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RELATIONSHIPS BETWEEN CIRCADIAN RHYTHMS 
AND ETHANOL INTAKE IN MICE

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

by 

Jennifer L. Trujillo

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Professor Michael Gorman, Chair
Professor Stephan Anagnostaras
Professor Sandra Brown
Professor Sean Drummond
Professor Amanda Roberts
Professor Gerhard Schulteis

2009
The Dissertation of Jennifer L. Trujillo is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009
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ABSTRACT OF THE DISSERTATION

RELATIONSHIPS BETWEEN CIRCADIAN RHYTHMS
AND ETHANOL INTAKE IN MICE

by

Jennifer L. Trujillo

Doctor of Philosophy in Psychology

University of California, San Diego, 2009

Professor Michael Gorman, Chair

This dissertation integrates methods from alcohol and circadian rhythms research to explore relationships between ethanol and circadian rhythms in mice. Ingesting alcohol at certain times of day differentially affects the body; circadian rhythms also impact preference for drinking alcohol at different times of day. The influence of circadian timing on development and maintenance of ethanol drinking patterns was studied in Chapter 2. This showed how establishing a history of ethanol exposure at preferred or non-preferred times of day can influence voluntary ethanol intake for several subsequent weeks. Chapter 3 asked if circadian period is causally related to alcohol intake and investigated this by creating non-24 hour light cycle and measuring ethanol intake in C57BL/6J as well as in high and low alcohol preferring (HAP and LAP) mice. Successful manipulations did not affect amount of ethanol drinking or preference indicating there is not a direct causal effect of circadian period on ethanol intake. Chapter 4 moved from cir-
cadian manipulations and voluntary ethanol intake to exploring the influence of chronic ethanol dependence and withdrawal on circadian rhythms. Daily body temperature and activity rhythms were recorded in mice exposed to repeated rounds of ethanol vapor. Ethanol vapor resulted in a reduction in amplitude of the core body temperature rhythm during withdrawal, an effect that was potentiated in subsequent vapor rounds. However, there were no lasting effects observed on body temperature or activity rhythms beyond the first 3 days following ethanol exposure. Together these studies have advanced knowledge about interactions between alcohol and the circadian system. They showed circadian scheduling of ethanol exposure can predict later ethanol intake, having implications that may be beneficial in the development of alcohol use disorder treatments. Experimentally induced changes to circadian period did not predict ethanol intake. This suggests that altering key aspects of the circadian system is not related to amount consumed or preference for ethanol. Finally, changes to circadian rhythms during withdrawal from ethanol signify changes to body function that are related to the degree of prior ethanol exposure; these might be useful in the future as indices of withdrawal or dependence severity.
Chapter 1

Introduction and Background

Literature

Alcohol is consumed prevalently by at least half the U.S. population. For many this is a leisure activity that does not intrude upon daily life functioning, but for approximately 5% of the U.S. population, this alcohol use includes alcohol abuse and dependence which greatly impacts their social and occupational lives [Grant et al., 2004]. Potential effects of alcohol on the body’s circadian rhythms either during or following intake have the potential to alter core processes of bodily functioning and might even influence continued alcohol intake. Additionally, ingesting alcohol at certain times day differentially affects the body (e.g., the potential lethality of alcohol varies according to time of day due to rhythmic differences in metabolism). The body’s circadian clock, the suprachiasmatic nucleus (SCN) coordinates daily rhythms in gene expression and organ function throughout the body. Circadian rhythms also impact preference for drinking alcohol at different times of day. Further, evidence suggests another role for circadian rhythms controlling
alcohol intake, that is, the endogenous length of the daily rhythm (such as a 23.4 hour rhythm opposed to a 24 hour rhythm) may influence alcohol intake.

This dissertation integrates methods from alcohol studies and circadian rhythms research to explore relationships at the intersection of these two areas. Separately, each of these areas has clear health implications; alcohol effects the body’s major neurotransmitters systems and circadian rhythms are present in both behaviors and organ function. Investigations on the relationship between alcohol and circadian rhythms have looked at numerous interactions, sometimes with clear findings, but more frequently with equivocal results. There are many reasons for this; the huge variety of methods employed to administer alcohol and to measure circadian rhythms no doubt contributes to discrepancies. In the current studies, a circadian perspective is highlighted in experiments that ask: 1) How does circadian timing influence the development and maintenance of alcohol drinking patterns? 2) Is circadian period causally related to alcohol intake? 3) Does ethanol dependence create changes to circadian rhythms beyond withdrawal, and how are rhythms affected after repeated rounds of ethanol dependence?

1.1 Overview of Circadian Rhythms

There are a number of circadian rhythms (activity, body temperature, hormonal levels, sleep pattern), each of which has a number of descriptive parameters (for this entire section, please refer to [Moore-Ede et al., 1982]), such as period (length of the rhythm) and phase (state of the rhythm within its period). Each rhythm can also be characterized in terms of its amplitude, i.e., point of high body temperature in late afternoon versus its low point during early morning. In the absence of external cues, these rhythms persist,
and free-run, that is, they run according to the body’s endogenous period length. A free running circadian rhythm has an endogenous period of approximately 24 hours. In an animal lab, a free running period (FRP) is assessed under constant conditions, i.e., constant darkness. However, in the natural world, rhythms are subject to the environment and are typically entrained or synchronized to an external stimulus; for instance, the 24 hour light-dark (LD) cycle entrains the body to a precise 24 hour rhythm. In the laboratory this is usually done with a 24 hour LD cycle with ratio of light to dark that varies according to study (i.e. summer, winter, or equatorial lighting conditions). Free running or entrained rhythms may be phase shifted; that is, something may cause the next phase to occur earlier than expected (a phase advance) or later than expected (a phase delay). Other stimuli may also affect a free running or entrained rhythm, such as a cage change (in animals) or activity, limited access to food, or dim light. Alternative to a traditional 24 hour period, animals can be entrained or exposed to a LD cycle longer or shorter than 24 hours, called a T-cycle. (Extremely long or short T cycles can even be used to force the body’s circadian rhythms to de-synchronize from one another.) Between individuals, the shape (i.e., unimodal, bimodal, etc.) and period of each rhythm may vary somewhat; however, the rhythms belonging to an individual are quite stable day to day (assuming they are not interfered with). Additionally within individuals, the shape of the rhythms may change with development or age.

In mammals, rhythms are considered under control of a central clock, namely, the suprachiasmatic nucleus. However, the clock may direct these rhythms directly or indirectly via peripheral or slave oscillators under its control; these slave oscillators, in turn, output the overt rhythm. Individual rhythms may not only differ in their phase
relationships to one another, but may also be dissociated. Thus, while one aspect of investigation focuses on alcohol’s effects on the overt rhythms, to determine whether these effects influence a clock output is a separate issue. The influence may be solely on the clock’s output with the underlying clock unaffected, or it may represent a real change in clock function. For example, alcohol is a sedative; it will temporarily lead to decreased activity and changes to body temperature. While this is an effect on rhythm output, to determine an effect on the body’s clock would require long-lasting changes. Specifically, this would mean continuous or long term monitoring of rhythms following alcohol exposure.

1.2 Circadian Effects on Alcohol Intake

Two of the empirical Chapters (2 and 3) address questions on how circadian rhythms contribute to ethanol intake. Below is a broad overview of research that has been conducted on this topic.

1.2.1 Time of Day Effects on Alcohol Preference

Alcohol consumed at different times of day produces differences in alcohol metabolism and susceptibility to lethal effects, time of day preferences, and cognitive effects. For example, mortality rate of rodents is highest early in the dark or active phase. Rodents voluntarily consume more alcohol in the dark, during their active phase [Danel and Touitou, 2004, el Guebaly, 1987, Smith et al., 1980]. Humans with alcoholism also reveal a circadian rhythm in their preference for their first drink of the day, also early into their active phase [Danel et al., 2003]. Circadian timing regulates alcohol
intake across species.

But how does it influence an individual’s long-term, subsequent intake? For instance, if an adolescent or young adult only has access to alcohol at a particular time of day such as early evening, how much do they drink in adulthood when they are able to drink at any time of day? Conversely, one could ask: given any history of alcohol exposure/intake, can it be altered by instituting a particular regimen of alcohol exposure based on a timed circadian schedule? An older study gave alcohol preferring (P) rats unrestricted access to alcohol for two weeks followed by a restricted schedule of intake for either 4 continuous or 4-1 hour periods per day to see if this would influence intake during another 2 week period of unlimited access [Murphy et al., 1986]. This study found no effects of the scheduled access on before and after unlimited alcohol intake; however, they did not manipulate the time at which alcohol was offered, nor did they mention the specific circadian time of the exposure in the study.

Although timing has been determined to play a large role in alcohol consumption, particularly in animal research, researching its role in development and control of alcohol use has been neglected. This leads to the question of whether administering ethanol at circadian preferred or non-preferred time of day can influence not only current but also subsequent ethanol intake. Chapter 2 will address this topic.

1.2.2 Circadian Manipulation and Alcohol Intake

Support for the possibility that altered circadian function is associated with alcohol intake comes from research that examines the relationship between shift work or jet lag, which disturb or shift the state of the circadian rhythm, and alcohol intake. In humans, surveys of international business travelers who commonly experience jet lag and
nurses working night or rotating shifts show greater amounts of alcohol intake [Rogers and Reilly, 2002, Trinkoff and Storr, 1998]. These are selected populations (people who chose to work in shift-work or jet-lag conditions) that may confound data.

In rodents, both single and chronic phase shifting of the clock is argued to produce higher amounts of alcohol intake in the day(s) following the shifts [Gauvin et al., 1997]. This is the dominantly cited study in reviews of years past e.g., see [Hiller-Sturmhofel and Kulkosky, 2001, Wasielewski and Holloway, 2001]. It is one of the few and most frequently cited study on phase shifting and subsequent alcohol intake in rodents. In it, rats were trained to receive alcohol at particular times of day, and when cycles were shifted, drinking was measured at the same clock time as prior to the shift [Gauvin et al., 1997]. This led to uninterpretable results as this intake 1) was not measured with consistent timing with respect to the shift, and 2) occurred at a different point of the circadian cycle. Thus, alcohol intake was measured at 6 am the following day whether a shift occurred at 11 pm or 2 pm. This led to inconsistent timing of alcohol testing with regard to the shift. While this may have been done to control for time of day effects on ethanol intake, shifting the circadian rhythm leads to this ethanol exposure time occurring at a different part of the circadian cycle. This confounds the results. More recently, this question has been re-visited in a more straight-forward study that looked at the effects of phase advancing male and female high alcohol drinking (HAD) rats on voluntary alcohol intake [Clark et al., 2007]. This study phase shifted the animals every few weeks and measured alcohol intake throughout; however it did not find any uniform effects of shifting on intake. Females showed decreased levels of alcohol drinking for the first several weeks compared with control rats whereas males showed no differences in
initial weeks, but several weeks into the manipulations began to drink more alcohol than control rats.

1.2.3 Circadian Disruption and Alcohol Intake

Clinical populations of humans with sleep problems (e.g., insomnia) that can also be equated to circadian disruptions, have an increased risk of developing alcohol use disorders [Brower, 2001]. Sleep problems (over-tiredness and difficulty sleeping) in children as young as 3-5 years predict early onset of alcohol drinking in adolescents [Wong et al., 2004]. This strong behavioral finding shows an early rhythm disturbance can serve as a risk factor for early onset alcohol intake. In part, this could potentially result from attempts to self-medicate sleep rhythm abnormalities. For example, in one study, adult humans with and without insomnia were given the choice of alcohol or water before bedtime; their choices and number of refills were monitored [Roehrs et al., 1999]. People with insomnia were more likely to choose alcohol before bedtime, and to take more alcohol refills. Intriguing EEG results from this study also found when these patients drank alcohol before bedtime it caused changes in that night’s sleep patterns. Their sleep consequently appeared more similar to control sleep patterns (controls who had not received alcohol). This result supports a self-medicating role of alcohol; intake temporarily led to rhythms that appeared closer to normal.

In rats, sleep problems have been induced through early postnatal exposure to antidepressants, which down-regulate monoamine neurotransmitters involved in sleep. As adults, these animals showed higher rates of alcohol self-administration compared to controls [Hilakivi et al., 1987]. However, inducing sleep problems without adding other confounds is a difficult task. It is unclear whether it was the early sleep disturbances, the
reduced monoamine levels, or both that were responsible for this effect. For example, in rats decreased monoamine levels associated with neonatal antidepressant exposure can also lead to depression later in life [Hilakivi and Hilakivi, 1987] which may have its own mediating effects on sleep and ethanol ingestion.

1.2.4 Period

New studies have looked at endogenous period length and alcohol preference in rats and mice [Hofstetter et al., 2003, Rosenwasser et al., 2005c]. Mice bred to have high alcohol preference (HAP) showed greater amounts of activity and a shorter period length (shorter tau) than low alcohol preferring (LAP) mice [Hofstetter et al., 2003]. Neither group actually received alcohol. Mice and rats bred for genetic preferences for high or low amounts of alcohol also show differences in activity and free running period length [Hofstetter et al., 2003]. This study adds to evidence that individual differences in rhythms can predispose one to alcohol intake. In combination with other studies [Roehrs et al., 1999, Wong et al., 2004] they show that circadian rhythm abnormalities may serve as a risk factor for increased drinking, for early onset drinking, or for a higher preference.

By focusing on an endogenous circadian rhythm in constant conditions, this study also used a more reliable measure of clock function. However, in these distinctly bred HAP and LAP mice, associated effects could be related to the alcohol preference, but they could also be due to one of the other many co-occurring characteristics in these strains. For instance, HAP mice are more active than LAP mice, which may have a feedback effect with running in wheels under constant conditions, causing a shorter period length. This effect is considered by the authors and the feedback effects of activity on period length have their own studies with discrepant results [Koteja et al., 2003, Mistlberger...
and Holmes, 2000].

In a later chapter we further investigate the relationship between length of period and alcohol intake, by manipulating period length to see if this affects alcohol intake. For this purpose, T cycles (a form of entraining that uses LD cycles that are shorter or longer than 24 hours) are used to induce period differences.

### 1.3 Alcohol Effects on Circadian Rhythms

Alcohol affects a variety of circadian rhythms. For instance, acute or chronic alcohol exposure may attenuate the amplitude of daily body temperature oscillations [Baird et al., 1998, Danel et al., 2001, Danel and Touitou, 2004, Taylor et al., 2006], and corticosteroid levels [Kakihana and Moore, 1976, Madeira et al., 1997]. Alcohol affects daily locomotor activity (intensity and duration) and sleep patterns (multiple parameters) e.g., [Rosenwasser et al., 2005a, Ehlers and Slawecki, 2000]. Other studies have examined effects of alcohol on SCN or related function in the brain [Allen et al., 2004, Chen et al., 2006, Janak et al., 2006, Madeira et al., 1997, Ruby et al., 2009].

Duration of alcohol administration in these studies ranges from none (in people who have a history of alcoholism but are currently abstinent), to single doses, to several months. Acute doses of alcohol are often used to investigate phase shifting effects. A phase shift would show the ability of alcohol to disrupt the rhythm’s timing, causing it to move to a different phase, forward or backward, before resuming its normal oscillation. Chronic alcohol regimens are important to model human alcohol consumption as well as to compare effects of long term exposure on circadian rhythms. It may be administered with repeated injections, liquid diet, or ethanol vapor; the goal is often to produce depen-
dence. To this end, vapor inhalation has the advantage of being non-stressful compared to the handling and shots accompanying injections, and the inconsistent rate or timing of ingestion associated with a liquid ethanol diet procedure. Two bottle choice (alcohol or water) self-administration does not usually result in dependence.

1.3.1 Period

Free running period and phase shifting effects are typically based on locomotor activity. While the information above deals with activity increases or decreases, here we are concerned with behavioral phenomena closely related to clock function. Observation of alcohol effects on free running period and phase shifting provides some of the strongest evidence that alcohol’s influence on circadian rhythms is due to a change in the clock itself rather than downstream effects. Effects on other rhythms that are not measured in constant conditions can be influenced by numerous factors. Thus, while there may be a clear effect on a rhythm, whether these are direct or downstream influences of the clock is difficult to distinguish. Downstream effects are no less interesting, but it is obviously important, ultimately, to understand underlying causes for clinical applications.

