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ORIGINAL INVESTIGATION

Deficit in brain reward function and acute and protracted anxiety-like behavior after discontinuation of a chronic alcohol liquid diet in rats

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Abstract
Rationale Discontinuation of chronic and excessive alcohol consumption leads to a dysphoric state in humans. It is not known if there are changes in brain reward function after the discontinuation of an alcohol liquid in rats.
Objectives The aim of these studies was to investigate the effect of withdrawal from an alcohol liquid diet on brain reward function and acute and protracted anxiety-like behavior.
Materials and methods The intracranial self-stimulation procedure was used to assess brain reward function, and the elevated plus maze test was used to assess anxiety-like behavior.
Results Discontinuation of chronic, 12 weeks, exposure to a 6.2% *v/v* alcohol liquid diet lead to a minor deficit in brain reward function and did not increase anxiety-like behavior. Discontinuation of chronic, 12 weeks, exposure to a 10% *v/v* alcohol liquid diet lead to a pronounced deficit in brain reward function and increased anxiety-like behavior. Two weeks after discontinuation of the 10% *v/v* alcohol liquid diet, the rats with a history of alcohol dependence did not display increased anxiety-like behavior. Restraint stress increased anxiety-like behavior in the rats with a history of alcohol dependence, but not in the control rats. Brain reward thresholds were assessed during the chronic 10% *v/v* alcohol exposure period. During this period, there were no differences between the brain rewards thresholds of the alcohol and control rats.
Conclusion These findings indicate that withdrawal from a 10% *v/v* alcohol liquid diet leads to a pronounced deficit in brain reward function and acute and protracted anxiety-like behavior in rats.

Keywords Alcohol · Withdrawal · Brain reward function · Anxiety · Rats

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Introduction

Alcohol addiction is a chronic disorder that is characterized by loss of control over alcohol consumption, development of tolerance, withdrawal symptoms, and relapse after periods of abstinence (American Psychiatric Association 2000). The positive and the negative-reinforcing effects of alcohol have been hypothesized to play a role in the development and maintenance of alcohol addiction (Solomon and Corbit 1974; Wise 1988). The positive reinforcing effects of alcohol (e.g., mild euphoria) have been suggested to play an important role in the initiation of alcohol abuse. In contrast, the negative-reinforcing effects of alcohol (i.e., alcohol self-administration to prevent withdrawal) have been proposed to play an important role in the maintenance of alcohol addiction (Andersohn and Kiefer 2004; Glenn and Parsons 1991). Abrupt cessation of chronic excessive alcohol consumption in humans is associated with negative affective symptoms such as anxiety and depression as well as somatic signs such as sweating, increased heart rate, nausea, fever, tremors, and convulsions (Becker 2000; Hall and Zador 1997). Although the somatic alcohol withdrawal signs can be debilitating, the negative affective components of alcohol withdrawal may play a more important role in the maintenance of alcohol addiction (Koob et al. 1997). In addition to an acute alcohol withdrawal syndrome,

recovering alcohol-addicted patients may experience a protracted abstinence syndrome that can persist for as long as 10 years (De Soto et al. 1985). Protracted alcohol withdrawal is characterized by anxiety, depression, and insomnia, and these symptoms can be potentiated by exposure to stressors (Smith and Aston-Jones 2008).

Animal models have been developed to improve the understanding of the neurobiological mechanism underlying the negative affective state associated with alcohol withdrawal. Bloom and colleagues demonstrated that alcohol dependence can be induced in rats by chronic intermittent exposure to alcohol vapor (Rogers et al. 1979). Discontinuation of alcohol vapor exposure has been shown to induce elevations in brain reward thresholds in a discrete-trial intracranial self-stimulation (ICSS) procedure and overt alcohol withdrawal signs such as tail stiffness and abnormal body posture (Schulteis et al. 1995). Elevations in brain reward thresholds are interpreted as a deficit in brain reward function (e.g., anhedonic state) as higher current intensities are required to maintain responding for rewarding electrical stimuli (Barr and Markou 2005). Elevations in brain reward thresholds or decreased response rates (rate-dependent ICSS methods) have also been reported during spontaneous or precipitated cocaine, amphetamine, fentanyl, morphine, and nicotine withdrawal (Bruijnzeel et al. 2006; Bruijnzeel et al. 2007; Glick et al. 1973; Kokkinidis and McCarter 1990; Wise and Munn 1995). The discontinuation of alcohol vapor exposure or alcohol liquid diet administration has been reported to increase anxiety-like behavior in a variety of tests such as the elevated plus maze test (Baldwin et al. 1991), acoustic startle procedure (Rassnick et al. 1992), and the social interaction test (Overstreet et al. 2002). In addition, repeated withdrawal sessions increase anxiety-like behavior associated with alcohol withdrawal (Overstreet et al. 2002). This is in line with studies which indicate that repeated cycles of alcohol intake and withdrawal potentiate the severity of alcohol withdrawal-induced seizures (Becker and Hale 1993).

Taken together, the above discussed studies indicate that withdrawal from intermittent alcohol vapor exposure leads to a deficit in brain reward function and that repeated cycles of alcohol intake and withdrawal potentiate anxiety-like behavior. Alcohol liquid diets are widely used to study the neurobiological mechanisms underlying alcohol dependence. It is, however, not known if chronic administration of an alcohol liquid diet or withdrawal from an alcohol liquid diet affects brain reward function. The first aim of our experiments was to investigate the effect of the alcohol concentration in a liquid diet, the exposure duration, and the effect of repeated cycles of alcohol administration on brain reward function after the discontinuation of alcohol administration. The second aim was to investigate the effect of the alcohol concentration in the liquid diet on anxiety-like behavior associated with the discontinuation of alcohol administration.

Materials and methods

Animals Male Wistar rats (Charles River, Raleigh, NC, USA) weighing 300–350 g at the onset of the experiments were used. Animals were single-housed in a temperature- and humidity-controlled vivarium and maintained on a 12-h reversed light/dark cycle (lights off 9 A.M.). All animals were treated in accordance with the NIH guidelines regarding the principles of animal care. The experiments were approved by the University of Florida IACUC committee.

Intracranial self-stimulation The rats were prepared with an 11-mm electrode in the medial forebrain bundle (anterior–posterior, −0.5 mm; medial–lateral, ±1.7 mm; dorsal–ventral, −8.3 mm from dura). The discrete-trial current-threshold ICSS procedure was a modification of a task developed by Kornetsky and Esposito (1979) and described in detail previously (Bruijnzeel et al. 2007; Markou and Koob 1992).

