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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Influence of Time of Day in the Development of Physiological and Behavioral
Drug Dependence in Mice**

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

in

Psychology

by

Amanda Susan Damaggio

Committee in charge:

Professor Michael Gorman, Chair
Professor Stephen Anagnostaras
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2014

The Dissertation of Amanda Susan Damaggio is approved, and it is acceptable in
quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2014

DEDICATION

For my dad.

*May I handle life with the same grace and dignity you handled death.
You will be in my thoughts and heart forever.*

TABLE OF CONTENTS

Signature Page.....	iii
Dedication.....	iv
Table of Contents.....	v
List of Figures.....	ix
List of Tables.....	xii
Acknowledgements.....	xiii
Vita.....	xiv
Abstract of the Dissertation.....	xv
Chapter 1: Introduction.....	1
1.1 Overview of Circadian Rhythms.....	2
1.1.1 Definition of Key Terms.....	3
1.1.2 The Core Clock and Its Output.....	4
1.2 Overview of Alcohol Addiction.....	5
1.2.1 The Withdrawal Reaction.....	6
1.2.2 Temporal Organization in Drug Addiction.....	7
1.3 Alcohol and Circadian Rhythms.....	8
1.3.1 Associations between Circadian Rhythmicity and Alcohol Addiction.....	9
1.3.2 Direct Evidence of Alcohol's Influence on Core Clock Functioning.....	11
1.4 Alcohol's Influence on Physiological and Behavioral Outputs.....	13
1.4.1 Body Temperature.....	13
1.4.2 Activity.....	16
1.4.3 Anxiolytic Behaviors.....	17
1.4.4 Consumption.....	19
1.5 Other Drugs of Abuse.....	20
1.6 Summary.....	20
1.7 Figures.....	22
Chapter 2: Circadian Phase Determines Effects of Repeated Ethanol Vapor Exposure and Withdrawal on Body Temperature and Activity Rhythms of Male Mice.....	24
2.1 Introduction.....	25
2.2. Methods.....	28

2.2.1 Subjects.....	28
2.2.2 Experimental Lighting Conditions.....	28
2.2.3 Surgery.....	29
2.2.4 Alcohol Vapor Inhalation and Rounds.....	29
2.2.5 Blood Ethanol Content.....	30
2.2.6 Data Analysis.....	30
2.3 Results.....	33
2.3.1 Multiple Rounds of Alcohol Exposure/Withdrawal.....	34
2.3.2 During Exposure, Alcohol Lowers Body Temperature Dependent upon the Time of Day.....	35
2.3.3 During Exposure, Alcohol Suppresses Activity in a Time of Day Dependent Manner.....	36
2.3.4 Independent of Activity, Alcohol Influences the Endogenous Body Temperature Rhythm During the Active Phase of the Circadian Cycle.....	37
2.3.5 Alcohol Withdrawal has Differential Effects on Core Body Temperature Depending upon the Circadian Cycle.....	38
2.3.6 Alcohol Withdrawal Increases Locomotor Activity Differentially Depending upon the Circadian Cycle.....	39
2.3.7 Alcohol Withdrawal Influences the Endogenous Body Temperature Rhythm, Independent of Activity, as a Function of Time of Day.....	40
2.4 Discussion.....	40
2.5 Figures.....	48
Chapter 3: Circadian Phase of Intoxication Modifies the Direction of the Core Body Temperature Response during Acute Withdrawal.....	59
3.1 Introduction.....	60
3.2 Methods.....	63
3.2.1 Subjects.....	63
3.2.2 Experimental Lighting Conditions.....	64
3.2.3 Surgery.....	64
3.2.4 Alcohol Vapor Inhalation and Rounds.....	65
3.2.5 Blood Ethanol Content.....	66
3.2.6 Data Analysis.....	66
3.3 Results.....	68
3.3.1 Multiple Rounds of Alcohol Vapor Inhalation Does Not Elicit Sensitization or Tolerance Compared to Baseline Levels.....	69
3.3.2 Chronic Alcohol Exposure Lowers Body Temperature Dependent upon the Time of Day.....	70
3.3.3 Alcohol Exposure Suppresses Activity during the Subjective Night.....	72
3.3.4 Independent of Activity, Alcohol Influences the Endogenous Body Temperature Rhythm during Alcohol Exposure.....	73

3.3.5 Alcohol Withdrawal Has a Varying Hyperthermic Response as a Function of the Circadian Cycle.....	76
3.3.6 Alcohol Withdrawal Increases Locomotor Activity Differentially Depending upon the Circadian Cycle.....	77
3.3.7 Alcohol Withdrawal Increases the Endogenous Core Body Temperature Rhythm.....	79
3.4 Discussion.....	81
3.5 Figures.....	87
Chapter 4: Circadian Phase of Passive Alcohol Vapor Exposure and Withdrawal Alters Subsequent Voluntary Alcohol Consumption in Male Mice.....	100
4.1 Introduction.....	101
4.2 Methods.....	106
4.2.1 Subjects.....	106
4.2.2 Limited Access Paradigm.....	106
4.2.3 72 Hour 2-Bottle Choice Test and Lickometer Collection.....	107
4.2.4 Alcohol Vapor Inhalation and Rounds.....	108
4.2.5 Elevated Plus Maze.....	108
4.2.6 Data Analysis.....	109
4.3 Results.....	110
4.3.1 Repeated Exposure and Withdrawal to Alcohol Vapor Inhalation Increases Consumption.....	111
4.3.2 Alcohol Exposure and Withdrawal Do Not Influence Anxiolytic Behaviors.....	112
4.4 Discussion.....	112
4.5 Figures.....	118
Chapter 5: Time of Day Does Not Act as a Conditioned Stimulus for Behavioral Sensitization in Mice	123
5.1 Introduction.....	123
5.2 Methods.....	126
5.2.1 Subjects.....	126
5.2.2 Experimental Lighting Conditions.....	126
5.2.3 Drugs.....	127
5.2.4 Apparatus.....	127
5.2.5 Sensitization.....	128
5.2.5.1 Habituation and Training.....	128
5.2.5.2 Testing.....	129
5.2.5.3 Behavioral Sensitization and Data Analysis.....	129
5.3 Results.....	130
5.3.1 The Acute Response to Amphetamine is Dependent on the Time of Day.....	130
5.3.2 Amphetamine Sensitization Across Training Days.....	131
5.3.3 Amphetamine Sensitization Test.....	132
5.4 Discussion.....	133

5.5 Figures.....	140
Chapter 6: Discussion.....	148
6.1 Time of Day Affects the Physiological Response to Chronic Alcohol Exposure.....	149
6.2 Circadian Phase of Alcohol Intoxication Modifies the Direction of the Acute Withdrawal Response.....	152
6.3 Circadian Phase of Alcohol Exposure Differentially Increases Alcohol Consumption.....	155
6.4 Time of Day is Not a Learned Environmental Cue for Behavioral Sensitization.....	158
6.5 Conclusions.....	162
References.....	164

LIST OF FIGURES

Figure 1.1:	Temporal patterns and symptoms of alcohol withdrawal.....	22
Figure 1.2:	Twenty-four hour alcohol consumption patterns.....	23
Figure 2.1:	Representative double-plotted actograms of locomotor activity and core body temperature during EtOH exposure and withdrawal in Experiment 1.....	48
Figure 2.2:	Mean core body temperature, locomotor activity, and activity independent body temperature rhythms averaged over the 3 days of EtOH exposure and withdrawal in round 4 of Experiment 1....	51
Figure 2.3:	Net change from baseline of the mean $\pm$ S.E.M. core body temperature, locomotor activity, and activity independent body temperature in the 10 lights off period averaged across all 4 rounds in Experiment 1.....	53
Figure 2.4:	Summed absolute deviation from baseline of the mean $\pm$ S.E.M. core body temperature, locomotor activity, and activity independent body temperature during EtOH exposure and withdrawal averaged across rounds in Experiment 1.....	55
Figure 2.5:	Mean $\pm$ S.E.M. influence of locomotor activity on the core body temperature rhythm (slope) averaged across all rounds of EtOH exposure and withdrawal in Experiment 1.....	57
Figure 3.1:	Representative double plotted actograms of a locomotor activity and core body temperature rhythms during EtOH exposure and withdrawal in Experiment 2.....	87
Figure 3.2:	Net change from baseline of the mean $\pm$ S.E.M. core body temperature, locomotor activity, and activity independent body temperature in both 10 lights off period during EtOH exposure averaged across all 4 rounds in Experiment 2.....	90
Figure 3.3:	Summed absolute deviation from baseline of the mean $\pm$ S.E.M. core body temperature, locomotor activity, and activity independent body temperature during EtOH exposure across rounds in Experiment 2.....	92
Figure 3.4:	Net change from baseline of the mean $\pm$ S.E.M. core body temperature, locomotor activity, and activity independent body temperature in both 10 lights off period during EtOH	

	withdrawal averaged across all 4 rounds in Experiment 2.....	94
Figure 3.5:	Mean $\pm$ S.E.M. influence of locomotor activity on the core body temperature rhythm (slope) averaged across all rounds of EtOH exposure and withdrawal in Experiment 2.....	96
Figure 3.6:	Mean core body temperature rhythm averaged over alcohol exposure or withdrawal across rounds in Experiment 2.....	98
Figure 4.1:	Mean hourly EtOH consumption pattern of mice exposed to EtOH vapor in the subjective night or the subjective day during baseline or averaged across the four consumption tests in Experiment 3.....	119
Figure 4.2:	Mean $\pm$ S.E.M. voluntary EtOH consumption during baseline or across the four consumption tests in Experiment 3.....	120
Figure 4.3:	Mean $\pm$ S.E.M. anxiolytic behaviors on an elevated plus maze measured by percent time in open arms, percent open arm entries, and mean closed arm entries in Experiment 3.....	121
Figure 5.1:	Mean $\pm$ S.E.M. horizontal locomotor response following an acute dose of amphetamine or saline administered either early or late in the light cycle in Experiment 4.....	140
Figure 5.2:	Mean $\pm$ S.E.M. vertical locomotor response following an acute dose of amphetamine or saline administered either early or late in the light cycle in Experiment 4.....	141
Figure 5.3:	Mean $\pm$ S.E.M. horizontal locomotor activity across the 7 days of training to either amphetamine or saline administered either early or late in the light cycle in Experiment 4.....	142
Figure 5.4:	Mean $\pm$ S.E.M. difference in horizontal locomotor activity from day 7 to day 1 in mice exposed to either amphetamine or saline either early or late in the light cycle in Experiment 4.....	143
Figure 5.5:	Mean $\pm$ S.E.M. vertical activity across the 7 days of training to either amphetamine or saline administered either early or late in the light cycle in Experiment 4.....	144
Figure 5.6:	Mean $\pm$ S.E.M. difference in vertical activity from day 7 to day 1 in mice exposed to either amphetamine or saline either early or late in the light cycle in Experiment 4.....	145

Figure 5.7:	Mean $\pm$ S.E.M. horizontal locomotor response by minute across the first 15 minutes of the sensitization test for animals previously exposed to either amphetamine or saline either early or late in the light cycle in Experiment 4.....	146
Figure 5.8:	Mean $\pm$ S.E.M. horizontal locomotor response across the first 15 minutes of the sensitization test for animals previously exposed to either amphetamine or saline either early or late in the light cycle in Experiment 4.....	147

LIST OF TABLES

Table 2.1:	Mean $\pm$ S.E.M. blood ethanol content (BEC) averaged within each round of EtOH vapor exposure in Experiment 1.....	50
Table 3.1:	Mean $\pm$ S.E.M. blood ethanol content (BEC) averaged within each round of EtOH vapor exposure in Experiment 2.....	89
Table 4.1:	Mean $\pm$ S.E.M. blood ethanol content (BEC) averaged within each round of EtOH vapor exposure in Experiment 3.....	118

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ABSTRACT OF THE DISSERTATION

Influence of Time of Day in the Development of Physiological and Behavioral Drug
Dependence in Mice

by

Amanda Susan Damaggio

Doctor of Philosophy in Psychology

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Professor Michael R. Gorman, Chair

Drug addiction is a spiraling and debilitating condition that has severe social and economic consequences for the individual and society. Recently, much work has examined the relationship between drug addiction and the circadian timing system. The master circadian pacemaker directs a daily temporal rhythm throughout the brain and body including the consumption of alcohol. Many drugs, including alcohol, can have myriad effects at different times of day. Conversely, alcohol can alter circadian physiology at multiple levels within the system. Few studies, however, have examined the role that the timing of chronic drug exposure and withdrawal plays in

the development and maintenance of drug addiction. Alcohol use, for example, follows a circadian pattern with consumption peaking in the early evening in non-dependent individuals and withdrawal likely occurring late at night. In the transition to dependence, however, craving and consumption expand earlier into the day. What consequences this altered timing of drug use plays in the physiological and psychological consequences of drug addiction remains to be seen. Herein, we specifically examine if: 1) controlling for time of day in alcohol exposure/withdrawal can alter the development of physical dependence, 2) the development of psychological dependence, and 3) if time of day can become a conditioned environmental cue that triggers craving and behavioral sensitization in an amphetamine model of addiction.

Chapter 1: Introduction

Alcoholism is both a debilitating and prevalent disease affecting approximately 17.6 million Americans (Grant et al., 2004). Nutt, King, Phillips, and Independent Scientific Committee on Drugs (2010) reported that alcohol was the most harmful drug overall to humans, more so than heroin and crack cocaine, yet alcohol use is pervasive. In western cultures alcohol is used both as a social facilitator and as a tool in formalized ceremonies. The overindulgence of alcohol, however, has significant socio- economic consequences. Damage (both physical and property) due to alcohol consumption was estimated to be about \$223.5 billion in 2011, the last year figures were available (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011). Moreover, in some individuals alcohol use develops into a spiraling addiction that has severe consequences for both the individual and society.

The development and progression of alcoholism involves a complex interaction between genetic predispositions and environmental influences. One environmental factor, the temporal context of drug exposure and withdrawal, is emerging as an important consideration in the addiction process. This temporal context encompasses a number of developmental, biological, and environmental timing components such as developmental phase, frequency and dose, time of day, season of the year, etc. (Becker & Lopez, 2004; Carpenter, 2003; Danel, Jeanson, & Touitou, 2003; Danel, Libersa, & Touitou, 2001; Griffin, Lopez, & Becker, 2009; Lopez & Becker, 2005; Nixon & McClain, 2010).

Specifically, this dissertation will explore the role of time of day in the development and maintenance of alcohol and amphetamine addiction. It is well established that the efficacy of a number of drugs vary as a function of time of day (Wasielewski & Holloway, 2001). *Chronotherapy*, the discipline of improving therapeutic treatments through the use of circadian timing, is highly clinically significant and is currently used in many fields to improve treatment outcomes (Danel & Touitou, 2004; Fu & Kettner, 2013; Wasielewski & Holloway, 2001). To date, time of day dependent differences in the behavioral and physiological responses to alcohol and amphetamine have not been thoroughly studied in a chronic model of addiction. The presented studies will investigate whether the timing of chronic alcohol exposure and withdrawal in rodents can alter physiological responses to the drug (Chapter 2), if the physiological withdrawal reaction to chronic alcohol exposure is based on the phase of exposure, the phase of withdrawal, or a combination of both (Chapter 3), whether the timing of chronic alcohol exposure and withdrawal alters the development of addictive-like behaviors (Chapter 4), and if time of day serves as a predictive contextual cue for a sensitized drug response (i.e., are time of day cues necessary for amphetamine sensitization; Chapter 5).

1.1 Overview of Circadian Rhythms

The internal time keeping system of mammals, the circadian clock, is located within the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. It governs the daily temporal organization of physiological and behavioral rhythms of the organism. At the simplest level, and sufficient for present purposes, the mechanism

depends on a transcriptional-translational feedback loop: the transcription factors Clock and Bmal1 are considered positive elements for driving transcription of 3 orthologous period and 2 Cryptochrome genes. Heterodimers of Per and Cry proteins indirectly inhibit their own transcription by interfering with the positive factors that drive it. These molecular clock mechanisms are present in cells throughout the body, but depend on a variety of signals from the SCN to maintain phase synchrony and thus to sustain circadian organization at a tissue level. Indeed, a major advance of the past decade has been the demonstration of the pervasive influence of circadian organization on cellular and tissue activity.

1.1.1 Definition of Key Terms

These rhythms are most commonly described by the *period* (the length of time it takes the rhythm to complete one full cycle), the *amplitude* (the difference between the lowest and highest point of the rhythm), and the *phase* of the rhythm (a reference interval within the rhythm). In the absence of environmental cues the endogenous circadian period is approximate to 24 hours and can be divided into two phases: *subjective day* and *subjective night* which roughly corresponds with intervals of rest or activity dependent on whether the animal is nocturnal or diurnal. For example, in nocturnal rodents the interval of *subjective night* includes elevated locomotor activity, increased core body temperature, feeding, drinking, melatonin secretion, etc., and the interval of *subjective day* favors inactivity, low body temperature and basal melatonin.

In the presence of environmental cues, the master pacemaker can be synchronized to match environmental time cues, the most prominent cue being the daily light/dark cycle. When the endogenous rhythm is out of sync with temporal

environmental cues that drive entrainment, *circadian desynchrony* occurs. Jet lag, for example, is an instance of circadian desynchrony where the body's internal rhythm does not match environmental time cues. As a result, a number of physiological symptoms occur as the internal rhythms readjust, shifting to the new time zone. During this process the rhythm is being *phase shifted* whereby the reference phase of the organism is occurring earlier (phase advance) or later (phase delay) than expected. Interestingly, the symptoms of alcohol withdrawal have been likened to a state of circadian desynchrony (Gauvin et al., 1997).

The environmental light/dark cycle plays such a crucial role in circadian entrainment that even brief periods of light can stabilize or alter the rhythm. For example, light pulses presented during the subjective night can phase advance or delay the rhythm depending if the light occurred early or late in the dark phase. Alternatively, brief periods of light that correspond with dawn and dusk can prevent the rhythm from free-running in an otherwise dark environment (i.e., absent of environmental cues). This *skeleton photoperiod* is a commonly used circadian laboratory manipulation as it prevents light from masking (i.e., altering or suppressing) the measured rhythmic output. This circadian manipulation is used in Chapters 2-4.

1.1.2 The Core Clock and Its Output

The core circadian clock within the SCN may have either a direct or indirect relationship with its downstream rhythmic outputs. In its most basic representation, the circadian system is composed of a central clock, or pacemaker, a set of input pathways that relay environmental synchronizers to the core clock, and a set of output

pathways that convey the rhythmic signal to systems throughout the organism (Rosenwasser, 2001). Stimuli can directly affect any of these components thereby altering the rhythmic output of the system. Alternatively, the stimuli may completely bypass these circadian components and instead act directly on the downstream system thereby indirectly altering the circadian output. For example, in humans, body temperature is a well-established circadian output which oscillates across a twenty-four hour period peaking during the daytime and reaching a nadir during the night. Alcohol consumption decreases core body temperature during the daytime, but increases it during the night (Danel et al., 2001). The site of action of alcohol on body temperature is unknown, but a few of many possibilities may include 1) alcohol interfering with the set point of body temperature within the hypothalamus (indirectly altering circadian rhythmicity), or 2) alcohol directly interfering with SCN functioning (i.e., directly altering circadian rhythmicity). In either scenario the rhythmic body temperature output would be altered.

1.2 Overview of Alcohol Addiction

Alcohol addiction is defined by a craving, preoccupation with, and compulsive use of the drug (American Psychiatric Association, 2013). Over time, and with repeated exposures to the drug, increased consumption occurs whereby greater amounts of the drug are needed to reach intoxication (i.e., tolerance occurs). This ‘dependence’ is considered to be a “maladaptive neurophysiological state that leads to a constellation of clinical signs and symptoms” (Becker, 2000, pg 105). These markers are characterized by physiological components that can be objectively

measured (e.g., seizures, changes in body temperature and heart rate) and psychological components reflecting changes in mood or affect that are often of a subjective nature (e.g., anxiety, irritability, and depression). Collectively, these elements are termed the *withdrawal reaction*. This reaction is experimentally used as a reflection of the underlying addiction of the organism since the biochemical changes of addiction are not fully understood and often cannot be objectively measured in either human or animal models.

1.2.1 The Withdrawal Reaction

The withdrawal reaction is composed of three distinct phases that encompass varying symptomologies and temporal periods (see Figure 1.1). In animals, the acute withdrawal period occurs over a 24-48 h period starting from the termination of active alcohol exposure. Physical symptoms predominate and are transient during this time. Psychological symptoms on the other hand manifest in the early and protracted abstinence periods and can continue for months into abstinence. The early abstinence period in animals is typically defined as 1-2 weeks after cessation of alcohol, while the protracted abstinence phase begins a month after the final exposure to alcohol and continues indefinitely (Heilig, Egli, Crabbe, & Becker, 2010). The term withdrawal, however, has been used loosely within the literature to encompass mere cessation of alcohol exposure to the periods previously defined. Within the confines of this dissertation the term *withdrawal* will refer to the period after the cessation of alcohol exposure to provide a universal definition that incorporates all withdrawal nomenclature.

Some have hypothesized that withdrawal, the period following active alcohol exposure, in addition to periods of drug exposure, is a crucial factor in the transition from moderate to severe alcohol consumption (Becker & Lopez, 2004; Healey, Winder, & Kash, 2008; Mello, 1973; Tang & Falk, 1983). For example, greater increases in ad lib consumption (Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005) and operant responding for alcohol (O'Dell, Roberts, Smith, & Koob, 2004) are reported in rodents when alcohol is administered in a repeated schedule of alcohol vapor exposure and withdrawal (an 'intermittent' schedule) compared to 'continuous' intoxication paradigms. Over time, and with successive withdrawals, the intensity of the physical withdrawal reaction progressively worsens via a kindling-like mechanism (Becker, 1998; Becker, Diaz-Granados, & Hale, 1997; Becker, Diaz-Granados, & Weathersby, 1997; Becker & Hale, 1993). This is supported in the clinical setting whereby individuals with a history of multiple detoxifications are likely to exhibit more severe withdrawal symptoms than those individuals without prior detoxification experiences (Duka et al., 2004). These findings suggest that the recurring abstinence periods, associated with failed rehabilitation attempts, lead to greater drug dependence (Booth & Blow, 1993; Lechtenberg & Worner, 1991).

1.2.2 Temporal Organization in Drug Addiction

The voluntary consumption of alcohol and withdrawal follows a temporal organization and thus the influence of temporal factors may contribute to the development of the addiction. In general alcohol administration is temporally organized by the length of exposure and withdrawal, and the time of day in which these components occur. For example, in humans, consumption of alcohol follows a

wide range of drinking patterns from intermittent binge drinking to chronic and continuous drinking in severe alcoholics (Ashley et al., 1976; Epstein, Kahler, McCrady, Lewis, & Lewis, 1995; Madden & Jones, 1972b). The abstinence period in these cases may last from several weeks or months in the binge drinker to a few hours in the addicted individual. Moreover, alcohol consumption is clearly temporally arranged in both humans and rodents with peak consumption occurring early within the subjective night (see Figure 1.2; Gibson & Shirreffs, 2013; Trujillo, Roberts, & Gorman, 2009). In the case of humans, during or following the transition to alcoholism, consumption occurs earlier in the day, shifting to late morning (Danel et al., 2003). As a consequence of the consumption pattern, withdrawal also occurs with a daily rhythm, likely peaking during the late night in alcohol-dependent subjects. The time of day in which withdrawal occurs may independently, or in conjunction with exposure, influence the development of the addiction. This notion will be explored more thoroughly in Chapters 2-4.

1.3 Alcohol and Circadian Rhythms

Given the ubiquity of clock influence on behavior, physiology and metabolism, and given the broad pharmacology of alcohol, it is perhaps not terribly surprising that there are intersections between the two. For example, shift-work, which disturbs several aspects of the circadian timing system, has been frequently associated with increased risk for alcohol abuse, although not completely uniformly (Hermansson et al., 2003; Trinkoff & Storr, 1998). Alternatively, alcohol administered to the SCN either in vivo or in vitro, can attenuate photic phase resetting in rodents (Brager, Ruby,

Prosser, & Glass, 2011; Prosser, Mangrum, & Glass, 2008; Ruby, Prosser, DePaul, Roberts, & Glass, 2009) providing clear evidence that alcohol directly influences SCN functioning. The following section will examine both direct and indirect evidence for a relationship between circadian rhythmicity and alcohol use.

1.3.1 Associations between Circadian Rhythmicity and Alcohol Addiction

Alcoholics exhibit many characteristics associated with disrupted circadian functioning such as aberrant sleep/wake cycles, sleep onsets, body temperature rhythms and melatonin secretion patterns (Brower, 2001; Danel, Cottencin, Tisserand, & Touitou, 2009; Reinberg, Touitou, Lewy, & Mechkouri, 2010; Roehrs & Roth, 2001; Wasielewski & Holloway, 2001). Conversely, substance use including alcohol is reportedly greater among people with later chronotypes (i.e., night-owls; Adan, 1994; Prat & Adan, 2011). As previously noted, an association between alcohol consumption and disrupted circadian rhythmicity is likely since pharmacologically alcohol acts broadly throughout the brain and central nervous system. Separating the direct effects of alcohol on the core circadian clock from the effects of clock-modulated outputs has been limited until recently, yet a number of productive lines of work in mutants suggest that there is a genetic relationship between alcohol consumption and circadian rhythmicity (Gamsby et al., 2013; McCulley, Ascheid, Crabbe, & Rosenwasser, 2013; Spanagel, Pendyala, et al., 2005; Zghoul et al., 2007).

Mutations in core circadian clock genes affect alcohol preference, intake, and sensitivity while perturbations to circadian rhythmicity may, in turn, increase alcohol consumption (for review see Perreau-Lenz & Spanagel, 2008; Perreau-Lenz, Zghoul, & Spanagel, 2007; Rosenwasser, 2001, 2010, Spanagel, Rosenwasser, Schumann, &

Sarkar, 2005). For example, a mutant of the *Per2* gene in mice (and *hPer2* in humans) is related to increased alcohol consumption (Spanagel, Pendyala, et al., 2005). This is presumably due to downregulation of glutamate transporter, leading to a hyperglutamtergic state that has previously been identified in the etiology of alcohol dependence (Gyetzai et al., 2011; Pulvirenti & Diana, 2001; Tsai & Coyle, 1998). Mutations of *mPer 1* (in some but not all studies), *mPer2*, or both genes increase ethanol intake and the reinforcing properties of alcohol in mice (Gamsby et al., 2013; Zghoul et al., 2007), while mutations in *mPer2* abolishes the circadian rhythm of alcohol sensitivity on a loss of righting reflex task (Perreau-Lenz et al., 2007). Conversely, selective breeding for ethanol related traits alters circadian phenotype in both mice (McCulley et al., 2013) and rats (Rosenwasser, Fecteau, Logan, et al., 2005). Mice bred to have high alcohol preference (HAP) have greater amounts of activity and shorter period lengths than low alcohol preferring (LAP) mice (Hofstetter, Grahame, & Mayeda, 2003).

Furthermore, perturbations of circadian functioning in rodents may also alter subsequent consumption. Exposure to constant light or constant dark alters ethanol consumption in rats and mice (Goodwin, Amir, & Amit, 1999; Hiller-Sturmhofel & Kulkosky, 2001; Rosenwasser & Fixaris, 2013) while repeated shifts in the light/dark cycle, mimicking rotating shift work, sometimes but not always increases ethanol intake in male rats (Clark, Fixaris, Belanger, & Rosenwasser, 2007; Gauvin et al., 1997; Logan, Seggio, Robinson, Richard, & Rosenwasser, 2010).

In humans, as in rodents, an association exists between altered circadian rhythmicity and alcohol consumption (Danel & Touitou, 2004; Rosenwasser, 2001;

Spanagel, Rosenwasser, et al., 2005). Chronobiological disruptions (e.g., perturbations in body temperature, sleep, and hormone rhythms) are a hallmark of alcohol addiction and predominate throughout alcohol withdrawal, extending even into periods of abstinence (Rosenwasser, 2001). Alcoholics exhibit a downregulation of core clock genes (hPer1, hPer2, hCry1, hCry2, hClock, and hBmal 1; Huang et al., 2010; Spanagel, Rosenwasser, et al., 2005). Alterations in circadian functioning may drive alcohol-seeking behaviors and increase the risk of relapse (Spanagel, Pendyala, et al., 2005). Higher consumption is even seen in non-dependent individuals with altered circadian phenotypes (Adan, 1994; Prat & Adan, 2011). Late chronotypes (“night-owls”), for example, consume more alcohol and stimulants than early chronotypes (“early birds”; Prat & Adan, 2011). Together, these lines of research suggest a bi-directional relationship between altered circadian functioning and alcohol addiction.

1.3.2 Direct Evidence of Alcohol’s Influence on Core Clock Functioning

Single acute doses of alcohol administered at varying times of day produce differences in metabolism (reviewed by Danel & Touitou, 2004), susceptibility to its lethal effects (Deimling & Schnell, 1980), changes in core body temperature (Baird et al., 1998), and differences in its cognitive effects (Reinberg, 1992), all suggestive that alcohol directly influences central clock functioning. For example, following an acute dose of alcohol, mortality is greatest early in the dark or active phase in mice (Deimling & Schnell, 1980). Furthermore, an increasing literature has exposed alcohol’s ability to directly influence core circadian functioning. Acute doses of alcohol applied directly to the SCN weaken photic and non-photic phase resetting in

vivo and in vitro (Brager et al., 2011; Prosser et al., 2008; Ruby, Prosser, et al., 2009). Blinded rats show phase shifts and changes in their free running period when administered acute doses of alcohol dependent upon the circadian phase of the animal (Egami, 1996). Some evidence, however, suggests that the circadian mechanisms may become tolerant to alcohol's effects over time (Lindsay, Glass, Amicarelli, & Prosser, 2014; Prosser & Glass, 2009). For example, (Prosser & Glass, 2009) report that tolerance to the attenuating effects of alcohol on phase resetting occurs if alcohol is applied in vitro to the SCN thirty minutes before the phase resetting stimuli.

Acute models of exposure, however, do not mirror human consumption patterns of the addicted individual. Additional studies are needed to elucidate the effects of chronic alcohol exposure on the circadian timing system and its outputs since tolerance or sensitization is reported in many downstream outputs following chronic exposure to the drug. For example, rats subjected to repeated doses of alcohol have shown tolerance to the induced changes in body temperature typically resulting from alcohol ingestion (Moore & Kakihana, 1978; Ristuccia & Spear, 2005), while time of day dependent sensitization of active phase hypothermia is reported in mice exposed to repeated i.p. injections of alcohol (Williams, Soliman, & Mizinga, 1993). Behavioral tolerance, following repeated alcohol exposures, is also reported in rodents on dowel performance tasks (Goldstein & Zaechelein, 1983; Rimondini, Sommer, Dall'Olio, & Heilig, 2008).

Overall, many studies suggest that chronic alcohol exposure influences circadian functioning (Brager, Ruby, Prosser, & Glass, 2010; Ruby, Brager, DePaul, Prosser, & Glass, 2009; Seggio, Fixaris, Reed, Logan, & Rosenwasser, 2009; Seggio,

Logan, & Rosenwasser, 2007). When administered chronically in a consumption model, alcohol attenuates photic resetting in rodents (Brager et al., 2010; Ruby, Brager, et al., 2009; Seggio et al., 2007), shortens free-running period in mice (Seggio et al., 2009), but has ambiguous effects on free-running period in rats (Rosenwasser, Fecteau, & Logan, 2005). To the best of this author's knowledge, however, no study has examined time of day dependent effects during and following chronic alcohol exposure.

1.4 Alcohol's Influence on Physiological and Behavioral Outputs

As previously discussed, the circadian system orchestrates a wide range of physiological and behavioral systems. Two physiological outputs in particular, body temperature and activity, have been extensively studied within circadian rhythms and addiction. The rhythmic output of these two systems can be simultaneously, continuously, and non-invasively measured via radio-telemetry. As such, they are ideally suited as a measurement of circadian rhythmicity within a chronic model of alcohol exposure and withdrawal. Alternatively, behavioral outputs of addiction, with the exception of alcohol consumption, have not been as extensively studied in a circadian model. Below two behavioral outputs, consumption and anxiolytic behaviors are discussed.

1.4.1 Body Temperature

Body temperature is a circadian regulated output whose similar responses to acute doses of alcohol in both humans and rodents are well documented (Baird et al., 1998; Danel & Touitou, 2004; Gordon, 2010; Wasielewski & Holloway, 2001). In

healthy humans a single acute dose of alcohol produces hypothermia during the subjective day in most (Danel et al., 2001; Devaney, Graham, & Greeley, 2003; O'Boyle, Van, & Hume, 1994), but not all studies (Yap, Mascord, Starmer, & Whitfield, 1993), while no change in body temperature is reported when alcohol is administered in the early evening (Devaney et al., 2003). Interestingly, a delayed hypothermic response is reported during the subjective night (sleep period) following an evening dose of alcohol in nondependent subjects (Eastman, Stewart, & Weed, 1994; Mullin, Kleitman, & Cooperman, 1933). When alcohol is administered throughout the course of a single day, an increased body temperature was reported during the non-active phase (i.e., sleep cycle) and decreased body temperature during the active phase (i.e., wake cycle; Danel et al., 2001). As a consequence of these effects, the overall amplitude of the core body temperature rhythm is dampened (Danel et al., 2001; Danel & Touitou, 2004). Together these findings suggest that the body temperature response to acute alcohol exposure is circadian dependent in humans.

Alcohol also shows phase dependent effects on body temperature rhythms in rodents receiving a single acute dose (Baird et al., 1998); that is, the greatest hypothermic effects occur when alcohol is administered early in the dark cycle (i.e., the active phase for rodents). Delayed hyperthermic responses in rodents are often not reported but are conceptualized to occur as 1) a “rebound” effect from the initial exposure induced hypothermia, as outlined by opponent process theories (Gallaher & Egner, 1987), 2) an independent product of the withdrawal reaction, or 3) a product of environmental factors such as stress or ambient temperature (Sinclair & Taira, 1988).

