

Assessing Anxiety and Reward-Related Behaviors Following Alcohol Administration or Chronic Stress

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Abstract

Dysregulation of stress- and reward-related neurocircuitry that results from experience with alcohol or chronic stress may underlie vulnerability to initiate or relapse to alcohol use. Related changes in behaviors associated with such dysregulation may provide insight into factors that increase or decrease vulnerability to alcohol dependence in humans. In the present study, anxiety-like behavior and the acquisition of conditioned place preference were assessed in male Wistar rats with differing histories of exposure to stress or alcohol two weeks following the termination of treatment. Thus, time spent on the open arms of the elevated plus maze as well as the number of days required to acquire alcohol-based place conditioning were evaluated in control animals vs. those with a history of exposure to chronic unpredictable stress, experimenter-administered intragastric alcohol, or self-administered alcohol liquid diet. Exposure to chronic unpredictable stress decreased open arm exploration on the elevated plus maze while no difference in open arm exploration was observed between the control group and alcohol-administration groups. However, animals with a history of intragastric ethanol administration or chronic unpredictable stress acquired alcohol-based place conditioning after 3 alcohol-context pairings while control animals and animals with a history of alcohol self-administration required 4 alcohol-context pairings to acquire a significant preference for the alcohol-paired compartment. Results reveal that 1) a history of alcohol contributes to the acquisition of conditioned place preference but is dependent on the administration regimen and 2) chronic unpredictable stress enhances the acquisition of alcohol-based conditioned place preference and produces anxiogenic performance. These findings suggest overlap in the neuroadaptive changes that result from chronic exposure to alcohol or stress which may contribute to vulnerability to alcohol use.

Keywords: Intragastric alcohol; Neuroadaptive changes; Chronic stress

Introduction

Exposure to stress as well as prior exposure to alcohol has been shown to predict subsequent alcohol abuse in humans [1]. Animal studies have demonstrated that stress and reward neurocircuitries are intimately associated and that stress is effective in promoting alcohol intake [2], conditioned stress [3], as well as reinstatement of previously extinguished responding for alcohol [4]. In addition to acute application of acute foot shock stress [5], a variety of stressors have been shown to have the ability to modify subsequent alcohol intake in a manner that is relevant to alcohol dependence including: maternal stress [6], early life stress [7], social isolation [8], and food restriction [9]. Similar findings on the effects of stressors on drug self-administration have been reported for other drugs of abuse [9]. Furthermore, administration of compounds that disrupt HPA axis activity, such as CRF antagonists, have been shown to be effective in preventing alcohol-motivated behaviors such as reinstatement elicited by a stressor [10] or by alcohol-associated cues [2].

Prior exposure to alcohol may also serve as an important predisposing factor with respect to the development of alcohol dependence as well as dysregulation of stress-related neurocircuitry [11,12]. Early alcohol experience has been shown to facilitate subsequent drinking as well as alcohol preference as indicated by “relapse-like” intake [13]. Breese and colleagues [11] have suggested that both stress and withdrawal function similarly to produce neuro adaptations that are related to alcohol dependence. This suggests strong integration between reward and stress-regulatory function as well as concurrent neuroadaptations that develop in these systems. Recent studies have revealed multiple candidate systems that may play an important role in these processes including CRF [14,15], cortisol [16], glutamate [5], nociceptin/NOP

[17,18] and BDNF [19,20].

While many studies have demonstrated the importance of early life experience and alcohol exposure on the development of alcohol seeking and self administration, exposure to mild, but chronic stress later in development, may also be an important factor in alcohol-related behavior. In particular, such stress exposure may provide a model for typical day-to-day stressors and, thus, present a compelling framework in which to examine the contribution of common stressors on subsequent alcohol-related behaviors. In rats, chronic unpredictable mild stress was originally used as a model of depression [21] but also found recent utility in the examination of reward-related performance [22,23] and stress-reactivity [24].

While the administration of alcohol, or other drugs of abuse, has been shown to alter stress- or reward-related performance, there have been few studies examining the effect of administration method on such performance, particularly in the case of alcohol despite differences being noted in the administration conditions for other drugs on subsequent anxiety-related performance, including cocaine [25]. Thus, an additional goal of the present study was to evaluate whether differences in stress- and reward-related performance were produced

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as a function of administration regimen (i.e. free-choice drinking of a alcohol liquid diet vs. intragastric administration of alcohol solution).

In the present study, we evaluated the effects of chronic stress exposure and chronic ethanol exposure, using voluntary and involuntary intake regimens, on stress- and reward-related behaviors. Based on previous research we hypothesized that both stress and ethanol exposure should enhance acquisition of alcohol-seeking and that involuntary ethanol exposure may produce a more pronounced effect than voluntary exposure. Thus we employed two different ethanol administration regimens, liquid diet and intragastric intubation, as well as the chronic unpredictable stress procedure as our exposure conditions [21]. Following two weeks from termination of the alcohol or stress procedure, alcohol-seeking was evaluated using number of acquisition sessions required to establish alcohol-based conditioned place preference while anxiety-related performance was evaluated using the elevated plus maze test [26].