Both increases and decreases in the active phase or FRP have been found following exposure to alcohol. This could result from the length of alcohol exposure and the length of time the rhythm is recorded e.g., a few days of withdrawal versus several months abstinent. Blinded hamsters (free running) receiving an alcohol solution in place of water showed a small but non-significant increase in period length compared to baseline [Zucker et al., 1976]. Hamsters receiving weeks of liquid alcohol exposure showed less activity and a slight, non-significant increase in period in constant dim compared with control hamster [Mistlberger and Nadeau, 1992]. The effect was maintained during weeks
of observation after alcohol was discontinued. Most rats also maintained in constant dim
with liquid alcohol showed period lengthening, though some showed no effect and oth-
ers a period decrease [Rosenwasser et al., 2005a]. These may be inconsistent, but they
investigated and found interesting individual differences. Specifically, those with shorter
periods at baseline tended to show lengthening while those with longer periods showed
decreases during alcohol exposure. Following alcohol, however, animals showed further
changes (increase or decrease) in period that were not stated to be predicted by prior
period length.

Prenatal exposure to alcohol has shown distal effects on FRP later in life. Ex-
posure to alcohol in neonates results in reduced length of FRP compared to controls as
measured during postnatal development [Allen et al., 2004]. Why this is different from
the more common increase in FRP seen in adults could be due to chance and/or develop-
mental factors. That is, effects with adults have not shown entirely consistent effects on
FRP. On the other hand, alcohol exposure on a young, developing circadian system may
have vastly different long term effects compared with those seen in adults. As these are
prenatal animals, perhaps their under-developed systems cannot resist alcohol’s effects;
instead, alcohol alters development.

Further evidence of alcohol’s distal effects comes from phase shifting studies.
Rats exposed to alcohol prenatally through a mother’s liquid diet exhibited less phase
shifting effects of light (when tested later in life) compared to controls [Sei et al., 2003].
Others have shown increased phase shifting effects in neonatal rats [Allen et al., 2005].
Thus it is unclear whether distal effects of ethanol exposure attenuate or potentiate phase
shifting effects; however the studies exposed animals to ethanol at different critical points
during development. Alcohol seemingly alters the way the circadian system responds to light both when it is administered developmentally or chronically to adult rats. In adult rats, chronic ethanol reduced the normal effects of a phase advancing light pulse on activity rhythms [Rosenwasser et al., 2005b]. Microdialysis of ethanol directly to the SCN of hamsters reduced phase advances in response to light [Ruby et al., 2009] providing compelling evidence that alcohol can influence the clock directly, at least when ethanol is delivered directly to it.

### 1.3.2 Hormone Rhythms

The SCN controls daily rhythms of cortisol release from the adrenal glands via signals to the paraventricular nucleus of the hypothalamus. Cortisol reaches its peak at the beginning of the active phase (in the early morning for diurnal animals and in the evening for nocturnal animals); it decreases over time and its lowest point is during the inactive phase. Corticosterone rhythms measured across time points in mice and rats with chronic alcohol exposure showed a shift and blunted amplitude [Kakahana and Moore, 1976, Madeira et al., 1997]. Normal humans showed no statistical differences in cortisol rhythms during alcohol exposure (over the course of a day) [Danel et al., 2006]. However, non-clinical patients may not have had sufficient time to endure any long-term effects accumulated in clinical patients. Thus, acute exposure in patients with alcoholism may look different. Surprisingly, a clinical population of humans with chronic alcoholism also showed no disturbance of cortisol rhythm or level [Angeli et al., 1982]. Cortisol function is altered in humans during withdrawal and one month following withdrawal. These reported increased cortisol levels in rhythms during acute withdrawal [Adinoff et al., 1991], and decreased levels in abstinent patients [Adinoff et al., 2005]. Some have
suggested that reduced cortisol reactivity following alcohol withdrawal is predictive of relapse [Junghanns et al., 2003].

Melatonin production is closely related to SCN function and can even be used as a phase marker of the SCN [Arendt, 2005]. Whether the animal is diurnal or nocturnal, melatonin levels rise and peak in the dark. They fall slowly during the night and remain low during the day. In addition, light has the ability to entrain melatonin production; this is a way light communicates day length information to the SCN. A non-clinical study that administered alcohol over a 24 hour period looked at acute effects on the melatonin rhythm during alcohol exposure and found no diurnal melatonin secretion (as suggested in some of the clinical literature), but a delayed nocturnal peak in half of its participants [Danel and Touitou, 2006]. Non-clinical participants given alcohol in the evening also showed decreased nocturnal melatonin secretion [Ekman et al., 1993, Rojdmark et al., 1993, Rupp et al., 2007]. Alcohol consistently decreased melatonin in healthy subjects in the nighttime immediately following an evening alcoholic drink; however, it is not apparent how this short-term effect extends with chronic exposure. Clinical patients uniformly show disturbances, but not predictable ones. Patients both during acute withdrawal [Schmitz et al., 1996] and at least 2 weeks abstinent [Kuhlwein et al., 2003] show decreased nocturnal melatonin secretion. However, others who fit into those same categories show an increase in both diurnal and nocturnal melatonin secretion [Majumdar and Miles, 1987, Fonzi et al., 1994]. Another sample found this also to be true in patients prior to withdrawal [Murialdo et al., 1991]. Thus far, clinical patients show non-predictable disturbances to their melatonin rhythms.
1.3.3 Sleep

Under the influence of the clock, there is a consolidated sleep-wake cycle, but when the SCN is lesioned, sleep is evenly distributed across day and night. It appears the SCN may have more control over slow wave sleep, but this is not entirely clear. One of the most common circadian rhythms used as a dependent measure is sleep pattern. Multiple sleep parameters are measured through electroencephalography (EEG) recordings subsequent to alcohol exposure.

Acute single doses of liquid alcohol in a non-clinical human sample resulted in more superficial sleep (with increased likelihood of wakefulness from sleep); this was based on one night of recording [Landolt et al., 1996]. Acute alcohol exposure intragastrically (IG) in rats also led to more superficial sleep and an increase in non-rapid eye movement (NREM) sleep based on one day of recording [Kubota et al., 2002]. In another study, rats given a single dose of alcohol IG were also recorded for a day [Rouhani et al., 1990]. Non-active wakefulness, number of slow wave sleep (SWS), and number of rapid eye movement (REM) sleep episodes all decreased in the period following administration. These effects are informative to the state of the body immediately following alcohol ingestion. However, in order to draw conclusions beyond one period post-exposure, more cycles of data need to exist in these studies to allow for the observation of real, persisting changes in rhythms.

Chronic alcohol vapor exposure in mice has resulted in decreases in sleep efficiency during early withdrawal, and an increase in REM sleep that persisted beyond the end of the four day study [Veatch, 2006]. Weeks of recording in rats after chronic exposure to alcohol via liquid diet saw increased NREM sleep, decreased amplitude of
REM sleep, and decreased spectral power [Kubota et al., 2002]. All measures returned to baseline within two weeks. Chronic exposure to alcohol via vapor also found decreased spectral power in rats [Ehlers and Slawecki, 2000]. In this study, however, spectral power remained low, not recovering to baseline by the end of the five week study.

In humans with a history of alcoholism, particular EEG rhythm parameters were associated with relapse [Drummond et al., 1998]. EEG rhythms measured at several time points over several months showed abnormal rhythms in all participants in the beginning of the study, shortly after withdrawal. At the start of the experiment each patient's sleep was disturbed. Over time some EEG sleep parameters returned to normal in the abstinent patients. One parameter remained disturbed in both abstinent and relapsed patients (proportion of REM sleep). In addition, in those patients who relapsed, total sleep time, proportion of SWS, and latency to REM also remained abnormal. These improvements or continued disruptions in sleep showed how individual rhythms can predict relapse or recovery. This study is highly informative and demonstrates the value of gaining information on the mechanisms for these effects. These advances would allow for more in depth understanding e.g., to see if the abnormal sleep rhythms are a result of abnormalities in the clock itself or in various mediators of clock function. What caused the sleep disruption? It may have always been disturbed, as studies have shown a relationship between baseline rhythms disruptions and alcohol intake [Roehrs et al., 1999, Wong et al., 2004]. Or maybe alcoholism led to the rhythm disturbances. In fact, animal studies have shown that chronic intake is bound to have long term effects on sleep [Ehlers and Slawecki, 2000, Kubota et al., 2002, Veach, 2006].
1.3.4 Locomotor Rhythms

Locomotor rhythms follow the animal’s active phase, and free run in constant conditions; they are measured continuously with running wheels, passive detectors, or biotelemetry. However, when the SCN is lesioned locomotor activity patterns becomes arrhythmic. Effects on circadian locomotor patterns also vary. Locomotor activity is decreased in rats during chronic exposure [Rosenwasser et al., 2005a, Taylor et al., 2002, Taylor et al., 2006] as well as in the hours following an ethanol injection [Baird et al., 1998]. Whereas, in the days of withdrawal immediately following chronic exposure, activity increased [Taylor et al., 2006]. These increases during withdrawal could simply be a re-bound effect that contrast with the decreased activity during alcohol exposure. Or increased activity may simply be a response to the hyper-excitability that manifests during ethanol withdrawal [Crawshaw et al., 1994]. Another study reported decreases in activity during the first day of withdrawal in mice with high sensitivity to ethanol withdrawal, but no differences in activity levels for a heterogeneous strain of mice [Kliethermes et al., 2005].

In constant conditions, locomotor activity is reliably used to determine and animal’s FRP. It is easy to collect and an especially valuable measure in animals because assessing FRP in humans is extremely laborious and difficult. However, mere handling of mice induces stress that can affect activity [Keeney et al., 2001]. In addition, activity rhythms can be affected variously by sedative effects of alcohol, amount of sleep, cage changes and overall health to name a few. So while it is a great measure to collect, the data can be noisy based on the amount of intruding factors in the environment.
1.3.5 **Body Temperature Rhythms**

Body temperature rhythms are robust to outside influences. However, until the recent use of telemeters, it has been a difficult measure in animals, and body temperature is difficult to record continuously in humans. An advantage of body temperature rhythms is that they can be measured remotely, continuously, and non-invasively in animals (once a telemeter is implanted).

Body temperature rhythms persist in constant conditions and may be phase shifted, the primary requirements for an endogenous circadian rhythm (for introductory paragraphs on rhythms, see [Moore-Ede et al., 1982]). Body temperature is set by the hypothalamus and with input from the SCN, it naturally oscillates between a high during the active phase (day in diurnal animals and night in nocturnal animals) and a low during the inactive phase. Body temperature rhythms in humans and rodents have shown similar responses to alcohol intake. Among patients with alcoholism (with chronic exposure to alcohol), and healthy humans (receiving several doses of alcohol across the course of one day), alcohol increases body temperature during the non-active phase and decreases body temperature during the active phase [Danel et al., 2001, Gross et al., 1975]. As a consequence of these effects, the overall amplitude of the core body temperature rhythm is dampened [Danel and Touitou, 2004]. Amplitude decreases may indicate a rhythm that is more flexible i.e., easier to shift. If this is the case, then those suffering from jet lag or shift work who increase their alcohol intake [Rogers and Reilly, 2002, Trinkoff and Storr, 1998] may be using alcohol to facilitate their shifting to the external environment.

The most commonly measured effect on body temperature in response to an ethanol injection is hypothermia in the hour or hours following in mice and rats [Craw-
Recently, the simple effect of hypothermia following an ethanol injection has been further complicated, showing either tolerance or desensitization in male versus female rats following chronic ethanol exposure [Taylor et al., 2009]. These are acute effects; however, a careful study with injections delivered across the day also found that that effects of ethanol on body temperature are time dependent [Baird et al., 1998]. That is, acute hypothermia is more pronounced during the active phase.

Rats subjected to repeated ethanol vapor exposure in one study showed tolerance to the induced changes in body temperature typically resulting from alcohol ingestion [Ristuccia and Spear, 2005]. In this experiment, tolerance to ethanol’s hypothermic effects on body temperature rhythms compared adult and adolescent age groups. Rats were exposed to alcohol vapor at the beginning of each day, and body temperature was monitored immediately following the dose. While the adults showed tolerance to these effects (the temperature decreased following exposure on the first day but not on subsequent days), adolescent rats did not habituate. They showed a hypothermic effect daily following each exposure session. Temperature was not monitored in the days following alcohol exposure in the above experiment and thus, we cannot infer long term effects on the body temperature rhythm in the absence of alcohol. Rats (males and females from 3 strains) receiving a liquid diet had their body temperature rhythms monitored during the last few days of during the first few days following ethanol exposure [Taylor et al., 2006]. Comparisons showed inconsistent effects on body temperature rhythm amplitude during withdrawal days compared with rhythms during alcohol exposure according to strain and sex of rats. They did not compare amplitude on withdrawal days directly with baseline
measures of amplitude, so it is unclear how measures during withdrawal compare with control conditions. While measures were observed for 3 days, the body temperature parameters were averaged across them for one withdrawal amplitude statistic.

These studies leave open the question of long term effects of chronic ethanol on circadian rhythms. Body temperature rhythm is a particularly beneficial measurement, resilient to outside influences. However, the above studies have not looked at circadian parameters beyond withdrawal or after multiple inductions of dependence, a comparison relevant to humans who are alcohol dependent and typically undergo multiple periods of excessive drinking, withdrawal, and relapse. Questions about chronic ethanol’s effects on circadian rhythms are pursued in Chapter 4.

1.4 Experimental Goals

The following empirical chapters build on the current literature and addresses questions about the interactions of ethanol and circadian rhythms that have translational relevance. Specifically, they ask about how circadian scheduling of alcohol intake influences subsequent drinking (Chapter 2), about the relationship between induced period differences and voluntary alcohol intake (Chapter 3), as well as effects of single and repeated rounds of ethanol vapor exposures on subsequent circadian body temperature and activity rhythms (Chapter 4).

Understanding the answers to these questions may translate to a number of human effects. The results have implications for how drinking at certain times of day may contribute to alcohol dependence or dysregulation of rhythms. Further, the characteristics of the circadian rhythm may relate to distinct responses to or preferences for alcohol.
Finally, specific rhythm properties in the absence of alcohol (i.e., circadian period) or rhythm disturbances resulting from chronic alcohol may be identified as risk factors for drinking problems.
Chapter 2

Circadian scheduling affects subsequent voluntary ethanol intake

There is a daily rhythm in the voluntary intake of ethanol in mice, with greatest consumption in the early night and lowest intake during the day. The role of daily timing of ethanol exposure on the development and control of long-term ethanol self-administration has been neglected. The present study examines these issues using C57BL/6J mice. Mice were repeatedly exposed to 10% ethanol for 2 h early in the night or day for several weeks. Subsequently, ethanol was available at the opposite time (Expt 1) or 24 h daily (Expts 1 and 2). Lick sensors recorded the patterns of drinking activity in Experiment 2. Mice exposed to ethanol during the night drink more than mice exposed during the day. Prior history did not affect ethanol intake when the schedule was reversed. Under 24 hour exposure conditions, mice with a history of drinking during the night consumed significantly more than mice drinking during the day. The circadian patterns of drinking were not altered. These results demonstrate that the daily timing
of ethanol exposure exerts enduring effects of self-administration of ethanol in mice. Understanding how circadian rhythms regulate ethanol consumption may be valuable for modifying subsequent intake.

2.1 Introduction

In many mammals, including humans, myriad aspects of physiology and behavior are characterized by robust and predictable 24 hour fluctuations. Among these are daily rhythms of ethanol consumption that have been observed in a number of different species (Danel and Touitou, 2004; el Guebaly, 1987, Smith et al., 1980). Mice and rats, for example, voluntarily consume more ethanol in the dark, during the active phase of their cycles than they do in the light or inactive phase (for review see (Hiller-Sturmhofel and Kulkosky, 2001)). In mice given temporally restricted daily access to ethanol, the peak for voluntary consumption has been further localized to a few hours into the dark phase (Rhodes et al., 2005). Humans with alcoholism likewise display a daily rhythm in their craving for their first drink of the day early into the active phase (Danel et al., 2003). In neither species has the temporal context of drinking been fully explored (but see Spanagel et al., 2005b), particularly with regard to its influence on ethanol self-administration and dependence. Thus, we examine the significance of scheduled ethanol intake on subsequent ad libitum consumption in a well established rodent model, the C57 mouse.