The effects of chronic alcohol exposure on body temperature in some cases, but not all, mirror the acute findings in rodents (Pohorecky, Brick, & Carpenter, 1986; Pohorecky & Roberts, 1991, 1992; Ristuccia & Spear, 2005; Taylor, Tio, Bando, Romeo, & Prolo, 2006; Williams et al., 1993). For example, repeated i.p. injections of alcohol in rats sensitized the hypothermic response during the dark cycle but animals become tolerant to its effects during the light cycle. In general, inter-laboratory differences in hypothermia may be due to sex and strain differences (Taylor et al., 2006) or the development of tolerance to some chronic alcohol exposure regimes (Pohorecky & Roberts, 1991, 1992; Ristuccia & Spear, 2005). Taylor et al. (2006) report that, in rats, fourteen days of exposure to an alcohol containing liquid diet (5% alcohol w/v) reduced the amplitude and the mean of the body temperature rhythm in two of the three male strains tested (LEW and F344 but not S-D), but only reduced the amplitude in two of the three female strains tested (S-D and F344). After the fourteen day exposure paradigm the alcohol containing liquid diet was removed. During this time the amplitude of the body temperature rhythm decreased in two of the three male strains (S-D and F344) but increased in female S-D and decreased in female LEW strains. Overall, active alcohol exposure decreased the mean body temperature and amplitude while alcohol withdrawal decreased the amplitude of the body temperature rhythm in a sex by strain dependent manner. It should be noted, however, that the body temperature rhythm during withdrawal was averaged over three days, thus the acute withdrawal effects (i.e., the first 24 hours of peak physiological withdrawal symptoms) may have been obscured. Conversely, Ristuccia and Spear (2005) found that four hours of vapor inhalation one hour into the light phase resulted in

hypothermia in adult rats both during and following active alcohol exposure on the first day, but after seven days of exposure the animals became tolerant to the hypothermic effect.

In general, these studies do not clearly elucidate the role of the long term alcohol exposure on the circadian body temperature rhythm in rodents. Human alcoholics typically undergo multiple periods of excessive consumption, withdrawal and relapse, yet most studies have not utilized rodent models that effectively mimic this behavior. One solution is the use of alcohol vapor inhalation. This model allows for extended periods of exposure (and withdrawal) while simultaneously ensuring elevated blood alcohol levels that can be reasonably controlled by the experimenter. Such a paradigm is ideally suited to the study of the circadian effects of chronic alcohol exposure and withdrawal on core body temperature. Chapter 2 of this dissertation will reveal that chronic exposure to alcohol vapor inhalation lowers the core body temperature rhythm during active vapor exposure; however, during acute withdrawal the body temperature rhythm is elevated during the subjective day and lowered during the subjective night. Chapter 3 will further elucidate these findings by revealing that the phase of alcohol exposure is the greatest directional influence to the acute withdrawal response, while the circadian phase of alcohol withdrawal indirectly influences the body temperature response via increased activity.

1.4.2 Activity

Another well documented circadian regulated output is the locomotor activity rhythm. Under standard conditions this rhythm mirrors the body temperature rhythm, peaking during the subjective night in nocturnal rodents and reaching a nadir in the

subjective day. Activity can also have a direct influence on the body temperature response (Weinert & Waterhouse, 1998), thus alcohol induced hypothermia may be secondary to the sedative effects of alcohol on activity (Wasielewski & Holloway, 2001). The influence of activity on core body temperature during and following chronic alcohol exposure will also be explored in Chapters 2 and 3. As in body temperature, the circadian effects of alcohol exposure and withdrawal on the circadian activity rhythm have not been fully explored.

Alcohol is a known sedative and decreases activity during exposure; conversely, hyperactivity is a hallmark of the withdrawal state in humans and in rodents. For example, Baird et al. (1998) showed phase and dose dependent difference in the hypoactivity of rats receiving an acute dose of alcohol. Higher doses of alcohol lowered general locomotor activity early in the subjective night more than during the subjective day. During chronic alcohol exposure Taylor et al. (2006) report that alcohol exposure decreases locomotor activity during the subjective night but increases activity during the first day of withdrawal. The hyperactivity during withdrawal is postulated to be either a “rebound” effect of reduced activity during alcohol exposure or a physiological manifestation of the withdrawal reaction (Crawshaw, O'Connor, Crabbe, & Hayteas, 1994).

1.4.3 Anxiolytic Behaviors

In addition to the physiological responses to alcohol previously discussed, a number of psychological responses can evolve from chronic alcohol use. Addiction in humans is characterized by the compulsion to obtain and use the drug and by the emergence of a negative affective state that drives drug reinforcement (Koob & Le

Moal, 2001). Relapse, thus, may result from the drive to relieve withdrawal-related anxiety in recovering alcoholics. For example, alcoholics are three times more likely to report drinking to alleviate feelings of anxiety or depressed mood as they are to drink to alleviate physical withdrawal symptoms (Hershon, 1977). In turn, anxiolytic drugs are often administered during withdrawal and are effective in reducing both anxiety and relapse (Soyka & Roesner, 2006)

The detection of anxiety related behaviors in rodents following alcohol exposure is sensitive to a number of experimental variables including the method of behavioral testing, regime of alcohol exposure (e.g., vapor inhalation versus 2-bottle choice), length of alcohol exposure, species, etc. and are reviewed elsewhere (Kliethermes, 2005). In brief, many but not all studies report increased anxiety-like behaviors in rodents following chronic alcohol vapor inhalation (Finn, Gallaher, & Crabbe, 2000; Kliethermes, Cronise, & Crabbe, 2004; Lal, Prather, & Rezazadeh, 1991; Pokk, Sepp, Vassiljev, & Vali, 2001; Valdez et al., 2002; Wilson, Watson, & Little, 1998). The elevated plus maze, the gold standard for anxiety-related behaviors, has proven more sensitive in rats than in mice. One possible explanation for this species difference may be the time point in which behavioral testing occurs whereby most mouse studies test anxiety 12 hours or less into acute withdrawal while in rats testing generally occurs at 12 hours or more (Kliethermes, 2005). Anxiolytic behaviors in mice may, therefore, not have fully emerged before testing occurred. Supporting this conclusion, Wilson and Little (1998) identify peak anxiolytic behaviors in mice occurring 16 hours into acute withdrawal; a time point that is employed in this dissertation for anxiolytic testing (Chapter 4).

1.4.4 Consumption

Alcohol consumption in nocturnal rodents follows a strong daily temporal organization with higher amounts consumed in the subjective night (i.e., active cycle) and reaching a nadir during the subjective day (inactive cycle; see Figure 1.2).

Increased alcohol consumption can be achieved by simply limiting access to a few hours early in the subjective night (i.e., a drinking in the dark paradigm). Previous work from our laboratory showed that this timing of alcohol exposure may have enduring effects on future ad libitum consumption of alcohol (Trujillo et al., 2009). Mice presented with an alcohol solution for 2 hours early within the subjective night consumed more alcohol when released into a 24 hour free choice condition than those initially presented with alcohol early within the subjective day.

Ad libitum access in a free choice paradigm, however, is generally not sufficient to induce physical dependence. Forced exposure to alcohol, such as passive alcohol vapor inhalation, can raise blood ethanol content (BEC), induce physical dependence, and increase voluntary alcohol consumption when repeatedly administered in an intermittent exposure paradigm (i.e., repeated exposure and withdrawal periods) when BECs are elevated to 175mg/dl or greater (Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005). Chapter 4 utilizes a similar vapor inhalation paradigm as reportedly used by the Becker laboratory to investigate if the timing of alcohol exposure and withdrawal influences voluntary alcohol consumption in a rodent model of dependence.

1.5 Other Drugs of Abuse

Time-of-day may serve as one of many critical environmental cues associated with drug use. Learning is known to play an important role in drug use and relapse. Environmental cues become associated with drug use and over time these cues can be used to predict drug exposure. In rodents, there is ambiguous evidence that time-of-day is a critical part of the environmental context for drug use and relapse (Arvanitogiannis, Sullivan, & Amir, 2000). The temporal context of this association, if present, may in turn influence drug use, craving, and relapse, and may provide a unique opportunity for therapeutic interventions. Prior studies suggest that the circadian system is implicated in mediating drug-seeking behavior, such as conditioned place preference and sensitization to cocaine (Akhisaroglu, Ahmed, Kurtuncu, Manev, & Uz, 2004; Sleipness, Sorg, & Jansen, 2007). For instance, Arvanitogiannis et al. (2000) found that amphetamine sensitization in rats depends upon testing and training occurring at the same time, but that in constant light, a condition that promotes arrhythmia, sensitization is independent of the training time. This work, however, lacks adequate circadian controls and has yet to be replicated or generalized to other drugs or species. Chapter 5 of this dissertation will attempt to replicate the Arvanitogiannis study in a mouse model of addiction that also properly controls for time of day dependent differences in sensitization.

1.6 Summary

This dissertation will address whether the circadian phase of drug exposure/withdrawal can modulate the development of addiction and addiction-related

behavior in rodents and if, in turn, time of day can serve as a predictive environmental cue to drug administration. This dissertation is the first, to our knowledge, to study the time of day dependent modulations in physiological and psychological dependence in a chronic rodent model of alcohol addiction. Chapter 2 of this dissertation will reveal that time of day manipulations of alcohol exposure and withdrawal in a chronic model of addiction can modify the core body temperature and locomotor activity responses. These responses will be further explored in Chapter 3 to clarify that the acute withdrawal response is related primarily to the phase of alcohol exposure, although the magnitude of the response may be influenced by the phase of acute withdrawal via increases in activity. Some evidence suggests that situations which promote more severe withdrawal severity are also associated with the development of addiction-like behaviors (Becker, Diaz-Granados, & Hale, 1997; Becker & Lopez, 2004; Lopez & Becker, 2005). Chapter 4 will provide limited evidence that controlling for the circadian phase of alcohol exposure/withdrawal increases voluntary alcohol consumption but not the development of anxiolytic behaviors. Lastly, time-of-day may also be a critical environmental cue associated with drug use; which may influence drug use, craving, and relapse, and may provide a unique opportunity for therapeutic interventions. Chapter 5 concludes, despite prior evidence to the contrary (Arvanitogiannis et al., 2000), time of day does not appear to be a salient conditioned environmental cue to drug sensitization.

1.7 Figures

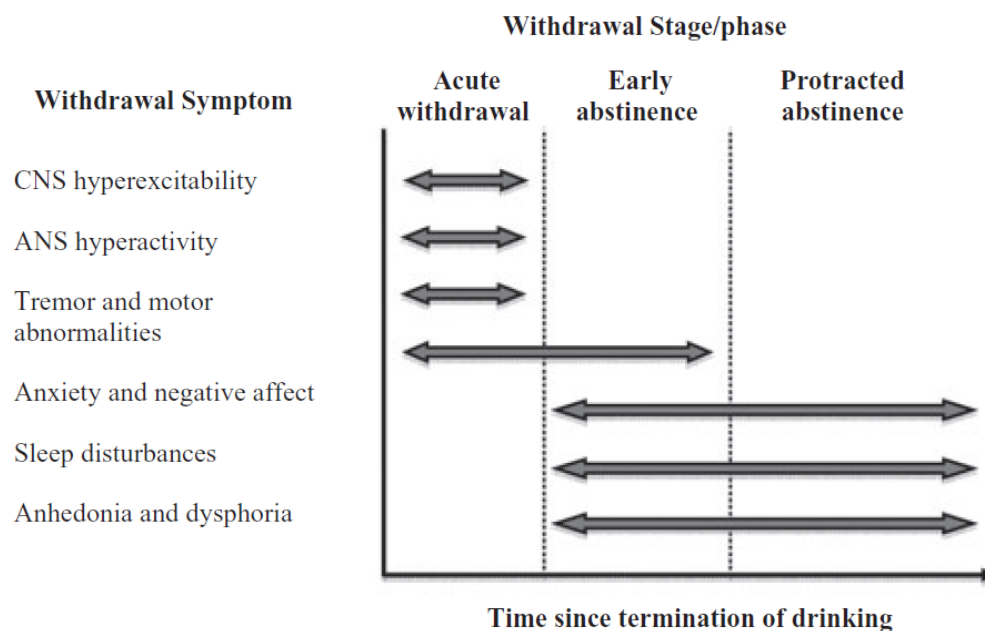
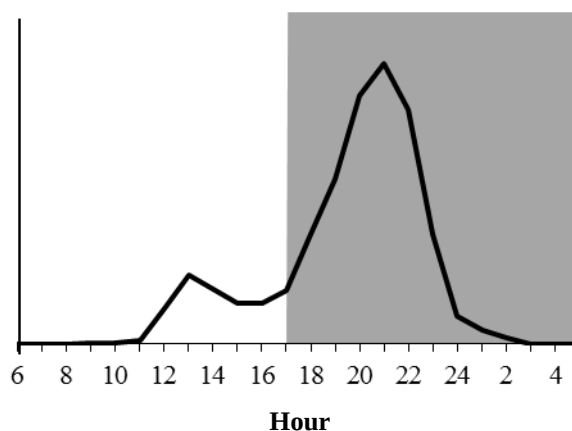


Figure 1.1: Temporal patterns and symptoms of alcohol withdrawal adopted from Heilig et al. (2010). In humans acute withdrawal (left) lasts 48-72 hours and in animals 24-48 hours. Early abstinence phase (middle) occurs over the course of 3-6 weeks in humans and 1-2 weeks in animals. The protracted abstinence phase (right) can last indefinitely, beginning around 3 months of abstinence in humans and one month of abstinence in animals.

24 Hour EtOH Consumption Rhythm

A: Male Humans



B: Male C57BL/J6 Mice

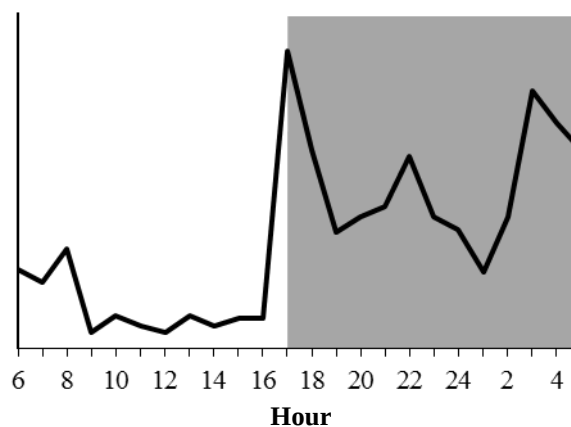


Figure 1.2: Twenty-four hour approximate consumption pattern of alcohol in male humans (A) and mice (B) adopted from Gibson and Shirreffs (2013) and Trujillo, Do, Grahame, Roberts, and Gorman (2011) respectively. Dark shading represents the estimated “evening” periods reported in each study.

Chapter 2: Circadian Phase Determines Effects of Repeated Ethanol Vapor Exposure and Withdrawal on Body Temperature and Activity Rhythms of Male Mice

Physiological responses to acute EtOH injection depend critically on the timing of their administration. Whether daily timing modulates effects of longer intoxication intervals characteristic of alcohol-dependent humans remains unknown. The present work examines time of day effects during EtOH exposure and withdrawal measured by locomotor activity and body temperature across multiple rounds of EtOH exposure/withdrawal. Two groups of mice, implanted with radio-telemeters, were entrained to opposite light: dark periods (14:10 LD cycle) so that their rest/activity cycles were 12 h apart. Under a 2-h skeleton photoperiod animals were simultaneously exposed to three daily cycles of EtOH vapor inhalation (14 h EtOH on) and withdrawal (10 h EtOH off). During this time, air-only control groups matched for entrainment were handled in a comparable manner. After the third cycle of EtOH vapor, the animals were left undisturbed for 11 days to recover. The protocol was repeated three additional times. During intoxication, mice exposed to EtOH in the subjective night exhibited greater hypothermia and more overall disruptions in the body temperature and locomotor activity rhythms. Acute withdrawal induced hypothermia during the subjective night and hyperthermia during the subjective day. Animals in both phases demonstrated significant disruptions in locomotor activity during withdrawal. Locomotor activity had little effect on body temperature during

EtOH exposure, but it significantly influenced core body temperature during acute withdrawal. These findings suggest that controlling for the circadian phase of exposure and/or withdrawal may mitigate the severity of symptomatic withdrawal.

2.1 Introduction

The temporal contexts of drug and alcohol exposure (e.g., developmental phase, frequency and dose, time of day, season of the year, etc.) are important considerations for understanding addiction processes (Danel et al., 2003; Eastwood & Stiasny, 1978; Griffin et al., 2009; Gunderson & Schuckit, 1975; Lopez & Becker, 2005; Nixon & McClain, 2010). In the non-dependent human population, for example, alcohol is preferentially consumed in the early evening, with the transition to dependence, alcohol craving and consumption increasingly expand into the morning hours (Danel et al., 2003). As a consequence of the consumption pattern, withdrawal, defined by dropping blood alcohol levels and pauses in the exposure to alcohol, will also occur with a daily rhythm, likely peaking during the late night in alcohol-dependent subjects. Physical withdrawal manifestations are characterized by a variety of disturbances in nervous system functioning (e.g., body temperature, hormone secretion, etc.) that, under standard conditions, fluctuate over the circadian cycle (Danel et al., 2009; Drummond, Gillin, Smith, & DeModena, 1998; Imatoh, Nakazawa, Ohshima, Ishibashi, & Yokoyama, 1986; Kalant & Le, 1983; Reinberg et al., 2010; Rosenwasser, 2001; Rupp, Acebo, & Carskadon, 2007). Withdrawal-related perturbations of the circadian body temperature rhythm are associated with an increased risk of mortality during acute alcohol withdrawal (Khan, Levy, DeHorn,

Miller, & Compton, 2008). A number of systems continue to show circadian desynchrony after weeks of alcohol abstinence (Drummond et al., 1998; Imatoh et al., 1986; Rosenwasser, 2001), and the continued disruption of sleep/wake rhythmicity into protracted withdrawal is predictive of future relapse (Drummond et al., 1998). As cycling of chronic ethanol exposure and abstinence has been suggested to contribute to an increase in alcohol consumption (Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005), the mitigation and treatment of temporally regulated disruptions may be clinically significant.

The master circadian pacemaker, located within the suprachiasmatic nucleus (SCN) of the hypothalamus, coordinates the daily organization of physiological and behavioral rhythms. In nocturnal rodents, for example, the SCN programs an interval of *subjective night* that includes elevated locomotor activity, increased core body temperature, feeding, drinking, melatonin secretion, etc. and an interval of *subjective day* that favors inactivity, low body temperature and basal melatonin. The biology of alcohol is likewise circadian. During the subjective night of mice ethanol preference is greatest (Freund, 1970) and more lethal (Deimling & Schnell, 1980). Acute ethanol injections (3 g/kg, i.p.) elicit a 5 °C drop in body temperature one hour before dark onset, but only 1.9°C twelve hours later (Williams et al., 1993). Compared to acute alcohol exposures, much less is known about how time-of-day modulates effects of long intoxication and withdrawal cycles typical of alcohol-dependent subjects.

Body temperature and locomotor activity are two circadian regulated systems that can be continuously recorded using radio-telemetry and display well documented responses to chronic alcohol exposure and withdrawal (Baird et al., 1998; Brick,

Pohorecky, Faulkner, & Adams, 1984). In the absence of alcohol, there is a well-established relationship between these systems. First, activity and body temperature are similar in the shape of their rhythmicity, both peaking during the subjective night in rodents. Secondly, activity can influence the body temperature response, masking the endogenous core temperature rhythm (Weinert & Waterhouse, 1998). For example, mice receiving acute i.p. injections of ethanol display a significant decrease in activity at the same time that pronounced hypothermia occurs (Papanicolaou & Fennessy, 1980). Thus, alcohol induced hypothermia could be secondary to the suppression of activity by the sedative influence of alcohol (Wasielewski & Holloway, 2001). An emerging literature, however, implicates the direct involvement of alcohol on circadian and, specifically, SCN functioning (Brager et al., 2010, 2011; Ruby, Brager, et al., 2009; Ruby, Prosser, et al., 2009).

The current study was undertaken to assess the significance of circadian phase in mediating the effects of alcohol intoxication and withdrawal on physiological indices of addiction. Body temperature measurement has been used as a high resolution index for withdrawal severity (Ritzmann & Tabakoff, 1976a). To isolate circadian phase as the independent variable -- apart from ambient temperature (Myers, 1981), light conditions (Geller, 1971; Sinclair & Geller, 1972) and handling-related stress that modulate alcohol's response (Crawshaw, Wallace, & Crabbe, 1998; Peris & Cunningham, 1986, 1987) -- mice with opposite circadian entrainment schedules were tested simultaneously, while maintained in identical "skeleton" photoperiods (see below). Additionally, because alcohol intoxication and withdrawal affect levels of locomotor activity that in turn influence body temperature, simultaneous measurement

of these two variables allowed the decomposition of the latter into activity-dependent and independent-components.

2.2 Methods

2.2.1 Subjects

Twenty-four male C57Bl/6J mice (Jackson, Sacramento, CA) six weeks of age were housed under standard temperature (22°C) and lighting (270 lux) conditions. Mice had ad libitum access to food (Purina) and water throughout the experiment. The animals were housed 3-4 per cage in plastic shoebox cages until the start of baseline data collection. All experimental procedures and animal care were approved by, and conducted under, the guidelines of the Institutional Animal Care and Use Committee at UCSD.

2.2.2 Experimental Lighting Conditions

Mice were randomly divided and initially entrained to one of two opposing 14:10 light:dark (LD) cycles (lights on from 0700-2100 or 1900-0900). After stable entrainment and recovery from telemetry implantation, all mice were individually housed and transferred to a common “skeleton photoperiod” consisting of two, 2 h light pulses (of the same intensity used during entrainment) that replaced the first and last fractions of the original 14 h light phase (lights on from 0700-0900 and 1900-2100). This yielded two groups of animals entrained to a single lighting and housing environment but with oppositely-phased subjective days and nights (sDay, sNight). Each of the two entrainment groups was subdivided to receive either ethanol (EtOH)

vapor or air only (EtOH sNight/Withdrawal sDay, n=8; Control sNight /Control sDay, n=4; EtOH sDay/Withdrawal sNight, n=8; Control sDay / Control sNight, n=4).

2.2.3 Surgery

One week after habituation to the original LD conditions, mice were anesthetized with isoflurane approximately 2 h into their respective light phases. Telemetry units (G2 E-Mitter, Respironics, Inc., Bend, OR) were implanted into the peritoneal cavity and attached to the abdominal wall. A single dose of buprenorphine (0.05 mg/kg) was administered for pain relief. Animals were returned to their LD condition once ambulatory and allowed to recover for seven days before being transferred to the skeleton photoperiod for baseline data collection.

2.2.4 Alcohol Vapor Inhalation and Rounds

The experimental groups received four rounds of EtOH vapor inhalation and withdrawal commencing at 10 weeks of age. Each round was a 14 day protocol that included three days of EtOH exposure and withdrawal, followed by an 11 day recovery period. Animals were run in two identical squads spaced seven days apart.

Following collection of seven or more days of baseline data where animals were left undisturbed under the skeleton photoperiod, mice received an acute i.p. injection of EtOH (1.5g/kg at 20% v/v) and pyrazole HCl (0.0681 g/kg) at 1900 and were immediately placed in modified cages attached to an EtOH vapor inhalation apparatus (La Jolla Alcohol Research Inc., La Jolla, CA). EtOH vapor was administered continuously for 14 h terminating at 0900, which in turn initiated a 10 h acute withdrawal phase. This cycle of injection/exposure/withdrawal was repeated on the following two days before the animal underwent an 11 day recovery period.

Controls, injected with an equivalent dose of pyrazole and saline, were housed on racks within the room. Prior pilot data confirmed no difference in the body temperature or activity rhythms of control animals housed in the modified caging attached to the vapor inhalation apparatus under air-only and those housed on the racks.

The generation of EtOH vapor is described elsewhere (Gilpin, Richardson, Cole, & Koob, 2008). Briefly 95% EtOH was dripped in a 2000-ml Erlenmeyer vacuum flask warmed to 50⁰C with air blown through the flask at 11 liters/min. The air/EtOH combination was in turn pumped into each attached chamber housing the experimental animals.

2.2.5 Blood Ethanol Content:

At the start of the 0700-0900 skeleton light pulse, tail blood (0.05 ml) was collected from each mouse in heparinized tubes prior to the end of each day of vapor inhalation and assayed for blood ethanol content (BEC). Target BEC was 175-250 mg/dl since prior work has implicated this range in the development of addiction-related behaviors in mice (Griffin et al., 2009). Because EtOH is a known analgesic, controls received a pinch and a milking motion to the tail, but no blood was collected. Blood samples were analyzed with Analox AM1blood analyzer (Analox Instruments Ltd; London, England)

2.2.6 Data Analysis:

Baseline telemetry data was collected for a minimum of one week prior to experimentation. Body temperature and general locomotor activity were sampled at six minute intervals using VitalView Data Acquisition System software (Respironics,

Inc; Bend, OR) and analyzed using Excel 2010 (Microsoft, Inc; Bellevue, WA) and SPSS (IBM Corp, Armonk, NY). To avoid acute effects of bleeding, handling, injecting, or exposure to light on body temperature or activity, data analysis was restricted to the 10 h intervals of darkness between skeleton pulses, when animals were always left completely undisturbed. Data were averaged across the three intervals of exposure or withdrawal within each round. Additional analyses were conducted with data collapsed across rounds.

The daily rhythms of body temperature and locomotor activity did not differ between the two air-exposed control groups, adjusting for their oppositely phased entrainment. Accordingly, the active phases of all control mice were combined for a composite sNight control group, and the inactive phases were combined for a composite sDay control group.

The effects of EtOH on body temperature and activity were calculated in two ways, to capture net changes in these variables as well as non-directional rhythm disruption. Two facts necessitate these analyses. First, because daily rhythms in these variables are not simple sinusoids or square waves but have complex waveforms (e.g., they are commonly bimodal in mice), it is important to consider whether there are perturbations in daily rhythmicity that might not be captured as a change in mean body temperature. Second, because daily patterns of locomotor activity and body temperature are mechanistically related (i.e., both generated by SCN activity, and increased activity can increase body temperature) it is valuable to use statistical analyses to estimate the portion of the body temperature rhythm that is not a secondary consequence of acute changes in activity levels. For each animal, values during

EtOH/air and withdrawal were calculated relative to a five-day baseline interval immediately prior to EtOH/air exposure and were averaged over the 10 h period of darkness. Rhythm disruption was calculated by smoothing data over 30 minute intervals and averaging the summed hourly absolute difference in values during EtOH/air or withdrawal relative to the five-day prior baseline.

To quantify the effect of activity on body temperature, we used linear regression analysis previously described by Weinert and Waterhouse (1998). Activity was summed over intervals of 6-60 min to determine the best correlation between the integrated effects of activity on the resulting body temperature. During intoxication and withdrawal the preceding 12 min of summed activity was most strongly correlated with the body temperature measurement. This 12 min integration time was used, in conjunction with linear regression, to calculate the hourly activity independent body temperature ($Act_{Ind} T_b$; i.e., the intercept is the expected body temperature if no activity was present) and to quantify the hourly influence of activity on body temperature (i.e., the slope indicates how much a unit of activity increases body temperature).

Data were analyzed using an ANOVA with circadian phase (sDay/sNight) and exposure (EtOH/Control) as between-subject variables and a significance level of $p < 0.05$ was set for all tests. Post-hoc tests were conducted using Tukey-Fisher least significant difference (LSD). All data from four experimental animals were excluded from analysis due to mortality ($n=1$ EtOH sDay/Withdrawal sNight; $n=2$ EtOH sNight/Withdrawal sDay) or telemetry failure ($n=1$ Control sDay/Control sNight).

2.3 Results

Representative actograms during the skeleton photoperiod are shown in Figure 2.1. Visual inspection confirmed in all animals that the circadian phases remained stable with respect to the skeleton photoperiod throughout the entire experiment. As expected, prior to EtOH administration, locomotor activity and core body temperature rhythms exhibit clear increases during the sNight and lower values during the sDay. A distinct bimodal peak in activity occurred during the sNight, which was further mirrored in the body temperature throughout baseline collection. During intervals of intoxication, EtOH-exposed animals show marked periods of decreased activity and body temperature in the sNight (Figure 2.1E and F). Following EtOH, mice exhibit clear perturbations in both the activity and body temperature rhythms dependent upon the circadian cycle. An initial increase in activity and body temperature was seen at the start of each round of EtOH exposure which also coincided with a cage change.

BEC was elevated into the target range, averaging 211.6 ± 4.6 mg/dl across rounds (Table 2.1). ANOVA yielded no main effects of either circadian phase or squad. Within-subject tests revealed a significant main effect of round ($F_{3,27}=55.2$, $p<0.001$) and a round by squad interaction ($F_{3,27}=9.5$, $p<0.001$). Post hoc comparisons indicated that there was a small magnitude but significant difference between the two squads (i.e., order in which the animals were run experimentally) in Round 1 ($p<0.003$) when BEC was lowest.

2.3.1 Multiple Rounds of Alcohol Exposure/Withdrawal

Core body temperature and general locomotor activity were analyzed across the four rounds to determine if EtOH exposure/withdrawal progressively disrupted these measures. Contrary to expectation, no significant effects of rounds were found for any measure during EtOH exposure nor were any effects seen in general locomotor activity or the $Act_{Ind} T_b$ during acute withdrawal. During withdrawal, however, a repeated measures ANOVA of core body temperature data indicated a significant main effect of round ($F_{3,69}=9.7$ $p<0.001$), the interaction of round by exposure ($F_{3,69}=4.6$, $p<0.006$), and an interaction of round by exposure by phase ($F_{3,69}=2.9$ $p<0.041$). Post-Hoc testing confirmed that the main effects of round ($F_{1,11}=27.1$, $p<0.001$) and round by exposure ($F_{1,11}=11.4$, $p<0.006$) were linear, reflecting a sensitized response, in animals withdrawing from EtOH vapor during the sDay, but there was no effect of round on body temperature during the sNight.

The summed absolute disruption in core body temperature was also analyzed across rounds during acute withdrawal. There was a significant round by group interaction in the absolute change of body temperature ($F_{3,33}=18.5$ $p<0.002$). Post hoc analysis indicated that the body temperature response became significantly more disrupted across rounds during the sDay ($F_{1,11}=11.4$, $p<0.006$) but not during the sNight.

Given the lack of effect of round on locomotor activity and $Act_{Ind} T_b$, and the possibility that the effects on body temperature were due to rising BEC across rounds (Table 2.1), all subsequent analyses were conducted with data averaged across rounds of intoxication/withdrawal.

2.3.2 During Exposure, Alcohol Lowers Body Temperature Dependent upon the Time of Day

In the interests of clarity, the responses to the repeated ethanol inhalation schedule are presented separately and sequentially for the intoxication and the withdrawal phases of the cycle. Core body temperature in the 10 h of darkness during the sNight was lowered 1.16°C in EtOH vapor-exposed mice compared to air controls, but was lowered by only 0.16°C when vapor coincided with the sDay (Figure 2.3A). The hypothermic effect was not constant throughout the sNight, but occurred primarily during the second portion of EtOH exposure (Figure 2.2A). An ANOVA of the body temperature during the 10 h of darkness yielded a significant main effect of exposure ($F_{1,23}=276.0$, $p<0.001$) and phase ($F_{1,23}=319.5$, $p<0.001$) as well as their interaction ($F_{1,23}=107.0$, $p<0.001$). Post-hoc analysis confirmed that EtOH lowered the average body temperature compared to air at both phases of the daily cycle ($p<0.001$), but that the reduction was greater for animals during the sNight ($p<0.001$).

The overall amount of perturbation in the body temperature rhythm from baseline (i.e., sum of deviations from baseline pattern regardless of sign) increased by 277% during the sNight of EtOH vapor-exposed mice compared to air controls in the 10 h period of darkness; EtOH exposure during the sDay increased the amount of body temperature disruptions by only 15% relative to air controls (Figure 2.4). An ANOVA of the absolute change in body temperature from baseline yielded a significant main effect of exposure ($F_{1,23}=295.1$, $p<0.001$), phase ($F_{1,23}=312.9$, $p<0.001$), and their interaction ($F_{1,23}=245.2$, $p<0.001$; Figure 2.4A). Post-hoc analysis

indicated that only animals in their sNight were disrupted by EtOH vapor compared to air controls ($p<0.001$).

2.3.3 During Exposure, Alcohol Suppresses Activity in a Time of Day

Dependent Manner

Locomotor activity averaged over the 10 h dark period of sNight was lowered by 12 counts/minute (a reduction of 54%) in vapor exposed animals, compared to air controls, but was increased by 2.3 counts/minute (an 11% increase) in intoxicated animals in their sDay (see Figure 2.3C). An ANOVA of the average change in activity indicated a significant main effect of exposure ($F_{1,23}=78.0$, $p<0.001$), phase ($F_{1,23}=78.6$, $p<0.001$), and their interaction ($F_{1,23}=35.9$, $p<0.001$). Post-hoc test confirmed that only animals exposed to EtOH during their sNight had a significant reduction in activity compared to air controls or animals in their sDay ($p<0.001$). No significant change in activity was seen during the sDay.

EtOH exposure greatly perturbed the locomotor activity rhythm during the sNight, increasing the overall amount of perturbations by 105% (Figure 2.4) from air controls. During the sDay the total amount of perturbation in activity was decreased by 28% compared to air controls. An ANOVA of the absolute change in activity from baseline confirmed a main effect of exposure ($F_{1,23}=14.5$, $p<0.001$), circadian phase ($F_{1,23}=57.7$, $p<0.001$), and their interaction ($F_{1,23}=35.4$, $p<0.001$; Figure S3C). Post-hoc analysis indicated that activity was only disrupted during the sNight compared to air controls ($p<0.001$).

2.3.4 Independent of Activity, Alcohol Influences the Endogenous Body

Temperature Rhythm During the Active Phase of the Circadian Cycle

The computed activity-independent body temperature ($Act_{Ind} T_b$) was lowered by $0.89^{\circ}C$ during the sNight of EtOH vapor-exposed mice compared to air controls, but only by $0.08^{\circ}C$ in intoxicated sDay animals (Figures 2.2E, 2.2F, and 2.3E). An ANOVA yielded a significant main effect of exposure ($F_{1,23}=72.3$, $p<0.001$), phase ($F_{1,23}=173.9$, $p<0.001$), as well as their interaction ($F_{1,23}=71.1$ $p<0.001$). Post-hoc comparisons indicated that only animals exposed to EtOH during their sNight displayed significant hypothermia in their $Act_{Ind} T_b$ compared to control or EtOH-exposed animals in their sDay ($p<0.001$).

