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Effects of Chronic Alcohol Exposure on Use of Incentive Value and Contribution of Central Amygdala

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Experimental Psychology

by

Chloe N. Shields

Committee in charge:

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2022

The dissertation of Chloe N. Shields is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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Chapter 1, in full, is a reprint of the material as it appears in Shields, C. N., Baltz, E. T., Lopez Valencia, M., & Gremel, C. M. (2022). Effects of chronic alcohol exposure on motivation-based value updating. *Alcohol, 101*, 53-64. https://doi.org/10.1016/j.alcohol.2022.03.004. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Effects of Chronic Alcohol Exposure on Use of Incentive Value and Contribution of Central Amygdala

by

Chloe N. Shields

Doctor of Philosophy in Experimental Psychology

University of California San Diego, 2022

Professor Christina Gremel, Chair

Chronic, high-level exposure to alcohol is associated with deficits in adaptive behavior, or the ability to flexibly adjust actions to achieve desirable outcomes. Such deficits can broadly impact quality of life, for instance by perpetuating alcohol consumption despite increasingly negative consequences. However, the specific components of adaptive behavior that are disrupted following chronic alcohol exposure are not entirely clear. One aspect that may be affected is the ability to use the incentive value – essentially, the subjective attractiveness – of possible outcomes to guide actions. This dissertation examines this possibility across two tasks crucially dependent on the use of incentive value, and investigates the contribution of central

amygdala (CeA), a brain region implicated in incentive processes. Chapter 1 examined the ability of mice previously exposed to chronic alcohol to update and use incentive value of food following a shift in motivational state. Results found alcohol mice were impaired following a mild shift in motivation but less so following a large shift, suggesting that deficits could be largely overcome with a salient enough motivational change. Chapter 2 examined the effect of incentive food cues on the actions of mice with a history of chronic alcohol exposure. Results found that the behavior of alcohol mice was subject to greater control by incentive cues, suggesting that the use of incentive value was enhanced in these animals. Chapter 3 examined this same cue-guided behavior during manipulation of CeA activity. Results suggested that alcohol enhancements in cue-guided behavior were countered by excitatory manipulation of CeA, but that CeA manipulation had opposite behavioral effects in alcohol-naïve mice. Together, these studies support that chronic alcohol exposure may enhance the use of incentive value in some circumstances but impair that ability in others, depending on crucial task features. Further, these findings suggest that a nuanced combination of major theories of addiction, such as the negative reinforcement and habit hypotheses, may be warranted. Greater understanding of how chronic alcohol exposure can impact behavior and the brain may prove useful in developing improved assessments and treatments for chronic alcohol use and alcohol use disorder in humans.

INTRODUCTION

Chronic alcohol use and alcohol use disorder (AUD) are prevalent phenomena worldwide, and are associated with deficits in adaptive behavior that can support continued alcohol use and broadly impact quality of life (Garbusow et al., 2014). One component of adaptive behavior that may be disrupted by chronic exposure to alcohol is the ability to use information about the subjective attractiveness of possible outcomes. Called incentive value, this subjective information is gained through experience and can be decoupled from homeostatic drives, such as basic caloric requirements (Balleine, 1992; Balleine & Dickinson, 1991). In a classic example of this principle, many of us may recognize the experience of eating a full dinner and yet suddenly finding ourselves craving dessert. Incentive value is also notably distinguishable from the actual pleasure derived from a particular outcome (e.g., Smith et al., 2011; Wassum et al., 2009). That is, in the case of alcohol use, incentive value can be understood as how much an individual "wants" to drink alcohol, independent of how much they may "like" the experience of drinking alcohol. Indeed, a dramatic dissociation of "wanting" and "liking" has been proposed as a major feature of substance use disorders (Robinson & Berridge, 1993; Robinson & Berridge, 2008).

Amplified incentive value for drugs and associated stimuli is thought to support drug seeking and misuse in substance use disorders, and may involve Pavlovian and instrumental learning mechanisms (Doñamayor et al., 2021). Briefly, Pavlovian learning is the process through which environmental cues paired with biologically relevant stimuli come to elicit responses, whereas instrumental learning is the process through which the likelihood of performing a particular behavior is influenced by its consequences. A major way in which these learning processes are involved in human alcohol misuse and AUD is that Pavlovian cues associated with alcohol can elicit craving, possibly motivating instrumental behaviors such as alcohol-seeking and consumption (Garbusow et al., 2014; Sjoerds et al., 2014; Witteman et al., 2015).

Human research suggests that alcohol-related disruptions in incentive processes and behaviors may also extend beyond alcohol and its related cues. One study found that in individuals with a history of AUD, non-drug reward cues had a greater influence on ongoing instrumental behaviors compared to controls (Garbusow et al., 2016, but see van Timmeren et al., 2020). A history of AUD is also associated with difficulty tracking shifting values over time (Brevers et al., 2014; Fein et al., 2004; Le Berre et al., 2014), appraising the value of future rewards (Boettiger et al., 2007; Mitchell et al., 2005; Bobova et al., 2009), and determining incentive value when faced with conflicting stimuli (Duka et al., 2011). These works suggest broad deficits in the ability to use incentive value to guide adaptive behavior following chronic alcohol exposure. However, research in humans often does not establish whether behavioral deficits pre-existed alcohol use or were produced by alcohol exposure itself.

Animal models provide an avenue to examine the causal role of ethanol exposure in disrupting incentive processes and their underlying neural mechanisms. Work in rodents corroborates many aspects of the incentive value deficits observed in human AUD. Cues and contexts predictive of alcohol can gain incentive salience in rodents, supporting cue-related behaviors such as conditioned reinforcement and alcohol seeking (Cunningham et al., 2006; Valyear et al., 2017). Beyond alcohol cues, a history of alcohol consumption can selectively increase value attribution for a non-drug reward cue in rodents (Kruse et al., 2017; Spoelder et al., 2015). In addition, chronic ethanol consumption and withdrawal impaired performance in a rodent version of an incentive conflict task where stimuli were rewarded individually but not when presented together (Borlikova et al., 2006). These findings suggest that incentive value deficits observed following chronic alcohol exposure are produced at least in part by the effects of alcohol.

Though these works establish a precedent for alcohol-induced disruptions in incentive processes, similar effects have not been thoroughly examined in animal models using methods that produce a high level of intoxication and withdrawal. Laboratory animals, and rodents in

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particular, tend not to consume to high levels when alcohol is voluntarily self-administered (Becker, 2000; Crabbe et al., 2010). A concern, therefore, is that voluntary administration procedures may fail to capture the extent of neurobiological adaptations that occur following the cycles of chronic, high-level alcohol exposure and withdrawal that are characteristic of AUD in humans (Sullivan et al., 2010; Zahr et al., 2011). In answer, the work reported in the following chapters examines incentive processes following chronic intermittent ethanol vapor exposure and withdrawal (CIE), a well-validated involuntary model of alcohol exposure designed to better replicate the patterns of alcohol use, changes in brain function, and behavioral phenotypes observed in AUD (Becker, 1994; Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005). Importantly, in all studies CIE was concluded prior to beginning any behavioral procedures, such that all training and testing occurred after acute withdrawal (Heilig et al., 2010). Thus, any effects of CIE may reflect long-term neuroadaptations produced by chronic alcohol exposure and withdrawal.

Numerous brain regions are implicated in processing incentive reward information, from determining value and assigning it to stimuli, to selecting actions to obtain desired outcomes. This circuitry is thought to involve complex interconnections between cortical, striatal, limbic, and midbrain regions (Haber & Behrens, 2014). Broadly speaking, cortical regions such as orbitofrontal cortex (OFC) project to the striatum, prominently including the nucleus accumbens (NAcc), which itself projects to midbrain regions such as the ventral tegmental area and substantia nigra (Groenewegen et al., 1999; Haber & McFarland, 1999). In turn, midbrain dopamine neurons in these regions output back to NAcc and the prefrontal cortex (Lynd-Balta & Haber, 1994; Williams & Goldman-Rakic, 1998). Another crucial component of this circuitry is the amygdala, which projects to NAcc and connects reciprocally with OFC and midbrain structures (Beckstead et al., 1979; Borrego et al., 2022; de Oliveira et al., 2011; Haber & Behrens, 2014; McDonald, 1991; Steinberg et al., 2020). Notably, activity in these regions can

be dissociated into signals for distinct aspects of reward, such as incentive value, hedonic impact, and associative reward prediction (Smith et al., 2011; Wassum et al., 2009).

The amygdala is of particular interest here as a region directly implicated in regulating the incentive value of outcomes (for review, see Warlow & Berridge, 2021). Located deep within the temporal lobe, the amygdala is comprised of several interconnected nuclei, with two major subdivisions including the basolateral amygdala (BLA, including the lateral, basal, and basomedial nuclei) and the central amygdala (CeA, including lateral and medial subdivisions). Principal neurons in the BLA are primarily glutamatergic, whereas neurons in the CeA are largely GABAergic (Janak & Tye, 2015). A general, highly simplified view of amygdala circuitry is that information flows serially through BLA to CeA, with cortical and thalamic sensory inputs into BLA being translated into behavioral output by the CeA; however, these subregions can also contribute to behavior independently (Corbit & Balleine, 2005; Holland & Gallagher, 2003; Janak & Tye, 2015). The CeA, in particular, is implicated in directing incentive motivation for outcomes (Mahler & Berridge, 2012; Robinson et al., 2014; Tom et al., 2019; Warlow et al., 2020), and is necessary for aspects of behavior guided by incentive environmental cues (Corbit & Balleine, 2005; Hall et al., 2001; Holland & Gallagher, 2003). CeA also demonstrates significant functional changes following chronic, high-level alcohol exposure, including enhanced baseline GABAergic transmission that has been proposed to produce a net inhibition of the area (Pleil et al., 2015; Repunte-Canonigo et al., 2015; Roberto et al., 2010; Roberto et al., 2004). However, it remains unclear how alcohol-induced changes to CeA function might relate to disrupted incentive processes following chronic alcohol exposure.

As previously stated, the exact incentive processes that are disrupted by alcohol dependence have yet to be fully characterized. One incentive process important for adaptive behavior is the ability to use one's current motivational state to infer the value of possible outcomes to one's actions. For instance, the incentive value of food is likely to be lower in a state of satiation compared to a hunger state. An impaired ability to infer the incentive value of

outcomes based on motivational state could, in the case of human alcohol use, contribute to alcohol seeking and consumption regardless of current motivation for alcohol. More broadly, such deficits could also affect seeking and consumption of non-alcohol rewards. Chapter 1 evaluates this possibility by examining the impact of chronic alcohol exposure on the ability to infer the incentive value of food following a shift in hunger state. Mice were first exposed to CIE procedures, and then were trained in an incentive learning task involving a sequence of lever presses under either mild or more severe food restriction. Half of the mice within each food restriction group were then shifted to a less hungry state, and their ability to update their behavior based on their shifted motivational state was evaluated in a series of test days. Results found that a history of CIE exposure led to deficits in using motivational state to update value and guide behavior accordingly, but that deficits could be overcome in part when the magnitude of motivational shift was particularly high. These findings suggest that one aspect of incentive processing that is disrupted by chronic alcohol exposure is the ability to infer value based on motivational state.

Chapter 1 demonstrates that chronic alcohol exposure can disrupt the use of internal information to guide incentive behavior. Another crucial incentive process that supports adaptive behavior is the ability to use external information, such as relevant environmental stimuli, to guide actions. Through association with salient rewarding or aversive outcomes, environmental stimuli can signal incentive value to direct decisions, elicit conditioned behaviors, and incentivize or deter actions. In the context of chronic alcohol use, cues for alcohol can gain amplified incentive value, potentially triggering craving, alcohol-seeking, and relapse to alcohol consumption (Garbusow et al., 2014; Sjoerds et al., 2014; Witteman et al., 2015). However, recent work in individuals with AUD suggests that responding may also be altered for reward cues in general, not just alcohol cues (Garbusow et al., 2016). Given the ubiquity of environmental cues that signal possible rewards, changes in how incentive cues guide actions could have broad implications for the ability to continually engage in adaptive behavior. Chapter

2 examines this possibility by investigating the effects of previous chronic alcohol exposure on incentive cue-guided behavior in mice using a Pavlovian-to-instrumental transfer (PIT) task. PIT is a process through which previously conditioned Pavlovian cues influence instrumental actions, and involves separate periods of Pavlovian and instrumental training followed by a test in which the instrumental action is performed in the presence and absence of a reward-associated Pavlovian cue (though no actual rewards are delivered). A working mouse model of PIT was first established, demonstrating transfer as an increase in lever pressing vigor during the presentation of a food-associated auditory cue. A separate cohort of mice was then exposed to full CIE procedures prior to behavioral training and testing. Results found that mice with a history of chronic alcohol exposure exhibited stronger PIT, as evidenced by augmented lever pressing during presentation of a food-associated cue, relative to control animals not exposed to alcohol. This evidence suggests that a history of prolonged alcohol exposure and withdrawal can enhance the influence of non-drug incentive cues on ongoing behavior.

The finding in Chapter 2 that CIE can enhance the use of non-drug incentive cues to guide behavior suggests that chronic alcohol exposure may alter the function of brain areas underlying cue-guided behavior. One region of interest is the CeA, as this area is not only heavily affected by chronic alcohol exposure but is also necessary to observe the type of PIT demonstrated in Chapter 2 (Corbit & Balleine, 2005; Hall et al., 2001; Holland & Gallagher, 2003). However, whether alcohol-induced neuroadaptations in CeA contribute to alcohol-induced disruptions in cue-guided behavior is unknown. The work reported in Chapter 3 examined the effects of chemogenetic CeA manipulation and CIE exposure on cue-guided behavior in a PIT task. Results found that excitatory and inhibitory chemogenetic manipulation of CeA during testing enhanced PIT in alcohol-naïve mice. However, mice with a history of CIE appeared to demonstrate enhanced PIT at baseline (replicating Chapter 2), and excitatory CeA manipulation in this group produced behavior consistent with a reduction in PIT strength. These results suggest that alcohol-induced alterations in CeA activity could contribute to concomitant

changes in PIT behavior, and that CeA manipulation may serve to "rescue" the enhanced PIT observed in alcohol mice.

Together, these three chapters identify important disruptions in incentive processes following prolonged alcohol exposure. They demonstrate that mice with a history of chronic alcohol exposure are impaired in using mild motivational state changes to infer incentive value (Chapter 1) and that non-drug incentive cues have a greater influence on the actions of these mice (Chapters 2 and 3). In the case of cue-guided behavior, the enhanced influence of incentive cues on behavior may involve alcohol-induced functional changes to CeA which are potentially countered with chemogenetic manipulation (Chapter 3). Disruptions in how incentive value is inferred and used to guide behavior could contribute to the broad deficits in decision-making and adaptive behavior that are associated with AUD. Better understanding these effects of chronic alcohol exposure on incentive processes and underlying neural structures may serve useful in improving methods of assessing and treating individuals with AUD.

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CHAPTER 1

Effects of Chronic Alcohol Exposure on Motivation-Based Value Updating

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Abstract

Dysfunctional decision-making has been observed in alcohol dependence. However, the specific underlying processes disrupted have yet to be identified. Important to goal-directed decision-making is one's motivational state, which is used to update the value of actions. As ethanol dependence disrupts decision-making processes, we hypothesized that ethanol dependence could alter sensitivity to motivational state and/or value updating, thereby reducing the capability for adaptive behavior. Here we employed a sequential instrumental learning task to examine this hypothesis. In two experiments, mice underwent chronic intermittent ethanol (CIE) or air (Air) vapor exposure and repeated withdrawal procedures to induce ethanol dependence. Mice were then trained on a sequence of distal and proximal lever pressing for sucrose under either mild or more severe food restriction. Half of all Air and CIE mice then underwent a motivational shift to a less hungry state and effects of this motivational shift were evaluated across three days. First, mice were re-exposed to sucrose, and effects of food restriction state and CIE exposure on lick and consummatory behavior were examined in the absence of lever pressing. Over the next two days, mice underwent a brief non-rewarded test and then a rewarded test where the ability to retrieve and infer sucrose value to guide lever pressing was measured. In the sucrose re-exposure session, prior CIE exposure altered sucrose-seeking in mice with a history of mild but not more severe food restriction, suggesting altered motivational sensitivity. During lever press testing, CIE mice were insensitive to decreases in motivational state and did not reduce proximal lever pressing regardless of food restriction state. Mildly restricted CIE mice, but not severely restricted CIE mice, also did not reduce distal pressing to the same degree as Air mice following a downshift in motivational state. Our findings suggest that ethanol dependence may disrupt motivational processes supporting value updating that are important for decision-making.

Key Words

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Introduction

Alcohol use disorder (AUD) is associated with long-lasting disruptions to decision-making processes. Decision-making often recruits our ability to use changes in our motivational state to appropriately adapt our behavior, often termed goal-directed control. In particular, deficits in goal-directed decision-making have been observed in human AUD (Gillan, Kosinski, Whelan, Phelps, & Daw, 2016; Sjoerds et al., 2013), as well as in animal models of alcohol dependence (Corbit, Nie, & Janak, 2012; Lopez, Becker, & Chandler, 2014; Renteria, Baltz, & Gremel, 2018). As these decision-making processes and their control by motivational state support ongoing adaptive behavior as well as the continued use and misuse of alcohol, understanding their disruption may shed light on obstacles toward recovery faced by those with AUD.

Control over decision-making processes is often evaluated with outcome devaluation procedures. Normally, the subject is trained to work for either a food or alcohol outcome, and then that outcome is devalued using sensory-specific satiation or aversive pairing of the reward with illness. Goal-directed control over decision-making is observed as a reduction in the frequency at which subjects work to get the reward following outcome devaluation procedures (Adams & Dickinson, 1981; Dickinson, 1985). In the alcohol field, often put forth is the habit hypothesis, which suggests that individuals chronically exposed to alcohol are insensitive to the negative consequences associated with continued alcohol consumption, and hence continue alcohol-seeking and show generally disrupted decision-making (Barker et al., 2015; Everitt & Robbins, 2005; Gremel & Lovinger, 2016). However, other bodies of work suggest alcohol-seeking can still be goal-directed (e.g., Samson et al., 2004), and raise questions about the contribution of habit-related processes to continued alcohol misuse and AUD (Hogarth, 2020). Understanding the specific behavioral mechanisms that may be disrupted could shed light on the above discrepancies.

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Rodent models of chronic alcohol use or exposure have provided some evidence to support the hypothesis that people with AUD may be insensitive to negative consequences. Long-term ethanol self-administration renders lever pressing for alcohol insensitive to outcome devaluation (Corbit et al., 2012), an effect termed habitual alcohol-seeking. This insensitivity to devaluation appeared to be driven by excessive alcohol exposure, as non-contingent ethanol exposure for the same extensive duration also left sucrose self-administration insensitive to outcome devaluation (Corbit et al., 2012). In addition, prior induction of ethanol exposure through vapor procedures rendered ethanol-seeking behavior insensitive to outcome devaluation, an effect not observed when subjects were instead exposed to air vapor (Lopez et al., 2014; Renteria, Cazares, & Gremel, 2020). Lastly, the effect of ethanol exposure seems largely due to direct actions on neural circuits that support goal-directed decision-making and not through other possibilities, such as an effect on ethanol valuation. For instance, prior ethanol exposure produced food responding that was insensitive to outcome devaluation by disrupting output of a neural circuit shown to support goal-directed control (Gremel et al., 2016; Renteria et al., 2018). It thus appears that chronic exposure to ethanol itself can alter mechanisms supporting and/or underlying goal-directed decision-making.

However, as stated above, the particular disruptions to behavioral mechanisms that support or underlie goal-directed decision-making are unknown. Outcome devaluation procedures used to assess goal-directed control depend upon numerous components, including (but not limited to) general sensitivity to changes in motivational state, the ability to update the value of the reward (i.e., is the reward devalued), and the ability to infer and assign the updated value as a consequence of the associated action (i.e., using the devalued reward to update the action value; Balleine, 2011). In outcome devaluation procedures, the former two are confounded, as motivational state is altered by consumption of the reward itself. Procedures aimed at examining incentive learning, the process by which motivational states are used to assign value to the goals of our actions, have been used to examine how a change in

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motivational state (achieved through shifts in the degree of food restriction) can influence the updating and inference of reward value to control actions (Baltz, Yalcinbas, Renteria, & Gremel, 2018; Wassum, Ostlund, Maidment, & Balleine, 2009). Importantly, effects of shifts in motivational state on reward-seeking and consumption can be examined in a re-exposure session. In this re-exposure session, the reward is delivered randomly in the absence of the normally associated action. Differences in seeking and/or consummatory behaviors during the re-exposure session can reflect differences in motivational state (often hunger state) and/or palatability, respectively (Balleine & Dickinson, 1998; Wassum et al., 2009). In subsequent test sessions, the ability to infer and use the updated value to control actions is assessed. Differences between groups in the frequency of actions can indicate a deficit in updating and/or inferring the updated value associated with a particular action, as long as behavior during the re-exposure session was similar between groups in the same motivational state (Balleine & Dickinson, 1998; Wassum et al., 2009). Otherwise, motivational and/or palatability differences could contribute to the apparent alteration in the ability to update and infer a value change. Thus, use of the incentive learning task allows for the potential to examine whether there are alterations in 1) sensitivity to changes in motivational state, 2) sensitivity to changes in palatability, and 3) the ability to update and infer a value change.

One hypothesis for how ethanol exposure may disrupt goal-directed decision-making is by impeding these motivational and/or incentive learning processes. That is, the mechanisms underlying sensitivity to shifts in motivational state, assigning a less desirable value to the reward based on this motivational state, and/or inferring that new representation to control behavior may be dysfunctional in ethanol dependence. In the present studies, we employed the widely used chronic intermittent ethanol exposure and repeated withdrawal (CIE) procedure (Becker, 1994; Griffin, Lopez, & Becker, 2009; Lopez & Becker, 2005) to induce ethanol dependence in mice, after which animals were trained in an incentive learning task. In a series of two experiments, we examined on separate days the capacity of mice to 1) use their current

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motivational state, 2) update incentive value, and 3) guide instrumental actions. Furthermore, we examined these abilities within the context of differing magnitudes of motivational state shifts, using either mild (16 hours/day, no access to food; 8 hours/day, unlimited access) or more severe food restriction (gram restriction to 85% of baseline body weight).

Materials and methods

Animals

Male and female C57Bl/6J and B6.129S2-*Emx1*tm1(cre)KrJ/J mice on a C57Bl/6J background (Emx1-Cre; bred in-house one generation from mice ordered from Jackson Laboratories, United States) were housed 2–4 per cage under a 14/10–hour light/dark cycle with access to food (Lab-diet 5015) and water *ad libitum* unless stated otherwise. C57Bl/6J mice were used as prior works have identified disrupted goal-directed decision-making processes following chronic ethanol exposure in this strain (Lopez et al., 2014; Renteria et al., 2018, 2020). Emx1-Cre mice were employed for the potential of future neurobiological manipulations. Mice were at least 8 weeks of age prior to the start of CIE procedures. The Animal Care and Use Committee of the University of California San Diego approved all experiments, and experiments were conducted according to NIH guidelines.