Methods and Materials

Apparatus

Elevated plus-maze: The elevated plus-maze apparatus was constructed of Plexiglas and consisted of four arms (10×50 cm) positioned at right angles and elevated 50 cm above the floor. Two arms were fitted with 40 cm high dark walls (enclosed arms). The other two arms had 0.5 cm high ledges (open arms). The elevated plus-maze was located in a quiet room that provided 1.5-2.0 lux of illumination for the open arms and <1 lux for the enclosed arms. On test day, rats were habituated for at least 2 h. Rats were then placed individually onto the center of the maze facing a closed arm. A FireFly MV camera (Point Grey, BC, Canada) placed directly above the maze acquired visual data that was sent to a computer running Open Control [27] tracking software that allowed quantification of time spent in each arm.

Conditioned place preference: Four rectangular Plexiglas boxes consisted of two compartments (38 cm × 27 cm × 36 cm) connected by a tunnel (8 cm × 8 cm × 8 cm). The tunnel could be closed by inserting removable Plexiglas block that completely occluded the tunnel, restricting movement to one of the main compartments. The two initially neutral compartments were visually distinct: each compartment wall consisted of either plain black Plexiglas surface or painted green vertical stripes (2 cm wide). The compartment floors were also distinct: one type was made of galvanized steel mesh, and the other was constructed of parallel stainless steel bars (1cm apart). Tunnel floors were smooth Plexiglas. The arrangement of walls and floor types was such that each box had a unique configuration. Fire Fly MV cameras (Point Grey, BC, Canada) were placed directly above each chamber to acquire visual data that was sent to a computer running Open Control [27] tracking software that allowed tabulation of time spent in each compartment.

Statistical Analyses

General: All statistical analyses were conducted using R (<http://www.r-project.org>). Shapiro-Wilk's tests of normality were conducted to check for any deviations from normality prior to analysis via parametric procedures.

Elevated plus maze: Unpaired t-tests were used to compare treatment conditions.

Conditioned place preference: Mixed designed 2 × 3 ANOVA with phase (i.e. preconditioning vs. test) as the within-subjects factor and number of acquisition sessions (2,3,or 4) as the between-subjects factor

were conducted separately for control and treatment groups in each experiment. Fischer's LSD tests were conducted following ANOVA to assess specific group differences and the false discovery rate procedure was used to adjust p values to control for type I error. An alpha level of 0.05 was used for all analyses.

Procedure

Conditioned Place Preference: Using an unbiased paradigm, the procedure consisted of three phases: pre-conditioning, conditioning and test. For all phases, animals received one session per day.

Preconditioning: During three ethanol-free 15 min sessions, rats were permitted free access to the entire apparatus. Rats were placed initially in the tunnel. The amount of time spent in each compartment was recorded.

Conditioning: During 4, 6 or 8, 20-min sessions, rats were confined to one compartment, different compartments on alternate days. Ethanol administration preceded placement into one compartment on odd days, while saline injections preceded placement into the other compartment on even days. The designation of the ethanol-paired compartment was counterbalanced.

Test: During one 15 min test session, rats were permitted free access to the entire apparatus with the tunnel occlusion removed. The amount of time spent in each compartment was tabulated.

Experiment 1: Liquid Diet (N=72)

Subjects: Animals were male Wistar rats (Charles River, St. Constant, QC, Canada), weighing 225-250 g on arrival. Subjects were housed in pairs on a 12-hour lights on and 12-hour lights off schedule (lights off at 2100). The rat colony room was maintained for temperature and humidity. Food and water were made available ad libitum at all times excepting behavioural testing. All behavioural tests were conducted between 1200 and 1900. Housing and testing procedures were conducted in strict adherence to the Canadian Council on Animal Care guidelines and approved by the University Animal Care Committee.

Solutions: Ethanol was made up in a volume of 15% w/v in Boost vehicle.

Liquid Diet: Rats received access to ethanol solution in addition to water and rat chow. Ethanol solution was made up in a volume of 15% w/v in Boost vehicle and was available for 16-h daily (1800-1000). During this time animals were separated using a Plexiglas divider that contained holes allowing for interaction between cage mates. Rats serving as controls received the same procedure with a Boost vehicle solution in place of the ethanol solution.

Elevated plus maze: Two weeks after the termination of the LD procedure, open arm exploration was evaluated during 5-min test session on the elevated plus maze.

Conditioned place preference: Rats underwent preconditioning as, described above, during the three days preceding the elevated plus maze test. Conditioning sessions began the day following the elevated plus maze test. Animals were divided into three groups receiving 2 (N=24), 3 (N=24), or 4 (N=24) ethanol-context pairings along with an equal number of vehicle-context pairings in 20-min sessions.

On the day following the last conditioning session, time spent in the ethanol-paired chamber was evaluated in a single 15-min test session.

