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Repeated moderate-dose ethanol bouts impair cognitive function in Wistar rats

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Abstract

The effects of repeated, intermittent administration of a moderate dose of ethanol (3.4 g/kg/day × 6 days, intragastrically via gavages) on cognitive function were examined in male Wistar rats. No significant differences in weight gain between the ethanol- and water-treated rats were found. Analysis of physical dependence revealed no signs of spontaneous withdrawal, whereas withdrawal signs exacerbated by Ro15-4513, an inverse benzodiazepine agonist, were apparent 5 hours but not 24 hours after the cessation of ethanol treatment. Spatial learning and memory, as assessed in the Barnes maze, were impaired 3–6 days following the treatment but recovered by the 11th–14th days. Reversal learning, however, was impaired throughout the 2-week observation period. Thus, bouts of moderate-dose ethanol administration transiently impair spatial learning and memory, and promote cognitive inflexibility. The employed ethanol exposure paradigm may provide a model of human cognitive deficits associated with alcohol binge drinking.

Keywords

Animal model; behavioral flexibility; binge drinking; cognitive impairment; ethanol; rat; spatial

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While this manuscript was in press two articles have been published by Cippitelli *et al.* demonstrating reversibility of object recognition but not spatial memory impairment following binge-like alcohol exposure in Wistar rats (a 4-day i.g. treatment with ethanol, 9–15 g/kg/day).

Cippitelli A, Damadzic R, Frankola K, Goldstein A, Thorsell A, Singley E, Eskay RL, Heilig M (2010) Alcohol-induced neurodegeneration, suppression of transforming growth factor-beta, and cognitive impairment in rats: prevention by group II metabotropic glutamate receptor activation. *Biol Psychiatry* 67:823–30.

Cippitelli A, Zook M, Bell L, Damadzic R, Eskay RL, Schwandt M, Heilig M (2010) Reversibility of object recognition but not spatial memory impairment following binge-like alcohol exposure in rats. *Neurobiol Learn Mem* 94:38–46.

Authors Contribution

AK and GB designed and directed the study. AK performed behavioural experiments. TS, VC and JM performed experiments with ethanol kinetics. AK, SL, TS and GB analyzed and discussed data. AK, TS, VC and GB wrote the paper and SL and TS edited the paper.

INTRODUCTION

In many societies, ethanol consumption is characterized by binge drinking. This consumption pattern can lead to transitory high blood ethanol concentrations (BECs) that may have both behavioral and cognitive consequences. Abstract thinking, cognitive flexibility and inhibition of competing responses are impaired after binge alcohol consumption (Leber, Jenkins & Parsons 1981; Tarter & Edwards 1986; Oscar-Berman, Hutner & Bonner 1992; Parsons & Nixon 1998). Binge drinking has also been proposed as a risk factor for the development of dementia (Brown *et al.* 2000; Mukamal *et al.* 2003; Järvenpää *et al.* 2005; Stephens & Duka 2008). Furthermore, it has been suggested that alcohol-induced deficits in cognition may be a critical factor underlying the habitual drug seeking and taking that characterizes addiction and dependence (Crews & Boettiger 2009; Robbins & Arnsten 2009).

There are several definitions for binge drinking, including those which rely on (1) quantity of consumption (five alcoholic drinks for men, four for women) in about 2 hours for a typical adult (NIAAA 2004); or (2) blood alcohol concentration; thus, the NIAAA recently redefined the term 'binge drinking' as anytime one reaches a peak blood alcohol concentration of 0.08% or higher (Quick Stats: Binge Drinking 2008; <http://pubs.niaaa.nih.gov>). Whatever a definition used, the consumption of substantial amounts of alcohol within a limited time is often implied when the term is used colloquially (Stephens & Duka 2008).