Liquid diet procedure For all experiments, regular lab chow was gradually replaced with the Lieber–DeCarli liquid diet (Lieber and DeCarli 1975). The alcohol liquid diet (LD-101A, TestDiet, Richmond, IN, USA) that was used in experiments 1 and 2 had an energy density of 1 kcal/g, and 36% of the calories were derived from alcohol; the alcohol liquid diet that was used in experiment 3 had an energy density of 1.2 kcal/g, and 48% of the calories were derived from alcohol. Prior to the onset of the liquid-diet procedures, the control rats and alcohol rats were pair-matched by weight. The control animals were pair-fed an identical amount of an alcohol-free isocaloric liquid diet (LD-101, TestDiet, Richmond, IN, USA). In the control diet, maltodextrin was isocalorically substituted for alcohol. The amount of liquid diet that the control rats received was based on the liquid diet intake of the alcohol rats during the previous day. Fresh diet was provided daily at the onset of the dark cycle. The body weights of the rats and the amount of liquid diet consumed were recorded daily.

Elevated plus maze testing The test apparatus consisted of four black polypropylene arms (Coulbourn Instruments, Whitehall, PA, USA). The two "open" arms had 0.5 cm ledges, and the two "closed" arms had 30 cm walls. The open arms were placed opposite of each other. The arms were 10 cm wide and 50 cm long and were placed on 55-cm-tall acrylic legs. Testing occurred in a quiet, dimly lit

(50 lux) room. The animals were allowed to acclimate to the testing room for at least 1 h prior to testing. At the beginning of each test, the animal was placed in the center of the apparatus facing an open arm. Animals were allowed to explore the apparatus for 5 min. Testing was recorded and analyzed using Observer 5.0 software (Noldus Information Technology, Wageningen, The Netherlands). The apparatus was cleaned with water and a Nolvasan solution (chlorhexidine diacetate) between animals. When the rats placed all four paws onto an arm, it was considered an entry into an arm.

Blood alcohol levels Tail blood (200 μl) was collected from anesthetized animals approximately 4 h after the onset of the dark cycle. Samples were centrifuged at 3,000 rpm for 5 min, and plasma was collected. The plasma samples were then frozen at −70°C until further processing. Alcohol levels were determined using an NAD-Alcohol dehydrogenase kit (Sigma, St Louis, MO, USA).

Experimental design

Experiment 1 First, the animals were stabilized on the ICSS procedure (less than 10% variation in brain reward thresholds within a 5-day period). After stable brain reward thresholds were achieved, the liquid diet was introduced (control, $n=9$; alcohol, $n=10$). All the animals received the control diet on day 1. The alcohol animals were fed a 1.3% *w*/*v* alcohol liquid diet on days 2 and 3, a 2.7% *w*/*v* alcohol diet on days 4 and 5, and a 4.0% *w*/*v* alcohol diet on day 6. The rats received the final concentration 4.8% *w*/*v* (6.2% *v*/*v*) on day 7. This alcohol concentration was maintained for the following 12 weeks, with the exception of the four 2-day withdrawal periods at the end of weeks 3, 4, 5, and 12. During each withdrawal session, the alcohol diet was replaced with the control diet at the onset of the dark cycle. All animals were then tested on the ICSS procedure at 6, 12, 24, 36, and 48 h post-alcohol removal. Brain reward thresholds and latencies were recorded. The control animals remained on the control liquid diet during withdrawal testing. Following the 48-h time point of the first three withdrawals, the alcohol diet was reintroduced. Blood samples for the determination of blood alcohol levels were collected on the day prior to each withdrawal session.

Experiment 2 The rats (control, $n=20$; alcohol, $n=20$) were prepared with electrodes, and after the animals were recovered from the surgeries, the liquid diets were introduced. The alcohol liquid diet was introduced as described in experiment 1, and the final alcohol concentration was 4.8% *w*/*v* (6.2% *v*/*v*). Approximately 2 weeks after the introduction of the liquid diet, the animals were trained on the ICSS procedure, and the brain reward thresholds were stabilized. Six control rats and four alcohol rats did not establish stable brain reward thresholds, and ICSS parameters were not further assessed in these animals. After the animals were stabilized, they were tested daily for the remainder of the liquid diet exposure period. The animals that were excluded for the ICSS component of this experiment were handled daily. After 12 weeks, the alcohol liquid diet was replaced by the control diet and brain reward thresholds, and response latencies were assessed 6, 12, 24, 36, 48, and 72 h after the discontinuation of the liquid diet (control, $n=14$; alcohol, $n=16$). All animals (control, $n=20$; alcohol, $n=20$) were tested in the elevated plus maze test approximately 30 h after the discontinuation of the alcohol liquid diet. Blood samples for the determination of blood alcohol levels were collected on the day prior to the withdrawal session.

Experiment 3 The animals were prepared with electrodes and stabilized on the ICSS procedure. After stable brain reward thresholds were achieved, the liquid diet was introduced (control, $n=18$; alcohol, $n=17$). All the animals received the control diet on day 1. The alcohol animals were fed a 2.7% *w*/*v* alcohol liquid diet on days 2 and 3, a 4.0% *w*/*v* alcohol diet on days 4 and 5, a 4.8% *w*/*v* alcohol diet on day 6, and the final concentration 7.9% *w*/*v* (10.0% *v*/*v*) on day 7. This alcohol concentration was maintained for the following 12 weeks. ICSS parameters were assessed for three consecutive days per week between 10:00 A.M. and 1:00 P.M. The electrodes of two animals became dysfunctional, and ICSS testing had to be discontinued. For the remainder of the experiment, these animals were handled daily and tested on the elevated plus maze test during acute and protracted withdrawal. After 12 weeks, the alcohol diet was replaced with the control liquid diet, and the brain reward thresholds and response latencies were assessed 6, 12, 24, 36, 48, and 72 h after the discontinuation of the alcohol liquid diet (control, $n=17$; alcohol, $n=16$). Some of the alcohol withdrawing animals had seizures after being handled. These animals were returned to their home cages and allowed to recover for at least 15 min before ICSS testing. None of the animals experienced seizures during ICSS testing. Blood samples for the determination of blood alcohol levels were collected on the day prior to the withdrawal session. All animals were tested in the elevated plus maze test approximately 30 h after the removal of the alcohol liquid diet. Both the control and the alcohol animals remained on the control liquid diet following testing. In order to investigate the effects of a history of alcohol dependence on the sensitivity to a stressor, the effect of restraint stress on the exploration of the elevated plus maze apparatus was investigated. The alcohol and control animals were divided into four groups: control/non-restraint

(n=9), control/restraint (n=9), alcohol/non-restraint (n=8), and alcohol/restraint (n=9). Animals in the restraint groups were placed in a restrainer (diameter, 8.8 cm; IITC Life Science, Woodland Hills, CA, USA) for 15 min immediately prior to being tested in the elevated plus maze. After the elevated plus maze tests, the alcohol liquid diet was reintroduced, and the alcohol rats received the 10% *v*/*v* alcohol liquid diet for an additional 8 weeks. At the end of these 8 weeks (total alcohol liquid diet exposure period was 20 weeks), the alcohol diet was replaced with the control liquid diet, and the brain reward thresholds and response latencies were assessed 6 h after the discontinuation of the alcohol liquid diet (control, n=17; alcohol, n=16).

