Pavlovian Cue-Driven Alcohol-Seeking: The Role of Dopamine and Impact of Vendor Differences in Long Evans rats

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Abstract

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Lindsay M. Sparks

Rationale Drug-associated environmental stimuli can acquire incentive and motivational properties through Pavlovian conditioning, and come to function as conditioned cues that elicit drug-seeking behavior. *Objectives* The current experiments tested the hypothesis that dopamine mediates alcohol-seeking driven by Pavlovian alcohol-predictive cues. Studies were conducted in Long-Evans rats obtained from two different sources, based on published reports that oral alcohol consumption can differ within-strain. *Method* Male, Long-Evans rats (220-240 g on arrival) from Charles River (St-Constant, Canada) and Harlan Laboratories (Indianapolis, USA) received intermittent, 24-h access to ethanol (15%; *v*/*v*) and water via 2 bottles on the home cage (21 sessions). Next, rats were trained to discriminate between 2 auditory stimuli (10-sec each; white noise or clicker); one stimulus (CS+) was paired with ethanol (0.2 ml per CS+; 3.2 ml per session; oral consumption) and the second stimulus (CS-) was not paired with ethanol. During 17 daily, 60-min Pavlovian discrimination training (PDT) sessions rats received 16 random presentations each of the CS+ and CS- delivered according to a variable-time 67-sec schedule. Entries made into a fluid port to consume ethanol were recorded before, during and after each CS. Following PDT, rats were habituated (5 sessions; 60-min) to a different, non-alcohol context where the cues and ethanol were withheld. At test, responding to the non-extinguished CS+ and CS- was measured in the second, non-

alcohol context in the absence of ethanol. Rats received injections of a dopamine D1-like receptor antagonist (SCH 23390; 0, 3.33 and 10 μg/kg; 1 ml/kg; s.c.) or a dopamine D2-like receptor antagonist (eticlopride; 0, 5, 10 μg/kg; 1 ml/kg; s.c.) 15-min before the test. In addition, we examined the impact of SCH 23390 (10 μg/kg; 1 ml/kg; s.c.); and eticlopride (10 μg/kg; 1 ml/kg; s.c.) on responding to the CS+ and CS- during PDT sessions when the CS+ was paired with ethanol. *Results* Rats from Charles River gained weight more rapidly and attained significantly higher overall weights than rats from Harlan. Across pre-exposure, ethanol consumption and preference were higher in Harlan rats. Across PDT sessions, rats from both vendors responded more to the alcohol-paired CS+ than the CS-. Total port-entry responses decreased across habituation in the second context. At test in a non-alcohol context, saline infused rats responded more to the CS+ than the CS-, indicating that discrimination between the two cues remained intact despite the absence of ethanol at test. Pre-treatment with SCH 23390 dose-dependently attenuated CS+ responding in rats from both vendors. However, eticlopride dose-dependently reduced CS+ responding in Harlan rats, but not in rats from Charles River. An infusion of SCH 23390, but not eticlopride reduced CS+ responding when cue presentations were paired with ethanol. *Conclusion* These results indicate novel differences in Long Evans rats obtained from different breeders. They also suggest that dopamine neurotransmission is required for responding to Pavlovian alcohol-predictive cues that are experienced in a non-alcohol context.

Keywords: Alcohol, Pavlovian conditioning, cues, context, dopamine, SCH 23390, eticlopride, vendor differences, Long-Evans

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General Introduction

Drug and alcohol addiction can be characterized by cycles of drug use, abstinence and relapse (Koob, Sanna, & Bloom, 1998). Nearly two-thirds of alcoholics relapse to drinking within weeks to months of first initiating treatment (Sinha, 2011). Additionally, 1-year outcome studies report that more than 85% of addicts relapse and resume drug use within one year of treatment (Sinha, 2011). Although considerable progress has been made in developing treatments for addiction, there are few interventions that consistently and effectively prevent relapse.

There is mounting support for the hypotheses that relapse can be facilitated by exposure to environmental stimuli that are associated with drug intake (O'Brien, Childress, McLellan, & Ehrman, 1992). Environmental stimuli that are repeatedly paired with the pharmacological effects of a drug can acquire incentive value through Pavlovian conditioning, and thereby function as conditioned cues that elicit behavioral, physiological and subjective reactions that may lead to drug use (Robbins & Ehrman, 1991; See, 2002; Weiss, 2005). These conditioned cues can be broadly divided into two categories, based on their relationship with drug intake. *Discrete drug cues* are stimuli that are closely linked with the act of drug intake, and reliably occur in close temporal proximity with the pharmacological effect of drugs. For example, the sight, smell and taste of an alcoholic beverage are sensory properties of alcohol that routinely precede intoxication, and have been shown to induce craving (McCusker & Brown, 1990).

Conversely, *contextual drug cues* are multimodal stimuli that are consistently present in an addict's environment during drug administration (Conklin, Robin, Perkins, Salkled, & McClernon, 2008). For example, individuals who regularly smoke cigarettes in a car might learn to associate the shared characteristics of cars with the pharmacological effects of nicotine. That drug-contexts can stimulate craving has recently been demonstrated (Conklin et al., 2008), suggesting that like discrete drug cues, contexts may have the potential to induce relapse.

Much of the progress in understanding the role of environmental cues in drug-seeking comes from animal models of relapse (Stewart & de Wit, 1981; de Wit & Stewart, 1983), which utilize instrumental conditioning procedures. Rats are trained to perform an instrumental response such as a lever-press or nose-poke in order to obtain drug, and drug delivery is paired with a discrete stimulus, such as a compound tone-light cue. Extinction is then conducted by withholding both the drug and the compound cue. At test, subjects are allowed to respond for the compound cue in the absence of drug delivery, and cue-induced reinstatement of drug-seeking is observed if responding is higher at test compared to extinction. Traditionally, all three phases of the experiment are conducted in the same environmental context, which is linked with the availability of drug during self-administration and the absence of drug during extinction.

Research conducted using instrumental conditioning models has found that discrete and contextual cues associated with alcohol consumption can reinstate alcohol-seeking in rats (Katner & Weiss, 1999; Liu & Weiss, 2002; Nie & Janak, 2003; Chaudhri, Sahuque, & Janak, 2009). Such models have been used to characterize the neurobiological underpinnings of cue- and context-induced reinstatement, and have

identified the neurotransmitter dopamine as a key player (Crombag, Grimm, & Shaham, 2002; Liu & Weiss, 2002; Hamlin, Newby, & McNally, 2007; Bossert, Poles, Wihbey, Koya, & Shaham, 2007; Chaudhri et al., 2009). While research conducted using instrumental conditioning procedures has provided valuable insight into the mechanisms that mediate reinstatement, it is noteworthy that in human addicts cue-induced craving is elicited by drug-predictive cues that are believed to have acquired incentive properties through Pavlovian learning (Robbins & Ehrman, 1991; Weiss, 2005). Dopamine neurotransmission mediates cue-induced reinstatement of instrumental drug-seeking; however, little is known about the role of dopamine in drug-seeking elicited by discrete, Pavlovian drug-predictive cues. Furthermore, discrete and contextual drug cues are believed to mediate drug-seeking through potentially distinct neurobiological mechanisms (Bossert et al., 2007; Chaudhri , Sahuque, Schairer, & Janak, 2010). Therefore, studies aimed at identifying the role of dopamine in responding to discrete drug cues should take into consideration the environmental context in which behavioral responding to such cues is tested.

Based on this rationale, the experiments in this thesis examined the role of dopamine in alcohol-seeking elicited by a Pavlovian-conditioned, alcohol-predictive cue in rats. In an effort to better isolate the requirement of dopamine in responding to discrete alcohol-cues, the effect of dopamine D1- and D2- receptor antagonists on Pavlovian cue-driven alcohol-seeking was tested in an environmental context that had never been associated with alcohol intake. The impact of dopamine antagonists on cue-driven alcohol-seeking during Pavlovian conditioning sessions where the cue was paired with alcohol was also assessed.

A second objective of these experiments was to investigate vendor differences between male, Long-Evans rats obtained from two different suppliers; Charles River (St-Constant, Canada) and Harlan Laboratories (Indianapolis, USA). This inclusion was made based on published reports that oral ethanol intake can vary within rats from the same strain that are obtained from different suppliers (Palm, Roman, & Nylander, 2011). In the present research, vendor differences were examined in oral alcohol consumption, Pavlovian-conditioned alcohol-seeking, and the effect of dopamine D1- and D2-receptor antagonists on responding to Pavlovian, alcohol-predictive cues.

The link between drug craving and relapse

Research conducted in human drug users and addicts indicates that drug craving can be elicited by several factors that include environmental stressors (Sinha, 2007), re-exposure to priming doses of drug (de Wit, 1996) and environmental stimuli associated with drug use (Robbins & Ehrman, 1991). A discussion on the behavioral, psychological and neurobiological mechanisms that mediate the impact of stress and priming on relapse is beyond the scope of this thesis, which focuses on the capacity of drug-predictive environmental cues to drive conditioned drug-seeking. There is substantial support for the hypothesis that previously neutral stimuli that are repeatedly associated with drug consumption can acquire incentive-motivational value through classical, Pavlovian-conditioning, a fundamental learning process that occurs in many animal species (Robinson & Berridge, 1993; Robinson & Berridge, 2003; Weiss, 2005; Berridge, Robinson, & Aldridge, 2009). As a result, environmental stimuli paired with drugs come

to function as conditioned cues that elicit craving and can motivate drug-seeking behavior (Ehrman, Robbins, Childress, & O'Brien, 1991; Milton & Everitt, 2010).

Environmental stimuli that predict drug availability can be broadly characterized as either *discrete drug cues* (proximal) or *contextual drug cues* (distal; Conklin et al., 2008). Discrete cues are central to an individual's drug use ritual. They can include sensory stimuli that are closely linked to, and therefore reliable predictors of, the pharmacological effects of a drug. For instance, the sight, smell and taste of a preferred alcoholic beverage serve as discrete cues that can elicit craving (McCusker & Brown, 1990). Contextual cues refer to stimuli that are consistently present in an addict's environment during drug use, but that are not directly tied to the act of drug administration. For example, the bar in which an individual frequently consumes alcohol might function as a context that could elicit craving (Conklin et al., 2008). Collectively, discrete and contextual drug cues function as Pavlovian-conditioned cues that can elicit reactivity. Furthermore, reactivity to discrete alcohol-cues is associated with an increased risk for relapse and predicts worse drinking outcomes in alcoholics (Rohsenow et al., 1994; Litt, Cooney, & Morse, 2000), suggesting that conditioned responses elicited by drug-predictive cues, discrete or contextual, may be an important component of relapse. The capacity of discrete drug cues to elicit conditioned reactivity such as craving and elevated physiological responses is well documented. Early studies report that alcoholics exhibit increased pupillary dilation in response to the smell of a preferred alcoholic beverage and are at greater risk for relapse (Kennedy, 1971). Additionally, alcoholics presented with the sight and smell of their preferred alcoholic beverage show increased salivation to such discrete alcohol cues, compared to non-alcoholics (Monti, Binkoff,

Abrams, Zwick, Nirenberg, & Liepman, 1987). Recent studies using a personalized imagery procedure report that alcohol-/drug-, and stress-related stimuli trigger craving, negative emotion, anxiety and physiological responses in abstinent alcoholics (Sinha, 2007). When compared to social drinkers, alcohol-dependent individuals exposed to alcohol-related cues report experiencing a persistent craving state, as well as increases in negative emotion, anxiety, systolic blood pressure and behavioral distress responses (Sinha, Fox, Hong, Bergquist, Bhagwagar, & Siedlarz, 2009). Together, these findings highlight the relationship between cue reactivity and discrete drug cues, providing evidence that they can pose a threat to abstinence.

The hypothesis that drug-associated environmental contexts can trigger conditioned responding was first proposed by Wikler (1965; 1984), who found that abstinent opioid users often reported experiencing the acute effects of withdrawal long after they ceased using the drug. Additionally, these symptoms were experienced when abstinent drug users encountered environments that were similar to those experienced during active drug use. Based on these reports, it was hypothesized that physical dependence might become conditioned to environmental stimuli that were present in the background during drug use, as they predict drug availability.

Though there is substantial evidence that discrete cues elicit craving and changes in physiological responses, few studies have investigated the independent influence of contextual drug cues on relapse. In a study aimed at investigating the associative strength of smoking-related cues, Conklin et al. (2008) examined whether tobacco-related contextual cues were sufficient to elicit cue reactivity in the total absence of discrete smoking cues. As expected, results indicated that discrete cues elicited strong cue

reactivity. Interestingly, tobacco contexts that were devoid of discrete smoking cues elicited higher craving when compared to neutral contexts. These results suggest that contextual cues can acquire associative strength and may serve to stimulate drug-seeking in the same manner as discrete drug cues.

Animal models of cue- and context-induced relapse to drug-seeking

The cue-induced reinstatement procedure is an established preclinical animal model that has been developed to investigate the role of discrete cues in drug-seeking. As described earlier, the ability of discrete drug cues to induce drug-seeking is assessed using operant conditioning, in which reinstatement of instrumental responding above extinction levels is observed upon response-contingent presentation of discrete cues that were previously associated with drug delivery (Ciccocioppo, Sanna, & Weiss, 2001; Liu & Weiss, 2002; Nie & Janak, 2003; Bossert et al., 2007; Hamlin et al., 2007; Liu et al., 2010).

Animal models have also been developed to investigate the role of contextual cues in relapse. These models are adapted from the ABA renewal procedure (Bouton & Bolles, 1979), which was first developed to study the influence of context in fear conditioning. In Pavlovian fear conditioning a conditioned stimulus (CS) is paired with an aversive, unconditioned stimulus (US; e.g., foot shock) in a distinctive context (A). Subjects learn to freeze during the CS, in anticipation of the US. Pavlovian conditioning is followed by extinction in an alternate context (B), where the CS is presented without

the US, and a reduction in freezing is observed. At test, presentations of the CS without the US in the prior training context (A) trigger a renewal of freezing to the CS.

The first study to test the renewal of drug-seeking was conducted by Crombag & Shaham (2002). Here, rats were trained to self-administer a combination of heroin and cocaine (i.e. speedball) in a self-administration context, referred to as context A. Following extinction in either context A or a novel context (B), rats were tested for renewal of speedball-seeking in the drug-associated (A) or novel context (B). At test, rats that received extinction in Context B demonstrated a renewal of instrumental responding upon exposure to the self-administration context (A), an effect that was not observed upon return to the novel context (B). This data, interpreted as context-induced reinstatement of drug-seeking, demonstrates the strong influence of drug-associated contextual cues on drug-seeking. The ABA renewal effect has since been replicated with other drugs of abuse, such as cocaine (Crombag et al., 2002), heroin (Bossert, Liu, Lu, & Shaham, 2004), and alcohol (Chaudhri, Sahuque, & Janak, 2008).

Studies that have used the renewal procedure to study context-induced relapse to drug-seeking typically assess this effect using an operant conditioning paradigm. However, drug-contexts can also trigger the renewal of Pavlovian-conditioned drug-seeking. In this task, rats are trained in a distinctive context (A) to discriminate between two auditory stimuli; one stimulus (CS+) is paired with alcohol, while the other stimulus (CS-) is not. As they learn the predictive value of the CS+, rats gradually check the fluid port where alcohol is delivered more frequently during the CS+, compared to the CS-. Following Pavlovian discrimination training, port entry responses are extinguished in a different context (B) where presentations of the CS+ no longer result in alcohol delivery.