Behavioral and neuropathological changes that are observed in human binge drinkers (e.g. physical withdrawal, neurotoxicity, and impairment of learning and memory) have been modeled in experimental animals (Obernier *et al.* 2002; Mitirattanakul *et al.* 2007; Faingold 2008; Stephens & Duka 2008). Studies examining ethanol-evoked alterations in learning and memory in rodents have employed high doses of ethanol administered either intragastrically over a period of days (from 7.6 g/kg/day to 15 g/kg/day) (Majchrowicz 1975; Obernier *et al.* 2002; Faingold 2008) or via a liquid diet for periods of weeks or months interrupted by withdrawal periods (Stephens *et al.* 2001). In these experiments, animals demonstrated deficits in learning and memory, and had BECs from 255 mg/dl to 400 mg/dl (Obernier *et al.* 2002; Faingold 2008). Although high BECs, above 250 mg/dl, were detected in human heavy drinkers (Bedford, O'Farrell & Howell 2006; Jones 2008; Lamminpää & Hopppu 2009), binge drinkers generally exhibit lower BECs (150–170 mg/dl; Jones 2008).

The aims of the present study were to examine if repeated intermittent exposure of rats to a dose of ethanol that produce BECs similar to those produced by binge ethanol intoxications in humans (i.e. between 80 mg/dl and 200 mg/dl) leads to a cognitive impairment. Ethanol (3.4 g/kg) was administered once daily by gavage for 6 days at the dose that was substantially lower than that used in the previous studies (from 7.6 g/kg/day to 15 g/kg/day; Majchrowicz 1975; Obernier *et al.* 2002; Faingold 2008). Gavage, rather than inhalation, was chosen for these studies to minimize physical dependence and the irritant effects of alcohol vapor, both of which can influence behavior. Spatial learning and memory were analyzed in the Barnes maze (BM) task (Barnes 1979; McLay *et al.* 1999). Previous works have demonstrated that performance in this task is hippocampally dependent (Williams *et al.* 2003; Seeger *et al.* 2004). Because clinical studies have shown that cognitive performance improves during the course of abstinence (Brandt *et al.* 1983; Loeber *et al.* 2009), performance was analyzed at two time points: 3–6 days and 11–14 days after the cessation of ethanol exposure.

MATERIALS AND METHODS

Animals

Male Wistar rats (B & K Universal, Sollentuna, Sweden), 14 weeks–16 weeks old and weighing 350–420 g at the beginning of the experiment were habituated for at least 7 days before initiation of experiments. Rats were housed four per cage in a temperature- and humidity-controlled room (19°C and 40–50% humidity), with food and water available *ad libitum* and a 12-hour light/dark cycle (lights on at 8:00 a.m.). All experiments were carried out between 10:00 a.m. and 3:00 p.m. Animal housing and the experimental procedures followed the relevant provisions and general recommendations of the Swedish animal protection legislation. All experimental protocols used in this study were carried out in accordance with the Swedish Legislation on Animal Experimentation (The Animal Welfare Act SFS 1998:56) and the European Union Legislation (Convention ETS 123 and Directive 86/609/EEC), and were approved by the local Ethical Committee for Use of Animal Subjects at the Karolinska Institutet. The study was designed as between-subjects (independent groups) experiment (i.e. each animal was used only once).

Ethanol treatment protocol

The protocol based on intragastric (i.g.) ethanol administration (Majchrowicz 1975; Collins, Corso & Neafsey 1996; Zou *et al.* 1996) was modified and used in the study. Rats received ethanol (40% v/v; 10 ml/kg; 3.4 g/kg) or tap water once per day for 6 days i.g. through gavages. The concentration of ethanol used is equivalent to that in the most prevalent type of alcoholic beverage (liquors, vodka, whisky) consumed by binge drinkers (MMWR Morb Mortal Wkly Rep 2007; Popova *et al.* 2007).

Determination of blood ethanol levels (BECs)

Rats were given a single i.g. water or ethanol (40% v/v; 10 ml/kg; 3.4 g/kg) infusion (acute group; $n = 8$), or treated as described above (binge group; $n = 8$). Blood samples (40 μ l) were taken from the tail vein 30, 60, 120, 240, 480 minutes and 48 hours after last ethanol administration, precipitated in 3% perchloric acid (200 μ l final volume), and stored at 4°C until analysis. Ethanol was quantified by the alcohol dehydrogenase assay. Samples (20 μ l) were incubated in duplicate in 1 ml of 0.5 M Tris-Cl buffer (pH 8.8) containing 5.5 μ g/ml of alcohol dehydrogenase and 1.5 mM β -nicotinamide adenine dinucleotide (β -NAD) for 40 minutes at room temperature. Accumulation of β -NADH was measured by reading sample absorbance at 340 nm. The ethanol concentration in the samples was estimated using a standard calibration curve.

