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Supplementing the Liquid Alcohol Diet with Chow Enhances Alcohol Intake in C57 BL/6 Mice

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Abstract

Background—Administration of alcohol-containing liquid diet is associated with body weight loss in rodents.

Aim—of this study was to modify the alcohol-containing liquid diet paradigm to reduce the body weight loss in mice during the alcohol treatment period.

Methods—Two sets of animals (Chow and No Chow groups) were exposed to chronic alcohol with a step-wise increase of alcohol in the diet. One set of control and alcohol exposed animals (Chow group) received chow during alcohol treatment. Food intake and body weight was measured every 24h. Level of intoxication was determined by measuring blood alcohol levels. Alcohol dependence of mice was determined by handling-induced convulsions (HIC) scoring. Chronic alcohol-mediated effects on brain and liver were examined.

Results—Body weight loss was attenuated in chronic alcohol exposed mice in Chow group as compared to No Chow group. Chow group mice consumed higher amounts of alcohol diet resulting in higher blood alcohol levels. Brain NMDAR1 protein levels and liver Cyp2E1 levels were significantly enhanced in chronic alcohol-exposed mice in Chow and No Chow groups suggesting that known medical consequences of alcohol are not interfered with in our modified alcohol treatment paradigm. HIC in Chow and No Chow group mice peaked between 3h and 5h after alcohol withdrawal. However, the severity of alcohol withdrawal was greater in Chow group mice.

Conclusions—Supplementing alcohol diet with chow not only attenuated body weight loss associated with alcohol intake in mice but also resulted in higher consumption of alcohol diet and higher blood alcohol levels.

Keywords

C57 BL/6 mice; chronic alcohol treatment; handling-induced convulsions; NMDA R1 subunit; Cyp 2E1 enzyme activity

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1. Introduction

Alcohol is one of the most abused drugs in the world. Several animal models including small rodents have been utilized to determine the effect of acute and chronic effects of alcohol on the function of many organ systems in the body. To administer alcohol to rodent models, a variety of methods such as feeding alcohol in a liquid diet or in agar gel diet, intragastric administration of alcohol using a feeding tube, intraperitoneal injection, or through inhalation have been used (Ponnappa and Rubin, 2000). One of the commonly used methods for rats and mice is the liquid diet method developed by DeCarli and Lieber (DeCarli and Lieber, 1967). In this method, animals are fed a nutritionally balanced liquid diet containing alcohol as their sole source of food and water (DeCarli and Lieber, 1967). Snell and colleagues (Snell et al., 1996b) modified the liquid diet method of DeCarli and Lieber for C57 BL/6 mice that are known to voluntarily consume alcohol (McClern, 1988). These investigators increased the alcohol content in the liquid diet in a step wise manner for C57 BL/6 mice (Snell et al., 1996b).

Utilizing the step-wise paradigm of alcohol administration (Snell et al., 1996b) we fed alcohol to C57 BL/6 mice in a commercially available nutritionally complete liquid diet and observed a loss of body weight during the alcohol treatment period. Although control mice receiving an isocaloric control liquid diet also experienced a reduction in body weight, the weight loss seen in alcohol mice was greater. The aim of the present study was to modify the liquid diet paradigm of alcohol administration such that the body weight loss is attenuated in alcohol mice during the alcohol treatment period. In this study, the alcohol containing liquid diet was supplemented with a known amount of regular rodent chow at a specific time during the step-wise administration of alcohol in C57 BL/6 mice. Here we demonstrate that supplementing the alcohol containing liquid diet with chow, results in attenuation of body weight loss in C57 BL/6 mice.

Supplementing the alcohol containing liquid diet with regular rodent chow did not alter known effects of chronic alcohol exposure such as alcohol-mediated increase in (i) hepatic cytochrome P450 2E1 (Cyp 2E1) enzyme activity and apoprotein levels (Forkert et al., 1991), and (ii) polypeptide levels of the NMDA R1 receptor subunit in hippocampus and cerebral cortex (Trevisan et al., 1994; Snell et al., 1996a; Kalluri et al., 1998).

