Repeated withdrawal from ethanol spares contextual fear conditioning and spatial learning but impairs negative patterning and induces over-responding: evidence for effect on frontal cortical but not hippocampal function?

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Abstract
Repeated exposure of rats to withdrawal from chronic ethanol reduces hippocampal long-term potentiation and gives rise to epileptiform-like activity in hippocampus. We investigated whether such withdrawal experience also affects learning in tasks thought to be sensitive to hippocampal damage. Rats fed an ethanol-containing diet for 24 days with two intermediate 3-day withdrawal episodes, resulting in intakes of 13–14 g/kg ethanol per day, showed impaired negative patterning discrimination compared with controls and animals that had continuous 24-day ethanol treatment, but did not differ from these animals in the degree of contextual freezing 24 h after training or in spatial learning in the Barnes maze. Repeatedly withdrawn animals also showed increased numbers of responses in the period immediately before reinforcement became available in an operant task employing a fixed-interval schedule although overall temporal organization of responding was unimpaired. Thus, in our model of repeated withdrawal from ethanol, previously observed changes in hippocampal function did not manifest at the behavioural level in the tests employed. The deficit seen after repeated withdrawal in the negative patterning discrimination and over-responding in the fixed-interval paradigm might be related to the changes in the functioning of the cortex after withdrawal.

Introduction
Repeated withdrawal from ethanol (RWD) reduces long-term potentiation (LTP) in lateral amygdala and hippocampus when tested 2 weeks postwithdrawal (Stephens et al., 2005). These findings add to the well established ability of repeated ethanol withdrawal to increase seizure susceptibility (Brown et al., 1988; Becker & Hale, 1993; Mhatre & Gonzalez, 1999; Stephens et al., 2001; Veatch & Becker, 2002) and to impair fear conditioning (Stephens et al., 2001; Ripley et al., 2003a; Ripley et al., 2003b; Stephens et al., 2005) in both binge drinkers and rodent models, deficits which, at least in the rat model, last for several weeks after the withdrawal experience. Electrophysiological studies indicate that electrical activity in the hippocampus changes with the number of withdrawals, giving rise to epileptiform-like activity that lasts up to 24 h postwithdrawal and then declines (Veatch & Becker, 2002; Duka et al., 2004), and previous withdrawal experience increases the ability of acute withdrawal (8 h) to induce c-fos expression in several limbic structures including the hippocampus (Borlikova et al., 2006). On the basis of these findings, it seems plausible that these changes in hippocampal function after repeated ethanol withdrawal might be paralleled by deficits in aspects of learning and memory mediated by the hippocampus. Data from human studies support this assumption. Thus, binge drinkers have impaired performance in selective cognitive tasks, including decreased spatial working memory (Weissenborn & Duka, 2003; Townshend & Duka, 2005), and parallels have been drawn between binge drinking in humans and the RWD model in rodents (Duka et al., 2004; Stephens et al., 2005; Townshend & Duka, 2005). Therefore it was of interest to test for potential learning deficits in RWD rats using tasks thought to be sensitive to hippocampal damage.

One such task is configural learning (Rudy & Sutherland, 1989). According to Rudy and Sutherland, some discrimination problems cannot be solved without construction of conjunctive representations of stimuli. They proposed the negative patterning discrimination problem as a task that requires such conjunctive representations to be formed. In the negative patterning task (Woodbury, 1943; Rescorla, 1972) the subject is rewarded for responding when either stimulus A or B occurs alone but is not rewarded for responding if A and B are presented in compound (A+, B+, AB–). Thus the task requires that the compound AB is not treated as either of its components. It is a property of the hippocampus that it acts to integrate information from different modalities to create complex stimuli and, in keeping with this idea, hippocampal lesions do disrupt performance of tasks based on configural cues (Rudy & Sutherland, 1989; Sutherland & McDonald, 1990; Alvarado & Rudy, 1995; Rudy & Sutherland, 1995).

Although others (Gallagher & Holland, 1992; Davidson et al., 1993; Bussey et al., 2000; Moreira & Bueno, 2003) have not found such an influence of hippocampal lesions, the inconsistency in results might arise from the different extents of hippocampal lesions and differences in the training procedures employed. Nevertheless, a more recent version of the configural association theory (Rudy & Sutherland, 1995) incorporates cortical representations of configural cues as a necessary requirement for solving configural problems.
In addition to configural learning, we investigated the consequences of RWD on the performance of a fixed-interval (FI) operant task. Hippocampal lesions disrupt the temporal patterning of responding on FI schedules of reinforcement by reducing the postreinforcement pause (Jaldow et al., 1989).

We have also studied the consequences of RWD in another task that requires information processing in the hippocampus. We have previously reported that repeated withdrawal from ethanol impairs fear conditioning in the conditioned emotional response paradigm (Stephens et al., 2001; Ripley et al., 2003a), thought to be dependent on the amygdalar processing (LeDoux, 2000). The hippocampus plays a complementary role to the amygdala in fear conditioning and, in keeping with previous outline, is essential in the conditioning of complex contextual cues (Phillips & LeDoux, 1992; Rudy et al., 2004). Therefore, it was of interest to test whether repeated experience of ethanol withdrawal would affect contextual freezing, a task widely recognized to be dependent on intact hippocampal functioning (Selden et al., 1991; Phillips & LeDoux, 1992; Fendt & Fanselow, 1999; Bannerman et al., 2001).