Effect of ethanol treatment on body weight

Body weights were evaluated immediately before the first ethanol/water treatment, immediately before the last treatment and 1 week after the last treatment. Repeated measures analysis of variance (ANOVA) was used to determine differences in weight gain between treatment groups.

Ethanol dependence and withdrawal

To determine if the ethanol treatment regimen induced physical dependence, locomotor activity and audiogenic seizures following administration of an inverse benzodiazepine agonist, Ro15-4513 [ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-1,4-benzodiazepine-3-carboxylate] was assessed. This compound can exacerbate ethanol withdrawal as evidenced by the induction of audiogenic seizures, increased locomotor activity and anxiogenesis in ethanol-dependent rats (Mhatre & Gonzalez 1999). Ro15-4513 (5.0 mg/kg; intraperitoneal (i.p.); RBI, Natick, MA) was dissolved in dimethyl sulfoxide and

diluted 1 : 1 with saline. Rats were treated with ethanol (groups 1 and 2) or water (groups 3 and 4) for 6 days. They were placed in locomotor activity cages 5 hours (groups 1 and 3) or 24 hours (groups 2 and 4) after the last treatment. Locomotor activity (horizontal activity counts), number of stereotypic movements and central time (time spent in the central zone: defined as area with a 10 cm or longer distance between the geometrical center of the rat and the walls) were monitored for 9-minute and for 3-minute intervals after each tone. Immediately after the initial 9 minutes of recording, rats were injected with Ro15-4513 or vehicle i.p., and returned to the activity boxes for 15 minutes. A loud tone (20 dB frequency) was introduced for 5 seconds twice, 5 and 10 minutes after injection, and the behavior was recorded for 3-minute intervals after each tone.

In addition to above-described activity measures, behavior of animals was rated by a trained observer as follows: 0—no response, 1—muscle tremor, 2—abnormal hind-limb posture, 3—mild limb extension, 4—severe limb extension with severe tremor and 5—tonic-clonic convulsion. Differences in drug-induced seizure scores and locomotion were tested for significance using non-parametric contingency table tests for categorical data (Fisher exact test) and the parametric Student *t*-test for numerical data.

Evaluation of learning and memory function in the BM task

The effects of ethanol administration on learning and memory were assessed in the BM task using four groups of rats ($n = 8/\text{group}$). Two groups were treated with water and two with ethanol. Behavioral testing of two groups was initiated on day 3 after the treatment with water or ethanol (experiment 1), and two remaining groups on day 11 (experiment 2). Rodents with hippocampal damage showed impaired performance in the BM (McLay, Freeman & Zadina 1998; Williams *et al.* 2003). In this task, animals escaped from a brightly lit circular open platform (1.5 m diameter) surface to a small dark recessed chamber located under 1 of the 12 holes around the perimeter of the platform. The upper surface of the maze was covered with white glacial polyvinyl chloride. Testing was conducted under intense illumination (6 · 32 W diffuse overhead fluorescent lighting and 4 · 125 W flood lamp, 1.5 m above the maze). Abundant visual cues (posters, etc.) were placed on the walls of the testing room in 1 m–2 m distance from the edge of the maze. The maze was raised 1 m from the floor and rested on a pedestal that can be rotated on a horizontal plane. Spatial reference points were fixed in relation to the escape hole. Data were acquired using a TSE digital analysis system linked to video camera and PC-compatible software (TSE GmbH, Homburg, Germany).