Chronic intermittent ethanol exposure

Multiple cohorts of mice were exposed to four rounds of ethanol vapor or air as previously described (hourly food restriction cohort $n = 3$, gram food restriction cohort $n = 2$; Renteria et al., 2018). Strain was kept consistent within each vapor cohort, with the first cohort of hourly food restriction including only Emx1-Cre mice and all other cohorts containing only C57 mice. Each round consisted of 16 hours of vapor (ethanol or air) exposure followed by an 8-hour withdrawal period. This was repeated for four consecutive days, with a three-day period in between rounds in which no vapor exposure occurred. Vapor exposure was done by placing mice in their home cages into Plexiglas chambers (Plas Labs Inc., United States), and passing air or ethanol vapor through the chambers. Ethanol was volatilized by bubbling air through a

flask containing 95% ethanol at a rate of 2.3 L/minute, and was combined with a separate air stream to give a total flow of approximately 10 L/minute. To avoid effects of stress on instrumental behaviors (Dias-Ferreira et al., 2009) and broad actions of pyrazole (including actions at the NMDA receptor; Pereira, Aracarva, Aronstam, Barreiro, & Albuquerque, 1992), no pyrazole or loading dose of ethanol was administered prior to placement in the chamber. Animals were monitored for ill effects of vapor exposure. Blood ethanol concentrations (BECs) were collected at the end of each round from 11 total sentinel mice (mean BEC = 40.34 ± 2.81 mM; Analox, United States). Use of sentinels prevented the ability to correlate BEC measurements with the magnitude of behaviors observed.

Behavioral training and testing procedures

Training was conducted as previously described (Baltz et al., 2018). In brief, mice were trained to press a distal "seeking" lever to gain access to a proximal "taking" lever that, when pressed, would produce delivery of 20% sucrose. In this paradigm, the distal lever has been shown to be sensitive to incentive learning processes, while the proximal lever is directly sensitive to changes in motivational state and does not rely on incentive learning processes to control responding (Balleine, Garner, Gonzalez, & Dickinson, 1995; Corbit & Balleine, 2003). Following training, mice were either kept in the same motivational state or underwent a shift in motivational state. To provide an opportunity to update the value of sucrose, mice were then given a re-exposure session where sucrose was delivered but no levers were presented. Testing then occurred across two days. First, mice were given a brief non-rewarded test where presses on the distal and proximal levers were recorded, but no sucrose was delivered. This test provided a measure of the ability to infer or retrieve and use the updated value gained on the re-exposure day to control responding. For the second test, mice were given a normal rewarded session and presses on the distal and proximal levers were recorded. This rewarded test provided an opportunity for mice to use the experienced updated value to control ongoing decision-making.

Experimental design and food restriction

Two experiments were run using different types of food restriction. In Experiment 1, the total time with access to food was restricted (hourly restricted), whereas in Experiment 2, the amount of daily food available was restricted (gram restricted). Prior to the onset of experimental procedures, mice in each experiment were assigned to one of two vapor groups (Air or CIE) and one of two restriction groups (shift or no shift). In Experiment 1, all mice underwent lever press training with 16 hours of food restriction that began ~3–4 hours before lights out and ended immediately prior to daily training. Mice within each Air or CIE group in Experiment 1 were further assigned to one of two groups: 16-16 Group or 16-2 Group. Mice in the 16-16 Group were kept at 16 hours of food restriction for the duration of the experiment. Mice in the 16-2 Group were shifted to a 2-hour food restriction the night prior to the re-exposure session, with the 2-hour food restriction beginning 1.5 to 3 hours into their light cycle (Vollmers et al., 2009) and ending immediately prior to daily sessions. Mice in this group were kept at 2-hour food restriction for the remainder of experimental procedures. In Experiment 2, mice were food-restricted to ~85% of their baseline body weight, and instrumental training was conducted under this gram restriction. Mice within each Air or CIE Group in Experiment 2 were further assigned to one of two groups: R-R Group or R-F Group. Mice in the R-R Group were kept gram-restricted and maintained at ~85% body weight for the duration of the experiment. Mice in the R-F Group were switched from gram restriction to free-feed the night before the re-exposure session, and were maintained at free-feed for the remainder of the experiment. Thus, there were a total of four groups in each experiment; in Experiment 1 the groups consisted of Air 16-16, Air 16-2, CIE 16-16, and CIE 16-2, while in Experiment 2 the groups consisted of Air R-R, Air R-F, CIE R-R, and CIE R-F. Group assignment was counterbalanced across cohort, sex, squad, and operant box. Given limitations of vapor and food restriction procedures, groups were kept consistent within a cage.

Instrumental training

Mice began instrumental training 3–5 days following the last CIE procedure. Mice were trained in standard operant chambers containing two levers situated around a food magazine containing a fluid well with contact lickometers and a house light on the opposite wall within sound-attenuating boxes (Med-Associates, Vermont, United States). On the first day, mice underwent magazine training on a random time (RT) schedule, with a 20% sucrose in water outcome (20–30 µL) delivered on average every 120 seconds for 60 minutes. For the next 3–4 days, mice had access to the right (proximal) lever, and right lever presses were rewarded on a continuous reinforcement (CRF) schedule (one lever press produces a sucrose delivery; equivalent to a fixed ratio 1 or FR1 requirement). The session continued until mice earned 30 sucrose deliveries or until 60 minutes had passed. After CRF training on the right lever, schedule training continued with introduction of the left (distal) lever into the chamber. During schedule training, the session began with the left lever out and the right lever retracted. Mice had to press the left (distal) lever under a random ratio (RR) schedule requirement to get access to the right (proximal) lever. A right lever press under an FR1 schedule would then result in the delivery of sucrose and retraction of the right lever. As gram restriction can support higher levels of lever pressing than hourly restriction, we used higher RR requirements in Experiment 2 than Experiment 1. The RR schedule requirement for the left lever increased across six days. For Experiment 1, schedule progression occurred as follows: RR1 for one day, RR2 for one day, and RR4 for four days. In Experiment 2, schedule requirements progressed from RR2 for one day, to RR4 for one day, to RR8 for the final four days. Sessions ended when a mouse earned 30 sucrose deliveries or 60 minutes had passed.

Re-exposure and testing

Prior to the re-exposure session, mice were or were not shifted in hunger state depending on group assignment, and were maintained at the assigned hunger state for re-exposure and testing sessions. In Experiment 1, mice in the 16-16 Group were kept at 16-hour food restriction, while mice in the 16-2 Group were food-restricted for just 2 hours prior to the re-

exposure session. In Experiment 2, R-R mice were kept at ~85% body weight by limiting food consumption. Mice in the R-F Group were allowed to free-feed starting ~16 hours prior to the re-exposure session. For the re-exposure session, mice were given re-exposure to sucrose during an RT session for 1 hour, with sucrose delivered on average every 2 minutes. The next day, mice were given a 5-minute non-reinforced test session where responses on the left lever under RR schedule requirements (same RR schedule as the last four days of training) would produce the right lever; however, right lever presses were not reinforced. The following day, mice were given a 60-minute rewarded session similar to the previous day, except that on this day right lever presses produced a sucrose delivery.

Behavioral and statistical analysis

The alpha level was set at 0.05 for all experiments. As all mice within an experiment experienced the same food restriction during training and there were no differences between final groups in acquisition, training data were collapsed across food restriction groups for ease of comparison. For behavior across training days, data were analyzed using two-way mixed ANOVAs (Vapor group × Day) performed on distal and proximal lever presses, lever press rates, head entries, and head entry rates. For re-exposure and test session data, the primary dependent variables were lick behavior and lever press rates during the test session (Baltz et al., 2018). Mice that experienced a reduction in hunger state were expected to reduce lick and response rates, but mice that were maintained at the same hunger state as training were not. For re-exposure lick and head entry behaviors, data were analyzed using two-way ANOVAs (Vapor group × Hunger state). Test data were analyzed using two-way ANOVAs (Vapor group × Hunger state) performed on distal and proximal lever press rates. *A priori* pairwise Bonferroni-corrected comparisons between hunger states were used to examine effects of motivational state on sucrose-seeking and consummatory behavior, as well as to determine the presence of incentive learning in each Vapor group (Baltz et al., 2018). Data were analyzed using Prism 6 (GraphPad, United States). Data are presented as mean ± standard error of the mean (SEM).

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Results

Attrition

As has been our prior experience (Baltz et al., 2018), the hourly food restriction used in Experiment 1 supported lower levels of behavior relative to more severe gram restriction (Experiment 2), with hourly food restriction not inducing weight loss (data not shown). In addition, in both experiments we shifted motivational state prior to testing by allowing mice increased food access, which also supported lower levels of behavior. Thus, not all mice had sufficient response rates (average rate of >0.25 left lever presses/minute to produce right lever access) during training or testing to be included in analyses (Experiment 1, $n = 12$ mice excluded; Experiment 2, $n = 10$ mice excluded). In Experiment 1, final Group ns are as follows: Air 16-16 = 9 (7F, 2M), Air 16-2 = 9 (9M), CIE 16-16 = 11 (5F, 6M), and CIE 16-2 = 10 (7F, 3M). In Experiment 2, final Group ns are as follows: Air R-R = 10 (8F, 2M), Air R-F = 6 (1F, 5M), CIE R-R = 10 (3F, 7M), and CIE R-F = 10 (6F, 4M). Lickometer technical malfunction resulted in excluding lick data from eight animals in Experiment 2. Final ns for analysis of re-exposure lickometer data in Experiment 2 are as follows: Air R-R = 8 (6F, 2M), Air R-F = 6 (1F, 5M), CIE R-R = 7 (2F, 5M), and CIE R-F = 7 (3F, 4M).

Weights

Following CIE exposure but prior to the start of behavioral procedures, Air and CIE mice showed similar weights [unpaired t tests, Experiment 1: $t(37) = 1.39$, $p = 0.17$; Experiment 2: $t(34) = 0.15$, $p = 0.88$]. Average weights in Experiment 1 were 23.26 ± 1.09 g in the Air group and 21.41 ± 0.81 g in the CIE group. In Experiment 2, average weights were 21.13 ± 1.02 g in Air mice and 20.95 ± 0.73 g in CIE mice.

Experiment 1

Acquisition. Five days after the last vapor exposure, all mice underwent magazine and CRF training (data not shown) prior to schedule training. Once schedule training began, Air and CIE mice similarly acquired distal lever pressing under an RR schedule (Figure 1A, D). Two-way

mixed ANOVAs (Vapor group × Day) conducted on distal lever presses and distal lever press rate revealed main effects of Day [distal lever presses: $F(4, 148) = 25.83$, $p < 0.0001$; distal lever press rate $F(4, 148) = 29.37$, $p < 0.0001$], but no main effect of Vapor group ($Fs < 0.17$, $ps > 0.05$) or significant interactions ($Fs < 1.12$, $ps > 0.05$). Furthermore, presses on the proximal lever were acquired similarly between Air and CIE mice (Figure 1B, E), with two-way mixed ANOVAs (Vapor group × Day) showing main effects of Day for proximal lever presses [$F(4, 148)$ = 36.33, $p < 0.0001$] and proximal lever press rate [$F(4, 148) = 31.79$, $p < 0.0001$], but no main effects of Vapor group ($Fs < 0.03$, $ps > 0.05$) and no significant interactions ($Fs < 1.47$, $ps > 0.05$). In addition to lever press behaviors, Air and CIE mice also showed similar levels of head entries [main effect of Day: $F(4, 148) = 9.19$, $p < 0.0001$; Figure 1C, F] and rate of head entries [main effect of Day: $F(4, 148) = 6.45$, $p < 0.0001$], with no other effects indicated (no main effects of Vapor group: $Fs < 2.04$, $ps > 0.05$; no interactions: $Fs < 0.95$, $ps > 0.05$).
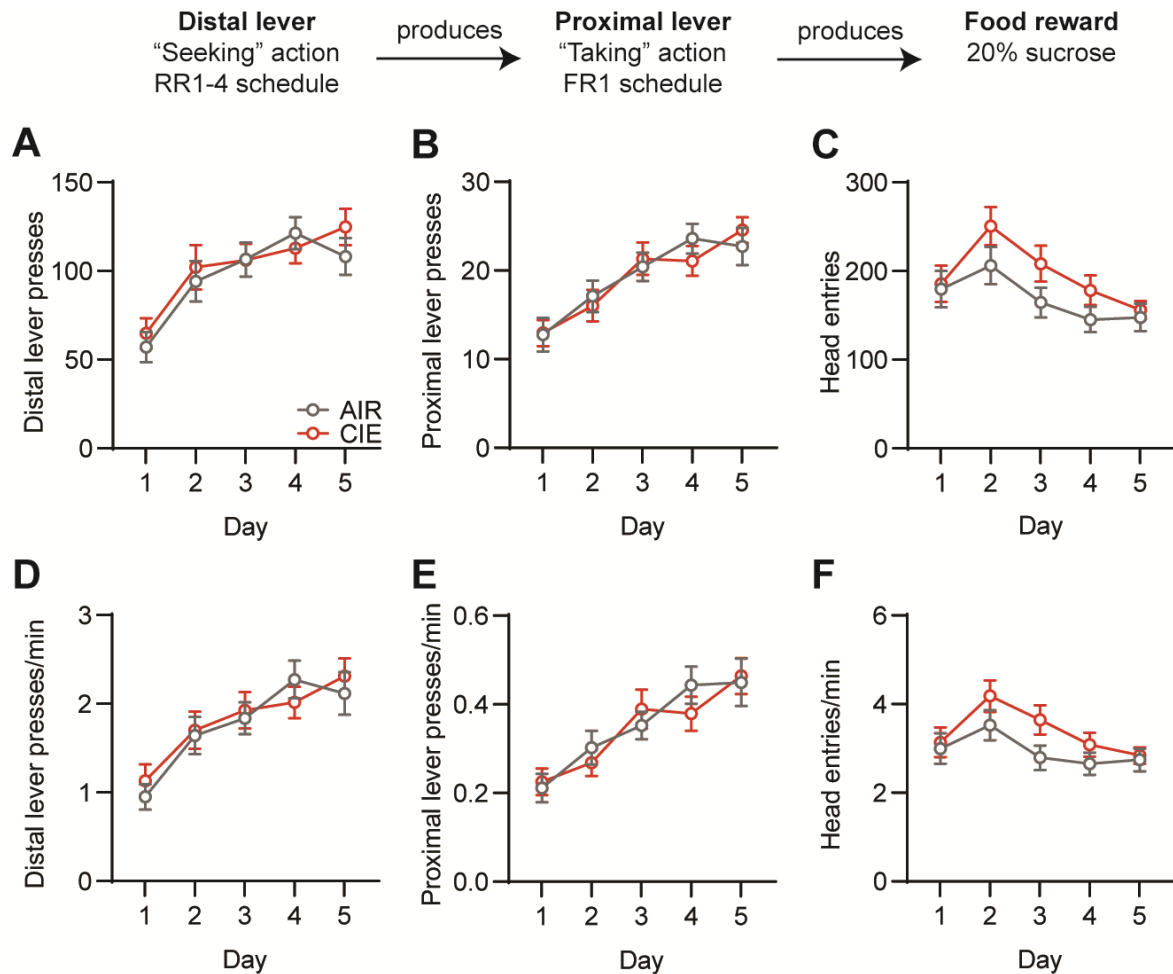
Figure 1.1: Acquisition of instrumental task in Experiment 1 with mice under 16-hour food restriction. Across five days of lever press training, chronic intermittent ethanol (CIE) and air control mice (**A**) increased the number of distal and (**B**) proximal lever presses. (**C**) Head entries mice made across days. Mice also increased the rate of (**D**) distal and (**E**) proximal presses. (**F**) Rate of head entries. RR = random ratio schedule; FR = fixed ratio schedule. Red circles (CIE mice) and gray circles (Air mice) are means ± SEM.

Re-exposure. The shift in hunger state was initiated prior to the re-exposure session, when Air 16-2 and CIE 16-2 mice were moved from a 16-hour restriction period to a 2-hour restriction period. Mice in the Air 16-16 and CIE 16-16 groups were maintained at 16-hour food restriction prior to the start of the re-exposure session. During the re-exposure session, sucrose was delivered on an RT schedule and licks and head entries were recorded. We found that a shift in hunger state altered the total number of head entries performed [main effect of Hunger state, $F(1, 35) = 18.63$, $p < 0.001$; no main effect of Vapor group or interaction: Fs < 0.83, ps > 0.05], with planned *post hoc* analyses revealing a decrease in head entries in the 16-2 group compared to the 16-16 group for both Air ($p < 0.01$) and CIE mice ($p < 0.05$; Figure 2A). The same pattern was observed for the rate of head entries (data not shown). We also found that a shift in hunger state altered the rate of licking [main effect of Hunger state, $F(1, 35) = 4.55$, $p < 0.05$; no main effect of Vapor group or interaction: Fs < 2.43, ps > 0.05]. A prior planned *post hoc* analysis showed a significant reduction in the 16-2 compared to the 16-16 lick rate for the Air group (Bonferroni-corrected $p < 0.05$) and not the CIE group ($p > 0.05$; Figure 2B). The same pattern was observed for total licks (data not shown). This finding suggests that while a reduction in hunger state decreased head entry and licking behavior, it may have done so to a larger extent in the Air group.

To investigate this further, we performed additional analyses on variables related to the patterning of licking across the session. Mice often organize their licking into bursts (defined as three or more sequential licks with an interlick interval of less than 1 second), and emit many bursts of licking behavior independent of whether or not sucrose has been delivered. We examined whether Vapor and Hunger assignment would differentially alter lick bursts and related lick patterns. When we examined the number of lick bursts made by Air and CIE mice, we found a main effect of Hunger state [$F(1, 35) = 7.32$, $p < 0.05$; no main effect of Vapor or interaction, Fs < 2.76, ps > 0.05; Figure 2C]. Planned comparisons within each group showed a significant reduction in the number of lick bursts for Air 16-2 compared to Air 16-16 mice ($p <$

0.05), but CIE mice showed similar numbers of lick bursts independent of hunger state ($p >$ 0.05). Further, Hunger state and Vapor group status did differentially affect the time in between licking bursts. The interburst interval was significantly longer in Air 16-2 mice compared to Air 16-16 mice, but CIE mice had similarly short interburst intervals regardless of hunger state. This was supported by a two-way ANOVA, which found a significant interaction between Hunger state and Vapor group [$F(1, 35) = 6.96$, $p < 0.05$], as well as main effects of Hunger state [$F(1, 35) = 10.32$, $p < 0.01$] and Vapor group [$F(1, 35) = 6.53$, $p < 0.05$; Figure 2D]. *A priori* Bonferroni-corrected comparisons confirmed that the difference between hunger state groups was significant in Air ($p < 0.05$), but not CIE ($p > 0.05$) mice. When we looked at lick behavior within a burst, we found similar average lick burst durations in all groups (no main effects or interactions, Fs < 2.9, ps > 0.5; Figure 2E) as well as similar interlick intervals during a burst (no main effects or interactions, Fs < 0.74, ps > 0.05; Figure 2F). Mice also emit bursts of licking following sucrose delivery, where licking behavior is more directly tied to consumption. When we examined the duration of the first burst following reinforcement delivery, we found that a reduction in hunger state on average tended to reduce lick burst duration [main effect of Hunger state, $F(1, 35) = 6.16$, $p < 0.05$], and there was no effect of Vapor group or interaction (Fs < 0.88, ps > 0.05; Figure 2G); however, prior planned comparisons found no significant differences between hunger state groups in either Air or CIE mice (ps > 0.05). These results suggest that though both Air and CIE mice showed similar lick behaviors when a reinforcer was likely present, CIE mice spent more time seeking reinforcement as indexed by lick rate, increased number of lick bursts, and reduced time between bursts even in the reduced motivational state.
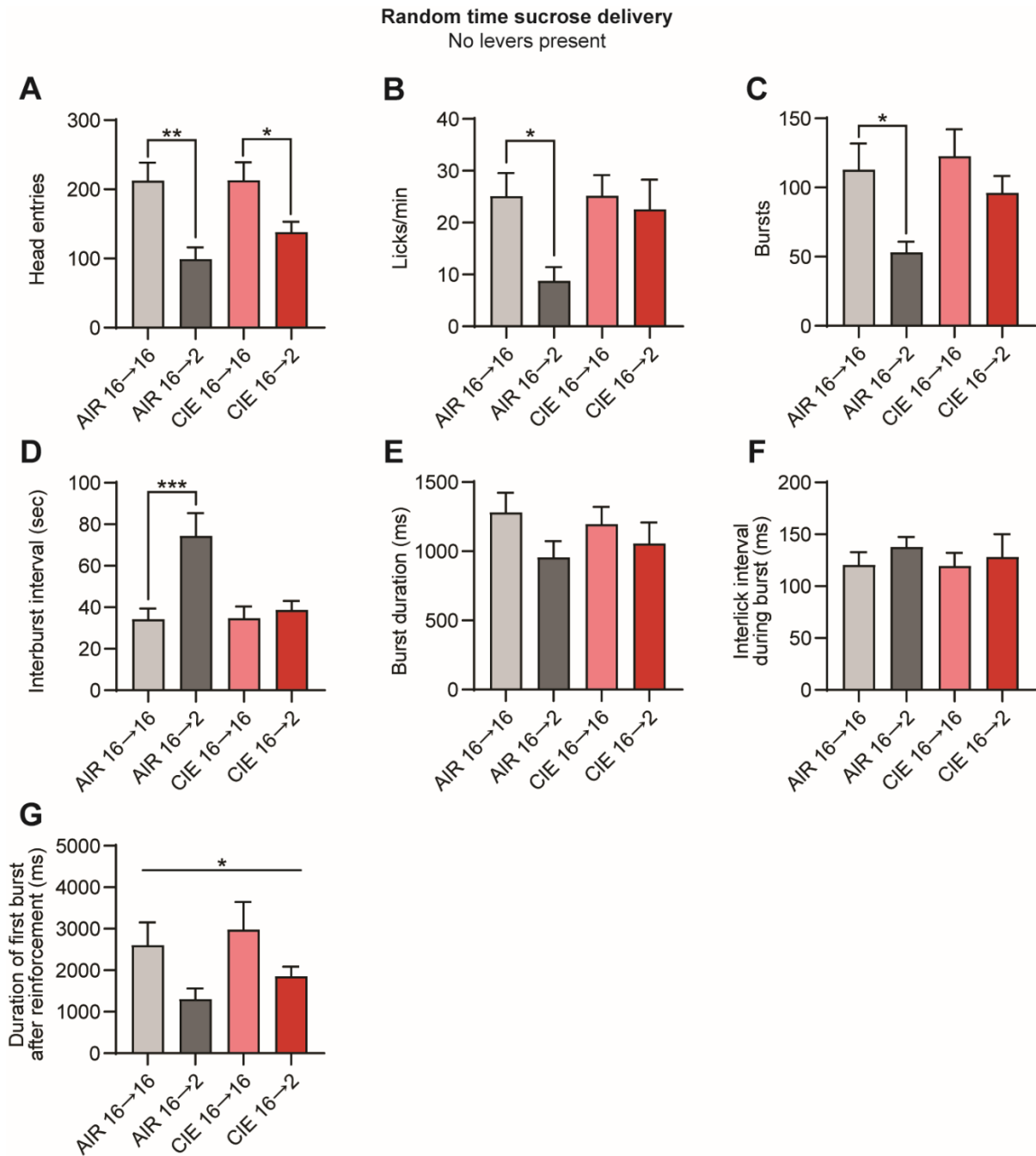
Figure 1.2: Re-exposure head entry and lick behavior for Experiment 1. (**A**) Total head entries during the 60-minute re-exposure session in chronic intermittent ethanol (CIE) and air control mice. (**B**) Rate of licking. (**C**) Total lick bursts, where a burst is defined as three or more sequential licks performed less than 1 second apart. (**D**) Time between bursts. (**E**) Duration of lick bursts. (**F**) Time between licks during a burst. (**G**) Duration of the first burst following delivery of a reinforcer. 16→16 = maintained at 16-hour restriction; 16→2 = shifted to 2-hour restriction. All data shown are means ± SEM. * = < 0.05, ** = < 0.01, and *** = < 0.001.