At test, rats are once exposed to the CS+ and CS- without alcohol in the prior drug-associated context (A). This manipulation causes a selective increase in responding to the alcohol-predictive CS+, with no change in responding to the CS- (Chaudhri et al., 2008; Chaudhri et al., 2010). The results of these studies suggest that contextual cues that have been paired with alcohol delivery have the potential to impact relapse to alcohol-seeking.

Drug associated contexts can also invigorate instrumental responding for discrete drug cues. Tsiang & Janak (2006) compared the interactive effects of discrete and contextual alcohol cues using an operant conditioning paradigm. Mice were trained to lever press for alcohol delivery paired with a compound cue presentation (tone-light stimulus) in context A. Behavior was then extinguished in context B, where a lever press no longer resulted in ethanol delivery or compound cue presentation. Reinstatement was then tested under three conditions; upon exposure to the self-administration context without the compound cue, and upon presentation of the compound cue in either the extinction context or the self-administration context. The level of active lever responses was significantly greater when the compound cue was presented in the alcohol-associated context relative to the alcohol-associated context alone, suggesting alcohol contexts can invigorate responding for discrete alcohol. Such findings lend further support to the significant role of context in drug-seeking, and suggest that the interactive effects of discrete cues and contexts may be a more powerful trigger for drug-seeking when compared to discrete or contextual cues alone.

The role of dopamine in alcohol reinforcement

Animal models have enabled researchers to study the neurobiological mechanisms that mediate drug reinforcement, and converging evidence suggest that many drugs of abuse share a common feature; they all act on the brain's reward circuitry. Specifically, a role for dopamine neurons has been identified in reward-related behavior and motivational processes (Wise & Bozarth, 1987). Dopamine is a catecholamine neurotransmitter that acts in the central nervous system (CNS) and is synthesized in neural cell bodies in the midbrain, originating from the substantia nigra (SN) and ventral tegmental area (VTA). Through the mesolimbic dopamine system, the VTA transmits dopaminergic projections to regions of the telencephalon that include the medial prefrontal cortex (mPFC) and the nucleus accumbens (NAc; Iversen, Iversen, Bloom, & Roth, 2009). More specifically, the projection from the VTA to the NAc has been strongly linked to motivated behavior, reward-seeking, attention and locomotor activity (Iversen et al., 2009). The NAc has been identified as a key area that mediates reinstatement of drug-seeking and its two subregions, the core and shell, are believed to be differentially important for cue- and context-induced reinstatement, respectively (Everitt & Robbins, 2005; Bossert et al., 2007; Chaudhri, Sahuque, Cone, & Janak, 2008).

As is the case with other drugs of abuse, dopamine release in the striatum is believed to be a putative mechanism for alcohol reinforcement (Wise & Bozarth, 1987). Mice that are genetically deficient in dopamine D1-receptors show reduced alcohol-seeking behavior. Additionally, blocking dopamine D1- and D2-receptors in wild-type mice reduces alcohol consumption, suggesting that both D1- and D2-dopamine receptor

mechanisms are involved in alcohol-seeking (El-Ghundi et al., 1998). In rats, dopamine release is stimulated by oral consumption of alcohol as well as by anticipation of access to alcohol (Katner & Weiss, 1999; Yoshimoto, McBride, Lumeng, & Li, 1992). Specifically, rats exposed to an alcohol-associated environment show increased activation of mesolimbic dopamine neurons during a period of time immediately before alcohol is made available, suggesting that dopamine is involved in conditioned responding to alcohol-associated contextual stimuli (Katner & Weiss, 1999).

The role of dopamine in cue- and context-induced reinstatement

Several lines of research suggest that dopamine neurotransmission is required for cue- and context-induced reinstatement of drug-seeking. Katner & Weiss (1999) investigated the impact of discriminative olfactory stimuli on instrumental alcohol-seeking, and used intracranial microdialysis to measure dopamine release in the NAc at test. Discriminative stimuli consisted of a banana odour (S+) that signalled alcohol availability during the self-administration, and an orange odour (S-) that signalled the availability of a quinine solution (alcohol non-availability). Following discrimination training and subsequent extinction of active lever responding, subjects were exposed to the operant conditioning chamber for a 20-minute "waiting period" before the start of the reinstatement test in order to prepare for the measurement of dopamine by microdialysis. At test, rats were exposed to the discriminative stimuli in the absence of alcohol delivery, and dopamine samples were collected at 5 minute intervals. A recovery of responding on the active lever was observed at test, with significantly higher responding upon

presentation of the S+ (i.e. banana odour) when compared to the S- (i.e. orange odour). These results suggest that alcohol-predictive discriminative stimuli can reliably elicit alcohol-seeking after extinction. An analysis of dopamine concentrations found a small but significant increase in dopamine release during the "waiting period", which confirms earlier reports that the anticipation of alcohol can activate mesolimbic dopamine neurons (Weiss, Lorang, Bloom, & Koob, 1993; Katner, Kerr, & Weiss, 1996). Interestingly, presentations of the S+ resulted in a decrease in dopamine release, an effect that was not observed in the S- condition. One explanation for this decrease might be due to a mismatch between in the predicted alcohol-availability at test and the absence of alcohol delivery (Schultz, Dayan, & Montague, 1997). Overall, these findings confirm the involvement of dopamine in responding to alcohol-predictive cues.

Using a similar procedure, the ability for dopamine D1- and D2-receptor antagonists to reverse the reinstatement of cue-induced alcohol-seeking was tested (Liu & Weiss, 2002). Here, the reinstatement of operant alcohol-seeking was observed upon presentation of the alcohol-predictive discriminative stimulus (S+; banana odour), but not the S- (anise odour). Furthermore, administration of a D1-receptor antagonist (SCH 23390) or a D2-receptor antagonist (eticlopride) dose-dependently attenuated the latency to initiate responding, and significantly reduced the number of lever presses during the reinstatement test. These data suggest that intact dopamine neurotransmission is required for responding to an alcohol-predictive discriminative stimulus, and that both D1- and D2-receptor subtypes are involved in this effect.

A role for dopamine in context-induced reinstatement of drug-seeking using the ABA renewal paradigm has also been identified. Here, the effects of SCH 23390 and

raclopride, a D2-receptor antagonist, were tested in rats trained to self-administer cocaine (Crombag et al., 2002). Context-induced renewal of lever-pressing was observed in saline pre-treated rats upon placement into the self-administration context at test. Both D1- and D2-receptor antagonists reduced context-induced renewal of cocaine-seeking, suggesting that dopamine neurotransmission at both receptor subtypes is required for this effect. Similar findings have been reported in cue-induced reinstatement of drug-seeking, where systemic administration of SCH 23390 or eticlopride significantly attenuated cue-induced reinstatement of nicotine-seeking in rats (Liu et al., 2010). Collectively, these findings support the involvement of dopamine in responding for drug-predictive discrete and contextual cues.

Vendor differences in rodent research: An important empirical consideration

Developing treatments for alcohol use disorders is challenging, as the behavioral and neurobiological mechanisms underlying alcohol reinforcement and alcohol-mediated behavioral effects remain unclear. These questions have been studied using various rat strains, some of which are selectively bred for high and low voluntary alcohol consumption (Bell, Rodd, Lumeng, Murphy, & McBride, 2006). Strain differences in voluntary alcohol intake can parallel drinking patterns in people suffering from alcoholism or alcohol abuse disorders, who exhibit varied patterns of drinking and can achieve different levels of intoxication (e.g. alcoholics, vs. heavy drinkers, vs. binge drinkers, vs. social drinkers). Making use of the genetic variability in rats enables the

development of animal models that can extend to the different patterns of human alcohol use.

While strain differences can provide useful information, research has shown that there are differences in alcohol consumption and alcohol-mediated behaviors from rats of the same strain that are obtained from different vendors. A study by Palm, Roman, & Nylander (2011) compared voluntary ethanol intake in Wistar rats obtained from five different suppliers (B&K Universal, UK [BK]; Charles River, Germany; Harlan Laboratories, Indianapolis, IN, USA [Hsd]; Harlan Laboratories, The Netherlands [RccHan^{TM}]; and Taconic, Denmark). Rats underwent voluntary ethanol consumption using a 3-bottle choice ethanol exposure procedure in which they received access to 5% ethanol, 20% ethanol and water for 6 weeks. Oral consumption of each solution was measured. Overall, RccHan^{TM} rats (Harlan Laboratories, The Netherlands) significantly differed from rats from other vendors. For example, they exhibited the highest ethanol preference percentage (80%), which was significantly higher than rats from BK (B&K Universal, UK) that had the lowest ethanol preference percentage of approximately 20%. Across 6 weeks of pre-exposure, only Hsd rats (Harlan Laboratories, Indianapolis) displayed an increase in ethanol consumption in 5% ethanol solution. In summary, although all rats used were of the Wistar strain, significant differences in ethanol consumption were observed depending on the supplier.

Preliminary data from our laboratory has also demonstrated differences in ethanol intake and preference between male Long-Evans rats obtained from Charles River Canada (St-Constant, Canada) or Harlan Laboratories (Indianapolis, USA). These differences in ethanol consumption suggest that the choice of vendor can have serious

implications for the outcome of ethanol studies, making the generalization of findings across research conducted in different laboratories increasingly difficult. Consequently, research presented in this thesis incorporated male, Long-Evans rats from two different sources in order to examine potential differences in oral ethanol consumption, the acquisition and expression of Pavlovian-conditioned alcohol-seeking, and sensitivity of cue-driven alcohol-seeking to dopamine.

Specific aims of the present research

The research presented above highlights an important role for dopamine in cue- and context-induced relapse to drug and alcohol-seeking. However, the majority of these studies in preclinical models have used instrumental conditioning procedures, in which the impact of dopamine D1- and D2-receptor antagonists are examined on lever pressing that previously resulted in drug delivery. Little is known about the role of dopamine in responding elicited by Pavlovian drug-predictive cues, which is important because reactivity to drug cues in humans is believed to be mediated through Pavlovian learning mechanisms. Furthermore, few studies attempt to isolate the behavioral and neurobiological underpinnings of cue- and context-induced relapse which, if understood, could be differentially targeted by pharmacotherapies against relapse.

Based on these considerations, the present experiments investigated the role of dopamine D1- and D2-receptors in responding to a non-extinguished, alcohol-predictive Pavlovian cue in rats. We hypothesized that dopamine neurotransmission is required for cue-driven alcohol-seeking, and predicted that blocking dopamine D1 and D2 receptors

with SCH 23390 and eticlopride, respectively, would dose-dependently attenuate cue-driven alcohol-seeking. In order to isolate the role of dopamine in responding to a discrete alcohol cue, the impact of dopamine antagonists on behavior was tested in a context that had never been associated with alcohol availability or consumption. A second objective was to investigate vendor differences in Long-Evans rats obtained from Charles River and Harlan Laboratories, with respect to oral alcohol consumption, the acquisition and expression of Pavlovian cue-driven alcohol-seeking, and the role of dopamine in responding to discrete alcohol-predictive cues.

Oral alcohol consumption was assessed during a 21-session pre-exposure phase, in which rats had access to alcohol and water via two bottles on the home cage. Sessions in which alcohol and water were provided were alternated with sessions in which both bottles contained water. This intermittent 24-hr access schedule has been shown to produce an escalation in alcohol consumption in rats (Wise, 1973; Simms et al., 2008).

Following pre-exposure, rats underwent Pavlovian Discrimination Training (PDT) sessions in which they were presented with 2 discrete, auditory cues in a distinct environmental context. One cue was consistently paired with alcohol delivery (CS+) and the second cue (CS-) was presented without alcohol. Subjects were then habituated in a second, different context where cue presentations and ethanol delivery were withheld. The purpose this phase was to reduce the frequency during which rats checked the fluid port where alcohol has previously been delivered during PDT sessions. At test, cue-driven alcohol-seeking was assessed by presenting both the alcohol-predictive cue (CS+) and CS- in the second, non-alcohol context without alcohol delivery. The role of dopamine was examined by systemically infusing rats with SCH 23390 (Exp. 1) or

eticlopride (Exp. 2) before test. We predicted that both the D1- and D2- receptor antagonists would dose-dependently attenuate Pavlovian cue-driven alcohol-seeking. Lastly, we examined the impact of SCH23390 and eticlopride on Pavlovian cue-driven alcohol-seeking during sessions in which the CS+ was paired with alcohol delivery. As operant responding for alcohol is reduced by dopamine antagonists (Rassnick, Pulvirenti, & Koob, 1992; Hodge, Samson, & Chappelle, 1997), we anticipated a similar reduction in Pavlovian alcohol-seeking at test.

General Methods

Subjects

Male, Long Evans rats (220-240 grams on arrival) were obtained from Charles River Canada (St-Constant, Canada) and Harlan Laboratories (Indianapolis, USA). Subjects were individually-housed in polycarbonate shoebox cages containing beta-chip bedding (colony room temperature, 21°C) and maintained on a 12-hour light/dark cycle (lights ON at 0700-hour). All experimental procedures were conducted during the light phase of the light/dark cycle. Access to rat chow (Charles River Rodent Animal Diet) and water was unrestricted throughout the experiment, unless otherwise indicated. All procedures were approved by the guidelines of the Canadian Council on Animal Care and the Concordia University Animal Research Ethics Committee.

Apparatus

Behavioral training and testing were conducted using twelve operant conditioning chambers (32.8 cm x 32.8 cm x 32.8 cm; Med Associates Inc., St-Albans, VT) each contained within a ventilated, sound-attenuating melamine cubicle (53.6 cm x 68.2 cm x 62.8 cm). Each chamber was composed of a stainless steel bar floor, paneled aluminum side-walls, and a clear, Plexiglas rear wall, ceiling and front door. The right wall of each operant chamber featured a central port, which contained a circular fluid receptacle into which fluid could be delivered. Fluid delivery occurred through a 20-ml syringe attached to a pump (Med Associates Inc., PMH-100, 3.33 rpm) that was located outside the cubicle. The upper left wall of the operant chamber featured a clicker stimulus (Med Associates, ENV-135M) and white noise stimulus generator (Med Associates, ENV-

225SM), as well as a white houselight (75W, 100mA). Port entries were measured by interruptions of infra-red beam across the entrance of the port and recorded to a computer using Med PC-IV software (Med Associates Inc.), which also controlled fluid delivery and stimulus presentations.

Drugs

Ethanol (15% *v*/*v*) was prepared by diluting 95% ethanol in tap water. Sweetened ethanol was prepared by dissolving sucrose (2% *w*/*v*) in 15% ethanol. SCH 23390 hydrochloride (R (+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; Sigma Aldrich) was dissolved in 0.9% sodium chloride to obtain a dose of 10 µg/ml. An additional dose of 3.33 µg/ml was obtained through serial dilution. Eticlopride hydrochloride (FLB 131, S-(−)-3-Chloro-5-ethyl-N-[(1-ethyl-2-pyrrolidinyl) methyl]-6-hydroxy-2-methoxybenzamide hydrochloride; Sigma Aldrich) was dissolved in 0.9% sodium chloride to obtain a dose of 10 µg/ml. An additional dose of 5 µg/ml was obtained through serial dilution. Doses of SCH 23390 and eticlopride were contained in 1 ml aliquots and stored at -20°C until use. SCH 23390 was protected from exposure to light at all times due to its light sensitive properties. Doses of SCH 23390 and eticlopride were selected based on previous reports that demonstrate behavioral effectiveness using similar behavioral procedures (Liu & Weiss, 2002; Bossert et al., 2007; Hamlin et al., 2007; Liu et al., 2010;).