Experiment 2: Intragastric Intubation (N=72)

Subjects: Animals were male Wistar rats (Charles River, St. Constant, QC, Canada), weighing 250-275 g on arrival. Subjects were housed in pairs on a 12-hour lights on and 12-hour lights off schedule (lights off at 2100). The rat colony room was maintained for temperature and humidity. Food and water were made available ad libitum at all times excepting behavioural testing. All behavioural tests were conducted between 1200 and 1900. Housing and testing procedures were conducted in strict adherence to the Canadian Council on Animal Care guidelines and approved by the University Animal Care Committee.

Alcohol intoxication by intragastric intubation: Ethanol dependence (Dependent, N = 36) was induced using an intragastric intubation procedure in which ethanol was administered four times daily for six consecutive days. On the first day, rats were intubated with a total of 11.0 g/kg ethanol in 4 fractional doses of 3.0, 3.0, 2.5, and 2.5 g/kg ethanol, administered at 4-hour intervals. During days 2 to 6, 12 hours after the last intubation on the preceding day, rats received a total of 10.0 g/kg ethanol in 4 fractional doses of 3.0, 2.5, 2.5, and 2.0 g/kg, again separated by 4-hour intervals. Rats serving as nondependent controls (Nondependent, N=36) were intubated with vehicle solution for ethanol (Boost®, Nestle) at time intervals and relative volumes identical to those in ethanol-dependent rats (22 ml/kg/day).

Withdrawal: Overt physical withdrawal was assessed 12 hours following last ethanol intubation. Using a withdrawal rating scale adapted from [28], ethanol withdrawal signs, including VentrO-medial Limb Retraction (VLR), irritability to touch (vocalization), tail rigidity, motor gating, and body tremors, were scored. Each sign was assigned a score of 0 to 2, based on the following severity scale: 0 = no sign, 1 = moderate, 2 = severe. The sum of the 4 observation scores (0 to 10) was used as to quantify withdrawal severity.

Elevated plus maze: Two weeks after the termination of the intragastric intubation procedure, open arm exploration was evaluated during 5-min test session on the elevated plus maze.

Conditioned place preference: Rats underwent preconditioning as, described above, during the three days preceding the elevated plus maze test. Conditioning sessions began the day following the elevated plus maze test. Animals were divided into three groups receiving 2 (N=24), 3 (N=24), or 4 (N=24) ethanol-context pairings along with an equal number of vehicle-context pairings in 20-min sessions.

On the day following the last conditioning session, time spent in the ethanol-paired chamber was evaluated in a single 15-min test session.

Experiment 3: Chronic Unpredictable Stress (N=72)

Subjects: Animals were male Wistar rats (Charles River, St. Constant, QC, Canada), weighing 225-250 g on arrival. Subjects were housed in pairs on a 12-hour lights on and 12-hour lights off schedule (lights off at 2100). The rat colony room was maintained for temperature and humidity. Food and water were made available ad libitum at all times excepting behavioural testing and during procedures within the CUS regimen that involved either food or water restriction. All behavioural tests were conducted between 1200 and 1900. Housing and testing procedures were conducted in strict adherence to the Canadian Council on Animal Care guidelines and approved by the University Animal Care Committee.

Chronic Unpredictable Stress: The chronic unpredictable stress regime consisted of six stressors (24 hour food restriction, 16 hour water restriction followed by one hour exposure to an empty bottle, 30

minute restraint sessions, prolonged light or dark cycles, 6 hour period in a soiled cage, 45 degree cage tilting) presented randomly once a day, five days a week. Subjects underwent the CUS regime for a period of six weeks, during which time half of the subjects received prandial exposure to alcohol for self-administration. Stressors, excepting food and water restriction, were presented between 1000 and 1600 and any given stressor was repeated during the week.

Elevated plus maze: Two weeks after the termination of the CUS procedure, open arm exploration was evaluated during 5-min test session on the elevated plus maze.

Conditioned place preference: Rats underwent preconditioning as, described above, during the three days preceding the elevated plus maze test. Conditioning sessions began the day following the elevated plus maze test. Animals were divided into three groups receiving 2 (N=24), 3 (N=24), or 4 (N=24) ethanol-context pairings along with an equal number of vehicle-context pairings in 20-min sessions.

On the day following the last conditioning session, time spent in the ethanol-paired chamber was evaluated in a single 15-min test session.