Statistical analyses For all the withdrawal experiments, ICSS parameters (brain reward thresholds and response latencies) were expressed as percentages of the pre-test day values. Percent changes in ICSS parameters were analyzed using a two-way repeated measures analysis of variance (ANOVA), with time (hours post-alcohol removal) as the within subjects factor and diet (control or alcohol) as the between subjects factor. For experiment 3, ICSS parameters over the course of the liquid-diet administration period were expressed as percentages of the values obtained on the day prior to the onset of the liquid diet administration. Percent changes in ICSS parameters were analyzed using a two-way repeated measures ANOVA, with time (weeks of diet administration) as the within subjects factor and diet (control or alcohol) as the between subjects factor. Elevated plus maze behavior during the acute withdrawal phase was analyzed using a one-way ANOVA with diet as the between subjects factor. Elevated plus maze behavior during the protracted withdrawal phase was analyzed using a two-way ANOVA, with restraint and diet (control or alcohol) as the between subjects factors. For all ICSS experiments, statistically significant results in the ANOVA were followed by the Newman–Keuls post hoc test. For the elevated plus maze experiments, significant results in the ANOVA were followed by the Fisher LSD post hoc test. Because of the large variation between animals in the elevated plus maze test, the more liberal Fisher LSD post hoc test was used. Statistical analyses were performed using SPSS for Windows version 16.0.

Results

Experiment 1 Statistical analyses indicated that there were no significant differences in the absolute baseline brain reward thresholds or the response latencies between the control group and the alcohol group prior to the administration of the liquid diets or prior to any of the withdrawal sessions (Table 1). The average alcohol intake (weeks 1–12) was 7.3±0.3 g/kg per day. The mean blood alcohol concentrations (BAC) on the day prior to each withdrawal session are shown in Table 2. Introduction of the liquid diets induced a small but significant increase in brain reward thresholds (prior liquid diet baseline vs. withdrawal 1 baseline; Time: $F_{1,17}$=5.595, p<0.03) and did not affect the response latencies. The baseline brain reward thresholds and the baseline response latencies remained stable from the first withdrawal session to the fourth withdrawal session. Throughout the experiment, the alcohol rats and the control rats were pair-fed, and there were no differences in the body weights of the control rats and the alcohol rats

Table 1 Absolute baseline brain reward thresholds and response latencies prior to the onset of the administration of the liquid diets and the alcohol withdrawal sessions

	Thresholds (μA) ± SEM		Latencies (sec) ± SEM	
	Control	Alcohol	Control	Alcohol
Experiment 1				
Prior onset liquid diet	119.03±13.44	120.42±9.90	3.72±0.13	3.43±0.14
Withdrawal 1 (3W)	130.09±14.78	123.71±10.19	3.61±0.16	3.43±0.11
Withdrawal 2 (4W)	134.07±17.82	119.00±8.95	3.66±0.17	3.35±0.11
Withdrawal 3 (5W)	129.35±16.10	118.96±11.12	3.77±0.17	3.38±0.10
Withdrawal 4 (12W)	135.93±16.25	124.12±8.95	3.51±0.17	3.35±0.13
Experiment 2				
Withdrawal 1 (12W)	94.29±6.35	111.04±10.96	3.30±0.10	3.40±0.10
Experiment 3				
Prior onset liquid diet	105.39±4.60	106.93±4.87	3.25±0.07	3.26±0.10
Withdrawal 1 (12W)	98.70±4.92	99.74±3.43	3.10±0.10[a]	3.43±0.09
Withdrawal 2 (20W)	103.70±5.06	99.82±5.55	3.07±0.10	3.27±0.07

W weeks. Data are expressed as means ± SEM

[a] Indicate a decrease in response latencies compared to the alcohol group (p<0.05)

Table 2 Mean blood alcohol concentrations (BAC) prior to each withdrawal session

Experiment	Liquid diet concentration (v/v)	Week of liquid diet administration	BAC (mg/dl)
1	6.4	3	48.91±15.89
		4	75.88±14.85
		5	114.44±7.62
		12	93.51±14.58
2	6.4	12	96.23±7.99
3	10.0	12	216.00±19.88

Data are expressed as means ± SEM

immediately prior or at the end of the liquid diet procedure (Table 3). After a period of 3 weeks, the alcohol liquid diet was replaced by the control diet (withdrawal session 1). Discontinuation of the alcohol liquid diet did not affect the brain reward thresholds (Table 4). During the withdrawal period, the response latencies of the control rats were slightly increased compared to those of the alcohol rats (Table 4; Treatment: $F_{1,17}$=10.904, p<0.004). After a period of 4 weeks, the alcohol liquid diet was again replaced by the control diet (withdrawal session 2). Discontinuation of the alcohol liquid diet did not affect the brain reward thresholds (Table 4). There were also no differences between the response latencies of the control rats and the alcohol rats during the withdrawal period (Table 4; Time: $F_{4,68}$=3.540, p<0.011). After a period of 5 weeks, the alcohol liquid diet was replaced by the control diet (withdrawal session 3). There were no differences between the brain reward thresholds of the control rats and the alcohol rats during the withdrawal period (Table 4; Time: $F_{4,68}$=2.577, p<0.045). There were also no differences between the response latencies of the control rats and the alcohol rats during the withdrawal period. After a period of 12 weeks, the alcohol liquid diet was replaced by the control diet (withdrawal session 5). During this final withdrawal period, the brain reward thresholds of the alcohol treated rats were elevated compared to those of the control rats (Table 4; Treatment: $F_{1,17}$=7.133, p<0.016; time: $F_{4,68}$=3.940, p<0.006). These findings suggest that elevations in brain reward thresholds are only detected after a prolonged (12 weeks) period of alcohol intake. Alcohol withdrawal did not affect the response latencies (Table 4).