General Behavioral Procedures

Ethanol pre-exposure

Two weeks after arrival, subjects were exposed to the taste and pharmacological effects of ethanol (15% *v*/*v*) in their home cages. Pre-exposure was conducted over 21 sessions, with 3 sessions per week (Monday, Wednesday and Friday) using a 24-hour intermittent-access schedule that has been shown to induce high ethanol consumption in rats (Wise, 1973; Simms, et al., 2008). At the start of each session rats were handled and weighed. They were then given access to ethanol in a pre-weighed, 100 ml graduated cylinder, as well as water in a pre-weighed 400 ml plastic bottle. Both containers were corked using rubber stoppers with metal sipper tubes that contained ball bearings to prevent spillage. At 24-hrs after placement onto the home cage both bottles were re-weighed, and then the ethanol-containing graduated cylinder was replaced with an alternate graduated cylinder containing water. In order to control for spillage that occurred while inserting and removing bottles from the home cages, a graduated cylinder and plastic bottle containing ethanol and water, respectively, were placed on two empty home cages in the animal colony. Water and ethanol bottles were placed on these cages, and weighed at the end of each 24-hour session. Spillage was calculated as an average between the two control cages. Subjects had continuous access to both the graduated cylinder and standard water bottle to monitor for bottle preference. Additionally, the left and right placement of bottles on the home cage was alternated daily to reduce the development or impact on consumption of a side preference. Body weight and fluid consumption were recorded daily. Ethanol intake was calculated by determining the amount of ethanol consumed (gm) as a function of body weight (kg). The distribution of

ethanol in solution as a function of its density was accounted for by multiplying the grams of ethanol consumed by 0.1185. Subjects with low ethanol intake values (g/kg ≤ 0.50, averaged across sessions 6 and 7 for experiment 1; sessions 13 and 14 for Exp. 2a) were given a sweetened ethanol solution (15% ethanol, 2% sucrose) for 3 sessions in order to increase consumption. In experiment 1, 15 rats (Charles River n = 13; Harlan n = 2) were given access to the sweetened ethanol solution. In experiment 2a, 12 rats (Charles River n = 8; Harlan n = 4) required the sweetened ethanol solution, and 2 rats were excluded due to self-mutilation.

Pavlovian discrimination training

Prior to experimental sessions, rats were exposed to the behavioral testing room and equipment. On day 1, they were transported on carts to the behavioral testing room where they remained in their home cages for approximately 20-min with the behavioral equipment turned on. Over the following 2 days, subjects were exposed to the operant conditioning chambers and exposed to the 2 distinctive contexts that would be used over the course of the experiment. On each day rats were brought into the behavioral testing room, weighed and then placed into the operant conditioning chamber for 20-min, during which the number of times they made port entries was recorded. Operant conditioning chambers featured context 1 on the first day and context 2 on the second day (see below for detailed description of each context).

Subsequently, rats underwent 17 daily, 1-hour Pavlovian Discrimination Training (PDT) sessions on a Monday to Saturday schedule for the first 28 days, followed by a

Monday to Friday schedule for the remainder of the experiment. At the beginning of each PDT session subjects were weighed and then placed in the operant conditioning chamber. At 5-min after starting the behavioral program, the houselight turned on to signal the start of the session. During each session rats were presented with 16 random presentations each of two 10-sec auditory stimuli (clicker or white noise) delivered independently according to a variable-time 67-second schedule. One stimulus (CS+) was consistently paired with the delivery of 15% ethanol (0.2 ml per CS+ presentation, delivered over the last 6-sec of the CS+; total of 3.2 ml per session) into the fluid port. The alternate stimulus (CS-) was not paired with ethanol delivery. Port entries were measured 10-sec prior to, during, and 10-sec following each CS presentation, as well as throughout the 1-hour session. Ports were verified at the end of each session to ensure that rats consumed the ethanol delivered during the session.

Subjects underwent PDT in one of two contexts that differed in visual, olfactory and tactile properties. Context 1 consisted of black cardboard walls placed over the Plexiglas walls of the operant conditioning chamber, with a smooth Plexiglas floor. A waste pan placed under the chamber floor contained a white absorbent sheet of paper (~9 cm x 18 cm), and 3 sprays of a lemon odour were sprayed onto the non-absorbent side of the white sheet of paper. Context 2 consisted of the clear, Plexiglas walls, with a perforated stainless steel floor. Brown paper towels (~9 cm x 18 cm) lined the inside of the waste pan and 3 sprays of almond odour were sprayed onto it. Odours were made from diluting lemon essential oil (SAFC Supply Solutions, St-Louis, USA) or benzaldehyde (almond odour; ACP Chemicals Inc., Montreal, Canada) with tap water to obtain a final concentration of 10%. The context designated for Pavlovian discrimination

training remained consistent across session, and was referred to as context A (alcohol-associated context).

Rats from each vendor (Charles River and Harlan) were counterbalanced across context types (1 and 2) and CS+ type (clicker or white noise) based on ethanol intake (g/kg) averaged across sessions 19-21 of pre-exposure. One rat was excluded from experiment 1, and 3 rats were excluded from experiment 2 based on low ethanol intake.

Habituation

Following the last PDT session rats underwent 5, daily 1-hour habituation sessions. Subjects were placed in the same operant conditioning chambers as in PDT: however, chambers that were configured as context 1 during PDT were re-configured as context 2 during habituation, and vice versa. During each habituation session the auditory cues were withheld and no ethanol was delivered. The total number of port entry responses made in each session was recorded. The context designated for habituation was referred to as context B, and served as a non-alcohol context.

Test

Following habituation, rats were counterbalanced into 3 groups based on performance during the last 4 sessions of PDT. At 24-hours after their final habituation session, responding to the CS+ and CS- in the absence of ethanol was assessed in context B, the non-alcohol context. At test, the cues were presented as during PDT, but ethanol

delivery was withheld. Fifteen minutes prior to the test session, rats received a subcutaneous injection of 0.9% sodium chloride (1 ml/kg), SCH 23390 (3.33 µg or 10 µg/kg; Exp. 1) or eticlopride (5 µg or 10 µg/kg; Exp. 2a).

Experiment 1

The purpose of this experiment was to investigate the role of dopamine D1-receptors in responding to a non-extinguished Pavlovian alcohol-predictive cue (CS+) and CS- in a non-alcohol context. A total of 30 male, Long-Evans rats were obtained from Charles River (n = 16) and Harlan Laboratories (n = 14). After 21 sessions of intermittent access to 15% ethanol and water, rats underwent 17 sessions of PDT in context A (alcohol-associated context) followed by 5 habituation session in context B (non-alcohol context). Saline sham injections were conducted in the animal colony to acclimate the rats to the injection procedure. The first sham injection was carried out within an hour after the end of the 9th session of PDT. The second sham was conducted before the 3rd session of habituation. At 24-hours after the final habituation session responding to the CS+ and CS- from PDT was tested in the non-alcohol context, without ethanol. Fifteen minutes prior to test, rats received an injection of 0.9% sodium chloride or SCH 23390 (3.33 µg/kg or 10 µg/kg, s.c.; 1 ml/kg). A within-subjects design was used such that each subject was tested in each treatment condition. After each test rats underwent 3 sessions of PDT re-training in context A and 4 sessions of habituation in context B (pre-session saline sham before session 4 of habituation).

Experiment 2a

The purpose of this experiment was to determine the role of dopamine D2-receptors in responding to an alcohol-predictive cue (CS+) and CS- in a context that had never been associated with ethanol intake. A total of 29 male, Long-Evans rats were obtained from Charles River (n = 16) and Harlan Laboratories (n = 13). Following 21 sessions of intermittent access to 15% ethanol and water rats underwent 17 sessions of PDT in context A (alcohol-associated context) followed by 5 habituation sessions in context B (non-alcohol context). Saline sham injections were conducted under the same schedule and conditions as in experiment 1. At 24-hours after the final habituation session responding to the CS+ and CS- was measured in the non-alcohol context, in the absence of ethanol. Fifteen minutes prior to test, rats received an injection of 0.9% sodium chloride or eticlopride (5 µg/kg or 10 µg/kg; s.c.; 1 ml/kg). A within-subjects design was used such that each subject was tested in each treatment condition. After each test rats underwent 3 sessions of PDT re-training in context A and 4 sessions of habituation in context B (pre-session saline sham before session 4 of habituation).

Experiment 2b

Following their last test from experiment 2a, 25 rats (Charles River, n = 18; Harlan Laboratories, n = 7) were used to investigate the impact of dopamine D1- and D2-receptor antagonists on Pavlovian-conditioned ethanol-seeking during PDT sessions in context A, where presentations of the CS+ were paired with 15% ethanol delivery.

Subjects underwent 2 sessions of PDT in context A (same context as in Exp. 2a). At 24-hours after the second PDT session responding to the alcohol-predictive cue (CS+) and CS- was tested in context A, where each CS+ presentation was paired with the delivery of ethanol. Fifteen minutes prior to test, rats received a subcutaneous injection of 0.9% sodium chloride (1 ml/kg), SCH 23390 (10 μg/kg; 1 ml/kg) or eticlopride (10 μg/kg; 1 ml/kg). A within-subjects design was used with 2 PDT re-training sessions between each test.

Statistical analyses

During ethanol pre-exposure, the dependent measures included body weight (gm), ethanol and water intake (ml), ethanol intake (g/kg) and ethanol preference (%; calculated as ratio of ethanol consumed in ml divided by the sum of water and ethanol intakes in ml).

During PDT and test the dependent measures consisted of port entries made into the fluid port during each CS+ and CS- presentation, as well as during a 10-sec interval before (pre-CS) and after (post-CS) each CS. The total number of port entries was also measured during each daily session. Normalized port entries were calculated to account for individual differences in baseline behavioral responding by subtracting the number of port entry responses made during the pre-CS period from responding during the corresponding CS interval.

Ethanol pre-exposure

Data from ethanol pre-exposure were analyzed using a repeated-measures analysis of variance (ANOVA) with Session (1-21) as a within-subjects variable and Vendor (Charles River, Harlan) as a between-subjects variable.

Experiment 1 and 2a

Data from PDT were analyzed using a repeated-measures analysis of variance (ANOVA) with Session (1-17) and CS (CS+, CS-) as within-subjects variables and Vendor (Charles River, Harlan), Context Type (1, 2), and CS Type (clicker, white noise) as between-subjects variables. Data from test were also examined using ANOVA with Dose (saline, low dose, high dose) and CS (CS+, CS-) as within-subjects variables and Vendor (Charles River, Harlan) as between-subjects variables. Port entries made during each CS+ trial were analyzed using ANOVA with CS+ trial (1-16) and dose (saline, low and high dose) as a within-subjects variable and Vendor (Charles River, Harlan) as a between-subjects variable.

Experiment 2b

Data from test were analyzed using a repeated-measures ANOVA with Drug (saline, SCH 23390 and eticlopride) and CS (CS+, CS-) as within-subjects variables and Vendor (Charles River, Harlan) as between-subjects variables.

Any significant violations of homogeneity as indicated by Mauchly's Test for Sphericity were corrected with the Huynh-Feldt test. Follow-up analyses were conducted using paired-samples or independent-samples *t*-tests where applicable. All statistical analyses were conducted using PASW Statistics software (version 18.0) with significance level of $\alpha = 0.05$.

Results

Experiment 1

Ethanol pre-exposure. Figure 1a depicts the weight (gm) of Charles River and Harlan rats across 21 sessions of ethanol pre-exposure during which rats had access to 15% ethanol and water via 2 bottles on the home cage. Rats from both vendors gained weight across sessions [Session, F (20, 700) = 1065.104, p = .000]. However, compared to rats from Harlan, rats from Charles River were heavier [Vendor, F (1, 35) = 99.925, p = .000], and gained more weight per session [Session x Vendor, F (20, 700) = 68.271, p = .000].

Water consumption (Fig. 1b) decreased in parallel for rats from both vendors across session [Session, F (20, 700) = 11.353, p = .000; Session x Vendor, F (20, 700) = 0.97, p = .840]. However, rats from Charles River consumed more water overall [Vendor, F (1, 35) = 50.219, p = .000]. An examination of ethanol consumption (Fig. 1b) revealed that rats from Harlan drank more ethanol than rats from Charles River [Vendor, F (1, 35) = 5.614, p = .023]. Across session, ethanol consumption increased in parallel for both vendors [Session, F (20, 700) = 12.352, p = .000; Session x Vendor, F (20, 700) = 0.838, p = .504].

Figure 1c depicts ethanol intake in grams of ethanol consumed per kilogram of body weight. Across session, g/kg increased in parallel for rats from both vendors [Session, F (20, 700) = 6.269, p = .000; Session x Vendor, F (20, 700) = 0.916, p = .466]. However, Harlan rats exhibited a higher g/kg overall [Vendor, F (1, 35) = 14.144, p = .001].

These levels of fluid consumption resulted in rats from Harlan exhibiting a higher ethanol preference (Fig. 1d) [Vendor, F (1, 35) = 26.009, p = .000]. Across session, ethanol preference increased in parallel for rats from both vendors [Session, F (20, 700) = 15.717, p = .000; Session x Vendor, F (20, 700) = 1.361, p = .187].

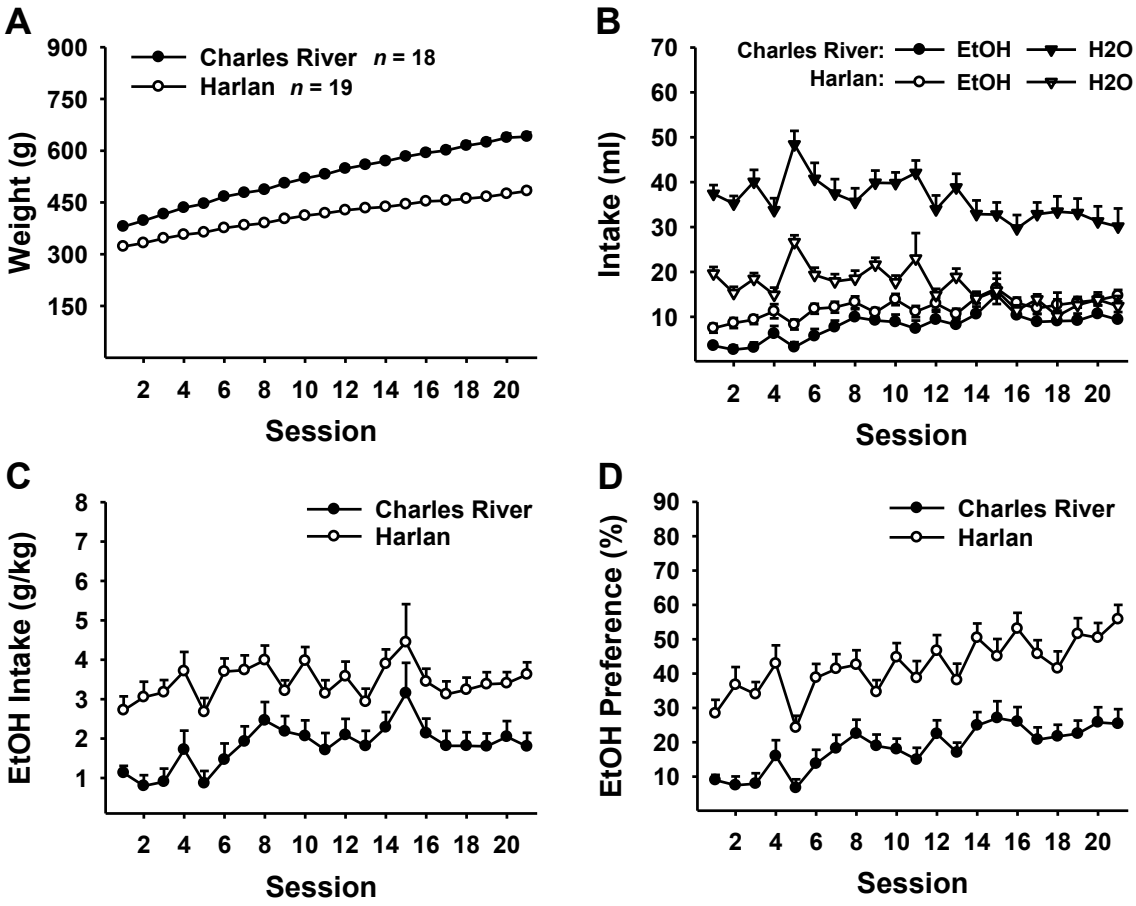

Figure 1. Weight gain and alcohol consumption across 21 sessions of alcohol pre-exposure in Charles River (filled symbols) and Harlan (open symbols) rats. **A** Mean (± SEM) body weight in grams. **B** Mean (± SEM) water and EtOH intake in ml. **C** Mean (± SEM) EtOH intake in g/kg. **D** Mean (± SEM) percent EtOH preference.