Results

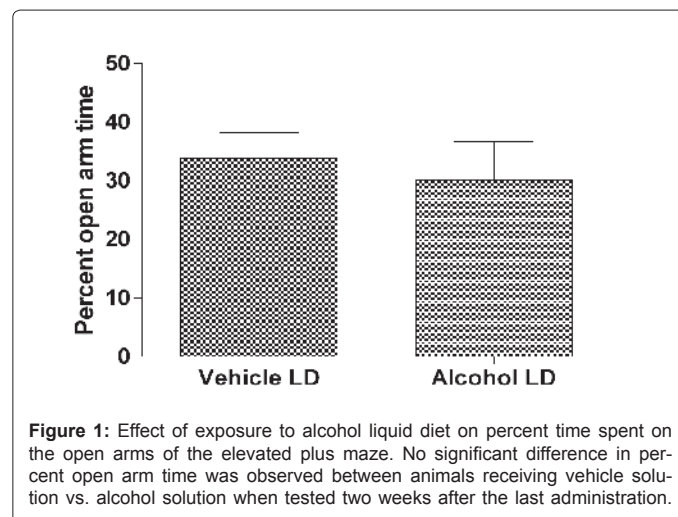
Experiment 1: Liquid Diet

Weight: No differences in weights of alcohol and control groups were observed during the administration phase (alcohol: 374.5 ± 27.1 ; control: 384.7 ± 30.4 ; $t(70) = 0.25$, $P = 0.80$), elevated plus maze (alcohol: 532.8 ± 42.7 ; control: 541.9 ± 40.5 ; $t(70) = 0.15$, $P = 0.88$), or conditioned place preference (alcohol: 539.2 ± 43.1 ; control: 546.2 ± 39.7 ; $t(70) = 0.12$, $P = 0.91$).

Intake: Animals receiving alcohol ingested an average of 11.7 ± 3.3 mg of alcohol per session. There was no difference between ($t(70) = 0.23$, $P = 0.82$) average intake during the first week (12.3 ± 3.8 mg) vs. the last week (11.1 ± 3.6 mg) of the procedure (Figure 1).

Elevated Plus Maze: At the two-week abstinence time point, no difference ($t(70) = 0.4714$, $p = 0.64$) in percent time spent on the open arms of the elevated plus maze was observed between alcohol (30.1 ± 6.5) and vehicle controls (33.8 ± 4.4). Shapiro-Wilk analysis did not reveal any significant deviation from normality in the percent open time data ($W = 0.985$, $P = 0.54$).

Conditioned Place Preference: With respect to control animals,



the overall split-plot ANOVA yielded a significant group by phase interaction ($F(2,64) = 4.43, p < 0.05$). Fischer's LSD post-hoc tests revealed that animals with four alcohol-context pairings spent more on the drug-paired side during the test vs. preconditioning phase, while those receiving two or three alcohol-context pairings did not differ in time spent in the alcohol-paired context between the pre-conditioning and test phases.

A similar pattern of results was observed in animals with a history of alcohol liquid diet. The overall split-plot ANOVA yielded a significant group by phase interaction ($F(2, 64) = 6.32, P < 0.05$). Fischer's LSD post-hoc tests revealed that animals with four alcohol-context pairings spent more on the drug-paired side during the test phase vs. preconditioning, while those receiving two or three alcohol-context pairings did not differ in time spent in the alcohol-paired context between the pre-conditioning and test phases.

Shapiro-Wilk analysis did not reveal any significant deviation from normality in time spent in the alcohol context during the preconditioning ($W = 0.956, P = 0.16$) or test phase ($W = 0.985, P = 0.88$).

Thus, no differences in the number of alcohol-context sessions that were required to support alcohol-based place conditioning were observed between the experimental and control treatment conditions (Figure 2).

Experiment 2: Intra-gastric Intubation

Weight: No differences in weights of alcohol and control groups were observed during the administration phase (alcohol: 423.5 ± 34.3 ; control: 467.7 ± 36.8 ; $t(70) = 0.88, P = 0.38$), elevated plus maze (alcohol: 519.4 ± 45.1 ; control: 527.2 ± 43.7 ; $t(70) = 0.12, P = 0.90$), or conditioned place preference (alcohol: 535.5 ± 43.2 ; control: 549.7 ± 40.8 ; $t(70) = 0.23, P = 0.81$).

Somatic Withdrawal: Effectiveness of the intra-gastric intubation regimen in producing acute somatic withdrawal, as evaluated 12 hours following the last intubation on Day 5, was confirmed by a significant

increase ($t(70) = 6.4846, p < 0.05$) in cumulative withdrawal score between alcohol (3.7 ± 0.4) and vehicle controls (0.8 ± 0.2).

Elevated Plus Maze: At the two-week abstinence time point, no difference ($t(70) = 0.5516, p = 0.58$) in percent time spent on the open arms of the elevated plus maze was observed between alcohol (32.1 ± 4.7) and vehicle controls (28.9 ± 3.4). Shapiro-Wilk analysis did not reveal any significant deviation from normality in the percent open time data ($W = 0.983, P = 0.44$) (Figure 3).

Conditioned Place Preference: With respect to control animals that had a history of intra-gastric intubation with boost vehicle, the overall split-plot ANOVA yielded a significant group by phase interaction ($F(2, 64) = 5.67, P < 0.05$). Fischer's LSD post-hoc tests revealed that animals with four alcohol-context pairings spent more on the drug-paired side during the test vs. preconditioning phase, while those receiving two or three alcohol-context pairings did not differ in time spent in the alcohol-paired context between the pre-conditioning and test phases.

In animals with a history of intra-gastric intubation of alcohol, the overall split-plot ANOVA yielded a significant group by phase interaction ($F(2, 64) = 4.98, P < 0.05$). Fischer's LSD post-hoc tests revealed that animals with three or four alcohol-context pairings spent more on the drug-paired side during the test phase vs. preconditioning, while those receiving two alcohol-context pairings did not differ in time spent in the alcohol-paired context between the pre-conditioning and test phases.

