1 and re-test 2).

Before the second re-test of diazepam, there was a 3-week period with 5% ethanol. This test showed that both groups DIC and CCC had the same level of intake with these two concentrations, which is an expected finding in these
psychologically dependent rats. However, the CIC group consumed significantly ($P < 0.01$) less ethanol when offered 5% ethanol, compared to the control periods on 10%. This shows, in the CIC group, an unexpected change in the usually very stable ethanol intake.

Blood samples were taken 18 to 42 min after a period of licking at the ethanol bottle in week 91 to measure ethanol concentrations. These samples gave results ranging from 0.10 mg/ml to 0.91 mg/ml with a mean of 0.25 ± 0.04 mg/ml (25 ± 4 mg/dl).

**DISCUSSION**

There has been much debate about the usefulness of different animal models of alcoholism (Cicero, 1979; Meisch, 1984; Stewart and Grupp, 1992). However, current interest in drug treatment of human alcoholism has changed the picture, and recently a need for standardization of experimental models of alcoholism has been pointed out (Myers, 1996). Many animal models utilize special techniques to achieve a high ethanol intake prior to testing of drugs, e.g. limited access to ethanol (Sinclair et al., 1992), fluid deprivation (Wahlström, 1972; June et al., 1992), or food restriction (Tang and Falk, 1983; Stewart and Grupp, 1992). Rats with a high ethanol intake can be selected, either prior to testing of drugs in outbred rats (Daoust et al., 1987; Meert, 1993), or as a basis for genetic selection (McBride et al., 1988; Balakleevsky et al., 1990; Carroll et al., 1990), which has resulted in several lines of rats with a high mean ethanol intake. These methods are appropriate for achieving a high ethanol intake, which will sometimes induce physical dependence. However, they do not show any sign of psychological dependence other than a high intake of ethanol, or a high craving for ethanol in behavioural tests. Such a high intake could also with time induce psychological dependence, but the chronic aspect of psychological dependence in rats is rarely incorporated in the design, and specific testing of the changed state is not included.

The main criticism of the current experimental models of alcoholism has been that rats do not drink enough ethanol voluntarily to achieve blood-ethanol concentrations high enough to be pharmacologically relevant (Cicero, 1979; Meisch, 1984; Stewart and Grupp, 1992). This has been attributed partly to ethanol’s aversive taste properties. The rats in our model of psychological dependence utilized in experiment 2 take the same daily dose of ethanol (in g/kg) independently of the offered concentration. This is not the case with rats with a continuous instead of intermittent exposure (Wahlström, 1987), or rats with less exposure to ethanol (Myers and Oblinger, 1977). Thus, the ingested volume of ethanol is not determined by the taste, but by the pharmacological potency, which is determined by the concentration. Furthermore, since the rats, according to our empirical definition of alcoholism, can take the same dose of ethanol (in g/kg) each day, they must have a control mechanism in the central nervous system that closely regulates ethanol intake to reach a certain daily dose. This means that the absolute values of blood-ethanol concentrations on single occasions are not a critical issue. However, blood-ethanol concentrations have been measured in experiment 2 and in earlier experiments using the same exposure to ethanol as in experiment 2. In earlier experiments, simultaneous samples from individual rats were definitely within the pharmacologically relevant range in some rats (Wahlström, 1987). In this experiment, when blood samples were taken close to a period of ethanol intake, all rats had measurable blood-ethanol concentrations.

There are two main ways to describe the amount of ethanol voluntarily taken by a rat. One is to multiply the ingested volume by the concentration (w/v) of the ethanol solution, and present it as a dose (in g/kg). The second is to divide the ingested volume of the ethanol containing fluid by total fluid intake, and present it as a preference ratio. Many investigators consider a high preference for ethanol (above 50%) as a sign of psychological dependence (Cicero, 1979), and when interpreting results from testing the effect of a drug, a decreased preference for ethanol is used as a measure of the effect (Beaman et al., 1984). The rats in our model of psychological dependence take a stable dose of ethanol each week, which is independent of the offered ethanol concentration (Fig. 5). When the concentration of the offered ethanol solution is increased, resulting in a decreased intake of the ethanol-containing fluid, the water intake shows a compensatory increase, resulting in a stable total fluid
intake. This gives a dramatic change in preference ratio, without a change in dose. Thus, in this model, the preference is a variable depending upon the offered concentration and is neither a sign of dependence, nor a reliable sign of the pharmacological effect of the tested drug.