Pavlovian discrimination training. Figure 2a depicts normalized CS+ and normalized CS- port entries across 17 sessions of Pavlovian Discrimination Training in Context A. Normalized port entries increased across Session [F (16, 448) = 19.791, p = .000], with responding to the CS+ stabilizing at higher levels than CS- responding [Session x CS, F (16, 448) = 10.344, p = .000]. This outcome was verified by a main effect of CS [F (1, 28) = 43.187, p = .000]. Rats from Charles River and Harlan showed similar acquisition of Pavlovian discrimination training. ANOVA indicated no main effect of Vendor [F (1, 28) = 0.025, p = .875] or significant interactions with Vendor [Session x Vendor, F (16, 448) = 0.796, p = .564; CS x Vendor, F (1, 28) = 1.680, p = .205; Session x CS x Vendor, F (16, 448) = 1.115, p = .355].

Follow-up paired samples t-tests collapsed across Vendor indicated that with the exception of PDT sessions 1 and 3, CS+ responding was significantly higher than CS- responding ($p < .05$ for each comparison).

Figure 2b depicts total port entries during PDT. Total port entries increased across the first 6 sessions and then decreased for the remaining sessions [Session, F (16, 448) = 3.873, p = .000]. There were no vendor differences in total port entries [Vendor, F (1, 28) = 1.561, p = .222], with rats from both vendors exhibiting similar patterns of responding across session [Session x Vendor, F (16, 448) = .964, p = .496]. There was no change in total port entries made across habituation [Session, F (4, 112) = 3.060, p = .058). Furthermore, there were no vendor differences in total port entries and rats from both vendors maintained similar patterns of responding across session [Vendor, F (1, 28) = .715, p = .405; Session x Vendor, F (4, 112) = .249, p = .910; Fig. 2b inset].

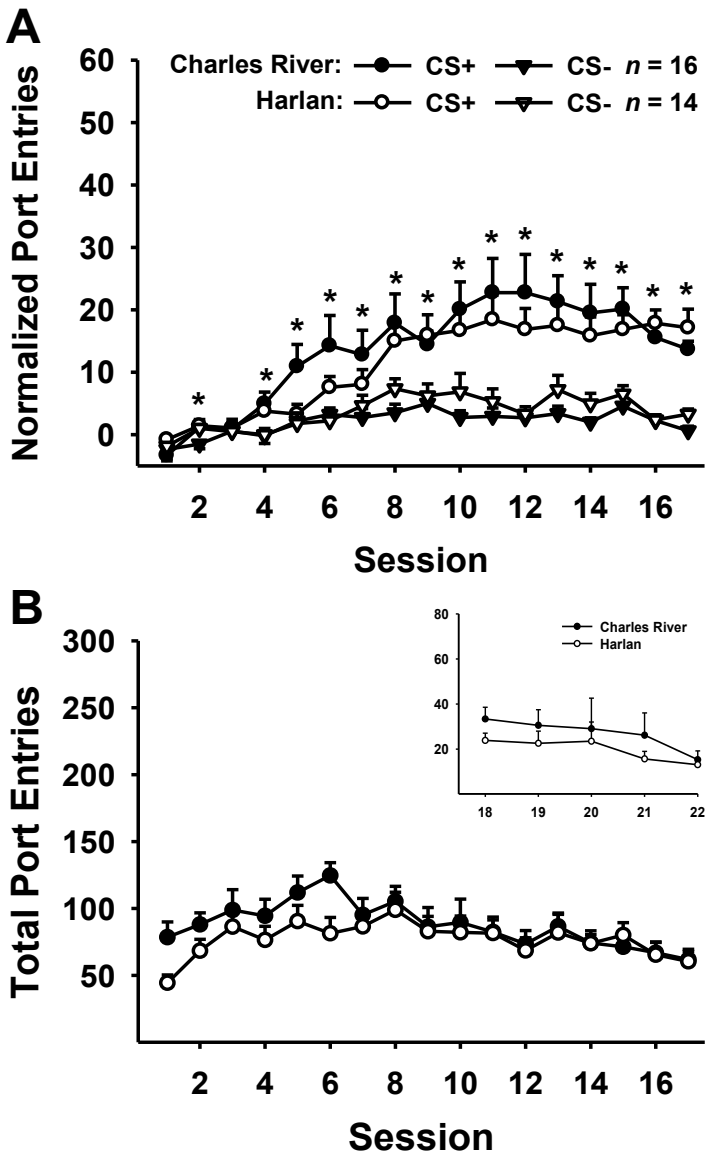

Figure 2. Behavioral discrimination between an alcohol-predictive CS+ and CS- across 17 sessions of Pavlovian Discrimination Training in Charles River (filled symbols) and Harlan (open symbols) rats. **A** Mean (± SEM) normalized port entries made during the CS+ (circles) and CS- (triangles), CS+ > CS- * $p < .05$. **B** Mean (± SEM) total port entries across PDT and 5 sessions of habituation in a non-alcohol context (inset).

Dose-dependent effects of SCH 23390 on Pavlovian cue-driven alcohol-seeking. Figure 3 depicts normalized port entries during the CS+ and CS- at test for rats from Charles River (3a) and Harlan (3b). At test both cues were presented as during PDT, but without ethanol and in the non-alcohol context. Overall, rats responded more to the CS+ than the CS- indicating that discrimination between the two cues remained intact [CS, F (1, 28) = 97.447, p = .000]. Blocking dopamine D1 receptors dose-dependently reduced CS+ responding, with no effect on CS- responding. These results are supported by a significant main effect of Dose [F (2, 56) = 10.601, p = .000] and a significant Dose x CS interaction [F (2, 56) = 10.359, p = .000].

There was no Dose x Vendor interaction [F (2, 56) = 1.529, p = .226] and no Dose x CS x Vendor interaction [F (2, 56) = 1.462, p = .240] suggesting that SCH23390 dose-dependently reduced CS+ responding in rats from both vendors. Follow-up t-tests for paired samples on normalized CS+ responding collapsed across Vendor indicated a significant difference between saline and 10 µg/kg [t (29) = 4.608, p = .000] and the 3.33 µg/kg and 10 µg/kg doses [t (29) = 3.822, p = .001], but no difference between saline and 3.33 µg/kg dose [t (29) = 0.992, p = .329].

Interestingly, the pattern of CS+ and CS- responding differed as a function of vendor. ANOVA indicated a significant main effect of Vendor [F (1, 28) = 5.245, p = .030] and a significant Vendor x CS interaction [F (1, 28) = 8.223, p = .008]. Follow-up t-tests for independent samples on data collapsed across Dose indicated that Harlan rats responded more to the CS- than did Charles River rats [t (88) = -2.328, p = .024]. However, rats from Harlan responded less to the alcohol-predictive CS+ at test compared to rats from Charles River [t (88) = 2.995, p = .004]. This difference appears to be driven

by a greater dose-dependent reduction in CS+ responding by SCH 23390 in Harlan rats (Fig. 3b).

Figures 3c and 3d depict port entries for each CS+ trial at test for Charles River and Harlan rats, respectively. Overall, the number of port entries per CS+ trial decreased across the test session [Trial, F (15, 420) = 17.009, p = .000]. Pre-treatment with SCH23390 dose-dependently reduced responding [Dose, F (2, 56) = 11.232, p = .000] and this effect was greater at the start of the test compared to the end [Trial x Dose, F (30, 840) = 1.974, p = .002]. There was no Dose x Vendor interaction [F (2, 56) = 1.438, p = .246] and no Trial x Dose x Vendor interaction [F (30, 840) = 1.154, p = .261] suggesting that the dose-dependent reduction in responding to each CS+ was similar for rats from both vendors. Follow-up 2-way ANOVAs collapsed across Vendor revealed a significant Trial x Dose interaction [F (15, 435) = 2.759, p = .006] for the comparison between saline and 10 µg/kg. Paired samples t-tests verified that saline pretreated rats responded more to the CS+ compared to rats pretreated with 10 µg/kg of SCH 23390 on trials 1, 3-6 & 9 (p < .05 for all comparisons). There was also a significant Trial x Dose interaction [F (15, 435) = 2.510, p = .020] for the comparison between 3.33 µg/kg and 10 µg/kg. Paired samples t-tests verified that rats pretreated with 3.33 µg/kg responded more to the CS+ compared to rats pretreated with 10 µg/kg of SCH 23390 on trials 1 & 3 (p < .05 for all comparisons).

There was a significant main effect of Vendor [F (1, 28) = 7.396, p = .011], which appears to be driven by lower levels of port entries per CS+ responding in Harlan rats. The pattern of CS+ responses across trial was also different as a function of vendor [Trial x Vendor, F (15, 420) = 2.059, p = .011]. Follow-up t-tests for independent samples on

data collapsed across Dose indicated that compared to rats from Charles River, rats from Harlan responded less on CS+ trials 3-5 and 16 ($p < .05$ for each comparison).

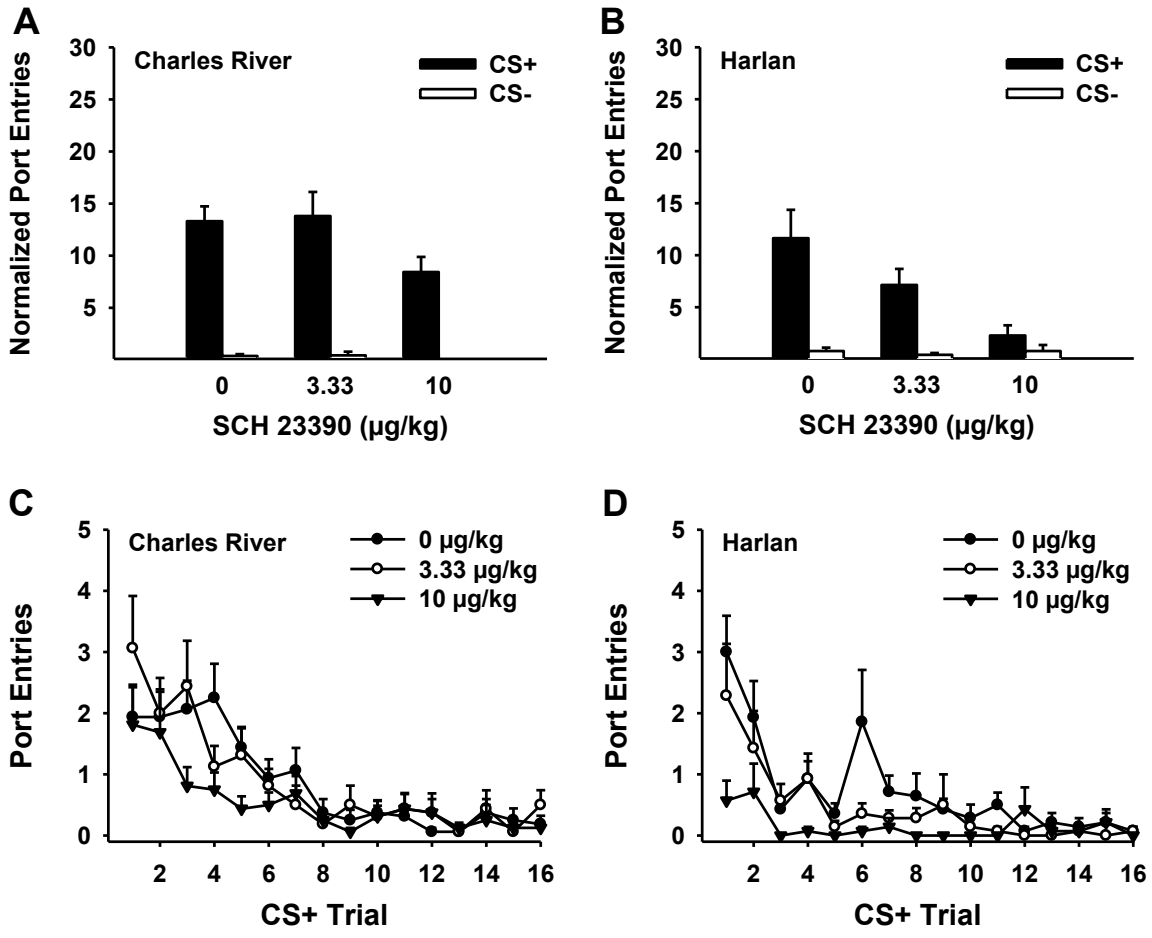

Figure 3. Pavlovian cue-driven alcohol-seeking in a non-alcohol context. Mean (± SEM) normalized port entries during the CS+ (filled bars) and CS- (open bars) following saline and SCH 23390 (3.33 µg/kg or 10 µg/kg) pre-treatment in Charles River (**A**) and Harlan (**B**) rats. Mean (± SEM) port entries made during each CS+ trial at test following saline and SCH 23390 (3.33 µg/kg and 10 µg/kg) infusions in Charles River (**C**) and Harlan rats (**D**).

Figures 4a and 4b depict total port entries during habituation and test for Charles River and Harlan rats, respectively. Habituation data represent means from the last 2 sessions before the corresponding test. At test, rats from Harlan made fewer total port entries than rats from Charles River [Vendor, F (1, 28) = 6.214, p = .019]. Pre-treatment with SCH 23390 dose-dependently reduced total port entries in rats from both vendors [Dose, F (2, 56) = 9.065, p = .000], with no Dose x Vendor interaction [F (2, 56) = 0.616, p = .544]. Follow-up t-tests for paired samples on data collapsed across Vendor indicated a significant difference between saline and 10 μg/kg [t (29) = 4.720, p = .000] and between 3.33 μg/kg and 10 μg/kg [t (29) = 2.623, p = .014]. There was no difference between saline and 3.33 μg/kg [t (29) = 1.585, p = .124].