Non-rewarded test. The following day, in a brief non-rewarded test session, we examined whether mice were able to retrieve and use the updated value of sucrose to control lever press behavior. Whereas Air mice lowered response rates on both distal and proximal levers following a decrease in hunger state, CIE-exposed mice did less so (Figure 3A, B). This magnitude effect was supported by a two-way ANOVA performed on distal lever press rate that showed a main effect of Hunger state [$F(1, 35) = 6.68$, $p < 0.05$], but no main effect of Vapor group or interaction ($Fs < 1.5$, $ps > 0.05$). *A priori* Bonferroni pairwise comparisons showed a significant reduction in distal lever press rate in Air 16-2 mice compared to Air 16-16 mice ($p < 0.05$), but not between CIE groups ($p > 0.05$). A two-way ANOVA performed on proximal lever press rate also showed a main effect of Hunger state [$F(1, 35) = 5.15$, $p < 0.05$], with *a priori* Bonferroni pairwise comparisons also showing a significant reduction in Air 16-2 mice compared to Air 16-16 mice ($p < 0.05$), but no difference between CIE 16-16 and 16-2 mice ($p > 0.05$). Once again, there was no effect of Vapor group and no interaction ($Fs < 1.38$, $ps > 0.05$). Hence, these data suggest that prior CIE exposure disrupted sensitivity to motivational shifts as evaluated in the re-exposure state that may have contributed to deficits in updating and inferring value to control both distal and proximal lever pressing.

Rewarded test. During the prior non-rewarded test, the sucrose value representation had to be inferred. In contrast, in the rewarded test, mice could use the observable value of sucrose for decision-making control over distal and proximal lever pressing. Further, the rewarded test provided another opportunity for mice to update reward value and use the experienced reduced value of sucrose to concurrently control responding. When CIE 16-2 mice were able to use the observable value of sucrose to control decision-making, they subsequently reduced both distal and proximal lever pressing (Figure 3C, D). A two-way ANOVA performed on distal lever press rate found a main effect of Hunger state [$F(1, 35) = 6.95$, $p < 0.05$; no main effect of Vapor group and no interaction, $Fs < 1.34$, $ps > 0.05$], with *a priori* pairwise comparisons showing a significant difference between CIE 16-16 and CIE 16-2 groups ($p < 0.05$) and no difference

between Air groups ($p > 0.05$), which had already retrieved and inferred the reduced value in the non-rewarded test session and reduced responding. There was also a main effect of Hunger state on proximal lever press rate [$F(1, 35) = 11.79$, $p < 0.01$], with only CIE 16-16 and CIE 16-2 groups differing as revealed by *a priori* pairwise comparisons ($p < 0.05$), and not Air groups ($p > 0.05$). There was again no main effect of Vapor group and no interaction (Fs < 1.76, ps > 0.05). These data suggest that CIE disrupted the ability to use motivational state to update and/or infer a relatively modest reduction in sucrose value to control lever press behavior. However, if able to experience the updated sucrose value during decision-making, CIE mice could use the experienced downshift in sucrose value to control decision-making.
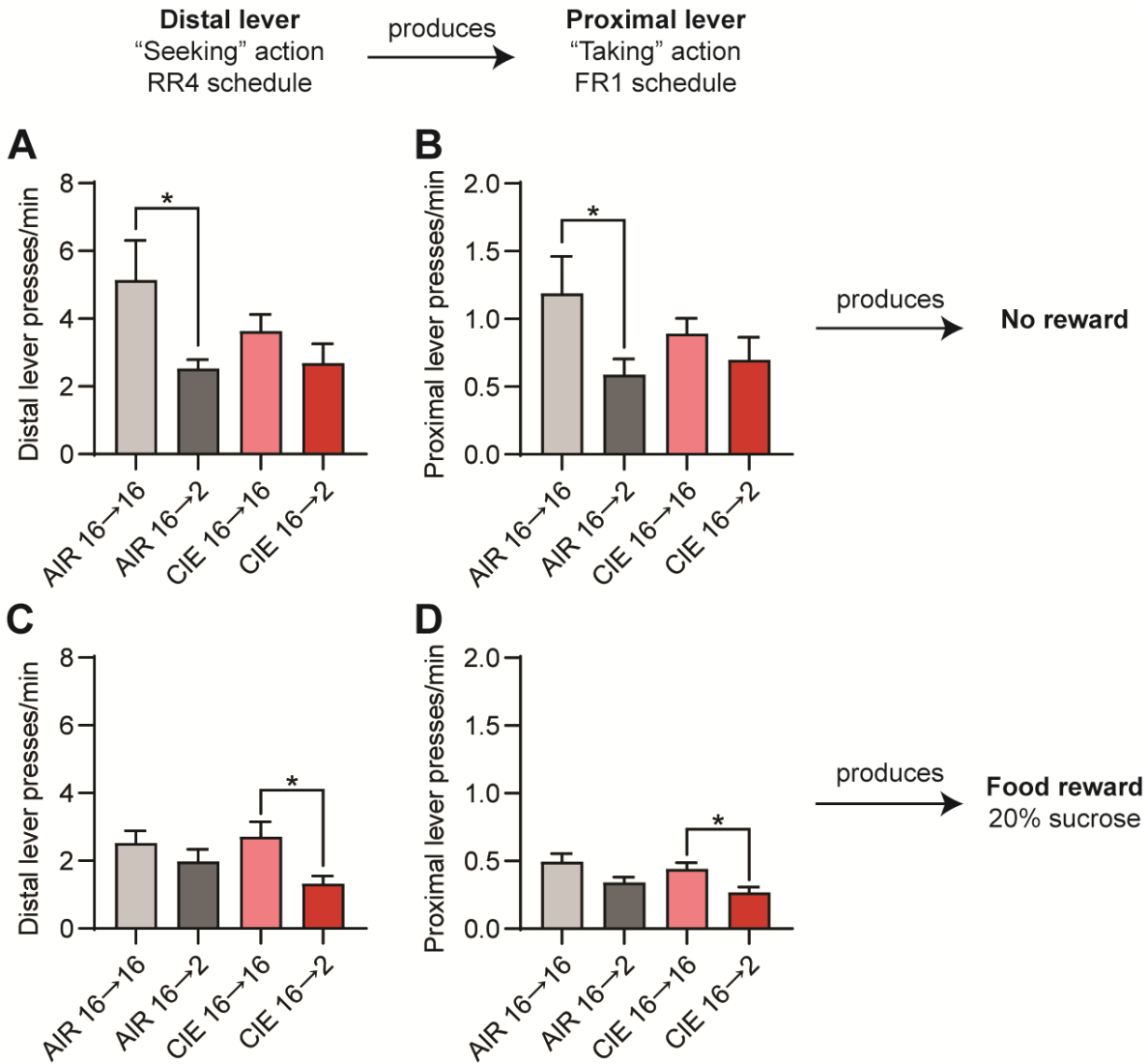
Figure 1.3: Non-rewarded test and rewarded test for Experiment 1. (**A**) Rate of distal and (**B**) proximal lever pressing in chronic intermittent ethanol (CIE) and air control mice during the 5-minute non-rewarded test, where lever presses went unrewarded. (**C**) Rate of distal and (**D)** proximal lever pressing during the 60-minute rewarded test session, where lever pressing did result in sucrose delivery. 16→16 = maintained at 16-hour restriction; 16→2 = shifted to 2-hour restriction. RR = random ratio schedule; FR = fixed ratio schedule. All data shown are means ± SEM. * = < 0.05.

Experiment 2

      Acquisition. Three days after the last vapor exposure, all mice were food-restricted and dropped to 85% of their baseline body weights across two days. Instrumental procedures began five days after the last vapor exposure. Again, Air and CIE mice similarly acquired lever press training. Following RT and CRF procedures, mice began RR training on the distal lever and maintained an FR1 schedule on the proximal lever. Air and CIE groups increased distal and proximal lever presses similarly across training (Figure 4A, B). This was supported by two-way mixed ANOVAs (Vapor group × Day), which showed main effects of Day for both distal [$F(5, 170) = 107.40$, $p < 0.0001$] and proximal [$F(5, 170) = 3.65$, $p < 0.01$] lever presses and no other main effects or significant interactions (Fs < 1.67, ps > 0.05). Furthermore, lever press rates were similar between Air and CIE mice, with a main effect of Day for both distal [$F(5, 170) = 92.08$, $p < 0.0001$] and proximal [$F(5, 170) = 18.05$, $p < 0.0001$] levers (no main effects of Vapor group or interactions: Fs < 1.08, ps > 0.05; Figure 4D, E). In addition, Air and CIE mice made similar numbers of head entries [main effect of Day: $F(5, 170) = 13.94$, $p < 0.0001$] at a similar rate [main effect of Day: $F(5, 170) = 4.57$, $p < 0.001$], with no other significant effects indicated (no main effects of Vapor group or interactions: Fs < 1.61, ps > 0.05; Figure 4C, F). Altogether, the data suggest that Air and CIE mice were able to similarly acquire instrumental chains of behavior.
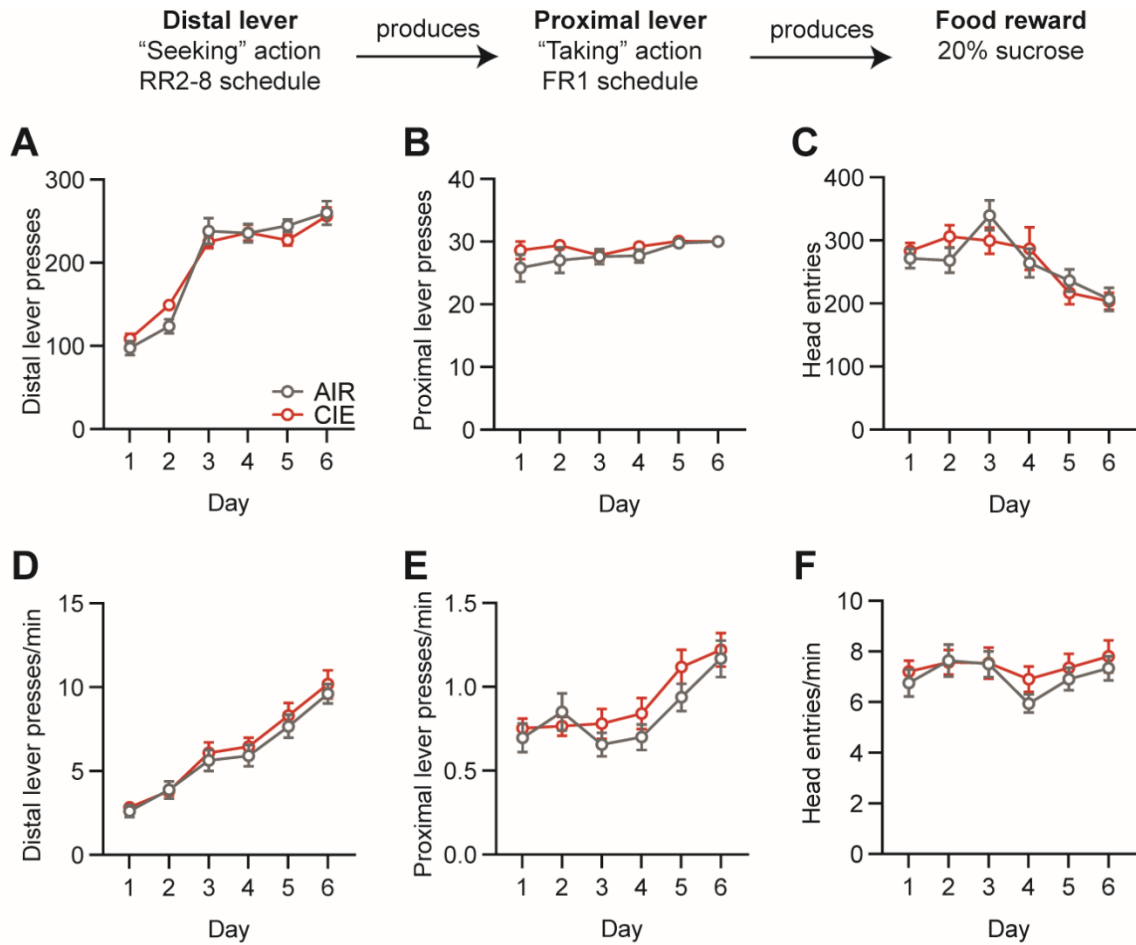
Figure 1.4: Acquisition of instrumental task in Experiment 2 with mice under gram-based food restriction. Across six days of lever press training, chronic intermittent ethanol (CIE) and air control mice (**A**) increased the number of distal and (**B**) proximal lever presses. (**C**) Head entries mice made across days. Mice also increased the rate of (**D**) distal and (**E**) proximal presses. (**F**) Rate of head entries. RR = random ratio schedule; FR = fixed ratio schedule. Red circles (CIE mice) and gray circles (Air mice) are means ± SEM.

Re-exposure. In Experiment 2, the reduction in hunger state was achieved by shifting mice from a gram-based food restriction state to a free-feed state. Mice in Air R-F and CIE R-F groups were allowed to free-feed in their home cages a minimum of 16 hours prior to the start of the re-exposure session and for the remaining duration of experimental procedures. During the first 16 hours of free-feed, shifted mice gained 3.73 ± 0.40 g – significantly more than R-R groups, which gained on average 0.14 ± 0.06 g [unpaired *t* test; $t(34) = 8.69$, $p < 0.05$]. Mice in Air R-R and CIE R-R were kept in the gram-restricted state for all experimental procedures.

During the 60-minute re-exposure session, head entry and licking patterns were similarly affected by hunger state in Air and CIE mice. We found that Vapor group and a shift in hunger state altered the number of head entries, as supported by a main effect of Hunger state [$F(1, 32) = 52.20$, $p < 0.0001$] and an interaction between Hunger state and Vapor group [$F(1, 32) = 6.32$, $p < 0.05$; no main effect of Vapor group: $F(1, 32) = 0.58$, $p > 0.05$; Figure 5A]. However, *post hoc* analyses for the interaction supported that head entries decreased in the R-F group compared to R-R group for both Air mice ($p < 0.0001$) and CIE mice ($p < 0.01$), albeit to a different degree. The same pattern was observed for head entry rates (data not shown). When we examined lick rate, a two-way ANOVA showed a main effect of Hunger state [$F(1, 24) = 114.20$, $p < 0.0001$; Figure 5B], with *a priori* pairwise comparisons showing a reduction in R-F compared to R-R in both Air and CIE groups ($ps < 0.0001$). There was no main effect of Vapor group and no significant interaction ($Fs < 1.18$, $ps > 0.05$). The same pattern was found for total licks (data not shown). The number of lick bursts made also followed a similar pattern. A two-way ANOVA revealed a main effect of Hunger state [$F(1, 24) = 111.00$, $p < 0.0001$] and Vapor group [$F(1, 24) = 4.34$, $p < 0.05$], but no interaction [$F(1, 24) = 3.00$, $p > 0.05$; Figure 5C]. Planned comparisons once again showed reductions in the number of lick bursts made in the R-F compared to the R-R group for both Air and CIE ($ps < 0.0001$). Finally, time between bursts was also significantly affected by hunger state regardless of Vapor group. A two-way ANOVA found a main effect of Hunger state [$F(1, 24) = 69.23$, $p < 0.0001$; Figure 5D], but no main effect

of Vapor group or interaction (Fs < 1.02, ps > 0.05). Bonferroni comparisons supported that in Air and CIE groups, less hungry R-F mice exhibited significantly longer interburst intervals compared to hungrier R-R mice (ps < 0.0001).

Once again, when we examined patterns of licking, the average burst duration was not affected by Vapor group or Hunger state (no main effects or interaction; Fs < 4.01, ps > 0.05; Figure 5E). Within a burst, the average interlick interval was similar across all subgroups, as supported by a two-way ANOVA that found no main effects of Vapor or Hunger groups and no interaction (Fs < 1.46, ps > 0.05; Figure 5F). Regardless of Vapor group, less hungry mice decreased their lick burst duration following reinforcement compared to hungrier mice. This was supported by a two-way ANOVA, which found a main effect of Hunger state [$F(1, 24) = 50.11$, p < 0.0001; Figure 5G] and no significant effect of Vapor group or interaction (Fs < 0.37, ps > 0.05). Bonferroni comparisons confirmed a significant reduction in lick burst duration in R-F mice compared to R-R mice for both Air (p < 0.001) and CIE (p < 0.0001) groups. These results suggest that following a severe shift in hunger state, CIE mice adjust seeking and consummatory lick behaviors similarly to Air mice.

Random time sucrose delivery
No levers present
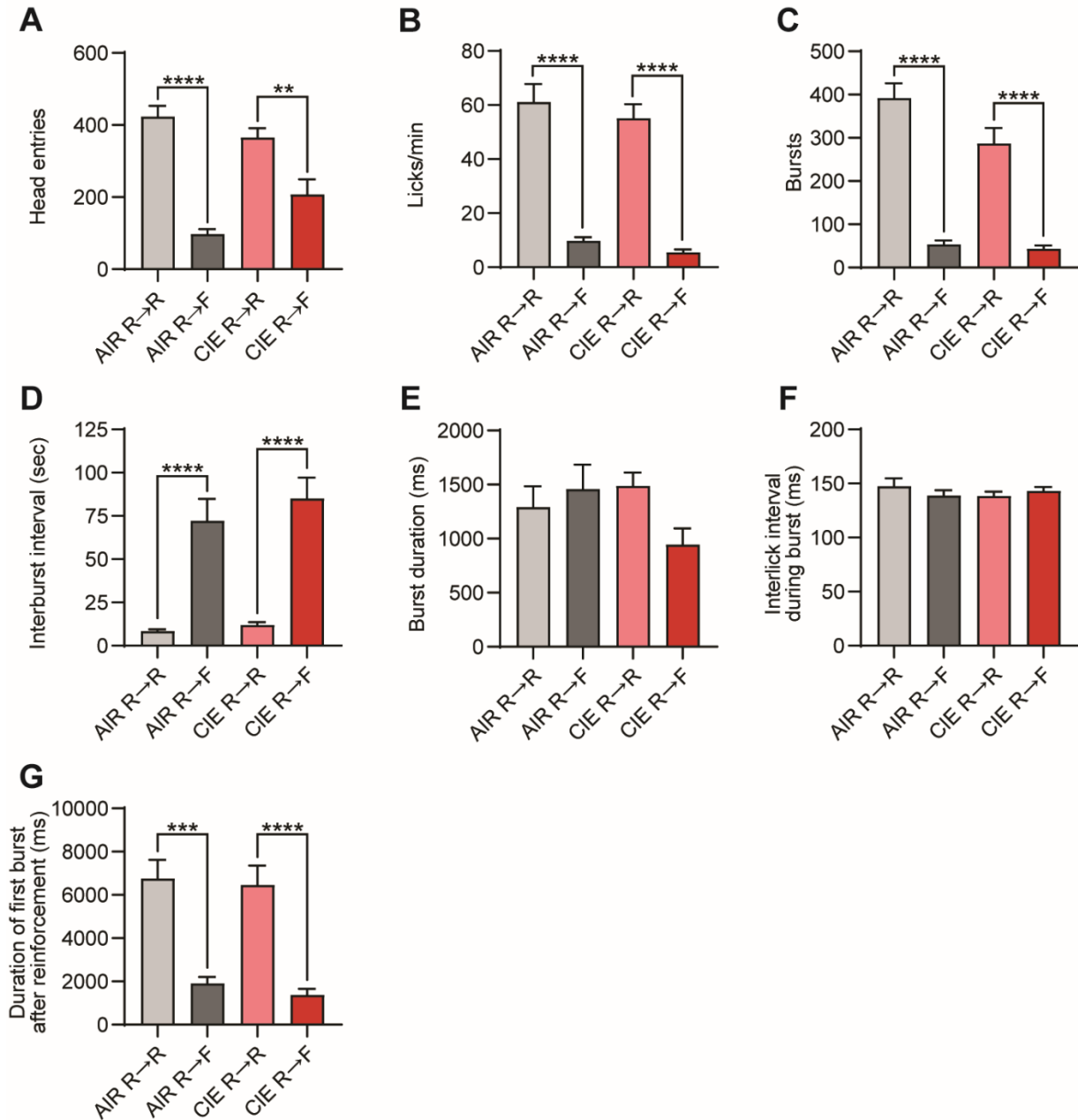
Figure 1.5: Re-exposure head entry and lick behavior for Experiment 2. (**A**) Total head entries during the 60-minute re-exposure session in chronic intermittent ethanol (CIE) and air control mice. (**B**) Rate of licking. (**C**) Total lick bursts, where a burst is defined as three or more sequential licks performed less than 1 second apart. (**D**) Time between bursts. (**E**) Duration of bursts. (**F**) Time between licks during a burst. (**G**) Duration of the first burst following delivery of a reinforcer. R→R = maintained at gram restriction; R→F = shifted to free-feed. All data shown are means ± SEM. ** = < 0.01, *** = < 0.001, and **** = < 0.0001.

Non-rewarded test. In the subsequent 5-minute non-rewarded test session the next day, the reduction in the more severe hunger state reduced distal responding for both Air and CIE mice (Figure 6A). A two-way ANOVA performed on distal lever press rate revealed a main effect of Hunger state [$F(1, 32) = 11.48$, $p < 0.01$], with *a priori* pairwise comparisons showing a reduction in R-F compared to R-R groups for both Air and CIE mice (ps < 0.05). In contrast, analysis of proximal lever press rates once again showed a main effect of Hunger state [$F(1, 32) = 8.04$, $p < 0.01$], and an *a priori* pairwise comparison showed a reduced press rate in the Air R-F group compared to the Air R-R group ($p < 0.05$), but no difference between CIE groups ($p > 0.05$; Figure 6B). For both distal and proximal lever press rates, no main effects of Vapor group and no significant interactions were observed (Fs < 2.03, ps > 0.05).