2. Materials and Methods

2.1. Materials

Liquid diet was purchased from Bioserv (Frenchtown, NJ). Alcohol dehydrogenase and NAD⁺ were obtained from Roche Diagnostic Corp. (Indianapolis, IN); Hybond polyvinylidene difluoride membrane and ECL plus detection system from GE Healthcare Life Sciences formerly Amersham-Biosciences (Piscataway, NJ); NMDA R1 monoclonal antibody (NR1pan); Cyp 2E1 polyclonal antibody from Millipore Corp formerly Chemicon International (Temecula, CA) and actin antibody from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

Experiments were performed with 6-8 week old male mice (strain C57 BL/6) bred in house. Animals were housed individually under a 12h-light/12h-dark illumination cycle (lights on at 9:00 AM). Animals were used in accordance with institutional guidelines and procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University.

2.3. Chronic Alcohol treatment of Mice

The chronic alcohol treatment paradigm used in the present study was adapted from Snell et al. (1996b) and Olive et al. (2001). Animals were acclimatized for one week (chow and water *ad libitum* for 3 days, control liquid diet and chow *ad libitum* for 2 days; and control liquid diet *ad libitum* for 2 days). Following this acclimation period, animals were introduced to the alcohol containing liquid diet with a step-wise increase in alcohol: days 1 and 2, 0% alcohol; days 3 and 4, 2.3% alcohol; days 5 and 6, 4.7% alcohol and days 7-16, 7% alcohol. Mice in Group I (No Chow group) received a measured volume of alcohol containing liquid diet containing 2.3 – 7% (v/v) alcohol as their sole source of food. Mice in Group II (Chow group) received the alcohol containing liquid diet exactly as Group I plus a **restricted** amount of Purina rat chow (~ 1.0 g/day) beginning day 9 through day 16. All animals were offered their pre-weighed food (Group I - the alcohol containing liquid diet; Group II - the alcohol containing liquid diet plus chow; and Group III and Group IV – see below for control animals) at 9:00 AM every 24 hours.

The amount of alcohol containing liquid diet consumed by both groups of animals was measured every morning (9:00 AM). Control animals in Group III were pair-fed and received the same volume of control liquid diet as the alcohol-exposed mice in Group I had consumed the previous day. Control animals in Group IV were pair-fed and received the same volume of control liquid diet as the alcohol-exposed mice in Group II had consumed the previous day and the same amount of **restricted** Purina rat chow (~ 1.0 g/day) as the alcohol - treated animals. Maltose-dextrin was substituted isocalorically for alcohol. Body weights of alcohol treated and control mice were monitored daily.

The level of intoxication of mice was determined by measuring blood alcohol levels on the day of sacrifice. Venous blood was obtained from the retro-orbital sinus or trunk. Blood samples were taken at 9:00 AM on the day of sacrifice; 24 h after the animals received their last liquid diet.

Note: Alcohol treatment was staggered (24 h time difference in initiating the alcohol treatment per set of control and alcohol exposed mice) when the animal number was more than six to ensure that all animals were sacrificed (between 9:00 and 10:00 AM) 24 h after the last alcohol treatment.

2.4. Determination of Blood Alcohol Levels

The blood alcohol levels were determined enzymatically as described (Draski et al., 2001). Briefly, a 40 µl venous blood sample was mixed with 2 ml of 3.0 % (v/v) perchloric acid. Samples were centrifuged at 716 x g for 10 minutes at 4 °C in a tabletop refrigerated centrifuge (Allegra, Beckman). A known volume of the supernatant from each sample was mixed with 2 ml of the reaction mixture (0.5 M Tris.HCl buffer, pH 8.8, containing alcohol dehydrogenase and NAD⁺) and incubated for 15 min at 30 °C. The alcohol content in the sample was determined by measuring the amount of NADH⁺ produced from NAD⁺. All the samples were read in a Beckman DU 640 spectrophotometer at 340 nm and blood alcohol levels were calculated from a standard curve (0-500 mg % alcohol) which was analyzed simultaneously with the unknown samples.

2.5. Tissue Lysate Preparation

A) Preparation of Brain Lysates: Mice were sacrificed by cervical dislocation and brains were dissected on ice. Hippocampi from control or alcohol treated animals were pooled and processed together respectively. The cerebral cortex from each animal was processed individually. Appropriate brain regions were homogenized in buffer A (50 mM Tris.HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 100 µg/ml leupeptin, aprotinin, 1 mM 4-(2-aminoethyl)-

benzenesulfonyl fluoride, and 1 mM dithiothreitol). Homogenates were centrifuged at 1000 x g for 10 min at 4 °C. Pellets containing nuclei were discarded and supernatants (S1 fractions) were recovered. Protein concentrations in tissue lysates were determined by Bradford method (Bradford, 1976). Aliquots of the supernatants were stored at -80 °C until further analysis.