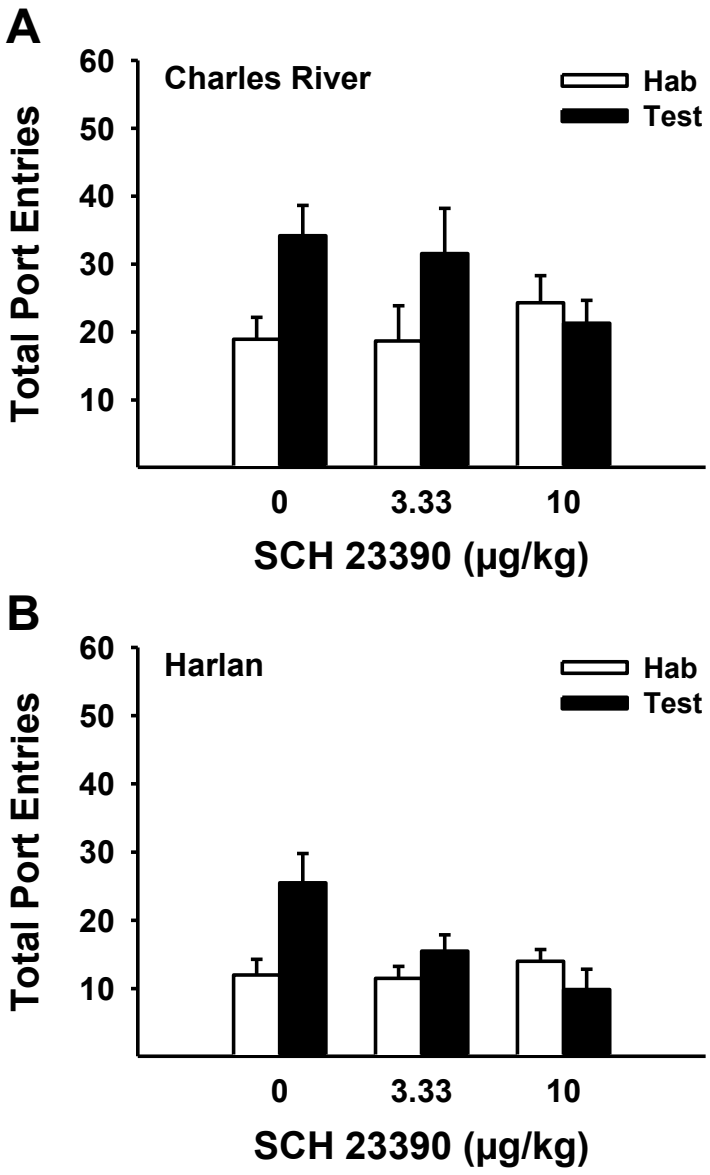

Figure 4. Mean (± SEM) total port entry responses during habituation and at test in a non-alcohol context. Habituation data (open bars) represent total port entries made across the last 2 sessions. Test data (filled bars) are averaged across a single session in saline and SCH 23390 (3.33 µg/kg or 10 µg/kg) pre-treated Charles River (**A**) and Harlan (**B**) rats.

Figure 5 depicts latency (in seconds) to the first port entry at test. ANOVA indicated a main effect of Dose [F (2, 56) = 5.877, p = .013], suggesting that SCH 23390 dose-dependently increased the time taken to make the first port entry. There was no main effect of Vendor [F (1, 28) = 0.131, p = .720] or Dose x Vendor interaction [F (2, 56) = 0.041, p = .959]. Follow up t-tests for paired samples on data collapsed across Vendor indicated a significant difference between saline and 10 µg/kg [t (29) = -2.703, p = .011] and between 3.33 µg/kg and 10 µg/kg [t (29) = -2.481, p = .019]. There was no difference between saline and 3.33 µg/kg [t (29) = 0.126, p = .900].

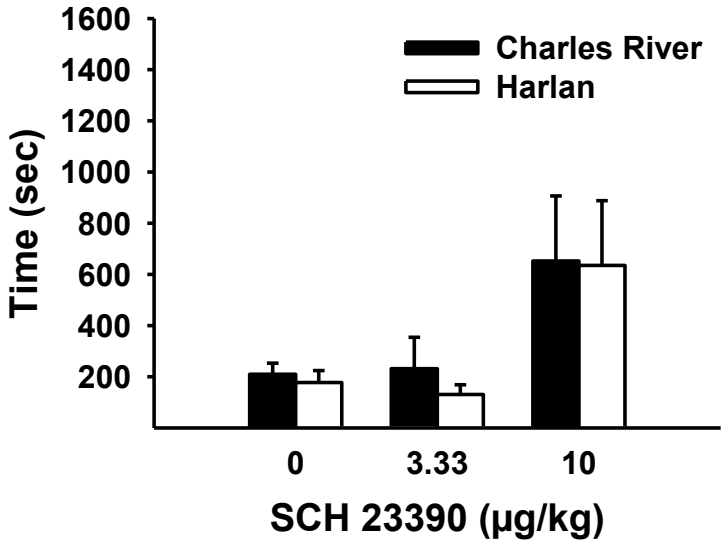

Figure 5. Latency to first port entry at test. Mean (± SEM) latency to first port entry at test in saline and SCH 23390 (3.33 µg/kg or 10 µg/kg) pre-treated Charles River (filled bars) and Harlan (open bars) rats.

Experiment 2a

Ethanol pre-exposure. Figure 6a depicts weight (gm) of Charles River and Harlan rats across 21 session of pre-exposure during which rats had access 15% ethanol and water via two bottles on the home cage. Rats from both vendors gained weight across session [Session, F (20, 740) = 1747.345, p = .000]; however, as with Experiment 1, rats from Charles River were heavier than rats from Harlan [Vendor, F (1, 37) = 36.362, p = .000] and gained more weight per session [Session x Vendor, F (20, 740) = 39.203, p = .000]. Independent samples t-tests confirmed a significant difference between vendors across all ethanol pre-exposure sessions ($p < 0.01$ for each comparison).

Water consumption (Fig. 6b) decreased across pre-exposure sessions [Session, F (20, 740) = 12.651, p = .000], although at a different rate for rats from each vendor [Session x Vendor, F (20, 740) = 4.099, p = .000]. Rats from Charles River drank significantly more milliliters of water than rats from Harlan [Vendor, F (1, 37) = 93.355, p = .000]. Alternatively, ethanol consumption increased across sessions [Session, F (20, 740) = 18.029, p = .000], with rats from Harlan drinking more milliliters of ethanol overall and escalating consumption across sessions at a faster rate than rats from Charles River [Vendor, F (1, 37) = 13.487, p = .001; Session x Vendor F (20, 740) = 3.202, p = .000]. Independent samples t-tests indicated significant differences between vendors on sessions 4 and 15-17 ($p < .05$) and sessions 1-3 and 5-14 ($p < .01$).

Ethanol intake in grams of ethanol consumed per kilogram of body weight (Fig. 6c) increased across sessions [Session, F (20, 740) = 8.150, p = .000] and differed as a function of vendor [Vendor, F (1, 37) = 24.234, p = .000], with Harlan rats exhibiting

higher overall ethanol intake values. There was a significant Session x Vendor interaction [F (20, 740) = 3.228, p = .000] likely attributable to the observation that ethanol intake in Harlan rats remained relatively stable across sessions, whereas ethanol intake increased in Charles River rats. Independent samples t-tests indicated significant differences between vendors on session 19 ($p < .05$) and sessions 1-3, 5-17 and 21 ($p < .01$).

Rats from Harlan developed a higher ethanol preference (Fig. 6d) than Charles River rats [Vendor, F (1, 37) = 24.234, p = .000]. Overall, ethanol preference increased across session [Session, F (20, 740) = 8.150, p = .000] with a significant Session x Vendor interaction [F (20, 740) = 3.228, p = .000]. Independent samples t-tests indicated significant differences between vendors on session 18 ($p < .05$) and sessions 1-17 and 19-21 ($p < .01$).

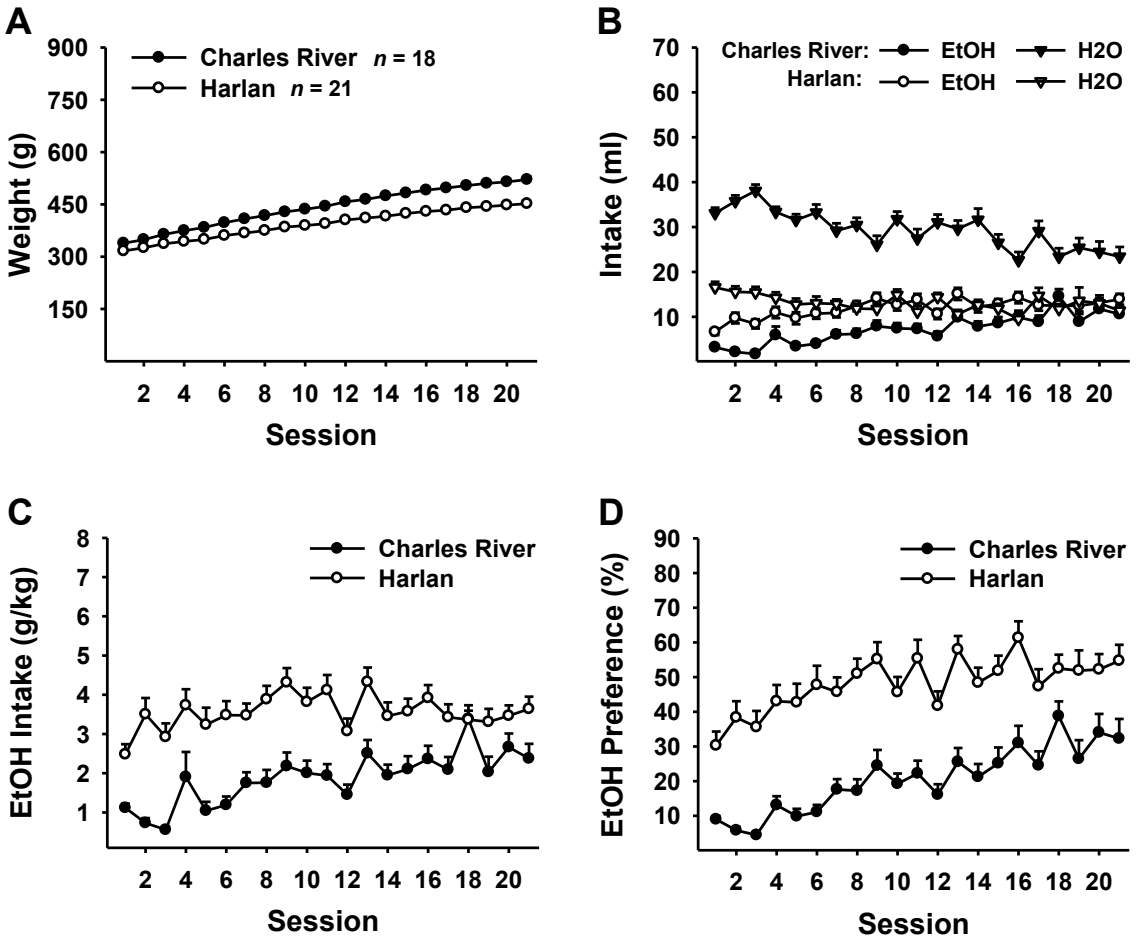

Figure 6. Weight gain and alcohol consumption across 21 sessions of alcohol pre-exposure in Charles River (filled symbols) and Harlan (open symbols) rats. **A** Mean (± SEM) body weight in grams. **B** Mean (± SEM) water and EtOH intake in ml. **C** Mean (± SEM) EtOH intake in g/kg. **D** Mean (± SEM) percent EtOH preference.

Pavlovian discrimination training. Figure 7a depicts normalized port entries during the CS+ and CS- across 17 sessions of Pavlovian discrimination training in context A. Normalized port entries increased across session [Session, F (16, 432) = 20.776, p = .000]. A main effect of CS revealed that rats responded significantly more to the CS+ than the CS- [CS, F (1, 27) = 68.395, p = .000]. A significant Session x CS interaction verified that while responding to both cues increased, rats achieved higher levels of responding to the CS+ [F(16, 432) = 11.574, p = .000].

Unlike in Experiment 1, vendor differences were observed in the acquisition of Pavlovian discrimination training. Rats from both vendors learned to discriminate between the CS+ and CS- [CS x Vendor, F (1, 27) = 3.822, p = .061]; however, there was a trend for the number of responses made to differ as a function of vendor [Vendor, F (1, 27) = 4.131, p = .052]. Furthermore, while the overall number of responses made increased across session, this measure differed significantly as a function of vendor [Session x Vendor, F (16, 432) = 2.141, p = .006]. Specifically, rats from Harlan achieved and maintained a higher level of CS+ responses than rats from Charles River [Session x CS x Vendor, F (16, 432) = 2.607, p = .001]. Follow-up t-tests for independent samples verified this higher level of CS+ responding in Harlan rats on sessions 13, 14, 16, and 17 ($p < .05$) and session 12 ($p < .01$).

Total port entries during PDT (Fig. 7b) decreased across session [Session, F (16, 432) = 4.283, p = .000]. Overall, while the total number of port-entries did not differ between Charles River and Harlan rats [Vendor, F (1, 27) = 3.881, p = .059] the pattern of responses across session varied as a function of vendor [Session x Vendor, F (16, 432) = 2.692, p = .000]. Independent samples t-tests confirmed that rats from Charles River

made more total port entries on sessions 14 and 15 ($p < .05$) and sessions 1-3 ($p < .01$). Total port entries decreased across habituation sessions for both vendors [Session, F (4, 108) = 6.933, p = .000; [Session x Vendor, F (16, 432) = 2.692, p = .000] and the total number of port entries did not differ between rats from Charles River and Harlan [Vendor, F (1, 27) = 1.528, p = .227; Fig. 7b inset].