Finally, we tested the effect of RWD on spatial learning and memory in the Barnes maze. The Barnes circular maze is similar in concept to the Morris water maze (Morris et al., 1982; Morris, 1984), in that an animal learns over the course of several trials to use spatial cues to locate a single target at the edge of the platform (Barnes, 1979; Barnes, 1988; Barnes et al., 1989; Mclay et al., 1999). Although less well-known than the Morris water maze, the Barnes maze has been successfully used by many researchers to assess hippocampal function (Bach et al., 1995; Mclay et al., 1998; Coburn-Litvak et al., 2003; Koopmans et al., 2003; Williams et al., 2003; Seeger et al., 2004).

Methods

Animals

The subjects of negative patterning and FI experiments were 28 male Lister hooded rats (250–300 g at the beginning of the diet treatment, bred at the University of Sussex). The subjects of the contextual fear conditioning and the Barnes maze experiments were 35 male Lister hooded rats (300–350 g at the beginning of the diet treatment, bred at the University of Sussex), though only a subset took part in the fear conditioning experiment. All animals were pair-housed and maintained on a 12-h light–dark cycle with lights off at 19.00 h; the temperature was kept within the range 21 ± 2 °C and the humidity was 50 ± 10%. The rats had ad libitum access to laboratory chow (Bekay Feeds, UK) and water, unless otherwise detailed in the protocol. Where diet intakes are expressed as per body weight, the actual body weight at the time of the measure was used. All experiments were carried out under the authority of the UK Animal (Experimental Procedures) Act, 1986.

Chronic alcohol treatment

Animals were randomly allocated into three groups, single withdrawal (SWD), repeated withdrawal (RWD) and control (CON) groups. The animals in the first two groups received a nutritionally complete liquid diet (Dyets, Bethlehem, PA, USA) containing 7% ethanol as their only food source. The controls were pair-fed with a calorifically matched control diet. In each experiment there was no initial difference in the body weight between the three groups and the pair-feeding system maintained body weights at a similar level. Animals in the RWD group were treated for 30 days. At 08.00 h on days 11 and 21 the ethanol-containing liquid diet was removed and animals were food-deprived for the following 8 h, although water was available ad libitum. For the following 3 days animals were fed with the control liquid diet. The amount of the control diet was restricted to the mean amount of the ethanol-containing diet that the animals had eaten over the preceding 3 days. The SWD group remained on ethanol-containing diet for 24 consecutive days. All animals were given fresh diet daily. Ethanol consumption was calculated as g of ethanol consumed per kg body weight.

On the final day, all animals were withdrawn from the liquid diet at 08.00 h and remained in their home cages with ad libitum access to water but no food for 8 h. Following this withdrawal period the animals were allowed free access to rodent chow until behavioural training began.

Negative patterning task

The apparatus included eight operant boxes housed in sound-attenuating chambers (Coulbourn Instruments, PA, USA). Each box was fitted with two retractable levers (Medical Associates Inc., Georgia, VT, USA) situated either side of a central magazine, connected with a pellet dispenser. Forty-five-milligram food pellets (Formula P purified rodent diet; Noyes, NJ, USA) served as reinforcement. Only one lever was used during the experiment for any given rat. The experimental boxes were fitted with a speaker located above the magazine and cue lights positioned above the levers. The floor of the chamber consisted of stainless steel rods. The boxes were illuminated for the duration of the session by a white light located above the magazine (housesight). Experimental contingencies and recording of behaviour were controlled by a computer running MedPC software through Medical Associates interfaces.

Acquisition of conditioned response

On day 10 after the final withdrawal animals (n = 8–10 per experimental group) were placed on a food-restriction (16 g/rat/day) schedule and were maintained on this schedule throughout the whole experiment. This amount of food was sufficient to maintain weights at ~85% of their free-feeding weight.

Two weeks after the final withdrawal, behavioural training began. All animals were randomly assigned to work with the left or right lever (counterbalanced) throughout the experiment. Initially animals underwent four sessions of lever-press shaping until they were obtaining a maximum of 50 pellet deliveries available on a fixed ratio (FR; one lever press providing one pellet) schedule. Following shaping, animals were subjected to training in 60-min sessions on a variable interval (VI) schedule for three consecutive days with VI 10, 20 and 20 s.

In the next stage of training, each session consisted of 50 trials with variable (40–80 s) intertrial intervals. During each trial a conditioned stimulus (CS; either light or tone) was presented for 15 s maximum; the first lever press after stimulus initiation terminated the CS and was immediately followed by delivery of a food pellet. Twenty-five light (located above the active lever) and 25 tone CS presentations were randomly mixed during each session.

On completion of the simple stimulus training, the compound stimulus was introduced. Now a session consisted of intermixed presentations of either light or tone simple CSs or the compound CS (simultaneous presentation of light plus tone). As before, each simple CS was presented for 15 s maximum and the first lever press during a simple CS presentation was reinforced and terminated the CS. Lever presses during compound CS presentations were not reinforced and did not terminate the CS, allowing us to record the number of lever presses during 15 s of compound presentation. Animals underwent
eight daily sessions consisting of 60 trials each (10 light, 10 tone and 40 light + tone compound CSs) and were then switched to 40-trial sessions (10 light, 10 tone and 20 light + tone compound CSs) for further training. This change in the training schedule was implemented to equalize the total number of potentially reinforced and nonreinforced stimuli presentations. Data from the 22nd day of compound element training were excluded from analysis due to disruption of the experiment by unplanned external noise.

The number of reinforced simple stimulus trials and the number of lever presses during compound stimulus trials, as well as the number of lever presses during intertrial intervals, were registered.

**Extinction of conditioned lever presses**

A test session consisting of 50 trials was given to test the ability of the animals from different treatment groups to extinguish conditioned lever pressing. In this session, the first 20 trials included randomly intermixed presentation of 10 light and 10 tone stimuli; the first lever press during these CS presentations was immediately reinforced and terminated the stimulus; the subsequent 30 trials (15 light and 15 tone mixed) were extinction trials, in which all task parameters were the same with the exception that no food pellets were delivered. Data on the number of trials with the lever presses were collected in bins of 10 trials.