Rewarded test. During the subsequent 60-minute rewarded test session, there was a significant reduction in lever press rate for both the distal and proximal lever in both Air and CIE groups (Figure 6C, D). A two-way ANOVA performed on distal lever press rate showed a main effect of Hunger state [$F(1, 32) = 37.63$, $p < 0.0001$], and *a priori* comparisons found significant reductions in distal press rate in R-F compared to R-R mice in both Air ($p < 0.01$) and CIE ($p < 0.0001$) groups. Unlike the non-rewarded test session, in the rewarded test session where mice were able to resample the sucrose delivered following completion of lever press requirements, CIE R-F mice reduced proximal lever press rate in a similar fashion to that of Air R-F mice. This was supported by a main effect of Hunger state [$F(1, 32) = 39.10$, $p < 0.0001$] and significant *a priori* pairwise comparisons between R-R and R-F mice in both Air ($p < 0.001$) and CIE groups ($p < 0.0001$). Once again, there were no main effects of Vapor group and no significant interactions for either distal or proximal lever press rates (Fs < 1.21, ps > 0.05). Together, this suggests that while CIE mice that had more severe food restriction still showed an insensitivity to devaluation on the proximal lever when value had to be inferred, goal-directed control over the more distal decision-making process remained intact. Furthermore, when allowed to use the current experienced action-outcome relationship to guide behavior, CIE mice did show

sensitivity to the reduced hunger state and decreased both the proximal as well as the distal

action.
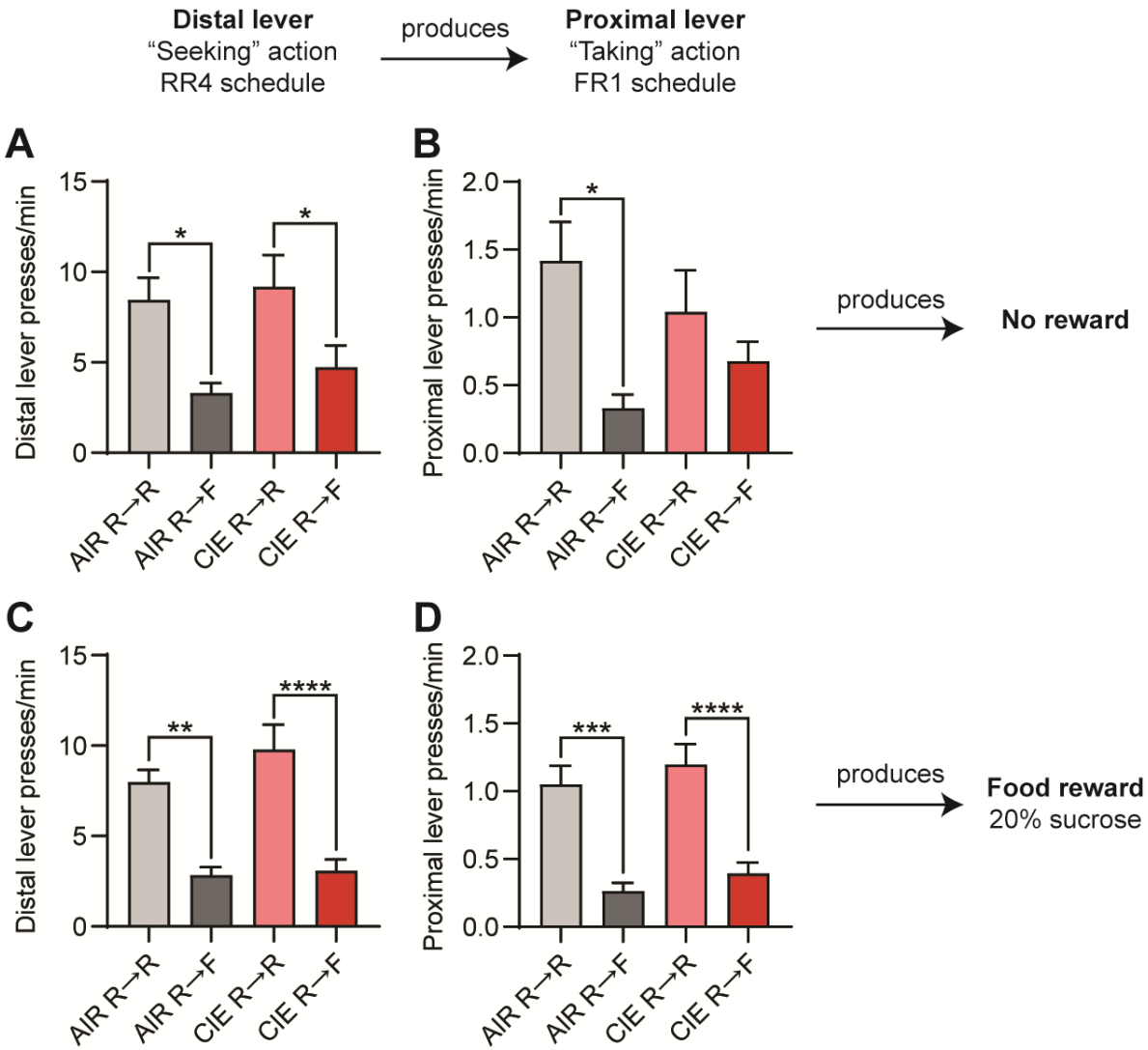
Figure 1.6: Non-rewarded test and rewarded test for Experiment 2. (**A**) Rate of distal and (**B**) proximal lever pressing in chronic intermittent ethanol (CIE) and air control mice during the 5-minute non-rewarded test, where lever presses went unrewarded. (**C**) Rate of distal and (**D**) proximal lever pressing during the 60-minute rewarded test session, where lever pressing did result in sucrose delivery. R→R = maintained at gram restriction; R→F = shifted to free-feed. RR = random ratio schedule; FR = fixed ratio schedule. All data shown are means ± SEM. * = < 0.05, ** = < 0.01, *** = < 0.001, and **** = < 0.0001.

Discussion

Here we report that the prior induction of ethanol dependence can, but does not always, result in long-lasting deficits in motivational processes supporting goal-directed decision-making. Most notably, differences in the degree of food restriction determined whether CIE mice showed sensitivity to motivational shifts and whether their lever press performance was consistent with an updated value change. The present data suggest that long-lasting deficits in motivational processes and potentially aspects of value updating could in part explain deficits in outcome devaluation previously observed in ethanol-dependent mice, and may in some cases support continued alcohol-seeking even in the face of decreased motivational states. However, our data also suggest that the ability to recruit and infer outcome value for goal-directed decision-making is still possible within a more salient motivational state. Thus, we have shown that goal-directed processes can also remain largely intact following the induction of alcohol dependence in mice.

There has been interest in examining whether alcohol use, misuse, and dependence, as well as drug addiction in general, causes a shift from goal-directed to habitual control (Barker & Taylor, 2014; Everitt & Robbins, 2005; Gremel & Lovinger, 2016). By employing a decision-making task that separates processes supporting motivational versus inference components of goal-directed control, we find evidence that prior CIE exposure can reduce sensitivity to shifts in motivational state that could contribute to the ability to update and infer a value change for adaptive behavior. The insensitivity of sucrose-seeking and related lick behaviors during re-exposure following a reduction in motivational state supports the hypothesis that CIE mice may be generally less sensitive to mild shifts in motivational state. During the re-exposure session, less-restricted 16-2 CIE mice licked at a similarly high rate as more-restricted 16-16 CIE mice, emitted a similar number of licks organized into bursts, and exhibited similarly low time between lick bursts. However, following a more severe shift in motivational state, CIE mice demonstrated patterns of licking that aligned with current motivational state and mirrored behavior seen in Air

mice. The fact that a more salient change in motivational state was able to support and recruit these same goal-directed processes for adaptive control lends support for this hypothesis.

Another possible explanation for changes in reward-seeking lick behaviors is that CIE produces a change in palatability, or the pleasantness of the outcome. Licking behavior has been proposed to reflect palatability (Berridge, 1991, 2000), and can be separated from incentive processes at both the behavioral and neural level (Wassum et al., 2009). Altered palatability in CIE mice seems less likely, however, considering that across experiments, CIE and Air groups demonstrated similar patterns of consumption licking during periods that directly followed reinforcement. Of note, we cannot be confident that mice consumed all the sucrose in the first bout following its delivery. However, this interpretation is consistent with prior work showing no difference in sucrose consumption between ethanol-dependent and control animals (Becker & Lopez, 2004). In light of these findings, we are prone to think a more likely explanation is that CIE induces changes in motivational sensitivity. Further, we found that impairment was notably specific to seeking lick behaviors; it was not seen in head entries or when consuming sucrose itself. The fact that impairment was not observed during a conditioned approach behavior (head entries) is somewhat surprising. It may be that mechanisms supporting conditioned approach behaviors and those supporting seeking licking are differentially affected by general motivational state shifts following alcohol exposure.

We were unable to determine whether the observed decreased sensitivity to shifts in motivational state were solely responsible for the deficits in updating and inferring a change in value to control behavior. Oftentimes, the presence of incentive learning is determined by examining test lever pressing following a shift in motivational state in a group of animals that have been re-exposed to the reward, compared to another group that underwent the same shift in motivational state but did not undergo a re-exposure session. What is typically observed is that subjects that have undergone the re-exposure use an updated value to control lever pressing, whereas subjects not given a re-exposure session do not (Balleine, 1992; Balleine &

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Dickinson, 1991; Baltz et al., 2018). The observation that mildly food-restricted CIE mice do not reduce lever pressing following a reduction in motivational state and re-exposure (akin to control animals that have not undergone re-exposure) could suggest deficits in updating or inferring an updated value to control lever pressing.

Of particular note, CIE mice showed impairments when incentive value had to be inferred to control lever pressing. This effect was consistently seen on the proximal lever across experiments. It has been proposed that the proximal response is influenced by immediate sensory and motivational aspects of the outcome to a greater extent than the distal response, which depends more on a diffuse representation of the outcome (Balleine, 2011). This hypothesis came in part from the finding that re-exposure to the outcome in the new motivational state was necessary for distal lever presses to be altered. However, proximal lever pressing in the incentive learning task often decreases following a downshift in motivation, independent of whether re-exposure occurred or not (Balleine, 1992; Balleine & Dickinson, 1991). Taking this hypothesis into account, one explanation for the present results in CIE mice is that the downshift in motivational state was not sufficient to reduce the value of immediate sensory or motivational aspects of reward used to control proximal lever pressing. However, CIE mice were able to use motivational state to guide lever pressing when the reward was directly available and consumable, such as in the rewarded test. This pattern of results notably reflects that observed in licking behavior during re-exposure, when mildly shifted CIE mice displayed normal consumption but also displayed seeking behaviors that were inconsistent with motivational state.

Thus, we cannot rule out the hypothesis that CIE induced impairments in encoding or retrieving value representations to control lever pressing in addition to any long-lasting change in motivational sensitivity. Mice exposed to CIE exhibited behavior consistent with deficits in encoding or retrieving incentive value to control distal decision-making, as well as deficits in encoding or retrieving more immediate properties of outcome value to control proximal pressing.

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Importantly, these were magnitude effects, and the overall direction of effects did not differ between Vapor groups. There was a difference between experiments in the extent of possible incentive learning deficits; namely, behavioral patterns consistent with incentive learning were intact in Experiment 2, as indexed by the decrease in distal lever response rate in both Vapor groups following the more severe reduction in motivational state. It is therefore possible that prior exposure to CIE produces deficits in encoding or retrieval processes – deficits that can be overcome with more salient shifts in motivational state.

As the habit hypothesis (or a lack of goal-directed control) is somewhat at odds with theories based on negative reinforcement, where continued drug use is supported by the outcome's ability to alleviate a negative state, and considering that there is a relative dearth of evidence for the habit hypothesis supporting substance misuse and abuse in humans (Hogarth, 2020), our data suggest more careful examination is warranted for parameters where goal-directed decision-making is used. The finding that a reduction in distal lever pressing following a shift in motivational state was observed in more severely restricted CIE mice in Experiment 2 confirms that there is not a complete absence of goal-directed decision-making in ethanol-dependent mice. The more salient the animal's motivational state, the more likely it is to be able to recruit and use goal-directed processes to control reward-seeking. It may be that a highly motivationally salient state may be sufficient to drive goal-directed alcohol-seeking, whereas in less motivationally salient states, subjects may appear habitual in their alcohol misuse. However, any interpretation should include caveats that stress and the potential for individual weight fluctuations across vapor exposure could be contributing to the observed effects, and the generalizability of our findings must be tempered as we only examined these processes in two strains of inbred mice. Further, though the distribution (after exclusions) of male and female mice was unbalanced across groups, final groups did not consistently over-represent either sex. While this lack of systematic variation in sex minimizes the concern for sex as a confound, our

findings should still be considered with the understanding that sex can have important implications for behavior.

Together, our data suggest that chronic ethanol exposure does induce long-lasting alterations to motivational processes supporting goal-directed decision-making. Interestingly, our findings also suggest that future investigation of how ethanol dependence interacts with motivational states to influence decision-making processes is warranted. Key neural circuits underlying the ability of varied motivational states to engage decision-making processes should be identified and investigated. Restoring motivational sensitivity in individuals with AUD may promote successful treatment by allowing them to use motivational states to appropriately control decision-making processes.

Declaration of Interest

The authors have no conflicts of interest to disclose.

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CHAPTER 2

Prior Chronic Alcohol Exposure Enhances Pavlovian-to-Instrumental Transfer

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Abstract

Alcohol dependence is associated with aberrant decision-making processes, particularly in the presence of alcohol-related environmental cues. For instance, alcohol cues can trigger alcohol seeking, consumption, and even relapse behavior. Recently, works have suggested that alcohol dependence may induce more general alterations in cued processes that support adaptive behavior, including enhanced cue control of volitional behavior unrelated to alcohol use. Here we examine this hypothesis by combining prior exposure to chronic intermittent ethanol and repeated withdrawal (CIE) procedures with a Pavlovian-to-instrumental transfer (PIT) task in mice. The PIT task entails training a Pavlovian association, separately training an instrumental contingency, and a final test during which the Pavlovian cue and instrumental action are combined for the first time. We first tested two variants of the PIT procedure in ethanol-naïve mice, differing in part in the duration of Pavlovian conditioned cues (short or long). We found in the PIT test that the short cue procedure produced negative transfer, whereas the long cue procedure produced positive transfer. We then used the long cue variant to examine PIT behavior in mice previously exposed to either CIE or air vapor. We found that prior CIE exposure strengthened PIT behavior, with enhanced instrumental responding during presentation of the food-associated cue. We further found that this enhancement in CIE mice persisted even after devaluation of the food outcome. Our findings suggest that ethanol dependence can enhance the influence of reward-predictive cues on ongoing behavior. Greater non-alcohol cue control of behavior may reflect the effect of chronic ethanol exposure on neural circuitry critical for cue-guided behavior in general.

Keywords

Introduction

       Alcohol use and dependence are associated with aberrant decision-making processes, particularly in the presence of alcohol-related cues. For example, viewing an alcoholic drink may trigger craving, alcohol-seeking, or even relapse to drinking behavior in individuals with alcohol use disorder (AUD). Animal models of ethanol use support that cues paired with ethanol can sustain ethanol-seeking and control behavior. In studies where animals are trained to self-administer ethanol, both discrete ethanol-paired cues and the self-administration context can reinstate instrumental ethanol-seeking behavior following extinction (Burattini et al., 2006; Tsiang & Janak, 2006). Ethanol-related cues and contexts also invigorate Pavlovian ethanol-seeking behavior (Cunningham et al., 2006; Remedios et al., 2014; Sciascia et al., 2015) and reinstate Pavlovian ethanol-seeking after extinction (Chaudhri et al., 2010). These altered responses to ethanol cues reflect ethanol's ability to reinforce learning and imbue predictive value to cues and contexts related to ethanol. However, another way that ethanol could alter behavior, particularly with chronic heavy use and dependence, is through ethanol-induced changes to neural circuits that underlie cue-guided behavior. Changes to neural circuitry produced by chronic ethanol exposure could disrupt responses guided by predictive cues in general, including cues associated with non-ethanol rewards. For drug-dependent individuals, such aberrations in cued processes could contribute to deficits in adaptive behavior throughout widespread aspects of life.

       Cues in our environment are thought to influence behavior in varied ways. By signaling salient rewarding or aversive outcomes, cues can direct decisions, elicit conditioned behaviors, and incentivize some actions or deter others. One way to examine the influence of environmental cues on ongoing actions is Pavlovian-to-instrumental transfer (PIT), a process through which previously conditioned Pavlovian cues influence instrumental behavior. A basic PIT procedure involves training a discrete Pavlovian conditioned association (e.g., auditory tone functions as a conditioned stimulus to predict an unconditioned stimulus, such as food reward),

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separately training an instrumental action (e.g., lever press produces same food reward), and then testing performance of the instrumental action in the presence and absence of Pavlovian conditioned cues. Transfer is demonstrated via energized instrumental behavior during presentation of the Pavlovian cue (e.g., rate of lever pressing increases during the auditory tone). Such transfer can be outcome-specific, where cues associated with a specific reward selectively enhance actions performed to earn the same reward. Another form of transfer is referred to as outcome-general, where reward-associated cues enhance instrumental actions in general, independent of which reward the action produces. Importantly, PIT has been demonstrated in rodents and humans (e.g., Garbusow et al., 2016; Lehner et al., 2017; Talmi et al., 2008; van Timmeren et al., 2020), making it a promising translational model.

Indeed, research in alcohol-dependent humans has used PIT models to investigate whether AUD affects cue-guided processes that support adaptive behavior. One prior finding suggested enhanced PIT in human alcohol dependence. In recently detoxified individuals, cues related to monetary wins and losses had a greater influence on volitional behavior compared to that observed in healthy controls (Garbusow et al., 2016). However, a different study examined PIT where cues predicted food rewards. There, they found that cues affected behavior similarly in abstinent individuals with AUD and controls in both general and specific PIT (van Timmeren et al., 2020). While the reason for this discrepancy is unclear, and could be related to properties of the disparate rewards used, this past work offers some support for the intriguing hypothesis that AUD may enhance the influence of general cues on behavior.

Past examinations of PIT in human AUD have been performed after disorder onset, thus necessarily encompassing both the acquisition and expression of learned associations and their interactions. It is therefore pertinent for animal work to model a similar time course. In the present work, we examined the influence of prior chronic ethanol exposure on food cue-guided PIT behavior, with ethanol exposure prior to behavioral procedures such that all training and testing occurred within the protracted phase of withdrawal (Heilig et al., 2010). Because PIT

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investigations in humans often rely on short discrete cues (e.g., Lehner et al., 2017; Talmi et al., 2008), but animal PIT models often require the use of longer duration cues (e.g., Crombag et al., 2008; Meltzer & Brahlek, 1970; Van Dyne, 1971), Experiment 1 tested two variants of the PIT procedure in ethanol-naïve mice using either short or long auditory conditioned cues to predict food delivery, with concomitant differences in reinforcement schedule, number of cue-reward pairings, and total number of reinforcers. Replicating previous findings (Crombag et al., 2008), we found that the short cue procedure produced negative transfer whereas the long cue procedure produced positive transfer in the PIT test. Then, in Experiment 2, mice were exposed to chronic intermittent ethanol (CIE) vapor exposure, a well-validated model to examine aspects of alcohol dependence in rodents (Becker, 1994; Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005), and underwent long cue PIT procedures. We found that prior CIE exposure selectively enhanced transfer in the PIT test relative to air controls. Our data suggest that protracted withdrawal from chronic ethanol exposure may enhance the ability of reward cues to guide ongoing actions in mice, perhaps reflecting ethanol-induced changes to neural circuitry critical for cue-guided behavior in general.

Materials and Methods

Animals

Adult female and male C57BL/6J and B6.129S2-*Emx1*[tm1(cre)Krj]/J (Emx1-Cre) mice were acquired from Jackson Laboratory (Bar Harbor, ME) or bred in-house 1 generation from mice acquired from Jackson Laboratory. Strain was kept consistent within each replication, such that Experiment 1A and the first replication of Experiment 2 used all Emx1-Cre mice, and Experiment 1B and the second replication of Experiment 2 used all C57BL/6J mice. There were no strain differences in behaviors examined. Average mouse ages at experiment start were 18.5 ± 0.52 weeks in Experiment 1A, 8.2 ± 0.45 weeks in Experiment 1B, and 17.2 ± 1.03 weeks in Experiment 2. Mice were group housed 2-5 per cage and maintained on a 14-hr light/10-hr dark cycle. Prior to experimental procedures, animals were provided with mouse chow (Lab-diet

5015) and water ad libitum. All experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee, and were conducted in accordance with NIH Guidelines.

Chronic Intermittent Ethanol Exposure

In Experiment 2, mice were exposed to 4 rounds of ethanol vapor or air control with repeated withdrawal (Becker, 1994; Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005). In each round, mice were exposed to 4 consecutive days of 16-hr vapor exposure followed by an 8-hr withdrawal period. After a round concluded, there were an additional 3 days of withdrawal before the next round began. Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 2.3 L/min. The resulting vapor was then combined with a separate air stream for a total flow rate of approximately 10 L/min. Ethanol or air vapor was delivered to mice in Plexiglas chambers (Plas-Labs Inc, Michigan, USA). Blood ethanol concentrations (BECs) were collected at the end of each round from sentinel mice (mean BEC = 43.1 ± 6.4 mM; Analox, USA). No injections of Pyrazole or ethanol were administered prior to placement in vapor chambers (Renteria et al., 2018), and animals were monitored for ill effects of vapor procedures.

Pavlovian-to-Instrumental Transfer Task

Overview. The PIT task, developed from Crombag et al. (2008), was used as a model of cue-guided behavior. The purpose of Experiment 1 was to test PIT transfer using two Pavlovian training procedure variants, differing in part by Pavlovian cue duration (either short, 8-sec, or long, 120-sec), in ethanol-naïve mice. The goal of Experiment 2 was to test the effect of prior chronic ethanol exposure on PIT transfer using the long cue procedure. Both experiments followed similar timelines, beginning with Pavlovian training with auditory tones, then instrumental lever press training, and finally testing for transfer (Figures 1A, 2A).

General Procedures. In all experiments, mice were food restricted to maintain 85% of baseline bodyweight starting 3 days prior to behavioral procedures. In Experiment 2, to limit the

effects of acute withdrawal and examine any lasting effects present in protracted withdrawal, food restriction began 3 days after CIE procedures concluded. Thus, in Experiment 2, behavioral procedures began a total of 6 days after the conclusion of CIE. All behavioral procedures were conducted in standard sound-attenuating operant boxes (Med Associates, Vermont, USA). Each operant box contained two retractable levers situated around a food magazine, and the chamber house light was illuminated during all procedures.

Pavlovian Acquisition. During Pavlovian training, both a food-predictive conditioned stimulus (CS+) and a conditioned stimulus predictive of an absence of food (CS-) were presented. The inclusion of a CS- allowed for disentanglement of whether any enhancement of instrumental behavior was based on the presence of environmental cues in general, or rather on the presence of cues specifically associated with rewards. During conditioning, two different frequency auditory tones (8 kHz and 15 kHz) were used as conditioned stimuli and were played for a duration of either 8-sec (Experiment 1A) or 120-sec (Experiment 1B, Experiment 2). Food reward (food pellet, Bio-Serve formula F05684) was delivered immediately following tone presentation for Experiment 1A, or on average every 30-sec during tone presentation for Experiment 1B and Experiment 2. During the CS- tone, the stimulus was played but nothing else occurred. Between each tone presentation was an intertrial interval (ITI) during which no tones were played for 120-sec on average. Mice were exposed to daily sessions of either 16 (Experiment 1A) or 10 (Experiment 1B, Experiment 2) CS+ tones and an equivalent number of CS- tones in each session, presented in pseudorandom order. Entries into the food receptacle were detected via infrared beam breaks and recorded. Pavlovian acquisition sessions were conducted for 8 days in Experiment 1 and 7 days in Experiment 2. One fewer Pavlovian training day was included in Experiment 2 so that the PIT test would occur within the 21-day time period in which disrupted instrumental decision-making has been documented in mice (Renteria et al. 2018).

Instrumental Acquisition. Following Pavlovian training, mice then underwent instrumental training in the same context used in Pavlovian training. Instrumental training involved daily sessions in which mice learned to press a lever for the same food earned during Pavlovian training. Throughout instrumental training and the transfer test, one lever was always available and the other lever was always retracted. One food pellet was delivered for every lever press under a continuous reinforcement schedule (CRF) for the first 3 days of training. On the following 5 days, 1 food pellet was delivered on average for every 10 lever presses (random ratio 10 schedule, RR). All instrumental training sessions ended when mice earned 30 pellets or 90-min elapsed. The number of lever presses was recorded, as were head entries into the food receptacle.

Pavlovian-to-Instrumental Testing. Following instrumental training, the influence of conditioned cues on lever press behavior was assessed. The approximately 30-min test was conducted in extinction, in that neither CS+ presentations nor lever presses resulted in food reward. During testing, the trained lever was present at all times. In Experiment 1A, CS+ and CS- tones were presented 8 times each, while in Experiments 1B and 2, CS+ and CS- tones were presented 4 times each. In both experiments, tones were presented in alternating order throughout the test, beginning with the CS+ tone. In addition, between each tone presentation was an ITI period lasting on average 120-sec. Lever presses and head entries into the food receptacle were recorded.