The overall amount of disruption in $Act_{Ind} T_b$ during EtOH vapor exposure increased by 137% in mice during the sNight but only by 11% during the sDay compared to air controls (Figure 2.4E). An ANOVA of the summed absolute change in the $Act_{Ind} T_b$ over the 10 h intoxication period indicated a significant main effect of exposure ($F_{1,23}=75.5$, $p<0.001$), phase ($F_{1,23}=162.6$ $p<0.001$), and their interaction ($F_{1,23}=62.3$ $p<0.001$). Post hoc tests indicated that the $Act_{Ind} T_b$ was only significantly perturbed in intoxicated sNight animals compared to air controls ($p<0.001$).

The mean correlation coefficient between activity and body temperature using the 12-minute integration time was 0.44, with a range of 0.04-0.68 between different animals. The slope of these linear regressions estimates the magnitude of the influence of activity on body temperature (i.e., how much a unit of activity increases body temperature). Compared to air exposure, this influence was reduced during EtOH vapor inhalation (reductions of 79% and 49% in sNight and sDay, respectively; Figure

2.5). An ANOVA revealed a significant main effect of exposure ($F_{1,23}=44.2$, $p<0.001$), but not of phase. Post-hoc analysis demonstrated that activity had less influence on the body temperature of mice exposed to EtOH (sNight, $p<0.001$; sDay, $p<0.001$) compared to the respective controls, but EtOH exposed animals did not differ from one another.

2.3.5 Alcohol Withdrawal has Differential Effects on Core Body Temperature Depending upon the Circadian Cycle

EtOH withdrawal, in the 10 h period of darkness and initiated during the sDay, increased core body temperature by 0.56°C . During the sNight, withdrawal had the opposite response, decreasing body temperature by 0.50°C (Figure 2.3B). An ANOVA yielded significant main effects of exposure ($F_{1, 23}=4.4$, $p<0.047$), circadian phase ($F_{1, 23}=177.3$, $p<0.001$), as well as their interaction ($F_{1, 23}=62.7$, $p<0.001$). Post-hoc analysis revealed that animals withdrawing from EtOH during the sNight had a suppressed body temperature compared to air controls ($p<0.001$), while animals in the sDay had significantly elevated the body temperature compared to controls ($p<0.001$).

The overall amount of disruption in the body temperature rhythm during the 10 h withdrawal interval was increased by 124% in mice withdrawing during the sNight and by 106% in mice withdrawing during the sDay compared to air controls (Figure 2.4B). An ANOVA of the summed absolute change in the body temperature from baseline during alcohol withdrawal revealed a significant main effect of exposure ($F_{1,23}=75.4$, $p<0.001$). The magnitude of the overall disruption in body temperature did not differ between the two experimental groups ($p<0.242$), but the disruption in

body temperature during withdrawal was significantly greater in the experimental animals compared to their respective air controls ($p < 0.001$).

2.3.6 Alcohol Withdrawal Increases Locomotor Activity Differentially

Depending upon the Circadian Cycle

During the 10 h dark period of EtOH withdrawal, mice became hyperactive, increasing their general locomotor activity by 9.5 counts/minute (a 252% increase) during the sDay and by 3.7 counts/minute (a 24% increase) during the sNight compared to air controls (see Figure 2.3D). An ANOVA indicated a significant main effect of exposure ($F_{1,23} = 47.2$, $p < 0.001$), circadian phase ($F_{1,23} = 30.7$, $p < 0.001$), and their interaction ($F_{1,23} = 9.7$, $p < 0.005$). Post-hoc analysis revealed that both groups withdrawing from EtOH were hyperactive compared to air controls (Withdrawal sDay, $p < 0.001$; Withdrawal sNight, $p < 0.032$), although the magnitude of this effect was greater during the sDay ($p < 0.001$).

Acute withdrawal in the 10 h dark period resulted in a 157% increase in the overall disruption of the locomotor activity rhythm during the sDay and a 44% increase during the sNight compared to air controls (Figure 2.4D). An ANOVA of the absolute summed change in locomotor activity from baseline during the acute withdrawal period yielded a significant main effect of exposure ($F_{1,23} = 58.5$, $p < 0.001$), phase ($F_{1,23} = 4.7$, $p < 0.042$) as well as their interaction ($F_{1,23} = 13.9$, $p < 0.001$). Post hoc tests revealed that the locomotor activity of mice withdrawing during their sDay was significantly more disrupted than the animals in their sNight ($p < 0.001$) or sDay controls ($p < 0.001$). Withdrawing sNight animals were, however, significantly more disrupted than their controls ($p < 0.001$).

2.3.7 Alcohol Withdrawal Influences the Endogenous Body Temperature Rhythm, Independent of Activity, as a Function of Time of Day

On average during the 10 h withdrawal period, the removal of EtOH during the sNight lowered the $Act_{Ind} T_b$ by $0.52^{\circ}C$ but increased $Act_{Ind} T_b$ by $0.21^{\circ}C$ in animals in the sDay (Figure 2.3F). An ANOVA yielded a significant main effect of phase ($F_{1,23}=70.6$, $p<0.001$) and an exposure by phase interaction ($F_{1,23}=26.5$, $p<0.001$). Post hoc analysis confirmed that, compared to air controls, withdrawal during the sDay significantly elevated the $Act_{Ind} T_b$ ($p<0.001$), but suppressed the $Act_{Ind} T_b$ during the sNight ($p<0.002$). An analysis of the slope of the linear regression estimates revealed no significant main effects or interactions (Figure 2.5B).

During acute withdrawal, the $Act_{Ind} T_b$ of animals previously exposed to ethanol vapor was 81% more disrupted during the sNight compared to air controls while animals in their sDay were only 46% more disrupted than controls (Figure 2.4F). An ANOVA of the summed absolute change from baseline of the $Act_{Ind} T_b$ revealed a significant main effect of exposure ($F_{1,23}=327.8$, $p<0.001$), phase ($F_{1,23}=21.0$, $p<0.001$) and their interaction ($F_{1,23}=5.1$, $p<0.034$). Post-hoc tests indicated that only the $Act_{Ind} T_b$ of animals withdrawing in the sNight was significantly more disrupted than air controls ($p<0.001$) and the magnitude of this disruption was greater in the sNight than during the sDay ($p<0.001$).

2.4 Discussion

To our knowledge, the current study is the first to critically examine time-of-day-dependent responses using an animal model of chronic alcohol dependence. Like

many drugs, alcohol has been shown to vary in efficacy according to the circadian phase. In humans, acute alcohol exposure is typically considered to have a hypothermic effect during the subjective day and to elicit hyperthermia during the subjective night (Danel & Touitou, 2004). Following alcohol dependence, withdrawal elicits a hyperthermic reaction (Kalant & Le, 1983) that predicts withdrawal severity and related mortality (Kalant & Le, 1983; Khan et al., 2008; Ritzmann & Tabakoff, 1976a). Gross, Lewis, Best, Young, and Feuer (1975) have suggested that withdrawing alcoholics may demonstrate temporally dependent temperature changes, but to date this has yet to be thoroughly studied in either a human or animal model of dependence. The current data indicate that withdrawal-related fluctuations in body temperature are indeed dependent upon the time of alcohol administration and withdrawal.

Core body temperature and locomotor activity were substantially decreased in the subjective night during repeated sessions of alcohol vapor intoxication. In rodents, acute i.p. injections of alcohol elicit greater amplitude hypothermic responses during the subjective day compared to the subjective night (Baird et al., 1998, Brick et al., 1984). With repeated exposures in the subjective day animals developed tolerance to alcohol's hypothermic effects, whereas repeated nighttime injections yielded sensitization (Williams et al. 1993). The intoxication and withdrawal periods associated with acute injections, however, are temporally indistinct; our protocol of prolonged vapor exposure allowed us to examine sustained effects of alcohol during the active exposure period, apart from the acute withdrawal effects. In the current

study repeated sessions of prolonged alcohol vapor exposure yielded hypothermia in both circadian phases across all rounds of vapor exposure.

The magnitude of the hypothermic effect during intoxication was more severe during the animal's subjective night. This suggests that during the subjective day animals may be more resilient to temperature disruptions, a notion supported by the lower mortality rate to many toxic insults during this phase (Gordon, 2010). At this phase, animals in the subjective day may have developed a greater degree of tolerance to the hypothermic effects of alcohol than animals in the subjective night (Moore & Kakihana, 1978; Williams et al., 1993). For activity levels, measurement of locomotor suppression would be highly constrained during the subjective day when activity levels are already low. The same is not true for temperature, however, as demonstrated elsewhere by potent hypothermic responses to acute alcohol injections (Moore & Kakihana, 1978), arguing against a "floor effect" during this circadian phase.

In contrast to the consistent, albeit of varying magnitude, hypothermic effects of alcohol during intoxication, acute withdrawal induced hypothermia or hyperthermia as a function of circadian phase. While hypothermia in rodents is observed primarily following an acute i.p. injection of alcohol (i.e., 1-2 g/kg) extending for approximately 2-4 hours (Baird et al., 1998), both hypothermic and hyperthermic effects are reported following chronic alcohol exposure 24 hours into withdrawal (Ritzmann & Tabakoff, 1976a; Taylor et al., 2006; Williams et al., 1993). The current data suggest that these previously reported withdrawal responses may be dependent on circadian phase. The present design, intended to mimic the intoxication/withdrawal schedule sometimes

experienced by human alcoholics, cannot distinguish whether the circadian response effects are attributable to the timing of ethanol exposure, withdrawal, or both. The hypo- versus hyperthermic response also cannot be attributed to subtle environmental variations that commonly go uncontrolled in studies of daily rhythmicity. Enabled by the use of skeleton photoperiods to equalize light exposure and the use of separate groups of phase-shifted animals held under identical environmental conditions, assures us that ambient temperature (Myers, 1981), lighting influences (Geller, 1971; Sinclair & Geller, 1972), or handling/husbandry (Crawshaw et al., 1998; Peris & Cunningham, 1986, 1987) cannot account for these directional differences.

Alcohol effects on body temperature were only partially dependent on changes in locomotor activity. Under standard lighting conditions, the activity and body temperature rhythms parallel one another, and are simultaneously regulated by the SCN. In the present study, activity levels accounted for a significant degree of variance in body temperature during any given hour, strongly suggesting that the depressant effects of alcohol on activity contributed to its hypothermic effects as previously proposed (Wasielewski and Holloway, 2001). However, statistically accounting for the relationship between activity and body temperature demonstrated that there remained a residual effect of treatment independent of the locomotor activity rhythms. Disruption of thermoregulatory functions was additionally apparent in the altered slope and significance of the activity/body temperature correlation in the presence of alcohol. Ethanol-induced disruption of thermoregulatory processes has been previously noted in wild-type animals (Crawshaw et al., 1998) and is sensitive to artificial selection (Crabbe, 1994; Crabbe, Feller, & Dorow, 1989; Crabbe, Kosobud,

Tam, Young, & Deutsch, 1987). During withdrawal, activity significantly influenced the temperature response in a circadian dependent manner. Acutely withdrawn animals in both the subjective night and day became hyperactive, although greater hyperactivity was seen during the subjective day. When the influence of activity on body temperature was accounted for, the magnitude of the effect was significantly lower during the subjective day. In humans hyperactivity and hyperthermia are common symptoms of withdrawal in dependency (White, Frewin, Kaur, Flavel, & McGregor, 1994). While a delayed hyperthermic effect is reported during the night in non-dependent humans kept in bed and administered alcohol throughout a 24 h period (Danel et al., 2001), in dependent individuals, a relationship between hyperthermia and activity may still exist, as both are well documented withdrawal responses (White et al., 1994).

Dependent and recovering alcoholics continue to exhibit perturbations in a number of circadian-regulated systems well into abstinence (Danel et al., 2009; Danel & Touitou, 2004; Drummond et al., 1998; Rosenwasser, 2001) suggesting that circadian disruptions either contribute to the development of the addiction or are instead a byproduct of the dependent state. In humans, situations that promote circadian desynchrony (e.g., shift-work) or abnormal rhythmicity are correlated with alcohol dependence (Trinkoff & Storr, 1998; Webb, Redman, Hennrikus, Rostas, & Sanson-Fisher, 1990), while in rodents, shifts of the LD cycle sometimes, but not always, promote voluntary alcohol consumption (Clark et al., 2007; Gauvin et al., 1997; Rosenwasser, Clark, Fixaris, Belanger, & Foster, 2010). Genetic studies have suggested that circadian gene mutations could also be associated with addiction

(Perreau-Lenz & Spanagel, 2008). Conversely, acute and chronic alcohol exposure directly influences SCN functioning and inhibits photic and non-photic phase shifts in rodents (Brager et al., 2010, 2011; Prosser et al., 2008; Ruby, Brager, et al., 2009; Ruby, Prosser, et al., 2009). The current data set demonstrated clear disruptions in the core body temperature rhythm during acute withdrawal, specifically during the subjective day. These perturbations may be suggestive of a robust change to circadian rhythmicity following extended alcohol exposure and withdrawal (however, we believe that the current design was not ideally suited to study sustained effects during protracted abstinence periods).

Some authors have interpreted changes in body temperature as a phase shift to the underlying circadian rhythm (Baird et al., 1998). With the present use of skeleton photoperiods this might be expected to lead to inversions of the rest/activity rhythms with respect to the lighting regime over the course of the experiment. No such inversions were noted. Instead our data support an alternative interpretation, that the amplitude of the body temperature rhythm is attenuated following ethanol exposure (Baird et al., 1998). Baird et al. (1998) found that animals exposed to an acute i.p. injection of alcohol one hour into the dark cycle shortened the period of temperature and activity and dose dependently attenuated the body temperature amplitude. Together, these findings imply alcohol dependency, generated by the schedule of alcohol administration and circadian context, may drive lasting alterations to circadian functioning.

One of our initial working hypotheses was that the schedule of alcohol administration and withdrawal would produce sensitized changes to the circadian body

temperature rhythm analogous to the kindling like effect on seizure activity (Becker, Diaz-Granados, & Hale, 1997); however, the evidence from the current study is not highly supportive of such a conclusion. In humans, prior detoxification experience is highly correlated with relapse (Duka et al., 2004), while in rodents repeated periods of intoxication and withdrawal increase operant responding (O'Dell et al., 2004), physical dependence (Becker, Diaz-Granados, & Hale, 1997; Becker, Diaz-Granados, & Weathersby, 1997), and consumption (Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005). Mice in the current study exhibited sensitization across rounds only in the hyperthermic response during acute withdrawal, but this response was not present in the unmasked body temperature, and the blood ethanol content exhibited an increasing pattern across rounds, likely contributing to this perceived sensitization effect.

In conclusion, withdrawal-related perturbations in body temperature are associated with an increased risk of mortality in humans (Khan et al., 2008), and some have suggested that these fluctuations may be circadian dependent (Gross et al., 1975). Unfortunately, many pharmacological treatments for alcoholism and symptomatic withdrawal do not fully alleviate withdrawal related changes in body temperature (Pietrzak & Czarnecka, 2005; Spanagel, Putzke, Stefferl, Schobitz, & Zieglansberger, 1996). Our findings suggest that body temperature fluctuations during withdrawal may be governed by the circadian phase of alcohol exposure/withdrawal. Control of the temporal context of alcohol exposure and withdrawal may, therefore, prove useful in the mitigation of circadian regulated physiological systems. In a clinical setting, this may involve the coordinated management of intoxication until the desired circadian

phase for withdrawal is reached. Management of intoxication is already used in the clinical setting and its use in conjunction with circadian phase may improve treatment outcomes during withdrawal.

Acknowledgements: Chapter 2, in part, is a reprint of the material as it appears in: Damaggio, A. S., & Gorman, M. R. (2014). Circadian phase determines effects of repeated ethanol vapor exposure and withdrawal on body temperature and activity rhythms of male mice. Alcohol Clinical and Experimental Research, 38(3), 879-888. The dissertation author was the primary investigator and author of this paper.

2.5 Figures

Figure 2.1 (opposite): Representative double-plotted actograms of locomotor activity (left) and core body temperature (right) during the 3rd round of EtOH vapor exposure and withdrawal for experimental (A, B, E and F) and control (C, D, G and H) animals over the subjective day (sDay) and subjective night (sNight). Black bars atop each panel indicate lights-off during the 2 h skeleton photoperiods. Note the shifted phase of activity and temperature rhythms in the top versus bottom four panels. Intervals of EtOH/air exposure and air/only in acute withdrawal are single-plotted in dark and light grey shading respectively. Each row of telemetry data illustrates a new day of recording with two-days of recording displayed per row. Four days of baseline data are depicted, followed by the 3-daily cycles of EtOH exposure and withdrawal and 6 of the 11 day recovery period.

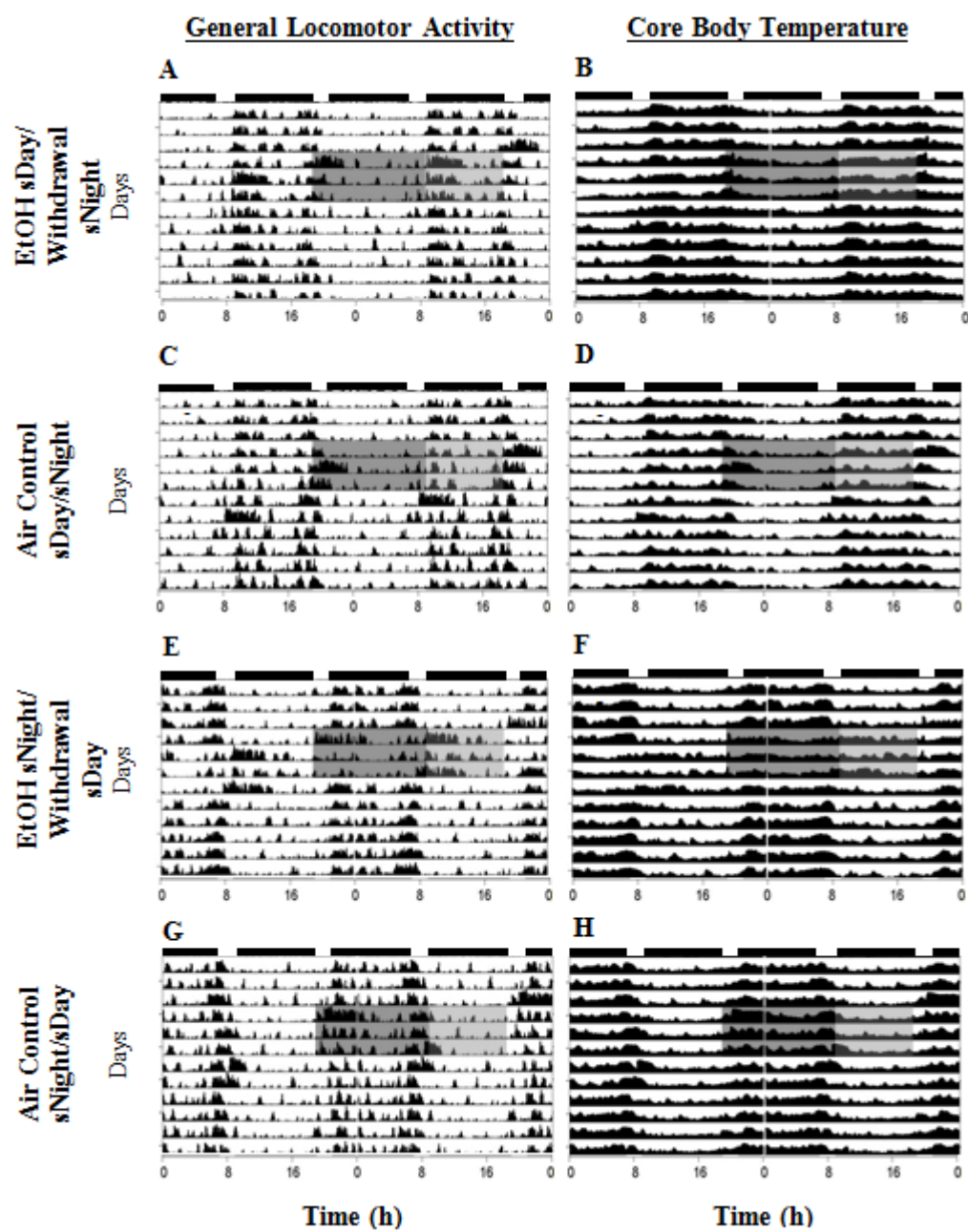


Table 2.1: Mean $\pm$ S.E.M. blood ethanol content (BEC) averaged within each round of ethanol (EtOH) vapor exposure. BEC was measured at the start of each 0700-0900 interval of the skeleton photoperiod during the final 2 h of the 14 h EtOH vapor inhalation. Mice exposed to EtOH during the subjective night (sNight) and subjective day (sDay) had significantly elevated BEC across all rounds, which progressively increased.

Mean Blood Ethanol Content (mg/dL)					
	Round				
	1	2	3	4	Average
EtOH sDay	160.8 $\pm$ 8.3	212.5 $\pm$ 12.5	211.6 $\pm$ 11.5	263.6 $\pm$ 9.8	212.1 $\pm$ 7.0
EtOH sNight	172.6 $\pm$ 11.6	206.7 $\pm$ 9.4	213.9 $\pm$ 10.9	247.2 $\pm$ 7.6	210.1 $\pm$ 6.1

Figure 2.2 (opposite): Mean core body temperature (top) locomotor activity (middle) and activity independent body temperature (bottom) rhythms averaged over the 3 days of exposure to EtOH vapor and withdrawal (experimental groups; solid line) or air-only (controls; dashed lines). The time of vapor is indicated by the black bar and coincided with the mice's subjective night (sNight; left) or day (sDay; right). Gray shading indicates lights off of the 2 hour skeleton photoperiod. Data shown are from the final of 4 rounds of vapor exposure. Mice receiving ethanol vapor inhalation (solid lines) in the sNight had less activity and lower body temperatures than controls but a greater amount of activity and an elevated body temperature during acute withdrawal. Conversely, animals intoxicated during the sDay closely mirrored their respective controls and demonstrated only slight hypothermia and increases in activity during acute withdrawal.

Averaged Rhythm During
EtOH Exposure and Withdrawal

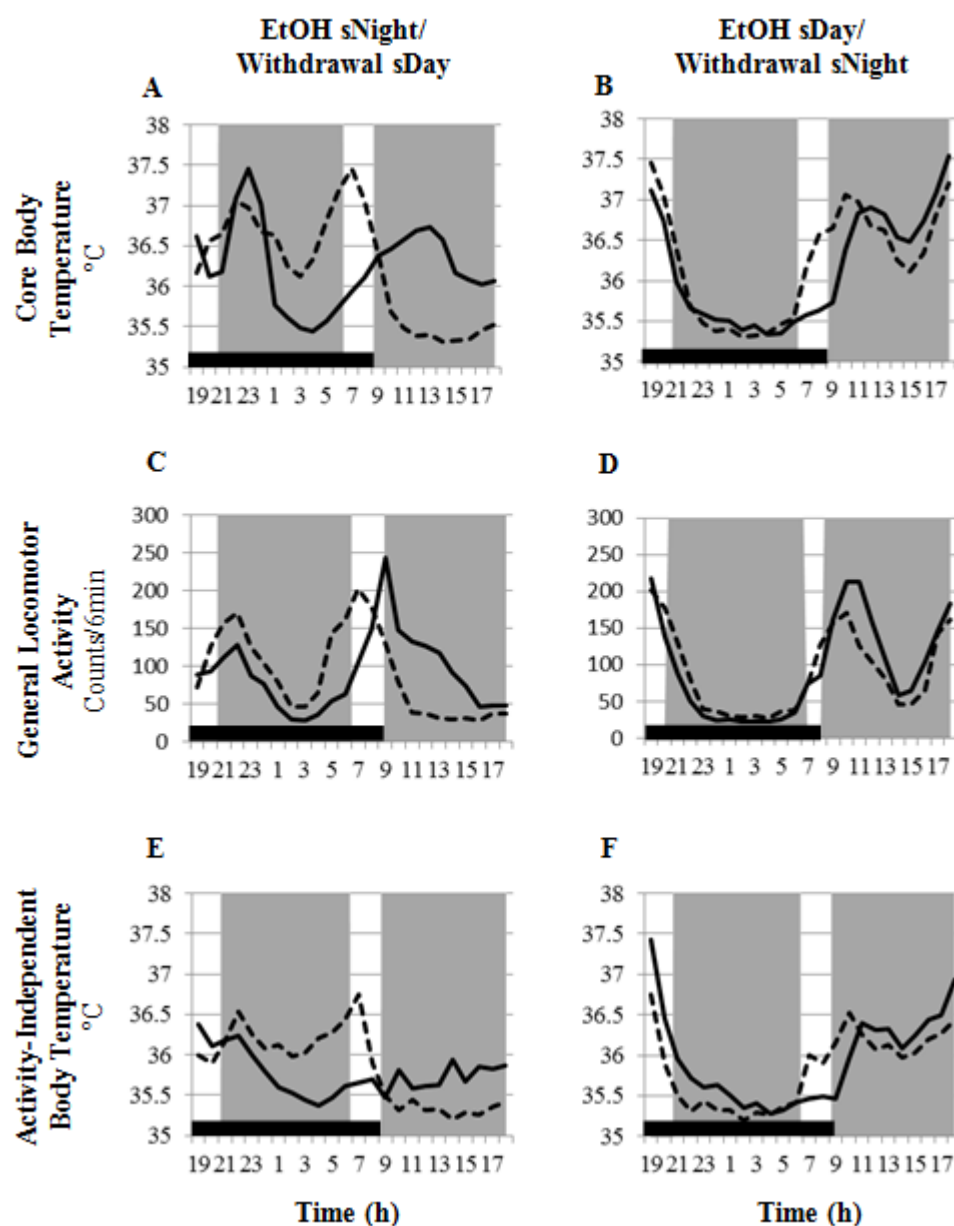


Figure 2.3 (opposite): Net change from baseline of the mean $\pm$ S.E.M. core body temperature (top), general locomotor activity (middle) and activity-independent body temperature (bottom) in the 10 h lights off period averaged across the four rounds of EtOH/Withdrawal. The same sets of mice are presented during both their active EtOH exposure (left) and withdrawal (right). Shading behind graph indicates subjective night (sNight). EtOH exposure in the sNight created significant reductions in activity and body temperature compared to their control and animals exposed to EtOH in the subjective day (sDay). In withdrawal, opposite effects for the same mice now in the sDay were seen for body temperature and the activity independent body temperature compared to their control and animals in the sNight. Animals became hyperactive during withdrawal in both circadian phases although the magnitude of the effect was greater during the sDay. * Significant difference between the experimental group and the respective control. ($p < 0.001$) ** Significant difference between the experimental groups ($p < 0.001$).

Net Change from Baseline

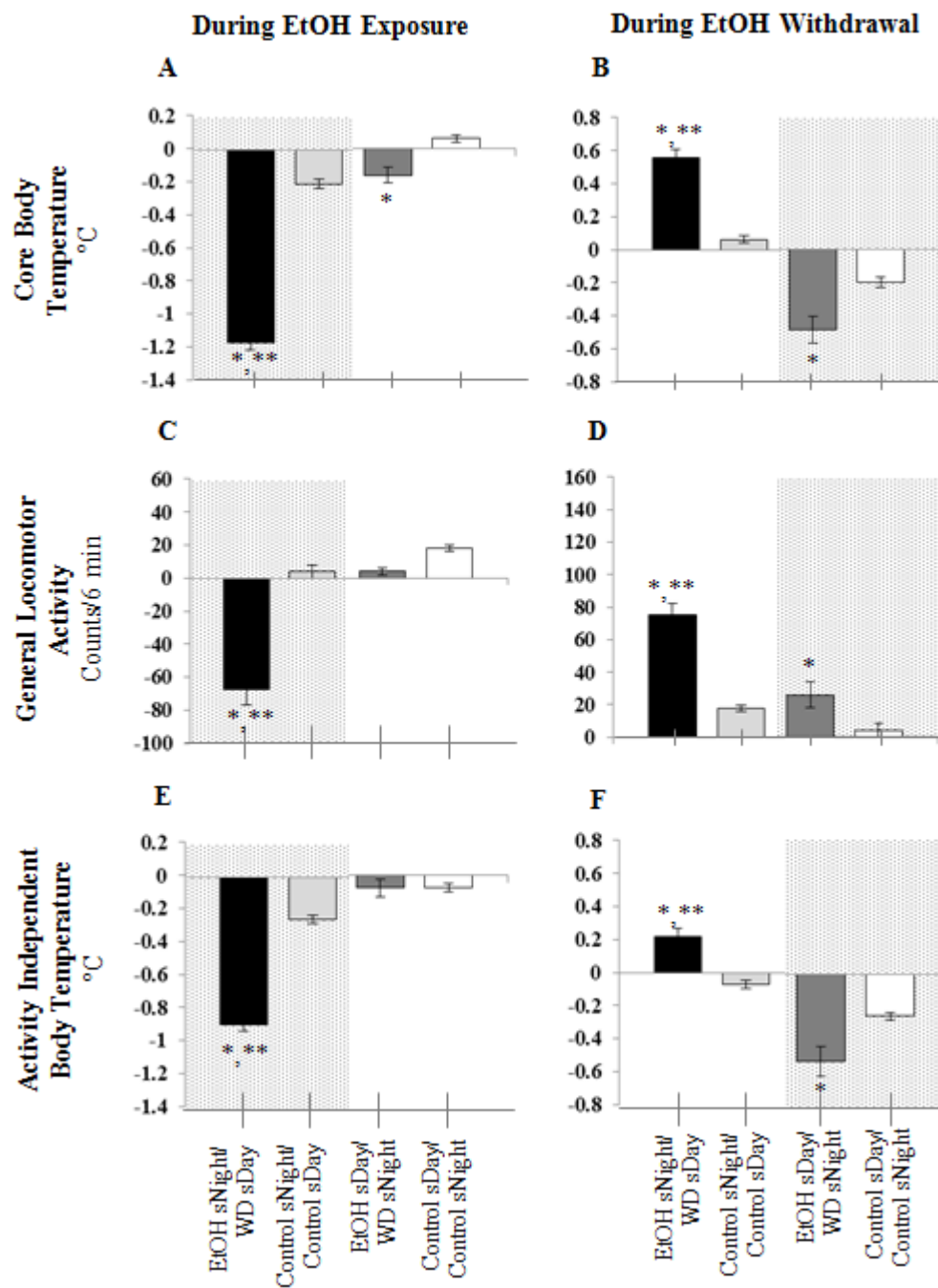


Figure 2.4 (opposite): Summed absolute deviation from baseline rhythm of the mean $\pm$ S.E.M. core body temperature (top), general locomotor activity (middle) and activity independent body temperature (bottom) during active ethanol (EtOH) vapor exposure (A, C, and E) and withdrawal (B, D, and F) in the 10 h lights off period of the skeleton photoperiod averaged across rounds. Shading behind graph indicates subjective night (sNight). Mice exposed to EtOH in the sNight had significant perturbations in activity and body temperature compared to their control and animals exposed to EtOH in the subjective day (sDay). During acute withdrawal, however, animals in both the sDay and sNight were significantly perturbed in activity and body temperature compared to their controls. * Significant difference in Tukey's LSD post-hoc ($p < 0.001$) between the experimental group and the respective control. ** Significant difference in Tukey's LSD post hoc ($p < 0.001$) between the experimental groups

Sum of Hourly Deviation from Baseline

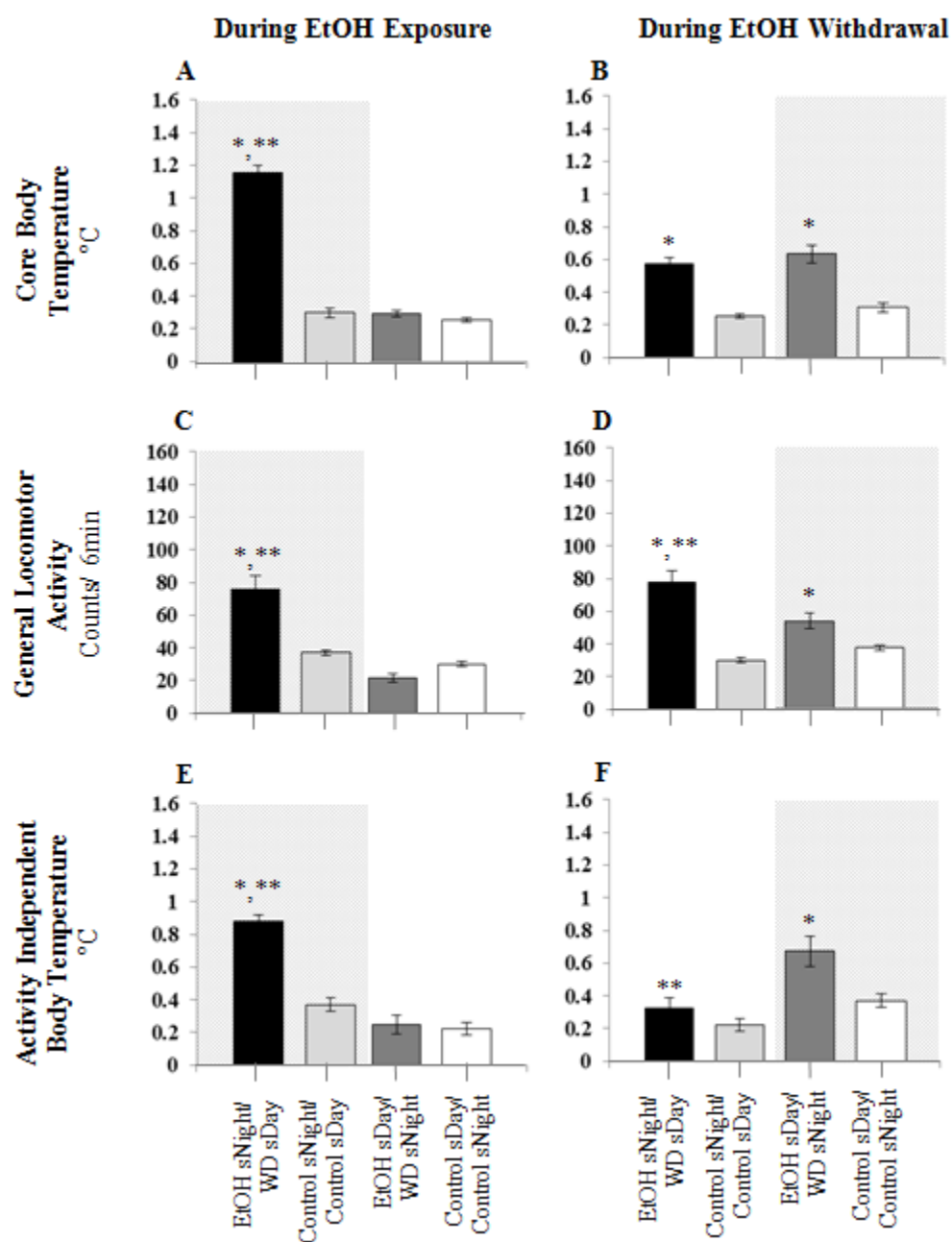
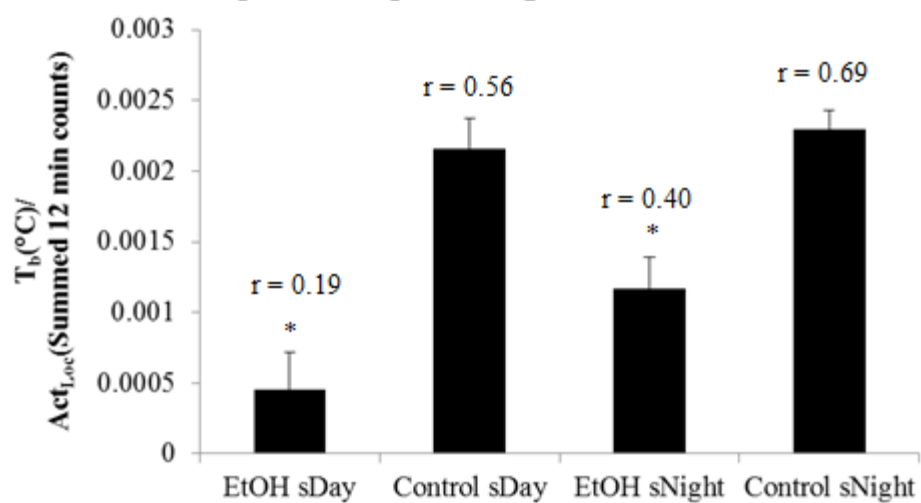
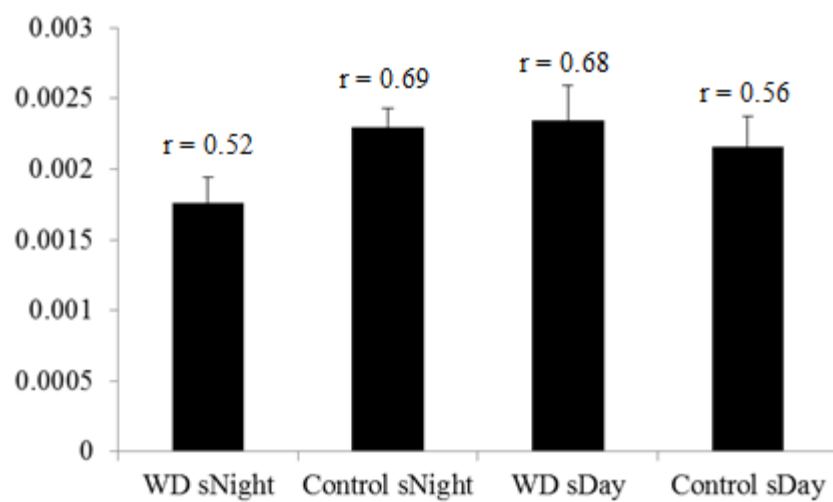


Figure 2.5 (opposite): Mean $\pm$ S.E.M. influence of locomotor activity on the core body temperature rhythm (i.e., slope of the activity independent body temperature) averaged across all rounds of ethanol (EtOH) vapor exposure (A) and acute withdrawal (WD; B). The correlation coefficient between activity and body temperature is reported above each bar. EtOH exposure significantly suppressed the influence of activity on body temperature during the subjective day and the subjective night compared to the respective controls, but this effect was not circadian dependent during active intoxication. *Significant difference between the experimental group and the air controls ($p < .033$).