Shapiro-Wilk analysis did not reveal any significant deviation from normality in time spent in the alcohol context during the preconditioning ($W = 0.982, P = 0.81$) or test phase ($W = 0.957, P = 0.18$).

Thus, alcohol-based place conditioning was established at three alcohol-context pairings in animals with a history of alcohol intubation, while control animals required an additional alcohol-context pairing (Figure 4).

Experiment 3: Chronic Unpredictable Stress

Weight: No differences in weights of alcohol and control groups

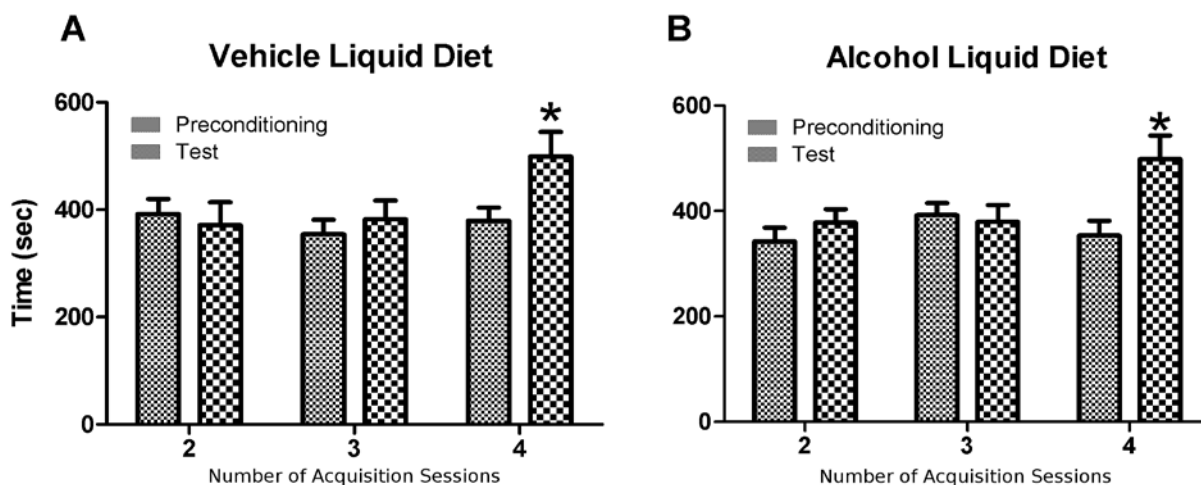


Figure 2: Mean time (sec) spent on drug-paired side during pre-conditioning and test phases across number of acquisition days. The symbol (*) indicates a significant difference ($p < 0.05$) from pre-conditioning in A) vehicle liquid diet controls and B) alcohol liquid diet animals.

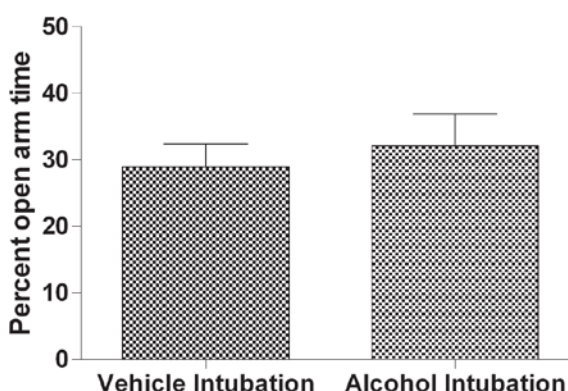


Figure 3: Effect of exposure to repeated alcohol intragastric intubation on percent time spent on the open arms of the elevated plus maze. No significant difference in percent open arm time was observed between animals receiving vehicle solution vs. alcohol solution when tested two weeks after the last administration.