Outcome Devaluation Procedures. In Experiment 2, the effect of outcome devaluation on PIT responding in Air and CIE mice was assessed. Following the first transfer test, outcome devaluation procedures were conducted. All mice were given free access to the same food pellets earned during training for 60-min. Immediately following this pre-feeding period, all mice went through a second transfer test identical to the first. Lever presses and head entries into the food receptacle were recorded. Of note, outcome devaluation procedures often involve comparing lever pressing or head entries in a devalued state with behavior in a valued state,

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where satiation has been achieved via pre-feeding of a non-paired reinforcer. In the present work, we excluded a valued comparison as the long nature of the test session can induce greater extinction learning, and the implementation of additional training days could alter either CS or lever press association strength.

Behavioral and Statistical Analysis

Data were analyzed using Prism 6 (GraphPad) and JASP (Version 0.13.1). The alpha level was set at 0.05 for all experiments. All data within groups was found to be normally distributed with the exception of CS+ food port entries during Pavlovian training in the Air group. Thus, for this group non-parametric analyses of Pavlovian training food port entry data are also presented. In Experiment 2, pre-training bodyweights were compared for Air and CIE mice with an unpaired t-test. For Pavlovian training, food port entry rate was analyzed with 2- or 3-way ANOVA examining the effects of the within-subjects factors Day and Trial Type (ITI/CS+/CS-), with the addition of the between-subjects factor of Group (Air/CIE) in Experiment 2. Rate of lever pressing was analyzed for instrumental training with 1- or 2-way ANOVA examining the effect of Day and, in Experiment 2, Group. For the PIT and devaluation tests, lever press rate was analyzed using 1- or 2-way ANOVA including the factor of Trial Type and, in Experiment 2, the factor of Group. Transfer was evidenced by an increase in lever pressing behavior during presentation of the food-associated CS+ compared to the CS- and the ITI. Planned post-hoc comparisons with Bonferroni correction were conducted to examine performance between trial types in Pavlovian training and in testing. Difference scores for the PIT test and devaluation test were computed as rate of lever pressing during the ITI subtracted from rate of lever pressing during the CS+ tone. Difference scores were compared to 0 in one sample t-tests for Experiment 1A and 1B, and for Air and CIE groups in Experiment 2. In addition, unpaired t-tests were performed to compare difference scores between Experiment 1A and 1B, and between Air and CIE groups in Experiment 2. Group consumption during Experiment 2 devaluation pre-feeding was compared with an unpaired t-test. To reveal any changes in Experiment 2 behavior

from the PIT test to the devaluation test, 2- or 3-way ANOVA were conducted with the factors of Group and Test for baseline ITI lever press rates and the additional factor of Trial Type for food port entry rates. Finally, the effects of sex and strain were examined in Experiment 2 using 2-way ANOVA for difference score, with the factors of Group and Sex or Strain.

Results

Attrition

In Experiment 1A, one mouse was excluded from all analyses for failing to learn to lever press, leaving a final *n* of 7 (2F, 5M). Experiment 1B was performed in two replicate cohorts, with a final *n* of 16 (16F) and no attrition. In Experiment 2, one mouse was excluded for failing to learn to lever press. Performed in 2 replicates, final group *n*s in Experiment 2 were *n* = 16 (8F, 8M) in the Air group and *n* = 17 (9F, 8M) in the CIE group. In addition, 2 mice did not consume food pellets during devaluation and so were selectively excluded from devaluation analyses. Final *n*s for devaluation analyses in Experiment 2 were *n* = 16 Air (8F, 8M) and *n* = 15 CIE (9F, 6M).

Sex and Strain

The effects of sex and strain on PIT were examined in Experiment 2, where strain differed by replication and both sexes were represented in Air and CIE groups. Analyses supported that transfer magnitude (difference between CS+ and baseline ITI lever press rates in the PIT test) and Air/CIE group differences in PIT were not affected by strain. This was supported by a 2-way ANOVA (Strain x Group) for difference score, which found no main effect of Strain ($F(1, 29) = 2.62$, $p = 0.12$), a marginal effect of Group ($F(1, 29) = 3.80$, $p = 0.06$), and no interaction ($F(1, 29) = 0.13$, $p = 0.72$). Strains were therefore combined for all further Experiment 2 analyses. Sex analyses revealed overall weaker PIT in females, as has been seen previously (Barker & Taylor, 2019). A 2-way ANOVA (Sex x Group) examining difference scores in the PIT test indicated a main effect of Sex ($F(1, 29) = 7.95$, $p < 0.01$) and a main effect of Group ($F(1, 29) = 6.53$, $p < 0.05$), but no interaction ($F(1, 29) = 1.14$, $p = 0.30$). The same

analysis for the devaluation test also revealed a main effect of Sex ($F_{(1, 27)} = 7.32$, $p < 0.05$) and a main effect of Group ($F_{(1, 27)} = 12.64$, $p < 0.01$), but no interaction ($F_{(1, 27)} = 0.01$, $p = 0.98$). Together, these results indicate overall weaker PIT in females relative to males, regardless of treatment group. In other words, CIE enhanced PIT strength in both sexes. Males and females were thus combined for all further Experiment 2 analyses.

Experiment 1A: Pavlovian-to-instrumental transfer with short conditioned stimuli

Mice were exposed to a Pavlovian conditioning procedure with 8-sec auditory tones. In this procedure, reward delivery occurred immediately after presentation of the CS+ tone. A benefit of this procedure is that it easily differentiates food port entries that are performed predictively (i.e., in expectation of food delivery) from food port entries that are performed to retrieve food. That is, predictive port entries are those that occur during CS+ presentation, and food retrieval port entries are those that occur soon after CS+ presentation.

Mice acquired Pavlovian conditioned behavior across training and learned that the CS+ tone was predictive of food delivery. This finding was supported by a 2-way ANOVA (Day x Trial Type) for rate of food port entries (port entries per min), which revealed significant main effects of Day ($F_{(7, 126)} = 6.26$, $p < 0.0001$) and Trial Type ($F_{(2, 18)} = 4.26$, $p = 0.03$), and a Day x Trial Type interaction ($F_{(14, 126)} = 4.08$, $p < 0.0001$) (Figure 1B). Post hoc comparisons for the main effect of Trial Type (collapsing across days) supported that the rate of predictive food port entries was significantly higher on average during the CS+ compared to the ITI ($p < 0.05$). There was no significant difference between food port entry rate during the ITI and CS- ($p = 0.09$) or between CS+ and CS- port entry rates ($p = 0.99$). However, post hoc day comparisons for the interaction between Day and Trial Type found that on the final day of training, there were significantly more food port entries performed during the CS+ compared to both the ITI ($p < 0.01$) and the CS- ($p < 0.05$). In subsequent instrumental training, mice learned to press a lever for the same food reward as earned in Pavlovian training and escalated their rate of lever

pressing across sessions. This finding was supported by a 1-way ANOVA (Day) for lever press rate, which found a significant main effect of Day ($F_{(7, 42)} = 18.34$, $p < 0.0001$) (Figure 1C).

However, PIT testing with short CS tones suggested negative PIT transfer. This finding was supported by a 1-way ANOVA (Trial Type) for lever press rate during the test, which revealed a significant main effect of Trial Type ($F_{(2, 12)} = 9.50$, $p < 0.01$) (Figure 1D). Post hoc comparisons indicated significantly lower lever pressing rates during the CS+ tone compared to both the ITI ($p < 0.05$) and the CS- tone ($p < 0.01$). There was no significant difference between lever press rates during the CS- and ITI ($p = 0.96$). Further supporting that negative transfer occurred, a one sample t-test found that the difference between lever press rates in the ITI and CS+ was significantly lower than 0 ($t_{(6)} = 4.03$, $p < 0.01$). Thus, the above data show that the short cue procedure resulted in a decrease in lever pressing during cue presentation.

Experiment 1B: Pavlovian-to-instrumental transfer with long conditioned stimuli

In Experiment 1B, we altered the Pavlovian training procedure described in Experiment 1A to use longer, 120-sec auditory tones. In this modified procedure, food pellets were delivered on average every 30-sec throughout the CS+ tone rather than immediately after tone presentation. This procedure reduces the precise temporal predictability of food delivery and provides larger windows to observe cue-influenced lever pressing behavior.

Animals demonstrated robust Pavlovian learning with long CS tones. This was supported by a 2-way ANOVA (Day x Trial Type) for rate of food port entries, which revealed a main effect of Trial Type ($F_{(2, 30)} = 15.36$, $p < 0.0001$) and Day ($F_{(7, 105)} = 7.31$, $p < 0.0001$), but no significant interaction between the factors ($F_{(14, 210)} = 1.71$, $p = 0.06$) (Figure 1E). Planned post hoc comparisons for the main effect of Trial Type found that food port entry rate was significantly higher during the CS+ tone compared to both the CS- ($p < 0.0001$) and the ITI ($p < 0.001$). There was no significant difference between food port entry rates in the ITI and CS- ($p = 0.99$). Next, animals were exposed to lever press training identical to the procedure described in Experiment 1A. Mice learned to lever press for the same food reward as earned

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during Pavlovian training and increased their rate of lever pressing across sessions, as demonstrated by a main effect of Day in a 1-way ANOVA ($F(7, 105) = 59.13$, $p < 0.0001$) (Figure 1F).

A PIT test conducted under extinction found that the long cue procedure produced positive PIT transfer (Figure 1G). This finding was supported by a main effect of Trial Type ($F(2, 30) = 8.64$, $p < 0.01$) in a 1-way ANOVA for lever press rate during the test. Post hoc comparisons found that lever press rate was selectively energized during the CS+ tone relative to the ITI ($p < 0.01$) and the CS- tone ($p < 0.01$). There was no significant difference between lever press rates in the ITI and CS- ($p = 0.99$). Further supporting that lever pressing was energized during the food-associated CS+, a one sample t-test found that the difference between ITI and CS+ lever press rates was significantly greater than 0 ($t(15) = 3.64$, $p < 0.01$). In addition, an unpaired t-test found that the PIT difference scores for Experiments 1A and 1B were significantly different, supporting that the short cue procedure produced negative transfer, whereas the long cue procedure produced positive transfer ($t(21) = 4.35$, $p < 0.001$) (Figure 1H). These findings suggest that certain aspects of Pavlovian conditioning can affect the direction of PIT transfer in mice. In particular, short cues with greater reward predictability and fewer cue-reward pairings may produce negative transfer, whereas long cues with less predictable reward delivery and more cue-reward pairings may enable a larger window to observe cue-energized instrumental behavior and positive transfer.

Figure 2.1: Long cue procedure produces positive Pavlovian-to-instrumental transfer (PIT) and short cue procedure produces negative PIT. (**A**) All mice first went through Pavlovian training including a food-associated tone (CS+), a non-food-associated tone (CS-), and intertrial intervals (ITI) where no tone was played; next, mice went through instrumental lever press training on continuous reinforcement (CRF) and random ratio (RR) schedules; finally, transfer was assessed in a PIT test. (**B-D**) Experiment 1A, short cue procedure. (**B**) Rate of food port entries across Pavlovian training days. (**C**) Rate of lever presses across instrumental training days. (**D**). Rate of lever pressing during each trial type in the PIT test. (**E-G**) Experiment 1B, long cue procedure. (**E**) Rate of food port entries across Pavlovian training days. (**F**) Rate of lever presses across instrumental training days. (**G**) Rate of lever presses during each trial type in the PIT test. (**H**) Average difference between rate of lever pressing during CS+ minus rate of lever pressing during ITI for Experiments 1A and 1B. Data points represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ represent significant comparisons.
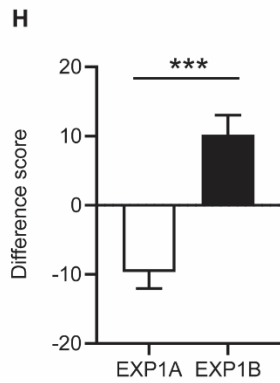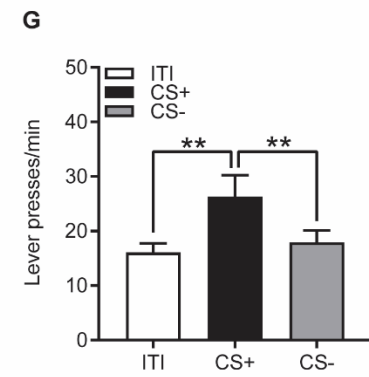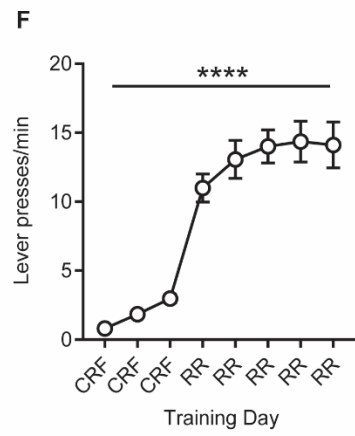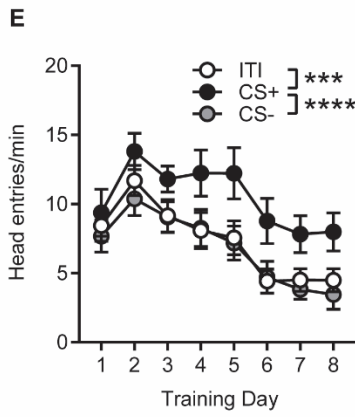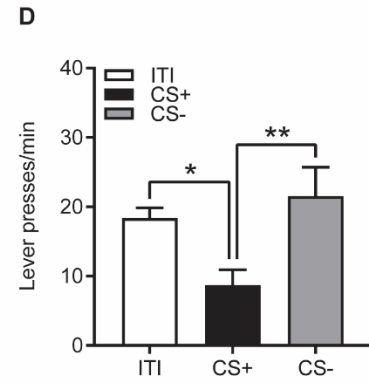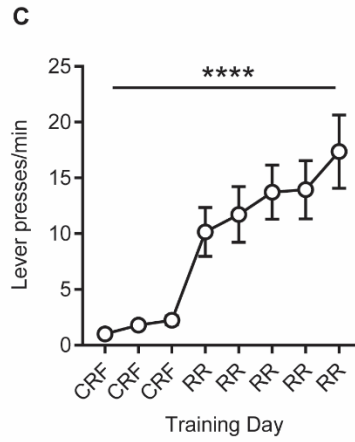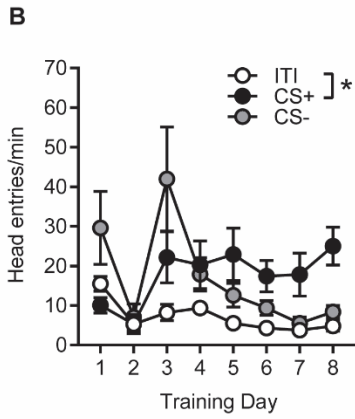
Experiment 2: CIE-induced enhancement of Pavlovian-to-instrumental transfer

We next examined the effect of chronic ethanol exposure on PIT behavior using the long cue protocol. A new cohort of mice was exposed to CIE procedures, followed by training conducted identically to Experiment 1B, except with 1 fewer day of Pavlovian training. Following CIE exposure but prior to food restriction, average weights were 31.24 ± 1.82 grams in the Air group and 28.78 ± 1.63 grams in the CIE group. An unpaired t-test supported that there was no significant difference in weight between groups (t(31) = 1.01, p = 0.32).

Robust Pavlovian conditioned behavior was observed across groups, with learning supporting similar levels of conditioning between Air and CIE mice (Figures 2B, 2C). A 3-way ANOVA (Day x Trial Type x Group) for rate of food port entries during training did not show a significant 3-way interaction (F(12, 372) = 2.62, p = 0.96), nor a significant 2-way interaction of Trial Type x Group (F(2, 62) = 0.68, p = 0.51). However, there was a significant Day x Trial Type interaction (F(12, 372) = 5.42, p < 0.001), and a significant Day x Group interaction (F(6, 186) = 2.66, p < 0.05). There were main effects of Trial Type (F(2, 62) = 39.06, p < 0.001) and Day (F(6, 186) = 4.78, p < 0.001), but no main effect of Group (F(1, 31) = 0.001, p = 0.98). Post hoc comparisons for the main effect of Trial Type showed that regardless of Air/CIE group, food port entry rates were significantly higher during the CS+ compared to both the ITI (p < 0.001) and the CS- (p < 0.001), whereas there was no significant difference between the ITI and CS- (p = 0.99). Further supporting similar levels of conditioning across groups, post hoc comparisons for the Day x Group interaction revealed no significant differences in overall food port entry behavior (collapsing across trial type) between Air and CIE on any given training day (ps = 0.99). Non-parametric tests also supported similar levels of training between groups. A Mann-Whitney test found no difference in CS+ food port entry rates between Air (*Mdn* = 8.00) and CIE (*Mdn* = 6.30) on the final day of training (U = 107, z = -1.03, p = 0.30). Confirming learning in the Air group, a Friedman test found a significant effect of Trial Type on food port entry rates during the last day of training in Air mice (χ^2(2) = 18.38, p < 0.0001), with Dunn's multiple comparisons

indicating significantly higher food port entries in the CS+ relative to the ITI and CS- ($ps < 0.001$) and no difference between ITI and CS- ($p = 0.99$).

Both Air and CIE groups acquired similar levels of lever press behavior across instrumental training, with no differences between groups (Figure 2D). This was supported by a 2-way ANOVA (Day x Group) for lever press rate, which revealed no main effect of Group ($F(1, 31) = 0.34$, $p = 0.56$) and no Day x Group interaction ($F(7, 217) = 0.76$, $p = 0.62$). A significant main effect of Day ($F(7, 217) = 79.71$, $p < 0.0001$) supported that mice increased lever pressing across training.

A subsequent PIT test revealed strengthened PIT transfer in CIE animals compared to Air animals (Figure 2E). A 2-way ANOVA (Trial Type x Group) for lever press rate found a significant main effect of Trial Type ($F(2, 62) = 21.89$, $p < 0.0001$) and a Trial Type x Group interaction ($F(2, 62) = 3.51$, $p < 0.05$). There was no main effect of Group ($F(1, 31) = 0.90$, $p = 0.35$). Post hoc comparisons for the Trial Type x Group interaction indicated that CS+ lever press rates were selectively enhanced in CIE animals compared to the CS- ($p < 0.0001$) and ITI ($p < 0.0001$), and there was no significant difference between the CS- and ITI ($p = 0.94$). In Air animals, CS+ lever press rates were selectively enhanced compared to the CS- ($p < 0.05$) but less so compared to the ITI ($p = 0.06$). There was no significant difference between the CS- and ITI periods ($p = 0.99$). An analysis of difference scores supported that transfer occurred in both groups. In one sample t-tests, the difference between CS+ and ITI lever press rates was found to be significantly greater than 0 for both the Air group ($t(15) = 2.28$, $p < 0.05$) and the CIE group ($t(16) = 5.12$, $p < 0.0001$). Supporting that PIT was enhanced in the CIE group, an unpaired t-test found that the difference between CS+ and ITI lever press rates was greater in CIE animals compared to Air animals ($t(31) = 2.27$, $p < 0.05$) (Figure 2F).

Outcome devaluation testing was performed to investigate whether devaluation procedures differentially influence PIT expression in Air versus CIE mice. We found that pre-feeding prior to PIT testing did not disrupt the enhanced PIT observed in CIE mice compared to

Air controls (Figure 2G). A 2-way ANOVA (Trial Type x Group) for lever press rate revealed a significant main effect of Trial Type ($F_{(2, 58)} = 18.32$, $p < 0.0001$) and a Trial Type x Group interaction ($F_{(2, 58)} = 5.21$, $p < 0.01$), but no main effect of Group ($F_{(1, 29)} = 0.45$, $p = 0.51$). Post hoc comparisons revealed that in the CIE group, CS+ responding remained significantly higher than during both the ITI ($p < 0.0001$) and the CS- ($p < 0.0001$). Lever press rates did not differ between the CS- and ITI in the CIE group ($p = 0.86$). In the Air group, CS+ responding was not significantly energized relative to the ITI ($p = 0.49$), but was significantly higher than during the CS- ($p < 0.05$). There was no significant difference between lever press rates during the ITI and CS- in the Air group ($p = 0.63$). Analysis of the difference between CS+ and ITI lever press rates for each group supported that PIT was still enhanced in the CIE group following devaluation. One sample t-tests revealed that whereas the CIE group difference score was significantly greater than 0 ($t_{(14)} = 4.90$, $p < 0.001$), the Air group difference score was not ($t_{(15)} = 1.55$, $p = 0.14$). Further, an unpaired t-test comparing difference scores between groups found that the CIE group score was significantly higher than the Air group score ($t_{(29)} = 3.05$, $p < 0.01$) (Figure 2H). Groups did not differ in consumption levels during the pre-feeding period (0.60 ± 0.11 grams in Air and 0.62 ± 0.07 grams in CIE; unpaired t-test, $t_{(29)} = 0.14$, $p = 0.89$). A 2-way ANOVA (Group x Test) also confirmed that across groups, baseline lever press rates in the ITI period decreased from the PIT test to the devaluation test. This was supported by a main effect of Test ($F_{(1, 29)} = 105.70$, $p < 0.0001$) and no main effect of Group ($F_{(1, 29)} = 0.12$, $p = 0.74$) or interaction ($F_{(1, 29)} = 0.30$, $p = 0.59$). Similarly, food port entries decreased from the PIT test to the devaluation test. This was supported by a 3-way ANOVA (Trial Type x Group x Test) for food port entry rate, which found a main effect of Test ($F_{(1, 29)} = 19.51$, $p < 0.001$) and no other significant main effects or interactions (ps > 0.05). Together, these results suggest that CIE-strengthened transfer in the PIT test was resistant to devaluation of the food reward outcome.
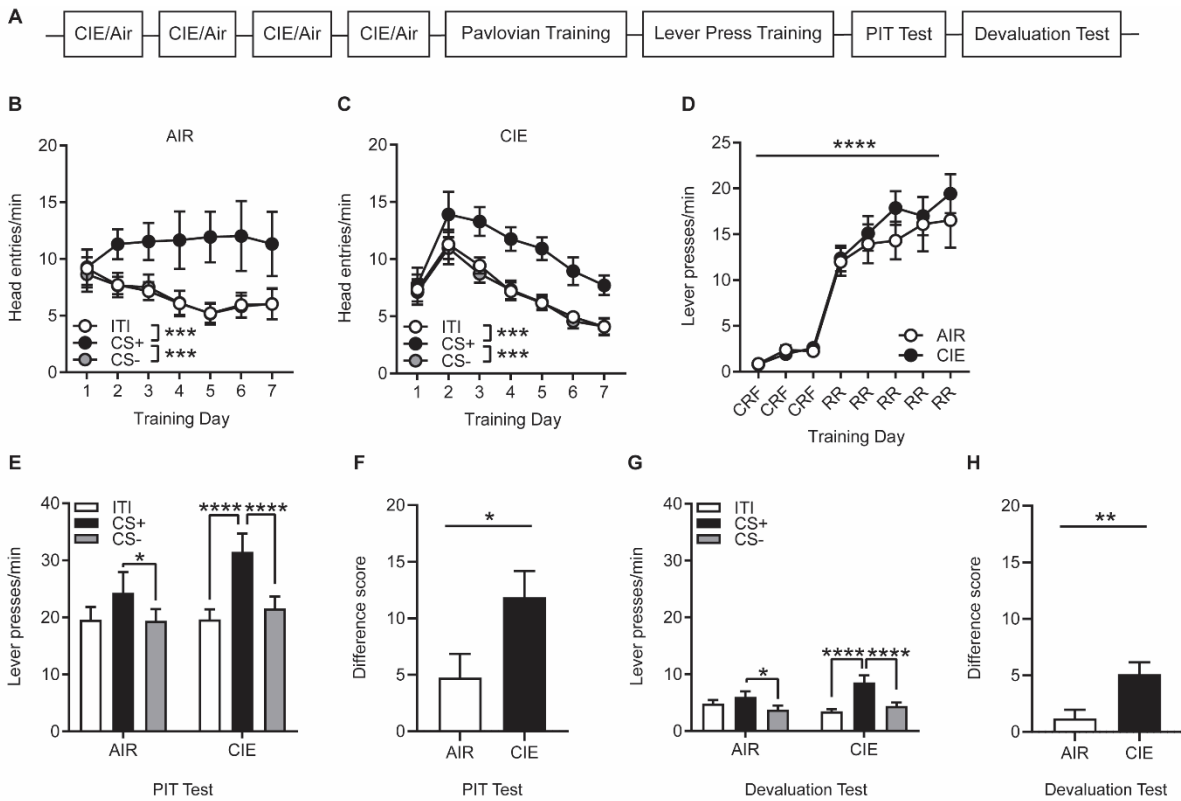
Figure 2.2: Chronic intermittent ethanol (CIE) exposure enhances Pavlovian-to-instrumental transfer (PIT). (**A**) All mice were exposed to either CIE or Air control procedures; next, animals went through long cue procedure Pavlovian training including a food-associated tone (CS+), a non-food-associated tone (CS-), and intertrial intervals (ITI) where no tone was played; next, mice received instrumental lever press training on continuous reinforcement (CRF) and random ratio (RR) schedules; transfer was then assessed in a PIT test; finally, a second PIT test was performed following devaluation of the food outcome. (**B**) Rate of food port entries across Pavlovian training days in Air group. (**C**) Rate of food port entries across Pavlovian training days in CIE group. (**D**) Rate of lever presses across instrumental training days. (**E**) Rate of lever pressing during each trial type in the PIT test. (**F**) Average difference between rate of lever pressing during CS+ minus rate of lever pressing during ITI in the PIT test. (**G**) Rate of lever pressing during each trial type in the devaluation test. (**H**) Average difference between rate of lever pressing during CS+ minus rate of lever pressing during ITI in the devaluation test. Data points represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ represent significant comparisons.