B) Preparation of Liver S10 Fractions: After sacrificing the animals, livers were dissected and homogenized in 50 mM Tris.HCl buffer (pH 7.5) containing 0.25 M sucrose, 25 mM KCl, and 3 mM MgCl₂. Homogenates were centrifuged at 2000 x g for 10 min at 4 °C. Pellets containing nuclei were discarded and supernatants were centrifuged at 10,000 x g for 10 min at 4 °C. Pellets containing mitochondria were discarded and supernatants (S10 fraction) were recovered. Protein concentrations in the S10 fractions were determined by Bradford method (Bradford, 1976). A known amount of protein was processed immediately to measure Cyp 2E1 enzyme activity and the rest of the sample was stored as aliquots at -80 °C until further analysis by Western blotting.

2.6. Cytochrome P450 2E1 Enzyme Assay

was performed using S10 liver fractions (n = 6-11 for each group) as described (Chang et al., 1998). Briefly, a known amount of protein from control and alcohol treated liver S10 fractions were incubated with 100 µM of *p*-nitrophenol in the presence of NADPH generating system at 37 °C in a water bath. At the end of the incubation, the enzyme reaction was stopped by the addition of trichloroacetic acid and samples were centrifuged at 10,000 x g for 5 min. A known volume of the supernatants was mixed with 2 M sodium hydroxide solution and samples were read at 535 nm in a Beckman DU640 spectrophotometer. Hydroxylation of *p*-nitrophenol (substrate) to *p*-nitrocatechol (product) in the unknown samples was calculated from a standard curve of authentic *p*-nitrocatechol (0, 1-20 nmol) which was analyzed simultaneously with the unknown samples. The Cyp 2E1 enzyme activity is expressed as nmol product formed/min/g S10 liver protein.

2.7. Western Blot Analysis

was performed as described (Kumari, 2001) to examine the effect of chronic alcohol treatment on the polypeptide levels of (i) NMDA R1 subunit of the NMDA receptor in the hippocampus and cerebral cortex and (ii) Cyp 2E1 apoprotein in liver. For NMDA R1 analysis, a known amount of tissue lysate (S1 fraction) (5 µg) from control and alcohol-exposed cerebral cortices or hippocampi (n = 6) and protein standards were separated on 7.5% SDS-PAGE. For Cyp 2E1 analysis, a known amount of S10 fraction (5 µg) from control and alcohol-exposed liver (n = 6) and protein standards were separated on 10% SDS-PAGE. Following electrophoretic transfer of proteins onto Hybond polyvinylidene difluoride membrane, membranes were incubated overnight at 4 °C with NMDA R1 antibody (NR1pan) that detects all the NMDA R1 splice variants or with Cyp 2E1 antibody. The next day, membranes were washed and incubated with an appropriate horseradish peroxidase conjugated secondary antibody. Immunoreactive bands were visualized using ECL plus detection system on PhosphorImager Storm 840 and data were analyzed using ImageQuantTL software.

2.8. Quantitation of Western Blot Results

Following visualization of immunoreactive NR1 band, PVDF membranes were stripped and re-probed with actin antibody. The relative changes in the NMDA R1 polypeptide levels were measured by quantifying the intensity of immunoreactive NMDA R1 bands using the ImageQuantTL software. To normalize the Western blot results, the density values of the immunoreactive NMDA R1 bands were divided by the density values of the immunoreactive actin bands from the corresponding gel lanes. Results are expressed as a

percent of control. Statistical analysis was performed using ANOVA and Fisher's least significant difference test.

The Cyp 2E1 apoprotein data was normalized in the same way as for immunoreactive NMDA R1 protein bands.

2.9. Convulsion Scoring

Handling-induced convulsion (HIC) scoring was performed as described (Crabbe et al., 1991). All animals (n = 5-10 for each group), both control (Groups III and IV) and alcohol-exposed (Groups I and II), were provided control liquid diet 24h after the last alcohol treatment (9:00 AM) and this time period was considered as time zero for HIC scoring. HIC scoring was performed every hour for 8 hours beginning at time zero and then at 24h after alcohol withdrawal. Animals were lifted by the tail and gently spun 180° if necessary. All animals were videotaped.