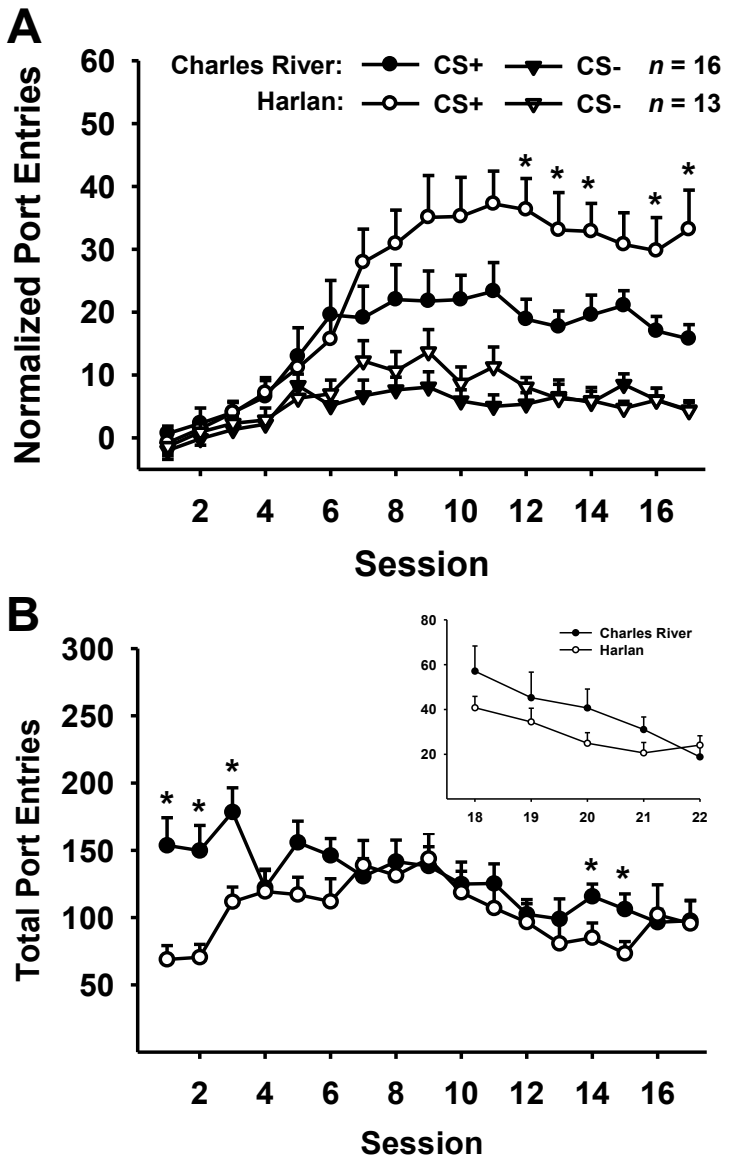

Figure 7. Behavioral discrimination between an alcohol-predictive CS+ and CS- across 17 sessions of Pavlovian Discrimination Training in Charles River and Harlan rats. **A** Mean (± SEM) normalized port entries made during the CS+ (circles) and CS- (triangles). Norm CS+, Harlan > Charles River * $p < .05$. **B** Mean (± SEM) total port entries across PDT and 5 sessions of habituation in a non-alcohol context (inset). Total port entries, Charles River > Harlan * $p < .05$.

Dose-dependent effect of eticlopride on Pavlovian cue-driven alcohol-seeking. Figure 8 depicts normalized port entries made during the CS+ and CS- at test for Charles River (Fig. 8a) and Harlan rats (Fig. 8b) when both cues were presented without ethanol in the non-alcohol context. Overall, rats responded more to the CS+ than the CS-, indicating that discrimination between the two cues remained intact [CS, F (1, 27) = 117.452, p = .000]. Blocking dopamine D2 receptors resulted in a dose-dependent reduction in CS+ responding, with no effect on CS- responding [Dose, F (2, 54) = 8.172, p= .001; Dose x CS, F (2, 54) = 6.077, p = .004]. This pattern of results was obtained in rats from both vendors [Vendor x CS, F (1, 27) = .005, p = .941], who also did not differ in the number of CS responses made at test [Vendor, F (1, 27) = .055, p = .817]. However, the dose-dependent reduction in responding varied as a function of vendor [Dose x Vendor, F (2, 54) = 3.439, p = .039]. As there was no Dose x CS x Vendor interaction [F (2, 54) = 1.361, p = .265] data were collapsed across CS for subsequent analyses.

Independent samples t-tests revealed no significant differences between vendors at any of the three doses (p > .05 for each comparison). Paired samples t-test revealed a significant difference in normalized port entries between the 5 µg/kg and 10 µg/kg doses [t (15) = 2.366, p = .032] in rats from Charles River. In Harlan rats, paired samples t-tests revealed significant differences between saline and the 10 µg/kg dose [t (12) = 3.645, p = .003], and the 5 µg/kg and 10 µg/kg dose [t (12) = 2.862, p = .014].

Figures 8c and 8d depict port entries made during each CS+ trial at test for Charles River and Harlan rats, respectively. In rats from both vendors, responding to the CS+ was higher at the start of the test when compared to the end [Trial, F (15, 405) =

32.074, $p = .000$; Trial x Vendor interaction, F (15, 405) = 1.566, $p = .080$]. Eticlopride dose-dependently attenuated the number of port entries [Dose, F (2, 54) = 7.372, $p = .001$] in both vendors [Dose x Vendor, F (2, 54) = 2.138, $p = .128$]. The attenuation in CS+ responding induced by eticlopride was greatest during the first half of the test session [Trial x Dose, F (30, 810) = 1.616, $p = .020$]. The effect of dose on responding across CS+ trials did not differ as a function of vendor [Trial x Dose x Vendor interaction [F (30, 810) = 1.329, $p = .113$], and nor was there a significant difference in levels of responding between vendors [Vendor, F (1, 27) = 0.011, $p = .917$].

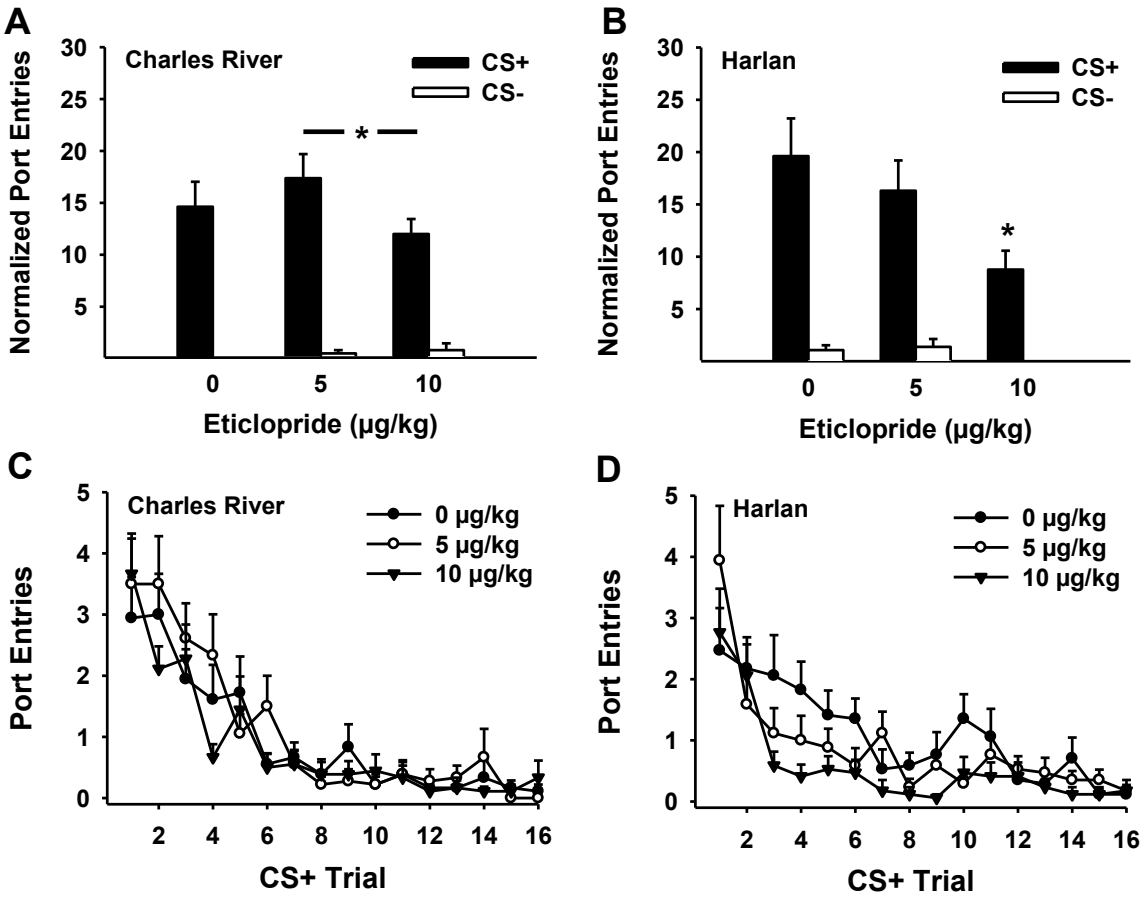

Figure 8. Pavlovian cue-driven alcohol-seeking in a non-alcohol context. Mean (± SEM) normalized port entries during the CS+ (filled bars) and CS- (open bars) following saline and eticlopride (5 µg/kg or 10 µg/kg) pre-treatment in Charles River (**A**) and Harlan (**B**) rats. Mean (± SEM) port entries made during each CS+ trial at test following saline and eticlopride (5 µg/kg and 10 µg/kg) infusions in Charles River (**C**) and Harlan rats (**D**). In Charles River, Norm CS+, eticlopride (5 µg/kg) > eticlopride (10 µg/kg); in Harlan, Norm CS+, eticlopride (10 µg/kg) < saline and eticlopride (5 µg/kg), * $p < .05$.

Figure 9 depicts total port entries made during habituation and at test for Charles River (Fig. 9a) and Harlan rats (Fig. 9b), respectively. ANOVA comparisons on test data revealed no main effect of Dose [F (2, 54) = 1.248, p = .295], Vendor [F (1, 27) = 1.800, p = .191] or Dose x Vendor interaction [F (2, 54) = 1.172, p = .317].

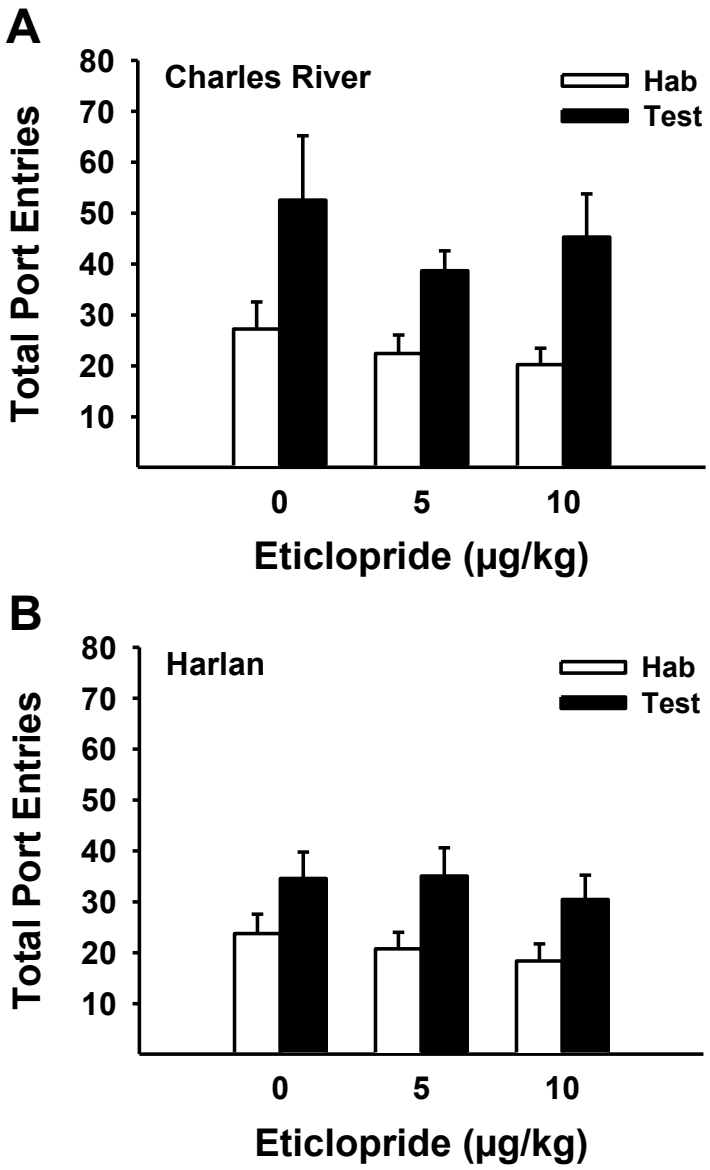

Figure 9. Mean (± SEM) total port entry responses during habituation and at test in a non-alcohol context. Habituation data (open bars) represent total port entries made across the last 2 sessions. Test data (filled bars) are averaged across a single session in saline and eticlopride (5 µg/kg or 10 µg/kg) pre-treated Charles River (**A**) and Harlan (**B**) rats.

Figure 10 depicts the latency to first port entry at test, represented in seconds. ANOVA comparisons indicated no main effect of Dose [F (2, 54) = 0.153, p = .858], Vendor [F (1, 27) = 1.773, p = .194] or Dose x Vendor interaction [F (2, 54) = 0.082, p = .922].

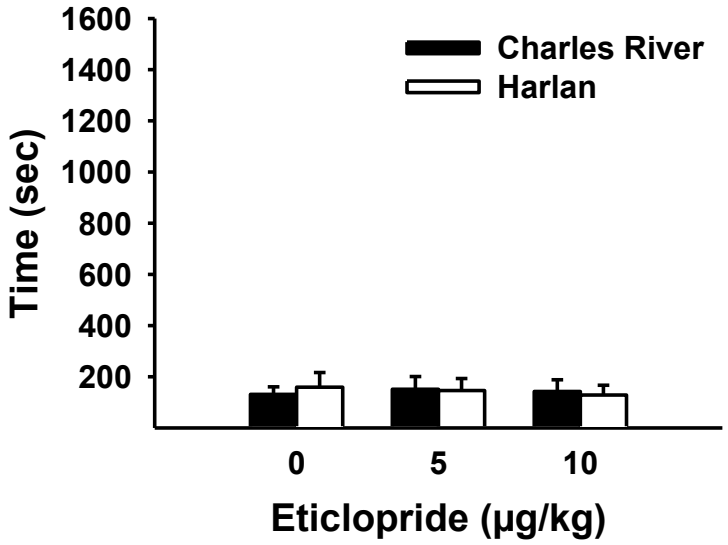

Figure 10. Mean (± SEM) latency to first port entry at test in saline and eticlopride (5 µg/kg or 10 µg/kg) pre-treated Charles River (filled bars) and Harlan (open bars) rats.

Experiment 2b

Effects of SCH 23390 and eticlopride in responding to an alcohol-predictive cue under alcohol-paired conditions. Figure 11a depicts normalized port entries made during the CS+ and CS- following treatment with saline, SCH 23390 (10 ug/kg) or eticlopride (10 ug/kg) in context A, when the CS+ was paired with ethanol delivery.

Overall, rats from both vendors responded more to the CS+ than the CS- [CS, F (1, 23) = 81.477, p = .000; CS x Vendor, F (1, 23) = 2.891, p = .103]. There was no main effect of Treatment [F (2, 46) = 2.300, p = .112] suggesting that there was no impact of D1 or D2 receptor antagonists on alcohol-seeking. However, a significant Treatment x CS interaction [F (2, 46) = 3.532, p = .037] indicated that treatment had an effect on one CS but not the other. This pattern was consistent for both vendors [Drug x CS x Vendor, F (2, 46) = 0.310, p = .735]. Response levels to the CS+ and CS- were comparable for rats from Charles River and Harlan [Vendor, F (2, 46) = 0.084, p = .919].

Follow-up ANOVA comparisons of normalized CS+ responding collapsed across Vendor revealed a main effect of Treatment [F (2, 48) = 3.854, p = .039]. Paired-samples t-tests indicated a significant difference in CS+ responding between saline and SCH 23390 ($p < .05$) with no significant differences between saline and eticlopride, or eticlopride and SCH 23390 ($p > .05$). With respect to normalized port entries made during the CS-, there was no main effect of Treatment [F (2, 48) = .420, p = .659].

Figure 11b illustrates total port entries made at test across saline, SCH 23390 and eticlopride treatment conditions. ANOVA revealed a main effect of Treatment [F (2, 46) = 4.782, p = .017], with no difference in the number of total port entries made as a

function of vendor [Vendor, F (1, 23) = 0.194, p = .663] and no Treatment x Vendor interaction [F (2, 46) = 0.057, p = .944]. Paired-samples t-tests on data collapsed across vendor revealed a significant difference in total port entries between saline and SCH 23390, and eticlopride and SCH 23390 ($p < .05$), with no significant differences between saline and eticlopride ($p > .05$).