The daily rhythm in ethanol intake likely derives from the joint influence of an endogenous circadian clock in the suprachiasmatic nuclei of the anterior hypothalamus (SCN) and environmental (for example, lighting) factors. The influence of endoge-
nously driven circadian rhythmicity on ethanol intake is suggested by altered ethanol intake following manipulations of the circadian clock. For example, repeated shifts in the light/dark cycle alter intake, albeit in different directions, in male versus female rats (Clark et al., 2007). Similarly, some (Spanagel et al., 2005a), but not other (Zghoul et al., 2007) genetic manipulations of the molecular circadian clock mechanism also affect ethanol drinking in mice. Correlations between circadian measures (e.g., period) and ethanol intake have also been reported (Hofstetter et al., 2003; Spanagel et al., 2005b). Conversely, alcohol consumption may feed back on the circadian pacemaker (Rosenwasser et al., 2005a-b). In addition to endogenous factors, some environmental factors, such as light can acutely affect ethanol intake and thereby contribute to the daily rhythm (Geller and Purdy, 1979).

While prior work has addressed the relationship between circadian clocks and concurrent ethanol intake (particularly in animals), and other research has found a role of (non-circadian) timing in increasing subsequent alcohol intake (e.g., intermittent is more effective than continuous ethanol exposure at increasing self-administration and producing dependence with liquids or vapor; Becker and Lopez, 2004; Finn et al., 2005; O’Dell et al., 2004; Wise, 1973), the role of circadian timing of ethanol exposure in subsequent or long term ethanol use has been neglected. Given the highly structured temporal context in which humans tend to consume alcohol, this issue warrants consideration.

Within a circadian context, timing of ethanol exposure may influence self-administration via two routes: first, self-administered drinking at preferred times of day may promote subsequent intake simply by virtue that these animals acquire a history of drinking more ethanol compared with animals offered ethanol at a non-preferred time
of day. In this case, drinking at the preferred time of day may be analogous to alcohol sweetening or dilution—two methods useful in establishing self-administration habits in animals that would not drink otherwise (Samson et al., 1999). Thus, drinking at a preferred time might be expected to lead to higher levels of subsequent self-administration, while drinking at a non-preferred time of day might not. Alternatively, repeated daily timed exposure may engage circadian oscillators involved in behavioral reinforcement. Daily timed feedings, for instance, have revealed the existence of a food entrainable oscillator (Pitt et al., 2003; Stephan, 1984) that generates increased activity in advance of the expected food/reward time. In this case, animals will re-entrain their food related activity such that they anticipate and eat according to a circadian rhythm that was determined by their prior circadian-timed schedule of feeding. Analogously, timed ethanol exposure might also entrain anticipatory activity and drinking despite drinking at a preferred or non-preferred time of day leading to equal self-administration. Comparable oscillatory mechanisms have been proposed to play a role in fear conditioning (Cain et al., 2008) as well as in the response to and reinforcement of drugs of abuse (Abarca et al., 2002; McClung et al., 2005), also see (Kosobud et al., 2007) for review.

Here, we ask what influence a history of drinking during a preferred (or non-preferred) time of day has on subsequent voluntary intake. To address this question and the hypotheses above, we present two experiments with C57BL/6J mice, a strain known for its high levels of voluntary ethanol intake. In Experiment 1 we assess whether a history of drinking during highly preferred times of day subsequently elevates drinking at less preferred times of day and vice versa. We then evaluate how these scheduled histories affect ethanol intake when it is available 24 hours/day. Experiment 2 replicates and
extends Experiment 1 by employing “skeleton” photoperiods to remove the potentially confounding factor of light exposure (Geller and Purdy, 1979), and further tests whether the timing, per se, of ethanol exposure (versus amount previously consumed) is critical in setting subsequent levels of self-administration. Lastly, we also monitor patterns of ethanol drinking during 24 hour ad lib access to determine how the circadian rhythm is altered by prior drinking history.

2.2 Materials and Methods

2.2.1 Subjects

Male C57BL/6J mice (Jackson Laboratories, Sacramento CA) were 10 weeks old at the start of each experiment. Mice were single housed with food (Purina chow) and water available ad libitum. All procedures and animal care was approved by and conducted under the guidelines of the Institutional Animal Care Use Committee at University of California, San Diego and at The Scripps Research Institute.

2.2.2 Lighting Conditions

In Experiment 1, mice were housed under a full 12 hour light/12 hour dark cycle. In Experiment 2, mice were housed under a 13 hour light/11 hour dark cycle prior switching to a skeleton light/dark cycle. During the skeleton light/dark cycle, mice received only two 1-hour pulses of light each followed by 11 hours of darkness. The two light pulses simulate dawn and dusk of a 13 hour day/11 hour night (Rosenwasser et al., 1983).
2.2.3 Drinking Procedures

Two hour two bottle choice ethanol exposure

A 10\% (w/v) ethanol solution was prepared using 95\% ethyl alcohol and water; a separate water bottle was also prepared for the procedure. Mice were given 50 ml conical tubes fitted with sipper tubes for two hours at a time for the two bottle choice portions of the study. At the end of each two hour exposure, the mouse’s normal 16 ounce water bottle was returned to the cage and the 50 ml ethanol and water bottles were weighed to determine g/kg intake as well as ethanol preference (preference calculated= ethanol intake/(ethanol + water intake)). This exposure lasted 5 days each week (Monday-Friday). Mice were weighed at two week intervals.

Twenty-four Hour Two Bottle Choice Ethanol Exposure

Mice were each given one 10\% (w/v) 50 ml ethanol bottle together with one 50 ml bottle of water for twenty-four hours at a time. Bottles were weighed every 2-4 days (and divided by number of days to obtain a 24 hour average) to determine g/kg intake and ethanol preference. The twenty-four hour ethanol choice procedure lasted 4 days during each week (starting Monday afternoon and ending Friday afternoon). Mice were weighed at two week intervals.

2.2.4 Experiment 1

Mice (N = 39) were randomly divided into 2 groups at the beginning of the experiment.
2 hour 2 BC: Initial Phase

Mice in the “Night” group (n = 20) received the two hour two bottle choice (2 hour 2 BC) procedure starting at 2 hours into the 12 hour dark phase, which corresponds to their active phase. Mice in the “Day” group (n = 19) were housed in a separate room on a reverse light/dark cycle. Mice in the Day group received ethanol (2 hour 2 BC) at the exact same time as the Night group, but because they were housed on a reverse lighting cycle this exposure started at 2 hours into the 12 hour light phase, when animals are generally inactive. The 2 hour 2 BC procedure lasted for 5 weeks.

2 hour 2 BC: Crossover Phase

After 5 weeks, mice were further subdivided so that half the mice from the Night and Day groups would continue to drink in their original lighting condition (Night/Night group and Day/Day group). The other half were transferred to the opposite lighting/drinking condition (Day/Night and Night/Day groups). Mice placed in opposite lighting conditions were transferred to a different room that was on a reverse light/dark cycle from their original room; they were allowed 2 weeks for re-entrainment to new lighting conditions. No animals received ethanol during this interval. Following this break, the Crossover Phase began and mice received 4 weeks of 2 hour 2 BC starting 2 hours into the dark or light.

24 hour 2 BC: Ethanol Phase

Three days following the last day of 2 hour 2 BC, all mice were given twenty-four hour two bottle choice ethanol exposure for three weeks. This began in the middle of the dark or light phase (10 am) on Monday and bottles were changed daily at non-consistent
times between 8 am and 3 pm.

2.2.5 Experiment 2

Mice (N = 30) were randomly divided into 3 groups at the beginning of the experiment. Animals were housed under a skeleton light/dark cycle; prior to skeleton cycles mice in the Subjective Day group were entrained to reverse light/dark cycles so that the Subjective Night and Subjective Day groups were 12 hours out of phase with each other. Locomotor activity was recorded throughout the experiment.

Initial Phase 2 hour 2 BC

Mice in the “Subjective Night” group (n = 10) received the two hour choice procedure during the subjective night starting 2 hours following the “dusk” light pulse (that signals the end of day). Mice in the “Subjective Day” group (n = 10) received the two hour choice procedure during subjective day starting 2 hours after the “dawn” light pulse (that signals the start of day). Subjective night and subjective day are terms used to describe an animal’s internal circadian phase as distinguished from environmental phase, which may not match. Under a skeleton light/dark cycle the internal sense of night and day was established by the mouse’s prior entrainment to a full light/dark cycle (thus, the subjective sense of night and day is reversed for the two groups of animals, even while they are housed in the same room). Mice in both groups received ethanol at exactly the same time, but because mice were earlier entrained to opposite light/dark cycles before being placed on the common skeleton light/dark cycle, ethanol exposure occurred either during the active (Subjective Night group) or inactive phase (Subjective Day group). Again, as mice were housed under a skeleton light/dark cycle, ethanol exposure took
place in darkness in all groups (during both night and day). Mice in the “Restricted
Subjective Night” group (n = 10) also received ethanol during subjective night starting
2 hours after the dusk light pulse; however the ethanol choice procedure for this group
lasted only 10-15 minutes in order to restrict intake to the same low levels seen in the
Subjective Day group during 2 hour 2 BC. This procedure lasted for 5 weeks.

Ad Lib 24 hour 2 BC

Three days following the last day of 2 hour 2 BC, mice were given 24 hour 2 BC
ethanol exposure for eight weeks. During Ad Lib 24 hour 2 BC, lickometers were also
used to record 24-hour ethanol drinking behavior.

2.2.6 Blood Alcohol Concentrations

One blood alcohol sample was taken from each mouse during the Initial and
Crossover Phase of Experiment 1. Tail blood (0.05 ml) was collected into heparinized
tubes immediately following the 2 hour exposure and assayed to determine BAL.

2.2.7 Activity Monitoring

In Experiment 2, locomotor activity was recorded continuously by a motion de-
tector mounted to the top of each cage lid (Coral Plus; Visonic, Bloomfield, CT). Drinking
activity for ethanol was measured by a contact sensing lickometer circuit. Lickometers
were used on each cage every other week, Monday afternoon through Friday afternoon
during 24 hour 2 BC. Both locomotor and lickometer data were recorded and compiled
into 6 minute bins by Vital View software (Mini Mitter, Bend, OR). Activity histograms
were made using Microsoft Excel by taking the average at each 6 minute interval across
the 24 hour period for the duration of the experiment. For locomotor data this includes all days throughout the experiment. As mentioned above, for drinking data this includes 4 days per week for weeks 1, 3, 5 and 7 of Ad Lib 24 hour 2 BC.

2.2.8 Data Analysis

Ethanol drinking and preference levels were analyzed by univariate repeated measures ANOVAs using SPSS 15.0 (Chicago, IL) to statistically compare between groups for each experimental phase (e.g. Initial, Crossover and Twenty-four hour phases). This was followed by post-hoc comparisons (corrected using the Bonferroni method). For activity rhythm analysis, data points were averaged over hourly intervals and reduced to 24 data points. Group values were compared at each time point across groups using ANOVAs corrected for multiple comparisons (Bonferroni method).

2.3 Results

2.3.1 Experiment 1

2 hour 2 BC

Ethanol intake (g/kg) for each group throughout Experiment 1 is represented in Figure 2.1. In the Initial 2 hour 2 BC, the Night group consumed approximately 3 times more ethanol than mice in the Day group (F(1, 37) = 415.5; p< 0.001). During Crossover, the Night/Night and Day/Night mice (that drank at night) consumed significantly more than the Day/Day and Night/Day mice (F(3, 35) = 84.5; p< 0.001). Drinking condition during the Initial phase had no effect on drinking in the Crossover phase; post-hoc comparisons showed no statistical differences between Day/Day and Night/Day groups (p =
or between the Night/Night and Day/Night groups (p = 0.27) during the Crossover phase. Mice in all groups preferred ethanol over water; however, mice who drank at night exhibited a stronger ethanol preference (for the Initial phase F(1, 37) = 32.96; p< 0.001; for the Crossover phase, F(3, 35) = 13.23; p< 0.001). In Crossover there were no differences between groups that drank during the same time of day (post-hoc comparisons between the Night/Night and Day/Night group were n.s. (p = 1.0) as were comparisons between the Day/Day and Night/Day groups (p = 1.0); see Fig. 2.2. Blood alcohol samples taken during Initial and Crossover phases following 2 hour 2 BC confirmed higher blood alcohol concentration (BAC) in the Night group compared to the Day group (mean ± SEM for Night = 84.0 ± 9.2 mg/dl, Day = 4.5 ± 0.7 mg/dl).

24 hour 2 BC

Under 24 hour, Ad Lib conditions Night/Night and Day/Night mice (that previously drank in night during Crossover) drank more (F(3, 35) = 17.87; p< 0.001) and had a higher preference (F(3, 35) = 4.65; p< 0.01) for ethanol during subsequent weeks of 24 hour exposure than Day/Day and Night/Day mice. Again, these results were influenced by the most recent timed history (Crossover) rather than by Initial conditions as post-hoc comparisons confirmed no statistical differences between the Night/Night and Day/Night groups or between the Day/Day and Night/Day group.
2.3.2 Experiment 2

2 Hour 2 BC

Figures 2.3 and 2.4 show ethanol intake and preference during Experiment 2. Mice drinking for 2 hours in the Subjective Night group consumed more (F(2, 27) = 68.53; p< 0.001) than mice drinking in the Subjective Day or Restricted Subjective Night groups. Post-hoc comparisons showed significant differences between the Subjective Night and Subjective Day groups (p< 0.001), and between the Subjective Night and Restricted Subjective Night groups (p< 0.001). By design, mice in the Restricted Subjective Night group drank comparable levels with those in the Subjective Day group (p = 0.56). Mice drinking in both the Subjective Night and Restricted Subjective Night groups showed higher preference for ethanol (F(2, 27) = 12.78; p< 0.001) than mice in the Subjective Day group. Specifically, post-hoc comparisons show differences between the Subjective Night and Subjective Day groups (p< 0.001) and between the Restricted Subjective Night and Subjective Day groups (p< 0.04).

24 Hour 2 BC

When ethanol was subsequently freely available 24 hours/day, mice in the Subjective Night and Restricted Subjective Night groups (who previously drank at night) consumed more than those in the Subjective Day group (F(2, 27) = 5.4; p< 0.02). Pair-wise comparisons showed significant differences between the Subjective Night group vs. the Subjective Day group (p< 0.02) and between the Restricted Subjective Night vs. the Subjective Day group (p< 0.05). As with volume ethanol intake, preference was also higher in the Subjective Night and Restricted Subjective Night groups than the
Drinking and Locomotor Activity Rhythms

Figure 2.5 shows each group's average daily ethanol drinking profile plotted across the 24 hour day. All groups showed bimodal licking patterns with peaks at the beginning and the end of the active phase. Consistent with volumetric measures, the number of licks was significantly reduced in the Subjective Day group compared to the others ($F(2, 27) = 5.45; p< 0.02$). Specifically, univariate ANOVAs of hourly average drinking levels across the day showed significantly higher intake during the early and middle hours of the night by mice in the Subjective Night and Restricted Subjective Night groups compared with the Subjective Day group ($p< 0.002$ at 2, 6 and 7 hours into the night phase). Average daily ingested ethanol volume was highly correlated with average daily number of licks (Pearson's $r = 0.86; p< 0.01$). Examination of individual records confirmed that all mice were clearly entrained to their respective dark period. Locomotor activity during Experiment 2 is shown in Figure 2.5 with each group's average daily locomotor activity plotted across 24 hours. Locomotor activity is also bimodal in all groups with peaks near the beginning and end of the active phase. All groups show similar amounts of daily activity (average daily counts for the Subjective Night group = 2250, Restricted Subjective Night = 2563, Subjective Day group = 1952). However, daily patterns of locomotor activity differed in the Subjective Day group compared with the Subjective Night and Restricted Subjective Night groups. Specifically, mice in the Subjective Night and Restricted Subjective Night groups showed more activity counts early and in the middle of the night phase when compared to the Subjective Day group (univariate ANOVAs showed that $p< 0.002$ at 1, 2, 5, 6 and 7 hours into the night).
phase). However, during the early part of the day, mice in the Subjective Day group showed greater amounts of activity when compared to the Subjective Night and Restricted Subjective Night groups (p < 0.002 at 1 and 2 hours into the day phase).