Experiment 2 For the second experiment, the mean alcohol intake was 8.7±0.2 g/kg per day, and this was significantly higher than the alcohol intake in the first experiment (7.3± 0.3 g/kg, $F_{1,22}$=13.482, p<0.01). The mean blood alcohol level on the day prior to the discontinuation of alcohol administration was 96.23±7.99 mg/dl (Table 2). The control rats were pair-fed, and there were no differences in the body weights of the control rats and the alcohol rats immediately prior to the onset of the liquid diet procedure or at the end of the liquid diet procedure (Table 3). Discontinuation of alcohol administration leads to an elevation in brain reward thresholds in the alcohol rats compared to the pair-fed control rats (Fig. 1a; Time: $F_{5,140}$=3.037, p<0.012; Treatment: $F_{1,28}$=4.793, p<0.037). Discontinuation of alcohol administration did not affect the response latencies (Fig. 1b; Time: $F_{5,140}$=2.735, p<0.022). All the animals were tested on the elevated plus maze 30 h after the discontinuation of the alcohol liquid diet. The animals that fell off the elevated plus maze during testing (one alcohol rat and four control rats) were excluded from the statistical analyses. The animals with a history of alcohol intake did not display increased anxiety-like behavior compared to the control rats. Alcohol withdrawal did not affect the number of closed arm entries, percentage of open arm entries, and protected head dips. There was a trend toward an alcohol withdrawal-induced decrease in the percentage of time spent on the open arms ($F_{1,33}$=2.905, p<0.098) and a trend toward a decrease in the number of unprotected head dips ($F_{1,33}$=3.827, p<0.059).

Experiment 3 The mean alcohol intake from weeks 1 to 12 was 10.3±0.2 g/kg. The mean blood alcohol level in the week prior to the discontinuation of alcohol administration was 216.00±19.88 mg/dl (Table 2). In order to investigate the effects of chronic alcohol intake on the brain reward system, brain reward thresholds (Fig. 2a) and response latencies (Fig. 2b) were assessed during the liquid diet administration period. Chronic administration of the liquid diets leads to a decrease in brain reward thresholds

Table 3 Absolute body weights (grams) of the control rats and alcohol rats immediately before the onset of the liquid diet procedures and at the end of the liquid diet procedures

Experiment	Prior		End	
	Control	Alcohol	Control	Alcohol
1	471.22±12.89	485.90±13.05	587.11±20.35	585.40±21.09
2	434.10±7.61	442.80±7.24	621.25±10.85	600.25±13.06
3	422.06±8.33	422.53±8.76	508.06±14.42	492.76±12.09

Data are expressed as means ± SEM

Table 4 Brain reward thresholds and response latencies (percentages) following removal of the alcohol liquid diet (6.4% *v*/*v*)

Weeks of alcohol	Hours post alcohol	Thresholds (μA) ± SEM		Latencies (s) ± SEM	
		Control	Alcohol	Control	Alcohol
3	6	99.65±1.38	103.97±2.79	102.62±2.18	97.22±2.00
	12	101.72±2.29	106.32±3.21	103.69±2.20	98.04±3.75
	24	105.21±2.54	107.38±2.91	105.51±2.05	101.12±2.31
	36	102.34±2.27	104.27±2.53	106.72±1.89	103.22±2.69
	48	105.55±2.68	107.48±3.03	108.23±2.11	97.54±1.59
4	6	100.47±2.69	101.22±3.03	104.14±2.57	96.40±1.70
	12	103.25±3.91	103.78±2.05	106.84±1.71	102.71±2.24
	24	104.87±1.80	106.35±3.15	105.32±2.52	102.13±1.77
	36	106.44±3.91	104.88±2.32	101.95±1.90	100.32±2.85
	48	103.49±3.42	105.43±3.42	99.47±1.77	100.34±2.32
5	6	103.25±1.41	105.99±3.28	99.01±2.60	100.36±3.53
	12	104.19±2.78	104.81±3.48	102.88±2.13	106.23±3.05
	24	103.70±3.01	109.78±3.33	97.28±2.33	105.09±2.75
	36	102.02±2.49	102.72±3.44	99.93±3.37	96.48±1.43
	48	106.24±1.63	110.96±3.90	99.99± 2.90	103.33±2.21
12	6	93.77±3.23	102.35±3.02	102.99±5.57	100.71±2.53
	12	99.27±1.80	102.25±2.11	103.81±4.00	101.59±2.76
	24	102.12±2.72	108.82±2.35	102.78±3.25	99.97±2.69
	36	101.41±1.93	104.98±1.03	103.03±2.77	98.68±2.17
	48	98.92±2.15	105.18±1.96	101.31±4.35	96.52±3.01

Data (means ± SEM) are expressed as a percentage of the values obtained on the day prior to withdrawal

in the alcohol group and the pair-fed control group (Time: $F_{12,372}$=9.078, p<0.0001). Chronic administration of the liquid diets decreased the response latencies in the pair-fed control rats and did not affect the response latencies of the alcohol rats (Time × Treatment: $F_{12,372}$= 3.978, p<0.0001). Newman Keuls post hoc tests indicated that the latencies of control animals were decreased compared to those of the alcohol treated during weeks 4, 7, 9, 11, and 12. Discontinuation of alcohol administration increased the brain reward thresholds of the alcohol rats and did not affect the brain reward thresholds of the pair-fed control rats (Fig. 3a; Time × Treatment: $F_{5,155}$=8.655, p<0.0001). Newman Keuls post hoc tests showed that the thresholds of alcohol withdrawing animals were significantly elevated 6, 12, and 24 h post-alcohol removal as compared to those of the control rats. Discontinuation of alcohol administration also increased the response latencies of the alcohol rats compared to those of the control rats (Fig. 3b; Time × Treatment: $F_{5,155}$=12.147, p<0.0001). Because there was a difference in the absolute response latencies of the control rats and the alcohol rats immediately prior to the withdrawal session (Table 1), the response latencies during this withdrawal session were expressed as a percentage of the values obtained on the test day prior to the onset of the liquid diet administration. Thirty hours after the discontinuation of the administration of the alcohol liquid diet, the alcohol withdrawing rats and the control rats were tested in the elevated plus maze (Fig. 4). Animals that fell off the elevated plus maze (two alcohol rats and one control rat) were excluded from the data analyses. The alcohol withdrawing rats displayed increased anxiety-like behavior compared to the control rats. The alcohol rats had a lower percentage of open arm entries ($F_{1,30}$=8.313, p<0.007), a decreased percentage time spent on the open arms ($F_{1,30}$= 5.646, p<0.024), a decreased number of protected head dips ($F_{1,30}$=4.180, p<0.0497) and unprotected head dips ($F_{1,30}$=5.905, p<0.021), and fewer entries in the closed arms ($F_{1,30}$=6.320, p<0.017). The effect of restraint stress on exploratory behavior in the elevated plus maze test was investigated in control rats and alcohol rats 2 weeks after the discontinuation of the alcohol liquid diet. The restraint-stress session decreased the percentage of open arm entries in the rats with a history of alcohol dependence and did not affect the percentage of open arm entries in the control rats (Fig. 5; Treatment × Restraint: $F_{1,31}$=4.972, p<0.033). There was a trend toward an increased sensitivity to the effect of restraint stress on the percentage of time spent on the open arms in the alcohol rats compared to the control rats (Treatment × Restraint: $F_{1,31}$=3.589, p<0.068). There were no differences in the number of closed arm entries, protected head dips, or unprotected head dips in the alcohol rats compared to the control rats. In order to investigate if repeated alcohol withdrawal sessions potentiate the deficit in brain reward function associated with alcohol withdrawal, the 10% *v*/*v* alcohol liquid diet was reintroduced after the last elevated plus maze test. The mean alcohol intake from weeks 13 to