Discussion

In the present study, we adapted a model of Pavlovian-to-instrumental transfer in mice to examine the influence of prior exposure to chronic intermittent ethanol on cue control of behavior. In ethanol-naïve mice, we found that a short cue procedure produced negative transfer and a long cue procedure produced positive transfer in the PIT test (Figure 1), supporting that procedural differences in Pavlovian training can influence the expression and direction of transfer in rodent models (Crombag et al., 2008). We then found that chronic ethanol exposure prior to all behavioral procedures resulted in enhanced PIT transfer compared to controls (Figure 2). These results suggest that protracted withdrawal following chronic ethanol exposure strengthened the ability of conditioned cues to guide ongoing instrumental actions. Importantly, as we used non-drug cues and rewards throughout all experiments, these findings suggest that ethanol dependence may alter behavior guided by reward cues in general, rather than just cues associated with ethanol.

We found that though performance did not differ significantly between groups during training, PIT transfer was selectively enhanced in ethanol animals compared to controls. This finding of CIE-enhanced PIT is consistent with one previous study showing increased influence of non-drug Pavlovian cues on behavior in abstinent humans with alcohol dependence (Garbusow et al., 2016). However, another recent report found no differences in transfer strength between abstinent individuals with AUD and controls (van Timmeren et al., 2020). Two notable differences between these studies are the type of reward and the type of PIT procedure used. Garbusow et al. (2016) used cues associated with monetary wins and losses, and van Timmeren et al. (2020) used cues associated with food rewards. However, as the present work also used food rewards, more investigation is necessary to understand any differences in performance between procedures using primary or secondary reinforcers. A second difference between these studies is the method of PIT used. Whereas van Timmeren et al. (2020) explicitly examined both specific and general PIT, finding no differences between patients and controls

with either form of PIT, Garbusow et al. (2016) used a PIT procedure that cannot determine which form of PIT was observed. These experimental differences may underlie the disparate results observed, or other undetermined factors may be involved.

We found that PIT transfer persisted in ethanol animals, but not control animals, following devaluation procedures. Though we did not directly test devaluation to avoid possible training and extinction effects, several factors support that devaluation occurred. Groups showed similar consumption during pre-feeding, and across all groups there was a reduction in baseline rate of lever presses and food port entries made during the devaluation test compared to the first PIT test. As CIE exposure has previously been shown to produce insensitivity to devaluation of ethanol self-administration as well as operant food-seeking (Lopez et al., 2014; Renteria et al., 2018; Renteria et al., 2020), the current findings suggest that cue-induced motivational influences on lever pressing are open to devaluation in control mice but not ethanol mice. Past works have revealed conflicting effects of devaluation on PIT, with procedures similar to those used here demonstrating devaluation susceptibility (Aitken et al., 2016; Corbit et al., 2007; Dailey et al., 2016). In contrast, outcome-specific PIT often shows no effect of devaluation in both rodents and humans (Holland, 2004; Rose et al., 2018; but see Seabrooke et al., 2019). Holland (2004) additionally found that sickness-induced devaluation did not alter presumed outcome-general PIT, perhaps indicating differing effects based on method of devaluation. As the procedure in the present work is thought to produce outcome-general PIT (Cartoni et al., 2016), and devaluation was induced via satiation, our results support that devaluation affected transfer in the control group but not the ethanol group.

It is well established that exposure to ethanol is associated with altered responses to cues for ethanol. In rodents, cues and contexts related to ethanol invigorate ethanol-seeking behavior (Cunningham et al., 2006; Remedios et al., 2014; Sciascia et al., 2015), and ethanol self-administration both augments ethanol-seeking (Remedios et al., 2014; Sciascia et al., 2015) and reinstates Pavlovian and instrumental ethanol-seeking following extinction (Burattini et al.,

2006; Chaudhri et al., 2010; Tsiang & Janak, 2006). Using PIT tasks, a number of rodent studies have found that ethanol-paired conditioned stimuli elicit increases in instrumental responding that was previously maintained by ethanol (Corbit & Janak, 2007; Corbit & Janak, 2016; Glasner et al., 2005; Krank, 2003; Krank et al., 2008; Milton et al., 2012). Though this effect has not been studied extensively in humans, one study of detoxified individuals with AUD interestingly found that alcohol cues had an inhibitory effect on instrumental behavior in PIT (Schad et al., 2019). This inhibitory effect may reflect the negative properties of alcohol and related cues experienced by patients during the process of detoxification (Schad et al., 2019). The present work moves beyond drug-specific cues to investigate the effect of chronic ethanol exposure on PIT behavior in the presence of reward cues in general, not just ethanol cues.

Notably, one prior study in mice using CIE and PIT procedures similar to those in the present work found that transfer was present in Air animals but abolished in CIE animals (DePoy et al., 2015). This study differed from the current work in that ethanol exposure was conducted after training had concluded, when associations were already learned (DePoy et al., 2015). In addition, DePoy et al. (2015) conducted testing immediately after the acute phase of ethanol withdrawal (2-3 days in nonhuman animals; Heilig et al., 2010). This timeline is markedly different from that used in the present work and previous work in humans with AUD, where ethanol exposure occurred prior to all behavioral procedures. Thus, DePoy et al. (2015) may have revealed an effect of more recent withdrawal on PIT performance, and would not have captured any possible effect of chronic ethanol exposure on training. Together, these results suggest that the sequence of drug exposure and conditioning may be an important factor in determining whether prior ethanol dependence enhances or inhibits PIT. Indeed, previous work supports that the timing of exposure to psychostimulants determines whether PIT is enhanced or impaired (Wyvell & Berridge, 2001; Hall & Gulley, 2011).

Though human PIT studies commonly utilize short, discrete Pavlovian cues (e.g., Garbusow et al., 2016; van Timmeren et al., 2020), previous work supports that procedures

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using short duration cues often do not produce positive PIT transfer in rodent models. Crombag et al. (2008) found that 10-sec Pavlovian tones did not produce transfer, whereas 120-sec Pavlovian tones produced robust transfer. Earlier work found similar results, with long Pavlovian cues producing positive transfer and short Pavlovian cues suppressing instrumental responding (Meltzer & Brahlek, 1970; Van Dyne, 1971). In the present work, mice trained with short cues demonstrated expected performance in both the Pavlovian and instrumental phases of training, but negative transfer in the PIT test. Mice trained with long cues, however, demonstrated expected performance in training and positive transfer in the PIT test. These findings could reflect several differences in the short and long cue procedures, including the temporal pairing of cue-food delivery, difference in the number of cue-reward pairings, and number of rewards delivered. Competition between lever press and food port entry behaviors may also be at play, as mice cannot perform these behaviors simultaneously; a short cue could bias checking of the food port in expectation of imminent food delivery, at the expense of lever pressing. Regardless of differences between procedures, these findings support using Pavlovian training procedures with long conditioned cues to examine the effect of ethanol dependence on transfer in rodent PIT.

Though several brain areas are thought to contribute to PIT, the two regions most heavily implicated are the amygdala and nucleus accumbens (NAcc; Blundell et al., 2001; Corbit et al., 2001; de Borchgrave et al., 2002; Hall et al., 2001; Holland & Gallagher, 2003), with relatively more recent studies uncovering a dissociation between select subregions. Lesions to the basolateral amygdala (BLA) and NAcc shell have been shown to abolish outcome-specific PIT, whereas lesions to central amygdala (CeA) and NAcc core abolish outcome-general PIT (Corbit et al., 2016; Corbit & Balleine, 2005; Corbit & Balleine, 2011). In amygdala, the dissociation between subregions has been corroborated in human PIT using high-resolution fMRI (Prévost et al., 2012). Further, lesions to CeA and substantia nigra pars compacta (SNpc) or ventral tegmental area (VTA) were shown to reduce transfer in presumed outcome-general

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PIT (El-Amamy & Holland, 2007), and VTA inactivation itself attenuates or abolishes transfer (Corbit et al., 2007; Murschall & Hauber, 2006).

Chronic alcohol exposure produces functional changes in amygdala and NAcc that could contribute to altered PIT behavior. In rodent CeA, CIE exposure was shown to augment GABAergic signaling and produce a net inhibition (Pleil et al., 2015; Roberto et al., 2004), potentially leading to disinhibition of downstream regions (Gilpin et al., 2015; Roberto et al., 2021). In rodent NAcc, CIE exposure increased glutamatergic activity (Griffin et al., 2014; Griffin et al., 2015), and in alcohol-dependent humans, NAcc activity in PIT was found to correlate with subsequent relapse (Garbusow et al., 2016). The overlap in brain regions implicated in PIT and affected by alcohol dependence is significant, and suggests that chronic exposure to alcohol could alter neural circuitry underlying the motivating influence of Pavlovian cues on instrumental behavior. As similar procedures to that used here are thought to produce outcome-general PIT (Cartoni et al., 2016), changes to subregions involved in that type of PIT are of greatest interest in regard to the present findings. For instance, an intriguing hypothesis is that CIE inhibition of CeA, and possible downstream disinhibition of areas such as SNpc and VTA, could underlie the facilitation of transfer demonstrated here in CIE mice.

One alternative explanation for enhanced transfer is that the CIE group could have undergone changes in motivational processes unrelated to ethanol. Though altered motivation can be difficult to measure directly, some evidence that could support increased motivation for the food reinforcer in CIE animals would be significantly lower weights, generally heightened responding, increased levels of consumption prior to the devaluation test, and possibly enhanced performance in other measures of motivational processes, such as incentive learning tasks. The present work, however, reflects a pattern of results that supports similar levels of food motivation between groups. There were no significant differences between Air and CIE groups in baseline weights, and across Pavlovian and instrumental training, animals exhibited very similar patterns of responding and no significant differences in behavior between groups.

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The two groups also did not significantly differ in consumption of the food reinforcer prior to the devaluation test. Though incentive learning was not examined here, recent work found that CIE-exposed animals actually exhibited impaired incentive learning although there was no weight loss during or after CIE (Galaj et al., 2020). Thus, though changes in motivational processes cannot be completely ruled out, findings in the present work do not appear to be consistent with CIE-increased food motivation.

Together, our data suggest that chronic exposure to ethanol and repeated withdrawal can enhance the control of cues on volitional behavior. Importantly, this change was induced by prior chronic ethanol exposure and observed in a non-ethanol related task state. This suggests that ethanol dependence and protracted withdrawal may be associated with changes to neural structures key for guiding cue responses in general, rather than just cues for ethanol. This work sets the stage for future research investigating the neural circuitry underlying altered cue control of behavior in ethanol dependence, and may suggest that ethanol-induced changes in brain areas such as the nucleus accumbens and amygdala are key to enhancements in cue control. Future work dissecting the distribution of lever presses and head entry behavior across a session could also be of interest, in particular to clarify any behavioral differences between varying training procedures. Elucidating the neural and behavioral effects of ethanol dependence on cue control of behavior is crucial for developing improved methods of assessing and treating individuals with AUD.

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Declaration of Interest

The authors have no conflicts of interest to disclose.

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CHAPTER 3

Effects of Central Amygdala Chemogenetic Manipulation and Prior Chronic Alcohol Exposure on Pavlovian-to-Instrumental Transfer

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Abstract

Background

      Recent works suggest that a history of chronic alcohol exposure can enhance the influence of non-drug reward cues on ongoing actions. This is often modeled in Pavlovian-to-instrumental transfer (PIT) tasks that examine the interaction between Pavlovian and instrumental learning processes, usually reflected as an increase in action vigor during the presentation of a reward-associated cue. Though prior chronic alcohol exposure has been shown to strengthen this type of cue-guided behavior, the neural mechanisms underlying such enhancements are not known.

Methods

      In the present work we examined the contribution of central amygdala (CeA), a region that is strongly implicated in PIT behaviors and is functionally altered by chronic alcohol exposure. We utilized Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to examine the impact of inhibitory and excitatory CeA manipulation on PIT behaviors in alcohol-naïve mice and mice with a history of chronic intermittent ethanol vapor exposure and withdrawal (CIE).

Results

      Replicating previous work, we found that a history of CIE strengthened baseline PIT, in the absence of any CeA manipulation. We also found that activation of both inhibitory and excitatory DREADDs expressed in CeA enhanced PIT in alcohol-naïve mice, though the latter markedly reduced response rates. However, in mice exposed to CIE, activation of excitatory DREADD receptors expressed in CeA appeared to weaken PIT.

Conclusions

      These results suggest that alcohol-induced disruptions in amygdala function may contribute to changes in appetitive behaviors, such as cue-guided responding, following chronic exposure to alcohol. Better elucidating the neural mechanisms underlying disrupted cue-guided

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behavior following chronic alcohol exposure may help understand and treat deficits in adaptive behavior associated with chronic alcohol use in humans.

Keywords

Pavlovian-to-instrumental transfer; chronic alcohol; central amygdala; chemogenetics; mice

Introduction

Chronic alcohol use is associated with changes in cue-guided behaviors. For instance, in alcohol use disorder (AUD), cues associated with alcohol can induce craving, potentially leading to consumption and relapse (Garbusow et al., 2014; Sjoerds et al., 2014; Witteman et al., 2015). However, recent work suggests that chronic exposure to alcohol may also alter responses to non-alcohol cues, thereby potentially disrupting everyday decision-making processes. Using Pavlovian-to-instrumental transfer (PIT) paradigms, which capture the process through which Pavlovian-conditioned reward cues alter the performance of voluntary instrumental actions, a history of AUD has been found to be associated with a greater influence of non-alcohol reward cues on behavior (Garbusow et al., 2016). However, the neural mechanisms underlying such disruption are not clear.

PIT has also been observed in more tractable models that readily allow for neural investigation (e.g., Corbit & Balleine, 2005; Crombag et al., 2008), including in rodent operant tasks using alcohol rewards and alcohol-paired cues (e.g., Alarcón & Delamater, 2019; Corbit & Janak, 2016; Glasner et al., 2005). In addition, we previously found in mice that a history of chronic alcohol exposure enhanced transfer in a food-based PIT task (Shields & Gremel, 2021), aligning with prior work in humans. One region thought to underlie such behaviors is the central nucleus of the amygdala (CeA), commonly known to be crucial for mediating physiological and behavioral responses to aversive cues and outcomes (e.g., Ciocchi et al., 2010; Han et al., 2015; Ozawa et al., 2017). However, the CeA is also strongly implicated in appetitive processes, including PIT-related behaviors (for review, see Warlow & Berridge, 2021). For example, a functioning CeA is necessary to observe outcome-general PIT (Corbit & Balleine, 2005; Hall et al., 2001; Holland & Gallagher, 2003; Prévost et al., 2012), where a cue predictive of one type of reward can enhance instrumental responding for rewards in general. CeA is also recruited to form cue-reward associations in Pavlovian learning (Gallagher et al., 1990; McDannald et al., 2004), and optogenetic excitation and μ-opioid stimulation of CeA have been demonstrated to

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direct incentive motivation for rewards as well as aversive outcomes (Mahler & Berridge, 2012; Robinson et al., 2014; Tom et al., 2019; Warlow et al., 2020). These works situate CeA as important for supporting the incentive and appetitive processes that underlie the influence of conditioned cues on behavior, as well as aversive states.

Changes to the CeA have also been heavily implicated in AUD, with adaptations in CeA function found following prolonged alcohol exposure. In rodents, chronic exposure to and withdrawal from ethanol has been found to increase baseline GABAergic transmission and produce a net inhibition of CeA (Pleil et al., 2015; Repunte-Canonigo et al., 2015; Roberto et al., 2010; Roberto et al., 2004). Considering that the CeA is comprised largely of GABAergic projection neurons, alcohol-induced inhibition of CeA is hypothesized to lead to downstream disinhibition that could contribute to behavioral effects observed following alcohol dependence (Gilpin et al., 2015; Roberto et al., 2012; Roberto et al., 2021). Indeed, the CeA is thought to mediate alcohol self-administration (Hyytiä & Koob, 1995; Möller et al., 1997; Roberts et al., 1996) as well as the negative affective state characteristic of alcohol withdrawal (Funk et al., 2006; Roberto et al., 2010; Sommer et al., 2008). However, effects on CeA function could also lead to changes in appetitive processes, beyond just alcohol-related behaviors and states.

Though the CeA exhibits functional changes following chronic alcohol exposure, and is known to be necessary for transfer in outcome-general PIT, it remains unclear whether the region underlies alcohol enhancement of cue-guided behavior in PIT. In the present work, we investigated the role of CeA in cue-guided appetitive behaviors, employing a PIT task most consistent with outcome-general transfer (Cartoni et al., 2016). In Experiment 1, we examined the effect of inhibitory chemogenetic CeA manipulation on PIT behaviors in alcohol-naïve animals. In Experiment 2, we investigated the effect of excitatory chemogenetic CeA manipulation on PIT behaviors in animals exposed to chronic intermittent ethanol (CIE) vapor exposure and withdrawal, a well-validated model of alcohol dependence.

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Materials and Methods

Animals

Adult (>6 weeks) female and male C57BL/6J mice were acquired from Jackson Laboratory (Bar Harbor, ME) or bred in-house 1 generation from mice ordered from Jackson Laboratory and were group housed 2-4 per cage. Mice were maintained on a 14-hr light/10-hr dark cycle, and provided with ad libitum water and, prior to experimental procedures, mouse chow (Lab-diet 5015). All experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee, and were conducted in accordance with NIH Guidelines.

Surgical Procedures

Under isoflurane anesthesia, mice were given stereotaxically-guided bilateral injections into CeA (coordinates from bregma: AP: -1.30 mm, ML: ± 2.90 mm, DV: -4.70 mm) via Hamilton syringe. All viruses were acquired from the UNC Vector Core. Mice in the experimental groups underwent bilateral co-injections of AAV5-hSyn-GFP-Cre and Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), either inhibitory DREADD AAV5-hSyn-DIO-hM4D(Gi)-mCherry (Experiment 1) or excitatory DREADD AAV5-hSyn-DIO-hM3D(Gq)-mCherry (Experiment 2; 100 nl of each virus per side). Control mice received bilateral co-injections of AAV5-hSyn-GFP-Cre and non-DREADD control virus AAV5-FLEX-tdTomato (100 nl of each virus per side). Clozapine-*n*-oxide (CNO; Sigma-Aldrich, USA) at a 10 ml/kg, 1 mg/kg dose was administered to all mice via intraperitoneal injection 30-60 min prior to the first transfer test, and 15 min prior to pre-feeding in the devaluation test. Mice were euthanized following testing, and brains were extracted and fixed in 4% paraformaldehyde. Brain slices at 50 μm thickness were examined for viral spread under a macro fluorescence microscope (Olympus MVX10).

Chronic Intermittent Ethanol Exposure

In Experiment 2, four cohorts of mice were exposed to four rounds of ethanol vapor or air control with repeated withdrawal (Becker, 1994; Becker & Lopez, 2004; Griffin et al., 2009;

Lopez & Becker, 2005; Renteria et al., 2018; Renteria et al., 2020; Shields & Gremel, 2021).

Each round consisted of four consecutive days of 16 hr of vapor exposure (ethanol or air) followed by an 8 hr withdrawal period. Between each round were an additional three days of withdrawal. During vapor exposure, mice in their home cages were placed into Plexiglass chambers (Plas Labs Inc., USA), and ethanol or air vapor was passed through the chambers. Ethanol was volatilized by bubbling air through a flask of 95% ethanol at a rate of 2.3 L/min, then combined with a separate air stream to produce a total flow of approximately 10 L/min. No pyrazole or loading doses of ethanol were administered prior to placement in the chamber to avoid effects of stress on instrumental behaviors (Dias-Ferreira et al., 2009) and broad actions of pyrazole, including at the NMDA receptor (Pereira et al., 1992). Animals were monitored for ill effects of ethanol exposure. At the end of each round, blood ethanol concentrations (BECs) were collected from a total of 16 sentinel mice (mean BEC = 37.13 ± .1.89 mM; Analox, USA).

Pavlovian-to-Instrumental Transfer Task

General procedures. The PIT task was used as a model of cue-guided behavior (Shields & Gremel, 2021). Across both experiments, 3 days prior to behavioral procedures, mice were food restricted to maintain 85% of baseline bodyweight. The induction of food restriction was followed sequentially by Pavlovian training, instrumental lever press training, and testing for transfer. In Experiment 2, food restriction started 3 days after CIE concluded, such that behavioral training began a total of 6 days after the end of all CIE procedures. This timeline was chosen to limit the effects of acute withdrawal while capturing any lasting effects of CIE on training and testing occurring within protracted withdrawal (Heilig et al., 2010), where we have previously observed exposure effects on decision-making processes and PIT responding (Renteria et al., 2018; Shields & Gremel, 2021). All behavioral procedures took place in standard sound-attenuating operant boxes containing two retractable levers surrounding a food magazine (Med Associates, Vermont, USA). The box house light was illuminated for all procedures.

Pavlovian Acquisition. Pavlovian training utilized a food-predictive conditioned stimulus (CS+) as well as a conditioned stimulus predictive of no food delivery (CS-). A CS- was included to uncover whether any alterations in behavior were specific to reward-predictive cues, or rather to environmental stimuli in general. Auditory tones at the frequency of 8 kHz or 15 kHz, discriminable for mice (De Hoz & Nelken, 2014), were used as conditioned stimuli and were presented for a duration of 120 sec. One food pellet (Bio-Serve formula F05684) was delivered on average every 30 sec throughout presentation of the CS+, whereas no food was delivered during the CS-. Between each CS presentation was an intertrial interval (ITI) when no tones were played and no food was delivered. The ITI duration varied pseudorandomly from 60 sec to 180 sec, with an average duration of 120 sec. Pavlovian training sessions occurred daily for a total of 7 days, with each session including 10 CS+ and 10 CS- tones presented in pseudorandom order. Mouse entries into the food magazine were recorded via infrared beam breaks.