Three training blocks, each consisting of three trials separated by a 10-minute intertrial interval, were run on subsequent days during the acquisition phase. At the start of each trial, the animal was gently released in the center of the maze. The direction that the animal initially faced varied randomly. On each trial, 11 of the 12 holes provided no escape, whereas the remaining hole provided access to the escape box, which was positioned on the underside of the maze. From the center of the maze, the rat could not visually distinguish the destination hole from the other 11 holes. Each rat was permitted to explore the maze freely for 180 seconds. After the rat entered the escape box, the hole was covered to prevent it from resurfacing onto the maze. The rat was left in the escape box for 30 seconds before being returned to its home cage. If the rat did not enter the escape box within 180 seconds, it was gently picked up by the experimenter and placed over the target hole, and allowed to enter the escape box. The hole was then covered for 30 seconds before the subject was returned to its home cage. The maze and escape box were cleaned carefully with a 10% (v/v) alcohol solution to dissipate odor cues and provide a standard olfactory context for each trial. A single 30-second probe trial, in which the latency to reach the virtual target hole was measured, was carried out 24 hours after the last learning trial (i.e. day 4). In addition, preference for the target hole was assessed by determining the number of mistaken pokes in

each hole. One hour after completion of the probe trial, three 180-second reversal learning trials were conducted. Reversal learning trials were identical to the training trials except that the position of the escape hole was rotated 180°. The rat was therefore unable to escape the maze using acquired spatial cues but had to relearn the new location of the hole.

For each day, data obtained at three daily learning trails as well as at reversal learning trials were separately pooled together and used for calculations of latency and number of wrong hole pokes. Changes in latencies and error rates were determined across sessions. Data were analyzed using a repeated measures ANOVA, followed by *post hoc* tests when appropriate.

RESULTS

BECs and body weight

BECs were analyzed in two groups of rats: (1) in the acutely treated naive rats; and (2) in rats exposed to ethanol for 6 days after the last ethanol treatment. BECs reached the peak values of 112 ± 27 mg/dl and 153 ± 27 mg/dl for acute and binge group, respectively, in 3–4 hours and then decreased to the basal levels by 8 hours in both groups of animals (Fig. 1a). BECs kinetic did not differ significantly between the two groups [$F(1, 6) = 0.94$; $P = 0.37$].

No significant differences in weight gain between the ethanol- and water-treated rats [repeated measures ANOVA, treatment–time interaction: $F(2, 56) = 1.4$; $P = 0.41$; Fig. 1b] were evident. All rats exhibited an increase in bodyweight during the 6-day treatment and the subsequent 7-day monitoring period. Total bodyweight gain for the 13-day period was 75 g (ethanol) and 60 g (water), respectively. No signs of inflammatory reaction on the lining of the stomach examined visually were evident.

Evaluation of physical dependence

Five hours following the last gavage, 6 of 11 ethanol-exposed rats and none of the water-exposed rats exhibited tone-induced seizures during the 15-minute period following the Ro15-4513 administration. Analysis of seizure intensity demonstrated that administration of Ro15-4513 resulted in virtually no behavioral responses in the water-treated animals; mean seizure intensity score $\pm$ standard error of the mean (SEM) was 0.3 ± 0.2 . In the ethanol-treated animals, Ro15-4513 induced muscle tremor, hind-limb extension and mild tonic–clonic convulsions of short duration (mean seizure intensity score $\pm$ SEM was 2.2 ± 0.4). The water- and ethanol-treated groups differed significantly in the observed seizure ratings ($P < 0.05$, Fisher exact test). No Ro15-4523-exacerbated audiogenic seizures were observed in rats 24 hours after the last ethanol or water administration, and only one of eight ethanol-treated rats exhibited seizures at a low score (0.9 ± 0.4).

Analysis of horizontal activity and time spent in the central zone of the arena 5 and 24 hours after the last ethanol or water infusion are presented in Fig. 2. At the 5-hour time point, ethanol-exposed rats displayed significantly lower basal horizontal activity compared with the water-treated animals ($P < 0.05$, *t*-test). However, after administration of Ro15-4513, ethanol-exposed rats responded to the tone stimulation with higher activity. These rats demonstrated higher levels of both spontaneous and Ro15-4513-precipitated anxiety-like behavior manifested as decreases in time spent in the central zone. Ethanol- and water-exposed rats displayed no significant differences in basal or Ro15-4513-evoked horizontal activity, at the 24 hours time point. Similarly, there was no difference between groups in time spent in the central zone.

Evaluation of cognitive function

In the first experiment, spatial learning and memory in the BM task were evaluated 3–6 days after the last ethanol or water exposure ($n = 8$ for each group) with three training trials conducted per day (Fig 3a–c).