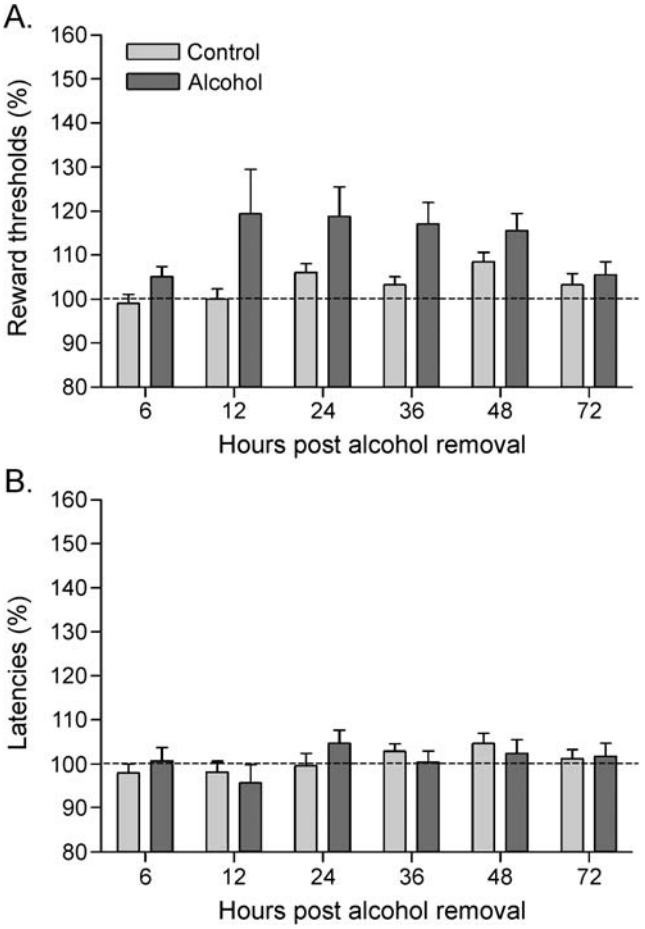

Fig. 1 Effect of the discontinuation of a chronic, 12 weeks, 6.2% *v/v* alcohol liquid diet on brain reward thresholds (**a** control, $n=14$; alcohol, $n=16$) and response latencies (**b**). Data are expressed as means ± SEM

20 was 9.5±0.5 g/kg. The control rats were pair-fed, and there were no differences in the body weights of the control rats and the alcohol rats immediately prior to the onset of the liquid diet procedure or at the end of the liquid diet procedure (week 20; Table 3). Discontinuation of alcohol administration leads to a significant elevation in brain reward thresholds (Fig. 6; $F_{1,32}=18.987$, $p<0.0001$) at the 6-h time point and an increase in the response latencies ($F_{1,32}=11.931$, $p<0.0016$). There was no significant difference in the alcohol withdrawal-induced elevations in brain reward thresholds or the increase in response latencies between the first (12 weeks) and second (20 weeks) withdrawal session. In order to investigate if there was a rank-order correlation between the severity of the deficit in brain reward function during the first and the second withdrawal session, a Spearman's rho correlation was conducted. There was a significant correlation between the rank order of the animals during the first and the second withdrawal session ($r=0.515$, $p<0.041$). This indicates that animals that had the most severe deficit in brain reward function during the first withdrawal session also had the most severe deficit in brain reward function during the second withdrawal session.

Discussion

The aim of the present studies was to investigate the effect of the alcohol concentration in a liquid diet and the exposure duration on brain reward function and anxiety-like behavior after the discontinuation of alcohol administration. These studies demonstrated that the discontinuation of alcohol administration mediates a deficit in brain reward function in rats that are fed an alcohol liquid diet for an

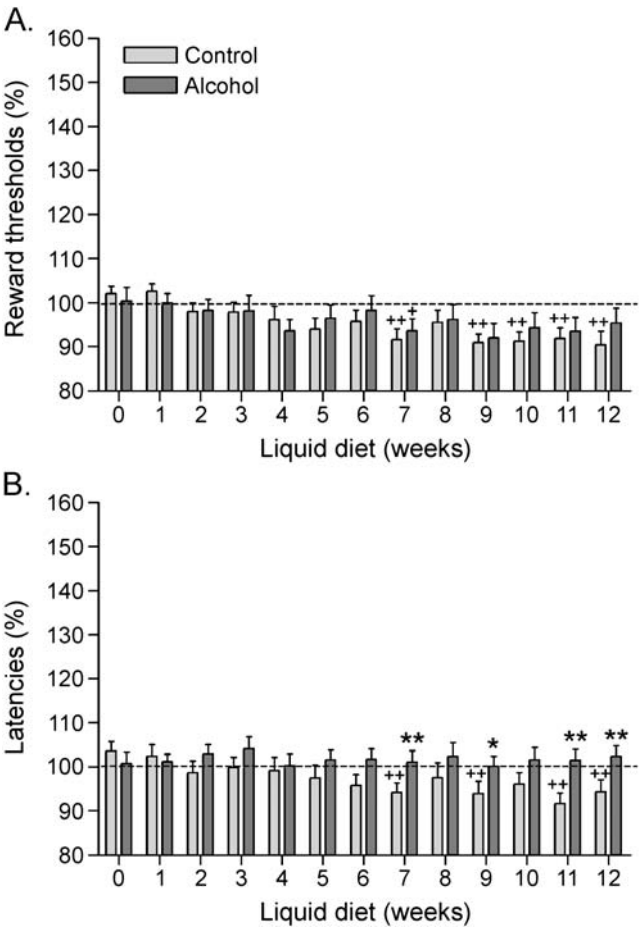

Fig. 2 Effect of chronic alcohol intake (10% *v/v*) on brain reward thresholds (**a** control, $n=17$; alcohol, $n=16$) and response latencies (**b**). Brain reward thresholds and response latencies are expressed as percentages of the values obtained on the day prior to the onset of the liquid diets. *Asterisks* (**$p<0.01$, *$p<0.05$) indicate a difference between the response latencies of the alcohol rats and the control rats. *Plus signs* (++$p<0.01$, +$p<0.05$) indicate a decrease in response latencies or lower brain reward thresholds compared to week 0. Data are expressed as means ± SEM