**FI operant task**

After completion of the negative patterning experiment, animals were trained on an FI task. This experiment was run in the same behavioural apparatus as the negative patterning task. On the FI schedule, the first lever-press response of the session was rewarded, and this response started the first 10-s interval. The rats received the next reward for the first lever press after 10 s had elapsed since the last rewarded response, and each rewarded response reset the interval. Responses occurring before the interval had elapsed were recorded, but had no specified consequences. The animals received 1 day of FI 10 s training, then 2 days of FI 30 s, and then 2 days of FI 60 s and, finally, FI was set up for 120 s for the remaining 18 days. Each FI 120 s session lasted until a maximum of 20 reinforcements had been earned or 60 min had elapsed, whichever came sooner.

The number and temporal pattern of lever presses were registered. The FI schedule produced a characteristic response pattern; responding accelerated throughout a response period (i.e. a ‘scalloped’ pattern of responding). To describe the temporal distribution of responses over the FI the mathematical index of curvature was used (Fry et al., 1960); for this calculation each 120-s interval was subdivided into 10 time bins of 12 s each. Running rate (presses per min) was calculated according to Jaldow et al. (1989) as the number of responses the animal made between two consecutive reinforcements divided by the actual time of the interval between these reinforcements and multiplied by 60.

**Contextual fear conditioning**

Contextual fear conditioning took place 2 weeks after the final withdrawal from the ethanol liquid diet. The rats \( (n = 6 \text{ per group}) \) had been extensively handled prior to the experiment.

The training was conducted in a clear Perspex \((36 \times 36 \text{ cm}, \text{ height } 36 \text{ cm})\) chamber. The removable floor of the chamber consisted of stainless steel rods \((0.5 \text{ cm diameter}, 1 \text{ cm apart})\). The floor was connected to Shock Source 521C and Shock Scrambler 521S (Campden Instruments Ltd, UK) operated by a computer running in-house software (courtesy of A. Mead). Both training and testing were conducted under diffuse overhead fluorescent lighting \((2 \times 32 \text{ W})\). The animal was placed in the chamber, the lid closed, and 15 s later received three foot-shocks \((0.7 \text{ mA}, 0.5 \text{ s}, \text{ with } 15-\text{s intervals}, \text{ before being removed from the chamber } 15 \text{ s later})\). The chamber was wiped with a cleaning liquid after each animal. Testing was conducted 24 h later in the same context; the animal was placed in the chamber and its behaviour was recorded for 3 min using a video camera fixed above the experimental chamber and connected to a video recorder (Sony, SLV-E280 UX, Sony Corp., Japan). The data were analysed by blinded observation of the videotapes and presented as the percentage of time spent freezing during the test session. The testing session was divided into 5-s bins and only those bins during which an animal did not show any movement except required for respiration were counted as freezing time.

**Spatial learning in the Barnes maze**

One month after the contextual fear conditioning the animals \((n = 11 \text{ or } 12 \text{ per group})\) started training in the Barnes maze. The maze consisted of a two-layer circular platform \((122 \text{ cm diameter})\) raised 60 cm above the floor. The upper layer, surfaced with white Plexiglas, had 18 holes \((9.5 \text{ cm diameter})\) evenly distributed around the circumference, 1.5 cm from the edge; the lower layer had only a single hole, which could be aligned with a hole in the upper layer, giving access to an ‘escape chamber’, a removable plastic box. The lower layer hole could be secured in position, allowing the upper layer to be rotated to give coincidence of one of the upper holes with the fixed hole in the lower layer. Testing was conducted under intense illumination \((4 \times 32 \text{ W} \text{ diffuse overhead fluorescent lighting and } 1 \times 500 \text{ W floodlamp, } 2 \text{ m above the maze})\). Abundant visual cues (posters, etc.) were placed on the walls of the testing room. The animals’ behaviour in the maze was recorded by a video camera fixed above the maze and connected with a video recorder (Sony, SLV-E280 UX). The maze was wiped with cleaning liquid after each trial and the upper platform layer rotated in a random fashion to prevent animals using any olfactory trails from their previous trial to locate the escape hole.

Animals were habituated to the maze 1 day prior to the beginning of training. Each daily session consisted of two trials, during which an animal was placed in a cylinder \((24 \text{ cm diameter}, 27 \text{ cm height})\) positioned in the centre of the maze; 15 s later, the cylinder was lifted and a trial began. A trial was terminated when the rat escaped through the hole into the escape chamber or when 3 min had elapsed, whichever occurred first. In the rare case when a rat did not find the escape hole within 3 min it was placed into the hole by the experimenter. Immediately after the animal escaped into the chamber the hole was closed with a wooden block and the animal was allowed to stay in the chamber for 15 s before being returned to its home cage. The second identical trial took place 10 min later. Training trials were given for 5 days, during which the escape hole was always in the same position. The escape latency and number of errors (an error was scored when a rat sniffed over or poked its nose in a hole other than the escape hole) were recorded.