2.4 Discussion

These results establish the importance of daily timing of ethanol exposure in the control of voluntary ethanol consumption in mice. As has been previously documented (Rhodes et al., 2005), mice demonstrated varying consumption and preference for ethanol at different times of day. This study breaks ground in exhibiting that the circadian timing of prior ethanol exposure has enduring effects on voluntary drinking during ad lib availability. In humans, we know alcohol consumption also follows predictable temporal patterns as people tend to drink near the end of the day (although as alcohol use disorders develop, drinking often begins earlier in the day). The mouse may be a convenient model to understand the relevance of temporal context for addictive behaviors.

In developing a mouse model of excessive ethanol intake, a number of laboratories have devised protocols to increase consumption by adding exteroceptive cues, for example sucrose, to enhance ethanol consumption, a cue that is faded out and leaves the animal with a self-administration habit that would not have otherwise occurred naturally. Alternatively, animals may be made physically dependent so that self-administration attenuates withdrawal symptoms. In the current experiments, mice showed a significant preference for ethanol over water in both their active and inactive phases, but this preference was significantly stronger at night. Thus, we tested whether the heightened preference of nighttime drinking would serve a role analogous to sweetening, such that high
voluntary drinking at night would render ethanol more attractive during the day. However, our Crossover phase in Experiment 1 showed no hint of a history effect on 2 hour daytime versus nighttime drinking. One methodological issue should be noted before fully rejecting this hypothesis. For logistical reasons, animals in Experiment 1 needed to be re-entrained to a reversed light/dark cycle in order to switch their times of ethanol exposure, thus introducing a 2 week break between phases of the experiment. This gap or the process of circadian re-entrainment itself could plausibly diminish the physiological memory or reinforcement associated with drinking during prior conditions. Consistent with such an interpretation is the finding that the alcohol deprivation effect in this same mouse strain is evident after one week of abstinence but not after two (Melendez et al., 2006). On the other hand, in both Experiments 1 and 2, effects of prior timed ethanol exposure persisted for much longer than 2 weeks following final scheduled ethanol access.

Whereas the prior timing of 2 hour scheduled ethanol access did not affect drinking when timing of that exposure was merely shifted, it markedly affected self-administration for up to 8 weeks when ethanol was subsequently available ad lib 24 hours per day. In Experiment 1, the critical aspect of timed exposure could have been the amount of ethanol consumed. But as Experiment 2 equalized the amount of ethanol ingested during the active versus the inactive phase, this explanation is ruled out. (Again, we note that animals scheduled to drink during the inactive phase nevertheless discriminated ethanol from water as indicated from their significant ethanol preference.) Thus, the daily timing of ethanol drinking produced an enduring effect on ongoing self-administration. This finding complements existing literature that documents an importance of ethanol exposure procedures in influencing voluntary alcohol
intake (e.g., continuous versus intermittent). The fact that the history effect persists for so long in Experiment 2 suggests either that there is an enduring physiological memory for the reinforcement of ethanol based on the timing of past exposure, or that the elevated 24 hour patterns of ad-lib are self-sustaining. The present data cannot distinguish these possibilities, although the waning of the effect in Experiment 2 suggests the former hypothesis. If the prior reinforcement effects are indeed remembered for 8 weeks, then it must also be the case that the temporal reinforcement effects were abolished or overridden in Experiment 1 by switching the time of daily exposure. That is, animals switched from drinking in the night to drinking in the day disregarded the earlier history in favor of the later despite the fact that fewer than 8 weeks elapsed between nighttime drinking and ad-lib exposure. This raises the intriguing, but yet to be rigorously tested idea that manipulations of circadian rhythms or timing of ethanol exposure could potentially contribute to treatments of alcohol use disorders. It should also be noted that we tested how a history of drinking at a preferred or non-preferred time of day produced differences in subsequent drinking but did not compare drinking levels to mice without pre-exposure who are allowed ethanol 24 hours/day. Thus, our data do not distinguish between a suppression of ad lib drinking by a history of alcohol at non-preferred times versus a facilitation by similar exposure at preferred times.

A central concern among circadian biologists is the degree to which daily rhythms in behavior are internally generated programs versus acute responses to a changing external environment. The use of skeleton photoperiods in Experiment 2 is an attempt to distinguish these possibilities since a literature exists that suggests an acute role of light on ethanol consumption in rats (Burke and Kramer, 1974; Geller, 1971). In contrast
to that literature, the main findings of the present study suggest that light during the inactive phase is of no or little significance. The skeleton photoperiods are also useful for examining whether timed ethanol exposure produces marked changes in the circadian activity rhythm. Time limited access to food, for example, induces a marked reorganization of circadian activity rhythms in a number of rodent species (Stephan, 1984). Scheduled ethanol, on the other hand, had no large effects on circadian activity rhythms either during scheduled drinking or after although the intensity of activity early in the active phase was somewhat reduced in mice drinking during their inactive period.

In several species, rewards available at certain times of day are time-stamped so that motivated behaviors are reinforced to be expressed around the time that the reward was scheduled (Kosobud et al., 2007; McClung et al., 2005). The scheduled 2 hour ethanol access in the present study might thus produce changes in the 24 hour ad-lib drinking by reinforcing ethanol consumption at a particular time of day. We found no evidence that scheduled drinking during the inactive phase led animals to drink preferentially at that time of day when ethanol was available around the clock. Instead, all mice avoided daytime drinking in favor of their active phase when ethanol consumption closely tracked the locomotor activity rhythm.

The mechanisms by which circadian timing affects ethanol preference and intake remain to be determined. While Experiment 2 controlled for the amount of ethanol consumed at different circadian phases, at this early stage of our investigation we cannot account for potential differences in pharmacokinetics or metabolism which are known to vary on a circadian basis (Kosobud et al., 2007). Thus, while we document that it is not the amount but the timing of ethanol consumed that influences subsequent 24 hour
intake we cannot exclude the possibility that the small amount of ethanol these mice consumed produced BALs transiently equivalent to those of unrestricted mice. The close association of locomotor and drinking activity suggests that circadian rhythms of arousal or alertness levels may mediate these effects. At a more reductionist level, at least one gene (Per2) that plays a central role in the generation of the circadian clock mechanism is associated with elevated ethanol intake in mutant mice (Spanagel et al., 2005a). There is a preliminary suggestion that a genetic variant of the Per2 gene in humans is related to increased alcohol consumption (Spanagel et al., 2005a). More broadly, an estimated 10% of the mouse transcriptome is under circadian control (Panda et al., 2002). Thus, there are myriad mechanisms by which the reinforcing potential of ethanol might be modulated on a temporal basis.

Once they are better understood, the effects reported here may have practical relevance for understanding development of and manipulating human alcohol intake. Behavioral cue exposure (CE) methods for reducing alcohol intake in humans expose subjects to alcohol without allowing them to drink to test how physiological responses to alcohol change over time (Glautier and Drummond, 1994). Though allowing alcohol in treatment is counter-intuitive, it is recognized that a long term change in behavior must utilize both proximate and ultimate goals that do not necessarily agree (DiClemente, 2007); others have made use of alcohol in treatment with positive findings (Sitharthan et al., 1997). For lasting improvements, CE methods incorporate contextual cues during alcohol exposures (Collins and Brandon, 2002; Stasiewicz et al., 2007). While this typically refers to environmental characteristics, we might extend this concept to temporal features. Timing characteristics of human alcohol drinking are currently being studied.
(Danel and Touitou, 2004), and time of day is a simple context for voluntary drinking that can be manipulated in behavioral treatments of alcohol use disorders. The treatment would not focus on trying to forget or extinguish the reinforcement of alcohol but rather on learning new, non-preferred schedules of intake that change the reinforcing properties of alcohol and ultimately reduce voluntary drinking. In fact, there is substantial evidence that associations are not extinguished as a classical behavioral theory would suggest, but that new alternative ones are learned (Bouton, 2002). Our Experiment 2 results show that the most recent schedule of alcohol exposure controls subsequent intake in an unrestricted environment. Behavioral treatments for alcohol first tried in animals have been extended to humans in the study of context cues (Collins and Brandon, 2002). Here we suggest another based on our results; human behavioral studies could use preferred circadian timing for alcohol ingestion as a context for alcohol use.

Chapter 2, in full, has been previously published in Alcoholism: Clinical and Experimental Research. Trujillo, Jennifer L., Roberts, Amanda J., Gorman, Michael R. "Circadian Timing of Ethanol Exposure Exerts Enduring Effects on Subsequent Ad Libitum Consumption in C57 Mice", Alcoholism: Clinical and Experimental Research, Vol. 33, 2009. The dissertation author was the primary investigator and author of this paper.
Figure 2.1  Mean ± SEM daily ethanol intake (g/kg) in each mouse group by week for all parts of Experiment 1. This includes 2 hour 2 BC for both the Initial (5 weeks) and Crossover (4 weeks) phases as well as for 24 hour 2 BC in the Ad Lib phase (3 weeks); the start of Crossover and Ad Lib phases are labeled and marked with arrows. The Dark/Dark group drank at night during both the Initial and Crossover phase; the Dark/Light group drank at night for the Initial phase and during the day (morning) for the Crossover phase; the Light/Light group drank during the day (morning) for both phases; the Light/Dark group drank during the day in the Initial phase and at night during the Crossover phase.
Figure 2.2 Proportion ethanol preference (versus water) for mice drinking during the dark or light in the Initial and Crossover phases, or with a history of drinking during the dark or light (Crossover phase) prior to Ad Lib 24 hour exposure. Mice show preference for ethanol when drinking in the dark (Initial and Crossover) and continue to show a preference during Ad Lib if they drank in the dark prior to 24 hour exposure (during the Crossover phase).
Figure 2.3  Mean ± SEM daily ethanol intake (g/kg) in each mouse group by week for 2 hour 2 BC (5 weeks) and 24 hour 2 BC (8 weeks) in Experiment 2; the start of Ad Lib intake is labeled on the figure. The Subjective Night group drank during the night (active phase) during 2 hour 2 BC; the Subjective Day group drank during the day (inactive phase) during 2 hour 2 BC; the Restricted Subjective Night group was allowed minimal ethanol access during the night (active phase) approximately 15 minutes starting at the same time as mice in the night group.
Figure 2.4  Proportion ethanol preference (versus water) exhibited by mice in each group during 2 hour 2 BC and 24 hour 2 BC. Mice show a higher preference for ethanol when drinking during the night versus the day for 2 hour 2 BC; groups also show a higher preference during Ad Lib with a history of drinking in the dark (Subjective Night and Restricted Subjective Night groups). Note that during 24 hour 2 BC ethanol is available at all times of day but animals still show a preference based on their history of drinking during the night or day during 2 hour 2 BC.
Figure 2.5  Average daily activity profiles by group for ethanol drinking (A) and locomotor (B) activity (sampled at 6 minute intervals). Axes are 24 hours across and show the average daily pattern of ethanol drinking (A) or locomotor activity (B) for animals in each group. Higher points on the line indicate higher levels of activity for that time of day. Drinking activity (A) was measured continuously every other week of 24 hour Ad Lib exposure (weeks 1, 3, 5 and 7 of Ad Lib). Locomotor activity (B) was measured all weeks throughout the entire experiment (during 2 hour and 24 hour 2 BC). Asterisks (*) on figure indicate the hours at which univariate ANOVAs showed significantly different activity or drinking levels in the Subjective Day group compared with the Subjective and Restricted Subjective Night groups (p< 0.002). Because locomotor activity profiles were largely similar during scheduled and ad lib drinking, data over both intervals are plotted together.
Chapter 3

The relationship of circadian period to ethanol intake

This chapter asks whether circadian period influences voluntary alcohol intake. In contrast to previous correlational studies, we test whether or not experimentally lengthening or shortening endogenous tau causes increases or decreases in ethanol consumption. In Study 1 this relationship is examined in C57BL/6J mice, an inbred strain known for high ethanol intake. Mice have 24 hour access to alcohol after entrainment to 22 or 26 hour periods in one of the following conditions: DD, 24 hour cycles (12:12 LD), 22 (11:11 LD) or 26 (13:13 LD) hour cycles. In Study 2, mice bred for high and low alcohol preference (HAP2 and LAP2) are used and tested for differences in voluntary ethanol intake in DD following entrainment to 22 or 26 hour cycles. Additionally, HAP2 and LAP2 mice are allowed to free run in DD to test for previously reported differences in tau, and for changes in alcohol drinking in this condition compared to standard 24 hour LD (12:12: LD) conditions. Despite strong effects of T cycles on entrained and free
running periods, these circadian period manipulations did not affect ethanol intake in C57BL/6J or in HAP2 and LAP2 mice. Together these results strongly argue against any direct effects of circadian period on ethanol consumption.

3.1 Introduction

Various research avenues suggest there may be a relationship between circadian period and alcohol intake. Animals with mutation of the Per2 gene (a clock gene with a key role in regulating the circadian clock) have shown an increased preference for alcohol [Spanagel et al., 2005]; endogenous period length (tau) also differs between high and low alcohol preferring mice and rats [Hofstetter et al., 2003, Rosenwasser et al., 2005]. Circadian period is driven by the endogenous master clock, the suprachiasmatic nucleus (SCN). Light cycles strongly influence the setting of the clock and resulting output of behavioral rhythms. However, under constant (i.e., constant dark) conditions the endogenous rhythmicity of the clock can be reliably assessed. HAP1 mice were found to have a tau of 0.3 hours shorter than LAP1 mice, a large robust difference in endogenous period length. This could be a chance genetic association between period-length and ethanol intake in these lines that does not stand up with successive derivations of selectively bred HAP and LAP mice, or the two could be mechanistically linked. For instance, it could be the case that high ethanol preference may be related to shorter endogenous periods. If the two are linked, there could be a simple relationship between tau and ethanol intake. Alternatively, it could be that the discrepancy between the animal’s shorter endogenous period and the 24 hour lighting conditions it lives under drive the increased ethanol preference. In two related studies, we explore this question
with C57BL/6J, HAP2 and LAP2 strains of mice. Mice were entrained to varying T cycles (periods shorter or longer than 24 hours) to see how altered period length or period after-effects influenced voluntary alcohol intake (Study 1 and 2). In HAP2 and LAP2 strains of mice, tau and voluntary alcohol intake during free-running conditions in DD were also measured (Study 2).

T cycles are often used to entrain animals to period lengths shorter or greater than 24 hours. Researchers have reliably been able to entrain animals to T cycles ranging from 20-28 hours [Pittendrigh & Daan, 1976, Stephan, 1983]. When released from T cycles into DD there are changes to the free running period. Thus, prior history can be used to transiently change tau. T cycles produce after-effects on tau (of approximately 0.5 hours) that typically last close to a month when animals are released into constant darkness (DD).

Study 1 asks if circadian period is mechanistically linked to alcohol intake in C57BL/6J mice. It tests this question by inducing period changes with T cycles to measure any resulting effects on ethanol intake and preference (comparable to the associations previously observed in HAP and LAP mice). Specifically, mice with shorter periods may drink more than 24LD controls. Or, lengthening endogenous periods with longer T cycles may influence ethanol intake in the opposite direction, reducing it. Testing ethanol intake in T cycles or in DD (with after-effects on circadian period) following T cycles tests this hypothesis in Experiments 1 and 2. Circadian period and ethanol intake could be related in an alternative manner, that is increased ethanol intake could be driven by the incongruency between the endogenous and external period. Releasing mice in 24 LD from T cycles can also lead to differences in entrainment to 24 hour lighting conditions. In this
sense, increased ethanol intake may be due to circadian disruption, stress or some other secondary mediator. This hypothesis is tested in Experiment 2 by measuring ethanol intake and preference in 24LD conditions following T cycles.

In both Studies, T cycles are used to entrain to periods longer or shorter than 24 hours. After T entrainment, voluntary ethanol intake was measured in various environments including under DD, 24 LD, or in T cycles in Experiments 1 and 2. Measuring ethanol intake in DD or in T cycles each has advantages; releasing animals into DD following T cycles allows for the observation of effects of T cycles on tau which typically last at least one month whereas keeping animals in T cycles stringently controls for period during ethanol exposure. Finally with C57BL/6J mice, assessing influence of T cycles on subsequent ethanol intake in 24LD is also a relevant comparison as HAP and LAP lines are bred for high and low ethanol preference exhibited under standard 24 LD conditions.