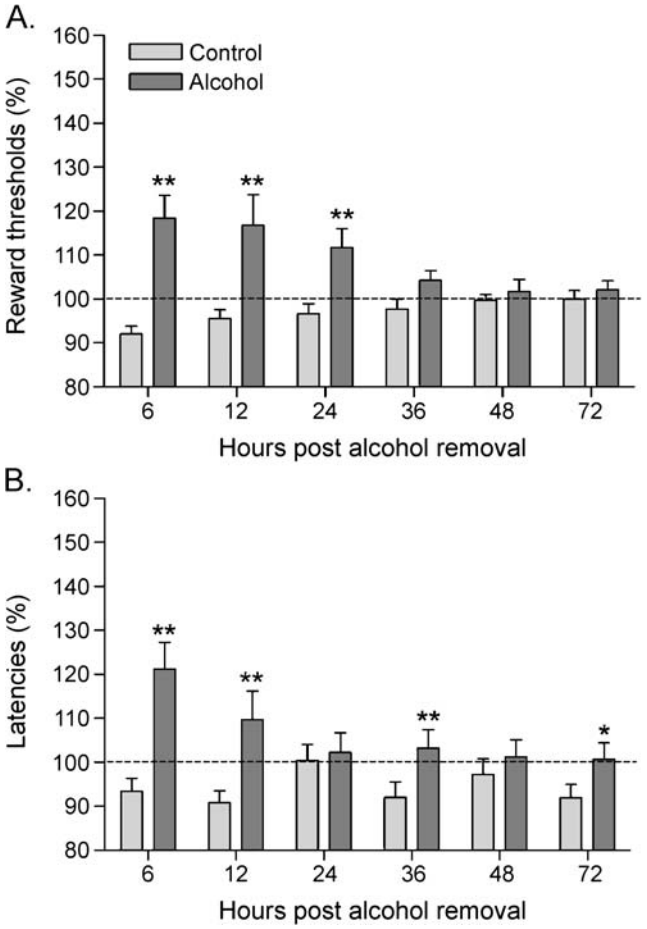

Fig. 3 Effect of the discontinuation of a chronic, 12 weeks, 10% *v/v* alcohol liquid diet on brain reward thresholds (**a** control, $n=17$; alcohol, $n=16$) and response latencies (**b**). *Asterisks* (**$p<0.01$, *$p<0.05$) indicate elevations in brain reward thresholds or increases in response latencies compared to the corresponding control group. Data are expressed as means ± SEM

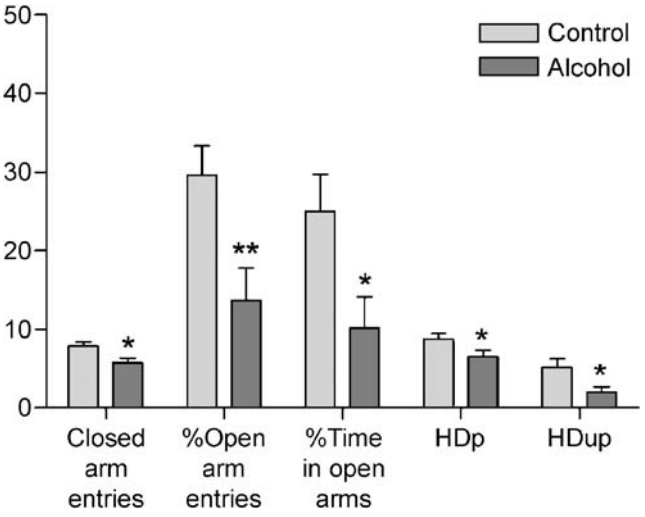

Fig. 4 Effect of the discontinuation of a chronic, 12 weeks, 10% *v/v* alcohol liquid diet on anxiety-like behavior in the elevated plus maze test (control $n=17$; alcohol $n=15$). The rats were tested in the elevated plus maze 30 h after the discontinuation of the alcohol liquid diet. *Asterisks* (**$p<0.01$, *$p<0.05$) indicate a difference between the control rats and the alcohol withdrawing rats. Abbreviations: *HDp* protected head dips, *HDup* unprotected head dips. Data are expressed as means ± SEM

extended period of time (12 weeks), but not in rats that are fed an alcohol liquid diet for a relatively short period of time (3–5 weeks). The duration of the deficit in brain reward function was dependent on the concentration of alcohol in the liquid diet. The alcohol withdrawal-induced deficit in brain reward function lasted longer in animals that were fed a 10% *v/v* alcohol liquid diet than in animals that were fed a 6.2% *v/v* alcohol liquid diet. To our knowledge, this is the first study to report that discontinuation of an alcohol liquid diet leads to a deficit in brain reward function. On a similar note, rats displayed increased anxiety-like behavior in the elevated plus maze test 30 h after the discontinuation of the 10% *v/v* alcohol liquid diet, but did not display increased anxiety-like behavior 30 h after discontinuation of the 6.2% *v/v* alcohol liquid diet. Two weeks after the discontinuation of the 10% *v/v* alcohol liquid diet, the rats did not display increased anxiety-like behavior in the elevated plus maze test. However, at this time point, restraint stress increased anxiety-like behavior in the elevated plus maze test in the rats with a history of alcohol dependence but not in the control rats. This indicates that chronic exposure to alcohol induces a long-term increased sensitivity to stressors. Taken together, these

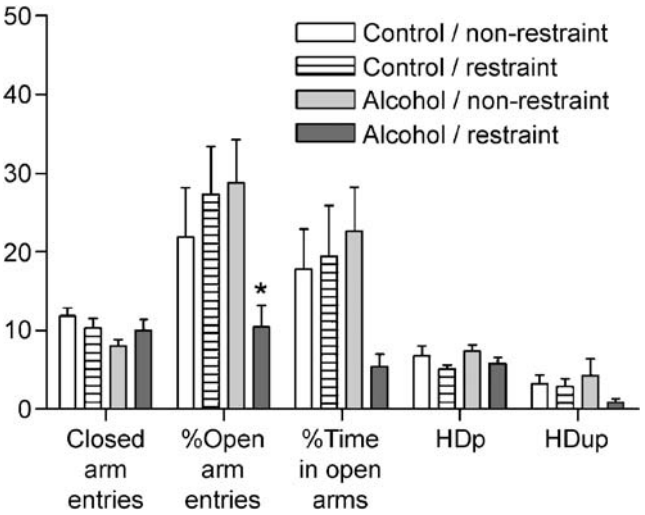

Fig. 5 Effects of 15-min restraint stress on anxiety-like behavior in control rats (control/non-restraint, $n=9$; control/restraint, $n=9$) and in rats that have been exposed to an alcohol liquid diet (12 weeks, 10% *v/v*; alcohol/non-restraint, $n=8$, and alcohol/restraint $n=9$). The rats were tested 2 weeks after the discontinuation of the alcohol liquid diet. Abbreviations: *HDp* protected head dips, *HDup* unprotected head dips. Data are expressed as means ± SEM