**Statistics**

SPSS (version 11.5, SPSS Inc., USA) was used for all statistical analysis. All data analysed using a repeated-measures ANOVA were initially analysed using Mauchly’s test of sphericity and the F-value on the next stage of the analysis was calculated using either the sphericity assumption or Greenhouse–Geisser epsilon depending on the result of Mauchly’s test. Significant interactions were followed by post hoc tests.
Results

**Chronic alcohol treatment**

In the subgroup of rats later tested on the negative patterning and FI, the mean ethanol consumption over the final week of chronic treatment was 13.16 ± 0.51 g/kg/day in the SWD group and 12.87 ± 0.49 g/kg/day in the SWD group (Fig. 1A). The repeated-measures ANOVA showed that, while consumption changed over days (day: \( F_{6,48} = 21.53, P < 0.001 \)), there was no significant difference in ethanol intake between the two groups (group: \( F_{1,8} = 0.20, P > 0.05 \); day × group interaction: \( F_{6,48} = 0.71, P > 0.05 \)). The animals later tested in the contextual fear experiment and the Barnes maze consumed, over the last week of treatment, 13.06 ± 0.42 g/kg/day (SWD group) and 13.97 ± 0.42 g/kg/day (RWD group) of ethanol (Fig. 1B) and there was no significant difference in the consumption level between the groups (day: \( F_{6,60} = 4.46, P < 0.05 \); group: \( F_{1,10} = 3.16, P > 0.05 \); interaction: \( F_{6,60} = 0.47, P > 0.05 \)).

**Negative patterning task**

**Single-element training**

The animals from all treatment groups successfully completed lever-press shaping and VI training. During the initial 3 days of simple stimuli conditioning (before introduction of the compound stimulus), all animals quickly learned to press the lever during light or tone presentation (Fig. 2). The number of trials with lever presses (Fig. 2A) significantly increased from day 1 to day 3 (repeated-measures ANOVA,
main effect of day: $F_{2.48} = 60.55, P < 0.001$), while the number of lever presses during the intertrial intervals (Fig. 2B) simultaneously decreased (main effect of day: $F_{2.48} = 29.12, P < 0.001$). The analysis confirmed that there was no difference between RWD, SWD and CON groups in the number of trials with lever presses (no effect of group: $F_{2.24} = 2.47, P > 0.05$ and no significant day $\times$ group interaction: $F_{4.48} = 0.63, P > 0.05$), and in responding during the intertrial intervals (no effect of group: $F_{2.24} = 0.427, P > 0.05$ and no significant day $\times$ group interaction: $F_{4.48} = 0.29, P > 0.05$).

Although a significant difference between stimuli was observed in the conditioned response acquisition (the animals learned to press the lever in response to light faster than in response to tone (main effect of stimulus: $F_{1.24} = 20.20, P < 0.001$ and significant day $\times$ stimulus interaction: $F_{2.48} = 7.66, P < 0.001$), there was no between-groups difference in this respect (no stimulus $\times$ group interaction: $F_{2.24} = 0.92, P > 0.05$ and no day $\times$ stimulus $\times$ group interaction: $F_{4.48} = 0.62, P > 0.05$).

Compound-element training

The pattern of responding after introduction of the compound stimulus (light $+$ tone) is shown on Fig. 3. The three-way ANOVA analysis of the number of trials with lever presses in response to the simple stimuli, during compound stimulus training the animals responded equally to light and tone stimuli; no effect of stimulus ($F_{1.25} = 0.018, P > 0.05$) and no significant interactions involving stimulus (no stimulus $\times$ group interaction: $F_{2.25} = 1.04, P > 0.05$, no day $\times$ stimulus interaction: $F_{23.575} = 1.03, P > 0.05$, and no day $\times$ stimulus $\times$ group interaction: $F_{46.575} = 1.28, P > 0.05$). Therefore, data on both simple stimuli were subsequently analysed together. Analysis of lever pressing during the compound stimulus training was complicated by the initial fluctuation of animals’ reactions following change in the session schedule on day 8. For this reason, only data from the second part of the training were analysed statistically. The three-way ANOVA on the data from the 10th to 24th days of training with type of stimulus (simple or compound) and day of training as within-subject factors and treatment group as between-subjects factor revealed that the number of lever presses in response to CS changed with training differently, depending on the type of stimulus (main effect of training day: $F_{13.325} = 7.95, P < 0.001$; main effect of stimulus: $F_{1.25} = 22.61, P < 0.001$; day $\times$ stimulus interaction: $F_{13.325} = 9.41, P < 0.001$); with training the animals reacted more and more on simple stimuli, while they gradually decreased responding for the compound stimulus. There was no significant main effect of group ($F_{2.25} = 2.56, P > 0.05$) and no day $\times$ group interaction ($F_{26.325} = 0.80, P > 0.05$) but the day $\times$ stimulus $\times$ group interaction was significant ($F_{26.325} = 1.90, P < 0.01$). Separate analysis of lever presses in response to presentation of the simple stimuli (Fig. 3A) confirmed that, with training, rats increased the number of trials with lever presses (main effect of day: $F_{13.325} = 2.72, P < 0.01$) and showed that animals from the RWD, SWD and CON treatment regimes did not differ in this respect ($F_{2.25} = 0.22, P > 0.05$ and no day $\times$ group interaction: $F_{26.325} = 1.12, P > 0.05$). At the same time, the analysis of the number of trials with lever presses in response to the compound stimulus (Fig. 3B) demonstrated a tendency towards difference between treatment groups (effect of group: $F_{2.25} = 2.76, P > 0.082$) in the speed of gradual suppression of responses with training (effect of training day: $F_{13.325} = 11.6, P < 0.01$), though no day $\times$ group interaction ($F_{26.325} = 1.25, P > 0.05$). RWD, SWD and CON animals pressed the lever in response to the compound stimulus with similar latencies: no main effect of group ($F_{2.25} = 0.92, P > 0.05$) and no significant interaction involving group (day $\times$ group $F_{26.325} = 0.71, P > 0.05$; stimulus $\times$ group $F_{2.25} = 0.11, P > 0.05$; day $\times$ stimulus $\times$ group $F_{26.325} = 1.51, P > 0.05$).