2.10. Statistical Analysis

Statistical analysis was performed using ANOVA and Fisher's Least Significant Difference test.

3. Results

3.1. Amount of alcohol containing liquid diet consumed and blood alcohol levels

The amount of alcohol containing liquid diet consumed was measured every day for mice in Groups I (No Chow group) and II (Chow group). The mean of the amount of liquid diet containing 7% alcohol consumed by mice in the two treatment groups is shown in Figure 1. For the first five days of 7% alcohol treatment, the daily alcohol consumption was comparable in Group I and Group II mice. From day 6 onwards, Group II mice (Chow group) received a **restricted** amount of rat Purina chow which the animals consumed completely. When the 7% alcohol containing liquid diet was supplemented with chow, ANOVA analysis revealed that the mean alcohol consumption was significantly higher in these animals (Group II) (Figure 1). For example, on day 7 of 7% alcohol treatment, No Chow group mice (Group I) consumed an average of 19.88 ± 1.3 g alcohol per kg body weight whereas Chow group mice (Group II) consumed an average of 27.59 ± 1 g alcohol per kg body weight.

To determine the level of intoxication in alcohol treated animals and to test whether the increased intake of alcohol containing liquid diet by animals in Group II had any effect on their blood alcohol levels, we measured blood alcohol content for mice in Groups I and II 24h after the last alcohol treatment. The blood alcohol levels were significantly higher in animals that received chow in addition to the alcohol containing liquid diet (Group I = 299.6 ± 34 ; Group II = 449 ± 44).

3.2. Effect of chronic alcohol exposure on the body weights

Control and alcohol-exposed mice in both treatment paradigms lost weight over the course of the treatment period. A comparison of body weights of the alcohol treated animals in the No Chow (Group I) versus the Chow (Group II) paradigms revealed that the loss of body weight was significantly less in chronic alcohol exposed mice in the Chow paradigm during the last four days (day 13 through day 16) of the 7% alcohol treatment (ANOVA, $p < 0.001$) (Figure 2).

3.3. Effect of chronic alcohol exposure on NMDA R1 polypeptide levels

To determine whether supplementing alcohol containing liquid diet with chow attenuates the known effects of alcohol on the body, we examined NMDA R1 receptor subunit protein levels in two brain regions, hippocampus and cerebral cortex. A known amount of tissue lysate (S1 fractions) of hippocampi and cerebral cortices from control groups (Group III and Group IV) and alcohol treated animals (Group I and Group II) was processed for Western blot analysis using NR1pan antibody. This antibody recognizes all the NMDA R1 splice variants. Quantitative analysis showed that chronic alcohol treatment of mice increased NMDA R1 polypeptide levels in the cerebral cortices of both groups of alcohol-treated mice (Groups I and II) (Figures 3 A, C) when compared to their respective controls. No significant difference was seen between the two alcohol-treated groups of mice. Similarly, NMDA R1 polypeptide levels increased in hippocampus (Figures 3 B, D) of both groups of alcohol-treated mice (Group I and II) when compared to their respective controls. No significant difference was observed between the two alcohol-treated groups of mice.

3.4. Effect of chronic alcohol exposure on liver cytochrome P450 2E1 enzyme activity and apoprotein levels

Chronic alcohol exposure *in vivo* is known to increase Cyp 2E1 enzyme activity (Forkert et al., 1991). That supplementing the alcohol containing liquid diet with chow produced similar effects on liver as has been observed previously (Forkert et al., 1991), we measured Cyp 2E1 enzyme activity using the *p*-nitrophenol method in freshly prepared S10 fractions of liver from control (Groups III and Group IV) and alcohol treated mice (Groups I and Group II). Cyp 2E1 enzyme activity is expressed as nM of *p*-nitrocatechol produced per minute per gram of protein in S10 fractions. As shown in Figure 4A, Cyp 2E1 enzyme activity in control livers of Group III was 347.33 ± 33 , and of Group IV was 504.5 ± 70.5 . By comparison, Cyp 2E1 enzyme activity in alcohol-treated livers of Group I was $1,875.66 \pm 105$ and of Group II was $3,506 \pm 203$. Although the Cyp 2E1 enzyme activity was increased in both alcohol-treated groups (Groups I and II), the increase in enzyme activity in Group II was significantly greater. The enzyme activity in two controls groups did not differ significantly from each other (Figure 4A).