Overall, rats from both vendors responded more during the 10-sec interval that followed the CS+ presentation than the 10-sec interval that followed the CS- presentation [PostCS, F (1, 23) = 22.290, p = .000; PostCS x Vendor, F (1, 23) = 0.810, p = .377]. There was no main effect of treatment, suggesting that there was no impact of the D1- or D2-receptor antagonist on alcohol-seeking; this finding was consistent for both PostCS+ and PostCS- intervals and was observed in rats from both vendors [Treatment x PostCS, F (2, 46) = 0.441, p = .646; Treatment x PostCS x Vendor, F (2, 46) = 0.103, p = .902]. Response levels during the PostCS intervals were comparable for rats from Charles River and Harlan [Vendor, F (1, 23) = 0.005, p = .944; data not shown].

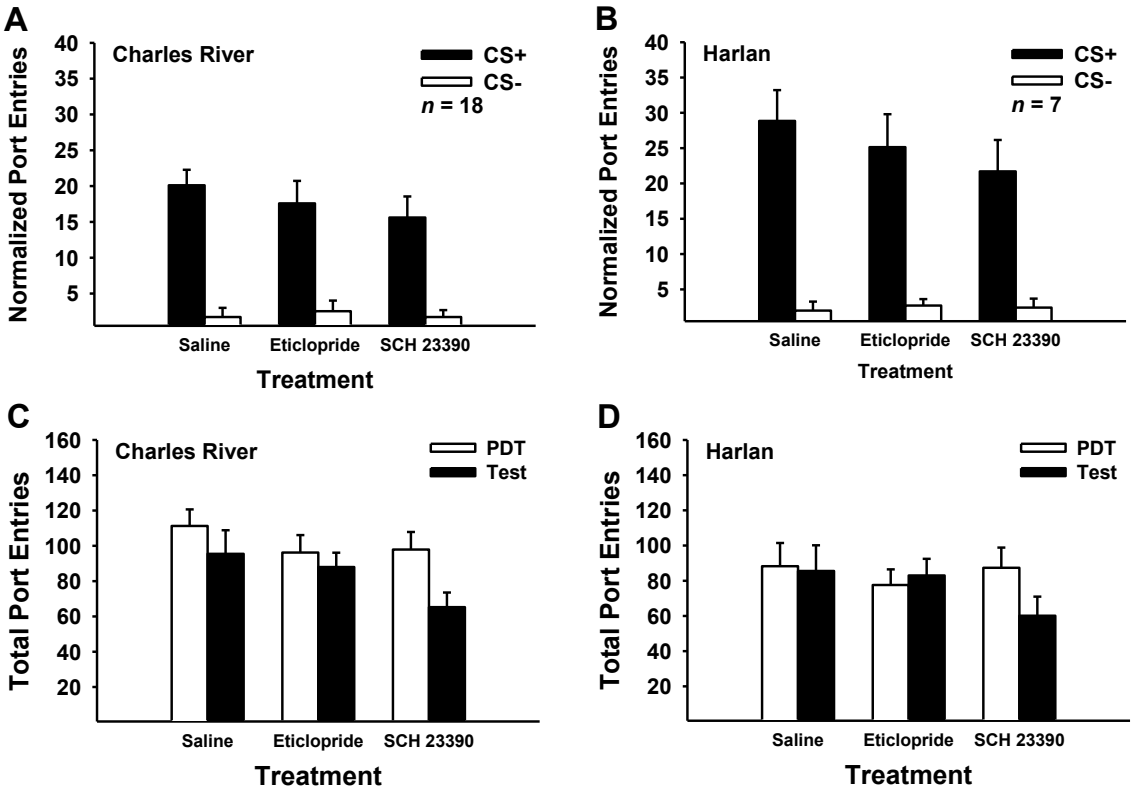

Figure 11. Behavioral outcomes during Pavlovian Discrimination Training sessions. Mean (± SEM) normalized port entries during the CS+ when paired with EtOH delivery (filled bars) and CS- (open bars) following saline, eticlopride (10 µg/kg) and SCH 23390 (10 µg/kg) pre-treatment in Charles River (**A**) and Harlan (**B**) rats. Mean (± SEM) total port entries made during the last 2 sessions of PDT (open bars) and at test (filled bars) following saline, eticlopride (10 µg/kg) and SCH 23390 (10 µg/kg) pre-treatment in Charles River (**C**) and Harlan (**D**) rats.

General Discussion

The present experiments examined the role of dopamine in responding elicited by a Pavlovian-conditioned alcohol-predictive cue. In order to isolate the role of dopamine in responding to discrete alcohol-cues from potentially separable mechanisms that might mediate the impact of alcohol-associated contexts on behavior, we tested the effects of dopamine receptor antagonists on cue-driven alcohol-seeking in a context that had never been associated with alcohol intake. At test, saline pre-treated rats consistently responded more to the alcohol-predictive cue (CS+) than to the CS-, demonstrating that discrete cues can trigger alcohol-seeking despite being experienced in a non-alcohol environmental context. Dopamine D1- and D2- receptor antagonists dose-dependently reduced Pavlovian cue-driven alcohol-seeking. Furthermore, the D1- but not D2-receptor antagonist significantly reduced responding to an alcohol-predictive cue when it was presented in combination with alcohol during re-training.

A second objective of these experiments was to investigate potential differences in ethanol consumption, Pavlovian conditioning and the role of dopamine in Pavlovian cue-driven alcohol-seeking based on which supplier the experimental subjects were obtained from. Vendor differences in male, Long-Evans rats obtained from Charles River and Harlan Laboratories were primarily observed in weight gain and oral alcohol consumption. Although rats from Charles River gained more weight throughout the study, rats from Harlan Laboratories developed a higher overall ethanol preference. There were no consistent differences in the acquisition and expression of Pavlovian discrimination training as a function of vendor. Neither were there any statistically significant vendor differences in the effect of the D1-receptor antagonist SCH 23390 on

responding to an alcohol-predictive cue. However, there was a more robust, dose-dependent attenuation of responding by the D2 dopamine receptor antagonist eticlopride on responding to the alcohol-predictive cue in rats obtained from Harlan. Overall, these results provide novel evidence for the involvement of dopamine in mediating Pavlovian cue-driven alcohol-seeking. Furthermore, the replicable differences between Charles River and Harlan rats in alcohol consumption highlight the importance of considering vendor selection in preclinical alcohol research.

Behavioral discrimination between an alcohol-predictive CS+ and CS-

Subjects from both experiments learned to discriminate between the alcohol-predictive cue (CS+) and a second stimulus (CS-) that was presented without alcohol. Discrimination was characterized by a significant increase across session in port entries made during the CS+ as rats learned to associate the availability of alcohol with this cue, and the comparatively lower rate of responding to the CS-. Behavioral discrimination occurs as the alcohol-predictive cue acquires incentive-motivational properties upon repeated pairing with the pharmacological effects of alcohol (See, 2002; Weiss, 2005; Chaudhri et al., 2008). In the present experimental design the CS- served as a within-subject control, providing a measure of specificity of conditioned responding to the alcohol-paired cue.

In both experiments the number of total port entry responses increased across initial sessions of PDT, which may be the result of subjects associating the context with alcohol availability. By mid-phase, a decrease in port entry responses suggests that rats

no longer made spontaneous entries to the fluid port; rather they became more selective indicating they learned the predictive relationship between the CS+ and alcohol delivery.

Following PDT, subjects underwent habituation in a second, different context, referred to as context B. The purpose of habituation was to expose the rats to an environmental context in which alcohol was never presented. The number of times that subjects checked the fluid port, putatively for alcohol, during this phase decreased across sessions, suggesting that they learned to stop checking the fluid port for alcohol delivery in the non-alcohol context.

Dose-dependent effects of SCH 23390 on cue-driven alcohol-seeking

Cue-driven alcohol-seeking was assessed by presenting the non-extinguished, alcohol-predictive CS+ as well as the CS- in a context that was not associated with alcohol. Saline pre-treated rats from Charles River and Harlan responded more to the CS+ than the CS-, indicating that discrimination between the two cues remained intact. Such results are consistent with recent findings that a non-extinguished Pavlovian alcohol-predictive cue can elicit alcohol-seeking when presented in a non-alcohol context (Chaudhri et al., 2010). Systemic infusions of the dopamine D1-receptor antagonist SCH 23390 resulted in a dose-dependent reduction in responding to the alcohol-predictive cue, with no effect on CS- responses. Specifically, we observed that an infusion of 10 μg/kg of SCH 23390 significantly reduced responding to the alcohol-predictive cue, relative to rats that were pre-treated with saline or 3.33 μg/kg of SCH 23390.

An analysis of the pattern of responses obtained at test revealed that CS+ trials at the start of the test session elicited more responses than subsequent CS+ trials. In particular, saline pre-treated rats consistently exhibited this pattern of behavior, suggesting that repeated presentations of the alcohol-predictive cue without alcohol delivery resulted in within-session extinction of port entry behavior. Specifically, the incentive value of the CS+ becomes increasingly weakened as it comes to predict non-reward (Wise, 2004), or the decrease in CS+ responding is attributed to learning that the CS+ no longer predicts alcohol delivery (Bouton, 2004). Systemic infusions of SCH 23390 dose-dependently reduced responding to each CS+ trial. Specifically, we observed that 10 µg/kg of SCH 23390 blocked responding on the first CS+ trial (Fig. 3). In addition, an examination of latency to first port entry indicated that SCH 23390 dose-dependently increased the time to make the first port entry, with significant differences in latency between saline and 10 µg/kg SCH 23390, and 3.33 µg/kg and 10 µg/kg doses (Fig. 5).

The most conservative explanation for the observed reduction in CS+ responding at test using the 10 µg/kg dose of SCH 23390 is that blocking dopamine D1 receptors reduced overall locomotor activity. For example, it has been shown that a subcutaneous injection of 1 mg/kg of SCH 23390 suppressed locomotor activity and rearing in rats (Hoffman & Beninger, 1985). However, we think this to be an unlikely explanation for the present results for the following reasons. First, the attenuation in port entry responding at test was specific to the CS+: SCH 23390 did not reduce CS- responding, albeit the levels of responding to the CS- might have been too low to detect an effect. Second, although the total number of port entries made by rats infused with SCH 23390

decreased dose-dependently at test, this measure did not differ between test and habituation for the 10 µg/kg dose (Fig.s 8a and 8b). That there was no difference between these two phases suggests that SCH 23390 did not reduce port entries to a level below what is typically observed in the absence of cues or ethanol, and instead blocked the specific increase in port-entries attributable to the presence of the CS+ at test. Third, we examined the effects of SCH 23390 (10 µg/kg) on port entry responding under conditions where the CS+ was paired with alcohol (Exp. 2b). Here, responding to the CS+ was significantly higher relative to CS- and SCH 23390 had no effect on responding to either cue, which suggests that the D1- receptor antagonist did not have an impact on alcohol-seeking or port-entry responses in general. Lastly, published studies that have investigated the effect of SCH 23390 on context-induced renewal of cocaine-, sucrose-, or ethanol-seeking (Crombag et al., 2002; Liu & Weiss, 2002; Hamlin et al., 2007), as well as on context- and discrete cue-induced heroin-seeking (Bossert et al., 2007) have reported that SCH 23390 doses similar to those used in the present experiments had minimal effect on high rates of instrumental responding for sucrose or food (Crombag et al., 2002; Nakajima, 1986).

Based on these studies, we can assume that the attenuation in responding caused by SCH 23390 is not attributable to locomotor deficits. Though dopamine has been implicated in motor function, it is also important for motivational processes (Wise & Rompré, 1989; Di Chiara, 2002). It has been reported that moderate doses of dopamine antagonists attenuate the motivation to act before they inhibit the ability to act. For example, rats treated with dopamine antagonists exhibit a gradual decline in well-learned responses. This suggests that antagonists reduce the motivation to act as opposed to the

ability to act, in which case rats would exhibit a sudden decrease in responding (Fouriezos & Wise, 1976; Wise, Spindler, de Wit, & Gerber, 1978; Wise, 2004). Dopamine is also involved in responding to and establishing incentive-motivational properties to previously neutral stimuli. Therefore, it is likely that a dopamine D1-antagonist might disrupt the incentive value attributed to the alcohol-predictive cue, leading to decreased motivation in cue-driven alcohol-seeking (Wise, 2004).

Previous studies provide a clear indication for the role of dopamine in drug-seeking. For example, blocking dopamine D1-receptors has been shown to reduce discrete cue-induced reinstatement of cocaine-, alcohol-,nicotine-, heroin-, and sucrose-seeking in rats (Ciccocioppo et al., 2001; Crombag et al., 2002; Hamlin et al., 2007; Liu et al., 2010; Bossert et al., 2007). While the NAc has been identified as a key brain area involved in drug-seeking, the NAc core and shell have different roles in reinstatement induced by discrete or contextual cues. Specifically, the NAc core and appears to be more important for cue-induced drug-seeking, whereas the shell is more involved in context-induced drug-seeking. For example, intracranial infusions of SCH 23390 into the medial and lateral NAc shell but not NAc core have been shown to reduce context-induced reinstatement of heroin-seeking (Bossert et al., 2007). Alternatively, blocking dopamine D1-receptors with SCH 23390 infused into the NAc core attenuates discrete cue-induced reinstatement of heroin-seeking, when tested in a context that has never been associated with heroin self-administration (Bossert et al., 2007). Similar findings have also been observed with respect to Pavlovian cue- and context-induced alcohol-seeking. Specifically, presentation of the CS+ and CS- in an alcohol-associated context elicited the renewal of port entry responding, and inactivating the NAc shell with muscimol/baclofen

attenuated this effect. Alternatively, cue-driven alcohol-seeking was observed when the CS+ and CS- were presented in a context that had not been associated with alcohol, and was attenuated by inactivating the NAc core. To our knowledge, the present findings are the first to identify a role for dopamine D1-receptors in Pavlovian-conditioned cue-driven alcohol-seeking, as SCH 23390 dose-dependently attenuated responding to an alcohol-predictive cue.

The involvement of dopamine in responding to Pavlovian drug-predictive stimuli is consistent with clinical reports. For example, positron emission tomography (PET) studies report dopamine increases in the dorsal striatum of cocaine addicts upon watching a video of cocaine-related proximal cues, suggesting that dopamine is involved in reactivity elicited by cocaine-conditioned cues (Volkow et al., 2006). Amphetamine-predictive stimuli have also been shown to elicit striatal dopamine release in humans (Boileau et al., 2007). Subjects received a pill which contained amphetamine on 3 separate occasions. Two weeks later, the amphetamine pill was switched to a placebo pill that looked identical to the first amphetamine-containing pill. Raclopride binding to dopamine D2- and D3-receptors was assessed during the amphetamine pill, placebo pill, and no pill (control) PET scan. Interestingly, amphetamine administration and placebo administration decreased raclopride binding potential with the same amplitude relative to what was observed in the control scan. Collectively, these studies indicate that drug-associated cues can elicit conditioned dopamine release and craving in humans, thereby increasing the propensity to relapse.