To further explore the effect of the different ethanol treatment regimens on negative patterning we analysed number of lever presses per minute during the compound stimulus presentation and during the intertrial intervals (Fig. 3C and D). The three-way ANOVA (with day of training and compound presentation or intertrial interval as within-subject factors and group as between-subjects factor) of data from the 10th to 24th days of training showed that the rats pressed the lever at different rates depending on the trial phase (i.e. compound stimulus presentation or intertrial interval; main effect of phase $F_{1,25} = 968.71, P < 0.001$). Also, the lever-pressing rates changed with training with different speed (main effect of training day: $F_{13.325} = 29.28, P < 0.001$; significant day $\times$ phase interaction: $F_{13.325} = 10.7, P < 0.001$). Moreover, there was a significant difference among the three treatment groups in the rate of lever pressing ($F_{2.25} = 6.15, P < 0.01$) and this difference, too, was phase-dependent (significant phase $\times$ group interaction: $F_{2.25} = 5.54, P < 0.05$). Post hoc Student–Newman–Keuls test revealed that the RWD group was different from both CON and SWD groups ($P < 0.05$). Separate analysis of the number of lever presses per minute during the compound stimulus presentation further highlighted a significant difference between

Fig. 2. Acquisition of responding for simple stimuli. (A) Number of trials with lever press (%). There was no difference between treatment groups in the percentage of reactions for light and tone stimuli; all animals acquired responding for the light stimulus faster than for tone ($P < 0.05$). (B) Number of lever presses per minute during intertrial intervals. There was no difference between treatment groups in the rate of intertrial lever presses. Data represent the mean ± SEM.
treatment groups (main effect of group: $F_{2,25} = 7.49, P < 0.01$) in the speed of reduction of the rate of lever presses with training but did not reveal a day $\times$ group interaction (main effect of day: $F_{13,325} = 23.37, P < 0.01$; no interaction: $F_{26,325} = 0.63, P > 0.05$). Post hoc test showed that RWD animals were slower in reducing the lever presses during the nonreinforced compound stimulus than animals from the CON and SWD groups ($P < 0.05$). During the intertrial interval the rate of lever presses also declined with training ($F_{13,325} = 23.49, P < 0.05$). The postreinforcement pause showed a significant increase in the index of curvature (Fig. 5C), which characterizes the distribution of lever presses over the time interval, showed a significant increase in the index with training (main effect of training day: $F_{17,425} = 33.72, P < 0.001$), but no difference between RWD, SWD and CON groups (main effect of group: $F_{2,25} = 1.46, P > 0.05$) and no day $\times$ group interaction ($F_{34,425} = 0.83, P > 0.05$). The postreinforcement pause (Fig. 5D) also increased with training (main effect of day: $F_{17,425} = 27.73, P < 0.001$) but there was no difference in the pause length between treatment groups (main effect of group: $F_{2,25} = 0.27, P > 0.05$; day $\times$ group interaction: $F_{34,425} = 1.27, P > 0.05$).

Extinction of conditioned lever presses

The final test showed (Fig. 4) that all animals were able to extinguish previously conditioned responses (two-way ANOVA, main effect of training bin: $F_{4,100} = 15.01, P < 0.01$) and RWD, SWD and CON groups were equally successful in response suppression in these circumstances (no significant difference between groups: $F_{2,25} = 0.03, P > 0.05$; no training bin $\times$ group interaction: $F_{8,100} = 0.35, P > 0.05$).

FI operant task

The results of FI responding can be seen in Fig. 5. Animals from all three treatment groups emitted the majority of lever presses close to the end of the FI, producing the characteristic ‘scalloped’ pattern of responding (Fig. 5A and B). The two-way ANOVA of the index of curvature (Fig. 5C), which characterizes the distribution of lever presses over the time interval, showed a significant increase in the index with training (main effect of training day: $F_{17,425} = 33.72, P < 0.001$), but no difference between RWD, SWD and CON groups (main effect of group: $F_{2,25} = 1.46, P > 0.05$) and no day $\times$ group interaction ($F_{34,425} = 0.83, P > 0.05$). The postreinforcement pause (Fig. 5D) also increased with training (main effect of day: $F_{17,425} = 27.73, P < 0.001$) but there was no difference in the pause length between treatment groups (main effect of group: $F_{2,25} = 0.27, P > 0.05$; day $\times$ group interaction: $F_{34,425} = 1.27, P > 0.05$).
FIG. 4. Effect of repeated withdrawal from ethanol on extinction of the lever pressing. The first 20 trials (trial bins 1 and 2) included presentation of the reinforced light or tone stimuli and the next 30 trials (trial bins 3, 4 and 5) were run in extinction. All animals successfully extinguished responding ($P < 0.05$); there was no difference between treatment groups in the rate of extinction. Data represent the mean ± SEM.

analysis of the running rate (number of lever presses per minute; Fig. 5E) showed significant fluctuation in the animals’ activity on different training days ($F_{17,425} = 10.22, P < 0.001$) in spite of the absence of any consistent increasing or decreasing trend in this measure. There was a slight tendency towards higher running rates in the RWD group, but it was not significant (no main effect of group: $F_{2,25} = 2.40, P > 0.05$; no day × group interactions: $F_{34,425} = 0.95, P > 0.05$).