A. EtOH Exposure Slope Averaged Across Rounds**B. EtOH Withdrawal Slope Averaged Across Rounds**

Chapter 3: Circadian Phase of Intoxication Modifies the Direction of the Core Body Temperature Response during Acute Withdrawal

We previously demonstrated that alcohol vapor inhalation and withdrawal could elicit either hypothermia or hyperthermia during acute withdrawal dependent upon the timing of the exposure/withdrawal cycle (Chapter 2). The current study further elucidates these findings by determining if these reactions are contingent on the timing of alcohol vapor exposure, the timing of acute alcohol withdrawal, or an interaction of both. Two groups of oppositely phased male mice (rest/activity phases were 12 h apart), housed under a 2-hour skeleton photoperiod, were simultaneously exposed to 24 hours of continuous alcohol vapor inhalation followed by a 24 hour acute withdrawal period. This series of exposure/withdrawal was repeated two more times followed by a three day recovery period. In total the mice were subjected to four rounds of the nine day exposure/withdrawal/recovery paradigm. Body temperature and activity were monitored continuously via radio-telemetry. During intoxication mice exhibited greater hypothermia in the subjective night regardless if this phase occurred in the first or second half of vapor exposure. The hypothermic reaction was accompanied by a decrease in locomotor activity. In the first period of acute withdrawal (1-11 hours) both groups exhibited increases in body temperature and activity irrespective of the circadian phase, although the largest effects were measured in the subjective day. Activity had little influence on body temperature

during alcohol exposure but significantly influenced the body temperature response during the acute withdrawal in the subjective day. We conclude that the circadian phase of alcohol exposure drives the acute withdrawal response; however, the magnitude of the effect may be influenced by the circadian phase of withdrawal. These findings suggest that in rodents chronic dependence to alcohol may be characterized by a hyperthermic withdrawal state as it is in humans. Moreover, this study provides further evidence that controlling for the circadian phase of exposure and withdrawal may mitigate the severity of the withdrawal reaction.

3.1 Introduction

In humans, consumption of alcohol follows a wide range of patterns from intermittent binge drinking to chronic and continuous, or near continuous, intoxication in severe alcoholics (Ashley et al., 1976; Epstein et al., 1995; Madden & Jones, 1972a). Abstinence periods may last from several weeks or months in the binge drinker to a few hours in the addicted individual. Like most behavioral, physiological or metabolic processes, however, alcohol consumption follows a strong daily temporal structure. In the non-dependent individual consumption occurs primarily in the early evening, but in the transition to dependence the onset of drinking occurs earlier in the day (Danel et al., 2003). As a consequence of the consumption pattern, alcohol withdrawal may also be temporally structured likely occurring during the late evening in the addicted individual. This patterning of consumption and withdrawal may elicit lasting physiological and behavioral changes for the addicted individual and/or may influence the development of the addiction (Damaggio & Gorman, 2014b).

Like many drugs, the response to alcohol depends critically on the timing of its administration in both humans and rodents (Baird et al., 1998; Danel et al., 2001; Eastman et al., 1994). In humans, acute alcohol exposure is typically considered to elicit hypothermia during the day and hyperthermia during the sleep cycle (Danel et al., 2001; Danel & Touitou, 2004; Eastman et al., 1994). In alcoholics, withdrawal from alcohol elicits a hyperthermic body temperature response (American Psychiatric Association, 2013; White et al., 1994), which some have suggested may be circadian dependent (Gross et al., 1975). In turn, withdrawal related hyperthermia is linked to withdrawal severity and mortality (Khan et al., 2008; Ritzmann & Tabakoff, 1976a) while repeated withdrawal episodes are associated with relapse (Duka et al., 2004). The attenuation of the withdrawal reaction is highly clinically significant. The employment of managed intoxication for alcoholics is already used in the clinical setting to ensure favorable treatment outcomes following injury (e.g., Hansbrough et al., 1984). Managed intoxication may, therefore, be used in combination with time of day to attenuate the subsequent withdrawal response by controlling for the circadian phase of exposure and/or withdrawal.

Conversely, in rodents hypothermia is typically reported following both acute and chronic alcohol exposures (Crawshaw et al., 1998; Ritzmann & Tabakoff, 1976a; Williams et al., 1993) while withdrawal related hyperthermia remains controversial (Spanagel et al., 1996). For example, chronic exposure to alcohol via liquid diets in rats is generally reported to induce hypothermia during withdrawal (Ritzmann & Tabakoff, 1976a, 1976b). Hyperthermia, on the other hand, is expected following alterations to environmental temperatures (Finn, Bejanian, Jones, Syapin, & Alkana,

1989; Finn, Syapin, Bejanian, Jones, & Alkana, 1994; Myers, 1981), environmental stress (Melchior & Allen, 1993; Peris & Cunningham, 1986, 1987; Sinclair & Taira, 1988), or as a so-called “rebound effect” following alcohol induced hypothermia (Gallaher & Egner, 1987; Sinclair & Taira, 1988). As in humans, mortality risks are greater in rodents following alcohol induced hyperthermia (Finn et al., 1989; Gordon, 2010); however, an animal model of alcohol dependence that elicits hyperthermia under standard environmental conditions has yet to be defined. An animal model of withdrawal related hyperthermia would provide a more accurate representation of the human condition and would allow for further research into possible therapeutic targets of dependency.

We previously showed that chronic alcohol vapor inhalation and withdrawal in rodents could elicit either hypothermia or hyperthermia during acute withdrawal dependent upon the circadian phase of the exposure/withdrawal cycle (Damaggio & Gorman, 2014a). Specifically, withdrawal during the subjective day (chronic exposure during the subjective night) appears to have a hypothermic effect on body temperature while the converse elicits hyperthermia. The exposure/withdrawal effects in this study were isolated by circadian phase (i.e., exposure during the subjective night/withdrawal during the subjective day or vice versa); however, it is unclear if the divergent body temperature response is driven by the temporal phase of the exposure and/or withdrawal. Specifically, these reactions may arise from 1) prolonged alcohol exposure within a specific circadian phase, 2) the time of day in which acute withdrawal occurred, or 3) an interaction of the exposure/withdrawal cycle.

In order to test these hypotheses, we utilized a 24 h exposure/withdrawal paradigm whereby alcohol exposure occurred in both circadian phases while limiting the acute withdrawal reaction (0-12 h into withdrawal) to a single circadian phase. If the previously reported differential acute withdrawal response (Chapter 2) was driven by phase of alcohol exposure then we would expect the body temperature response to be limited to one direction (i.e., hypothermia or hyperthermia only) as exposure to alcohol in the current experiment occurred equally in both circadian phases. Conversely, if the responses were due to the timing of acute withdrawal, then we would expect contrasting withdrawal responses (i.e., hyperthermia and hypothermia) since acute withdrawal was limited to a single circadian phase that differed between the two groups.

3.2 Methods

3.2.1 Subjects

Twenty-four male C57Bl/6J mice (Jackson Laboratories, Sacramento, CA) six weeks of age were housed under standard temperature (22°C) and lighting (270 lux) conditions. Mice had ad libitum access to food (Purina chow) and water throughout the experiment. The animals were group housed (3-4 per cage) in plastic shoebox cages until the start of baseline data collection. All experimental procedures and animal care were approved by and conducted under the guidelines of the Institutional Animal Care and Use Committee at University of California, San Diego.

3.2.2 Experimental Lighting Conditions

The mice were randomly divided and entrained to one of two opposing 14:10 light:dark (LD) cycles as previously described (see Chapter 2). After stable entrainment and recovery from telemetry implantation, all mice were individually housed and transferred to a common “skeleton photoperiod” consisting of two, 2 h light pulses that replace the first and last fractions of the original 14 h light phase (lights on from 0800-1000 and 2000-2200). This yielded two groups of animals entrained to a single lighting regime and housing environment but with oppositely-phased subjective days and nights (sDay, sNight). Each of the two entrainment groups was subdivided to receive either ethanol (EtOH) vapor or air only (EtOH sNight/Withdrawal sDay, n=8; Control sNight /Control sDay, n=4; EtOH sDay/Withdrawal sNight, n=8; Control sDay / Control sNight, n=4). All data from two animals was excluded from analysis for faulty telemetry data (n=1 EtOH sNight/Withdrawal sDay) or mortality (n=1 Control sNight/Control sDay).

3.2.3 Surgery

One week after habituation to the original LD conditions, mice were anesthetized with isoflurane gas approximately 2 h into their respective light phases. Telemetry units (G2 E-Mitter, Respironics, Inc., Bend, OR) were implanted into the peritoneal cavity and attached to the abdominal wall. A single dose of buprenorphine (0.05 mg/kg) was administered for pain relief. Animals were returned to their LD condition once ambulatory and allowed to recover for seven days before being transferred to the skeleton photoperiod for baseline data collection.

3.2.4 Alcohol Vapor Inhalation and Rounds

The experimental groups received four rounds of EtOH vapor inhalation and withdrawal commencing at 10 weeks of age. Each round was a nine day protocol that included six days of EtOH exposure and withdrawal, followed by three additional days of recovery. Animals were run in two identical squads spaced one day apart.

Following collection of baseline data, mice received an acute i.p. injection of EtOH (1.5g/kg at 20% v/v) and pyrazole HCl (0.0681 g/kg) at 0900 and were immediately placed in modified cages attached to an EtOH vapor inhalation apparatus (La Jolla Alcohol Research Inc., La Jolla, CA). EtOH vapor was administered continuously for 24 h terminating at 0900 the next day. Controls were injected with an equivalent dose of pyrazole and saline but did not receive EtOH vapor inhalation.

At the end of vapor inhalation the cages of EtOH exposed animals were moved to racks within the room and allowed a 24 h withdrawal period before the cages were once again connected to the EtOH vapor machine. Control cages were disturbed in a similar manner as the experimental groups. This cycle of exposure/withdrawal was repeated two additional times before the animals underwent a three day recovery period. In total the mice received 4 rounds of the nine day protocol of vapor inhalation, withdrawal, and recovery. Cage changes occurred at the start of each round of vapor.

The regulated production of EtOH vapor is described elsewhere (Gilpin et al., 2008). Briefly, EtOH vapor was created by dripping 95% EtOH in a 2000-ml Erlenmeyer vacuum flask warmed to 50°C. Air was blown through the flask at 11

liters/min. The air/EtOH combination was in turn pumped into each attached chamber housing the experimental animals.

3.2.5 Blood Ethanol Content

At the start of the 0800-1000 skeleton light pulse, tail blood (0.05 ml) was collected from each mouse in heparinized tubes prior to the end of each day of vapor inhalation and assayed to determine blood ethanol content (BEC). Target BEC was between 175-250 mg/dl. Because EtOH is a known analgesic, controls received a pinch and a milking motion to the tail, but no blood was collected.

3.2.6 Data Analysis

Baseline telemetry data was collected for a minimum of one week prior to experimentation. Core body temperature and general locomotor activity were sampled at six minute intervals using VitalView Data Acquisition System software (Respironics, Inc; Bend, OR) and analyzed using Excel 2010 (Microsoft, Inc; Bellevue, WA) and SPSS (IBM Corp., Armonk, NY). To avoid any effects of bleeding, handling, injecting, or exposure to light on body temperature or activity, data analysis was restricted to the 10 h intervals of darkness between skeleton pulses, when animals were left undisturbed. Data were averaged across the three days of exposure or withdrawal within each round. Additional analyses were conducted with data collapsed across rounds when appropriate.

The daily rhythms of body temperature and locomotor activity did not differ between the two air-exposed control groups, adjusting for their oppositely phased entrainment. Accordingly, the active phases of all control mice were combined for a

composite sNight control group, and the inactive phases were combined for a composite sDay control group.

In order to quantify the effect of activity on body temperature we used linear regression analysis previously utilized by Weinert and Waterhouse (1998). Activity was summed over the course of 6-60 min to determine the best correlation between the integrated effects of activity on the resulting body temperature. A 12-minute integration time was used with a mean correlation between activity and body temperature of 0.59, with a range of 0.19-.78 between different animals. During intoxication and withdrawal the preceding 12 min of summed activity was correlated with the body temperature measurement. This 12 min integration time was used, in conjunction with linear regression, to calculate the hourly activity-independent body temperature ($Act_{ind} T_b$; intercept) and to quantify the hourly influence of activity on body temperature (slope).

The effects of EtOH on body temperature, activity, and the $Act_{ind} T_b$ were calculated using 1) the net changes in these variables from baseline, 2) as a non-directional rhythm disruption from baseline, and 3) as a correlation between the BEC and the resulting exposure/withdrawal response within each round. For each animal, values during EtOH/air and withdrawal were calculated relative to a three-day moving baseline interval (i.e., the three-day recovery period) immediately prior to EtOH/air exposure and were averaged over the 10 h periods of darkness. Rhythm disruption was calculated by summing the average hourly absolute difference from baseline across both 10 hour periods of darkness (i.e., 20 h total). An additional analysis was also conducted using these two techniques to examine any lasting changes to the body

temperature and activity rhythms during the three-day recovery period. Lastly, correlations were calculated between BEC at the end of vapor inhalation and net change from baseline during 1) the second 10 h dark alcohol exposure period, 2) the first 10 h dark period of acute withdrawal, and 3) the second 10 h dark period of acute withdrawal during each round of vapor inhalation/withdrawal.

Data were analyzed using an ANOVA with circadian phase (sDay/sNight) and exposure (EtOH/Air Control) as between-subject variables, and a significance level of $p < 0.05$ was set for all tests. Post-hoc tests were conducted using Tukey-Fisher least significant difference (LSD).

3.3 Results

Representative actograms during the skeleton photoperiod in the third round of EtOH vapor inhalation and withdrawal are shown in Figure 3.1. Visual inspection confirmed in all animals that the circadian phases remained stable with respect to the skeleton photoperiod throughout the entire experiment. Prior to EtOH administration, locomotor activity and body temperature rhythms exhibited clear increases during the subjective night (sNight) and lower values during the subjective day (sDay). A bimodal peak in activity occurred during the sNight, which was further mirrored in the body temperature throughout baseline collection. During intervals of intoxication, EtOH-exposed animals showed marked periods of decreased activity and body temperature in the sNight (Figure 3.1 A, B, E, F). Immediately following EtOH, mice exhibited increases in both the locomotor activity and body temperature rhythms in both circadian phases (i.e., sNight and sDay).

Blood ethanol content (BEC) was elevated into the target range, averaging 196.2 mg/dl in the sDay and 241.0 in the sNight (Table 3.1). Within-subject tests revealed a significant main effect of round ($F_{3,33}=8.471$, $p<0.001$) and a round by squad interaction ($F_{3,33}=6.638$, $p<0.001$). Post-hoc comparisons revealed no significant difference between the squads across rounds ($t_{1,13} = -0.582$ $p<0.570$). Animals were subsequently collapsed across squads. An ANOVA revealed a significant main effect of round ($F_{3,39} = 6.116$ $p<0.002$), which visual inspection of the data confirmed to be linear, but not the interaction of round by group. A between group analysis indicated a significant main effect for group ($F_{1,13} = 4.758$ $p<0.048$) revealing that on average animals starting vapor inhalation in the subjective night (ending in the sDay) had significantly lower BECs at the time of collection than animals starting in the sDay (ending in the sNight) despite equivalent exposure levels of EtOH.

3.3.1 Multiple Rounds of EtOH Vapor Inhalation Does Not Elicit Sensitization or Tolerance Compared to Baseline Levels

Groups were initially analyzed across rounds to determine if multiple rounds of vapor inhalation sensitize the net change in activity, body temperature, or activity independent body temperature responses from baseline in either the first or second dark portion of the skeleton photoperiod. No round effects were seen either during EtOH exposure or during acute withdrawal for any of these measurements.

Multiple rounds of alcohol exposure and withdrawal, however, did perturb these rhythms overall during active alcohol vapor exposure. A repeated measures ANOVA of the total rhythm disruption during EtOH exposure across the 20 hours of

darkness revealed a significant main effect of round for the body temperature ($F_{2,3,57.5}=5.975$ $p<0.003$), activity ($F_{2,3, 57.8} = 6.975$ $p<0.001$), and activity independent body temperature ($F_{2,1, 52.6} =4.389$ $p<0.016$); however, visual inspection indicated that these effects were not linear. Conversely, during acute withdrawal a repeated measures ANOVA did not reveal any significant findings for the body temperature or activity independent body temperature rhythm. A significant main effect of round was detected in the general locomotor activity ($F_{1,9, 46.2} = 7.917$ $p<0.001$). Visual inspection confirmed a progressive tolerance to perturbation in activity during acute withdrawal.

Data was subsequently collapsed across rounds for all future analyses because 1) a round effect was not found in the net change from baseline in our measurements, 2) BECs intensified across rounds likely contributing to any alterations in rhythm perturbations, and 3) the perturbations across rounds were not clearly linear (with the exception of withdrawal related perturbations in activity).

3.3.2 Chronic Alcohol Exposure Lowers Body Temperature Dependent upon the Time of Day

In the interest of clarity, the responses to repeated ethanol inhalation schedules are presented separately and sequentially for the intoxication and withdrawal phases of the cycle. The average core body temperature in the first 10 h of darkness during EtOH exposure was lowered by 0.92°C in animals in their sNight compared to air controls, but was lowered by only 0.30°C when vapor coincided with the sDay compared to controls (Figure 3.2A). During the first 10 h of darkness, an ANOVA of the core body temperature yielded a significant main effect of exposure

($F_{1,25}=121.055$, $p<0.001$) and phase ($F_{1,25}= 54.527$, $p<0.001$), as well as their interaction ($F_{1,25}=31.279$, $p<0.001$). Post-hoc analysis indicated that EtOH lowered the average body temperature compared to air controls at both phases of the daily cycle (sDay, $p<0.004$; sNight, $p<0.001$), but that the reduction was greatest when exposure coincided with the sNight ($p<0.001$).

In the second 10 h of darkness, the body temperature was lowered by 1.14°C in animals in the sNight and 0.50°C in animals in the sDay compared to air controls (Figure 3.2B). An ANOVA indicated a significant main effect of exposure ($F_{1,25}=149.813$, $p<0.001$), phase ($F_{1,25}=40.637$ $p<0.001$), and their interaction ($F_{1,25}=23.822$, $p<0.001$). Post-hoc analysis revealed that EtOH vapor exposed animals had a significantly lower body temperature compared to air controls ($p<0.001$), but that the greatest decreases occurred during the sNight (in animals previously exposed to EtOH in the sDay; $p<0.001$).

Further analysis of the net change in body temperature compared across the first and second 10 h period of darkness indicated that the magnitude of the sDay hypothermia was greater following longer intervals of EtOH exposure (i.e., sDay hypothermia was greater during 13-23 h of EtOH exposure compared to 1-11 h of EtOH exposure; $t_{1,13} = -2.424$, $p<0.031$). Conversely, there was only a trending difference in the amount of hypothermia that occurred during the sNight ($t_{1,13}= 2.128$, $p<0.053$). BEC, measured at the end of vapor inhalation, was not significantly correlated with exposure related hypothermia in the second period of darkness.

The overall perturbation in the body temperature rhythm (i.e., area under the curve) across both 10 h periods of darkness during EtOH exposure was increased by

65% during the sNight and by 68% during the sDay compared to air controls (Figure 3.3 A). An ANOVA of the absolute change in body temperature from baseline summed over both 10 h periods of darkness yielded a significant main effect of exposure ($F_{1,25}=168.411$, $p<0.001$), but not of phase or their interaction. Post-hoc analysis indicated animals exposed to EtOH vapor inhalation were significantly more disrupted in their body temperature rhythm than their respective air controls ($p<0.001$), but animals exposed to EtOH did not differ from one another.

3.3.3 Alcohol Exposure Suppresses Activity During the Subjective Night

General locomotor activity, averaged over the first 10 h period of darkness during EtOH vapor inhalation, was lowered by 36.1 counts/6 min (a reduction of 620%) in animals in the sNight compared to air controls but was increased by 1.8 counts/6 min (an increase of 18%) in animals exposed during the sDay (Figure 3.2C). An ANOVA of the average change in locomotor activity from baseline indicated a significant main effect of exposure ($F_{1,25}=4.621$, $p<0.041$), phase ($F_{1,25}=8.318$, $p<0.008$), and their interaction ($F_{1,25}=5.643$, $p<0.026$). Post-hoc test confirmed that animals exposed to EtOH during their sNight had a significant net reduction in activity compared to air controls ($p<0.045$) and animals exposed during the sDay ($p<0.025$).

In the second 10 h period of darkness and EtOH vapor exposure animals had a 24% decrease in activity in the sDay and a 977% decrease in activity in the sNight compared to air controls (Figure 3.2D). An ANOVA indicated a significant main effect of exposure ($F_{1,25}=31.292$, $p<0.001$), phase ($F_{1,25}=34.868$, $p<0.001$), and their interaction ($F_{1,25}=26.411$, $p<0.001$). Post-hoc testing indicated a significant reduction in the activity of animals in the sNight (previously in the sDay) compared to air

controls ($p < 0.001$) and animals in the sDay (previously in the sNight; $p < 0.001$). Further analysis revealed no significant difference in the amount of hypoactivity across the two 10 h periods corresponding with sNight (i.e., hypoactivity was equivalent during the sNight regardless if the sNight occurred in the first or second phase of EtOH vapor inhalation). The severity of the ending BEC was not correlated with a reduction in activity during the second portion of alcohol exposure in either the sNight or sDay.

The summed absolute deviation in activity from baseline yielded a 23% increase in the overall perturbation of the activity rhythm in animals starting EtOH exposure in the sNight compared to air controls, while those exposed to EtOH starting in the sDay exhibited a 39% increase in rhythm disruption compared to controls (Figure 3.3B). An ANOVA confirmed a main effect of exposure ($F_{1,25}=24.893$, $p < 0.001$), but not of phase. Post-hoc analysis indicated that animals exposed to EtOH vapor had significantly more disrupted activity rhythms compared to their air controls (starting EtOH sDay, $p < 0.001$; starting EtOH sNight $p < 0.044$), but no significant difference in the amount of perturbation between the experimental groups was found.

3.3.4 Independent of Activity, Alcohol Influences the Endogenous Body

Temperature Rhythm during EtOH Exposure

The computed activity-independent body temperature ($Act_{Ind} T_b$; see methods) averaged across the first 10 h period of darkness was lowered by 0.66°C when EtOH vapor inhalation was initiated during the sNight compared to air controls, but only by 0.20°C when vapor exposure began during the sDay (Figure 3.2E). An ANOVA yielded a significant main effect of exposure ($F_{1,25}=83.049$, $p < 0.001$), phase

($F_{1,25}=42.580$, $p<0.001$), as well as their interaction ($F_{1,25}=23.378$, $p<0.001$). Post-hoc comparisons indicated that animals exposed to EtOH in either circadian phase displayed hypothermia compared to air controls (sDay, $p<0.004$; sNight $p<0.001$), but that the severity of the hypothermia in the $Act_{Ind} T_b$ was greater when EtOH was administered during the sNight compared to the sDay ($p<0.001$).

During the second 10 h period of EtOH exposure and darkness the $Act_{Ind} T_b$ was lowered by 0.40°C in the sDay and by 0.90°C in the sNight compared to air controls (Figure 3.2F). An ANOVA indicated a significant main effect of exposure ($F_{1,25}=140.029$, $p<0.001$), phase ($F_{1,25}=36.840$, $p<0.001$), and their interaction ($F_{1,25}=21.298$, $p<0.001$). As in the first portion, animals exposed to EtOH vapor in either circadian phase had significantly lower $Act_{Ind} T_b$ compared to air controls ($p<0.001$), however, the magnitude of this effect was greater during the sNight ($p<0.001$) compared to EtOH exposure in the sDay. Additional analysis revealed that the severity of hypothermia was greater in the second portion of EtOH vapor inhalation in both the sNight ($t_{1,13}=2.326$, $p<0.037$) and in the sDay ($t_{1,13} = -0.542$, $p<0.004$). The BEC at the end of alcohol exposure correlated with the severity of the hypothermic response in only one out of eight tests (four rounds and two treatment groups).

The total amount of disruption in the $Act_{Ind} T_b$ during 20 h of EtOH vapor exposure increased by 62% in mice starting vapor inhalation during the sNight and by 66% during the sDay compared to air controls (Figure 3.3E). An ANOVA of the summed absolute change in the $Act_{Ind} T_b$ during the dark portions of the skeleton photoperiod and EtOH intoxication indicated a significant main effect of exposure

($F_{1,25}=113.942$, $p<0.001$), but not of phase or their interaction. Post hoc tests indicated that the $Act_{ind} T_b$ was disrupted in intoxicated animals compared to air controls ($p<0.001$), but did not differ between the EtOH exposed mice.

The slope of the linear regression model estimated the magnitude of the influence of activity on body temperature (i.e., how much a unit of activity increases body temperature; see Figure 3.5). Compared to air exposure, this influence was reduced during EtOH vapor inhalation by 62% in the sNight and 30% in the sDay in the first 10 h period of darkness. An ANOVA of the slope data in the first 10 hours of darkness during active EtOH exposure revealed a significant main effect of exposure ($F_{1,25}=59.280$, $p<0.001$), phase ($F_{1,25}=19.043$, $p<0.001$), and their interaction ($F_{1,25}=5.243$ $p<.0310$). Post-hoc analysis indicated that activity had less influence on the body temperature of mice exposed to EtOH during the sNight ($p<0.001$) and the sDay ($p<0.002$) compared to their respective air controls, however, during intoxication activity had the least amount of influence on the body temperature if EtOH vapor coincided with the sNight ($p<0.001$) in the first 10 h period of darkness (Figure 3.5A).

In the second 10 hours of darkness during EtOH exposure the influence of activity on body temperature was reduced by 24% in the sNight and 30% in the sDay compared to air controls. An ANOVA of the slope indicated a significant main effect of exposure ($F_{1,25}=15.385$ $p<.001$) and phase ($F_{1,25}=4.242$ $p<.050$), but not their interaction. Post-hoc analysis revealed that activity had less influence on the body temperature of animals exposed to EtOH in the sNight (previously in the sDay) compared to air controls ($p<0.002$). There was no significant difference between

experimental animals or animals in the sDay (previously in the sNight) compared to their air controls (Figure 3.5C).

3.3.5 Alcohol Withdrawal Has a Varying Hyperthermic Response as a Function of the Circadian Cycle

EtOH withdrawal, in the first 10 h period of darkness and initiated during the sNight, increased core body temperature by 0.26°C compared to air controls. During the sDay, the magnitude of the hyperthermia was greater, increasing body temperature by 0.54°C (Figure 3.4A) compared to controls. An ANOVA yielded significant main effects of exposure ($F_{1,25}=62.588$, $p<0.001$), circadian phase ($F_{1,25}=14.952$, $p<0.001$), as well as their interaction ($F_{1,25}=7.995$, $p<0.009$). Post-hoc analysis revealed that animals in the first 10 h darkness period of acute withdrawal from EtOH had an elevated body temperature in both the sDay and sNight compared to air controls (sNight, $p<0.001$; sDay, $p<0.001$), but this increase was greatest in animals during the sDay ($p<0.001$). Moreover, BEC was not significantly correlated with the degree of the hyperthermic reaction.

During the second 10 h period of darkness withdrawal increased core body temperature by 0.18°C during the sDay and by 0.04°C during the sNight compared to air controls (Figure 3.4B). An ANOVA indicated a significant main effect of phase ($F_{1,25}=11.924$, $p<0.002$) and exposure ($F_{1,25}=9.397$, $p<0.005$), but not their interaction ($F_{1,25}=3.890$, $p<0.060$). Post-hoc analysis revealed that animals in the sDay (previously withdrawing in the sNight) had an elevated body temperature compared to controls ($p<0.009$) and animals in the sNight ($p<0.001$).

Further analysis revealed that the acute withdrawal responses were more severe during the first half of acute withdrawal. A comparison of the identical circadian phases between the first and second 10 hour periods of darkness revealed that withdrawal related hyperthermia was greater in both the sNight ($t_{1,13}= 4.205$, $p<0.001$) and sDay ($t_{1,8,803}= -5.906$, $p<0.001$) immediately following EtOH exposure.

The overall amount of perturbation in body temperature from baseline during both 10 h dark periods of acute withdrawal increased by 12% in the sNight and by 40% in the sDay compared to air controls (Figure 3.3B). An ANOVA of the absolute change in body temperature from baseline indicated a significant main effect of exposure ($F_{1,25}=7.884$, $p<0.010$), but not of phase or their interaction. Post-hoc analysis revealed that animals withdrawing first in sDay (then in the sNight) were significantly more disrupted than air controls ($p<0.011$) or the reverse experimental group ($p<0.014$).

3.3.6 Alcohol Withdrawal Increases Locomotor Activity Differentially

Depending upon the Circadian Cycle

During the first 10 h dark period of EtOH withdrawal, mice previously exposed to EtOH vapor became hyperactive, increasing their locomotor activity by 1316% during the sDay and by 235% percent during the sNight compared to air controls (Figure 3.4C). An ANOVA indicated a significant main effect of exposure ($F_{1,25}= 47.307$, $p<0.001$), circadian phase ($F_{1,25}=13.213$, $p<0.001$), and their interaction ($F_{1,25}= 6.135$, $p<0.020$). Post-hoc analysis showed that both groups withdrawing from EtOH were hyperactive compared to air controls (sDay, $p<0.001$; sNight, $p<0.007$), although the magnitude of this effect was greater during the sDay ($p<0.002$). BECs

prior to acute withdrawal were not correlated with the resulting locomotor withdrawal reaction.