In Study 2 HAP2 and LAP2 mouse lines are also obtained to test for circadian period differences in DD, to measure effects of DD on ethanol intake compared with standard 24LD conditions, and to find out if shorter or longer entrained periods (created with T cycles) change ethanol consumption in high and low alcohol preferring mice. The first goal of this study was to again observe whether there is a difference in tau in HAP2 and LAP2 mice as compared with the previously derived HAP1 and LAP1 mice. If the difference in tau holds, this strengthens the argument that endogenous period and alcohol intake may be related. However, if there are no differences in tau between lines then we know this connection was coincidental in the prior derivation; this provides evidence that endogenous tau is not related to ethanol intake in these mice. It does not rule out the possibility that circadian period can be causally related to ethanol intake, and so these
mice are also tested for ethanol intake and preference in DD following entrainment to T cycles.

HAP2 and LAP2 mice are also allowed ethanol access in DD to compare intake with controls under LD conditions. This investigates the possibility that living in 24 hour lighting conditions in combination with a significantly different endogenous period (in the case of HAP1 mice) may contribute to ethanol preference. (Study 1 contains a complimentary experiment wherein ethanol intake is measured in 24LD following entrainment to shorter or longer periods in T cycles.) If this combination contributes to increased ethanol preference, then it may be alleviated (and ethanol intake may be changed) by allowing ethanol exposure under DD (with no imposed period).

3.2 Methods: Study 1

This study was composed of two related experiments. Experiment 1 looked at ethanol intake following T cycle entrainment in DD; Experiment 2 looked at ethanol intake while living in T cycle conditions or in 24 LD following entrainment to T cycles. Tables 3.1 and 3.2 outline methods in Experiments 1 and 2, respectively.

3.2.1 Subjects and Husbandry

Male C57BL/6J mice (Jackson Laboratories, Sacramento CA) were approximately 10 weeks old at the start of each experiment. Mice were group housed during entrainment to T cycles and single housed prior to the start of alcohol exposure with food (Purina chow) and water available ad libitum throughout. The mice were weighed at the start of alcohol exposure, and once every two-weeks thereafter. Cage changes happen
once per week at the same time to allow for the experimenters to detach bottles prior to cage change, and to re-attach them following the cage change. Control mice in 24LD always had cage changes during the light phase; however as experimental mice were on non-24 hour T cycles it varied whether their cage changes were during the active or inactive phase of their cycle. All procedures and animal care was approved by and conducted under the guidelines of the Institutional Animal Care Use Committee at University of California, San Diego.

3.2.2 Experiment 1 Procedure: Drinking in DD Following T Cycles

Mice drank in DD following T cycle entrainment. Mice (N=23) were entrained to 22 or 26 hour T cycles (T22 or T26) or 24 LD (control condition) for at least 3 weeks. Mice were then released into DD and allowed 24 hour 2 BC ethanol exposure 7 days a week for 28 days. Recording of licking and locomotor activity was alternated at various points throughout ethanol exposure. Licking was measured the majority of the time; however, since this was the first time the procedure with T cycles and ethanol was carried out, locomotor activity (the traditional measure to confirm entrainment and monitor rhythms) was recorded on and off to ensure that licking and locomotor activity rhythms were similar.

3.2.3 Experiment 2 Procedure: Drinking in T Cycles or in 24 LD from T cycles

Mice drank in T cycles or in 24 LD following T cycle entrainment. Methods for Experiment 2 are displayed in Table 3.2. Mice (N=40) were entrained to T22 or T26 cycles or 24 LD (control condition) for at least 3 weeks. Next, mice either continued in
T22 or T26 conditions, or were transferred to 24 LD from T cycles, or continued in 24LD and allowed 24 hour 2 BC ethanol exposure for 30 days. Ethanol licking activity was continuously recorded throughout the ethanol exposure phase.

### 3.2.4 T Cycle Entrainment

All mice were housed under a full 12 hour light/12 hour dark cycle prior to T cycle entrainment; controls remained under this 24 LD cycle throughout T cycle entrainment. At the start of entrainment, the new light dark cycle coincided with the time of lights on for mice under 24 LD conditions. This ensured no sudden phase shift due to a change in lighting cycle. Circadian periods were lengthened or shortened by 1 hour (to T25 or T23 from 24 LD with equal time in light and dark phases); mice were allowed at least a week to entrain before cycles were stretched an additional hour to T22 (11 hour light/11 hour dark) or T26 (13 hour light/13 hour dark). From this point mice were allowed at least 3 weeks to stably entrain prior to the start of ethanol exposure.

### 3.2.5 Activity Monitoring

Locomotor or ethanol licking activity was recorded continuously and compiled into 6 minute bins by Vital View software (Mini Mitter, Bend, OR). Drinking activity for ethanol was measured by a contact sensing lickometer circuit. Locomotor activity was recorded by a contact sensing unit activated when the mouse touched the wire cage lid and a metal platform on the cage floor. Switches between licking and locomotor activity in Experiment 1 are indicated on representative actograms in Figure 3.1.
3.2.6 24 Hour 2 Bottle Choice

A 10% (w/v) ethanol solution was prepared using 95% ethyl alcohol and water; a separate water bottle was also prepared for the procedure. Mice were given 50 ml conical tubes fitted with sipper tubes for 24 hours at a time. Bottles stayed on 7 days/week and were weighed 1-2 times per week (and divided by number of days to obtain a 24 hour average) to determine g/kg intake and ethanol preference (preference calculated = ethanol intake/(ethanol + water intake)).

3.2.7 Statistical Analyses

Statistical analyses were completed with SPSS (Chicago IL) and ClockLab (Actimetrics, Wilmette, IL). All statistically significant values are at a level of p= 0.05 or lower; post-hoc comparisons used the Bonferroni correction method to control for alpha level. Figures and tables were prepared using Microsoft Excel®, Microsoft Powerpoint®, and ClockLab.

Drinking in DD following T cycles

Individual taus based on combined licking and locomotor activity were calculated using a Chi-square periodogram analysis in ClockLab which determines the circadian periodicity within a wide range of hours (20 to 30 hours) with a significance level of p< 0.01. In Experiment 1 taus are based on 28 days of activity, in Experiment 2 they are based on 30 days (the duration of ethanol exposure for each experiment). Taus were compared across groups using SPSS.

Because mice in different entrainment conditions experienced a different number
of cycles or days, average ethanol intake was compared per cycle as well as per 24 hours. When the two analyses produced the same results, subsequent analyses (i.e., correlations between average ethanol intake and other measures) were done with average ethanol intake per 24 hours. Analyses of variance (ANOVAs) compared overall average daily and per cycle ethanol intake (g/kg) as well as preference by entrainment conditions. Repeated measures analyses of variance (ANOVAs) compared daily average ethanol (g/kg) intake across week by entrainment condition.

**Drinking in T cycles or in 24 LD from T cycles**

Individual taus based on licking activity were calculated using a Chi-square periodogram analysis in ClockLab which determines the circadian periodicity within a wide range (20 to 30 hours) with a significance level of p < 0.01. Taus were compared across groups using SPSS.

ANOVAs compared overall average daily ethanol intake (g/kg) and per cycle ethanol intake (g/kg) as well as preference by drinking condition. Repeated measures analyses of variance (ANOVAs) compared daily average ethanol intake (g/kg) across 7-9 day time periods by mouse by drinking condition.

### 3.3 Results: Study 1

#### 3.3.1 Experiment 1–Drinking in DD from T cycles

Figure 3.1 shows representative actograms for mice in each condition. Actograms are double plotted, 48 hours across with subsequent days beside and below the current day. The start of the actograms are coincident with the start of ethanol exposure.
As expected, effect of condition on circadian period was highly significant \[F(2,20)= 40.9; p< 0.001\] and showed after effects on period. Specifically, mice drinking in DD following T22 showed free running periods shorter than 24 hours and mice drinking in DD following T26 showed free running periods longer than 24 hours; see Figure 3.2.

Univariate ANOVAs showed no significant effect of entrainment condition on: average daily ethanol intake \[F(2,19)= 0.3; p= \text{n.s.}\], average per cycle ethanol intake \[F(2,19)= 0.3; p= \text{n.s.}\], or preference \[F(2,19)= 0.2; p= \text{n.s.}\]; see Figures 3.3 and 3.4. Not surprisingly, there was no statistical difference between average ethanol intake per 24 hours and average ethanol intake per cycle; a paired samples t-test showed no difference between average ethanol intake per 24 hours Vs. per cycle \([p=0.9]\); the two values were highly correlated \([\text{Pearson’s } r= 1.0; p< 0.001]\). A repeated measures ANOVA also showed no effect of week \[F(1,19)=2; p= \text{n.s.}\] or effect of week by entrainment condition \[F(1,19)= 0.1; p= \text{n.s.}\] on daily average ethanol intake across weeks (Figure 3.5).

As shown above there was no effect of condition on ethanol intake; a test for a correlation (in all mice collapsed across groups) between circadian period and daily average ethanol intake and confirmed there was none \([\text{Pearson’s } r= 0.1; p= \text{n.s.}]\).

### 3.3.2 Experiment 2–Drinking in T cycles or in LD from T cycles

Effect of entrainment condition on entrained period values was large, mean entrained period values (as determined by Chi-square periodograms–see Methods) for mice drinking in 24 LD, T22 or T26 were 24, 22, and 26 respectively. There was no within group variation, thus additional statistics to determine the strength of between group differences (ANOVA) were not performed on these large differences. Figures 3.6 and 3.7 show mean circadian period by group as well as representative actograms for
mice in each group in Experiment 2.

Univariate ANOVAs showed no significant effect of entrainment condition on: average daily ethanol intake \([F(4,35) = 0.6; p = n.s.]\), average per cycle ethanol intake \([F(4,35) = 0.5; p = n.s.]\), preference \([F(4,35) = 1.6; p = n.s.]\), or average ethanol licking \([F(4,35) = 1.4; p = n.s.]\). Average ethanol intake and preference are shown in Figures 3.8–3.9. As in Experiment 1, a paired samples t-test showed no difference between average ethanol intake per 24 hours Vs. per cycle \([p = n.s.]\). Ethanol intake generally declined across weeks; a repeated measures ANOVA showed a main effect of week on daily average ethanol intake \([F(1,35) = 12.3; p = 0.001]\), but no interaction of entrainment condition by week \([F(4,35) = 1.5; p = n.s.]; see Figure 3.10.

A correlation of all mice (collapsed across groups) provided further confirmation that there was no relationship between circadian period and average daily ethanol intake \([\text{Pearson’s } r = -0.1; p = n.s.}\].

### 3.4 Discussion: Study 1

Changing period through entrainment to T cycles had no effect on ethanol g/kg intake or preference in C57BL/6J mice drinking in DD, in 24 LD, or in T cycles. There was also no influence of entrainment condition on average amount of ethanol licking measured in Experiment 2. We did see strong effects of T cycles on activity with T cycle after-effects in DD of 0.5 hours in the desired direction. In short, we did not observe any evidence for an influence of period on ethanol intake male C57BL/6J mice despite strong differences among entrained period based on T cycle conditions.

Manipulating circadian period in C57BL/6J mice showed large variability in
ethanol intake and no differences across groups entrained to T cycles and allowed ethanol under numerous lighting conditions. Power calculations for the ability to detect a 50% change in ethanol intake between these groups was between 72% and 82% (depending on the comparison) reflecting the large variability displayed within groups. Noteworthy is that variability in Experiment 2 (drinking in T cycles or in 24 LD following T cycles) was higher for experimental groups than the control group. This hints to the possibility that while circadian period had no uniform effect on ethanol intake, it may have had a non-specific effect of increasing intake in some mice, or decreasing it in others, creating a wider range of variability within experimental groups in this Experiment. If this were the case, we would expect a similar finding in Experiment 1, drinking in DD following ethanol intake; however, these mice showed similar variability and mean daily drinking values within 1 g/kg between groups.

The C57 BL/6J strain of mice are a genetically homogeneous strain with a high tendency to drink alcohol compared with other inbred mouse strains [Rhodes et al., 2007, Yoneyama et al., 2008]. Any changes in alcohol intake produced by T cycles, in mice that are similar in both their genetics and alcohol intake would provide strong evidence for a circadian effect. However, in Experiments 1 and 2 we found no evidence for an influence of entrained period on ethanol intake after entrainment to T cycles despite robust differences in entrained period in T cycles and in DD following T cycles. Mice released into 24 LD from T cycles re-entrained to 24 LD in 4-5 days and appear to also demonstrate some differences in entrainment to 24 LD though differences in the form of their activity rhythms was not compared. They also did not show any differences in ethanol intake from control mice, even during the first several days of ethanol exposure
during re-entrainment to the 24 LD cycle. These results strongly suggest that there is no strong direct effect of period on ethanol intake in C57BL/6J mice. It is possible that period can in fact affect intake, but these effects are not present in this inbred strain. It could be that average ethanol intake levels are at a ceiling level and potential effects of period manipulation on increasing ethanol intake are not adequately testable in this strain of mice.

High and low alcohol preferring (HAP and LAP) mice are bred for their alcohol intake from the heterogeneous HS/Ibg mice (created by crossing 8 inbred strains). HAP and LAP lines are uniform with respect to ethanol preference but not homogeneous. It was these lines of mice which showed tau differences in DD [Hofstetter et al., 2003] and this finding along with tau differences shown in high and low alcohol preferring rats [Rosenwasser et al., 2005] that motivated the present experiments. Next, Study 2 turns directly to HAP2 and LAP2 mice to further test for a causal effect of period on ethanol intake and preference.

3.5 Methods: Study 2

3.5.1 Subjects and Husbandry

Male and Female HAP2 and LAP2 mice (N=59) were approximately 16 weeks old at the start of the experiment. HAP2 and LAP2 lines (supplied by the University of Indiana) were created by bidirectional selection from HS/Ibg mice, which were originally derived using an 8-way cross of other inbred strains. Successive generations of HS/Ibg mice were bred for high or low voluntary ethanol drinking that was tested for 4 weeks. HAP2 and LAP2 mice used in the current study were 30th generation of selective breeding
for ethanol preference (for additional details on the creation of HAP and LAP mouse lines see Grahame et al., 1999). Mice were housed and weighed as in Study 1; cage changes were handled as in Study 1. All procedures and animal care was approved by and conducted under the guidelines of the Institutional Animal Care Use Committee at University of California, San Diego.

### 3.5.2 Experiment 3 Procedure

**Part A**

Methods for Experiment 3 part A are outlined in Table 3.3. Following acclimation to the laboratory, male and female HAP2 and LAP2 mice were housed in 24LD or allowed to free run in constant dark (DD) for 2 weeks. During this time locomotor activity was recorded on all mice. Subsequently, mice stayed in the same lighting conditions with the addition of 24 hour 2 BC ethanol exposure for 21 continuous days (Part A ethanol exposure). At this time, control mice in 24LD had their ethanol licking activity recorded while experimental mice in DD continued to have locomotor activity recorded. At the end of Part A ethanol exposure mice in DD were returned to normal 24LD conditions to re-entrain for 2 weeks prior to the start of Part B.

**Part B**

Methods for Experiment 3 part B are outlined in Table 3.4. Control mice continued in 24 LD conditions while experimental mice were divided in half and entrained to either T22 or T26 for at least 3 weeks. During this time locomotor activity was recorded in experimental animals living in T cycles. Subsequently, mice were released into DD
from T cycles and allowed 24 hour 2 BC ethanol for 21 days (Part B ethanol exposure); locomotor activity continued to be recorded in these mice. Control mice received alcohol under 24 LD conditions.

3.5.3 T Cycle Entrainment

T cycle entrainment was handled as in Study 1.

3.5.4 Activity Monitoring

Recording of locomotor and licking activity was measured as in Study 1.

3.5.5 24 Hour 2 Bottle Choice

Twenty-four hour ethanol exposure procedures were the same as in Study 1.