Despite the absence of differences in the abovementioned measures, the analysis of the distribution of the lever presses during the last day of training (day 18; Fig. 5B) revealed significant differences between treatment groups (main effect of group: $F_{2,25} = 3.46, P < 0.05$) and significant time bin × group interaction ($F_{18,225} = 3.32, P < 0.05$). It also confirmed larger number of responses emitted by all animals regardless of the treatment prehistory closer to the end of the 120-s interval (main effect of time bin: $F_{9,225} = 146.56, P < 0.001$). Post hoc comparison showed that distribution of the lever presses of the RWD group was significantly different from that of control animals ($P < 0.05$). Subsequent separate analysis of the data from each time bin showed that RWD animals emitted more lever presses than CON and SWD animals specifically during the last time bins ($F_{2,25} = 4.83, P < 0.05$; bin 10: $F_{2,25} = 4.57, P < 0.05$; post hoc $P < 0.05$). For comparison, on day 1 (Fig. 5A) of training there were no differences between treatment groups (no main effect of group: $F_{2,25} = 1.66, P > 0.05$; no significant time bin × group interaction: $F_{18,225} = 0.67, P > 0.05$; though there was a significant main effect of time bin: $F_{9,225} = 73.24, P < 0.001$). For further analysis, data on the cumulative number of lever presses during last two bins of the interval (9th and 10th bins) were compared with the cumulative number of responses emitted during the 4th and 5th bins (Fig. 5F). For simplification, the original data were grouped in blocks of 3 days (averaged). The ANOVA, with groups as between-subjects factor and day-blocks and bins as repeated measures, showed that the animals emitted significantly more responses during the last bins of the interval (bin: $F_{2,25} = 203.69, P < 0.001$) and the number of responses depended on the group (bin × group interaction: $F_{2,25} = 5.28, P < 0.05$); it also reconfirmed dependence of the number of responses on training and bin (day-block: $F_{3,125} = 10.35, P < 0.001$; day-block × bin: $F_{3,125} = 79.43, P < 0.001$), as well as giving rise to an overall day-block × bin × group interaction ($F_{10,125} = 3.31, P < 0.01$); there was no interaction between day-block and group (day-block × group: $F_{10,125} = 1.25, P > 0.05$) and no significant difference between treatment groups (group: $F_{2,25} = 2.81, P > 0.05$). Subsequent separate analysis of the responses during the last (9th and 10th) bins not only confirmed a significant effect of day-block ($F_{2,25} = 31.08, P < 0.001$) but also showed a significant difference between groups ($F_{2,25} = 22.23, P < 0.05$) and a day-block × group interaction ($F_{10,125} = 2.68, P < 0.05$). Subsequent analysis of each day-block with one-way ANOVA showed that, on the last two day-blocks of FI training, RWD animals emitted more responses than CON and SWD animals (13th–15th days: $F_{2,25} = 6.36, P < 0.01$; 16th–18th days: $F_{2,25} = 4.46, P < 0.05$; post hoc $P < 0.05$).

### Contextual fear conditioning

There was no difference between treatment groups in the total time spent freezing during test sessions (Fig. 6). The one-way ANOVA of the percentage of time spent freezing during testing sessions showed no difference between treatment groups ($F_{2,15} = 0.38, P > 0.05$; Fig. 6A). Repeated-measures analysis of the percentage of freezing during three 1-min time bins demonstrated clear increases in the amount of freezing during the testing session (significant main effect of time bin: $F_{2,30} = 45.92, P < 0.001$) but failed to find any difference between different groups (no effect of group: $F_{2,15} = 0.38, P > 0.05$; no time bin × group interaction: $F_{3,30} = 1.89, P > 0.05$; Fig. 6B).

### Spatial learning in the Barnes maze

The escape latency decreased with training (trial: $F_{2,288} = 39.50, P < 0.001$; Fig. 7A), indicating that rats became faster in finding the escape hole in the course of training. Animals from all treatment groups were equally successful in reducing the searching time (groups: $F_{2,32} = 0.56, P > 0.05$) and the decrease was similar in all groups (group × trial interaction: $F_{18,288} = 0.76, P > 0.05$). The number of errors also gradually decreased in the course of training (trial: $F_{2,88} = 9.86, P < 0.001$; Fig. 7B) at the same rate (group × trial interaction: $F_{18,288} = 0.69, P > 0.05$) and to the same extent in all three groups (group: $F_{2,32} = 0.09, P > 0.05$).

### Discussion

The main observation of the present study was that animals which had previously experienced repeated withdrawal from ethanol showed impaired discrimination performance when a compound stimulus, made up of two previously reinforced simple stimuli, was not reinforced, a so-called negative pattern discrimination. While all groups gradually learned to suppress responding during the nonreinforced compound cue, the RWD group was slower in learning to suppress nonrewarded responses. Importantly, there was no difference between treatment groups in the acquisition of a conditioned response for the single-element stimuli. Thus RWD rats appear to show a specific deficit in learning about compound stimuli, consistent with a deficit in hippocampal function.

These observations thus suggest that, following repeated experience of withdrawal from ethanol, rats have a deficit in their ability to use configural cues to guide their behaviour. As outlined in the introduction, such a deficit might be attributable to a deficit in hippocampal functioning (Rudy & Sutherland, 1989; Sutherland & McDonald, 1990; Alvarado & Rudy, 1995) and would be consistent with the
evidence that RWD from the same ethanol treatment protocol results in impairment of hippocampal LTP 2 weeks after final withdrawal (Stephens et al., 2005), and in spontaneous epileptoform activity in hippocampus during the 24 h following withdrawal (Veatch & Becker, 2002; Duka et al., 2004). However, configural learning involves other brain structures in addition to hippocampus (Rudy & Sutherland, 1995) and, in another test that is known to require intact processing in the hippocampus (Selden et al., 1991; Phillips & LeDoux, 1992; Fendt & Fanselow, 1999; Hall et al., 2000, 2001), we found no effect of RWD treatment in contextual fear conditioning, i.e. all groups showed similar increases in the amount of freezing during the test session. We also found that freezing increased as the test progressed, a feature which has been previously reported (Bannerman et al., 2001), but again there was no difference between experimental groups. As freezing occurred for all groups during 24 h, it seems unlikely that the failure to find group differences was attributable to floor or ceiling effects. Thus, in the contextual freezing experiment, there was no evidence that the RWD rats showed a deficit that might be related to impairment of hippocampal functioning.