In the second 10 h period of darkness during withdrawal animals in the sDay decreased their activity by 21% while animals in the sNight decreased their activity by 250% compared to air controls (Figure 3.4D). An ANOVA yielded a significant main effect of phase ($F_{1, 25} = 17.972$, $p < 0.001$), exposure ($F_{1, 25} = 8.935$, $p < 0.006$), and their interaction ($F_{1, 25} = 7.847$, $p < 0.010$). Post-hoc analysis indicated that animals in the sNight (previously in the sDay) were hypoactive compared to the sDay animals and air controls ($p < 0.001$; $p < 0.001$). Additional analysis revealed that hyperactivity was greatest in the first portion of acute withdrawal in both the sNight ($t_{1,13} = 7.128$, $p < 0.001$) and the sDay ($t_{1,8.202} = -7.243$, $p < 0.001$) when compared to the equivalent circadian phase in the second 10 h period of darkness and withdrawal.

Acute withdrawal over the course of both 10 h dark periods perturbed general locomotor activity in mice first withdrawing during the sNight by 22% compared to air controls. Mice in the sDay were disrupted by 54% compared to air controls (Figure 3.3D). An ANOVA of the absolute change in activity from baseline averaged over both 10 h periods of darkness during EtOH vapor inhalation confirmed a main effect of exposure ($F_{1,25} = 31.944$, $p < 0.001$), phase ($F_{1,25} = 12.136$, $p < 0.002$), and their interaction ($F_{1,25} = 12.136$, $p < 0.002$). Post-hoc analysis revealed that animals first withdrawing in the sDay had significantly more disrupted activity rhythms compared to their air controls ($p < 0.001$) or those in the sNight ($p < 0.001$).

3.3.7 Alcohol Withdrawal Increases the Endogenous Core Body Temperature Rhythm

In the first 10 h period of darkness of acute withdrawal the $Act_{Ind} T_b$ was elevated compared to air controls by $0.33^{\circ}C$ in the sNight and by $0.34^{\circ}C$ in the sDay (Figure 3.4E). An ANOVA revealed a significant main effect of exposure ($F_{1, 25}=38.053$, $p<0.001$), but not of phase ($F_{1, 25}=4.030$, $p<0.056$) or their interaction ($F_{1, 25}=0.008$, $p<0.930$). Post-hoc analysis indicated that EtOH withdrawal in either circadian phase significantly elevated the $Act_{Ind} T_b$ compared to air controls ($p<0.001$), but no difference was seen between the experimental animals in acute withdrawal. As in the case of body temperature and activity, the prior BECs were not correlated with the severity of the $Act_{Ind} T_b$ reaction.

In the second 10 h period of darkness and withdrawal the $Act_{Ind} T_b$ was elevated by $0.20^{\circ}C$ in the sDay and by $0.12^{\circ}C$ in the sNight compared to air controls (Figure 3.4F). An ANOVA indicated a significant main effect of phase ($F_{1, 25}=13.829$, $p<0.001$) and exposure ($F_{1, 25}=16.607$, $p<0.001$). Post-hoc tests revealed that the $Act_{Ind} T_b$ was significantly elevated during the sDay (in animals previously withdrawing in the sNight) compared to air controls ($p<0.005$) and animals withdrawing in the sNight (previously in the sDay; $p<0.001$). Moreover, hyperthermia during the sNight was greatest in the first portion of acute withdrawal compared to the second 10 h period of withdrawal and darkness ($t_{1,13}=3.973$, $p<0.002$) while no difference in the magnitude of the hyperthermia was seen in the sDay periods ($p<0.062$).

During both 10 h periods of darkness and withdrawal the $Act_{Ind} T_b$ was disrupted in both experimental groups by 25% compared to air controls (Figure 3.3F). An ANOVA of the summed absolute change in the $Act_{Ind} T_b$ over both 10 h withdrawal periods in darkness indicated a significant main effect of exposure ($F_{1,25}=5.743$, $p<0.024$), but not of phase or their interaction. Post hoc tests, however, yielded no significant differences between the experimental groups and air controls.

During acute withdrawal the influence of activity on body temperature (measured by the slope of the $Act_{Ind} T_b$) was reduced during the first 10 hour period of acute withdrawal by (reductions of 36% and 12% in sNight and sDay, respectively; Figure 3.5B) compared to air controls. An ANOVA of the 1st 10 hour period of darkness revealed a significant interaction between phase and exposure ($F_{1,25} = 14.190$, $p<0.001$) and a main effect of exposure ($F_{1,25} = 35.832$ $p<0.001$) but not phase. Activity had less of an influence on the body temperature response during acute withdrawal in animals in the sNight compared to their air control ($p<0.001$) or animals withdrawing from EtOH during the sDay ($p<0.001$).

In the second 10 hour period of darkness in acute withdrawal the influence of activity on body temperature was reduced by 12% in the sDay and 18% in the sNight compared to air controls (Figure 3.5D). An ANOVA revealed a significant main effect of phase $F_{(1,25)}=8.153$ $p<0.009$), exposure ($F_{1,25}=8.844$ $p<0.006$), but not their interaction. Post-hoc analysis indicated that activity had less influence on animals withdrawing in the sNight (previously withdrawing the sDay) than air controls ($p<.030$) or animals withdrawing in the sDay ($p<0.020$)

3.4 Discussion

This study further establishes that the temporal context of chronic alcohol exposure and withdrawal influences activity and core body temperature rhythms. The current paradigm of intermittent and chronic alcohol vapor inhalation is known to initiate dependence and addiction-like behaviors in rodents (Becker, Diaz-Granados, & Hale, 1997; Becker & Lopez, 2004; Lopez & Becker, 2005; O'Dell et al., 2004), and loosely models consumption patterns in human alcoholics. Following the transition to alcohol dependence, withdrawal elicits a hyperthermic reaction in humans (Kalant & Le, 1983) that predicts withdrawal severity and related mortality (Kalant & Le, 1983; Khan et al., 2008). An animal model of alcoholism that induces withdrawal related hyperthermia, as shown here, provides a more accurate representation of the human condition and allows for further research into possible therapeutic targets of alcohol dependency such as chronotherapy. Managed intoxication for alcoholics is already used in the clinical setting to ensure favorable treatment outcomes following injury, and its wider adaptation using chronobiological principles may prove clinically significant.

The current study replicates our prior findings and underscores the importance that circadian phase plays in autonomic related responses to chronic alcohol exposure. It is well established in rodents that acute i.p. injections of alcohol produce more severe hypothermia during the subjective day compared to the subjective night (Baird et al., 1998; Brick et al., 1984; Williams et al., 1993). Repeated i.p. injections of alcohol, however, which models chronic alcohol use, elicit tolerance to hypothermia

during the subjective day but sensitize the response during the subjective night (Williams et al., 1993). We previously demonstrated that chronic intermittent alcohol vapor inhalation, a model of alcohol exposure and withdrawal that simulates the drinking pattern of some individuals with advanced alcohol use disorders and allows us to examine sustained effects during active alcohol exposure, produces more severe hypothermia during the subjective night than the subjective day (Chapter 2), a finding replicated herein. In the current experiment, when alcohol exposure was administered for an additional 12 hours (24 hours total) in two oppositely phased groups, greater amplitude hypothermia occurred consistently during the subjective night demonstrating a circadian rhythm to the exposure response.

Comparison of the hypothermic response across similar circadian periods, but under varying lengths of alcohol exposure, demonstrated that subjective night hypothermia appears to depend primarily on the circadian phase of the animal, while the length of alcohol exposure sensitizes the hypothermic response during the subjective day. Repeated withdrawal periods did not sensitize the exposure response across rounds; however, we did observe a sensitized response when alcohol vapor was extended for an additional 12 hours. Specifically, subjective day hypothermia was more severe in the second portion (12-24 hours) of exposure compared to the first (0-12 h). In contrast, hypothermia occurring in the subjective night was of an equivalent magnitude regardless if it occurred in the first or second half of exposure.

The major aim of the current study was to determine if the bi-directional acute withdrawal response (Damaggio & Gorman, 2014a) reported in Chapter 2 was dependent on the circadian phase of exposure and/or withdrawal. We previously

demonstrated that animals withdrawing in the subjective day (and exposed to alcohol in the subjective night) exhibited severe *hyperthermia* while animals withdrawing in subjective night (exposed to alcohol in the subjective day) exhibited withdrawal related *hypothermia*. If this differential withdrawal response was due solely to the circadian phase of alcohol exposure, by exposing animals to equal amounts of alcohol in both circadian phases while limiting their acute withdrawal to a single phase, similar unidirectional acute withdrawal reactions would be expected. In the current study, mice withdrawing in either phase exhibited *hyperthermia* which was not invoked by either changes in ambient temperature (Myers, 1981) or environmental stressors (Crawshaw et al., 1998; Peris & Cunningham, 1986) since animals were left undisturbed in their home cage under normal laboratory conditions.

The magnitude of the hyperthermic withdrawal response varied suggesting that differences in the severity of the prior alcohol exposure (i.e., the exposure response in the prior 12 hours) or the phase of acute withdrawal may influence the subsequent withdrawal reaction. Our data point to circadian dependent alterations in activity during withdrawal as the mediator of hyperthermic withdrawal response. Animals in acute withdrawal were hyperactive, a common characteristic of acute withdrawal in both humans and rodents (Taylor et al., 2006; White et al., 1994). Unlike the exposure period, where changes in activity had little influence on the body temperature response, increased locomotor activity during withdrawal in the subjective day, as in our prior study, increased the magnitude of the hyperthermia. When activity was considered there was no difference in the magnitude of the hyperthermic response regardless of the circadian phase of the animal during acute

withdrawal. Although greater withdrawal hyperthermia was preceded by severe intoxication effects (measured by higher BECs and lower body temperatures), differences in activity clearly account for the variance between the hyperthermic responses in withdrawal. The equivalent response of the activity independent body temperature suggests that the circadian phase of alcohol exposure drives the hyperthermic response in withdrawal. Moreover, when considered with the results of Chapter 2 (alcohol exposure in the subjective night produced severe hypothermia and withdrawal in the subjective day yielded severe hyperthermia) our findings suggest that 1) subjective night exposure drives a hyperthermic withdrawal response and 2) the severity of the temperature response is regulated by the circadian phase of the activity cycle in withdrawal (e.g., less severe withdrawal reactions occur in the subjective night when fewer abnormal increases in activity occur).

It must be noted that BECs did differ between the experimental groups despite exposure being equalized between the two groups. Mice measured for BEC near the start of the inactive phase (i.e. towards the end of the subjective night) had higher BECs than the converse group. BECs show circadian fluctuations with the greatest levels of intoxication occurring near the start of the inactive phase (i.e., subjective day), though whether these differences are significant remains to be seen (Brick et al., 1984; Soliman & Walker, 1979; Sturtevant & Sturtevant, 1981). Many have concluded that the behavioral and biochemical responses to alcohol instead appear to reflect differences in CNS sensitivity and not differences in alcohol absorption (Brick et al., 1984; Yap et al., 1993), an interpretation we believe our findings support since BECs were not correlated with the severity of the withdrawal response.

Two points must be addressed regarding our study design. First, because the hypothermic response during alcohol exposure was in part circadian dependent, we cannot fully conclude if the withdrawal response was dependent solely on the circadian phase of exposure, was mediated by the severity of the exposure hypothermia, or both. One possibility to untangle this relationship would be to use ambient temperature to drive a severe hypothermic reaction during alcohol exposure in the subjective day and then allow the animal to withdraw during the subjective night to measure the acute withdrawal response (e.g., does the animal exhibit withdrawal hypothermia or hyperthermia?). If this response is primarily driven by the circadian system then unequal withdrawal reactions would be expected. Secondly, we cannot exclude that the hyperthermic withdrawal reaction may be exacerbated by the length of alcohol exposure.

The presence of clear time of day dependent differences in the response to alcohol suggests that core circadian mechanisms may be involved. Considerable evidence implicates the direct involvement of alcohol on core circadian functioning (Brager et al., 2011; Prosser et al., 2008; Ruby, Brager, et al., 2009; Ruby, Prosser, et al., 2009). In hamsters, EtOH attenuated light induced phase advances but not phase delays (Seggio et al., 2007). Similarly, alcohol administered via a 2-bottle choice, acute i.p. injections, or via localized application to the SCN using reverse microdialysis probes attenuated photic phase advances in hamsters (Ruby, Brager, et al., 2009; Ruby, Prosser, et al., 2009) and attenuated photic phase delays in mice (Brager et al., 2010). How these alteration translate to behavioral changes is unclear since the SCN develops rapid tolerance to the actions of alcohol (Lindsay et al., 2014;

Prosser & Glass, 2009). Together, these findings provide clear evidence that in both acute and chronic alcohol exposure paradigms circadian functioning is altered and this modification may in part be transmitted to downstream systems.

Lastly, we did not observe any robust changes to activity or body temperature during protracted abstinence. Rebound hyperthermia can last for several days (Gallaher & Egner, 1987); however, hyperthermia, in the current study, was observed only during acute withdrawal (i.e., 0-24 h into abstinence) and had abated by the second portion of the acute withdrawal cycle (12-24 h into withdrawal) in animals in their subjective night. While some hyperthermia was still detectable during this time period in the subjective day, all experimental groups had returned to baseline levels by the second day of withdrawal.

In conclusion, the physiological response to alcohol depends critically on the timing of its administration. Our findings suggest that in a rodent model of dependence the acute withdrawal response is due in part to the timing of alcohol exposure. Chronic vapor inhalation during the subjective night, which elicits the most severe hypothermia during exposure, may in turn drive withdrawal hyperthermia. Interestingly, in both humans and rodents this consumption time coincides with the natural alcohol drinking pattern in non-dependent individuals (Danel et al., 2003; Gauvin et al., 1997) and may be a key in the transition to dependence.

Acknowledgements: Chapter 3, in part, is a reprint of the material as it appears in Damaggio, A. S., & Gorman, M. R. (2014). The circadian timing system in ethanol consumption and dependence. Behavioral Neuroscience, 128(3), 371-386. The dissertation author was the primary investigator and author of this paper.

3.5 Figures

Figure 3.1 (opposite): Representative double-plotted actograms of locomotor activity (left) and core body temperature (right) during the third round of ethanol (EtOH) vapor exposure and withdrawal for experimental (A, B, E, and F) and control (C, D, G, and H) mice over the subjective day (sDay) and subjective night (sNight). Black bars atop each panel indicate lights-off during the 2 h skeleton photoperiods. Note the shifted phase of activity and temperature rhythms in the top versus bottom four panels. Each row of telemetry data illustrates a new day of recording with two-days of recording displayed per row. The three intervals of 24 h of EtOH/air exposure (green shading) and 24 h of acute withdrawal (red shading) are single-plotted. The 3-day recovery period of Round 2 is depicted, followed by the 9-day exposure/withdrawal/recovery period in Round 3. In panel B, there is evidence of pronounced hypothermia during sNight EtOH exposure and moderate hypothermia during the sDay. Withdrawal periods are characterized by hyperthermia. Panels E and F illustrate pronounced sDay hyperactivity and hyperthermia during withdrawal.

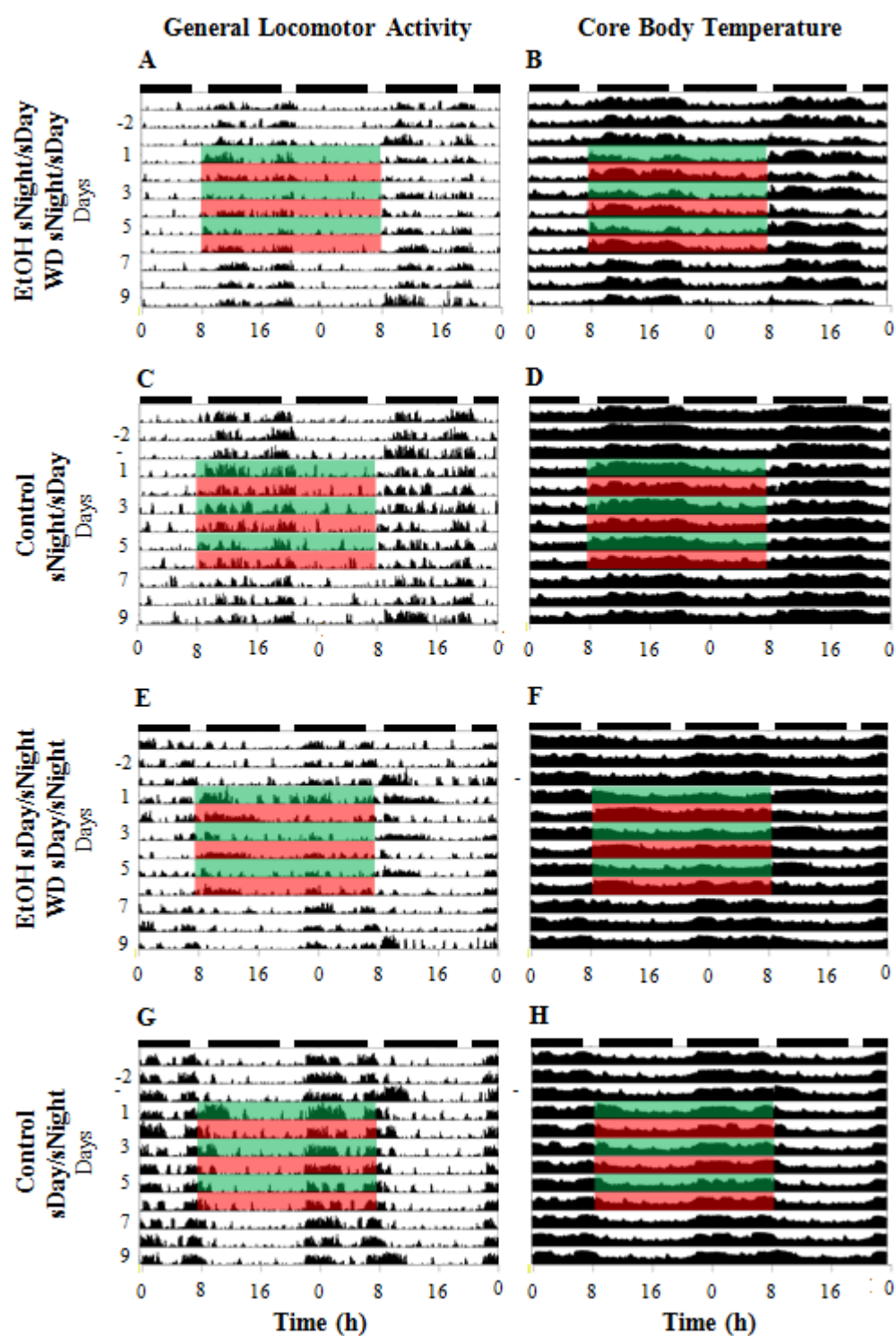


Table 3.1: Mean $\pm$ S.E.M. blood ethanol content (BEC) averaged within each round of ethanol (EtOH) vapor exposure. BEC was measured at the start of each 0800-1000 interval of the skeleton photoperiod following 24 h of continuous EtOH vapor inhalation. BECs became significantly more elevated across multiple rounds of vapor inhalation. Mice starting EtOH exposure in the subjective night (sNight) and ending in the subjective day (sDay) had significantly lower BECs than the oppositely phased group. *Significant difference between the experimental groups ($p < 0.048$).

Mean Blood Ethanol Content (mg/dL)					
	Round				
	1	2	3	4	Average
sNight EtOH start/ sDay EtOH end	185.6 $\pm$ 22.3	177.3 $\pm$ 18.5	208.9 $\pm$ 23.0	212.9 $\pm$ 22.3	196.2 $\pm$ 19.6*
sDay EtOH start/ sNight EtOH end	223.7 $\pm$ 6.7	223.9 $\pm$ 11.8	241.7 $\pm$ 11.2	274.7 $\pm$ 20.8	241.0 $\pm$ 8.8

Figure 3.2 (opposite): Net change from baseline of the mean $\pm$ S.E.M. core body temperature (top), general locomotor activity (middle) and activity-independent body temperature (bottom) in either the first (left) or second (right) 10 h dark period of the skeleton photoperiod averaged across the four rounds of ethanol (EtOH) exposure. Bar colors of animals exposed to EtOH vapor inhalation (darker shading) or air-only (lighter shading) were maintained in the first and second 10 h period of darkness to indicate animals of the same group despite a change in the circadian cycle. Shading behind the graph indicates subjective night (sNight). During the first half cycle of exposure (left), ethanol (EtOH)-exposed mice were hypothermic in both portions of their circadian phases with the greatest decreases occurring during the sNight. Alcohol suppressed activity only in the subjective night during the first and second portion of exposure. In the second half cycle of exposure (right), hypothermia was again more pronounced in the subjective night compared with the subjective day (sDay), and hypoactivity occurred only during subjective night. * Significant difference between the experimental group and control ($p < 0.045$); ** significant difference between animals exposed to EtOH during the subjective night versus the subjective day ($p < 0.025$). *Note.* Deviations from baselines plotted in Figure 3.2 are not in all cases evident in the average daily temperature rhythm in Figure 3.6.

Net Change from Baseline during EtOH Exposure

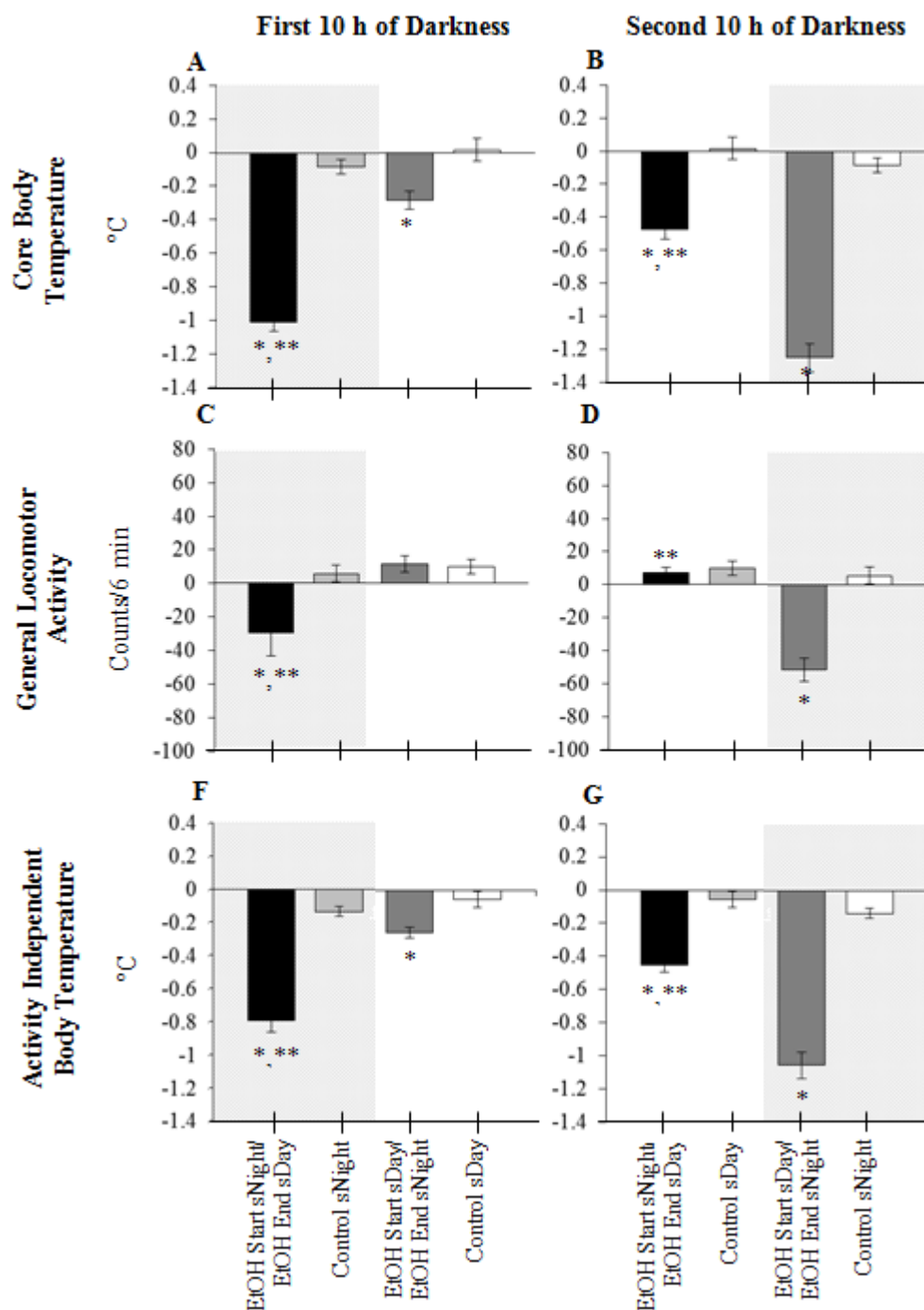


Figure 3.3 (opposite): Summed absolute deviation from baseline rhythm of the mean $\pm$ S.E.M. core body temperature (top), general locomotor activity (middle) and activity independent body temperature (bottom) during active ethanol (EtOH) vapor exposure (A, C, D and E) and withdrawal (B, D, and F) in the 20 h lights off periods of the skeleton photoperiod averaged across rounds. During EtOH exposure all rhythms were significantly perturbed compared to air controls. Following 24 h of continuous EtOH exposure, mice withdrawing starting in the sDay had significantly perturbed body temperature and activity rhythms compared to controls or animals starting withdrawal in the sNight. * Significant difference between the experimental group and control ($p < 0.011$); ** significant difference between animals exposed to EtOH during the subjective night versus the subjective day ($p < .014$).

Absolute Deviation from Baseline

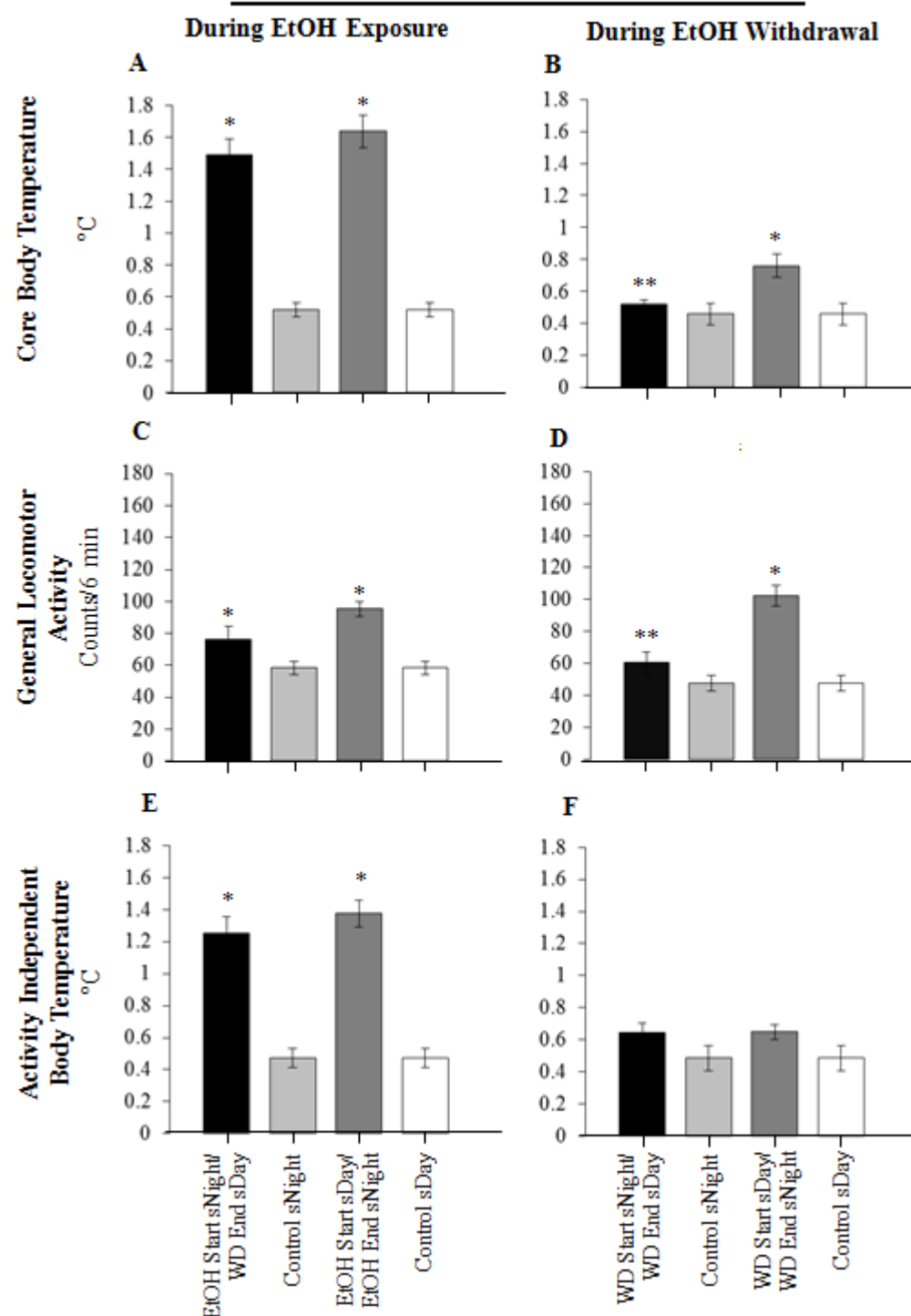


Figure 3.4 (opposite): Net change from baseline of the mean $\pm$ S.E.M. core body temperature (top), general locomotor activity (middle) and activity-independent body temperature (bottom) in either the first (left) or second (right) 10 h dark period of the skeleton photoperiod averaged across the four rounds of ethanol (EtOH) withdrawal. Bar colors of animals exposed to EtOH vapor inhalation (darker shading) or air-only (lighter shading) were maintained in the first and second 10 h period of darkness to indicate animals of the same group despite a change in the circadian cycle. Shading behind the graph indicates subjective night (sNight). EtOH exposed mice were hyperactive and exhibited hyperthermia during the first portion of acute withdrawal *Significant difference between the experimental group and control ($p < 0.045$); ** significant difference between animals exposed to EtOH during the subjective night versus the subjective day ($p < 0.025$). *Note.* Deviations from baselines plotted in Figure 3.4 are not in all cases evident in the average daily temperature rhythm in Figure 3.6.

Net Change from Baseline during EtOH Withdrawal

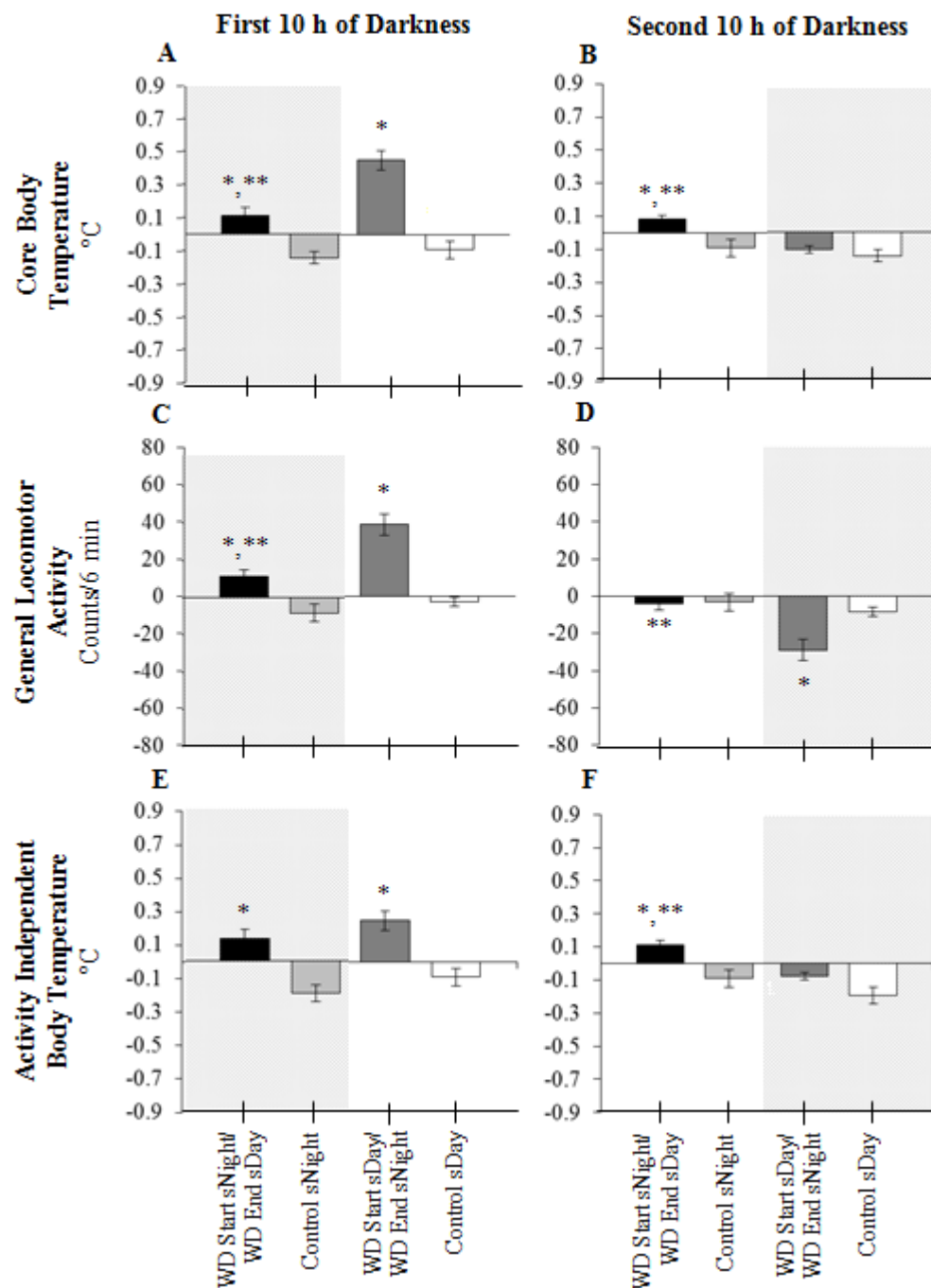


Figure 3.5 (opposite): Mean $\pm$ S.E.M. influence of locomotor activity on the core body temperature rhythm (i.e., slope of the activity independent body temperature) averaged across all rounds of ethanol (EtOH) vapor exposure (left) and acute withdrawal (right) in the first (top) and second (bottom) 10 hour period of darkness in the 2 hour skeleton photoperiod. Bar colors of animals exposed to EtOH vapor inhalation (darker shading) or air-only (lighter shading) were maintained in the first and second 10 h period of darkness to indicate animals of the same group despite a change in the circadian cycle. Shading behind the graph indicates subjective night (sNight) and white background indicates subjective day (sDay). EtOH exposure significantly suppressed the influence of activity on body temperature in both experimental groups during the first phase of EtOH exposure but only in the sNight in the second phase of EtOH exposure. Acute withdrawal suppressed the influence of activity on body temperature during the sNight. *Significant difference between the experimental group and control ($p < 0.002$); ** significant difference between the experimental groups ($p < .001$).