3.5.6 Statistical Analyses

Statistical analyses were completed with SPSS (Chicago, IL) and ClockLab (Actimetrics, Wilmette, IL). All statistically significant values are at a level of p< 0.05 or lower; post-hoc comparisons used the Bonferroni correction method to control for alpha level. Figures and tables were prepared using Microsoft Excel® and ClockLab.

Activity

Individual taus based on locomotor activity were calculated using a Chi-square periodogram analysis in ClockLab which determines the circadian periodicity within a wide range (20 to 30 hours) with a significance level of p< 0.01. ANOVAs compared tau measures by mouse line, sex, and entrainment condition in Parts A and B. ANOVAs also
tested for differences in average locomotor activity (counts/min) by mouse line and in sex in LD compared to DD conditions. An ANOVA also compared a measure of rhythm robusticity (Q) between LD and DD conditions. Animals with obvious abnormalities in their data had these days excluded from activity analyses; these animals were excluded from any Q analyses as this measure is sensitive to number of days it is based on. Analyses of activity prior to ethanol is based on the 10 days preceding the start of ethanol exposure; analyses of any activity measures during ethanol is based on the entire 21 days of ethanol exposure. Correlations (Pearson’s r) between observed period and ethanol intake were also performed.

**Ethanol Consumption**

Alcohol consumption (g/kg) and proportion preference values were analyzed using SPSS (Chicago, IL). ANOVAs compared overall average daily and per cycle ethanol as well as preference by mouse line, sex, and entrainment condition. Values for average daily or per cycles g/kg ethanol intake in Parts A and B were based on 21 days of ethanol exposure. Repeated measures analyses of variance (ANOVAs) compared daily average intake across 3-6 day time periods by mouse line, sex, and entrainment condition for ethanol exposures in Part A and B.
3.6 Results: Study 2

3.6.1 Experiment 3 Part A: Living in LD or DD

Activity

Figure 3.11 presents representative actograms for HAP2 and LAP2 mice in LD and in DD for Experiment 3 part A. There was a significant effect of lighting condition on rhythm robusticity in Part A prior to alcohol exposure between LD and DD conditions with mice in LD exhibiting significantly higher Q values in LD compared with DD [F(1,47)=4.6; p= 0.04].

There were no period differences under DD conditions by mouse line or sex [F(3,35)= 0.8; p= n.s.]. However circadian periods for both strains in DD were significantly shorter than 24 hours (as in 24 LD conditions), [F(1,50)= 33.1; p< 0.001]; see Figure 3.12. If animals had exhibited a 0.3 hour difference in period, we had enough power to detect it; a power analysis calculator showed 87% power to detect such a difference with the sample size and variance present in the present study. Repeated measures ANOVA showed no significant differences in circadian period for mice in DD prior to ethanol compared with ethanol exposure [F(1,35)= 1.3; p=n.s.]. Additionally, there were no significant correlations between ethanol intake per day with either circadian period in DD preceding ethanol exposure [Pearson’s r= -0.2; p= n.s.] or with circadian period during ethanol exposure [Pearson’s r= 0.2; p= n.s.].

Average locomotor activity counts prior to ethanol exposure were significantly different between mouse line and sex [F(3,50)= 15.4; p< 0.001] but was not significantly affected by LD or DD lighting conditions [F(1,50)= 1.9; p= n.s.]. HAP2 mice showed
significantly greater average levels of locomotor activity (counts/min) than LAP2 mice, with female HAP2 mice showing even greater levels of activity than male HAP2 mice [pairwise comparisons p= 0.02]. Repeated measures ANOVA showed no significant differences in average levels of locomotor activity prior to alcohol compared with average activity during concurrent ethanol exposure for mice in DD [F(1,35)=0.9; p= n.s.].

**Ethanol Consumption**

There were no significant differences between average ethanol intake levels when calculated per day versus per cycle (based on individual tau). The values were highly correlated [Pearson’s r= 1.0; p< 0.001], so for simplicity, only average daily ethanol intake levels are presented in analyses.

Average ethanol intake and preference are shown in Figures 3.13-3.14. As expected, an ANOVA confirmed HAP2 mice had significantly higher ethanol preference [F(1,50)= 126.9; p< 0.001], and exhibited significantly higher average ethanol g/kg intake per day [F(1,50)= 153.0; p< 0.001] than LAP2 mice in LD and DD. Pairwise comparisons showed that female HAP2 mice drank more than male HAP2 mice [p< 0.01]. This was mirrored by measurements of ethanol licking activity in LD control mice showing a highly significant effect of line on licking [F(1,15)= 89.9; p < 0.001] and female HAP2 again licking more than male HAP2 [p< 0.05]. There was also a main effect of lighting condition such that mice drank more on average per day [F(1,50)= 8.0; p< 0.01] in LD compared with DD conditions. Post-hoc pairwise comparisons showed that this effect was largely driven by female HAP2 mice who drank more than male HAP2 mice in LD conditions.

Repeated measures ANOVA showed no significant difference in average daily
ethanol intake across time based on daily average ethanol intake levels for 3-6 day time epochs \( F(1,50) = 0.5; p = \text{n.s.} \); see Figure 3.15.

### 3.6.2 Experiment 3 Part B: T cycles

**Activity**

Representative actograms for HAP2 and LAP2 mice prior to and during ethanol exposure in part B of Experiment 3 are shown in Figure 3.16. As expected, there was a main effect of entrainment condition on observed circadian period in the days preceding Part B ethanol exposure \( F(2,43) = 119.5; p < 0.001 \) as well as during ethanol exposure \( F(2,45) = 89.7; p < 0.001 \); see Figure 3.17. Mice entrained to T cycles and then free-ran near 24 hours (following T26) or 23 hours (following T22) in DD. There were no significant differences within entrainment condition on mouse line and sex either prior to \( F(3,43) = 0.7; p = \text{n.s.} \) or during ethanol exposure \( F(3,45) = 1.9; p = \text{n.s.} \). There was no significant association between entrained period during ethanol exposure with average daily ethanol intake \( \text{Pearson’s } r = 0.1; p = \text{n.s.} \). This is convergent with the null effect of entrainment condition on average daily ethanol intake reported in the above subsection.

A univariate ANOVA on Q values by entrainment condition revealed no significant effects for experimental mice in T cycles either prior to \( F(1,24) = 0.5; p = \text{n.s.} \) ethanol exposure or for mice following T cycle entrainment in DD during ethanol exposure \( F(1,30) = 0.05; p = \text{n.s.} \).
Ethanol Consumption

There were no significant differences between average ethanol intake levels when calculated per day versus per cycle (based on individual periods). The values were highly correlated [Pearson’s r = 1.0; p << 0.001]; for simplicity, only average daily ethanol intake levels are presented in analyses.

As in Part A, univariate ANOVAs showed a significant effect of mouse line on ethanol preference [F(1,45)=212.7; p << 0.001] and average daily [F(1,45)= 118.2; p << 0.001] g/kg ethanol intake. As in Part A, HAP2 mice were higher on all of these dimensions and post-hoc comparisons showed female HAP2 consumed more g/kg ethanol than male HAP2 [p << 0.001]. See Figure 3.18-3.19 for average ethanol intake and preference during part B of Experiment 3.

There was however no effect of entrainment condition (24 LD, T22 or T26 entrainment) on ethanol preference [F(2,45)=0.7; p = n.s.], or average daily [F(2,45)=0.4; p = n.s.] ethanol g/kg intake during Part B.

Repeated measures ANOVA showed no significant difference in average daily ethanol intake across time based on daily average ethanol intake levels for 3-6 day time epochs [F(1,45)= 2.3; p = n.s.]; see Figure 3.20.

3.7 Discussion: Study 2

Both HAP2 and LAP2 mouse line have a FRP shorter than 24 hours, closer to that of LAP2 mice in the originally cited Hofstetter et al., 2003 study. This suggests that the correlation between tau and ethanol preference in the HAP1 and LAP1 lines was coincidental. However, this might be influenced by the way tau was measured in
the Hofstetter study compared to the current study. That is, the Hofstetter et al., 2003 study used running wheels to assess activity while the current used recordings of general locomotion. Specifically, wheel running may feedback and affect period in highly active animals [Koteja et al., 2003, Mistlberger & Holmes, 2000]; we observed significantly higher average activity levels in HAP2 compared with LAP2 mice. Our lab is currently exploring this mediating factor of tau in HAP2 mice.

There were strong effects of T cycles on activity with T cycle after-effects in DD of 0.5 hours in the desired direction. These are large differences in period; the significant difference between HAP and LAP lines in the prior Hofstetter et al. (2003) study was approximately 0.3 hours. If the difference in tau were related to ethanol intake in HAP2 and LAP2 lines, these tau differences should have been replicated in the current study, or successful T cycle manipulations affecting period should have influenced ethanol intake.

As mentioned above, there was no effect of tau or T cycle history on ethanol intake in HAP2 or LAP2 mice in Part A or B. There was an effect of lighting in Part A such that mice in LD conditions drank more on average than mice in DD. This was largely due to female HAP2 mice drinking more in LD than in DD. Female mice do consistently show higher ethanol intake than males [Grahame et al., 1999]. However, the reason female mice drank more in LD than in DD in Part A of the current study is unknown. Speculatively, it could be housing in DD reduced drinking through some other mediating factor such as reduction of stress. It might be suggested that decreased tau in DD led to a decrease in ethanol intake in female HAP2 mice. This is unlikely as this effect was not seen when period was shortened in Part B; drinking in Part B showed no differences according to circadian period conditions. It is also possible the effect in Part
A could be due to large variation in a small sample size for this group.

As expected, HAP2 mice drank much higher levels of ethanol than LAP2 mice in Part A and B. Average activity levels in mice prior to ethanol exposure in Part A mirrored these effects with HAP2 mice showing greater activity levels than LAP2, and female HAP2 mice more active than male HAP2 mice. The specific reason for greater amounts of locomotor activity in these mice is undetermined. HAP2 mice also run more in wheels than LAP2 mice; recent literature has framed wheel running as a rewarding activity [Ozburn et al., 2008], which could be a particularly relevant comparison to HAP and LAP mice.

The current Study showed no evidence for an influence of tau on ethanol intake in male or female HAP2 and LAP2 mice despite robust differences in tau in T cycles and in DD following T cycles. These results strongly suggest that there is no strong direct effect of tau on ethanol intake in HAP2 and LAP2 mice. Using both high and low alcohol preferring strains allowed for high sensitivity to potential increases or decreases in ethanol intake or preference.

3.8 Summary and Conclusions

All Experiments in Chapter 3 conducted successful T cycle manipulations resulting in different entrained periods. However, Experiments 1-3 showed no effects of period on ethanol intake in C57BL/6J and high and low (HAP2 and LAP2) alcohol preferring mice. Together these results strongly argue against any direct effects of tau on ethanol consumption. In Study 1 the effect of tau on ethanol intake received no support with C57BL/6J mice, an inbred strain that is known for high ethanol intake. Mice had
24 hour access to alcohol after entrainment to 22 or 26 hour periods in: DD, 24 hour cycles (12:12 LD), 22 (11:11 LD) or 26 (13:13 LD) hour cycles. There were no differences in alcohol intake or preference among any of these groups although each was successfully entrained to its target tau. In Study 2 high and low alcohol preferring mice (HAP2 and LAP2) were tested for differences in voluntary ethanol intake in DD following entrainment to 22 or 26 hour cycles. These mice also successfully entrained to short or long T cycles but showed no differences in alcohol intake according to entrained condition. Analyses of ethanol intake across days and weeks of exposure ruled out the possibility of a transient difference in ethanol intake for all experiments.

HAP and LAP mice were also looked at for differences in activity levels, rhythm robusticity, and FRP differences previously reported in DD. Like previous studies with HAP and LAP mice, HAP2 showed greater levels of activity compared to LAP2 mice. There were no differences in robusticity of circadian rhythms between the two lines, nor were there any observed differences in tau in free running conditions. One important distinction is that our study used general locomotor activity while the study reporting tau differences used running wheels, which may show tau shortening effects as a byproduct of greater amounts of wheel running. Our lab is currently following up this possibility by allowing HAP2 and LAP2 mice running wheels to see if the lack of inter-strain tau differences are a function of the strain replicate (using HAP2 and LAP2 mice rather than HAP and LAP mice) or possibly due to the way the period was measured with mediating effects of running wheels on tau.

On a related point, in the future wheel running behavior may also be studied in relation to reward in HAP and LAP mouse strains. Wheel running has been described as
a potent reinforcer in rodents (Ozburn et al., 2008); it is possible that wheel running may interact with ethanol influencing the reward or addiction system in mice. HAP and LAP mouse lines are beginning to be put forth by researchers as a translational phenotype associated with a tendency towards higher ethanol intake; for example, HAP mice were recently shown to be more impulsive than LAP mice [Oberlin & Grahame, 2009]. If this is the case, it may be interesting to see if we can change ethanol intake in these mice with other procedures such as circadian scheduling described in Chapter 2.

Prior studies have shown inconsistent effects of ethanol exposure on tau (for example, Mistlberger et al., 1992), and it was not an effect we tested or controlled for extensively in the current experiments. However, there were no differences on tau in DD prior to compared with during ethanol exposure in Part A of Experiment 3. Nor were there differences in tau between HAP2 mice (that consumed large amounts of ethanol) compared with LAP2 mice (that consumed only small amounts of ethanol) in DD suggesting that the ethanol consumption in the current study did not have a significant effect on tau in HAP2 and LAP2 mouse lines.

C57 mice showed more enduring after-effects in DD following long (T26) T cycles compared with HAP2 and LAP2 mice. When released into DD from T26, HAP2 and LAP2 mice started reverting to shorter period within a couple days whereas C57 mice continued to run with periods longer than 24 hours for several weeks. The robust T cycle after-effects seen in the C57BL/6J mice is the typically expected response. The almost non-existent after-effect of long T cycles on tau in HAP2 and LAP2 lines qualifies how our data are able to speak to lengthening tau effects on ethanol intake in these mice. The evidence remains that no tau manipulation in HAP2, LAP2 or C57 mice influenced
ethanol intake; this provides strong evidence against the possibility that tau has a direct effect on ethanol intake and preference.

Besides observed after-effects on period, T cycles also create changes to the circadian system that affect the phase relationships with peripheral body tissues such as organs [Molyneaux et al., 2008]. Thus entrainment to T cycles potentially has far reaching effects on the circadian system, creating a powerful conclusion that disrupting these components nevertheless does not affect ethanol intake and preference. Thus, people whose circadian rhythms are disturbed either through endogenous period differences or through other means (such as travel across time zones) are not expected to show a direct effect of these disturbances on alcohol intake.
Table 3.1. Methods for Experiment 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Sex</th>
<th>n</th>
<th>Light cycle prior to ETOH</th>
<th>Light cycle during ETOH</th>
<th>Activity recorded during ETOH (28 days)</th>
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<tbody>
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<td>M</td>
<td>8</td>
<td>24LD</td>
<td>DD</td>
<td>Alternated between</td>
</tr>
<tr>
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<td>C57BL/6J</td>
<td>M</td>
<td>7</td>
<td>T26</td>
<td>DD</td>
<td>licking and locomotor</td>
</tr>
<tr>
<td>T22 to DD</td>
<td>C57BL/6J</td>
<td>M</td>
<td>8</td>
<td>T22</td>
<td>DD</td>
<td>(see Fig 3.1)</td>
</tr>
</tbody>
</table>
Figure 3.1 Representative double-plotted actograms of locomotor and licking activity by C57BL/6J mice during 28 days of 24 hour ethanol exposure in DD for Experiment 1. Activity recording was switched between licking and locomotor; licking is recorded at the start and successive stars on the actograms indicate approximate time on days when recording is switched to the alternate activity. 3.4A. Mice in DD following 24LD. 3.4B. Mice in DD following 26 hour T cycles. 3.4C. Mice in DD following 22 hour T cycles.
Figure 3.2 Circadian period (average ± SEM) for C57BL/6J mice in DD following 24 LD, 26, or 22 hour T cycles in Experiment 1. Individual taus were calculated by Chi-square periodogram analysis.
Figure 3.3 Average (± SEM) ethanol intake (g/kg) per 24 hours by C57BL/6J mice drinking in DD following 24LD or T cycles in Experiment 1.
Figure 3.4 Ethanol preference for C57BL/6J mice drinking in DD following 24 LD or T cycles in Experiment 1 (bars indicate SEM).
Figure 3.5  Daily average ethanol intake (g/kg) across weeks by C57BL/6J mice drinking in DD following 24 LD or T cycles in Experiment 1 (bars indicate SEM).
Table 3.2. Methods for Experiment 2.