The animals in our RWD group had received their treatment before testing in the conditioned freezing experiment, so that any hippocampal...
impairments induced by the treatment were already present during acquisition. There is some evidence that, in similar circumstances, animals with a more severe insult, hippocampal lesions, adopt a different, nonconfigural strategy, so that they may instead associate individual features of the shock context with the shock; during test, these discrete features may summate to elicit a strong but hippocampus-independent ‘contextual’ fear (Anagnostaras et al., 2001). Thus, it cannot be excluded that our RWD rats also adopt such an alternative strategy to overcome the consequences of any hippocampal dysfunction [though, as the use of such discrete features depends upon intact amygdala processing (Rudy et al., 2004), we might have anticipated a deficit in this aspect of fear conditioning too].

In a further experiment designed to investigate the consequences of RWD on performance of a task thought to depend upon processing in hippocampal pathways, we studied spatial learning in rodents. In this experiment, we compared the effects of RWD treatment with SWD and CON treatments on the ability to learn the location of an escape hole using the Barnes platform, a task which has been shown to be sensitive to hippocampal lesions. No differences were found between RWD and other groups in this test of spatial learning, again suggesting that hippocampal functioning is not severely impaired following RWD treatment. Hence the deficits in configural learning that we observed in the RWD rats may be attributable to deficits in other brain areas, or in other behavioural processes than learning itself.

What, then, might account for the deficits seen in the negative patterning experiment? The fact that RWD, SWD and CON animals had similar response latencies to cues signalling food suggests that the slowed rate of decline of the RWD group in responding following introduction of the nonreinforced compound stimulus was not simply attributable to increased motivation for food (Sclafani, 1972). At the same time this fact further argues against hippocampal deficit, as hippocampectomy decreases rats’ latency to respond (Whishaw & Tomie, 1991; Richmond et al., 1997). Another possibility is that the deficit seen in RWD animals in negative patterning might be due, not to a learning deficit, but to a generally reduced ability of these rats to suppress responding of a previously established behaviour (i.e. responding to the light or tone). This notion might be supported by the observation that the RWD animals emitted more lever presses during intertrial intervals, though this difference was not significant. Furthermore, we found no difference between treatment groups in the rate and speed of extinction, when the simple conditioned stimuli no

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**Fig. 6.** Effect of repeated withdrawal from ethanol on contextual fear conditioning. (A) Time (%) spent freezing during a 3-min testing session. RWD animals did not differ from CON and SWD animals in the total time spent freezing 24 h after the training. (B) Time (%) spent freezing during testing in 1-min time intervals. All animals showed an increase in freezing over time during testing ($P < 0.05$), and there was no difference between treatment groups. Data represent the mean ± SEM. Freezing was counted in 5-s bins; only those bins during which an animal did not show any movement except required for respiration were counted as freezing time.

**Fig. 7.** Effect of repeated withdrawal from ethanol on spatial learning in the Barnes maze. (A) Escape latency (s). Animals from all treatment groups with training became faster in finding the escape hole ($P < 0.001$) and there was no difference between groups in this regard. (B) Number of errors (approaches to an incorrect hole). Animals gradually learned location of the escape hole and approached wrong holes on fewer occasions ($P < 0.001$); both ethanol-treated groups showed the same efficiency as did control animals.

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longer predicted food. Nevertheless, withholding responses during extinction differs from withholding responses during the compound presentation, as the compound presentation takes place in the context of sometimes obtaining food (as reinforced and nonreinforced trials were interpolated during the session), so that an alternative potential cause for the deficit could be the inability of these animals to withhold inappropriate responding in the context of forthcoming reinforcement.

We tested this possibility by training our animals on the FI schedule. All animals produced a characteristic pattern of responding with training. The index of curvature (Fry et al., 1960) reached 0.75, indicating that the majority of the responses were emitted towards the end of the FI. There was no difference in the index of curvature between treatment groups, confirming that chronic ethanol treatment with neither repeated nor single withdrawal affected temporal organization of responding under this schedule. The same postreinforcement pause length in RWD, SWD and CON groups further supported this interpretation. Nevertheless, RWD rats (relative to SWD and CON rats) showed increased rates of responding in the period immediately before reinforcement became available. These results support our suggestion that the deficit seen in RWD animals on negative patterning might be caused by the inability of these animals to withhold inappropriate responding in the vicinity of expected reinforcement. At the same time, data from the FI responding further question involvement of the hippocampus in the deficits seen after the repeated withdrawal, as animals with lesions of hippocampus show shorter postreinforcement pauses and faster overall rates of responding (Gray & McNaughton, 1983; Jaldow et al., 1989). In our experiment, although there was some tendency towards higher response rates it did not reach significance and, moreover, RWD animals clearly showed the same duration of postreinforcement pauses as CON and SWD rats.