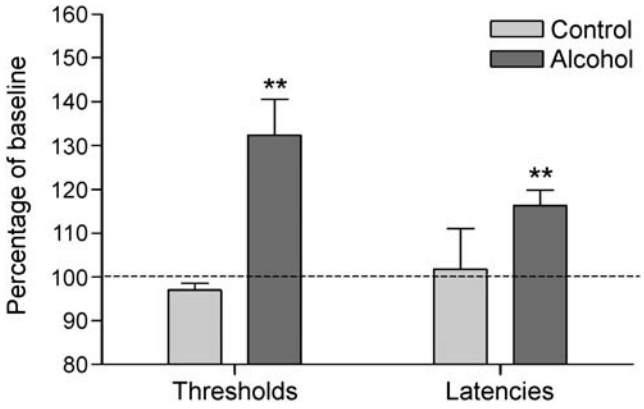

Fig. 6 Effect of the discontinuation of a chronic, 20 weeks, 10% *v/v* alcohol liquid diet on brain reward thresholds (control, $n=17$; alcohol, $n=16$) and response latencies. ICSS parameters were assessed 6 h after the discontinuation of the alcohol liquid diet. *Asterisks* (**$p<0.01$, *$p<0.05$) indicate elevations in brain reward thresholds or increases in response latencies compared to the corresponding control group. Data are expressed as means ± SEM

studies indicate that discontinuation of alcohol administration after prolonged (12 weeks) exposure to a 10% *v/v* alcohol liquid diet induces a deficit in brain reward function and increased acute and protracted anxiety-like behavior.

Numerous clinical and preclinical studies have demonstrated that there is a positive relationship between the number of previous alcohol withdrawals and withdrawal-induced seizures (Becker and Hale 1993; McCown and Breese 1990; Rogawski 2005). Furthermore, alcohol withdrawing subjects who have experienced multiple alcohol withdrawal episodes have more severe cravings during the withdrawal phase, are more likely to have a heavy drinking episode during the first few days after quitting, and display a slower decline in Clinical Institute Withdrawal Assessment for Alcohol-Revised (CIWA-Ar, includes among others nausea, tremor, anxiety, and agitation) scores compared to subjects who experience their first or second alcohol withdrawal episode (Lukasiewicz et al. 2005; Malcolm et al. 2000). Preclinical studies suggest that repeated alcohol withdrawals may potentiate anxiety-like behavior associated with alcohol withdrawal. Overstreet and colleagues investigated the effect of repeated alcohol withdrawal episodes on anxiety-like behavior in the social interaction test (Overstreet et al. 2002). They showed that withdrawal from 15 days of an alcohol liquid diet (4.5% *w/v*, which is similar to the alcohol concentration in experiment 1 and 2 [4.8% *w/v* or 6.2% *v/v*]) did not decrease social interaction in rats. However, rats displayed a decrease in social interactions after three alcohol cycles that each consisted of 5 days of an alcohol liquid diet and 2 days of a control diet. In experiment 1, we investigated the effect of repeated cycles of alcohol intake and withdrawal on brain reward function. The results of this study demonstrated that the rats did not display a deficit in brain reward function during the first, second, third, or fourth withdrawal session. A similar result was obtained in the third experiment. It was demonstrated that there was no difference in the elevations in brain reward thresholds at the 6-h time point during the first (12 weeks) and second (20 weeks) withdrawal session. Taken together, the above discussed literature and our findings suggest that repeated withdrawal cycles may potentiate alcohol withdrawal-induced seizures and anxiety-like behavior, but does not affect the deficit in brain reward function associated with alcohol withdrawal.

In the first experiment, the rats were exposed to a 6.2% *v/v* alcohol liquid diet, and the effect of discontinuation of alcohol administration on brain reward function was investigated 3, 4, 5, and 12 weeks after the onset of the alcohol liquid diet. Only during the last withdrawal session were the brain reward thresholds of the alcohol group elevated compared to those of the control group, but differences between the alcohol group and control groups were marginal (<10%). In the second experiment, the rats were also exposed to a 6.2% *v/v* alcohol liquid diet; however, in this experiment, the rats were continuously exposed to the alcohol liquid diet, and alcohol withdrawal was investigated after 12 weeks of exposure to the liquid diet. Discontinuation of the alcohol liquid diet lead to a 19% elevation in brain reward thresholds at the 12-h time point. This observation suggests that chronic continuous exposure to a 6.2% *v/v* alcohol liquid diet induces a slightly more severe deficit in brain reward function than intermittent exposure to an alcohol liquid diet. In the third experiment, the alcohol group was continuously exposed to a 10% *v/v* alcohol liquid, and alcohol withdrawal was investigated after 12 weeks of exposure. Discontinuation of alcohol administration lead to a significant increase in brain reward thresholds, and the brain reward thresholds of the alcohol withdrawing rats were elevated at the 6-, 12-, and 24-h time points. This pattern of results suggest that the deficit in brain reward function is most severe in rats that have been continuously exposed to an alcohol liquid diet with a relatively high, 10% *v/v*, alcohol concentration. The present results also indicate that there is a positive relationship between the alcohol concentration in the liquid diet and the blood alcohol levels. The blood alcohol levels in the rats exposed to the 6.2% *v/v* alcohol liquid diet were between 90 and 100 mg/dl at the end of the exposure period, and the blood alcohol level in the group that received the 10% *v/v* alcohol liquid diet was 216 mg/dl at the end of the exposure period. The response latencies were significantly increased after the discontinuation of the 10% *v/v* alcohol liquid. Increased response latencies can be indicative of motor impairments or sedative effects (Baldo et al. 1999; Markou and Koob 1992). It is, however, unlikely that the elevations in brain reward thresholds were

completely due to motor impairments or sedative effects. A close look at the data suggests that there was no relationship between the brain reward thresholds and the response latencies. For example, at the 24-h time point the brain reward thresholds were elevated, but the response latencies were not increased.