Escape latencies declined over the three training days in both groups [time effect $F(2, 24) = 5.48$, $P = 0.01$]. Repeated measures ANOVA revealed a significant time–treatment interaction, $F(2, 24) = 3.08$, $P = 0.035$, in escape latencies (Fig. 3a). *Post hoc* analyses revealed significant differences between treatment groups on the second and third training days ($P = 0.009$ and $P = 0.006$, respectively). Repeated measures ANOVA analysis of hole poke behavior revealed a significant treatment–time interaction [$F(2, 24) = 3.8$, $P = 0.04$] but no time effect ($P = 0.69$) (Fig. 3b). Also, as above, differences between the two groups were significant on the second and third day ($P = 0.01$ and $P = 0.002$, respectively).

A probe test, in which the target hole was disconnected from the escape chamber, was conducted 24 hours after the last learning trial. This test revealed significantly longer latencies ($P = 0.0006$) and increased error poking ($P = 0.008$) in the ethanol-treated group. Reversal learning trials were performed after the probe test; longer latencies ($P = 0.01$) and increased error poking ($P = 0.003$) were found in the ethanol-treated group (Fig 3a,b).

In the second experiment, two new groups of rats ($n = 8$ /group) were analyzed in the BM task 11–14 days after the last exposure to water or ethanol (Fig 3d–f). Escape latencies declined over the 3 days of training [$F(2,28) = 13.8$, $P = 0.006$] with no apparent differences between the water- and ethanol-treated groups (treatment–time interaction: NS). The number of error pokes did not decrease during the 3 days of training (time effect, NS), and no differences between the ethanol- and water-treated rats (treatment $\times$ time: $P = 0.65$) (Fig. 3e) were found. Analysis of individual trials on day 1 showed that this group of animals learned quickly the task, with the decrease in the number of hole pokes from 5.8 ± 0.7 in the first trial to 2.5 ± 0.2 in the third trial on the first day of learning; probe test also revealed no differences in spatial memory between treatment groups.

Reversal learning trials showed robust differences between the ethanol- and water-treated rats; longer latencies ($P = 0.01$) and increased error poking ($P = 0.01$) were found in the ethanol-treated animals compared with the control rats (Fig 3c,d).

DISCUSSION

The principal findings of the present study are that repeated, intermittent exposure to a moderate dose of ethanol results in (1) the transient impairment of spatial learning and memory, and (2) a prolonged deficit in reversal learning. The ethanol-treated rats displayed deficits in spatial learning and memory, revealed in learning trials and probe test, respectively, within the first week after the cessation of ethanol, whereas impairments in reversal learning were observed for at least 2 weeks after the last ethanol exposure. Recovery of spatial learning is consistent with the previous BM study (Borlikova, Elbers & Stephens 2006) that demonstrated no spatial impairment in rats in 1 month after repeated ethanol withdrawals.

Reversal learning is a type of discrimination learning that measures cognitive flexibility (Stalnaker *et al.* 2009). Our data extend the observations of Oboernier *et al.* (2002), who described impairment of reversal learning in the water maze following high-dose ethanol (7.6 g/kg/day $\times$ 4 days) administration. In that study, however, spatial learning and memory were not affected, at least when measured during the second week of withdrawal. Impairment of reversal learning has been interpreted as perseveration of behavior, i.e. the

rats repeatedly return to the quadrant, which had been associated with successful completion of the task in the prior learning trials (Obernier *et al.* 2002). The reversal learning task in the BM is not a classical test of reversal learning and perseveration. However, the greater number of entries and increased amount of time spent in the original escape zone demonstrated by the ethanol-treated animals indicate perseverative responses toward the original escape direction. This behavior resembles the deficits in executive functioning that are often manifested by alcoholics. This type of deficit in response to inhibition is considered as a component of alcohol addiction in humans related to their difficulties to change behavior even though alcohol use is associated with negative consequences (Bowden *et al.* 2001; Crews & Boettiger 2009). The observed spatial learning deficit might be attributed to impaired hippocampal function (Sutherland & McDonald 1990) and is consistent with impairment of hippocampal long-term potentiation observed in rats following prolonged high-dose ethanol exposure in combination with intermittent periods of withdrawal (Stephens & Duka 2008). In contrast, deficits in reversal learning may relate to a dysfunction in the frontal cortex (Crews & Boettiger 2009). Such dysfunction may be relevant for addictive behavior, and altered neurotransmission in the frontal cortical areas may underlie both impaired cognitive function and aggravated ethanol-seeking behavior. At this moment, we can not rule out that ethanol-induced alterations in emotionality or motivation may contribute to changes in BM behavior. It would be unlikely, however, that such alterations differentially affect spatial learning and reverse learning.