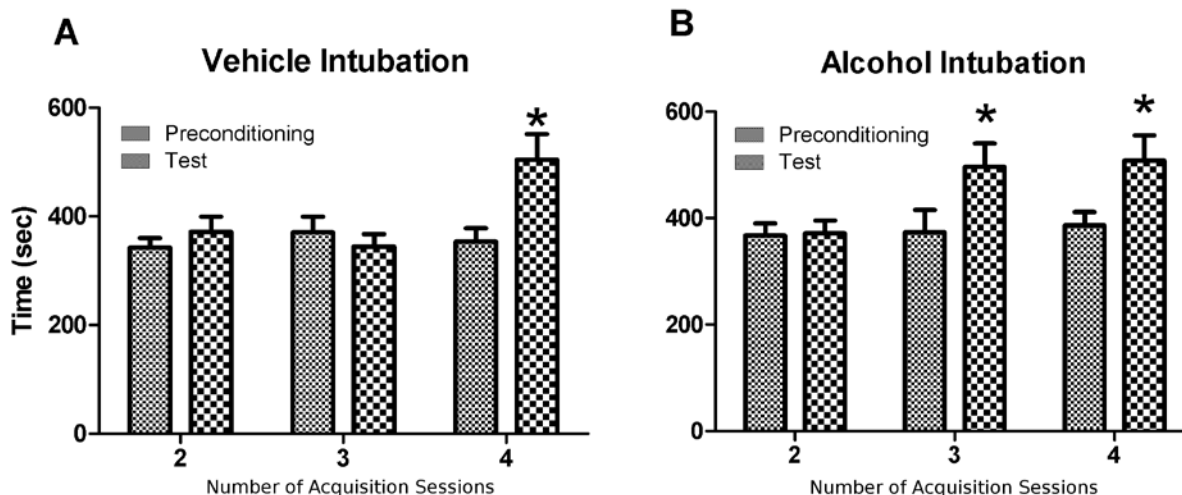


Figure 4: Mean time (sec) spent on drug-paired side during pre-conditioning and test phases across number of acquisition days. The symbol (*) indicates a significant difference ($p < 0.05$) from pre-conditioning in A) vehicle intubated controls and B) alcohol intubated animals.

were observed during the administration phase (CUS: 365.2 ± 25.4 control: 377.6 ± 28.7 ; $t(70) = 0.32$, $P = 0.75$), elevated plus maze (CUS: 508.1 ± 35.9 ; control: 539.5 ± 39.2 ; $t(70) = 0.59$, $P = 0.56$), or conditioned place preference (CUS: 520.4 ± 38.6 ; control: 548.1 ± 37.2 ; $t(70) = 0.52$, $P = 0.61$).

Elevated Plus Maze: At the two-week abstinence time point, animals with a history of chronic unpredictable stress exhibited a significantly lower ($t(70) = 2.0$, $p < 0.05$) proportion of time spent on the open arms (34.3 ± 3.8) vs. non-stress controls (24.3 ± 3.1) (Figure 5). Shapiro-Wilk analysis did not reveal any significant deviation from normality in percent open time data ($W = 0.984$, $P = 0.51$).

Conditioned Place Preference: With respect to control animals, the overall split-plot ANOVA yielded a significant group by phase interaction ($F(2, 64) = 4.67$, $P < 0.05$). Fischer's LSD post-hoc tests revealed that animals with four alcohol-context pairings spent more on the drug-paired side during the test vs. preconditioning phase, while those receiving two or three alcohol-context pairings did not differ in

time spent in the alcohol-paired context between the pre-conditioning and test phases.

In animals with a history of exposure to chronic unpredictable stress, the overall split-plot ANOVA yielded a significant group by phase interaction ($F(2, 64) = 5.24$, $P < 0.05$). Fischer's LSD post-hoc tests revealed that animals with three or four alcohol-context pairings spent more on the drug-paired side during the test phase vs. preconditioning, while those receiving two alcohol-context pairings did not differ in time spent in the alcohol-paired context between the pre-conditioning and test phases.

Shapiro-Wilk analysis did not reveal any significant deviation from normality in time spent in the alcohol context during the preconditioning ($W = 0.971$, $P = 0.44$) or test phase ($W = 0.989$, $P = 0.97$).

Thus, alcohol-based place conditioning was established at three alcohol-context pairings in animals with a history of exposure to

chronic unpredictable stress, while control animals required an additional alcohol-context pairing (Figure 6).

Discussion

The results from the present set of experiments indicate that following a two-week withdrawal period 1) animals receiving alcohol via access to a liquid diet required the same number of alcohol-context pairings to established place conditioning as controls (Experiment 1,2) a history of alcohol established via intragastric intubation decreased the number of alcohol-context pairings required to support place conditioning vs. controls (Experiment 2). Exposure to chronic mild stress over a period of six weeks produced anxiety-like performance on the elevated plus maze and also decreased the number of alcohol-context pairings required to support place conditioning vs. controls (Experiment 3). Levels of alcohol intake were comparable in the liquid diet and intubation condition which suggests that the

different pattern of results observed with respect to alcohol-based place conditioning in experiments 1 and 2 was not a function of the amount of alcohol consumed. No differences from their respective controls on anxiety-like performance on the elevated plus maze were found for either alcohol history group. With respect to the chronic unpredictable stress experiment, the findings suggest a history of chronic unpredictable stress results in dysregulation of both stress- and reward-related neurocircuitry in a persistent manner.

No effect of alcohol administered via intragastric intubation (Experiment 2) was observed on time spent on the open arms of the elevated plus maze. This finding is consistent with a previous report that suggests emergence, dissipation and re-emergence of anxiety-like behaviour following alcohol [29]. In the case of intragastric intubation, previous findings have demonstrated anxiety-like performance on the elevated plus maze following one week [30] but not three weeks of abstinence [18]. The lack of difference from controls at the two-