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<th>Group</th>
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<th>Light cycle during ETOH (30 days)</th>
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<td>M</td>
<td>8</td>
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</tr>
<tr>
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<td>M</td>
<td>8</td>
<td>T26</td>
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</tr>
<tr>
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<td>8</td>
<td>T26</td>
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<td>Licking</td>
</tr>
<tr>
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<td>8</td>
<td>T22</td>
<td>T22</td>
<td>Licking</td>
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</table>
Figure 3.6 Representative double-plotted actograms of licking activity by C57BL/6J mice during 30 days of 24 hour ethanol exposure in 24LD or in T cycles for Experiment 2. 3.6A. Control mice in 24LD from 24LD. 3.6B. Mice in 24LD following 26 hour T cycles. 3.6C. Mice in 24LD following 22 hour T cycles. 3.6D. Mice in 26 hour T cycles. 3.6E. Mice in 22 hour T cycles. Light dark bars above A-C represent times of light and dark for the 24 hour light/dark cycle; light schedules shift daily in D-E in 26 and 22 hour T cycles.
Figure 3.7 Circadian period (average ± SEM) for C57BL/6J mice living in T cycles or in 24LD following T cycles in Experiment 2. Individual taus were calculated by Chi-square periodogram analysis.
Figure 3.8 Average (± SEM) ethanol intake (g/kg) per 24 hours by C57BL/6J mice drinking in T cycles or in 24LD following T cycles in Experiment 2.
Figure 3.9 Ethanol preference for C57BL/6J mice drinking in T cycles or in 24LD following T cycles in Experiment 2 (bars indicate SEM).
Figure 3.10  Average ethanol intake per 24 hours (g/kg) across days by C57BL/6J mice drinking in T cycles or in 24LD following T cycles in Experiment 2 (bars indicate SEM).
Table 3.3. Methods for Experiment 3 part A.

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<th>Light cycle during ETOH</th>
<th>Activity recorded prior to ETOH (10 days)</th>
<th>Activity recorded during ETOH (21 days)</th>
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<td>licking</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>LAP2</td>
<td>M</td>
<td>24LD</td>
<td>24LD</td>
<td>locomotor</td>
<td>licking</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>10</td>
<td>HAP2</td>
<td>M</td>
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<td>DD</td>
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<td>locomotor</td>
</tr>
<tr>
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<td></td>
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<td></td>
<td>10</td>
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<td>M</td>
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<tr>
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<tr>
<td></td>
<td>10</td>
<td></td>
<td>F</td>
<td>DD</td>
<td>DD</td>
<td>locomotor</td>
<td>locomotor</td>
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Figure 3.11  Representative double-plotted actograms of locomotor and some licking activity by HAP2 and LAP2 mice prior to (first 10 days) and during 21 days of 24 hour ethanol exposure in 24LD or in DD for Experiment 3 part A. The start of ethanol exposure is indicated by a star.  3.11A. HAP2 mouse in 24LD. 3.11B. LAP2 mouse in 24LD. 3.11C. HAP2 mouse in DD. 3.11D. LAP2 mouse in DD. Locomotor activity is recorded throughout in DD. In 24LD, locomotor activity is recorded in the 10 days prior to and for the first few days of ethanol exposure; starting a few days into ethanol exposure (indicated by an arrow) ethanol licking activity is recorded in the 24LD group. Light/dark bars above actograms indicate times of lights on and off for 24 LD and lights off in DD.
Figure 3.12  Circadian period (average ± SEM) for HAP2 and LAP2 mice living in 24 LD or in DD prior to and during ethanol exposure in Experiment 3 part A. Individual tans were calculated by Chi-square periodogram analysis.
Figure 3.13 Average (± SEM) ethanol intake (g/kg) per 24 hours by HAP2 and LAP2 male and female mice in either 24LD or DD for Experiment 3 part A. 3.13A shows average ethanol intake for males and females combined. 3.13B shows average ethanol intake broken down for male and female HAP2 mice.
Figure 3.14 Ethanol preference for HAP2 and LAP2 mice drinking either in 24LD or in DD in Experiment 3 part A (bars indicate SEM).
Figure 3.15 Average ethanol intake per 24 hours (g/kg) across days by HAP2 and LAP2 mice drinking in 24LD or in DD in Experiment 3 part A (bars indicate SEM).
Table 3.4. Methods for Experiment 3 part B.

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<th>Part B Group</th>
<th>Part A history</th>
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<th>Strain</th>
<th>Sex</th>
<th>Light cycle prior to ETOH</th>
<th>Light cycle during ETOH</th>
<th>Activity recorded prior to ETOH (4-10 days)</th>
<th>Activity recorded during ETOH (21 days)</th>
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</thead>
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<td>24LD</td>
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<td>HAP2</td>
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<td>licking</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
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<td>F</td>
<td>24LD</td>
<td>24LD</td>
<td>locomotor</td>
<td>licking</td>
</tr>
<tr>
<td>4</td>
<td>LAP2</td>
<td>24LD</td>
<td>5</td>
<td>M</td>
<td>24LD</td>
<td>24LD</td>
<td>locomotor</td>
<td>licking</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td></td>
<td>F</td>
<td>24LD</td>
<td>24LD</td>
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<td>licking</td>
</tr>
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<td>T26</td>
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<td>HAP2</td>
<td>M</td>
<td>T26</td>
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<td>locomotor</td>
</tr>
<tr>
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<td></td>
<td>5</td>
<td></td>
<td>F</td>
<td>T26</td>
<td>DD</td>
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<td>4</td>
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</tr>
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<td></td>
<td></td>
<td>5</td>
<td></td>
<td>F</td>
<td>T26</td>
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<tr>
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<td></td>
<td>5</td>
<td></td>
<td>F</td>
<td>T22</td>
<td>DD</td>
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Figure 3.16  Representative double-plotted actograms of activity by HAP2 and LAP2 mice prior to and for 21 days of ethanol exposure for Experiment 3 part B. Prior to ethanol exposure experimental mice were housed in 22 or 26 hour T cycles; these mice were released into DD on the first evening of ethanol exposure. Control mice remained in 24LD throughout. Locomotor activity was recorded for all mice prior to ethanol exposure; during ethanol exposure experimental mice from T cycles continued to have locomotor activity recorded whereas control mice in 24LD had ethanol licking activity recorded. The start of ethanol exposure is indicated by a star; the switch from recording locomotor to licking activity in control mice is also marked by this star. 3.16A. HAP2 mouse in T22 followed by DD. 3.16B. LAP2 mouse in T22 followed by DD. 3.16C. HAP2 mouse in T26 followed by DD. 3.16D. LAP2 mouse in T26 followed by DD. 3.16E. HAP2 mouse in 24LD. 3.16F. LAP2 mouse in 24LD.
Figure 3.17  Circadian period (average ± SEM) for HAP2 and LAP2 mice living in 22 or 26 hour T cycles prior to ethanol exposure, and in DD during ethanol exposure in Experiment 3 part B. Individual taus were calculated by Chi-square periodogram analysis.
Figure 3.18  Average (± SEM) ethanol intake (g/kg) per 24 hours by HAP2 and LAP2 male and female mice in either 24LD or in DD following 22 or 26 hour T cycles in Experiment 3 part B. 3.13A shows average ethanol intake for males and females combined. 3.13B shows average ethanol intake broken down for male and female HAP2 mice.
Figure 3.19  Ethanol preference for HAP2 and LAP2 mice drinking in 24LD or in DD following 22 or 26 hour T cycles in Experiment 3 part B (bars indicate SEM).
Figure 3.20  Average ethanol intake per 24 hours (g/kg) across days by HAP2 and LAP2 mice drinking in 24LD or in DD following 22 or 26 hour T cycles in Experiment 3 part B (bars indicate SEM).
Chapter 4

Effects of repeated withdrawal from ethanol dependence on circadian temperature and activity rhythms

Various biological rhythms are disrupted during alcohol administration and withdrawal. Humans with a history of alcoholism show particular sleep disruptions which are predictive of relapse [Drummond et al., 1998], and rodents show long term changes to sleep EEG function weeks following ethanol withdrawal [Ehlers and Slawecki, 2000]. Ethanol has also been shown to affect body temperature during ethanol ingestion as well as during withdrawal; however, chronic ethanol effects on circadian rhythms have not been carefully studied. In the current chapter, we introduce an exploratory study to test for effects of multiple inductions of ethanol dependence on subsequent circadian measures of body temperature and activity rhythms. Following each of three ethanol vapor sessions, circadian body temperature and activity rhythms are recorded via biotelemetry
for at least one week. Results indicate a cumulative effect of vapor exposures on reducing body temperature amplitude during withdrawal suggesting a potentiating effect on rhythm disturbances in the days immediately following subsequent ethanol exposures.

### 4.1 Introduction

As reviewed in Chapter 1, various biological rhythms are disrupted during alcohol administration and withdrawal. Studies on sleep/wake rhythms have found that relapse in abstinent alcoholic patients is related to sleep dysfunction [Drummond et al., 1998]. Further, rodent sleep EEG studies show disturbed sleep in dependent animals weeks after withdrawal [Ehlers and Slawecki, 2000]. Sleep, although rhythmic, is influenced by many processes and is not as closely tied to circadian function as other rhythms such as core body temperature. Core body temperature is typically robust to exogenous influence, and can often be used as a reliable indicator of circadian function. It can also be used to probe the possibility that these disturbances long after ethanol ingestion has ended is unique to sleep and other factors influencing sleep, or if they are indicative of disruption in the circadian system. Thus, employing an alternative markers of circadian function can assess this issue.

So how does ethanol affect body temperature? Acutely, injections of ethanol cause hypothermia for up to a few hours [Baird et al., 1998, Crawshaw et al., 1997, O’Connor et al., 1989]. An effect that differs with age (in rats) and with history of ethanol exposure [Ristuccia and Spear, 2008, Taylor et al., 2009]. This does not involve investigation of the daily body temperature rhythm; though the hypothermic effect of ethanol on body temperature also differs according to time of day. Thus, the timing of
ethanol ingestion modulates the body’s physiological response to it.

In contrast to acute, single doses of ethanol, chronic ethanol exposure allows for dependence and enduring changes to the body in response to ethanol. During chronic ethanol exposure, body temperature and activity rhythms typically display blunted amplitudes [Danel et al., 2001, Devaney et al., 2003, Ristuccia and Spear, 2005, Taylor et al., 2002, Taylor et al., 2006]. However, to find out whether chronic ethanol and dependence affect circadian rhythms it is important to measure rhythms in the absence of ethanol’s confounding effects, following ethanol exposure. Recording circadian rhythms after inductions of ethanol dependence allows for the examination of long-term effects on rhythms during withdrawal and beyond. During the first few days of withdrawal the body is undergoing a shift in the altered homeostasis or allostasis achieved during ethanol dependence. At this time a host of compensatory changes to deal with the sudden absence of ethanol are taking place, and it is possible that observed changes to circadian rhythms could be peripheral to the circadian clock. Long-lasting changes following ethanol exposure likely indicate a change to core circadian clock mechanisms.

Research exposing subjects (mainly rodents) to chronic ethanol has often recorded rhythms following ethanol exposure for 1 day, or 3 days (in other words, during withdrawal) though even these studies are few. Research with chronic ethanol vapor and body temperature from a non-circadian perspective measured body temperature for 1 day following chronic ethanol exposure [Crawshaw et al., 1994]. No effect on body temperature following ethanol (using the overall temperature average) was reported; however, in a graph of body temperature across the day it appears as though the amplitude of the rhythm is decreased. A human study similarly did not report decreased amplitude dur-
ing withdrawal from ethanol but presented data indicating decreased body temperature amplitude for the first 3 days (average) of withdrawal [Gross et al., 1975]. One study with rats compared circadian rhythm parameters of body temperature including amplitude following ethanol exposure but found inconsistent changes to amplitude between male and female rats and across strains of rats [Taylor et al., 2006]. However, that study compared these measures during 3 days of withdrawal to 3 days during ethanol exposure, a time period that is commonly associated with decreased amplitude. Similarly, amplitude of activity rhythms following ethanol are also unsettled. Activity was found to be decreased during the first day of withdrawal in mice with a high sensitivity to withdrawal but not in outbred mice (without high withdrawal sensitivity), but increased in the first day (only the first, of 3 days) of withdrawal in multiple strains of rats [Kliethermes et al., 2005, Ristuccia and Spear, 2005, Taylor et al., 2006]. Thus, effects of chronic ethanol on subsequent circadian body temperature rhythms are unclear. It remains unknown whether a history of ethanol dependence disrupts circadian rhythms, a practical question relating to humans. Known circadian rhythm disruptions during withdrawal may be a useful index of withdrawal severity, and beyond withdrawal they may be an indicator of relapse, similar to other rhythmic behaviors such as sleep.

Practically in humans, alcohol use disorders involve multiple periods of abuse, sobriety or attempts at recovery, and relapse. Multiple inductions of ethanol dependence using vapor are a useful method for modeling this behavior in rodents. Multiple inductions have also been shown to lead to higher voluntary ethanol intake [Becker and Lopez, 2004, O’Dell et al., 2004]. Thus, the goal of the current study is to examine the neglected effect of single as well as multiple inductions of alcohol dependence on circadian
body temperature and activity rhythms during withdrawal and beyond. This is done by focusing on specific parameters of circadian rhythms that are reliable measures of circadian function, amplitude and entrainment. Amplitude, the size of daily variation in the rhythm is determined by the circadian system, although outside factors may influence it, consistent and lasting differences reflect either a change to the peripheral mechanisms that output the rhythm or even to the clock itself. Entrainment is the timing of the start and end of the active phase with respect to the environmental light:dark cycle. The beginning and end of the active phase, the dark or night for mice, are described as the onset and offset. The pattern of entrainment is determined by the circadian clock; indeed changes to the clock, elicit predictable changes to entrainment. For instance, light at certain times during the circadian cycle is a potent agent in shifting or re-setting the circadian clock. When a pulse of light is delivered at a specific time, its effects on the circadian clock are measured by looking at the difference in entrainment or onsets and offsets in the days subsequent to the stimulus. Differences seen only during withdrawal are more likely to be mediated by ethanol’s influence on temperature regulation (acute ethanol leads to hypothermia) as effects on circadian clock mechanisms would be expected to last for multiple cycles. Similarly, for activity, changes seen only during withdrawal may indicate an excitatory or depressive influence of withdrawal symptoms. This study focuses on these parameters and asks specifically, if there are differences in amplitude or entrainment in the days immediately following ethanol (withdrawal) or beyond. Further, we ask how these differences are impacted following multiple inductions of dependence.
4.2 Methods

4.2.1 Animals

Adult male (N = 15) C57BL/6J mice (n = 8 experimental, n = 7 control) were 11 weeks old at the start of the experiment (at the time of surgery). Mice were individually housed during biotelemetry recording phases only. At all other times mice were group housed. Throughout, lights were on a 12 hour light/12 hour dark cycle. Mice had ad libitum access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committees at The Scripps Research Institute.

4.2.2 Biotelemetry

Mice were anesthetized with isoflurane gas and telemeters were implanted into the peritoneal cavity and attached to the abdominal wall; analgesia was given post-operatively. Ample recovery time (>3 weeks) was allowed prior to baseline recording. Telemetry data was recorded using Oxymax software (Columbus, OH) designed for the comprehensive lab animal monitoring system (CLAMS; Columbus Instruments, Columbus, OH) where the animals were housed. Before the first exposure to ethanol vapor, baseline biotelemetry was recorded for one week. Mice were again placed into the CLAMS following the last session of each vapor bout. Body temperature and locomotor activity were recorded continuously during and were sampled every 18 minutes throughout recording sessions.

Body temperature and locomotor activity were recorded for a baseline and three post-vapor recording sessions that lasted at least 7 days each. However, recording following vapor session 2 was corrupted and data was lost between days 4 and 10; recording
continued for days 11-13 following vapor exposure 2.