Instrumental Acquisition. Instrumental training followed Pavlovian training and was conducted in the same behavioral context with daily sessions for a total of 8 days. In each session, mice were able to press a lever for the same food reward that was earned in Pavlovian training. Throughout training and testing, one lever was always available whereas the other lever was always retracted. For the first 3 days of instrumental training, 1 food pellet was delivered for every lever press (continuous reinforcement schedule, CRF). Following CRF, the schedule changed to random ratio (RR), such that one food pellet was earned for every 10 lever presses on average. Instrumental training sessions ended when a mouse earned 30 food pellets or 90 min elapsed, and lever presses and entries into the food receptacle were recorded.

Pavlovian-to-Instrumental Testing. After instrumental training concluded, the influence of conditioned stimuli on lever pressing was assessed in a transfer test lasting approximately 30 min. The length of the test varied slightly, depending on the exact durations of ITI periods within a given test. The test was conducted in extinction, such that no food pellets were delivered at

any time throughout the session. During the test, the trained lever was continuously available

and the CS+ and CS- tones were presented a total of 4 times each, with each tone presentation

separated by an ITI lasting on average 120 sec, but varying pseudorandomly from 60 sec to 180

sec. Tones were presented in alternating order, beginning with the CS+ tone, and lever presses

and food port entries were recorded. Notably, this procedure results in relatively weak PIT in

control mice (Shields & Gremel, 2021), allowing observation of PIT enhancement and reducing

concern for ceiling effects.

Outcome Devaluation Procedures. The day after the transfer test, a second test

evaluated the influence of outcome devaluation on cue-guided lever pressing. Mice were first

given free access to the food pellets earned during training for a total of 60 min. Immediately

following this pre-feeding session, mice underwent a second transfer test identical to the first. It

is important to note that outcome devaluation procedures commonly compare behavior in a

devalued state to behavior in a valued state, following pre-feeding with a non-paired reinforcer.

Here, we excluded a valued comparison to limit the effects of extinction and to avoid the

possibility that additional training days could alter the strength of CS or lever press associations.

Behavioral and Statistical Analysis

Data were analyzed using Prism 6 (GraphPad) and JASP (Version 0.13.1.0), and the

alpha level was set at 0.05. In Experiment 2, a 2-way ANOVA (CIE round x CIE group) was

conducted to compare weight in grams between groups on the first day of each CIE round.

Pavlovian training was analyzed with 2- or 3-way mixed ANOVAs to examine how food port

entry rates on the final day of training were affected by the within-subjects factor of Trial Type

(ITI/CS+/CS-) and the between-subjects factor of Treatment (control/DREADD), with the

addition of the between-subjects factor of CIE group (Air/CIE) in Experiment 2. Lever press

rates on the final day of instrumental training were analyzed with an unpaired t-test for

Treatment in Experiment 1, and a 2-way ANOVA (CIE group x Treatment) in Experiment 2. For

the PIT and devaluation tests, 2- and 3-way ANOVAs were conducted to examine the effect of

Trial Type and Treatment on lever press rates, with the addition of CIE group as a factor in Experiment 2. Transfer was evidenced by an increase in lever pressing vigor during the food-associated CS+ relative to ITI and CS- periods. In the case of a 3-way interaction, follow-up 1-way ANOVAs (Trial Type) for lever press rate were conducted to examine the presence of transfer within Air and CIE groups. Throughout training and testing, planned post-hoc comparisons with Bonferroni correction were conducted to follow up on significant main effects or interactions involving the factor of Trial Type. More direct comparisons between groups were conducted using difference score as a measure of PIT magnitude, calculated by subtracting test ITI lever press rate from test CS+ lever press rate. Difference scores were examined in unpaired t-tests for Treatment in Experiment 1, and in 2-way ANOVAs (CIE group x Treatment) in Experiment 2. Each group's difference score was further compared against 0 in one-sample t-tests to confirm the presence of transfer. In Experiment 2, we also tested whether difference score correlated with lever press rates on the final day of training. Finally, consumption in grams during the devaluation pre-feeding session was examined with an unpaired t-test for Treatment in Experiment 1, and a 2-way ANOVA (CIE group x Treatment) in Experiment 2.

Results

Histology

Brains were examined for viral spread and only animals with bilateral viral expression in the CeA were included in analyses. The final ns for Experiment 1 were $n = 11$ (5F, 6M) in the control virus group and $n = 11$ (6F, 5M) in the H4 group. In Experiment 2, the final ns were $n = 10$ (5F, 5M) in Air control mice, $n = 8$ (6F, 2M) in the Air H3 group, $n = 10$ (4F, 6M) in the CIE control group, and $n = 10$ (3F, 7M) in the CIE H3 group.

Experiment 1: Chemogenetic inhibition of CeA in PIT

Experiment 1 examined the effects of chemogenetic inhibition of CeA on PIT behaviors. The experimental timeline began with surgeries, followed by Pavlovian and instrumental training, then a test for transfer and a devaluation test (Figure 1A). Mice first received bilateral

injections of an inhibitory DREADD (H4 group, n = 11; 6F, 5M) or control virus (control group, no DREADD, n = 11; 5F, 6M) into the CeA (Figure 1B and C). Acquisition began with Pavlovian conditioning, where mice demonstrated robust Pavlovian conditioning regardless of treatment group. A 2-way ANOVA (Trial Type x Treatment) for food port entry rate on the final day of training revealed a significant main effect of Trial Type (F(2, 40) = 19.58, p < 0.0001) but no significant effect of Treatment or interaction (Fs < 0.75, ps > 0.05). Post hoc comparisons for the main effect of Trial Type confirmed that food port entry rates were significantly higher during the food-associated CS+ (food port entries/min in control group: 6.45 ± 1.03 and H4 group: 5.42 ± 0.53) compared to CS- (food port entries/min in control group: 3.52 ± 0.50 and H4 group: 3.26 ± 0.52) and ITI periods (food port entries/min in control group: 3.30 ± 0.52 and H4 group: 2.90 ± 0.36; ps < 0.0001). The comparison between the CS- and ITI was not significant (p > 0.05). Following Pavlovian training, mice acquired lever press behavior in instrumental training. An unpaired t-test for lever press rate on the final day of training found no significant difference between Treatment groups (t(20) = 1.63, p > 0.05; lever presses/min in control group: 13.32 ± 2.07 and H4 group: 9.40 ± 1.25). These results support that the control and H4 groups acquired similar levels of Pavlovian and instrumental behavior in acquisition.
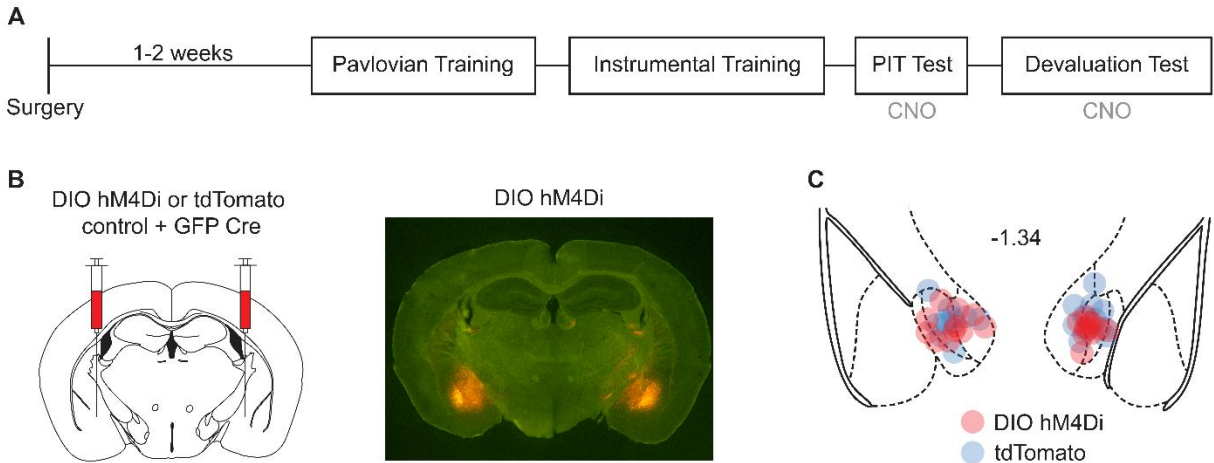
Figure 3.1: Experimental timeline and surgical procedures in Experiment 1. (**A**) After recovering from surgical procedures, mice underwent Pavlovian training, followed by instrumental training, a PIT test for transfer, and a second PIT test following devaluation of the food outcome. Clozapine-*n*-oxide (CNO) was administered prior to testing only. (**B**) Schematic of central amygdala (CeA) injection site (left) and representative mCherry expression (right). (**C**) Schematic of viral injection sites in CeA. Each circle indicates the center of viral expression in a single animal (-1.34 mm from bregma), with red circles indicating the DIO hM4Di group and blue circles indicating the control tdTomato group.

After instrumental training a transfer test was performed, with CNO administered to both groups 60 min before the test began. We found that activation of H4 receptors expressed in CeA enhanced PIT transfer without affecting overall response rates (Figure 2A). This was supported by a 2-way ANOVA (Trial Type x Treatment) performed on lever press rates, which found a Trial Type x Treatment interaction ($F(2, 40) = 3.48$, $p < 0.05$) and a main effect of Trial Type ($F(2, 40) = 14.96$, $p < 0.0001$) but no effect of Treatment ($F(1, 20) = 0.28$, $p > 0.05$). For the Trial Type x Treatment interaction, post hoc comparisons for the H4 group found that CS+ responding was significantly higher compared to CS- and ITI responding in the H4 group (ps < 0.0001), while the control group showed similar press rates across trial types (ps > 0.05). Regardless of Treatment group, lever press rates were not significantly different between the CS- and ITI (ps > 0.05). Group difference scores, calculated as CS+ lever press rate minus ITI lever press rate, supported that PIT was present in both groups (Figure 2B). One sample t-tests found that the difference between CS+ and ITI responding was significantly greater than 0 in both control mice ($t(10) = 2.61$, $p < 0.05$) and H4 mice ($t(10) = 3.64$, $p < 0.01$). Further, an unpaired t-test comparing difference scores between groups found a trend toward higher PIT in CeA H4-expressing mice ($t(20) = 1.89$, $p = 0.07$).
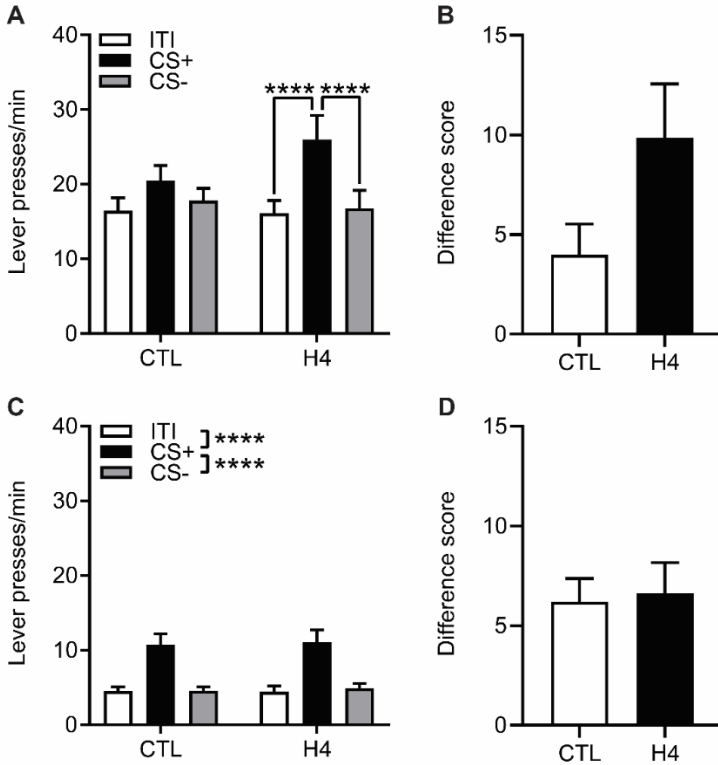
Figure 3.2: Inhibitory chemogenetic central amygdala manipulation enhances Pavlovian-to-instrumental (PIT) transfer in ethanol-naïve animals. (**A**) Rate of lever pressing during each trial type in the PIT test, which included a food-associated auditory tone (CS+), an auditory tone not associated with food (CS-), and an intertrial interval (ITI) when no tones were played. (**B**) Average difference between the rate of lever pressing during CS+ trials and the rate of lever pressing during ITI periods in the PIT test. (**C**) Rate of lever pressing during each trial type in the devaluation test. (**D**) Average difference between the rate of lever pressing during CS+ trials and the rate of lever pressing during ITI periods in the devaluation test. Data points represent mean ± SEM. **** $p < 0.0001$ represents significant comparisons. CTL = control virus group; H4 = inhibitory chemogenetic virus group.

A devaluation test was conducted after the first transfer test, and consisted of a 60 min pre-feeding session with the same reinforcer earned during training immediately followed by a second test for transfer conducted in the same fashion as the first. CNO was administered to all mice 15 min before the pre-feeding session began. Activation of the H4 receptor did not affect consumption during pre-feeding, as supported by an unpaired t-test ($t(20) = 0.85$, $p > 0.05$; average in control group: 0.42 ± 0.07 g; average in H4 group: 0.50 ± 0.06 g). While test lever press rates were decreased from the first test, transfer was still present in both treatment groups (Figure 2C). This was supported by a 2-way ANOVA (Trial Type x Treatment) for lever press rate, which found only a significant main effect of Trial Type ($F(2, 40) = 35.60$, $p < 0.0001$) and no main effect of Treatment or interaction (Fs < 0.05, ps > 0.05). Post hoc comparisons confirmed that responding was higher in the CS+ compared to the ITI and CS- (ps < 0.0001; no difference between ITI and CS-, $p > 0.05$). Analyses of difference scores supported this pattern of results (Figure 2D). One sample t-tests found that the difference between CS+ and ITI responding was significantly greater than 0 in both control mice ($t(10) = 5.34$, $p < 0.001$) and H4 mice ($t(10) = 4.34$, $p < 0.01$). An unpaired t-test comparing difference scores found no significant difference between groups ($t(20) = 0.23$, $p > 0.05$). These results suggest that DREADD enhancement of PIT was limited to the first transfer test, and that H4 activation did not alter the effects of devaluation procedures and/or subsequent testing.

Experiment 2: Chemogenetic excitation of CeA in PIT following CIE exposure

As chemogenetic inhibition of CeA enhanced PIT in alcohol-naïve mice, we hypothesized that chronic alcohol exposure might strengthen PIT via a similar mechanism and that CeA excitation could potentially counteract this effect. Thus, Experiment 2 examined the effects of CeA chemogenetic excitation (control or H3 virus) on PIT behaviors in animals previously exposed to CIE or air control procedures. There were four groups in total: Air control ($n = 10$; 5F, 5M), Air H3 ($n = 8$; 6F, 2M), CIE control ($n = 10$; 4F, 6M), and CIE H3 ($n = 10$; 3F, 7M). The timeline was identical to Experiment 1, except that four rounds of CIE were performed

after recovery from surgery but before all behavioral procedures (Figure 3A). Mice received

bilateral CeA injections of an excitatory DREADD (H3) or control virus (Figure 3B and C), and

were allowed to recover for 1-2 weeks before CIE procedures began. Ethanol exposure did not

significantly affect weights in the CIE group, and mice gained weight on average throughout CIE

procedures. This was supported by a 2-way ANOVA (CIE round x CIE group) comparing weight

in grams between groups on the first day of each CIE round, which found a significant main

effect of CIE round (F(3, 108) = 15.71, $p < 0.001$) but no main effect of CIE group or interaction

(Fs < 0.99, ps > 0.05). Across all rounds, the average weight for CIE mice was 24.90 ± 0.44 g,

and the average weight for Air mice was 23.80 ± 0.54 g.
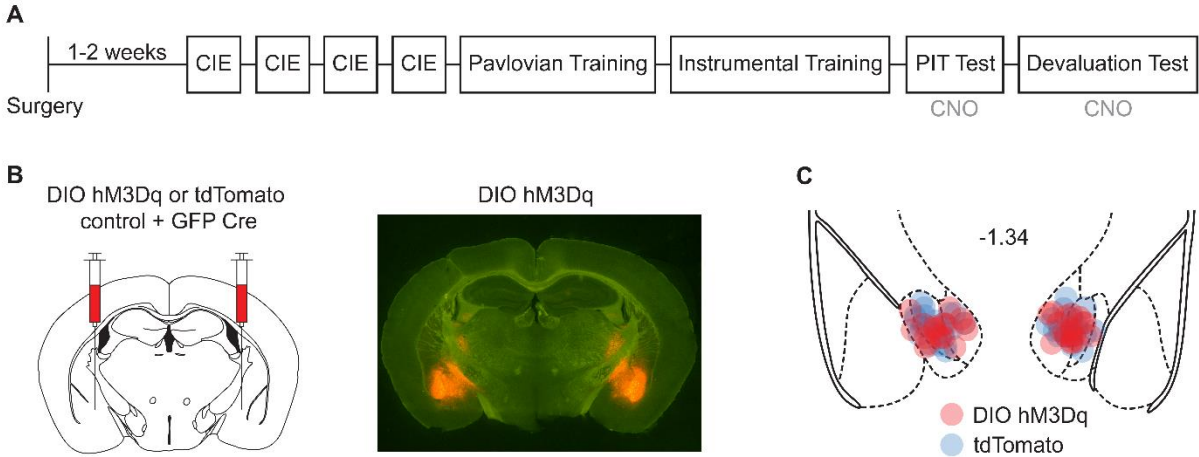
Figure 3.3: Experimental timeline and surgical procedures in Experiment 2. (**A**) After recovering from surgical procedures, mice underwent four rounds of chronic intermittent ethanol (CIE) vapor exposure and repeated withdrawal. This was followed by Pavlovian training, instrumental training, a PIT test for transfer, and a second PIT test following devaluation of the food outcome. Clozapine-*n*-oxide (CNO) was administered prior to testing only. (**B**) Schematic of central amygdala (CeA) injection site (left) and representative mCherry expression (right). (**C**) Schematic of viral injection sites in CeA. Each circle indicates the center of viral expression in a single animal (-1.34 mm from bregma), with red circles indicating the DIO hM3Dq group and blue circles indicating the control tdTomato group.

Animals again demonstrated robust Pavlovian conditioning. This was supported by a 3-way ANOVA (Trial Type x CIE group x Treatment) for food port entry rates on the final day of training, which found a main effect of Trial Type ($F_{(2, 68)} = 29.78$, $p < 0.001$) but no other significant main effects or interactions ($Fs < 3.68$, $ps > 0.05$). Post hoc comparisons for the main effect of Trial Type found that food port entry rates were higher during the CS+ (Air: 9.52 ± 1.39; CIE: 6.47 ± 0.95) compared to the ITI (Air: 5.30 ± 0.88; CIE: 3.57 ± 0.53) and CS- (Air: 4.81 ± 0.70; CIE: 3.64 ± 0.57; $ps < 0.001$), but that the comparison between ITI and CS- types was not significant ($p > 0.05$). After Pavlovian training, mice were trained to press a lever for the same reinforcer. A 2-way ANOVA (CIE group x Treatment) for lever press rates on the final day of training found a significant main effect of CIE group ($F_{(1, 34)} = 5.12$, $p < 0.05$), but no main effect of Treatment and no interaction ($Fs < 0.77$, $ps > 0.05$). For the main effect of CIE group, response rates supported that lever pressing was on average higher in the CIE group (13.43 ± 0.76 presses/min) compared to the Air group (9.41 ± 0.79 presses/min). Together, these findings suggest that mice acquired similar levels of Pavlovian behavior, but that mice previously exposed to CIE had higher response rates during instrumental training.

A transfer test followed the conclusion of lever press training. CNO was administered to all mice 30-60 min before the test began. We found a PIT effect across groups, and that responding was overall lower in H3 mice compared to controls (Figure 4A). This was supported by a 3-way ANOVA (Trial Type x CIE group x Treatment) for lever press rates, which found main effects of Trial Type ($F_{2, 68} = 22.82$, $p < 0.001$) and Treatment ($F_{(1, 34)} = 34.51$, $p < 0.001$) but no other main effects or interactions ($Fs < 2.68$, $ps > 0.05$). Post hoc comparisons for the main effect of Trial Type supported that responding was higher during the CS+ compared to the ITI and CS- ($ps < 0.001$; no difference between ITI and CS-, $p > 0.05$). For the main effect of Treatment, response rates overall were higher in the control group (17.33 ± 0.88 presses/min) compared to the H3 group (8.94 ± 0.72 presses/min) when averaging across Trial Type and CIE group.
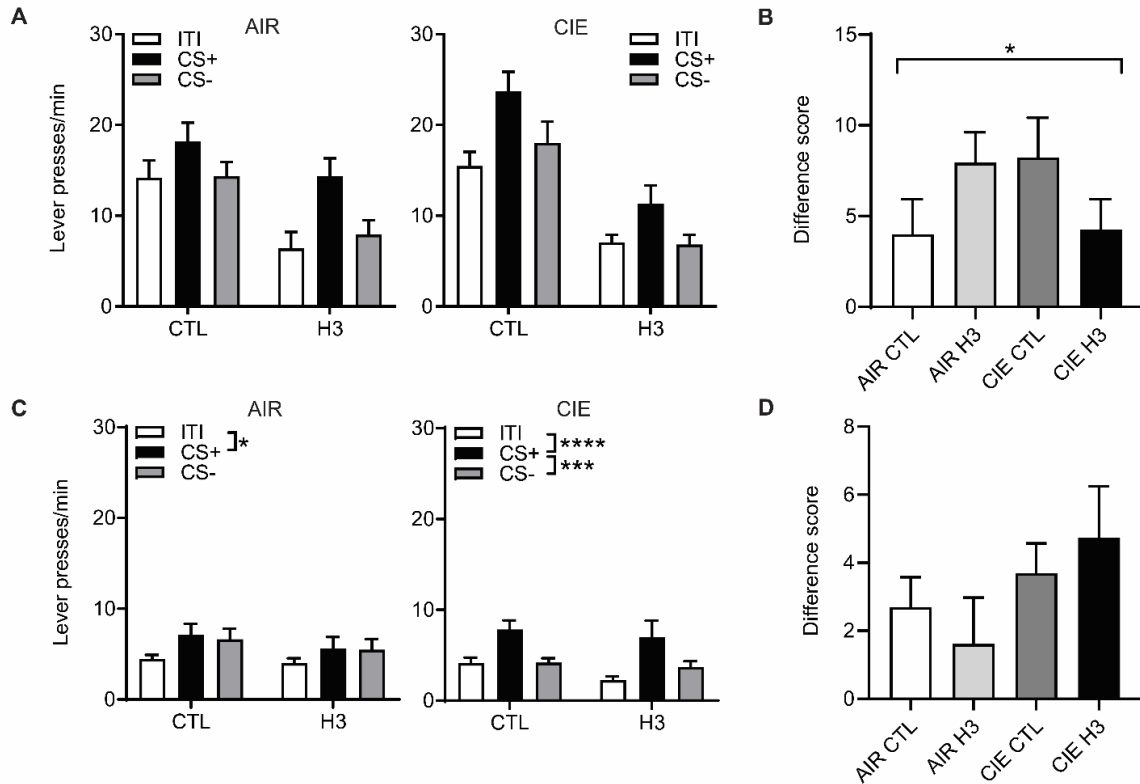
Figure 3.4: Excitatory chemogenetic central amygdala manipulation differentially affects Pavlovian-to-instrumental (PIT) transfer depending on history of ethanol exposure. (**A**) Lever press rates during each trial type in the PIT test in ethanol-naïve mice (left) and mice exposed to chronic intermittent ethanol (CIE; right). Trial types during the test included a food-associated auditory tone (CS+), an auditory tone not associated with food (CS-), and an intertrial interval (ITI) when no tones were played. (**B**) Average difference between the rate of lever pressing during CS+ trials and the rate of lever pressing during ITI periods in the PIT test. (**C**) Lever press rates during each trial type in the devaluation test in ethanol-naïve mice (left) and CIE mice (right). (**D**) Average difference between the rate of lever pressing during CS+ trials and the rate of lever pressing during ITI periods in the devaluation test. Data points represent mean ± SEM. * = < 0.05, *** = < 0.001, and **** $p < 0.0001$ represent significant comparisons or interactions. CTL = control virus group; H3 = excitatory chemogenetic virus group.