Mean Slope Averaged Across Rounds

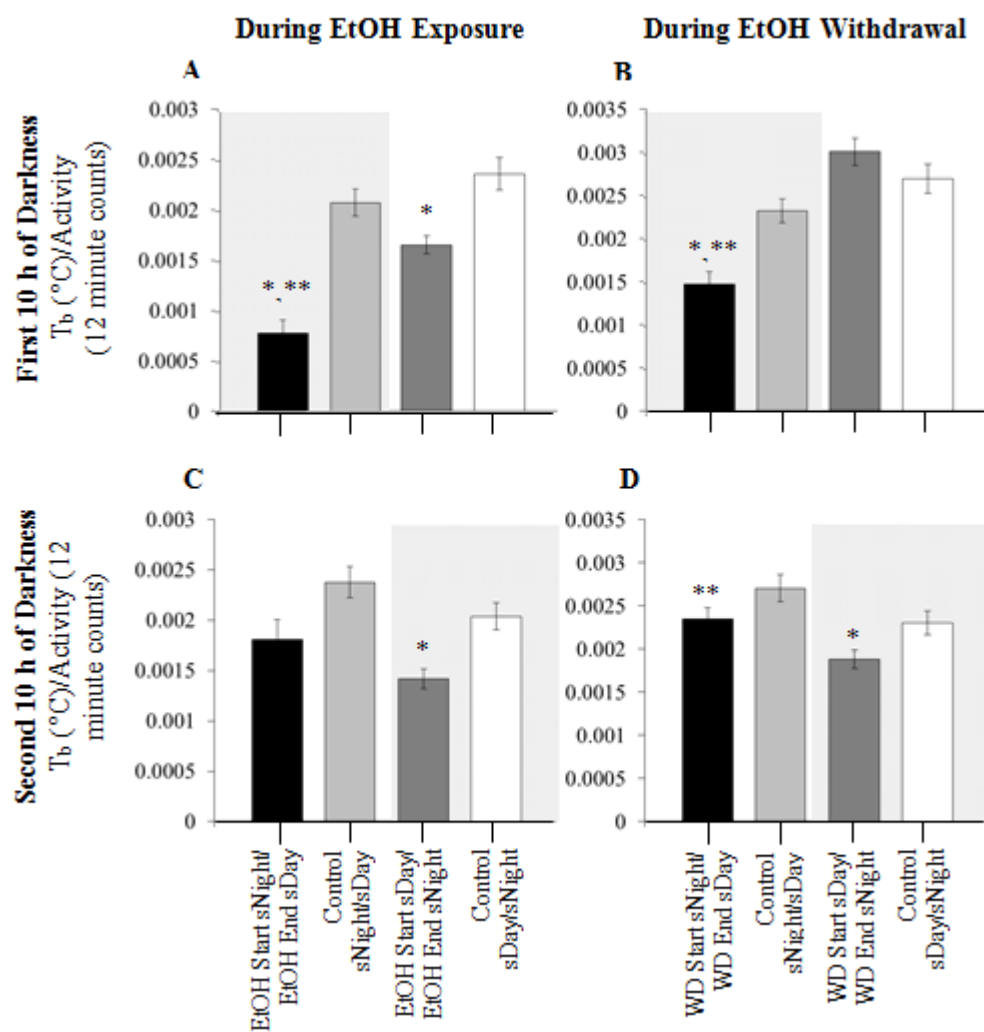
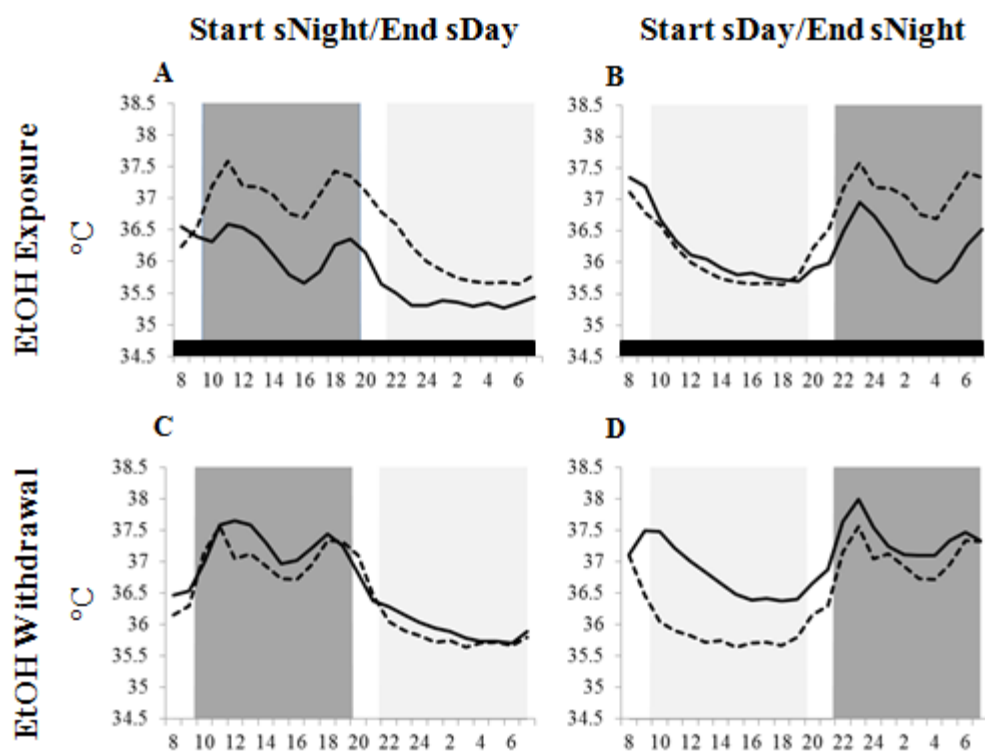


Figure 3.6 (opposite): Mean core body temperature rhythm averaged across the four rounds of 24 h ethanol (EtOH) exposure (top) and acute withdrawal (bottom) initiated either in the subjective night (sNight; left) or subjective day (sDay; right). Shading indicates lights off of the 2-hr skeleton photoperiod: light shading corresponds with the animals' subjective day while darker shading corresponds with the subjective night. Black bar indicates period of EtOH vapor inhalation. Experimental animals (solid line) were hypothermic during alcohol exposure compared with air controls (dotted line). During the first 12 hr of acute withdrawal animals became hyperthermic with more pronounced increases occurring during the subjective day. *Note.* Treatment-induced deviations from baselines that are plotted in Figures 3 and 5 are not in all cases evident in the average daily rhythms in temperature.

Average Core Body Temperature Rhythm Across EtOH Exposure and Withdrawal



Chapter 4: Circadian Phase of Passive Alcohol Vapor Exposure and Withdrawal Alters Subsequent Voluntary Alcohol Consumption in Male Mice

The emergence of many addictive-like behaviors co-occurs with a sensitized withdrawal response (Becker, Diaz-Granados, & Hale, 1997; Lopez & Becker, 2005; Ritzmann & Tabakoff, 1976a). The severity of the withdrawal reaction can be mitigated by controlling for the circadian phase of the exposure/withdrawal cycle (Chapters 2 & 3). Specifically, greater hyperthermia is elicited if alcohol exposure occurs during the subjective night and withdrawal occurs during the subjective day compared to the converse circadian conditions. The aim of this experiment was to determine if the development of addictive-like behaviors, measured by increased ethanol consumption and increased anxiety, can also be mitigated by controlling for the circadian phase of alcohol exposure and withdrawal. Male C57BL/6J mice were entrained to oppositely phased light/dark cycles so that their active cycles occurred 12 hours apart. Mice were then housed under a 2-hour skeleton photoperiod and exposed to four rounds of alcohol vapor inhalation and withdrawal during either their subjective day (i.e., inactive cycle) or subjective night (i.e., active cycle). Each round consisted of three days of fourteen hours of continuous alcohol vapor inhalation followed by a 10 hour acute recovery period. Following each round, mice were tested for voluntary alcohol consumption 36 hours into acute withdrawal in a 72 hour 2-bottle choice test of a 10% w/v EtOH solution versus water. Mice were allowed one

additional day of recovery before initiating the next round of vapor inhalation. After the final round of alcohol vapor inhalation, a behavioral anxiety test was administered on an elevated plus maze 16 hours into acute withdrawal. Mice exposed to alcohol vapor inhalation during the subjective day (and withdrawing during the subjective night) voluntarily consumed more alcohol than their controls or animals exposed to alcohol vapor inhalation during the subjective night. Animals in the subjective night displayed more anxiety-like behavior than animals in the subjective day irrespective of drug exposure. Overall, this study suggests that controlling for the circadian phase of alcohol exposure and withdrawal may mitigate the development of increased alcohol consumption characteristic of alcohol abuse disorder.

4.1 Introduction

Like most behavioral, physiological, or metabolic processes that are examined in detail, alcohol consumption follows a strong daily temporal structure. In humans, the consumption of alcohol peaks in the early evening (Danel et al., 2003; Gibson & Shirreffs, 2013). Likewise, mice and rats voluntarily consume higher amounts of alcohol in the subjective night (i.e., active cycle) than during the subjective day (i.e., inactive cycle) though, unlike in humans, this consumption pattern closely follows the general activity rhythm. In the transition to dependence, the drinking pattern of humans shifts with onset occurring earlier within the day (Danel et al., 2003). Many drugs, including alcohol, have myriad effects at different times of day. Thus, the timing of the drug's exposure prior to, and/or during the transition to, dependence may be a causal factor in the shift and increased use of the drug.

One of the hallmarks of the addictive state is increased consumption of the drug. In rodents, stable and increased levels of alcohol consumption can be simply achieved by limiting access to a few hours early in the subjective night (i.e., a drinking in the dark paradigm). Previous work from our laboratory indicates that this time-dependent exposure can have enduring effects on ad libitum consumption (Trujillo et al., 2009). Mice allowed a choice of alcohol or water during a 2 h interval either early in the day or early in the night preferentially drank alcohol compared to water, but preference and consumption were higher in mice exposed to alcohol during the night. When mice were subsequently allowed 24 h access to ethanol and water, those that had nighttime alcohol experience consumed 25-40% more than mice experiencing consumption during the day. The effect of the prior drinking history endured up to 8 weeks under ad libitum 24 h conditions. A separate experiment confirmed this effect was not due to greater drug exposure in the night as the effect was replicated if total consumption amounts in either the night or day were equalized. Controlling for the time of day in which the animal can voluntarily consume alcohol can, therefore, alter ethanol consumption and preference.

Ad libitum access to ethanol in a choice situation (as described above), however, is generally not sufficient to maintain intoxication or induce physical dependence in most rodents, although mild dependence may be achieved in alcohol-preferring (P) rats (McBride et al., 2013; McBride, Rodd, Bell, Lumeng, & Li, 2014) and crossed high alcohol preferring (cHAP) mice (Matson & Grahame, 2013; Matson, Kasten, Boehm, & Grahame, 2014). Alternatively, a forced exposure condition such as passive alcohol vapor inhalation can raise blood alcohol levels in a highly

controlled fashion, will induce alcohol dependence that generates both physiological and psychological withdrawal symptoms, and can ensure intoxication during times of day the animal would not voluntarily consume alcohol. Moreover, elevated and sustained blood ethanol levels, as induced by vapor inhalation, motivate higher rates of subsequent ad lib consumption of ethanol that are physiologically relevant in a choice paradigm (Gilpin et al., 2008; Griffin et al., 2009; Schulteis, Hyytia, Heinrichs, & Koob, 1996; Valdez et al., 2002). Thus, vapor alcohol inhalation provides an ideal paradigm to investigate time of day dependent effects of alcohol exposure on subsequent ad libitum consumption.

Because alcohol consumption follows a strong temporal organization it follows that withdrawal also is temporal constrained, likely occurring in humans in the late subjective night. Considerable evidence indicates that the withdrawal reaction is not simply a marker of dependence, but may be a causal factor in the transition from moderate to severe alcohol consumption (Healey et al., 2008; Mello, 1973; Tang & Falk, 1983). For example, greater increases in ad lib consumption (Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005), operant responding for alcohol (O'Dell et al., 2004), and anxiolytic behaviors (Valdez et al., 2002; Valdez, Sabino, & Koob, 2004) have been observed in rodents that have been administered alcohol vapor interrupted by intervals of withdrawal (an 'intermittent' schedule) compared to animals receiving the same amount of vapor exposure administered continuously. Analogously, in the clinical setting, individuals with a history of multiple detoxifications have been noted to exhibit more severe withdrawal symptoms than those individuals without prior detoxification experiences suggesting that the recurring

abstinence periods, associated with failed rehabilitation attempts, may lead to greater drug dependence (Booth & Blow, 1993; Lechtenberg & Worner, 1991).

In rodents, withdrawal periods of both short and long duration can alter subsequent ethanol response: withdrawals lasting longer than a day increase consumption and the rewarding properties of alcohol as demonstrated in the well-studied alcohol deprivation effect (Sparta et al., 2009), while short duration withdrawal reactions (e.g., 6-12 h) triggered by daily cycles of exposure and withdrawal is sufficient to increase drinking and seizure activity (Becker, Diaz-Granados, & Hale, 1997; Becker, Diaz-Granados, & Weathersby, 1997; Becker & Lopez, 2004). Some evidence suggests that in rats periods of alcohol deprivation/withdrawal may alter the circadian consumption rhythm of alcohol under ad libitum conditions (Vengeliene, Noori, & Spanagel, 2013) whereby drinking bouts, normally occurring primarily in the active cycle, expand into the inactive cycle. Together these findings indicate that withdrawal periods, in both animals and humans, influence the physiological and psychological properties of alcohol dependence and that situations that promote greater physical dependence may also promote increased addictive like behaviors. In Chapters 2 and 3 we demonstrated that alcohol exposure during the subjective night (and withdrawal during the subjective day) elicited a severe hyperthermic state during withdrawal. Thus, a similar exposure/withdrawal schedule may in turn elicit increased alcohol consumption and alter the voluntary consumption pattern of alcohol.

Moreover, controlling for the time of day which rodents are exposed to, and withdrawn from, alcohol may also provoke alterations in other psychological states

such as anxiety-related behaviors. Increased anxiety in humans is a characteristic of the acute alcohol withdrawal reaction and is considered a contributing factor to the development of alcohol use disorder and relapse (Becker, 2012). Treatment for anxiety, however, is not universally effective in the treatment of alcoholism (Spanagel & Kiefer, 2008). The elevated plus maze is a well-validated test of anxiety in rodents (Lister, 1987; Pellow & File, 1986) and is considered the “gold-standard” test for anxiety-related behaviors. Rats have shown consistent increases in anxiety-related behaviors following intermittent alcohol exposure (Rasmussen, Mitton, Green, & Puchalski, 2001; Rassnick, Heinrichs, Britton, & Koob, 1993) though these effects often co-occur with decreases in general locomotor activity (Lal et al., 1991; Wilson et al., 1998). These anxiety-related behaviors, however, have not been reliably replicated in a mouse model of addiction (see Kliethermes, 2005 for review).

The following experiment will determine if voluntary alcohol consumption in rodents can be amplified by controlling the time of day in which alcohol vapor inhalation and withdrawal induces dependence. Anxiolytic behaviors in these mice will also be tested following repeated rounds of alcohol vapor inhalation occurring either during the subjective night or the subjective day. Since repeated withdrawals are suggested to lead to greater physiological and psychological drug dependence, increases in consumption and anxiolytic behaviors may be greater if the animal is withdrawn from alcohol during a time of day that elicits more severe withdrawal reaction.

4.2 Methods

4.2.1 Subjects

Thirty-two male C57Bl/6J mice (Jackson, Sacramento, CA) six weeks of age were housed under standard temperature (22°C) and lighting (270 lux) conditions and entrained to one of two opposing 14:10 light: dark cycles (as described in Chapter 2) so that the active cycles were 12 hours apart. Following stable entrainment mice were transferred to the same room and housed under a 2 hour skeleton photoperiod which coincided with each group's dawn/dusk transition into subjective night (sNight) or subjective day (sDay). Mice had ad libitum access to food (Purina) and water throughout the experiment unless otherwise stated. The animals were housed 3-4 per cage until the start of the limited access paradigm described below, at which time mice were individually housed, placed in a modified shoebox cage, and equally divided into one of four experimental groups based on treatment and original entrainment schedule (n=8 per group; EtOH sNight, EtOHsDay, Control sNight, Control sDay). All experimental procedures and animal care were approved by and conducted under the guidelines of the Institutional Animal Care and Use Committee at UCSD.

4.2.2 Limited Access Paradigm

To ensure stable baseline alcohol consumption all mice were given nineteen days of a 2 hour limited access paradigm (including a five day sucrose fading procedure) commencing 30 minutes before lights off of the subjective night period (at 0930 for the sDay group or 2030 for the sNight group). During this time water bottles were replaced with a 10% w/v EtOH solution held in a 50mL graduated tube. After a

2 hour period the EtOH solution was replaced with water bottles until the procedure was repeated the following day. The amount consumed was recorded after each session and fresh solution was made approximately every 2-3 days. Animals were run in two identical squads spaced 3 days apart.

4.2.3 72 Hour 2-Bottle Choice Test and Lickometer Collection

Once stable consumption occurred, mice were presented with a 24 hour 2-bottle choice (H₂O or 10% w/v EtOH) paradigm, presented in a counterbalanced manner, for three days to measure baseline consumption. One hour prior to lights off (2000), a 50ml graduated cylinder filled with water and another filled with a 10% w/v EtOH solution was added to each cage. The position of the EtOH solution and the water was counterbalanced across animals. Each modified shoebox cage contained a metal platform located under the water and EtOH solution. A contact sensing circuit was attached to each EtOH cylinder and platform so that when the animal stepped onto the platform and consumed EtOH the circuit was completed and the number of licks could be recorded. Mice from both the experimental and control groups were left undisturbed for 72 hours to measure consumption and the daily drinking pattern of EtOH. At the end of the test the solutions were removed and replaced by a water bottle. The amount consumed was measured to calculate g/kg of EtOH intake and EtOH preference. Mice were allowed one day to recover before exposure to alcohol vapor inhalation as described below. Thirty-six hours into the final withdrawal period of each round of alcohol vapor inhalation the voluntary consumption test was repeated for a total of 5 tests (baseline and Rounds 1-4)

4.2.4 Alcohol Vapor Inhalation and Rounds

The experimental groups received four rounds of EtOH vapor inhalation and withdrawal. Each round was an eight day protocol that included three days of EtOH vapor inhalation and withdrawal (as described in Chapter 2), one recovery day, a three day consumption test (described above), and an additional one day recovery period. Mice from the two experimental groups were simultaneously administered alcohol vapor inhalation on a 14:10 schedule so that alcohol exposure coincided with either the animals' subjective night (n=8, EtOH sNight) or the subjective day (n= 8, EtOH sDay). Controls received air only during this time (n=8, Control Active Phase; n=8, Control Inactive Phase). Mice received an acute i.p. injection of EtOH (1.5g/kg at 20% v/v) and pyrazole HCl (0.0681 g/kg) at 2000 and were immediately placed in modified cages attached to an EtOH vapor inhalation apparatus (La Jolla Alcohol Research Inc., La Jolla, CA). EtOH vapor was administered continuously for 14 h terminating at 1000, which in turn initiated a 10 h acute withdrawal phase. This cycle of injection/exposure/withdrawal was repeated on the following two days before the animal underwent a one day recovery and EtOH consumption test. Controls, injected with an equivalent dose of pyrazole and saline, were housed within the same experimental room. Blood ethanol content was collected each morning immediately prior to the acute withdrawal period and was analyzed as described in Chapter 2.

4.2.5 Elevated Plus Maze

Following the final round of EtOH exposure/withdrawal an anxiolytic test was administered sixteen hours into withdrawal using an elevated plus maze. The maze consisted of two open and two enclosed arms (6.5cm by 36 cm each) joined at a center

hub (6.5 cm x 6.5 cm) elevated 74 cm from the ground. Testing was performed under 25 lux red light in a quiet room with a white noise machine providing approximately 65dB of background noise. A camera was mounted above the maze to record activity. Each mouse was placed on the center of the maze (counterbalanced for direction) and allowed five minutes to explore while behavior was recorded. Animals were then returned to their home cage to receive the final consumption test.

4.2.6 Data Analysis

The average voluntary ethanol intake (g/kg) following the four rounds of alcohol vapor inhalation was calculated and analyzed by ANOVA during each test period (baseline, and Round 1-4) with exposure (EtOH/Control) and phase (sNight/sDay) as between subject variables. Due to the high variability in baseline consumption levels, difference scores were also calculated and analyzed by ANOVA for the average consumption amount following alcohol vapor exposure compared to baseline levels.

Lickometer data was recorded and compiled into 6 minute bins by Vital View software (Mini Mitter, Bend, OR) and analyzed in two ways: 1) Averaged into two hour bins and collapsed across the three days of lickometer data. Bonferonni corrections were made for multiple comparisons. 2) Average licks per hour were summed across the three days of recording and correlated with the total amount of EtOH g/kg consumed.

Video from the elevated plus maze was analyzed using Smart Software (Smart Technologies; Calgary, Canada) and the following measurements were computed for each mouse: (1) percent time spent in the open arms, (2) percent entries in both open

and closed arms, and (3) number of closed entries. An ANOVA with circadian phase (sNight/sDay) and exposure (EtOH/Control) as between-subject factors was analyzed for each measure.

A significance level of $p < 0.05$ was set for all tests. Post-hoc test were conducted using Tukey-Fisher least significant differences (LSD). Two animals died during the course of the experiment (EtOH sNight group) and their data was subsequently excluded from analysis.

4.3 Results

The consumption of EtOH during baseline ad libitum access followed a circadian pattern with clear bi-modal increases in the subjective night and reaching a nadir during the subjective day (see Figure 4.1). No significant difference was seen in the baseline EtOH consumption rhythm between the controls and the experimental groups. Following multiple rounds of EtOH vapor inhalation visual inspection revealed no significant changes in the consumption rhythm following repeated exposures to alcohol vapor inhalation and withdrawal. The consumption rhythms were subsequently collapsed across rounds. Consumption of the EtOH solution spiked immediately following its initial presentation on day 1 as expected due to the novelty of the bottle but quickly returned to normal. Visual inspection of the lickometer data confirmed that under the skeleton photoperiod mice remained entrained to their original light:dark cycle. An analysis of the 2-hour average number of licks, conducted with a Bonferonni correction for multiple tests, revealed a significant difference between animals exposed to EtOH vapor in the sDay and controls at the

start of sNight ($t_{1,14} = 3.613$ $p < 0.003$), coinciding with the species' natural peak in EtOH consumption. Neither experimental group significantly differed from controls at any other 2 hour time point. The total number of licks summed over the three days of the consumption test did not correlate with the total amount consumed in any of the four rounds and was not further analyzed.

Blood ethanol content (BEC) averaged 174.73 ± 9.6 mg/dl for animals exposed to EtOH during the sDay and 194.47 ± 11.8 mg/dl in the sNight across the four rounds of EtOH vapor inhalation. BEC increased across rounds with the lowest values occurring in the first round of EtOH exposure/withdrawal (see Table 4.1). A repeated measures ANOVA revealed a significant main effect of round $F_{1,9, 26.0} = 55.138$, $p < 0.001$) but not the interaction of round by phase.

4.3.1 Repeated Exposures and Withdrawals to Alcohol Vapor Inhalation Increases Consumption

Prior to EtOH vapor inhalation mice voluntarily consumed on average 24.7g/kg of EtOH. There was no significant difference between the groups in their baseline consumption levels (see Figure 4.2), although a high degree of inter-animal variability occurred in the sNight Control group. Following EtOH vapor inhalation mice exposed to sNight EtOH vapor on average voluntarily consumed 4% more EtOH during the three day 2 bottle choice test than controls while mice exposed to sDay EtOH vapor consumed 19% more EtOH than controls. An ANOVA revealed a significant main effect of exposure ($F_{1,26} = 8.051$ $p < 0.009$; Figure 4.2). Post hoc tests indicated that animals exposed to alcohol vapor inhalation during the subjective day voluntarily drank significantly more alcohol than their controls ($p < 0.003$) and animals

exposed to alcohol vapor during the subjective night ($p < 0.048$). An ANOVA of the difference score between baseline consumption levels and the average consumption across the four rounds of consumption test revealed no significant interactions or main effects.

4.3.2 Alcohol Exposure and Withdrawal Does Not Influence Anxiety

Anxiolytic behaviors as measured on the plus maze are depicted in Figure 4.3. An ANOVA of the percent time spent in the open arms revealed a significant main effect of phase ($F_{1, 26} = 4.255$ $p < 0.049$) but not an interaction or other main effects. Post hoc analysis revealed that control animals in the subjective day spent less time in the open arms than controls in the subjective night ($p < 0.033$). There were no significant main effects or interactions for the percent of open arm entries or for the mean closed arm entries.

4.4 Discussion

This study is the first to critically investigate if the development of anxiolytic behaviors differ as a function of the circadian cycle in which alcohol dependence is induced. A number of studies have highlighted the role withdrawal specifically plays in the development of addiction-related behaviors (Becker & Lopez, 2004; O'Dell et al., 2004; Valdez et al., 2002). Chapters 2 and 3 revealed that controlling for the time of day in which alcohol exposure and withdrawal occurs can mitigate the severity of the physical withdrawal reaction. In the current study we provide limited evidence that voluntary consumption of alcohol, but not anxiolytic behaviors, may be influenced by the circadian phase of alcohol exposure and withdrawal.

Previously, Becker and Lopez (2004) demonstrated increased voluntary alcohol consumption in a 2-hour limited access paradigm following repeated intermittent alcohol vapor inhalation in the subjective night and the withdrawal periods occurring in the subjective day. The timing of the Becker study coincides with severe exposure related hypothermia and withdrawal related hypothermia as reported in Chapter 2. Moreover, much work from the Becker laboratory has shown that the escalation of voluntary alcohol consumption is dependent on repeated withdrawal experiences (Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005) which, in turn, increase withdrawal severity measured by handling induced convulsions via a kindling-like mechanism (Becker, Diaz-Granados, & Hale, 1997; Becker, Diaz-Granados, & Weathersby, 1997; Becker & Hale, 1993). Therefore, we originally speculated that situations which promote more severe withdrawal related physical dependence would in turn promote more severe addiction-related behaviors. We instead found no evidence supportive of this hypothesis and limited support for the converse.

Ad libitum consumption of alcohol was increased during the 72 hour 2-bottle choice paradigm if alcohol vapor exposure occurred during the subjective day (withdrawal in subjective night), and lickometer data indicated that this increase occurred primarily at the start of subjective night. This effect, however, did not hold when baseline consumption was considered. Even with repeated exposures to alcohol vapor inhalation and withdrawal, voluntary consumption levels of alcohol did not differ from baseline levels using almost an identical paradigm as the Becker

laboratory to induce alcohol dependence (Becker & Lopez, 2004; Griffin et al., 2009; Lopez & Becker, 2005). Our findings must therefore be cautiously interpreted.

The failure to replicate Becker and Lopez (2004) is likely due to the already high consumption levels of this species and procedural differences between studies (e.g., ad libitum access to alcohol during testing, the use of the sucrose fading procedure, diluted ethanol solution, etc.) A primary difference in our study from the Becker and colleagues' study was the use of ad-libitum access to alcohol during testing instead of a limited access paradigm. Two-hour limited access paradigms employ 1) a mouse's tendency to naturally consume higher levels of alcohol at the start of the dark cycle (e.g., a drinking in the dark paradigm) and 2) a period of alcohol deprivation following the 2-hour period which increases the reinforcing properties of alcohol (i.e., the alcohol deprivation effect; Sparta, 2009). Evidence of the latter is demonstrated in the Becker study as mice consumed higher levels of EtOH on the second day compared to the first. Thus, our study may have failed to make alcohol rewarding enough to induce increased consumption levels. Due to the nature of the circadian paradigm, though, ad libitum access was necessary to ensure both groups (sDay and sNight) had access to alcohol at the same point in acute withdrawal even if it didn't coincide with peak consumption times in their circadian cycle. Furthermore, we cannot rule out the possibility that the controls were able to consume enough alcohol during the sucrose fading procedure to influence dependence and that a 10% w/v EtOH solution was not sensitive enough to increase consumption in experimental mice while discouraging consumption in controls. Experimental mice were, however, sufficiently intoxicated with alcohol vapor inhalation to induce higher

consumption levels, as sustained elevated BEC above 175 mg/dl are considered critical in the escalation of subsequent voluntary alcohol consumption (Giffin et al 2009).

Furthermore, we did not observe a robust change in the circadian drinking profile of alcohol dependent mice. Human alcoholics consume alcohol starting earlier in the day than non-dependent individuals (Danel et al., 2003). This shift in the consumption pattern may be due to the timing of the prior drinking history (under non-dependent conditions) or augmented by the timing of consumption/withdrawal under the shifted conditions. Some evidence suggests that alterations also occur to the circadian drinking pattern of alcohol in rats (Vengeliene et al., 2013) and mice (Trujillo et al., 2009) after prolonged alcohol exposure and withdrawal. Previous work from our laboratory assessed whether the timing of prior alcohol exposure would exert a marked influence on the 24 hour consumption pattern in mice (Trujillo et al., 2009). Mice were initially presented with a two-hour two bottle choice paradigm during the early subjective day or subjective night. When mice were given the opportunity to drink in either phase their prior consumption history determined their consumption under the 24 h ad lib conditions. Mice previously exposed during the sNight continued to drink more than animals exposed initially during the sDay and had greater increases in their drinking profile specifically during the early and middle portions of the subjective night. Using lickometer data in the current study we were able to examine the overall voluntary consumption pattern of alcohol in mice following repeated cycles of vapor inhalation and withdrawal, a paradigm that models the chronic alcohol consumption and withdrawal in severely dependent human

alcoholics. Interestingly, forced alcohol vapor did not expand or alter the circadian drinking repertoire of these mice. It is not clear why voluntary conditions (2-hour limited access) induced lasting changes to the 24-hour ad libitum drinking repertoire but forced conditions (alcohol vapor inhalation) did not.

A negative emotional state is the most commonly reported reason for relapse in humans (Annis, Sklar, & Moser, 1998). In rats, anxiety-like behaviors are often reported during withdrawal from chronic alcohol exposure (Baldwin, Rassnick, Rivier, Koob, & Britton, 1991; File, 1994; Lal et al., 1991; Schulteis et al., 1996) and may persist for many weeks into abstinence (Baldwin et al., 1991; Rasmussen et al., 2001; Rassnick et al., 1993; Valdez et al., 2002; Valdez et al., 2004). Conversely in mice some but not all studies report an increase in anxiety following alcohol exposure and withdrawal (Finn et al., 2000; Kliethermes, 2005; Kliethermes et al., 2004; Onaivi, Todd, & Martin, 1989; Pokk et al., 2001). In the current study mice repeatedly exposed to alcohol vapor inhalation and withdrawal did not differ from air controls with anxiety-related behaviors on an elevated plus maze. As in other studies (Beeler, Prendergast, & Zhuang, 2006), a circadian dependent difference in the expression of anxiolytic behaviors did exist independent of drug exposure. Animals testing in the subjective night were more anxious than their subjective day counterparts. Anxiolytic behaviors, following chronic alcohol exposure, may emerge at distinct time points following abstinence (Kliethermes, 2005; Stevenson et al., 2009) that were not captured here; though, testing occurred at a peak time point for withdrawal related anxiolytic behaviors in mice (Wilson et al., 1998). Alternatively, anxiolytic behaviors may not be reliably produced in a vapor model of addiction as Kliethermes (2005)

report that no studies that utilize vapor inhalation in mice have found increased anxiolytic behaviors, and those that do using other methods of induction (e.g., liquid diet) often co-occur with decreased locomotor activity complicating the interpretation of these findings.

In conclusion, controlling for the time of day of alcohol exposure and withdrawal provided limited evidence that increased alcohol consumption occurs in a circadian dependent manner. Additional studies are needed to parse out this finding from the literature that supports greater consumption levels following chronic intermittent alcohol exposure than demonstrated herein. Time of day may yet play an important role in the development of addiction related behaviors and could potentially provide a prevention strategy for those at risk for alcohol use disorder.

4.5 Figures

Table 4.1: Mean $\pm$ S.E.M. blood ethanol content (BEC) averaged within each round of ethanol (EtOH) vapor exposure. BEC was measured at the start of each 0700-0900 interval of the skeleton photoperiod during the final 2 h of the 14 h EtOH vapor inhalation. Mice exposed to EtOH during the subjective night (sNight) and subjective day (sDay) had progressively increasing BEC across rounds.