In earlier experiments using drugs that activate the GABA/BDZ receptor complex, both increases and decreases, as well as no change in voluntary ethanol intake have all been reported (for references, see Introduction). If this means that different doses have different effects on ethanol intake, this could explain why the dose–response curve in experiment 1 (Fig. 2) was not linear. On the first day of the diazepam treatment in experiment 2, all diazepam-treated rats decreased their voluntary intake of ethanol (Fig. 3). The main reason for this strong effect, compared with that seen in experiment 1, is probably the induction of psychological dependence in experiment 2. It is possible that activation of the GABA/BDZ receptor complex decreases ethanol intake, because this activation produces effects that resemble those of ethanol. If the rats seek a certain effect from ethanol, they have to drink less ethanol to reach this effect. The psychologically dependent rats in experiment 2 seek a defined effect of ethanol each day, and if a part of this effect is accomplished by the diazepam injection, they should consume less ethanol. This basic idea is supported by the smaller variability in the psychologically dependent rats, recorded as a smaller standard deviation, after the diazepam injection. In experiment 1, where the rats had had limited access to ethanol, voluntary ethanol intake after the 20 mg/kg dose of diazepam decreased from $3.2 \pm 1.9 \text{ g/kg/day}$ to $2.3 \pm 1.5 \text{ g/kg/day}$ ($\pm 1 \text{ SD}$). In experiment 2, where the rats were psychologically dependent and took the same dose of ethanol each week, voluntary ethanol intake after 20 mg/kg of diazepam decreased from $2.7 \pm 0.8$ to $1.2 \pm 0.3$ ($\pm 1 \text{ SD}$).

In experimental preclinical testing of drugs on voluntary ethanol intake, it is important to evaluate the effect of prolonged treatment. However, in many experimental settings, only single doses are tested (Beaman et al., 1984; McBride et al., 1988; Meert, 1993; Rassnick et al., 1992). The importance of the treatment concept is well illustrated by the present experiments. At the end of the 3-week diazepam treatment period, when the diazepam-treated rats regained access to ethanol, they consumed more ethanol than before the treatment (Fig. 3). When compared to the initial effect of diazepam, this shows that a clear tolerance had developed to the effect of diazepam on ethanol intake. This effect (140% of the pretreatment intake) is not the ideal effect for a drug that is used by alcoholics, and would not have been discovered by single-dose testing. Such a development of tolerance has been found with all drugs tested so far in our model, such as atropine (Wahlström, 1995), citalopram (Wahlström and Hedlund, 1996) and buspirone (Hedlund and Wahlström, 1997). All these drugs initially significantly decreased ethanol intake, but on the last day of the treatment ethanol intake was at the same level as before the treatment, or even higher.

The tolerance to the effect of diazepam on ethanol intake probably developed through the action of diazepam on a system regulating ethanol intake. This system had already been changed by the intermittent and long-term ethanol treatment, resulting in a stable ethanol intake. Since the decrease in ethanol intake after the first dose of 20 mg/kg of diazepam in experiment 2 (56%) was larger than the corresponding decrease after 20 mg/kg in experiment 1 (approximately 30%), the sensitivity to diazepam in this system had increased at the time of the first dose in experiment 2. Thus, 20 mg/kg could have been supramaximal as an acute dose in experiment 2, and a lower dose than the one chosen from the results of experiment 1 could have been equally effective. However, the way in which tolerance would develop to a lower dose of diazepam is very hard to predict, both from the results with 20 mg/kg and from earlier studies, since the rats have a changed regulation of ethanol intake. Unfortunately it was not possible to include more than one dose of diazepam in the present experiment.