Dose-dependent effects of eticlopride on cue-driven alcohol-seeking

In experiment 2a, rats acquired discrimination between both cues and the acquisition and habituation phases were identical to experiment 1. Eticlopride, a dopamine D2-receptor antagonist dose-dependently attenuated responding to the alcohol-predictive CS+, with no effect on responding to the CS-. In addition, we observed that this effect differed as a function of vendor: neither the 5 μg/kg nor the 10 μg/kg of eticlopride reduced responding to the alcohol-predictive cue relative to saline pre-treated rats from Charles River. Alternatively, in Harlan rats an infusion of 10 μg/kg of eticlopride significantly reduced responding to the alcohol-predictive cue when compared to pre-treatment with saline or 5 μg/kg of eticlopride.

As in Experiment 1, an analysis of the pattern of responses at test revealed that CS+ trials elicited more responses at the start of the session compared to consecutive CS+ trials. Saline pre-treated rats showed this pattern of responding suggesting within-session extinction of port entry responding similar to experiment 1. Again, this provides evidence that the incentive value of the CS+ becomes weakened as it comes to predict non-reward (Wise, 2004) or that subjects have now learned that the CS+ no longer predicts alcohol delivery (Bouton, 2004). A systemic infusion of eticlopride dose-dependently attenuated responding to each CS+ trial (Fig. 8c and Fig. 8d). Additionally, the number of total port entry responses did not significantly differ between rats that were pre-treated with saline or eticlopride. Unlike experiment 1, an investigation of latency to first port entry (in seconds) suggests that an infusion of eticlopride had no effect on the rats' ability to make a first port entry response: there were no significant differences in

latency between the saline pre-treated rats and the 5 μg/kg or 10 μg/kg doses of eticlopride (Fig. 10).

Although D2-receptor antagonists are known to produce motor deficits (Smith, Smith, Zigmond, Amalric, & Koob, 2000), we do not attribute the reduction in CS+ responding to a locomotor impairment based on these findings. In addition, the 10 μg/kg dose of eticlopride did not affect responding to the alcohol-predictive cue when paired with alcohol delivery Exp. 2b). These results are consistent with previous findings that eticlopride does not cause a locomotor deficit within this dose range (Liu & Weiss, 2002) and that the D2-receptor antagonist induces motoric impairments at a 20 μg/kg or higher (Bardo, Valone, & Bevins, 1999; Bevins, Besheer, & Pickett, 2001). Similar to experiment 1, we therefore suggest that blocking dopamine D2-receptors reduces responding to the alcohol-predictive cue by attenuating the motivation to response to alcohol-predictive cues (Wise, 2004).

That dopamine D2-receptors are required for Pavlovian cue-driven alcohol-seeking is consistent with reports that D2-receptors are involved in reinstatement to alcohol-seeking induced by a discriminative olfactory cue which signals the availability of alcohol (Liu & Weiss, 2002). The capacity of discriminative cues to elicit alcohol-seeking can be dose-dependently reduced by SCH 23390 and eticlopride, suggesting that both dopamine receptors subtypes are also involved in discriminative-cue-induced alcohol-seeking. This study used a reinstatement procedure, in that the discriminative olfactory cue was extinguished before test. Interestingly, our findings suggest that similar neural mechanisms are required for responding to extinguished and non-extinguished drug-predictive cues whether acquired through operant or Pavlovian conditioning.

However, a recent study investigating the role of dopamine D1- and D2-receptors in discrete cue-induced food seeking reported opposing roles for these receptor subtypes. Whereas systemic injections of SCH 23390 attenuated discrete cue-induced reinstatement of food seeking, systemic injections of eticlopride significantly increased responding to a food-related cue during reinstatement tests (Ball, Combs, & Beyer, 2011). Similar effects of D2 antagonists have been observed in cue-induced reinstatement of cocaine seeking (Berglind, Case, Parker, Fuchs, & See, 2006). These results suggest that effect of D2-receptor antagonists on discrete cue-induced reinstatement might differ as a function of reward.

Studies that have examined the intracranial locus of D2-mediated effects indicate that infusions of raclopride in the basolateral amygdala (BLA) attenuated cue-induced reinstatement of cocaine seeking, but exclusively at a high dose (5.0 μg/side). Interestingly, a lower dose of raclopride (1.25 μg/side) potentiated cue-induced reinstatement. Proposed explanations for the inverted-U dose response were the location of D2-receptors within the BLA, the temporal changes in D2-receptor occupancy and the nature of raclopride in that it increases extracellular dopamine. Specifically, the effect of a high dose of raclopride combined with cocaine infusions likely produced increasing levels of extracellular dopamine, displacing raclopride and increasing post-synaptic dopamine receptor stimulation in the BLA at the D1- and D2-receptors (Berglind et al., 2006). Although our drug was administered systemically and we used eticlopride and not raclopride, it is interesting that the low dose resulted in a small increase (although not significant) in responding to the alcohol-predictive cue in rats from Charles River.

Here, we found that D1-receptor antagonists produced a dose-dependent decrease in responding to an alcohol-predictive cue, suggesting that intact dopamine neurotransmission is necessary in cue-driven alcohol-seeking. Alternatively, we observed that D2-receptor antagonists produce different effects, which might depend on several factors. For example, we might expect differences in the effects of eticlopride depending on the dosage administered, and the reinforcer or unconditioned stimulus, as was evidenced by the potentiation of cue-induced reinstatement in food-seeking and cocaine-seeking (Ball et al., 2011; Berglind et al., 2006). Differences might also occur depending on whether acquisition is controlled by Pavlovian or instrumental learning mechanisms. With respect to our findings, we suggest that the D2-receptor antagonist might produce differences in its effect based on vendor, as eticlopride dose-dependently attenuated cue-driven alcohol-seeking in Harlan rats, with no effect in rats from Charles River. In addition, we observe that the interactive influence of the alcohol-related contextual and discrete cues paired with alcohol delivery reliably trigger alcohol-seeking under these conditions. The dopamine D1- but not D2-receptor antagonist significantly reduced responding to the alcohol-predictive cue when paired with alcohol delivery. However, treatment with SCH 23390 and eticlopride did not affect responding during the 10-sec interval that followed the CS+ presentation when rats were likely consuming alcohol. These results suggest that the D1-receptor antagonist specifically reduced responding to the alcohol-predictive cue, suggesting that blocking dopamine D1-receptors might have altered the incentive value of the alcohol-predictive cue even when paired with alcohol delivery. Additionally, fluid ports were dry at the end of the test sessions indicating that the the D1- and D2-receptor antagonist did not affect alcohol consumption.

Vendor differences in alcohol pre-exposure, Pavlovian Discrimination Training and the role of dopamine in Pavlovian cue-driven alcohol-seeking

Results from both experiments revealed several interesting and replicable findings with respect to vendor differences in weight gain and ethanol consumption. Despite starting off at the same weight and being housed under identical conditions, male, Long-Evans rats from Charles River gained weight at a significantly faster rate and consequently became significantly heavier than rats from Harlan Laboratories (Figures 1 ab and 6ab). Ethanol consumption (ml) increased significantly across pre-exposure sessions for rats from both vendors: however, Harlan rats consumed a greater volume of ethanol per session than their counterparts from Charles River, particularly across the first 10 sessions of pre-exposure. Ethanol intake measured in grams of ethanol consumed per kilogram of body weight (g/kg) also increased significantly in rats from both vendors. However, g/kg was significantly higher in rats obtained from Harlan, as was to be expected since Harlan rats drank significantly more ethanol than rats from Charles River and displayed a significantly lower body weight. Interestingly, we found that ethanol preference was more than twice as high in rats from Harlan compared to rats from Charles River, which could be expected as Harlan rats drank less water than Charles River rats.

These data are consistent with vendor differences found in male, Wistar rats obtained from 5 different suppliers. Significant differences in body weight were reported in Wistar rats obtained from the United Kingdom (B&K Universal), Germany (Charles River Europe), U.S.A (Harlan Laboratories), The Netherlands (Harlan Laboratories) and Denmark (Taconic Farms Europe). Wistar rats from Harlan Laboratories in The

Netherlands differed significantly from most of the other vendors. They drank significantly more ethanol than rats from the other suppliers, with 80% of the total number of rats exhibiting a mean ethanol intake of more than 3.0 g/kg/day. By the end of the experiment, 90% of the Harlan rats from The Netherlands had an ethanol preference above 60% (Palm et al., 2011). Overall, these results highlight that selection of vendor can have serious implications in studies that measure alcohol consumption. Collectively, results from both studies demonstrate that although all rats are of the same strain, they strongly differ with respect to ethanol consumption and preference based on vendor. Several factors have been proposed to explain such differences. For instance, they could result from differences in genetic makeup from years of breeding rats at different facilities. Vendor differences can also be attributable to environmental early life experiences such as maternal separation or weaning age which can impact alcohol consumption in adulthood in certain rat strains (Roman, Gustafsson, Hyytia, & Nylander, 2005; Gustafssson & Nylander, 2006; Ploj, Roman, & Nylander, 2003). Because differences between rats are always a risk with outbred strains, we emphasize the importance of understanding these differences and considering them when designing alcohol studies (Palm et al., 2011).

Unlike the replicable differences observed during ethanol pre-exposure, there were no consistent vendor differences with respect to the acquisition of Pavlovian discrimination training. Charles River and Harlan rats from both experiments learned to discriminate between the alcohol-predictive cue (CS+) and the cue that was presented without ethanol. The observed increases in CS+ responding across session suggest that rats from both vendors learned the predictive relationship between the CS+ and ethanol

availability. That said, while Charles River and Harlan rats from experiment 1 displayed similar levels of responding during acquisition, rats obtained from Harlan learned to discriminate between both cues at a faster rate and achieved higher levels of responding than rats from Charles River in experiment 2, resulting in higher level of responding to the CS+ at test. These results suggest that behavioral inconsistencies might also exist in rats obtained from the same vendor but at different times.

At test, we observed differences in Charles River and Harlan rats with respect to the effect of SCH 23390 and eticlopride. In experiment 2a, blocking dopamine D2-receptors did not attenuate responding to the alcohol-predictive cue (CS+) or the cue that was presented without ethanol (CS-) in rats from Charles River. Relative to saline, rats infused with the low dose (5 μg/kg) or high dose (10 μg/kg) did not exhibit a reduction in responding to either cue. Therefore, dopamine neurotransmission at D2 receptors might not be required for responding to an alcohol-predictive cue in rats obtained from Charles River. Conversely, we found a significant difference in responding between saline and eticlopride (10 μg/kg) pre-treated rats from Harlan, indicating that dopamine D2-receptors might be involved in cue-driven alcohol-seeking in rats from this vendor. One possibility is that a higher dose of the D2-receptor antagonist would be required to attenuate cue-driven alcohol-seeking in Charles River rats. Interestingly, differences in the effect of SCH 23390 followed a similar pattern in rats from Charles River and Harlan. More specifically, Figure 3a depicts a reduction in responding to the alcohol-predictive cue in the highest dose (10 μg/kg) of SCH 23390 in rats from Charles River. In Harlan rats however, we observe a reduction in port entry responses in the low (3.33 μg/kg) and high (10 μg/kg) doses of SCH 23390, relative to pre-treatment with saline (Fig. 3b).

Therefore, it is possible that male, Long-Evans rats from Charles River and Harlan differ in terms of dopamine sensitivity. While differences in dopamine sensitivity across rats of the same strain but from different vendors have not been studied, differences in dopamine sensitivity have previously been identified in different strains of rats. For example, Wistar rats are less sensitive to the effects of morphine, an opioid agonist, compared to Sprague Dawley rats. An early study reported that Wistar rats needed a dose of morphine twice as high than Sprague Dawleys to induce conditioned place preference and comparable increases in dopamine activity, suggesting that these strains show differential sensitivity to the opioid agonist (Shoaib, Spanagel, Stohr, & Shippenberg, 1995). The fact that these differences in sensitivity exist between strains might suggest that they can also differ across vendors.

Methodological strengths and limitations

The current studies demonstrate that blocking dopamine D1- and D2-receptors with pharmacological antagonists can attenuate Pavlovian cue-driven alcohol-seeking. Our data were obtained using a procedure that attempts to isolate the neural circuits required for responding elicited by discrete alcohol-predictive cues from potentially differentiable mechanisms that mediate the impact of alcohol-associated environmental contexts on alcohol-seeking. That contexts are an important factor to take into account is suggested by an extensive literature demonstrating that extinguished Pavlovian-conditioned responses can be renewed in contexts where CS-US associations are initially

formed (Bouton, 2004; Crombag, Bossert, Koya, & Shaham, 2008; Chaudhri et al., 2010).

Furthermore, preliminary data from our laboratory has provided evidence of the interactive influence of contextual and discrete cues. A higher level of responding to the alcohol-predictive cue was observed when rats were exposed to the alcohol-associated context relative to non-alcohol or novel contexts. These data provide further evidence that the collaborative effects of discrete and contextual cues can impact craving and lead to relapse, perhaps having a greater influence on relapse than the independent effects of discrete or contextual cues. Therefore, our procedure isolates the role of the alcohol-predictive cue by examining behavior in a non-alcohol context as opposed to an alcohol-associated context.

It should be noted however, that testing the ability of discrete cues to trigger alcohol-seeking in a non-alcohol context might present certain limitations. For example, the non-alcohol context may have acquired inhibitory properties resulting in lower levels of responding. In contrast, an additional strength of our procedure is that we explore non-extinguished cues, which might translate more accurately to human addicts that do not undergo cue-exposure therapy during rehabilitation. Understanding the neurobiological mechanisms that mediate cue-driven alcohol-seeking can lead to the development of effective therapeutic interventions that target responding to non-extinguished cues.

Future studies

It remains a goal for future studies to examine the brain areas in which dopamine acts to mediate responding to Pavlovian-conditioned alcohol-predictive cues. The nucleus accumbens (NAc) has been identified as an important brain region responsible for the ability of environmental cues to facilitate drug-seeking (Crombag et al., 2002; Bossert et al., 2007; Hamlin et al., 2007; Chaudhri et al., 2008) and has been implicated in cue-driven alcohol-seeking (Chaudhri et al., 2010). More specifically, the NAc core and shell have been implicated in discrete-cue- and context-induced drug-seeking, respectively (Bossert et al., 2007; Chaudhri et al., 2010). Therefore, it would of great interest to examine the effect of infusing SCH 23390 and eticlopride in the NAc core and shell to localize the specific brain areas required for responding to a Pavlovian-conditioned alcohol-predictive cue in a non-alcohol context. Future directions should also investigate vendor differences in dopamine D1- and D2-receptor sensitivity as vendor selection has proven not only to be an important consideration when examining ethanol intake and preference, but also in the effects of D1- and D2-antagonist on cue-driven alcohol-seeking.

In summary, our findings demonstrate that alcohol-predictive cues can consistently and reliably elicit Pavlovian-conditioned alcohol-seeking in rats, even when those cues are experienced in a context that has never been associated with alcohol intake. Dopamine D1- and D2-receptor antagonists attenuate this effect, suggesting a role for both receptor subtypes in Pavlovian cue-driven alcohol-seeking. The existence of vendor differences with respect to weight gain, ethanol consumption and preference indicate that vendor selection should be an important consideration when designing

alcohol studies. Therefore, our findings might help explain some discrepancies in response to ineffective pharmacological manipulations in animal models of addiction research. Furthermore, we expect that our conclusions will help researchers be mindful in their interpretations of preclinical models, and their application to the study of human addiction.

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