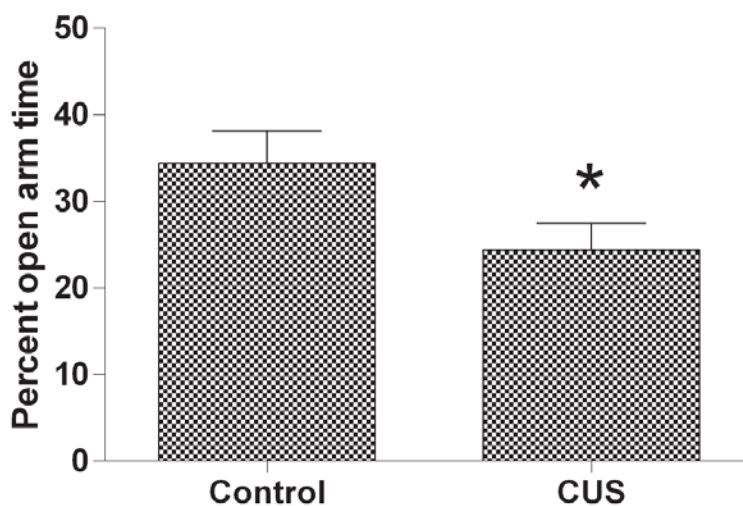


Figure 5: Effect of exposure to chronic unpredictable stress on percent time spent on the open arms of the elevated plus maze. A significant decrease in percent open arm time was observed in animals receiving chronic unpredictable stress vs. non-stress controls when tested two weeks after the last administration (* denotes $p < 0.05$ vs controls).

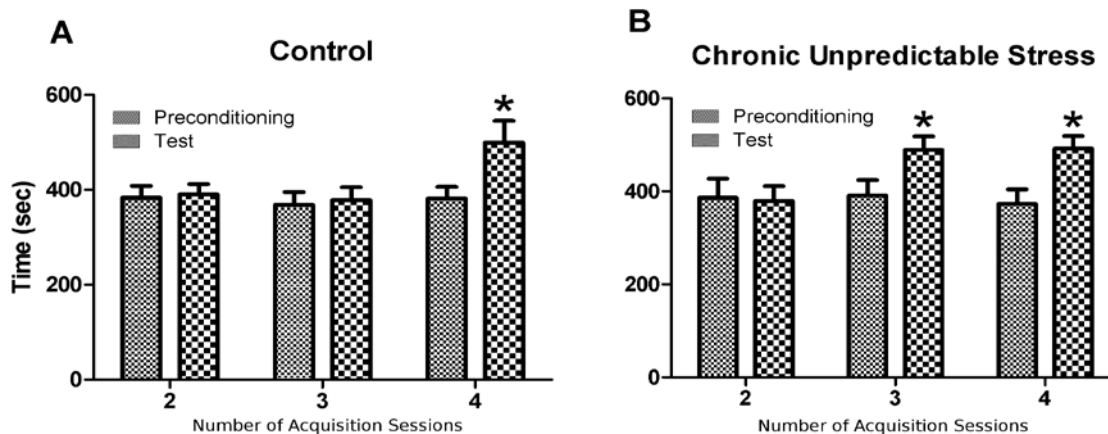


Figure 6: Mean time (sec) spent on drug-paired side during pre-conditioning and test phases across number of acquisition days. The symbol (*) indicates a significant difference ($p < 0.05$) from pre-conditioning in A) non-stress controls and B) chronic unpredictable stress animals.

week abstinence period utilized in the present experiment suggests a dissipation of anxiety-like performance on the plus-maze between one and two weeks of abstinence. A similar time course on overt expression of anxiety-like behaviour has also been observed in the case of cocaine. Mutschler and Miczek [25] found that rats trained to self-administer cocaine under binge-like conditions produced fewer ultrasonic vocalizations vs. yoked controls up to five days following the last cocaine administration. At two weeks of abstinence, no differences in the production of anxiety-like ultrasonic vocalizations [25]-consistent with our present findings with the effects alcohol history in both the voluntary (i.e. Alcohol Liquid Diet) and involuntary (i.e. Alcohol intubation) groups on anxiety-like performance on the elevated plus maze. Alternatively, failure to detect an alteration in anxiety-like performance may also be task-specific. Santucci and colleagues [31] found that rats receiving alcohol voluntarily in a liquid diet for 26 days exhibited thigmotactic tendencies in the Morris Water maze, indicative of increased anxiety, up to four months from the last alcohol administration.

While intragastric intubation decreased the number of alcohol-context pairings to establish place conditioning vs controls (Experiment 2), animals receiving alcohol liquid diet did not differ from controls in this regard (Experiment 1). Numerous differences between these administration regimens may have contributed to the different pattern of place conditioning results observed in these two experiments. One difference between experiments is that intubation of alcohol is involuntary while administration of a liquid diet is voluntary. Controllability of drug intake (i.e. voluntary vs. involuntary) may differentially alter stress responsivity during withdrawal [25]. While the overt anxiety-like response may dissipate by the two-week abstinence point, residual alterations in stress-regulatory function may drive drug-seeking behaviour more strongly through a negative reinforcement mechanism [32,33]. The present finding that chronic unpredictable stress exposure, in which no drug is administered, also subsequently increased the rate of alcohol-based place conditioning, supports this view.