For a more direct comparison of PIT magnitude between subgroups, we analyzed difference scores across Treatment and CIE groups. A 2-way ANOVA (CIE group x Treatment) found H3 activation differentially affected PIT difference scores in Air and CIE mice (CIE group x Treatment interaction: $F(1, 34) = 4.26$, $p < 0.05$; main effect Fs < 0.02, ps > 0.05). Visual inspection of the data suggested that in non-DREADD control animals, difference scores were higher in CIE compared to Air mice, similar to our prior observations (Shields & Gremel, 2021). H3 activation appeared to increase PIT in Air mice, whereas H3 activation in CIE mice appeared to reduce the strength of PIT (Figure 4B). Though follow-up post hocs accounting for all comparisons were not significant, a one sample t-test found that average difference score in the Air control group was not significantly greater than 0 ($t(9) = 2.07$, $p > 0.05$), suggesting Air control mice did not show PIT. Difference scores were significantly greater than 0 for all other groups (Air H3: $t(7) = 4.72$, $p < 0.01$; CIE control: $t(9) = 3.77$, $p < 0.01$; CIE H3: $t(9) = 2.56$, $p < 0.05$), indicating significant PIT. We did not find evidence that stronger PIT in CIE mice was related to higher rates of responding, as across all mice lever press rate on the final day of training did not correlate with test difference score ($r(38) = 0.08$, $p > 0.05$). Together, these results suggest that DREADD activation differentially affected PIT magnitude in CIE and Air control mice.

A devaluation test was conducted after the first test. CNO was administered to all mice 15 min before beginning the 60 min pre-feeding session, which was then immediately followed by a second transfer test. An examination of amount consumed during the pre-feeding session found that CNO activation of H3 receptors expressed in CeA reduced pre-feeding consumption. In addition, increased consumption was observed in CIE animals relative to Air animals. This was supported by a 2-way ANOVA (CIE group x Treatment) for consumption in grams during the pre-feeding session, which found a main effect of Treatment ($F(1, 34) = 20.60$, $p < 0.0001$) and a main effect of CIE group ($F(1, 34) = 6.87$, $p < 0.05$). Average consumption within each subgroup was 0.40 ± 0.02 g in Air control, 0.19 ± 0.04 g in Air H3, 0.49 ± 0.06 g in CIE control,

and 0.32 ± 0.04 g in CIE H3. This pattern of results still held when we examined amount consumed per kg of body weight (data not shown). Of note, low consumers were included in all devaluation analyses in light of the effect of H3 excitation on consumption, though the pattern of lever pressing during testing also did not change depending on their inclusion (data not shown).

We found that patterns of lever press responding in the devaluation test differed depending on CIE group (Figure 4C). A 3-way ANOVA (Trial Type x CIE group x Treatment) for lever press rate revealed an interaction between Trial Type and CIE group ($F_{(2, 68)} = 3.73$, $p < 0.05$) and a main effect of Trial Type ($F_{(2, 68)} = 14.99$, $p < 0.001$; all other Fs < 2.09, ps > 0.05). Follow-up 1-way ANOVAs (Trial Type) within each CIE group confirmed main effects of Trial Type for both Air ($F_{(2, 34)} = 4.26$, $p < 0.05$) and CIE ($F_{(2, 38)} = 15.89$, $p < 0.0001$). Post hocs for this effect in Air mice found only a significant difference between CS+ and ITI responding ($p < 0.05$; other comparisons ps > 0.05), whereas in CIE mice CS+ responding was significantly greater than ITI ($p < 0.0001$) and CS- ($p < 0.001$; no difference between CS- and ITI, $p > 0.05$). Difference scores suggested no significant variation in PIT magnitude between subgroups (Figure 4D). This was supported by a 2-way ANOVA (CIE group x Treatment) for the difference between ITI and CS+ responding, which found no significant effects or interaction (Fs < 3.03, ps > 0.05). However, when comparing each group's difference score against 0 in one sample t-tests, we found that transfer was no longer significant in Air H3 mice ($t_{(7)} = 1.19$, $p > 0.05$) but was significant in other groups (Air control: $t_{(9)} = 3.07$, $p < 0.05$; CIE control: $t_{(9)} = 4.24$, $p < 0.01$; CIE H3: $t_{(9)} = 3.12$, $p < 0.05$). These results suggest that CIE mice were somewhat more resistant to devaluation procedures.

Discussion

In the present work we examined the effects of CeA chemogenetic manipulation and prior chronic ethanol exposure on cue-guided behavior in the PIT task. In Experiment 1, we found that activation of DREADD H4 receptors expressed in CeA strengthened PIT without affecting response rates (Figure 2). In Experiment 2, we found that activation of DREADD H3

receptors expressed in CeA differentially affected PIT depending on history of ethanol exposure (Figure 4). In the Air group, activation of DREADD H3 in CeA facilitated transfer, whereas in the CIE group, this activation of CeA appeared to weaken PIT. Importantly, this decrease in PIT strength was relative to CIE animals that received control virus, who themselves demonstrated strong PIT, whereas Air control animals did not show significant PIT (similar to prior work, Shields & Gremel, 2021). These results support that manipulation of CeA activity can enhance cue-guided behavior in ethanol-naïve mice, but may produce an opposite effect in animals with a history of ethanol dependence and withdrawal, who already show augmented PIT at baseline.

Previous work has established the crucial role of CeA in outcome-general PIT transfer, when associative reward cues elicit increases in instrumental responding for rewards in general. The CeA is a major output nucleus of the amygdala and is comprised mostly of inhibitory GABAergic interneurons and projection neurons. Studies in rats support that CeA lesions abolish outcome-general PIT (Corbit & Balleine, 2005; Hall et al., 2001; Holland & Gallagher, 2003), and in humans this type of PIT is associated with fMRI activity within the bounds of the CeA (Prévost et al., 2012). The present work employs a chemogenetic strategy to manipulate (rather than eliminate) CeA activity in an inhibitory manner (Experiment 1) and an excitatory manner (Experiment 2). As an inhibitory nucleus, the effects of inhibition and excitation of CeA are likely complex and could both lead to increased inhibition in CeA (Gilpin et al., 2015; Roberto et al., 2012; Roberto et al., 2021). Our finding that both decreasing and increasing the activity of inhibitory local and projection populations in CeA strengthened PIT behavior in ethanol-naïve animals likely reflects this complexity.

The precise effects of chemogenetic manipulation on CeA activity and output are not well understood. Because the CeA is an inhibitory nucleus, a straightforward possibility is that chemogenetic inhibition of the area produces downstream disinhibition, whereas excitation of the area strengthens the inhibitory influence of the CeA on downstream regions. This explanation, however, seems somewhat at odds with our finding that PIT in alcohol-naïve

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animals was enhanced in both cases. Another possibility is that excitatory and inhibitory DREADD activation exerts differential effects on neural subpopulations within the CeA. As past work has shown that distinct cell types within CeA play opposing roles in behavior (for review, see Warlow & Berridge, 2021), it is possible that preferential inhibition of one population could produce similar behavioral results as excitation of another population. A third possibility is that chemogenetic excitation augments local inhibitory circuitry within the CeA. As past work supports that CeA projection neurons are gated by inhibitory interneurons (Hunt et al., 2017), amplified local inhibition via chemogenetic excitation could affect CeA activity in a functionally similar way to chemogenetic inhibition. Future work will be necessary to fully understand the effects of DREADD manipulation on CeA function.

Though we found that H3 and H4 DREADD activation in CeA produced similar effects on PIT strength in alcohol-naïve animals, there was a marked difference in overall response rates and the way in which PIT was strengthened. DREADD inhibition did not alter baseline response rates, but selectively increased responding during the reward-associated cue. This behavioral pattern reflects our previous finding that animals exposed to CIE, a procedure which is thought to produce a net inhibition of CeA (Pleil et al., 2015; Repunte-Canonigo et al., 2015; Roberto et al., 2010; Roberto et al., 2004), also showed a selective enhancement of responding during a reward-associated cue but no change in baseline responding (Shields & Gremel, 2021). In contrast, DREADD excitation reduced responding, and stronger PIT occurred not because response rates selectively increased during the reward-associated cue, but rather because response rates during baseline periods were substantially reduced. Optogenetic stimulation of CeA can narrow and direct incentive motivation for specific rewards, and even aversive outcomes, over other possible outcomes (Robinson et al., 2014; Warlow et al., 2017; Warlow et al., 2020). Thus, stronger PIT in the context of reduced responding could reflect directed incentive motivation, such that responding effort decreased in the absence of a reward-associated cue.

Activating DREADD H3 receptors in CeA appeared to strengthen PIT in alcohol-naïve animals, but an opposite pattern was observed for animals with a history of chronic ethanol exposure. The CIE control group, which was exposed to ethanol but received a non-DREADD control virus, appeared to demonstrate stronger PIT compared to the Air control group, where significant PIT was absent. This finding is consistent with prior work showing that CIE exposure can itself enhance PIT (Shields & Gremel, 2021). Comparing to this already-enhanced PIT in CIE control animals, visual observation of the data suggested the CIE H3 group exhibited relatively weaker transfer, which was notably closer to the level of transfer observed in Air control animals. This finding could be consistent with the "rescue" of a CIE-induced behavioral effect, possibly achieved via disruption of the ethanol-induced CeA inhibition that has been demonstrated following chronic ethanol exposure and withdrawal (Pleil et al., 2015; Repunte-Canonigo et al., 2015; Roberto et al., 2010; Roberto et al., 2004).

Devaluation testing revealed varied effects of DREADD activation and alcohol exposure on consumption and lever pressing-related behaviors. Whereas activation of DREADD H4 receptors did not affect consumption, activation of DREADD H3 receptors substantially decreased consumption. Again, this finding likely reflects complex differential effects of CeA manipulation; thought to modulate feeding behaviors, subpopulations within the CeA have been shown to enhance or inhibit consumption depending on the cell types affected (Cai et al., 2014; Douglass et al., 2017; for review, see Izadi & Radahmadi, 2021). In the devaluation test itself, however, there were no differences in lever pressing behavior between DREADD and control virus groups in either experiment. This finding suggests that despite any effects on baseline feeding behavior, the process of devaluing the food outcome was not affected by CeA manipulation.

The only group difference observed for lever pressing behaviors during devaluation testing was between Air and CIE groups, where PIT in alcohol mice was less affected by devaluation. This finding is consistent with prior work showing insensitivity to devaluation in

mice exposed to CIE (Lopez et al., 2014; Renteria et al., 2018; Renteria et al., 2020; Shields & Gremel, 2021). Enhanced PIT was also accompanied by significantly higher pre-feeding consumption in CIE animals relative to Air animals. However, we found that this effect was driven by three particularly low consumers in the Air H3 group. Inclusion of these mice influenced only whether there was an effect of CIE on consumption, not whether there was an effect of DREADD treatment on consumption, and did not change the pattern of lever pressing in the devaluation test. In light of the observed effect of H3 treatment on consumption, however, these low consumers were not excluded from analyses. Combined with our finding that weights did not differ between Air and CIE mice, this suggests that any effect of CIE on consumption was minimal or nonexistent (as shown previously, Shields & Gremel, 2021).

Together, our data suggest that CeA manipulation can enhance PIT, and confirm our previous finding that a history of CIE exposure also enhances PIT. Importantly, the way in which PIT was strengthened differed depending on the direction of CeA manipulation: an inhibitory DREADD selectively enhanced responding for reward-related cues, whereas an excitatory DREADD lowered responding during baseline periods and reduced responding overall. These findings suggest that a possible neural mechanism underlying CIE-enhanced PIT is alcohol-induced inhibition of the CeA, and that excitatory DREADD manipulation of CeA can "rescue" this behavioral effect. An important extension of this work would be to further investigate the exact effects of excitatory and inhibitory DREADD manipulation on CeA projection activity, as well as to examine the possible roles of specific cell types within the area. Elucidating the neural mechanisms underlying disrupted cue-guided behavior in alcohol dependence is crucial to better understand and treat the decision-making deficits that can greatly impact the lives of those with AUD.

Conflict of Interest

The authors have no conflicts of interest to declare.

Acknowledgements

Chapter 3, in full, is a reprint of the material submitted for publication as it may appear in Shields, C. N. & Gremel, C. M. Effects of central amygdala chemogenetic manipulation and prior chronic alcohol exposure on Pavlovian-to-instrumental transfer. The dissertation author was the primary investigator and author of this paper.

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DISCUSSION

Summary

Three sets of studies examined the effects of chronic alcohol exposure on the ability to use incentive value to guide behavior. In Chapter 1, a history of chronic alcohol exposure produced deficits in the ability to use motivational state to update the incentive value of a reward and guide behavior accordingly, though these deficits could be overcome in part by a particularly salient motivational shift. In Chapter 2, prior chronic exposure to alcohol enhanced the influence of an incentive environmental cue on instrumental actions. In Chapter 3, chemogenetic manipulation of central amygdala (CeA) dampened this alcohol enhancement of cue-guided behavior, but had the opposite effect of strengthening cued behavior in alcohol-naïve animals. In combination, these studies identify two major ways in which incentive processes are disrupted by a history of chronic, high-level alcohol exposure – by interfering with the ability to use motivational state to determine incentive value, and by enhancing the ability of incentive stimuli to guide actions. They further support that disruption of CeA function may contribute to alcohol-induced changes in how environmental cues influence ongoing behavior.

How Are Incentive Processes Disrupted by Chronic Alcohol Exposure?

Conceptually, the results reported herein could support at least two possible ways in which a history of chronic alcohol disrupts incentive processes. One possibility is that the incentive value of rewards is broadly amplified, as suggested by greater responding to a reward cue (in Chapters 2 and 3) and continued responding despite a recent reduction in motivation (in Chapter 1). However, this explanation is not fully consistent with the results observed in Chapter 1, where reward seeking and consumption behaviors were not consistently elevated in alcohol animals compared to alcohol-naïve animals. Instead, this study found alcohol-related deficits specifically when performance depended upon inferring, rather than directly experiencing, a reduction in the incentive value of food. From this perspective, a better explanation may be that

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prior alcohol dependence can enhance some incentive processes (e.g., cue-guided behavior) but impair others (e.g., motivation-based value updating).

Distinct features of the tasks used in these studies suggest conditions under which chronic alcohol exposure may differentially affect the use of incentive value. One major feature is the observability of crucial task components. In the Pavlovian-to-instrumental transfer (PIT) task used in Chapters 2 and 3, performance of transfer depends on an externally observable environmental cue (an auditory tone that was previously associated with food reward). In contrast, in the incentive learning task used in Chapter 1, deficits were found when performance depended on internal information (a recent shift in motivational state) that could not be determined using external stimuli. This finding suggests that beyond any broad effects on motivational sensitivity, chronic alcohol exposure may specifically interfere with the ability to use unobservable information to infer incentive value. Indeed, incentive learning tasks like the one used in Chapter 1 have been shown to recruit the orbitofrontal cortex (OFC; Baltz et al., 2018; Malvaez et al., 2019). This is notable as the OFC is thought to be crucial for representing unobservable task states (Schuck et al., 2016; Wilson et al., 2014), and its function is markedly altered by chronic alcohol exposure (Renteria et al., 2018; Nimitvilai et al., 2016; Nimitvilai et al., 2017; Nimitvilai et al., 2018).

Another notable task feature is the stability of incentive value over time. In the incentive learning task (Chapter 1), the value of the food reward was not constant across training and testing but rather was expected to decrease after subjects became less hungry. In the PIT task (Chapters 2 and 3), however, incentive value remained relatively consistent. That is, once value was established during training, no obvious updating was required to perform the task. These findings suggest that chronic alcohol exposure could impair the ability to track shifts in incentive value, whether signaled by internal state changes (e.g., hunger) or external information (e.g., reward cues). This notion is consistent with work in humans, where a history of chronic alcohol

exposure has been associated with difficulty tracking shifting values over time (Brevers et al., 2014; Fein et al., 2004; Le Berre et al., 2014).

Another important consideration is the type of learning recruited in each task. The incentive learning task explicitly trains only instrumental actions, whereas the PIT task tests the interaction between Pavlovian and instrumental learning processes. The incentive learning findings in Chapter 1 support that chronic alcohol exposure can, but does not always, impair the ability to adjust instrumental actions in light of a change in outcome value. In the context of alcohol use, such impairment could contribute to maladaptive instrumental behaviors, such as drinking despite already being highly intoxicated. Results from the PIT studies in Chapters 2 and 3 suggest that chronic alcohol exposure may also increase the ability of environmental stimuli to regulate instrumental actions. The ability to engage in adaptive behavior often involves determining which reward cues to respond to and which to ignore. For instance, individuals whose actions are more influenced by reward cues like billboard advertisements may find themselves purchasing far more fast-food meals than intended. From this perspective, even "enhanced" incentive processes could contribute to maladaptive behavior in chronic alcohol use and alcohol use disorder (AUD).

Implications for Theories of Addiction

The findings reported in the preceding chapters have important implications for major theories of addiction. One prominent proposal is the habit hypothesis, which holds that addiction involves a transition from goal-directed behavior, when actions are flexibly performed with the goal of acquiring specific outcomes, to habitual behavior, when actions are performed more rigidly and are not tightly controlled by outcomes (Belin et al., 2013; Everitt & Robbins, 2016). Practically, this may involve a transition from initial voluntary and recreational drug use to compulsive drug use despite negative consequences. The results from the incentive learning study in Chapter 1 support the habit hypothesis to an extent, but provide an important caveat. In this study, animals with a history of chronic alcohol exposure displayed impaired goal-directed

control in that their behavior was not affected by a relatively mild downshift in outcome value. However, after a relatively large shift in outcome value, alcohol animals were able to adjust behavior similarly to non-alcohol animals, consistent with goal-directed control. These findings suggest that a more nuanced interpretation of the habit hypothesis may be warranted, and that goal-directed behavior may still be recruited in particularly salient states.

Further implications for the habit hypothesis come from the PIT studies in Chapters 2 and 3. The habit phenotype has been defined as a stimulus-response relationship, where actions are elicited by environmental stimuli rather than being influenced by the outcomes they produce (Belin et al., 2013; Everitt & Robbins, 2016). In the PIT task utilized here, enhanced control of reward cues over actions could indicate an overreliance on habitual stimulus-response relationships. Notably, this enhanced PIT was also relatively resistant to outcome devaluation, a classic test of goal-directed behavior where responding is examined following devaluation of the reward (via satiation or pairing with illness; Friedel et al., 2014). Continued responding after devaluation of the outcome supports that goal-directed control was indeed diminished in alcohol animals. These results align with prior work in rodents showing that chronic alcohol exposure can disrupt goal-directed behavior (e.g., Lopez et al., 2014; Renteria et al., 2018).

Another major theory of addiction is the notion that drug consumption is sustained by negative reinforcement (Koob, 2013; Wise & Koob, 2014). Negative reinforcement describes the process through which a particular behavior leads to the removal of an unpleasant state or stimulus, thus strengthening the behavior. For example, drug use may be sustained by the drug's ability to relieve withdrawal symptoms. This theory is somewhat counter to the habit hypothesis, since it suggests that drug consumption is maintained by actions directed toward a specific goal (i.e., removing unpleasant states like withdrawal). Results of the incentive learning study in Chapter 1 suggest that animals chronically exposed to alcohol may still recruit goal-directed behavior given a sufficiently salient motivational state. In that study, the motivational

state in question was a large reduction in food deprivation; in the context of alcohol use, acute withdrawal may also be a highly salient motivational state. Together, the findings across these three chapters support a nuanced combination of the negative reinforcement and habit hypotheses, where behavior is subject to habitual control under mild motivational states but goal-directed control is recruited in sufficiently salient states, such as acute withdrawal. This interpretation could help to explain discrepancies in human AUD literature, where some findings may support that goal-directed decision-making is intact (for review, see Hogarth, 2020).

Future Directions

Although the studies reported here provide significant insight into how incentive processes are disrupted by chronic alcohol exposure, several questions remain. From a behavioral perspective, further investigation into the importance of specific task components could be intriguing. Since the ability to use incentive value appears to be impaired in some contexts but enhanced in others, future work could examine whether these different outcomes depend on the observability of task features (whether incentive value is signaled by internal or external information), the stability of incentive value (whether value shifts throughout the task or remains static), the type of learning recruited (instrumental and/or Pavlovian), or other factors. In the context of PIT, it would also be valuable to establish whether alcohol exposure enhances the influence of reward cues on reward-seeking behavior in general (outcome-general PIT) or more specifically enhances only actions directed toward acquiring the same reward as associated with the cue (outcome-specific PIT). Since these subtypes of PIT implicate dissociable neural substrates (Corbit et al., 2016; Corbit & Balleine, 2005; Corbit & Balleine, 2011), it is conceivable that chronic alcohol exposure could enhance one type but not the other.

Another valuable extension of this work would be to examine in greater detail how CeA contributes to alcohol-induced changes in PIT behavior. It would be useful to know whether alcohol exposure affects specific cell types in CeA, where distinct populations have been shown to contribute differentially to behavior (Warlow & Berridge, 2021). For example, food-seeking

behavior can be increased or decreased depending on which CeA neurons are stimulated (Kim et al., 2017; Venniro et al., 2017). From the circuit level, future work could investigate whether alcohol exposure affects the inhibitory influence of CeA on downstream regions to produce behavioral changes. Projections to substantia nigra pars compacta and ventral tegmental area are of particular interest, as these connections are implicated in the formation of associations between cues and rewards (Fadok et al., 2018; Lee et al., 2010; Lee et al., 2011). In addition, future work could investigate the impact of temporally-specific CeA manipulation (e.g., via optogenetics) to examine targeted effects on PIT behaviors.

Future work could also examine how other relevant brain areas contribute to disrupted incentive processes. Major regions of interest include the OFC, crucial for encoding and retrieving updated value to control responding (e.g., in incentive learning tasks; Baltz et al., 2018; Malvaez et al., 2019), and subregions of the amygdala and nucleus accumbens (NAcc), which play dissociable roles in subtypes of PIT. More specifically, CeA and NAcc core are implicated in outcome-general PIT, whereas and basolateral amygdala (BLA) and NAcc shell are implicated in outcome-specific PIT (Corbit & Balleine, 2005; Corbit & Balleine, 2011; Corbit et al., 2016; Lichtenberg, 2017; Prévost et al., 2012). OFC and BLA-OFC projections are also implicated in outcome-specific PIT (Lichtenberg, 2017; Ostlund & Balleine, 2007). Given that chronic alcohol exposure functionally alters the amygdala (Kryger & Wilce, 2010; Pleil et al., 2015; Repunte-Canonigo et al., 2015; Roberto et al., 2010; Roberto et al., 2004), the NAcc (Griffin et al., 2014; Griffin et al., 2015), and the OFC (Renteria et al., 2018; Nimitvilai et al., 2016; Nimitvilai et al., 2017; Nimitvilai et al., 2018), these regions represent strong candidates for further study. Elucidating these and other remaining questions will help to better understand the role of alcohol in disrupting incentive processes and underlying neural circuitry, potentially contributing to the development of improved treatments for AUD in the future.

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