The results of our study are in line with a study by Koob and colleagues in which they investigated the effect of alcohol withdrawal on brain reward thresholds (Schulteis et al. 1995). Alcohol dependence was induced by passive exposure to alcohol vapor for 17 days, and during the last week of exposure, the blood alcohol levels were approximately 200 mg/dl immediately after the exposure sessions. Withdrawal from alcohol vapor exposure leads to an elevation in brain reward thresholds, which lasted approximately 48 h. In this study, alcohol withdrawal leads to somewhat higher elevations in brain reward thresholds and of longer duration than in our study (experiment 3) in which alcohol dependence was induced by using a 10% *v/v* alcohol liquid diet. A possible explanation for the fact that withdrawal from alcohol vapor induces a more severe deficit in brain reward function than withdrawal from an alcohol liquid diet is that in the animals that received the alcohol liquid diet, there may be a more gradual decrease in blood alcohol levels due to a reservoir of alcohol in the stomach. It has been estimated that the half maximal gastric emptying time in rats receiving a 4.8% *w/v* liquid diet is 1 h (Sankaran et al. 1996). There is a strong relationship between the rate of gastric emptying and alcohol absorption, and therefore, the absorption of alcohol will continue for hours after the discontinuation of the alcohol liquid diet (Holt 1981).

In the third experiment, the effect of chronic exposure to alcohol on brain reward thresholds and response latencies was investigated. It was shown that chronic exposure to the liquid diets lead to a gradual lowering of brain reward thresholds in the control rats and the alcohol rats. A lowering in brain reward thresholds is interpreted as a potentiation of brain reward function. It is interesting to note that chronic exposure to the liquid diets lead to a gradual lowering in brain reward thresholds. Previous research indicates that prolonged access (6 h per day) to cocaine leads to an elevation in brain reward thresholds (Ahmed et al. 2002). Our observation suggests that chronic access to alcohol has less deleterious effects on brain reward function than psychostimulants such as cocaine. We suggest that the lowering in brain reward thresholds in the alcohol and control rats is due to a diet-induced decrease in caloric intake. Previous research has demonstrated that rats that are fed a 36% alcohol liquid diet consume 30% fewer calories per day and have a 75% reduction in body weight gain compared to rats that have unlimited access to an isocaloric liquid diet that does not contain alcohol (Goheen et al. 1980; Reidelberger et al. 1996). Carr and colleagues demonstrated that severe food deprivation, 10 g of lab chow per day, leads to a rapid decrease in body weights and an increased sensitivity to rewarding electrical stimuli 5–7 days after the onset of the food deprivation (Abrahamsen et al. 1995; Carr et al. 2000). In our study, a potentiation of brain reward function was detected 7 weeks after the onset of the liquid diet. This discrepancy might be due to the fact that the rats in our study were mildly food-deprived compared to the rats in Carr's studies. In order to able to draw firm conclusions about the effects of food deprivation on brain reward thresholds, additional control groups are needed. Future studies may add ad libitum liquid diet or lab chow groups to control for the effects of chronic mild food deprivation on brain reward function.

During the second and third experiments, it was investigated if withdrawal from an alcohol liquid diet would increase anxiety-like behavior. The present findings indicated that withdrawal from a 10% *v/v* alcohol liquid diet (average BAC 216±19.88 mg/dl), but not withdrawal from a 6.2% *v/v* alcohol liquid diet (average BAC 96.23± 7.99 mg/dl), mediates an increase in anxiety-like behavior in the elevated plus maze test 30 h after the discontinuation of the alcohol liquid diet. Anxiety-like behavior was assessed at only one time point, and it cannot be completely ruled out that increased anxiety-like behavior might have been detected in the rats withdrawing from the 6.2% *v/v* alcohol liquid diet at an earlier time point. We are not aware of any studies that did a thorough investigation of the time course of alcohol withdrawal-induced anxiety-like behavior in rats receiving relatively low doses of alcohol. Research by Pandey and colleagues suggests that anxiety-like behavior is most severe 24 h after the discontinuation of a 9% *v/v* alcohol liquid diet; however, increased anxiety-like behavior was detected up to a least 72 h after the discontinuation of the alcohol liquid diet (Pandey et al. 1999). The observation that withdrawal from a 10% *v/v* alcohol liquid diet increased anxiety-like behavior in the elevated plus maze test is in line with previous studies that reported that withdrawal from 8.7 to 11.5% *v/v* alcohol liquid diets increases anxiety-like behavior (Baldwin et al. 1991; File 1994; Pandey et al. 1999; Rassnick et al. 1993). The present finding also suggests that the alcohol withdrawal-induced anxiety-like behavior is dependent on the concentration of alcohol in the liquid diet, and blood alcohol levels as discontinuation of alcohol administration did not increase anxiety-like behavior in rats that had been exposed to the 6.2% *v/v* alcohol liquid diet. Withdrawal from the 10% *v/v* alcohol liquid diet induced a small but significant decrease in the number of closed arm entries. Therefore, a decrease in activity could confound the interpretation of the data (i.e., decrease in open arm exploration due to a decrease in overall activity). It should be noted, however, that the alcohol

withdrawing rats had a lower open arm entry/total arm entry ratio than the control rats. This indicates that the alcohol withdrawing rats made relatively fewer entries into the open arms than into the closed arms compared to the control rats. In the third experiment, we also investigated if exposure to the 10% *v*/*v* liquid diet would have long-term effects on anxiety-like behavior and if a history of alcohol dependence would increase the sensitivity to restraint stress. Two weeks after withdrawal from the 10% *v*/*v* alcohol liquid diet, the rats with a history of alcohol dependence did not display increased anxiety-like behavior in the elevated plus maze test compared to the control animals when tested under baseline conditions. However, exposure to restraint stress increased anxiety-like behavior in the animals with a history of alcohol dependence, but did not increase anxiety-like behavior in the control rats. This observation suggests that chronic alcohol intake does not have a long-term effect on baseline anxiety-like behavior but potentiates stress-induced anxiety-like behavior.

Taken together, the present findings indicate that withdrawal from a 10% *v*/*v* alcohol liquid diet leads to a deficit in brain reward function, acute anxiety-like behavior, and an increased sensitivity to restraint stress 2 weeks later. The present 10% *v*/*v* alcohol liquid-diet model allows the investigation of the effects of novel compounds on alcohol withdrawal-induced deficits in brain reward function and anxiety-like behavior in the same subset of animals. Compounds that attenuate the negative affective state associated with acute and protracted alcohol withdrawal may decrease alcohol craving and thereby improve relapse rates in alcohol-addicted patients.

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