The S10 fractions from control and alcohol exposed livers (Groups I-IV) were analyzed for the Cyp 2E1 apoprotein levels by Western blot analysis (Figure 4B). Quantitation of Western blot analysis showed a three fold increase in Cyp 2E1 apoprotein levels in liver extracts (S10 fractions) of both groups of alcohol-exposed mice (Groups I and II) as compared to their respective controls (Figure 4 C).

3.5. Scoring of handling-induced convulsions

Handling-induced convulsions are exhibited by mice that experience withdrawal from alcohol (Goldstein and Pal, 1971). To ensure that alcohol treated mice in Group I and Group II were dependent on alcohol we determined alcohol withdrawal severity by scoring handling-induced convulsions (HIC). Scoring of HIC convulsions was performed according to the criteria listed in Table 1 (Crabbe et al., 1991). Symptoms of alcohol withdrawal started to appear with 1h of alcohol withdrawal in Chow group mice (Group II). By comparison, No Chow mice (Group I) exhibited alcohol withdrawal symptoms only 2h after alcohol withdrawal. The time-course of withdrawal severity recorded for mice in Group I and Group II are shown in Figure 5. The severity of withdrawal increased as a function of time. The peak withdrawal severity scores were reached between 3 and 5 hours following alcohol withdrawal for mice in both alcohol treatment groups (Groups I and II). However, alcohol withdrawal severity was significantly greater in Group II mice (alcohol, chow) as compared to Group I mice (alcohol, no chow) (Figure 5). At 5 and 6 hours respectively, two animals in Group II experienced severe spontaneous seizures and died instantaneously.

4. Discussion

C57 BL/6 mice consume alcohol voluntarily (McClearn, 1988). A step wise increase in alcohol content in a balanced liquid diet is known to make C57 mice dependent on alcohol with some loss of body weight (Piano et al., 2001). While trying to attenuate this body weight loss we serendipitously discovered that C57 male mice consumed higher amounts of alcohol when a known amount of solid food was provided with the alcohol diet. Here we show that increased intake of alcohol diet resulted in high blood alcohol levels with a moderate loss of body weight. Such mice experienced handling-induced seizures within one hour of alcohol withdrawal. Interestingly, mice that received the alcohol liquid diet supplemented with chow achieved higher handling induced withdrawal scores than animals that had only been exposed to the alcohol liquid diet. We believe that this phenomenon can be explained by the fact that the chow supplemented alcohol animals consumed more of the alcohol containing liquid diet. Similar observations were made in alcoholic patients undergoing alcohol withdrawal. Patients that had consumed larger amounts of alcohol experienced more severe symptoms of alcohol withdrawal (Saitz, 1998).

During our initial experiments, we observed that alcohol treated animals experienced a greater loss of weight than their pair-fed controls. Necropsy of the alcohol-fed mice on the day of sacrifice revealed that the animals had experienced intestinal hemorrhage during the alcohol treatment phase. Consumption of alcohol is known to result in gastric hemorrhagic lesions as well as mucosal injury to the small intestine (Bhandare et al., 1990; Bode and Bode, 2003). However supplementing the alcohol containing liquid diet with chow abolished the intestinal hemorrhage. It is likely that the presence of chow alleviated some of the mucosal injury of the gastrointestinal tract that occurs with chronic alcohol consumption.

By providing a small **restricted** amount of chow simultaneously with the alcohol containing liquid diet we observed that the weight loss of alcohol-exposed mice was attenuated. However, the loss of body weight in the pair-fed control mice was less than that seen in mice receiving the alcohol containing liquid diet (Group I). This phenomenon of 'energy wastage' is believed to develop, at least in part, as a result of hepatic induction of Cyp 2E1 (Lieber, 1991). Body weight loss can affect the physiological function of many organ systems (Piano et al., 2001) and may introduce non-experimental variables. In our chow and alcohol diet paradigm the weight loss experienced by chronic alcohol exposed animals (Group II) mirrors more closely the weight loss observed in control animals (Group IV) and may therefore attenuate potential experimental confounds arising from differential weight loss between treatment groups.