Considering the development of tolerance to 20 mg/kg/day of diazepam, it would have been interesting to test other doses. Tolerance might not develop as rapidly with the lower dose, e.g. with 2 mg/kg/day, that was effective in experiment 1. However, the rats in experiment 2 had a very long exposure to ethanol. This long exposure could certainly have changed many neurotransmitter systems, e.g. the GABA system. This suggests that the effect of a GABA agonist, such as diazepam, could be hard to predict from results.
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obtained from rats with a shorter exposure to ethanol.

After 3 weeks of treatment with the 20 mg/kg dose of diazepam twice a day, rats that have not been exposed to ethanol have developed signs of physical dependence on diazepam, as determined by an increased dose of hexobarbital when tested with an EEG threshold method (Korkmaz et al., 1994). This dose is twice that used in experiment 2. Since no convulsions were observed in experiment 2, there is no direct evidence of physical dependence. However, ethanol intake was increased in the week after diazepam treatment ($P < 0.05$). Hypothetically, this could be regarded as a self-medication of weak abstinence symptoms directly after the diazepam treatment. Furthermore, this self-medication could be the source of the increase in variance of ethanol intake seen in the week after the treatment.

In experiment 2, when both the pre-treatment intake was compared to intake after treatment (Fig. 4), and the rats were tested with 5% ethanol, the pattern of ethanol intake in the diazepam-treated rats (DIC group) resembled that of control rats with a continuous choice during the diazepam treatment period (CCC group) more than that of control rats with an interrupted choice (CIC group). This suggests that diazepam could act as a substitute for ethanol when given during ethanol withdrawal, not only in relation to withdrawal-associated seizures and anxiety, but also as regards the regulation of voluntary ethanol intake. The result is an intake pattern resembling that of control rats on a continuous choice. A similar result has been reported earlier (Deutsch and Walton, 1977). In this latter experiment, rats were treated intragastrically with ethanol to induce a high voluntary oral intake of ethanol. Diazepam treatment could then substitute for the forced ethanol exposures to maintain a high voluntary ethanol intake during short test sessions. However, this is not a surprising result, since the rationale for the use of benzodiazepines in withdrawal is their cross-tolerance with ethanol (Sellers and Kalant, 1976).

As long as 19 weeks after a 3-week treatment, the effect of diazepam on ethanol intake remained changed, indicating a long-lasting tolerance to diazepam, probably due to changes in the GABA/BDZ system. Whether these changes are irreversible cannot be ascertained at present. In earlier analysis done on rats participating in our rat model of alcoholism without receiving any drug treatment, no changes have been observed in binding sites or in affinity for flunitrazepam (Harro et al., 1994). This has been confirmed in later, more detailed, analysis using autoradiography (G. Wahlström, L. Hedlund and B. B. Fredholm, unpublished results). This indicates that the BDZ receptors of these rats are intact, but does not totally rule out changes e.g. in receptor subunit composition. In similar analysis on psychologically dependent rats, not exposed to drugs, a bimodal distribution of GABA-transaminase has been found (Sherif et al., 1993), with a doubled activity of GABA-transaminase in some rats. In these analyses, no correlation was found between ethanol intake and activity of GABA-transaminase. In experiment 2 of the present study, no bimodal distribution of ethanol intake or the effect of diazepam was found. However, the critical issue is the long-lasting tolerance induced by the diazepam treatment and the consequences of this possibly irreversible change.

In conclusion, a single dose of diazepam can decrease ethanol intake in psychologically dependent rats, but when diazepam is given chronically for 3 weeks, a tolerance to this effect develops. Although the increase in ethanol intake seen immediately after the end of the diazepam treatment lasts for only 1 week, the tolerance to the ability of diazepam to decrease ethanol intake is certainly long-lasting and may be irreversible. These results point to consequences of treatment with diazepam in psychologically dependent rats that could be of clinical importance, but further investigations are certainly needed.

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