Mean Blood Ethanol Content (mg/dL)					
	Round				
	1	2	3	4	Average
EtOH sDay	102.1 $\pm$ 22.8	226.0 $\pm$ 8.1	241.8 $\pm$ 15.6	256.9 $\pm$ 12.3	194.5 $\pm$ 11.8
EtOH sNight	94.8 $\pm$ 8.2	212.7 $\pm$ 5.2	214.0 $\pm$ 8.6	234.7 $\pm$ 14.8	174.7 $\pm$ 9.6

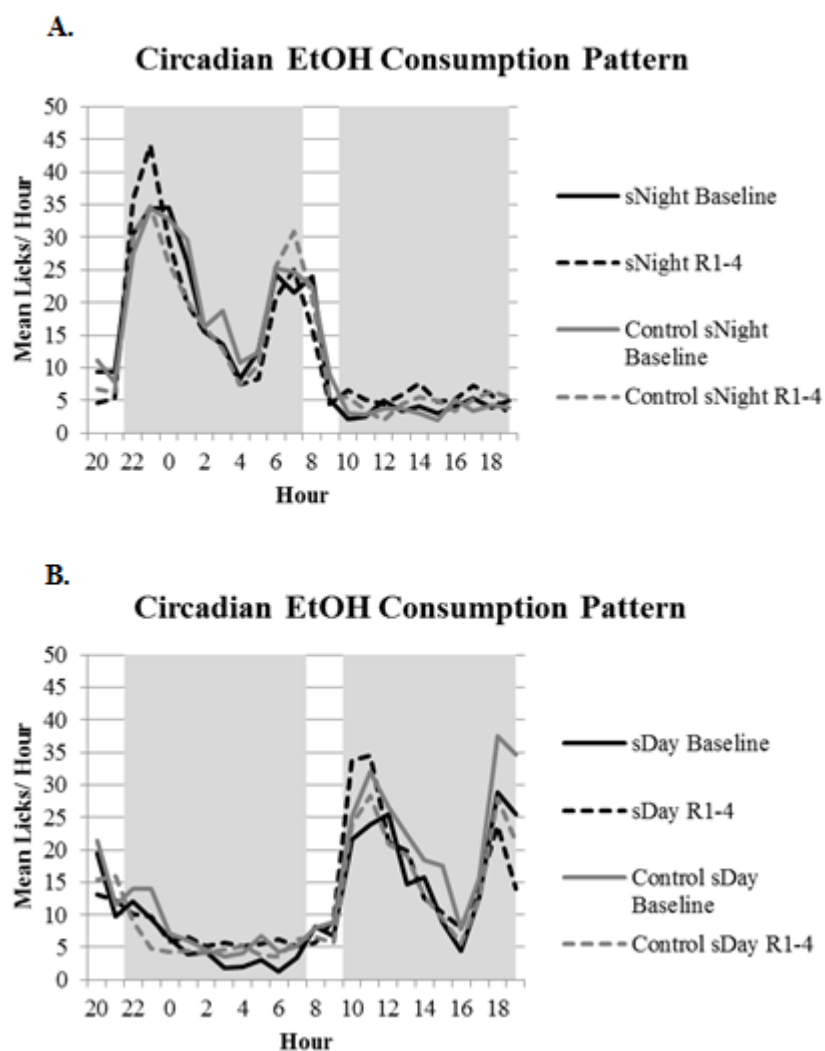


Figure 4.1: Mean hourly ethanol (EtOH) consumption pattern of mice exposed to EtOH vapor inhalation in the subjective night (sNight; A) or the subjective day (sDay; B) during baseline (solid lines) or across the four consumption tests (dashed lines). Mice were exposed to three days of EtOH vapor inhalation (black lines) or air only (grey lines) prior to each consumption test. Shading behind the graph indicates lights out during the 2 h skeleton photoperiod. Mice exhibited a bimodal peak in consumption, but the consumption patterns did not significantly change over the course of the experiment.

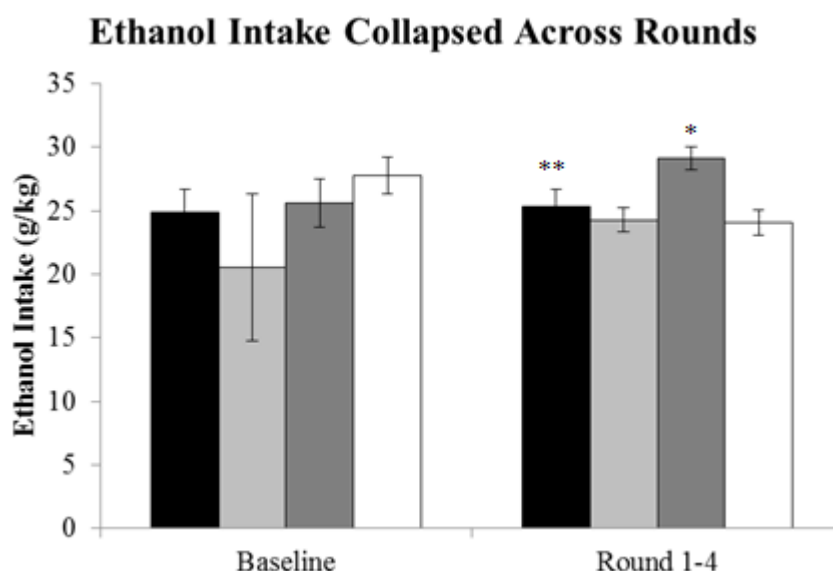
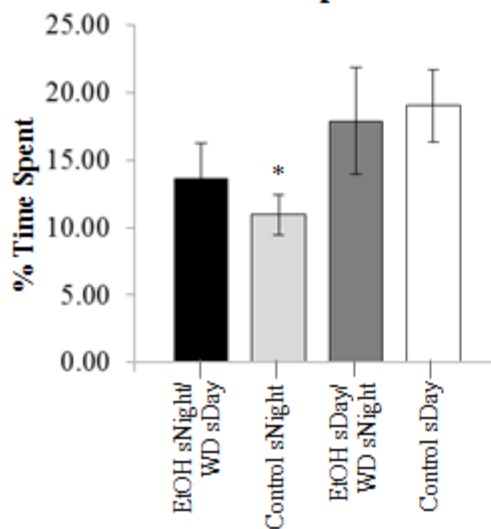
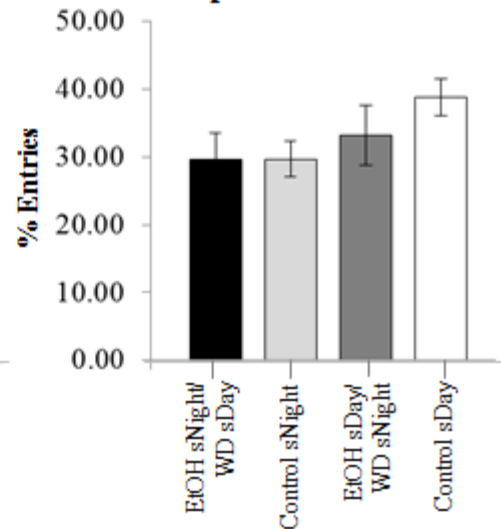
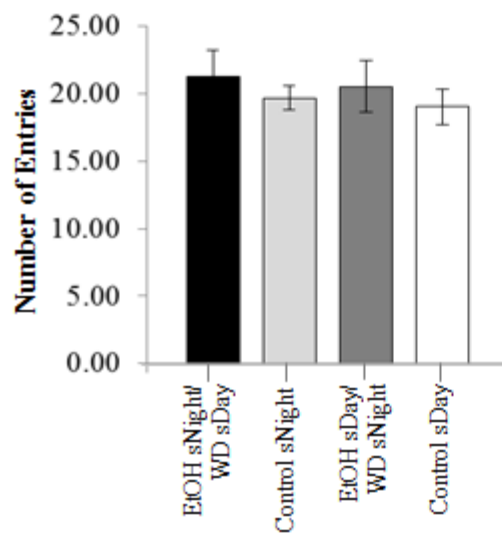


Figure 4.2: Mean $\pm$ S.E.M. voluntary ethanol (EtOH) consumption during baseline (left) or averaged across the four consumption tests (right). Mice exposed to EtOH vapor inhalation in the subjective day (sDay; dark grey bars) increased their voluntary consumption of alcohol compared to air exposed sDay controls (white bars) while no difference was observed between animals exposed to EtOH vapor in the subjective night (sNight; black bars) and their respective sNight control (light grey bars). The increase in consumption in the sDay experimental group was not significant compared to baseline consumption levels, however. * Significant difference between experimental group and air controls ($p < 0.003$); ** significant difference between the experimental groups ($p < 0.049$).

Figure 4.3 (opposite): Mice were exposed to four rounds of ethanol (EtOH) vapor inhalation in either the subjective night (sNight; black bars) or the subjective day (sDay; dark grey bars) before anxiolytic behaviors were measured on an elevated plus maze 16 hours into the final withdrawal session. Controls for the sNight group (light grey bars) or the sDay group (white bars) received air only. During testing mice were in the same phase as during EtOH exposure (e.g., EtOH sNight mice were in the sNight during the plus maze test). A) Control mice in the sDay spent more time in the open arms of elevated plus maze than controls in the sNight. B) The percent of open arm entries of EtOH vapor exposed mice did not differ from controls. C) The number of entries into the closed arms did not differ between groups. * Significant difference between the controls ($p < 0.033$).

A. Percent Time in Open Arms**B. Percent Open Arm Entries****C. Mean Closed Arm Entries**

Chapter 5: Time of Day Does Not Act as a Conditioned

Stimulus for Behavioral Sensitization in Mice

Environmental cues play an important role in drug use and relapse. Little work, however, has examined whether time of day is a critical component of the environmental context. The aim of this experiment is to investigate whether time-of-day serves as a predictive environmental cue for behavioral sensitization to amphetamine (AMPH). C57BL/6J x129S1/SvImJ mice were given an i.p. injection of AMPH or saline at either 2 or 10 hours into the light cycle and general locomotor activity was recorded. This paradigm was repeated daily for seven days. After a one week abstinence period mice were tested for behavioral sensitization at either the same time of day as training or a novel time of day. During training the initial acute injection of AMPH showed clear time of day dependent differences in the horizontal locomotor response with greater increases in activity occurring when AMPH was administered late in the day. Testing at either the same or a different time of day as training had no differential effect on the sensitized response. We conclude that time of day is not a salient environmental cue in the presence of strong contextual cues in this strain of mice.

5.1 Introduction

Classical conditioning plays an important role in drug use and relapse. Environmental cues become associated with drug use, and over time, these cues can elicit drug craving. This is important in the treatment of addiction whereby

environmental cues can initiate relapse and may provide a unique opportunity for therapeutic interventions. One environmental factor, time of day, is emerging as an important consideration in the addiction process (Damaggio & Gorman, 2014b); though, for the most part, time has been ignored as a component of the environmental context of drug use.

The repeated administration of a psychostimulant drug, such as amphetamine, leads to progressive increases in behavioral responses, commonly measured by locomotor activity in rodents. This “behavioral sensitization” is thought to model the transition between casual and compulsive drug use and has both associative and non-associative learning components. Relevant to the current purposes, behavioral sensitization is influenced by time of day dependent manipulations; however, the response may be mitigated by factors such as dose (Gaytan, Lewis, Swann, & Dafny, 1999; Gaytan, Swann, & Dafny, 1996), drug (Akhisaroglu et al., 2004; Gaytan, Swann, & Dafny, 1998), species (Akhisaroglu et al., 2004; Gaytan et al., 1998; Uz, Javaid, & Manev, 2002), and length of abstinence (Sleipness, Sorg, & Jansen, 2005). For example, in CBA/J mice, repeated amphetamine administration yields greater behavioral sensitization during the light cycle than during the dark cycle (Akhisaroglu et al., 2004) while in rats the converse is true (Gaytan et al., 1999).

In addition to differences in drug sensitivity, time of day may also serve as a predictive cue to drug onset. It is well established that the behavioral expression of sensitization can be regulated by environmental cues such as context (Badiani, Anagnostaras, & Robinson, 1995; Robinson, Browman, Crombag, & Badiani, 1998). In rats, cocaine or amphetamine can elicit robust behavioral sensitization when the

drug is administered in a novel drug-paired environment instead of the home environment (Badiani et al., 1995). Alcohol and psychostimulant use has a naturally occurring pairing with specific times of day. In humans cocaine abuse occurs primarily in the late afternoon/early evening with cocaine related health problems peaking soon thereafter (Morris, 1987; Raymond, Warren, Morris, & Leikin, 1992). In rats, voluntary alcohol and psychostimulant use peaks during the dark phase (Gauvin et al., 1997; Roberts, Brebner, Vincler, & Lynch, 2002; Sorg, Stark, Sergeeva, & Jansen, 2011). Drug craving and relapse may therefore be driven, in part, by the time of day drug use is expected.

Few studies, however, have examined whether the time of drug exposure can serve as a predictive environmental cue to behavioral sensitization. Arvanitogiannis et al. (2000) found that rats tested for amphetamine sensitization at the same time as training were significantly more active than controls (activity counts differed by approximately 150%), but when rats were tested at a novel time of day there was no difference compared to controls. This study, however, fails to properly control for diurnal differences in amphetamine sensitization which, as discussed above, are well documented in rodents (Akhisaroglu et al., 2004; Gaytan et al., 1999; Gaytan et al., 1998). Thus, in the Arvanitgoiannis study differences in sensitization, as a factor of testing time, may simply reflect diurnal differences in the sensitized response and not time of day as a learned environmental cue to sensitization. Moreover, this effect may be limited to specific species and strains. While the utilized rat strain was not reported in the Arvanitgoiannis study, time of day learning in conditioned place preference paradigms occurs in Wistar not Long Evans rat strains (Cain, Ko, Chalmers, & Ralph,

2004). The use of additional training and testing times, as well as generalization to other species, can more fully elucidate the role of temporal context in sensitization.

In conclusion, this chapter will investigate if the development and expression of sensitization to chronic amphetamine is dependent upon time of day serving as a predictive environmental cue to sensitization in mice. After receiving repeated injections of amphetamine either early or late in the light cycle, mice will be tested for behavioral sensitization either at the same or a novel time of day as training to determine if sensitization differs as a factor of training/testing time.

5.2 Methods

5.2.1 Subjects

Eighty-nine male and female C57BL/6J x129S1/SvImJ mice inbred from Jackson Laboratory (West Sacramento, CA) and at least 10 weeks of age were group housed (3-5 mice per cage). Mice were handled for at least 5 days prior to experimental manipulations and had continuous access to food and water. All animal care and testing procedures were approved by the UCSD IACUC and were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals

5.2.2 Experimental Lighting Conditions

Animals were randomly assigned to one of two opposing 14:10 light:dark (LD) conditions (lights on from 2100-1100 or lights on from 0700-2100) and allowed two weeks to stably entrain to their respective LD conditions before habituation to the

testing chambers. The groups remained on their respective LD cycle throughout the course of the experiment.

5.2.3 Drugs

Amphetamine (1.5mg/kg) was dissolved in physiological (0.9%) saline. All saline and drug injections were administered intraperitoneally (i.p.) in a volume of 10ml/kg. Mice received either X mg/kg of amphetamine or X mg/kg saline throughout the course of training. During testing, all animals received a 10ml/kg injection of saline and a 1.5 mg/kg injection of amphetamine.

5.2.4 Apparatus

Eight mice were tested concurrently in individual modified place preference chambers (43.2 x 43.2 x 30.5 cm; Med-Associates Inc., St. Albans, VT) housed in a windowless room (in total 12 squads of mice were run every day of training). Each chamber consisted of two compartments (21.6 x 43.2 x 30.5 cm; Med-Associates Inc., St. Albans, VT) bisected by an opaque wall with a removable insert. The compartments each had distinct visual, tactile, and odor cues, designed to maximize their contextual differences. One compartment had walls decorated with colorful stickers, while the other compartment had standard clear polycarbonate walls. Mice were counterbalanced across compartments so that only one compartment of the preference chamber was in use. Animals were consistently trained and tested in the same compartment across days. The compartments were cleaned and scented with 7% isopropyl alcohol before each use. Each of the chambers was equipped with two rows of 16 x 16 beam infrared (I/R) arrays and sensors that were evenly spaced and juxtaposed around the four peripheral sides of the chamber. The data acquisition and

analysis software (Activity Monitor v 5.5; Med-Associates Inc., St. Albans, VT) used the interruption of breaks in the X and Y coordinate defining I/R beams at its corresponding sensor to detect the presence or absence of subjects and to derive other behavioral parameters (i.e., distance traveled and time spent in a compartment) generated by software algorithms.

5.2.5 Sensitization

5.2.5.1 Habituation & Training

Habituation and training took place at the same time each day with either saline or amphetamine (AMPH), but depending upon the animals' LD schedule, training occurred either early or late in the light cycle. Animals entrained to the 0700-2100 LD cycle were habituated and trained two hours into the light phase (Early) while the 2100-1100 LD animals were habituated and trained two hours prior to lights off (Late).

Animals were habituated to the testing chambers for 20 minutes on two consecutive days prior to training. On each of the seven training days mice were run in an identical counterbalanced manner. Mice received an i.p. injection of either 1.5mg/kg amphetamine dissolved in physiological (0.9%) saline (AMPH group) or saline only (Saline group) and were immediately placed into the testing chamber with the insert in place for 15 minutes. Mice were counterbalanced between the left and right side of the chamber and run consistently on the same side in both training and testing. Animals were then removed and the chamber was cleaned and re-scented

prior to use by the next squad. Mice were allowed a one week drug abstinence period before testing occurred.

5.2.5.2 Testing

Seven days after the final training session, mice were tested for behavioral sensitization. Testing occurred at either the same time (Standard) or at a novel time (Novel) from training. As in training, the novel testing time differed between the groups as a function of the entrained photoperiod occurring either 2 or 12 hours after lights on. Specifically, novel testing times for animals trained early in the day (2 h after lights on) occurred 12 hours after lights on (the same time point as those trained late in the day) and vice versus for animals trained late in the day (i.e., trained at 12 hours after lights on but tested 2 hours after lights on). At the start of testing all animals received an 1.5mg/kg dose of saline and were placed in chamber for five minutes to measure locomotor activity. Mice were then immediately removed and received an 1.5mg/kg dose of amphetamine. Locomotor and vertical activity was then recorded for 45 minutes.

5.2.5.3 Behavioral Sensitization and Data Analysis

Mice were randomly assigned to one of eight conditions that differed by drug administration (Saline or AMPH), time of day training occurred (Early or Late), and novelty of testing time (Standard or Novel). In total the groups were as follows: AMPH/Early/Standard, n=15; AMPH/Early/Novel, n=15; AMPH/Late/Standard,

n=13; AMPH/Late/Novel, n=15; Saline/Early/Standard, n=8; Saline/Early/Novel, n=7; Saline/Late/Standard, n=8; Saline/Late/Novel, n=8.

Horizontal locomotor activity (general locomotor activity on the x axis) and vertical locomotor activity (locomotor activity on the y axis), such as rearing behaviors, were measured during training for each of the 7 training days by Activity Monitor software (Med-Associates Inc.; St. Albans, VT). Data was analyzed using an ANOVA with training time (Early/Late) and drug (AMPH/Saline) as between-subject variables. The sensitization test was analyzed using an ANOVA with training time (Early/Late) and testing time (Standard/Novel) as between-subject variables. A repeated measures ANOVA was used when appropriate to determine any effects across training days or across minutes of the sensitization test. A significance level of $p < 0.05$ was set for all tests. Behavioral sensitization was measured as the difference between the response on Day 7 (sensitized response) and Day 1 (acute response) of training. Post-hoc tests were conducted using Tukey-Fisher least significant difference (LSD).

5.3 Results

5.3.1 The Acute Response to Amphetamine is Dependent on Time of Day

On the first day of training, mice administered an acute i.p. dose of AMPH late in the day increased their horizontal locomotor activity by 150% (i.e., traveled 48 cm/min more) compared to saline controls (see Figure 5.1) while animals administered an equivalent dose of AMPH early in the day only differed from controls by 2% (i.e., traveled only 1 cm/min more). Analysis of the acute response to AMPH on the first

day of training revealed a significant interaction of time of day by drug ($F_{1,85} = 5.808$ $p < 0.018$) and a main effect of drug ($F_{1,85} = 6.311$ $p < 0.014$). Post hoc test revealed that animals exposed to AMPH late in the day were significantly more active than their controls ($p < 0.002$) while no difference was seen early in the day or between animals exposed to AMPH.

Figure 5.2 depicts the average vertical activity of the mice following the first acute dose of amphetamine. When AMPH was administered early in the day vertical activity decreased by 51% compared to saline controls while an equivalent dose administered late in the day increased vertical activity by only 2% compared to controls. An ANOVA indicated a significant interaction between time of day and drug ($F_{1,85} = 4.881$ $p < 0.030$) and a main effect of drug ($F_{1,85} = 4.444$ $p < 0.038$). Post-hoc analysis revealed that vertical activity was attenuated in animals exposed to AMPH early in the day compared to controls ($p < 0.019$).

5.3.2 Amphetamine Sensitization across Training Days

Visual inspection of Figure 5.3 revealed that animals receiving AMPH became progressively sensitized across the seven days of training while controls did not. A repeated measures ANOVA revealed a drug by day interaction ($F_{2,3,295,4} = 14.606$, $p < 0.001$) and a significant main effect of day ($F_{2,3,295,4} = 15.762$ $p < 0.001$). A difference score (day 7 versus day 1) was calculated to quantify the amount of sensitization that occurred across training days. Mice exposed to AMPH early in the day had a 13% increase (a difference of 81.1 cm/min) in locomotor activity while mice trained late in the day had a 519% increase (a difference of 76.8 cm/min) compared to their respective controls (see Figure 5.4). An ANOVA revealed a significant main

effect of drug ($F_{1,85} = 25.919$ $p < .001$). Post hoc analysis indicated a significant difference between the experimental groups compared to their respective controls ($p < .001$).

Vertical activity across the 7 days of training is depicted in Figure 5.5. A repeated measures ANOVA revealed a drug by day by time of day interaction ($F_{3,4,282.0} = 2.829$ $p < 0.033$) and a main effect of day ($F_{3,4,282.0} = 19.145$ $p < 0.001$). On average, mice exposed to AMPH early in the day *increased* their vertical activity by 16.58 counts compared to controls between the day 7 and the day 1 response. Conversely, animals exposed to AMPH late in the day *decreased* their vertical activity by 8.36 counts compared to controls (see Figure 5.6). An ANOVA revealed a drug by time of day interaction ($F_{1,84} = 5.127$ $p < 0.026$), but no other main effects. Post-hoc analysis indicated that animals exposed to AMPH early in the day had significantly more vertical activity than their respective controls ($p < 0.046$).

5.3.3 Amphetamine Sensitization Test

Visual inspection of Figure 5.7 indicates that locomotor (horizontal) activity for animals previously exposed to AMPH during training progressively increased in the first 5-6 minutes of the sensitization test before plateauing. This was confirmed by a repeated measures ANOVA which revealed a significant interaction of minute by testing time ($F_{3,3,177} = 5.326$ $p < 0.001$) and a main effect of minute ($F_{3,3,177} = 6.821$ $p < 0.001$). Data was collapsed across the first 15 minutes of the sensitization test and is presented in Figure 5.8. An ANOVA revealed no significant interactions or main effects of either time of day or time of prior training. Vertical activity was not analyzed due to data loss.

5.4 Discussion

This study demonstrates that time of day is not a predictive environmental cue for behavioral sensitization following chronic amphetamine exposure in mice. Many drug-induced behavioral responses show diurnal differences such as locomotor activity, drug sensitivity, behavioral sensitization, self-administration, and conditioned place preference (Falcon & McClung, 2009). The data presented herein report 1) diurnal differences in acute drug sensitivity to amphetamine (measured by behavioral sensitization), 2) no diurnal differences in long term drug sensitivity (also measured by sensitization), and 3) no evidence that time of day acts as a learned environmental cue to behavioral sensitization. Below we explore hypotheses, including melatonin deficiency and overshadowing, which may impair time of day dependent sensitization and the learning of temporal context.

Surprisingly, in the current study the acute locomotor response to amphetamine differed as a function of time of day on the first day of training suggestive of a diurnal difference in sensitivity. Despite the well documented time of day effects of psychostimulants on behaviors such as feeding (Dobrzanski & Doggett, 1976) and conditioned place preference (Manev & Uz, 2009), the general locomotor response following an acute dose of psychostimulant is generally not considered circadian dependent (Gaytan et al., 1998; Hutchinson, Hudson, & Dubocovich, 2012; Manev & Uz, 2009; Uz et al., 2002). For example, in mice, with or without melatonin (C3H/HeJ and C57BL/6J strains respectively), an i.p. injection of cocaine increased locomotor behavior regardless of the time of drug administration (Uz et al., 2002).

Similarly in rats, acute doses of amphetamine increased locomotor activity equally across four circadian time points, though time of day dependent differences in stereotypic behaviors were seen (Gaytan et al., 1998). In the current study, mice exposed to an acute dose of amphetamine early in the day did not differ from controls, while those exposed to amphetamine late in the day were more active than controls. Thus, our data indicate that C57BL/6J x129S1/SvImJ mice display diurnal differences in the acute locomotor response to amphetamine. Follow-up studies are needed to conclude if the acute response to amphetamine is truly circadian dependent in this strain of mice (i.e., occurs in the absence of light/dark cues). Conversely, vertical rearing behavior, on the first day of training, was attenuated early in the light cycle but not late in the light cycle which coincides with previous findings that stereotypic behaviors, but not locomotor activity, are time dependent following acute amphetamine exposure in rats (Gaytan et al., 1998)

Contrary to the acute response, mice from our stock exhibited no diurnal differences in behavioral sensitization following chronic amphetamine exposure. It is well documented that drug seeking behavior varies throughout the day but is dependent on a number of factors such as dose, drug, species, and length of abstinence (Akhisaroglu et al., 2004; Gaytan et al., 1999; Gaytan et al., 1998; Manev & Uz, 2009; Sleipness et al., 2005; Uz et al., 2002). For example, C3H/HeJ mice repeatedly administered cocaine showed greater short term behavioral sensitization (sensitization measured at the end of training or prior to a 1 week abstinence period) early in the light phase than at the start of the dark phase (Uz et al., 2002). Similarly, long term

sensitization (sensitization measured after a one week abstinence period) also displays diurnal differences. In rats, amphetamine produces greater long term behavioral sensitization if repeated doses are administered in the middle of the dark cycle compared to the light cycle (Gaytan et al., 1999) in contrast to cocaine, which produces greater sensitization during the light cycle (Akhisaroglu et al., 2004). Some evidence, though, suggests time of day dependent sensitization to psychostimulants may not be robust across strains (Abarca, Albrecht, & Spanagel, 2002; Akhisaroglu et al., 2004; Hutchinson et al., 2012) or laboratories (Abarca et al., 2002; Uz et al., 2002). C3H/HeN mice, for example, displayed no short or long term time of day dependent differences in behavioral sensitization to methamphetamine (Hutchinson et al., 2012). Moreover, drug seeking paradigms show similar findings whereby time of day dependent modulations of conditioned place preference depend on the strain of rat used (Cain et al., 2004). In the current study, time of day did not influence behavioral sensitization following amphetamine treatment in either short term or long term behavioral sensitization providing further support that time of day dependent sensitization may be strain specific.

A major aim of the current study was to determine if time of day acts as a conditioned stimulus for behavioral sensitization to amphetamine. Previously, Arvanitogiannis et al. (2000) found that amphetamine sensitization in rats depends upon testing and training occurring at the same time of day. The Arvanitogiannis study, however, did not properly control for time of day dependent differences in behavioral sensitization. Thus, the Arvanitogiannis study may merely reflect differences in the diurnal sensitized response and not differences in training/testing

time. Furthermore, the rat strain that was utilized by Arvanitogiannis and colleagues was not reported. This makes generalizations to the literature difficult as time of day learning in conditioned place preference paradigms differs among rat strains, as previously discussed (Cain et al., 2004).

In the current study time of day did not act as a conditioned stimulus for behavioral sensitization. Mice displayed equivalent levels of drug sensitization if testing corresponded to either a standard or novel time of day compared to training. Thus, contrary to hamsters, which imprint training time even if it is not required for rewarding or avoidance related tasks (Cain, Chalmers, & Ralph, 2012; Cain & Ralph, 2009), time of day, in our study, was not a salient cue in the environmental context of drug use. An unpublished replication of this study with cocaine in our laboratory showed similar results suggesting these results are not due to drug differences. A number of methodological factors, however, such as species and procedural differences could account for the difference in our findings versus the Arvanitogianni and colleagues' study. Specifically, we identify three possible alternative hypotheses: 1) our mice may be melatonin deficient which may inhibit time of day dependent learning, 2) time of day dependent learning is species and/or strain specific, and/or 3) the environmental cues overshadowed the learning of temporal cues.

The circadian pacemaker is a major regulator of the pineal gland, which generates the daily rhythmic melatonin levels of mammals: levels peak at night in both nocturnal and diurnal mammals and reach a nadir during the day. Melatonin can, thus, be considered a neuroendocrine representation of night within mammals. Furthermore, the pineal N-acetylserotonin/melatonin system is a likely agent of time

of day dependent learning as it is necessary for diurnal differences in behavioral sensitization (Hutchinson et al., 2012; Hutchinson, Ma, Liu, Hudson, & Dubocovich, 2014; Manev & Uz, 2009; Uz et al., 2002) and conditioned place preference (Clough, Hutchinson, Hudson, & Dubocovich, 2014; Kurtuncu, Arslan, Akhisaroglu, Manev, & Uz, 2004). For example, melatonin synthesizing rats (Sprague-Dawley) and mice (CBA/J) become sensitized to daily injections of cocaine if administered during the day (low melatonin) but not during the night (high melatonin; Askisaroglu et al., 2004). Conversely, MT1 melatonin receptor knockout mice become sensitized to cocaine in either circadian phase, but not MT2 melatonin knockouts (Hutchinson et al., 2012). C57BL/6 mice, a strain that does not synthesize melatonin, becomes equally sensitized in both portions of the light/dark cycle, in some (Hutchinson et al., 2014; Uz et al., 2002) but not all studies (Abarca et al., 2002), following repeated daily injections of cocaine suggesting that high melatonin levels at night may be responsible for the suppression of behavioral sensitization in melatonin synthesizing mice (Uz et al., 2002). Supporting this conclusion, Sircar (2000) found that pretreatment of melatonin prior to cocaine administration in the subjective day prevented the development of behavioral sensitization in rats. Though these studies have been conducted primarily using cocaine, amphetamine is considered to work via a common mechanism. Since melatonin rhythms have not been measured in our mice, but our mice derive from C57BL/6J and 129S1/SvImJ stock, it is possible our mice have altered melatonin synthesization. This deficiency, in turn, may interfere with circadian dependent differences in behavioral sensitization or learning.

Alternatively, the salience of environmental cues in relation to temporal cues may have played a role in our null findings. Sensitization is conceptualized, in part, to involve associative cues. In the simplest sense, environmental cues become associated with drug use and over time predict the onset of the drug (Anagnostaras & Robinson, 1996). The environmental context of drug use, in particular, is a strongly salient cue in the prediction of drug administration, and its expression in some circumstances can be fully governed by the environmental context (Anagnostaras & Robinson, 1996; Badiani et al., 1995; Robinson et al., 1998). In the current experiment animals were administered amphetamine outside of their home cage in a specific environment that would predict drug onset in conjunction with time of day. It is, therefore, possible that the environmental cues overshadowed learning of the temporal context. Conversely, time of day may have been learned, but our test was not sensitive enough to detect time of day dependent differences (i.e., placement in the environment triggered maximum sensitization). Future experiments are needed to parse out environmental learning versus temporal learning.

If time of day is a relevant contextual cue, it remains to be seen where the site of this action is occurring: if the temporal context of drug use is governed directly by the central circadian pacemaker located within the suprachiasmatic nucleus (SCN) of the hypothalamus or is part of an endogenous circadian oscillator that operates outside of the SCN. Mounting evidence suggests that the temporal context of drug use occurs in the latter (Cain et al., 2012; Cain & Ralph, 2009; Tataroglu, Davidson, Benvenuto, & Menaker, 2006). In the case of methamphetamine use, a methamphetamine sensitive circadian oscillator (MASCO) controls methamphetamine

seeking behaviors and functions outside of the SCN (Honma & Honma, 2009; Mohawk, Baer, & Menaker, 2009). Furthermore, the SCN is not necessary for time of day learning in conditioned learning paradigms (Mistlberger, de Groot, Bossert, & Marchant, 1996), food entrainment (Boulos, Rosenwasser, & Terman, 1980), and conditioned place preference (Cain et al., 2012; Cain & Ralph, 2009); though, much work does demonstrate that drugs of abuse directly interfere with core clock functioning (Brager et al., 2010, 2011; Prosser et al., 2008; Prosser et al., 2014; Ruby, Prosser, et al., 2009)

In conclusion, temporal context does not appear to be a salient environmental cue for amphetamine sensitization in mice. Moreover, in contrast to other studies (Arvanitogiannis et al., 2000), our mice displayed clear time of day dependent differences in locomotor activity following an acute dose of amphetamine, but this circadian difference was eliminated following repeated exposures. We hypothesize that a possible deficiency in melatonin and/or overshadowing may have led to our findings.

5.5 FIGURES

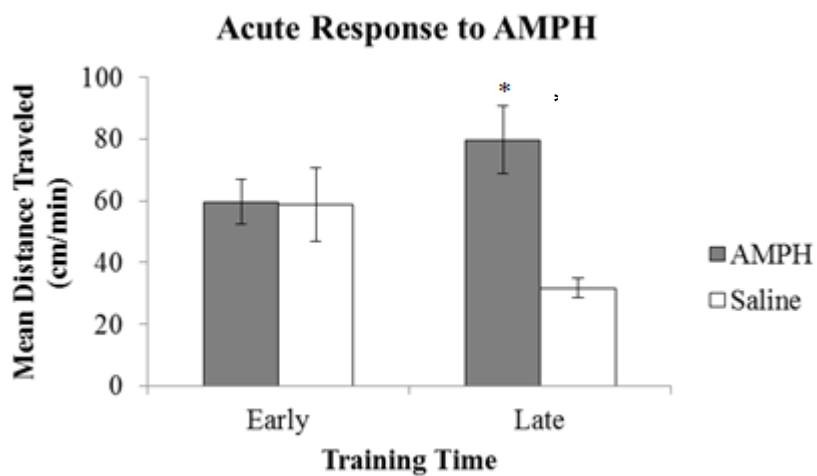


Figure 5.1: Mean $\pm$ S.E.M. horizontal locomotor response following an acute dose of amphetamine (AMPH; grey bars) or saline (white bars) administered either early (left) or late (right) in the light cycle on training day 1. Mice exposed to AMPH late in the day were significantly more active than their respective controls ($p < 0.020$).