Surprisingly, the daily consumption of alcohol containing liquid diet was significantly increased in mice when chow was provided with the alcohol containing liquid diet (Figure 1). This increase in alcohol intake was reflected by an increase in blood alcohol levels (449 ± 44 mg% in Group II mice as compared to 299.6 ± 34 mg% in Group I mice).

At this time it is not clear why animals in Group II consumed more of the alcohol containing liquid diet as compared to the animals in Group I. We speculate that perhaps the consumption of solid food (i.e. chow) increased the animal's need for fluid. Since the alcohol containing liquid diet is the only source of liquid for the animals they consumed more of it. Alternatively, perhaps the presence of food slowed the absorption of alcohol, and as a result the animals consumed more alcohol. In light of this hypothesis it is interesting to note that free access to water during chronic administration of alcohol containing liquid diet to Long-Evans rats has no effect on the consumption of the alcohol containing liquid diet (de Fiebre and de Fiebre, 2003).

Regardless of the underlying mechanism(s), it is important to note that supplementing alcohol containing liquid diet with **a restricted amount** of rodent chow enhances the daily consumption of alcohol containing liquid diet in mice

We also performed a number of experiments to confirm the medical consequences of chronic alcohol exposure in our alcohol/chow treatment paradigm. Chronic alcohol treatment *in vivo* is known to up regulate NMDA receptor number with a concomitant increase in NMDA R1 polypeptide levels in several different regions of the brain (Trevisan et al. 1994; Snell et al., 1996a; Kalluri et al., 1998). Therefore we examined, by Western blotting, the effect of chronic alcohol exposure on the NMDA R1 polypeptide levels in hippocampus and cerebral cortex of mice. Our Western data showed a statistically significant increase in NMDA R1 polypeptide levels in hippocampus and cerebral cortex of both alcohol-exposed mice (Groups I and II) as compared to their respective controls (Groups III and IV). This data confirms the previously reported alcohol-mediated up regulation of NMDA R1 polypeptide levels in hippocampus and cerebral cortex of mouse. Also, this data demonstrated that by providing chow with alcohol does not interfere with alcohol's effects on the brain.

To assess the effect(s) of chronic alcohol exposure on an organ other than brain we chose to examine liver function, specifically Cyp 2E1 enzyme activity and Cyp 2E1 apoprotein levels. Our data demonstrated a several fold increase in Cyp 2E1 enzyme activity (Figure 4A) and Cyp 2E1 apoprotein levels (Figure 4C) in the livers of both alcohol-exposed mice (Groups I and II as compared to their respective control groups (Groups III and IV). These results are in agreement with the observations of Forkert and colleagues who reported chronic alcohol-mediated induction of Cyp 2E1 in mice (Forkert et al., 1991). Although Cyp 2E1 apoprotein levels were comparably increased in both alcohol – treated groups of animals, Cyp 2E1 enzyme activity was significantly greater in animals that were treated with alcohol and chow (Group II). Increase in enzyme activity without increase in protein levels can perhaps be explained by the fact that phosphorylation of Cyp 2E1 enzyme alters the activity of the Cyp 2E1 enzyme without altering the polypeptide content (Oesch-Bartlomowicz et al., 1998). Taken together, biochemical analyses showed that feeding a known amount regular chow with alcohol containing liquid diet does not interfere with the effects of alcohol on various organs in the body.

In summary, we have provided evidence that supplementing the alcohol containing liquid diet with some amount of rodent chow increased the consumption of the alcohol containing liquid diet without a major loss of body weight. Increased consumption of alcohol containing liquid diet when provided with chow resulted in higher blood alcohol levels. Such mice experienced handling induced seizures within 1h of alcohol withdrawal. Consumption of chow with alcohol did not interfere with some of the known effects of alcohol on different organs in mice.

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Figure 1. Mean daily alcohol consumption (g/kg body weight) of experimental Group I (No Chow group) and Group II (Chow group). Data points represent mean $\pm$ SEM ($n = 12$). From day 6 of the 7% alcohol containing liquid diet, ANOVA indicated a significant difference in the consumption of liquid diet between the two experimental (* $p < 0.001$). Arrow marks the day when Group II mice began receiving chow in addition to alcohol containing liquid diet.

Figure 2.