Another difference is that while overall intake levels of alcohol were similar, intubated animals received widely distributed, large quantities of alcohol while those receiving alcohol via liquid diet were able to administer in smaller quantities but more frequently. Thus, peak Blood Alcohol Concentrations (BACs) may not have been similar. Following the same intragastric intubation regimen, it has been previously demonstrated that this procedure produces BACs of 200-250 mg/dl [18,30] while BACs of 94.3 mg/dL [28] have been observed in animals receiving liquid diet in a manner similar to the present experiment. Furthermore, animals receiving alcohol in a liquid diet did so over a longer duration, and thus, received a greater total amount of alcohol. Given that these animals were indistinguishable from controls, it appears that increased total alcohol intake is not critical in the development of alterations in reward-related learning as examined in the present investigation. Interestingly, animals subjected to chronic unpredictable stress required fewer alcohol-context pairings to support alcohol place conditioning vs. controls. This finding lends some support to the notion that repeated withdrawal-related stress in alcohol intubated animals may provide an explanation for the decreased number of required alcohol-context pairings required for CPP. Thus, it may not be the alcohol intake, per se, that produces this effect, but rather repeated alcohol-withdrawal stressors (See [11] for a review of alcohol withdrawal conceptualized as stress "kindling"). Similarly, withdrawal from intermittent exposure to alcohol vapour has been shown to increase alcohol self administration during acute withdrawal [34]. Interestingly, O'Dell and colleagues [34]

also demonstrated that the critical variable may be repeated withdrawal rather than the total alcohol intake. While rats that received two weeks of continuous exposure did not differ from controls on subsequent self administration, those receiving two weeks of intermittent exposure (i.e. 14 hours on/10 hours off) exhibited a significant increase vs controls despite have decreased total alcohol exposure. Furthermore, increased self-administration was specific to alcohol as animals receiving either intermittent alcohol vapour exposure did not differ from controls in self administration of a saccharin solution [34]. These findings are consistent with the present finding that total alcohol consumption did not account for alterations in the number of alcohol-context pairings required to support place conditioning.

Consistent with a previous report [24], animals exposed to Chronic Unpredictable Stress (CUS) exhibited decreased open arm exploration and increased burying in the defensive burying test two weeks after termination of the stress procedure. It is important to note the parameters under which CUS is conducted, as well as the period between the last stress exposure and test, appear to be critical in determining whether alterations in open arm exploration on the elevated plus maze are observed. Matuszewich and colleagues [24], for example, did not observe any difference on plus maze or defensive burying performance in CUS animals one or seven days following termination of the stress procedure. The importance of time course is underscored by the findings of [35] that demonstrated anxiolytic elevated plus maze performance at two days following the last stress exposure. An additional consideration is the variation on the specific stressors used across implementations of chronic unpredictable stress in different laboratories which make it difficult to establish precisely how this procedure affects anxiety-like performance. Thus, it is somewhat unsurprising that while a number of studies find anxiogenic effects, consistent with the present investigation [25,36] others do not [37,38].

As previously indicated, results from the present investigation appear to suggest that the common feature which predicts more rapid development of alcohol-based CPP may be repeated stress exposure rather than a history of ethanol intake per se. This explanation is consistent with a large clinical literature which has linked stress exposure to enhanced craving and seeking of alcohol and other drugs in humans and non-human animals, including maternal stress [6], early life stress [7], social isolation [39], and food restriction [9]. Hedonic dysregulation provides a framework in which to understand the impact of stress on drug-seeking behaviour and offers clues to neural substrates which may mediate the interaction of these systems [12]. Breese and colleagues [11] suggest that stress mimics acute withdrawal from drugs of abuse and, further, that this state becomes sensitized upon repeated presentations of the stressor. Thus, in the case of both alcohol intubation as well as chronic unpredictable stress, animals may experience sensitization of a stress-like state that may facilitate later acquisition of place conditioning for alcohol. It is also important to note that while we and others have shown that prior exposure to stress increases alcohol-seeking behaviour, the relationship between stress and motivation is complex, particularly when examining alcohol consumption. Increases in alcohol consumption have been reported as a consequence of exposure to repeated stress [39] as well as exposure to an enriched environment [40].

Chronic unpredictable stress has been widely utilized as a model of depression and has been linked to decreased reward function [41]. Paradoxically, this may also provide an explanation for the present finding of enhanced effectiveness within the CUS group of alcohol-context pairings in establishing CPP. 1) Decreased dose sensitivity may

have enhanced reward value as there is a biphasic effect of alcohol on place conditioning with high doses producing an aversion [42]. Thus, if a dose between 0.5 mg/kg and 1.0 mg/kg is more effective in establishing place preference, decreased sensitivity may have resulted in more robust place conditioning. 2) Decreased motivation for conventional reinforcers may have enhanced the reward-value of alcohol, and associated stimuli, in a relative manner. Exposure to forced swim test [43] has been shown to enhance morphine-based place conditioning preferentially over food-based place-conditioning. Furthermore, Lepsch and colleagues [23] found that chronic stress exposure increased cocaine-induced locomotor output.

The present findings support the view that prior exposure to chronic mild stress or alcohol administration present significant risk factors to the subsequent development of alcohol-seeking behaviours [11,32,33]. Furthermore, the present findings offer insight into key features of alcohol administration which may be critical in such development. Future studies further elucidating the common and dissociable neural substrates underlying these observations may provide important clues towards development of pharmacotherapeutic targets of alcohol abuse.

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