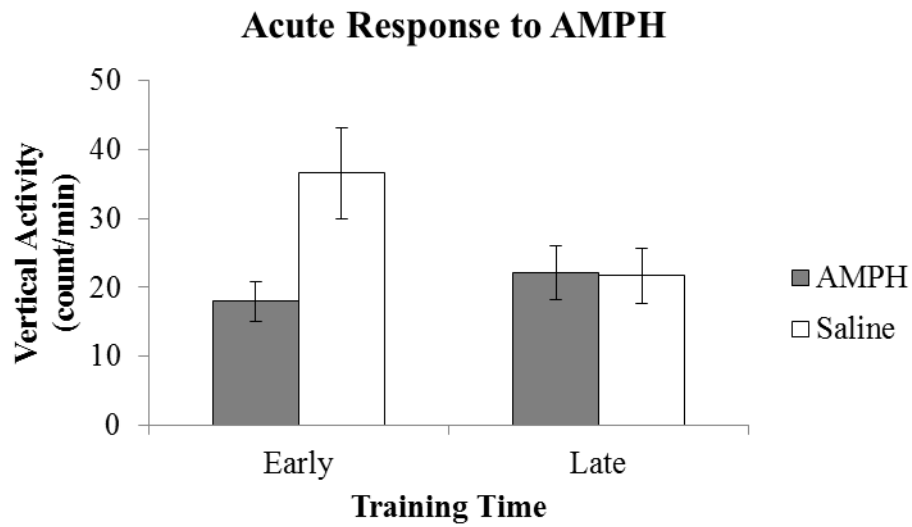


Figure 5.2: Mean $\pm$ S.E.M. vertical locomotor response following an acute dose of amphetamine (AMPH; grey bars) or saline (white bars) administered either early (left) or late (right) in the light cycle on training day 1. Mice exposed to AMPH early in the day had significantly more rearing behaviors than their respective controls ($p < 0.019$).

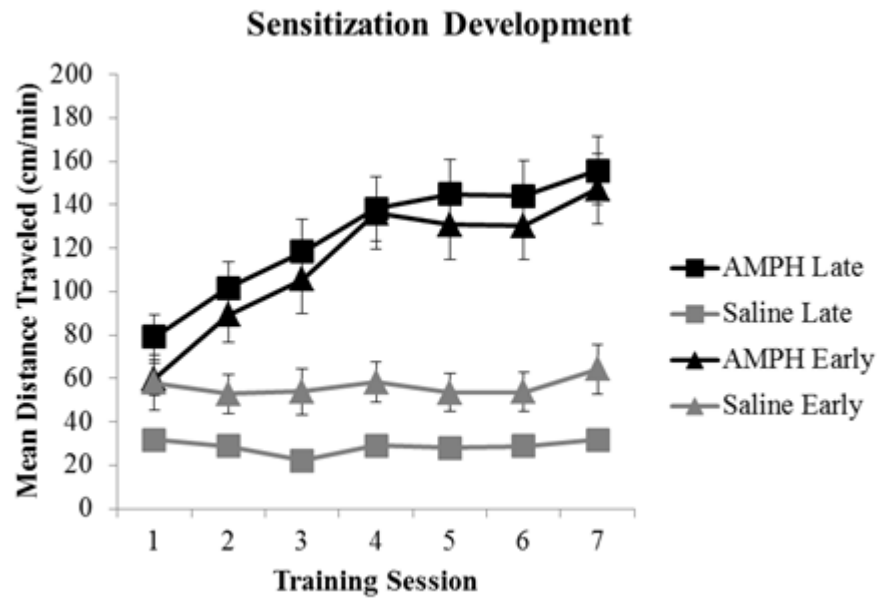


Figure 5.3: Mean $\pm$ S.E.M. horizontal locomotor activity across the 7 days of training to either amphetamine (AMPH; black lines) or saline (grey lines) administered either early (triangles) or late (squares) in the day. The locomotor response to AMPH became progressively more sensitized across training sessions irrespective of the time of day in which training occurred ($p < 0.001$).



Figure 5.4: Mean $\pm$ S.E.M. difference in horizontal locomotor activity from Day 7 to Day 1 in animals exposed to amphetamine (AMPH, grey bars) or saline (white bars) either early (left) or late (right) in the day. Mice exposed to AMPH were significantly more sensitized on the final day of training compared to controls, however, equal amounts of sensitization developed in both experimental groups irrespective of training time. * Significant difference between experimental group and controls ($p < 0.001$).

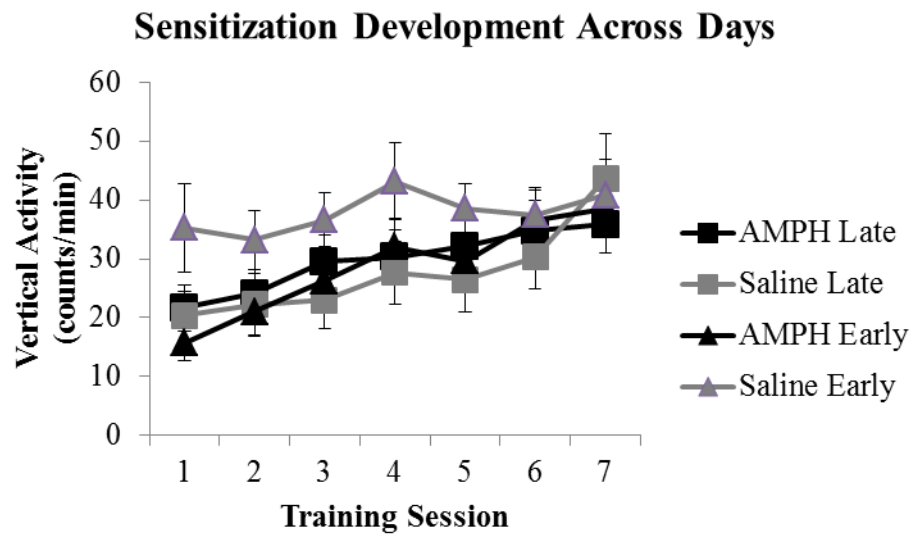


Figure 5.5: Mean $\pm$ S.E.M. vertical activity across the 7 days of training to either amphetamine (AMPH; black lines) or saline (grey lines) administered either early (triangles) or late (squares) in the subjective day. Vertical activity on average increased across training days.

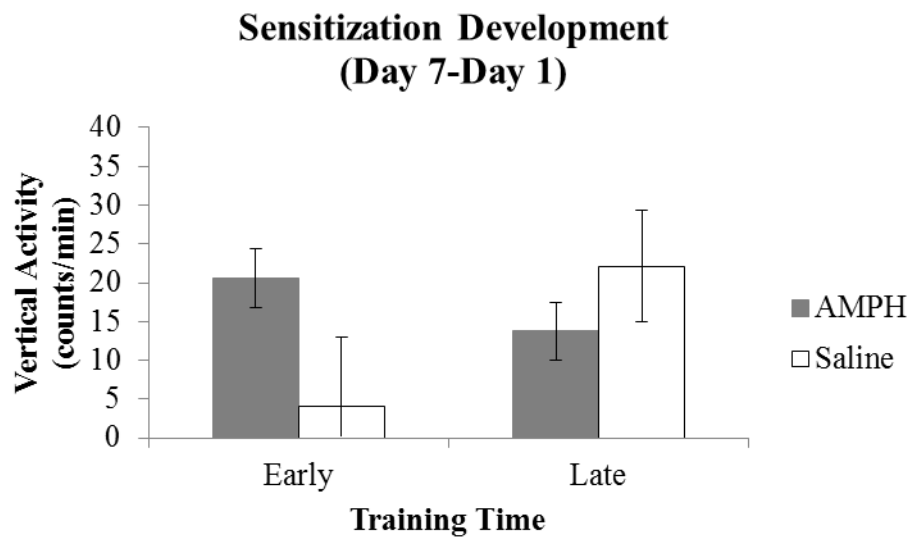


Figure 5.6: Mean $\pm$ S.E.M. difference in vertical activity from Day 7 to Day 1 in animals exposed to amphetamine (AMPH, grey bars) or saline (white bars) either early (left) or late (right) in the day. Mice exposed to AMPH early in the light cycle increased their rearing behaviors compared to controls over the course of training. Conversely, mice that were trained late in the day increased their rearing behavior irrespective of the drug condition. * Significant difference between experimental group and controls ($p < 0.046$).

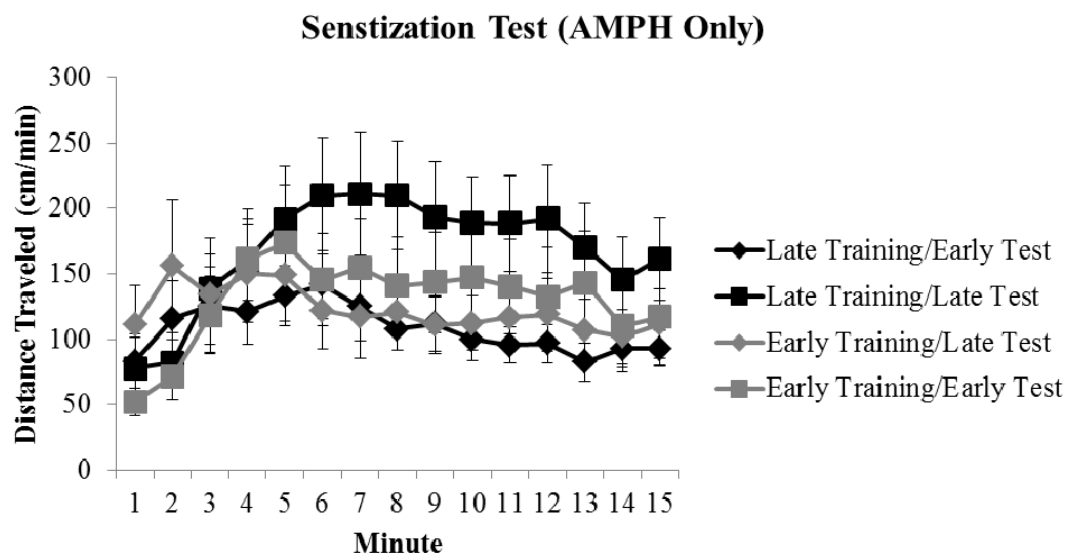


Figure 5.7: Mean $\pm$ S.E.M. horizontal locomotor response across the first fifteen minutes of the sensitization test for animals previously exposed to amphetamine (AMPH) either early (grey lines) or late (black lines) in the day and subsequently tested one week later for behavioral sensitization at either the original time of training (squares) or a novel time of day (triangles). Locomotor activity on average increased across the first 5-6 minutes of the sensitization test before plateauing.

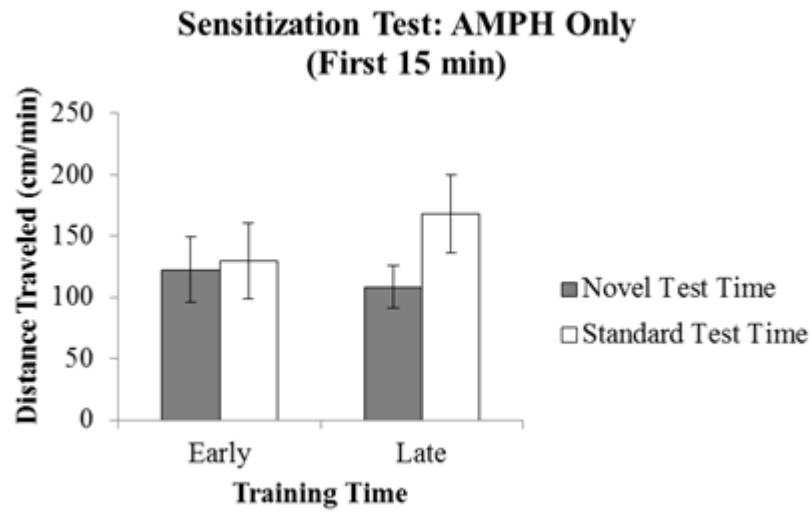


Figure 5.8: Mean $\pm$ S.E.M horizontal locomotor response across the first fifteen minutes of the sensitization test for animals previously exposed to amphetamine either early (left) or late (right) in the subjective day and tested at either the same time (grey bars) or a novel time (white bars) as training. Mice exhibited statistically equivalent locomotor responses regardless of prior training time or testing time.

Chapter 6: Discussion

The ingestion of alcohol varies widely as a factor of both socio-economic and cultural components. In western countries, alcohol is frequently used as a facilitator of social interactions as well as an implement of formal ceremonies. Unfortunately, in some individuals the consumption of alcohol becomes a spiraling addiction that has severe consequences for both the individual and the society. Approximately 18 million people in the US alone suffer from alcohol use disorder (Grant et al., 2004) and alcohol related damage (physical and property) is estimated to be about \$223.5 billion a year as of 2011 (Bouchery et al., 2011). While the development and progression of alcoholism involves a complex interaction of genetic and environmental influences, one environmental factor - the temporal context of drug exposure and withdrawal - is emerging as an important consideration in the addiction process. Understanding whether the timing of drug use and the subsequent withdrawal influences the response and development of alcohol use disorder (AUD) may be a valuable tool in the clinical setting. Chronotherapy (the discipline of improving therapeutic treatments through the use of circadian timing principles) may be useful in the prevention and treatment of AUD and relapse avoidance. Little work, however, has explored the significance of circadian timing in chronic alcohol use and addiction; a void this dissertation addresses.

Specifically, this dissertation asked 1) if the timing of chronic alcohol exposure and withdrawal could alter the development of physiological dependence to the drug,

2) if the physiological withdrawal reaction to chronic alcohol exposure is based on the circadian phase of exposure, the phase of withdrawal, or a combination of both, 3) if the timing of chronic alcohol exposure and withdrawal alters the development of addictive-like behaviors (i.e., psychological dependence), and 4) if time of day serves as a predictive contextual cue for a sensitized drug response (i.e., are time of day cues necessary for amphetamine sensitization).

6.1 Time of Day Affects the Physiological Response to Chronic Alcohol Exposure

Alcohol consumption is, in part, regulated by time of day in both humans and rodents (Danel et al., 2003; Gibson & Shirreffs, 2013; Trujillo et al., 2009). In the non-dependent human, for example, alcohol is preferentially consumed in the early evening; however, in the transition to alcoholism, alcohol craving and consumption increasingly expand into the morning hours (Danel et al., 2003). As a consequence of the consumption pattern, alcohol withdrawal also follows a daily rhythm, likely peaking late at night in the dependent individual. The physiological response to acute doses of alcohol, like many drugs, depends critically on the timing of its administration. Little work, though, has examined the influence of time of day in a chronic model of addiction that more closely models consumption patterns of individuals with advanced AUD. Chapter 2 of this dissertation addressed whether in mice the physiological response to alcohol both during and following chronic alcohol intoxication is modulated by the timing of the intoxication and withdrawal intervals.

The data presented in Chapter 2 indicate that fluctuations in body temperature and activity are dependent upon the time of day of alcohol administration and withdrawal. Mice entrained to two oppositely phased photoperiods and housed together under a 2 hour skeleton photoperiod were repeatedly exposed to 14 h of EtOH vapor inhalation, coinciding with either the animal's subjective night or subjective day, followed by a 10 hour withdrawal period (occurring in the opposite circadian phase as the exposure period). Core body temperature and locomotor activity was substantially decreased when alcohol exposure occurred during the subjective night. Decreases in the body temperature, albeit of a lesser magnitude, also occurred during the subjective day. Circadian dependent differences in the body temperature response are suggestive of either an increased resilience of the subjective day to toxic insults (Gordon, 2010) or a differential enhancement of tolerance and/or sensitization (Moore & Kakihana, 1978; Williams et al., 1993). In rodents, acute i.p. injections of alcohol elicit greater amplitude hypothermia during the subjective day compared to the subjective night (Baird et al., 1998), yet the subjective day is associated with a lower mortality rate to challenge doses of alcohol (Gordon, 2010). Furthermore, repeated daily i.p. injections of alcohol in the subjective day produce tolerance to the hypothermic effects of alcohol, while injections during the subjective night elicit sensitization {Williams, 1993 #14}.

Interestingly, in contrast to the hypothermic effects of alcohol exposure, acute withdrawal induced hypothermia or hyperthermia as a function of the circadian phase. Mice withdrawing from alcohol during the subjective day became *hyperthermic* while mice withdrawing in the subjective night were *hypothermic*. Moreover, as in humans,

mice became hyperactive during acute withdrawal although the magnitude of this effect was greater during the subjective night. The experimental design of Chapter 2 could not distinguish if the withdrawal effects were attributable to the timing of alcohol exposure, withdrawal, or both. The response, however, cannot be attributed to changes in environmental stimuli such as temperature (Myers, 1981), lighting influences (Geller, 1971; Sinclair & Geller, 1972), or handling/husbandry (Crawshaw et al., 1998) as use of the skeleton photoperiod equalized these environmental conditions. While hypothermia is primarily observed in rodents following an acute dose of alcohol extending for approximately 2-4 hours (Baird et al., 1998), both hypothermic and hyperthermic effects are reported following chronic alcohol exposure extending up to 24 hours into withdrawal (Ritzmann & Tabakoff, 1976a; Spanagel et al., 1996; Taylor et al., 2006; Williams et al., 1993). Our data suggest that these varied withdrawal responses may be dependent upon the circadian phases of the exposure/withdrawal cycle.

One of our initial working hypothesis was that the schedule of alcohol administration and withdrawal would produce a sensitized withdrawal response analogous to the kindling-like effect on seizure activity (Becker, 1998), our findings, however, were not supportive of such a conclusion. In humans, prior detoxification experience is highly correlated with relapse (Duka et al., 2004) while in rodents repeated periods of intoxication and withdrawal increase consumption (Becker & Lopez, 2004; Griffin et al., 2009), physical dependence (Becker, Diaz-Granados, & Hale, 1997; Becker, Diaz-Granados, & Weathersby, 1997), and operant responding (O'Dell et al., 2004). The body temperature response was sensitized across rounds

during acute withdrawal; however, this effect was not present when the influence of activity on the body temperature rhythm was accounted for using linear regression (i.e., the *activity independent body temperature rhythm*). Thus, in the predicted absence of activity, alcohol does not sensitize the body temperature response, but alcohol may alter activity over time, which masks the endogenous withdrawal reaction in body temperature. Moreover, the blood ethanol content progressively increased across rounds likely contributing to a perceived sensitization effect. Together these findings suggest that the 1) the circadian phase of alcohol exposure drives the severity of exposure-related hypothermia, 2) bi-directional differences in body temperature can be provoked by controlling for the phase of alcohol exposure and withdrawal and, 3) withdrawal-related hyperactivity drives a sensitized withdrawal reaction in body temperature. These findings provide the first evidence in rodents that in a chronic model of addiction physical dependence may be, in part, governed by the circadian system.

6.2 Circadian Phase of Alcohol Intoxication Modifies the Direction of the Acute Withdrawal Response

Chapter 3 of this dissertation explored whether the bidirectional body temperature response in acute withdrawal (reported in Chapter 2) was driven by the circadian phase of exposure, withdrawal, or both. Utilizing a similar procedural design as the prior experiment, oppositely phased mice were exposed to a repeating paradigm of 24 hours of alcohol vapor inhalation followed by a 24 hour withdrawal period. This pattern of alcohol exposure ensured alcohol intoxication in both circadian

phases while limiting the acute withdrawal reaction (1-11 hours following the removal of alcohol vapor inhalation) to a single phase (subjective night or subjective day). The presented data replicated exposure-related hypothermia and provided additional evidence that the circadian phase of alcohol exposure and withdrawal alter the withdrawal response.

The magnitude of the decreases in body temperature and activity during active alcohol exposure was modified by the circadian phase of the animal. Mice in the subjective night had significantly lower body temperatures and decreases in activity than controls or animals in the subjective day. Not surprisingly, most of these circadian effects were more pronounced when the mouse was exposed to longer periods of alcohol vapor inhalation (1-11 hours versus 13-23 hours).

Contrary to our prior findings, mice withdrawing in either circadian phase (i.e., subjective day or subjective night) were hyperthermic, but mice withdrawing in the subjective day had more severe hyperthermia than those in the subjective night. Moreover, hyperactivity, a hallmark of the withdrawal reaction, was more severe during the subjective day. Using linear regression, the influence of activity on the body temperature rhythm was calculated. The activity independent body temperature rhythm yielded identical acute withdrawal responses between the experimental groups suggesting that increased activity during the subjective day masked the endogenous body temperature rhythm leading to the higher measured core body temperature output. Thus, because the hyperthermic withdrawal response was equivalent in the experimental groups, which received alcohol exposure in both circadian phases but were limited to one circadian phase during acute withdrawal, we can conclude that the

circadian phase of alcohol exposure appears to mediate the acute withdrawal response even though some influence of activity, via the circadian phase of withdrawal, may regulate the body temperature response.

When considered with the findings from Chapter 2, prolonged alcohol exposure in the subjective night appears to drive more severe physical withdrawal reactions than the subjective day. Mice become hyperthermic which is associated with an increased risk of mortality (Finn et al., 1989; Gordon, 2010). Withdrawal related hyperactivity may, in turn, worsen the severity of this reaction during the subjective day, but only if the animal is already hyperthermic. Clinically, this study underlines the importance of attenuating activity during withdrawal to improve survival rates due to hyperthermia related mortality in humans. Moreover, it provides the first evidence that in a detoxification setting detoxification should be started immediately if consumption has only recently occurred in the inactive cycle but managed intoxication should be administered until the active cycle if intoxication occurred for a prolonged indefinite period.

We cannot, however, separate the link between exposure-related hypothermia and circadian phase in our study, nor exclude the influence of prolonged alcohol exposure on the acute withdrawal response. It is possible that severe exposure-related hypothermia alone drove the hyperthermic withdrawal response and not circadian phase (which elicited a hypothermic exposure response). Moreover, alcohol vapor exposure in Chapter 3 was of a longer duration than in Chapter 2 (24 versus 14 hours) and may have influenced the direction of the withdrawal reaction. Additional studies are necessary to test these alternative hypotheses.

6.3 Circadian Phase of Alcohol Exposure Differentially Increases Alcohol Consumption

The emergence of many addictive-like behaviors (e.g., increased consumption, operant responding, and increased anxiolytic behaviors) in rodents appears to co-occur with a sensitized withdrawal response (Becker, Diaz-Granados, & Weathersby, 1997; Griffin et al., 2009; Heilig et al., 2010; O'Dell et al., 2004; Valdez et al., 2002).

Models that promote greater physical dependence may therefore promote greater psychological dependence to alcohol. Chapter 4 assessed whether psychological dependence to alcohol, measured by voluntary consumption and anxiolytic behaviors, could be modified by controlling for the circadian phase of alcohol exposure and withdrawal.

It is well established that repeated cycles of chronic intermittent alcohol vapor exposure increases alcohol consumption in mice (Becker & Lopez, 2004; Griffin et al., 2009). Chapter 4 utilized a similar alcohol vapor protocol as the Becker laboratory and thus, we had two a priori expectations based on the literature: 1) mice in both experimental groups would increase consumption compared to controls and 2) mice exposed to alcohol vapor in the subjective night (a time that induces greater physical dependence) would have greater alcohol consumption than those exposed in the subjective day. We, however, found that intermittent alcohol exposure did not increase consumption in both experimental groups and found limited evidence that the circadian phase of exposure escalates alcohol consumption. Specifically, animals exposed to alcohol vapor inhalation in the subjective day (withdrawal in the subjective

night) had greater ad libitum consumption of alcohol compared to controls, but this effect was not significant when compared to the baseline voluntary consumption levels. Lickometer data indicated that this increase occurred primarily at the start of subjective night.

A major difference between our study and the studies of Becker and colleagues was the use of ad libitum access to alcohol during the 3 day consumption test instead of a 2 hour limited access paradigm. Two-hour limited access paradigms employ 1) a mouse's tendency to naturally consume higher levels of alcohol at the start of dark cycle (e.g., a drinking in the dark paradigm), and 2) a period of alcohol deprivation, which increases the reinforcing properties of alcohol (i.e., the alcohol deprivation effect; Sparta, 2009). Due to the nature of circadian manipulations mice had to have continuous access to alcohol in order for testing to occur at the same time in withdrawal and to ensure both groups had access to alcohol during peak consumption times (i.e., the subjective night). While few studies to date have measured continuous voluntary alcohol consumption in alcohol dependent mice, the current study is not the only to measure decreased voluntary alcohol consumption in a 24 hour two-bottle choice paradigm. Allen, Fantom, and Wilson (1982) found that mice made physically dependent to alcohol (following an eight day alcohol liquid diet protocol) voluntarily consumed less alcohol when it was presented for six days under 24 hour two-bottle choice conditions (alcohol/water solution and water) compared to their pre-dependent baseline voluntary consumption levels. Thus, increased alcohol consumption may not generalize to situations where the reinforcing effects of alcohol, such as alcohol deprivation, are not present. Furthermore, continuous access to EtOH may, in fact,

lower voluntary consumption of alcohol after a withdrawal period. Alternatively, controls in our study may have consumed enough alcohol during the sucrose fading procedure to influence dependence and that a 10% w/v EtOH solution was not sensitive enough to increase consumption in experimental mice while discouraging consumption in controls. Additional studies that control for these methodological differences are needed to clarify our findings from the literature and to provide insights into whether the timing of alcohol consumption facilitates future increased consumption in humans.

Furthermore, we did not observe any anxiolytic differences in our experimental mice from controls on the elevated plus maze. As in other studies (Beeler et al., 2006), a circadian dependent difference in the expression of anxiolytic behaviors did exist independent of drug exposure. Animals tested in the subjective night were more anxious than their subjective day counterparts. Anxiety-like behaviors following chronic alcohol exposure are well established in rats (Baldwin et al., 1991; File, 1994; Lal et al., 1991; Schulteis et al., 1996), but not reliably replicated in mice (Finn et al., 2000; Kliethermes, 2005; Kliethermes et al., 2004; Onaivi et al., 1989; Pokk et al., 2001). Moreover, no studies using alcohol vapor inhalation have found increased anxiolytic behaviors, and those that do, using other methods of induction (e.g., liquid diet), often co-occur with decreased locomotor activity complicating the interpretation of these findings (Kliethermes, 2005). Thus, mice do not appear to be a reliable species to model anxiolytic behaviors in a chronic model of addiction.

6.4 Time of Day Is Not a Learned Environmental Cue for Behavioral Sensitization

Chapter 5 investigated whether time of day is a salient environmental cue for drug sensitization. Limited evidence suggests that time of day is coded as part of the environmental context of drug use and can elicit drug seeking behaviors (Cain et al., 2004) and a sensitized locomotor response (Arvanitogiannis et al., 2000) to psychostimulants. For example, (Arvanitogiannis et al., 2000) reported that rats administered cocaine in the morning only exhibited a sensitized behavioral response when tested in the morning, but not in the afternoon (a novel time of day), suggesting that time of day served as a salient environmental cue to behavioral sensitization. Behavioral sensitization to psychostimulants, however, differs as a function of time of day (Akhisaroglu et al., 2004; Gaytan et al., 1999; Manev & Uz, 2009) and may account for differences in locomotor sensitization between training and testing time in the Arvanitogiannis study.

In this dissertation, C57BL/6J x129S1/SvImJ mice were repeatedly administered i.p. injections of amphetamine either early or late in the day then tested, following a one week abstinence period, either at the same time of day as training or at a novel time of day. Measurement of behavioral sensitization by changes in locomotor activity lead to three relevant findings: 1) mice displayed diurnal differences in acute drug sensitivity to amphetamine, 2) the animals did not exhibit diurnal differences in long term drug sensitivity, and 3) no evidence indicated time of day acts as a learned environmental cue to behavioral sensitization. We suggest mice

either do not use time of day as a learned environmental cue to behavioral sensitization, that a possible melatonin deficiency in our strain may have inhibited the learning of time of day as a relevant environmental cue, and/or overshadowing may have impaired time of day dependent sensitization and the learning of temporal context in the current study.

Mice were hyperactive following an acute dose of amphetamine administered late, but not early, in the light cycle. Following repeated exposures to amphetamine, the general locomotor response was equally sensitized irrespective of the time of drug administration. The acute and sensitized responses in the current study, however, stand apart from the general literature which reports 1) increased locomotor activity following an acute dose of psychostimulant irrespective of the time of day (Gaytan et al., 1998; Hutchinson et al., 2012; Manev & Uz, 2009; Uz et al., 2002) and 2) time of day dependent differences in the sensitized locomotor response following chronic psychostimulant administration (Gaytan et al., 1999; Manev & Uz, 2009; Uz et al., 2002). The divergent findings (both chronic and acute) in the literature compared to our study are not overly surprising since time of day dependent sensitization may not be robust across mice strains (Abarca et al., 2002; Akhisaroglu et al., 2004; Hutchinson et al., 2012) or laboratories (Abarca et al., 2002; Uz et al., 2002). C3H/HeN mice, for example, display no short or long term time of day dependent differences in behavioral sensitization to methamphetamine (Hutchinson et al., 2012) and studies in C57BL/6J mice report no diurnal differences in behavioral sensitization in some (Hutchinson et al., 2014; Uz et al., 2002), but not all studies (Abarca et al., 2002). Strain differences in other drug behavioral paradigms, such as conditioned

place preference are also seen, whereby time of day dependent modulations of conditioned place preference depends on the strain of rat used (Cain et al., 2004).

Time of day did not act as a conditioned stimulus for behavioral sensitization in our study. Mice displayed equivalent levels of drug sensitization if testing corresponded at a standard or novel time of day compared to training. Thus, contrary to hamsters, which imprint training time even if it is not required for rewarding or avoidance related tasks (Cain et al., 2012; Cain & Ralph, 2009), time of day, in the current study, was not a salient cue in the environmental context of drug use. An unpublished replication of this study with cocaine in our laboratory showed similar results suggesting these results are not due to drug differences. A number of methodological factors, however, such as species and procedural differences could account for the difference in our findings versus Arvanitogiannis et al. (2000).

Specifically, we propose a possible melatonin deficiency in our mice, and/or environmental cues overshadowing the learning of temporal cues as possible hypotheses for our mice not coding time of day as a salient environmental cue to drug use. Much evidence has pointed to the pineal N-acetylserotonin/melatonin system as a likely regulator of time of day dependent learning as it is necessary for diurnal differences in behavioral sensitization (Hutchinson et al., 2012; Hutchinson et al., 2014; Manev & Uz, 2009; Uz et al., 2002) and conditioned place preference (Clough et al., 2014; Kurtuncu et al., 2004). For example, melatonin synthesizing rats (Sprague-Dawley) and mice (CBA/J) become sensitized to daily injections of cocaine if administered during the day (low melatonin) but not during the night (high melatonin; Askisaroglu et al., 2004). Conversely, MT1 melatonin receptor knockout

mice, but not MT2 melatonin knockouts, become sensitized to cocaine in either circadian phase (Hutchinson et al., 2012). Furthermore, as mentioned above, C57BL/6 mice, a strain that does not synthesize melatonin, become equally sensitized in both portions of the light/dark cycle, in some (Hutchinson et al., 2014; Uz et al., 2002), but not all, studies (Abarca et al., 2002) following repeated daily injections of cocaine. This suggests that high melatonin levels at night may be responsible for the suppression of behavioral sensitization in these mice (Uz et al., 2002). Since our mice derive from C57BL/6J and 129S1/SvImJ stock, it is possible our mice have altered melatonin synthesization, though this has not been measured.

Alternatively, the salience of environmental cues in relation to temporal cues may have played a role in our null findings. The environmental context of drug use is a strongly salient cue in the prediction of drug administration as much work has shown that environments paired solely with drug administration can exert greater behavioral sensitization than in non-paired environments (Anagnostaras & Robinson, 1996; Badiani et al., 1995; Robinson et al., 1998). In the current experiment animals were administered amphetamine in an environment that would predict drug onset in conjunction with time of day. It is possible that the environmental cues overshadowed learning of the temporal context. Conversely, time of day may have been learned but our test was not sensitive enough to detect time of day dependent differences (i.e., placement in the environment triggered max sensitization). Future experiments are needed to parse out environmental learning versus temporal learning in order to fully determine if time of day is a learned environmental cue to drug sensitization. One

possible way this could be achieved would be to run a similar experimental paradigm as presented herein but train and test animals within their home cage environment.

6.5 Conclusions

This dissertation provides evidence that the timing of drug exposure and withdrawal is an important consideration in the addiction process and suggests future lines of research into time of day as a chronotherapeutic tool in the prevention of relapse in psychostimulant addiction. In a rodent model of addiction, prolonged repeating periods of alcohol exposure (i.e., alcohol vapor inhalation), which models the consumption of pattern of severe alcoholics, elicits more severe withdrawal reactions if alcohol is administered during the active cycle. Much evidence suggests that physical dependence is associated with the development of greater psychological dependence to the drug (Becker & Hale, 1993; Griffin et al., 2009; Heilig et al., 2010; Lopez & Becker, 2005; Valdez et al., 2002; Valdez et al., 2004). Transitionally, this suggests that in a clinical setting, in addition to counseling and other preventative tools, those at risk for developing AUD may have a greater chance at prevention by limiting intake to specific circadian phases. Utilization of chronotherapeutic tools may curtail the physical withdrawal reaction and curb escalating consumption (though additional studies are needed to verify and clarify a relationship between circadian phase and consumption). For example, consumption during a human's inactive cycle may mitigate withdrawal symptoms potentially improving the prevention of AUD. In the case of those with AUD, our study stresses the importance of limiting activity during alcohol withdrawal, especially in the inactive cycle. Hyperactivity during the

inactive cycle increases the hyperthermic withdrawal response which, in turn, increases the risk of mortality in withdrawal. Moreover, the transition of the circadian drinking to earlier within the day (i.e., the active cycle) in individuals with AUD, may help maintain the addiction as it increases the severity of the physical withdrawal reaction. For those in recovery from drug addiction, we did not find compelling evidence that time of day may illicit drug sensitization or cravings in a mouse model of addiction; however, additional research is needed to clarify our results. In conclusion the timing of drug administration and withdrawal may be a viable chronotherapeutic tool in the prevention, treatment, and survival of human alcoholics.

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