Comparison of body weights (% of initial body weight) of control and chronic alcohol exposed mice in No Chow group and Chow group during the alcohol treatment period. The step-wise increase in concentration of alcohol in the liquid diet is shown by solid arrows on the x-axis. The open arrow on the x-axis indicates the day when alcohol diet was supplemented with regular chow for control and alcohol exposed mice in Chow group. Data points represent mean $\pm$ SEM ($n = 8-11$ animals per treatment). ANOVA indicated that body weights of mice in two experimental groups differed significantly from day 13 of the alcohol treatment period (* $p < 0.001$).

Figure 3.

Western blot analysis of NMDA R1 (NR1) polypeptide levels in two brain regions. Cell lysates were prepared using cerebral cortices (A) and hippocampi (B) obtained from control and alcohol treated mice in Chow and No Chow group mice. Lysates were separated on SDS-PAGE, blotted onto PVDF membrane and probed with NR1pan antibody. Immunoreactive NR1 polypeptide bands were visualized using ECL Plus solution as described in Materials and Methods. The apparent molecular weight of NMDA R1 polypeptide is indicated on the left. To normalize the data, membranes from NR1 Western blotting were stripped and re-probed with actin antibody. Quantitation of NR1 polypeptide levels in the cerebral cortex (C) and hippocampus (D) was performed using ImageQuantTL software. Intensities of NR1 bands were normalized by dividing the density of NR1 bands with the density of actin band from the respective lane and data is expressed as percent of control (mean $\pm$ SEM of six independent experiments). Statistical analysis was performed by ANOVA and Fisher's Least Significant Difference Test, * $p < 0.0001$.

Figure 4.

Cytochrome P450 enzyme activity and Cyp 2E1 apoprotein levels.

A: Cytochrome P450 enzyme activity was determined by incubating a known amount of liver extracts (S10 fractions) with the enzyme substrate, *p*-nitrophenol in the presence of an NADPH generating system. Hydroxylation of *p*-nitrophenol to *p*-nitrocatechol (product) by Cyp 2E1 enzyme was measured by reading optical density at 535 nm. Generation of *p*-nitrocatechol in the unknown samples was calculated from a standard curve of authentic *p*-nitrocatechol (see Materials and Methods). The calculated Cyp 2E1 activity (nmol product formed/min/g S10 liver protein) was significantly up regulated in alcohol treated mice in both Chow and No Chow groups of mice. However, the calculated Cyp 2E1 activity was significantly higher in alcohol exposed mice in Chow group as compared to alcohol exposed mice in No Chow group. Data points represent mean $\pm$ SEM of six animals. Statistical analysis was performed by ANOVA and Fisher's Least Significant Difference Test (* $p < 0.0001$, ** $p < 0.001$, # $p < 0.0001$).

B: Cyp 2E1 apoprotein levels in liver extracts of control and alcohol treated mice in Chow and No Chow groups mice were determined by Western blotting using Cyp 2E1 antibody. Immunoreactive Cyp 2E1 apoprotein bands were visualized using ECL Plus detection system on PhosphorImager.

C: Quantitation of Cyp 2E1 apoprotein levels was performed using ImageQuantTL software exactly as for the NMDA R1 subunit in Figure 3. Data points represent mean $\pm$ SEM of 6-11 animals. Statistical analysis (ANOVA and Fisher's Least Significant Difference Test) showed a three fold increase in Cyp 2E1 apoprotein levels in alcohol exposed mice as compared to their respective controls (* $p < 0.01$, ** $p < 0.05$).

Figure 5. HIC scores during the 24 hour period following removal of alcohol-containing diet for mice exposed to chow and alcohol (Chow) and mice exposed to alcohol only (No chow). Data points represent mean $\pm$ SEM of 5-11 animals in each group.

Table 1

Handling-induced convulsion rating scale. This rating scale is adapted from Crabbe and colleagues (Crabbe et al., 1991).

Symptom	Score
Severe, tonic-clonic convulsion elicited before lifting by the tail, with quick onset and long duration: spontaneous, or elicited by mild environmental stimulus, such as lifting the cage	7
Severe, tonic-clonic convulsion when lifted by the tail, with quick onset and long duration, often continuing for several seconds after the mouse is released	6
Tonic-clonic convulsion when lifted by the tail, often with onset delayed by as much as 1 to 2 s	5
Tonic convulsion when lifted by the tail	4
Tonic convulsion after gentle 180° spin	3
No convulsion when lifted by the tail, but tonic convulsion elicited by gentle 180° spin	2
Only facial grimace after gentle 180°	1
No convulsion	0