As mentioned in the introduction, the data from the literature regarding whether or not negative patterning discrimination and, more broadly, configural learning is sensitive to lesions of the hippocampus and hippocampal formation are contradictory. While Sutherland, Rudy and colleagues consistently showed that hippocampal lesions disrupted negative patterning discrimination (Rudy & Sutherland, 1989; Sutherland & McDonald, 1990; Alvarado & Rudy, 1995; Rudy & Sutherland, 1995), other researches have not found such deficits (Davidson et al., 1993; Papadimitriou & Wynne, 1999; Bussey et al., 2000; Moreira & Bueno, 2003). Whishaw & Tomie (1991) and Richmond et al. (1997) reported that hippocampectomised rats, although impaired, could acquire a configural task that did not require animals to withhold responding (i.e. successive discrimination). It is important to note that different research groups employed rather different training procedures and, in particular, those experiments that have found deficits in configural learning (e.g. Rudy & Sutherland, 1989; Sutherland & McDonald, 1990; Alvarado & Rudy, 1995) have tended to use go/no-go negative patterning task like that used here.

The findings from the Blackburn & Hevenor (1996) experiments using amphetamine are of particular interest. They found that amphetamine disrupted negative patterning, which was run as a go/no-go task, by increasing responses to the unrewarded compound; at the same time, amphetamine had little impact on performance of a task in which the rats had to respond to either of the single stimuli with one response (lever-press or chain-pull) and to the compound stimulus with the other response. This observation is difficult to reconcile with a deficit in ability to learn configural cues, and indicates that disruption of performance of the ‘classical negative patterning task’, which is run as go/no-go task, must be attributable to other factors. Indeed, Whishaw & Tomie (1991) noted that motor demands of the task should be considered, and Davidson et al. (1993) suggested that impairments in the negative patterning paradigm might be caused by increased responsiveness rather than by disruption of configural association. Other researchers also pointed to the importance of an impaired ability to withhold responding and/or increased responsiveness in this task (Blackburn & Hevenor, 1996; Papadimitriou & Wynne, 1999). It therefore seems possible that the deficits we observed in the RWD rats reflect changes in responsiveness.

This kind of deficit might have more in common with alterations in frontal function, and there are several reasons to suggest that the impairment of the negative patterning discrimination after RWD might be related to the changes in prefrontal cortex functioning rather than to a hippocampal deficit. Studies in human alcoholics have shown that repeated experience of withdrawal from alcohol is associated with impaired cognitive function in executive control tasks sensitive to dysfunction of prefrontal cortex, which include lower ability to inhibit a prepotent response and inability to wait before a response to receive a reward (Duka et al., 2003). Binge drinkers (for whom RWD rats may serve as a model; Duka et al., 2004), when compared with nonbingers, are impaired on a pattern recognition task and a spatial working memory task, both of which depend on cortical processing (Weissenborn & Duka, 2003; Duka et al., 2004; Townshend & Duka, 2005). In rats, using the same treatment procedure as one employed in this study, we have observed a mild retardation of extinction of the conditioned emotional response acquired prior to ethanol exposure (Ripley et al., 2003a), which might also be considered as a sign of RWD effects on prefrontal function (Morgan et al., 1993).

In the present experiments we have observed that RWD rats (relative to SWD and CON rats) show increased rates of responding on an FI schedule in the period immediately before reinforcement became available. Similar premature responses in operant tasks employing differential reinforcement of low-rate responding (DRL) have been considered as a sign of impaired prefrontal function (Peterson et al., 2003). The results shown by the RWD group on the FI responding resemble impaired ability of repeatedly detoxified human alcoholics to inhibit a prepotent response and to wait before emitting a response (Duka et al., 2003). All these data suggest that effect of RWD on prefrontal cortex function needs further careful examination.

To summarize, the present study demonstrated that RWD impairs negative patterning discrimination, increases the number of responses in the period immediately before reinforcement became available on the FI schedule but does not affect contextual fear conditioning and spatial learning in the Barnes maze. Overall, these data suggest that in our model of repeated withdrawal from ethanol any changes in hippocampal function do not manifest at the behavioural level in the tests employed. The deficit seen after repeated withdrawal in the negative pattern discrimination and over-responding in the FI paradigm might be related to the changes in the functioning of the cortex after withdrawal. These findings parallel data from human patients with a history of repeated detoxifications and hence support the RWD procedure as a useful model for studies of processes happening in human alcoholics.

Finally, the implications of the present observations for human alcoholism may be outlined. The pattern of data suggests repeated withdrawal experience results in increased tendency to respond for rewards, especially in the vicinity of expected reinforcement. If these observations can be applied to humans, then the present findings may suggest that repeated detoxifications may contribute to the reduced ability of alcoholics to control their alcohol consumption in the presence of cues indicating alcohol availability. Secondly, the data may suggest that repeated detoxification may reduce the ability to suppress inappropriate behaviours, perhaps reflected in other forms of disinhibited behaviour associated with alcoholism.
It is widely recognized that alcoholics display morphological abnormalities in the frontal lobe system (for a review, see Moselhy et al., 2001) and that deficits in frontal function leading to loss of control of behaviours may predispose to alcohol, and other drug, abuse. Consistent with the idea that repeated withdrawal may impair frontal lobe function, alcoholic patients with two or more previous experiences of medically supervised detoxifications from alcohol were more impaired than patients with a single, or no, previous experience of detoxification in tasks measuring frontal lobe function (Duka et al., 2003; Townshend & Duka, 2005). However, in the case of humans it is difficult to determine whether impaired frontal function is a cause or a consequence of heavy drinking, and thus frequent withdrawal attempts. The present animal experiments imply that such behavioural deficits may arise as a consequence of alcohol abuse and its treatment. Consistent with this view, data from a related animal procedure suggest that binge drinking can indeed induce cortical damage and lead to cognitive deficits such as perseverative responding (Obernier et al., 2002).

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Abbreviations

CON, control; CS, conditioned stimulus; FI, fixed interval; FR, fixed ratio; LTP, long-term potentiation; RWD, repeated withdrawal from ethanol; SWD, single withdrawal; VI, variable interval.

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