Repeated infusions of ethanol can rapidly produce physical dependence in rodents that is proportional to the duration of exposure and dose of ethanol administered (Wallgren, Kosunen & Ahtee 1973; Majchrowicz 1975; Sircar & Sircar 2005). Repeated cycles of ethanol intoxication and withdrawal result in audiogenic seizures (for review, see Faingold 2008) and increased sensitivity to the effects of the GABA-A receptor inverse agonist Ro15-4513 in dependent animals (Mehta & Ticku 1989; Mhatre & Gonzalez 1999). In our experiments, no signs of spontaneous withdrawal and withdrawal signs triggered by Ro15-4513 were no longer apparent 24 hours after the cessation of ethanol treatment, e.g. 24 hours prior to the initiation of BM training and other behavioral testing. Therefore, it is unlikely that physical withdrawal can account for the cognitive deficits observed in the present study. However, the ethanol-treated rats may bear some aspects of ethanol dependence that were not detected by the presented behavioral techniques.

In summary, the present study provides evidence that repeated intermittent exposure of rodents to a moderate ethanol dose causes cognitive deficits. The employed ethanol exposure paradigm produces BECs documented in inebriated drivers apprehended in Scandinavia and, hence, may provide a model of human binge drinking. Impairment of spatial learning/memory and reversal learning in rats, which are thought to result from functional aberrations in hippocampal/cortical neurotransmission, may model, respectively, transient and long-lasting cognitive impairment in human binge alcohol drinkers. The present findings have important implications for understanding of the sequelae of binge alcohol consumption. They also highlight the importance of studies assessing (1) the mechanisms by which repeated ethanol binges affect cognitive functions; and (2) a role of impairments in cognitive flexibility by moderate ethanol doses in predisposition toward alcohol abuse.

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All the funding sources mentioned above had no involvement in the study design, in the collection, analysis and interpretation of data, or in the decision to submit the manuscript.

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Figure 1.

(a) Blood ethanol concentrations (BECs) and weight gain in rats treated with water or ethanol. BECs [mean $\pm$ standard error of the mean (SEM)] were measured either after acute intragastric (i.g.) ethanol (EtOH) administration or after the six repeated i.g. ethanol infusions ($n = 8$ animals/group). (b) Weight gain in rats treated with water or ethanol for 6 days. Rats' body weight was measured prior to the first (1) and last (2) administration of water or ethanol, and 1 week after the last treatment (test 3). Body weight (g) is shown as mean $\pm$ SEM ($n = 8$)

5 hours after last treatment

24 hours after last treatment

□ Water ■ EtOH

Figure 2.

Evaluation of physical dependence. Data on horizontal locomotor activity (a,b) (activity counts) and central time (c,d) (time in seconds spent in the central area) are shown. $n = 6$ for both groups of water-treated animals, and $n = 11$ and $n = 8$ for ethanol (EtOH)-treated animals. The rats were treated with water or EtOH for 6 days and then analyzed in 5 hours and 24 hours. * $P < 0.05$ (Student t -test)

Figure 3.

Evaluation of learning and memory function by the Barnes maze task. Data on escape latencies (a,d) and a number of wrong hole pokes (b,e) recorded at the three learning days (D1, D2, D3), retention trial (Ret) and reversal learning day (Rev) are shown as mean $\pm$ standard error of the mean of the three daily trials ($n = 8/\text{group}$). $*P < 0.05$ (Student t -test). (c,f) Typical tracks from the water- and ethanol (EtOH)-treated rats recorded at the last learning trial and last reversal learning trial. Data were collected on the first (c) or second (d) week after treatment