4.2.3 Ethanol Vapor Sessions

Each ethanol vapor session consisted of 3 days (bouts) of ethanol vapor; Figure 4.1 illustrates the 3-day ethanol vapor session procedure. On ethanol vapor days, ethanol vapor lasted for 16 hours/day (controls received air vapor) interspersed with 8 hours of rest (air) in between. Each day immediately prior to the start of ethanol, experimental mice were injected (i.p.) with an ethanol (1.5 g/kg at 20% v/v) and pyrazole HCl (0.0681 g/kg) solution; controls were also injected with pyrazole (0.0681 g/kg) in a 0.9% saline solution. The pyrazole was added to achieve and help maintain stable blood ethanol levels. Immediately following each ethanol bout, tail blood samples were collected (0.05 ml). Vapor bouts started 1 hour prior to lights off. Mice were placed into CLAMS and telemetry recording began within 1-2 hours following the last vapor bout and blood sampling of the session. There were a total of 3 vapor sessions, each followed by a phase of biotelemetry recording that lasted at least 7 days.

4.2.4 Procedure

Mice were implanted with telemeters and following adequate recovery time (>3 weeks) there was a baseline recording session for 1 week. Mice were split into experimental or control conditions. Experimental mice were subject to three ethanol vapor bouts lasting 3 days (controls received air vapor), each of which was immediately followed by at least a week of biotelemetry recording in CLAMS. There was at least a 1 week break between recording in CLAMS and the subsequent ethanol vapor bout. Table 4.1 outlines the experimental timeline.
4.2.5 Daily Body Temperature Analysis

Daily body temperature rhythms were subject to amplitude and entrainment analyses. For the first 5 days following vapor bouts, amplitude for each mouse was calculated per day (as maximum-minimum temperature) and entered into a repeated measures ANOVA. Temperature rise or onset was determined daily as the first time at which body temperature was above a threshold of 36 degrees Celsius for 3 consecutive time samples (body temperature was sampled every 18 minutes). Onset of the temperature rise is a reliable circadian marker indicating the start of the active phase or subjective night. Temperature fall or offset was determined daily as the time at which temperature fell below a threshold of 36 degrees Celsius for 3 consecutive time samples at the end of subjective night. Offset or the temperature decline is a circadian marker indicating the end of subjective night and start of subjective day; it is typically more ambiguous than onset. Temperature onsets and offsets were calculated for the first 8 days following ethanol vapor bouts (except following vapor session 2 when there was a recording error and only the first 3.5 days were recorded). Amplitude, temperature onset, and temperature offset were all analyzed separately in repeated measures ANOVAs and for each recording session (baseline, post vapor 1, post vapor 2, and post vapor 3). Data were prepared and analyzed using ClockLab (Actimetrics, Wilmette, OR), SPSS (Chicago, IL), and Microsoft Excel®. Planned comparisons were done between subjects for all days corresponding to repeated measures ANOVAs and examined whenever a repeated measures ANOVA revealed significant effect. Days where parameters were indeterminable, i.e. due to a cage change artificially affecting measures, were excluded from analysis (this did not affect more than one day per analysis).
4.2.6 Daily Locomotor Activity Analysis

Daily locomotor activity rhythms were also subject to amplitude and entrainment analyses. For the first 5 days immediately following vapor bouts (in addition to baseline), amplitude for each mouse was calculated and entered into a repeated measures ANOVA. Activity onsets and offsets for the first 7-8 days following vapor bouts were determined by eye-fit visual analysis of the activity data using ClockLab (except following vapor session 2, when data was corrupted and therefore only usable for days 1-3 and 11-13). Activity onset and offsets are reliable circadian markers indicating the start and end of the active phase, respectively. However, they are less reliable markers of circadian phase than body temperature onsets and offsets as activity is subject to more exogenous factors i.e., a cage change has large effects on activity for that day. Data was prepared and analyzed using ClockLab (Actimetrics, Wilmette, OR), SPSS (Chicago, IL), and Microsoft Excel®. Planned comparisons were done between subjects for all days corresponding to repeated measures ANOVAs and examined whenever a repeated measures ANOVA revealed a trend towards or a significant effect. As with temperature analyses, days where parameters were indeterminable were excluded from analysis (this did not affect more than one day per analysis).

4.3 Results

The text will focus on statistically significant results; however, for reference, resulting statistics for all repeated measures ANOVAs are displayed in Table 4.2. At the end of vapor sessions, mice were withdrawn from ethanol chambers exhibiting average blood ethanol levels of 200 mg/dl for session 1, 158 mg/dl for session 2, and 124 mg/dl
for session 3.

4.3.1 Body Temperature

Average body temperature rhythm profiles for experimental and control groups for the first 4 days of each recording session are represented in Figure 4.2.

Baseline

Figure 4.3 graphs temperature amplitude for each telemetry recording session. Prior to ethanol exposure, there were no significant effects of assigned experimental condition or day on Baseline temperature amplitude.

Figure 4.4 shows body temperature entrainment (onsets and offsets) for each telemetry recording session. There were no differences by experimental condition on temperature rise onsets during Baseline recording; however there were significant differences in temperature entrainment across days for both groups \(F(6,78) = 17.6; p < 0.001\). There were no differences in temperature offsets between experimental groups or across days.

Post-Vapor Session 1

Following the first vapor session, amplitudes were different across time for both the experimental and control groups \(F(4,52) = 32.5; p < 0.001\) during Post-vapor 1 recording, as well as an interaction with experimental condition \(F(4,52) = 3.7; p < 0.05\); amplitude increased across days for both groups, but more for the experimental group, which started off with a lower amplitude. However, there was no significant main effect of experimental condition.

There were no differences for temperature onsets following the first vapor session.
Temperature offsets were significantly different, although inconsistently, across days for both experimental and control mice \(F(5,60) = 4.0; p < 0.01\); there were no differences between experimental and control groups.

**Post-Vapor Session 2**

Temperature amplitude following vapor session 2 showed significant differences in amplitude across days \(F(2,26) = 62.2; p < 0.001\) as well as an interaction with experimental condition \(F(2,26) = 5.0; p < 0.05\)–amplitude increased over time for both groups but more for experimentals. Although there was no main significant difference between groups, planned comparisons showed that temperature amplitude was significantly different between groups on day 1 following vapor session 2 \(F(1,13) = 10.1; p < 0.01\)–amplitude was higher for controls.

Temperature onsets showed no differences between groups or across days following vapor session 2. Temperature offsets were significantly different across days for both groups \(F(2,20) = 11.9; p < 0.001\); there were no differences between experimental and control groups.

**Post-Vapor Session 3**

Temperature amplitudes were significantly different between experimental and control groups following vapor session 3 \(F(1,13) = 5.7; p < 0.05\)–amplitudes were higher for control mice; planned comparisons showed significant differences between groups on days 1 \(F(1,13) = 28.2; p < 0.001\) and 2 \(F(1,13) = 13.6; p < 0.01\) following vapor session 3. There were also significant differences across days for both groups \(F(4,52) = 17.3; p < 0.001\) as well as an interaction with condition \(F(4,52) = 9.5; p < 0.001\)–amplitude
increased across days, but much more so for the experimental group who started with lower amplitude than the control group.

Temperature onsets showed no differences across days or between groups following vapor session 3. Temperature offsets were different across days for both groups \[ F(7,77) = 3.5; p < 0.01 \]; however there were no differences between experimental and control groups.

### 4.3.2 Activity

Average activity rhythm profiles for experimental and control groups for the first 4 days of each recording session are represented in Figure 4.5.

**Baseline**

Activity amplitudes for each telemetry recording session are represented in Figure 4.6. Activity amplitudes were not different between groups prior to vapor during Baseline recording; however, both groups showed a difference in amplitude across days \[ F(4,52) = 13.0; p < 0.001 \]-amplitude was highest on the last day of recording.

Activity entrainment during each telemetry recording session are shown in Figure 4.7. Activity onsets and offsets during baseline were not different between groups during Baseline; however, both groups showed a difference in activity onset \[ F(6,78) = 22.6; p < 0.001 \] and offset \[ F(6,72) = 3.7; p < 0.01 \] across days.

**Post-Vapor Session 1**

There were no differences for activity onsets between groups following vapor session 1.
Activity onsets showed no differences between groups or across days following vapor session 1; however activity offsets were different between experimental and control groups [F(1,13) = 10.4; p < 0.05].

Post-Vapor Session 2

Activity amplitudes following vapor session 2 were significantly different between experimental and control groups [F(1,13) = 7.2; p < 0.05]. One-way ANOVAs showed that mean amplitude was lower for experimental mice for all days, (1-3) following vapor session 2, and that days 1 [F(1,13) = 6.5; p < 0.05] and 3 [F(1,13) = 8.7; p < 0.05] were significantly different between groups.

Activity onsets following vapor session 2 showed no differences between experimental and control groups; however both groups showed different activity onsets across days 1-3 [F(2,26) = 5.5; p < 0.05]. There were no differences between activity onsets for days 11-13. Activity offsets were not different between groups or across days.

Post-Vapor Session 3

Activity amplitudes were not different between experimental and control groups following vapor session 3; however both groups showed differences in activity amplitude across days [F(4.52) = 3.6; p < 0.05].

Activity onsets were not different between experimental and control groups or across days following vapor session 3. Activity offsets were also not different between experimental and control groups although both groups showed different offsets across days [F(6,72) = 2.4; p =< 0.05].
4.4 Discussion

Results indicate a cumulative effect of ethanol vapor exposures on reducing body temperature amplitude during withdrawal. This effect was primarily due to hypothermia during the night or active phase, when temperature reaches its peak. Our results show a potentiating effect, with the difference in amplitude greater following subsequent vapor sessions. This change in amplitude returns to normal within 3 days, although it does take longer to repair itself with subsequent vapor sessions. Our data indicate that this effect does not last beyond the initial days of withdrawal. However, a future study could test whether amplitude remains narrowed beyond the first few days of withdrawal by inducing additional episodes of ethanol dependence.

Patterns were similar but weaker with activity rhythms, which did not show statistically significant differences between groups except during 1 day following vapor session 2. Overall, groups did not show large differences in measures of activity amplitude or entrainment following ethanol vapor sessions. The activity data of course do not contradict the temperature data, but provide no evidence for an influence of ethanol on activity entrainment. This measure was also more variable and was susceptible to exogenous influences such as cage changes. This may have been a large factor influencing activity results as mice are transferred from group housing in the ethanol chambers to single new cages for biotelemetry recording sessions. In combination with body temperature results that suggest the effects of ethanol did last beyond the first few days, the lack of effects on activity may not be a surprise. These results support the use of body temperature as a preferential measure to activity rhythms.

Entrainment onsets and offsets did show some within subject differences across
days during recording sessions, but these were inconsistent with regard to direction. The only significant differences between experimental and control mice for body temperature onsets occurred for 2 days following vapor session 2. Although we saw no significant effects on entrainment, it is possible that effects on temperature and or activity onsets and offsets are being masked by the light/dark cycle. That is, living under standard lighting conditions provides strong cues to the clock and circadian behavior, and the presence of light may inhibit an onset from occurring that would otherwise be seen in the dark. Apparent in Figures 4.2 and 4.4, temperature rise onsets occurred normally in the light (just prior to lights off), suggesting that our lack of onset differences is not due to masking effects of light. However, as entrainment is a reliable measure of clock function, it would be informative to re-implement this paradigm in constant conditions such as constant dark, without the confounding influence of light. Prior research has found a distal influence of chronic ethanol on entrainment after neonatal ethanol exposure [Allen et al., 2005]. It would be interesting to further explore why ethanol during sensitive developmental periods affects circadian rhythms differently than ethanol in adulthood.

Ethanol dependence creates a state of allostatic or altered homeostasis as a result of physiological changes caused by the prolonged, high amounts of ethanol. Dependence together with tolerance (the reduced bodily response to ethanol) are cornerstones of alcohol addiction or alcoholism, afflicting tens of millions of people in the U.S. and other countries. Exploring changes to the circadian system in response to chronic ethanol may provide additional clues on how the process of ethanol addiction is remembered by the body and could also provide useful indicators of the severity of addiction, of resilience or susceptibility to relapse.
Together these data suggest that the effects of repeated inductions of ethanol dependence induce differences to circadian rhythms that last for a few days, at most, following the discontinuation of ethanol. Sustained effects would indicate a role of the circadian clock, but transient effects are ambiguous and suggestive that mechanisms of temperature differences are peripheral to the clock. Potentiated effects following cumulative vapor sessions signify the body’s memory and sensitization to the effects of ethanol on some circadian parameters. These enhanced effects occurred despite decreased blood ethanol levels across vapor sessions. Thus, blood ethanol levels signified prolonged tolerance to ethanol vapor sessions that occurred weeks apart while at the same time effects on circadian rhythms during withdrawal showed sensitization. Future studies could integrate behavioral methods such as self-administration of ethanol to investigate possible associations or feedback effects of these variables.
Table 4.1. Experiment timeline. The table overviews the progression of the experiment from surgery, followed by baseline telemetry recording, vapor session 1 and subsequent post-vapor session 1 recording, etc. The numbers under the heading "Telemetry days analyzed" represent the days that were included in various statistical analyses of body temperature and activity amplitude and entrainment.

<table>
<thead>
<tr>
<th>Procedural phase</th>
<th>Age</th>
<th>Telemetry recording</th>
<th>Telemetry days analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>11 wks</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Baseline</td>
<td>20 wks</td>
<td>Baseline recording</td>
<td>1-8</td>
</tr>
<tr>
<td>Vapor session 1</td>
<td>22 wks</td>
<td>Post-vapor 1 recording</td>
<td>1-8</td>
</tr>
<tr>
<td>Vapor session 2</td>
<td>25 wks</td>
<td>Post-vapor 2 recording</td>
<td>1-3, 11-13</td>
</tr>
<tr>
<td>Vapor session 3</td>
<td>31 wks</td>
<td>Post-vapor 3</td>
<td>1-9</td>
</tr>
</tbody>
</table>
Each ethanol session consists of 3 days of ethanol vapor; each day consists of 16 hours of ethanol and 8 hours off. Immediately preceding the start of ethanol exposure each day Experimental mice were injected with ethanol and pyrazole. At the end of each 16 hour vapor bout, blood samples were taken to establish blood ethanol concentrations. Following the final 16 hour vapor bout, mice were transferred into the biotelemetry recording cages and the post-vapor recording session began.
Table 4.2. Results of repeated measures ANOVAs. The table details F values for between subjects effects (Experimental ethanol versus Control air-vapor exposed mice), within subjects effects (differences across days), and the interaction of group with days. Statistically significant values are marked with * and all others are marked as p = n.s.

<table>
<thead>
<tr>
<th></th>
<th>BODY TEMPERATURE</th>
<th>ACTIVITY</th>
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<tbody>
<tr>
<td></td>
<td>Between subjects</td>
<td>Within subjects</td>
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<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amp</td>
<td>F(1,13)=1.5; p=n.s.</td>
<td>F(1.8, 23.3)=3.1; p=n.s.</td>
</tr>
<tr>
<td>onset</td>
<td>F(1,13)=0.02; p=n.s.</td>
<td>F(6.76)=1.6; p&lt;0.001*</td>
</tr>
<tr>
<td>offset</td>
<td>F(1,11)=0.04; p=n.s.</td>
<td>F(6.66)=1.2; p=n.s.</td>
</tr>
<tr>
<td><strong>Post-vapor 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amp</td>
<td>F(1,13)=0.7; p=n.s.</td>
<td>F(4.52)=32.5; p&lt;0.001*</td>
</tr>
<tr>
<td>onset</td>
<td>F(1,13)=1.5; p=n.s.</td>
<td>F(5.65)=0.9; p=n.s.</td>
</tr>
<tr>
<td>offset</td>
<td>F(1,12)=0.4; p=n.s.</td>
<td>F(5.66)=4.0; p&lt;0.01*</td>
</tr>
<tr>
<td><strong>Post-vapor 2</strong></td>
<td></td>
<td></td>
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<tr>
<td>amp</td>
<td>F(1,13)=3.5; p=n.s.</td>
<td>F(2.26)=32.2; p&lt;0.001*</td>
</tr>
<tr>
<td>onset</td>
<td>F(1,13)=4.3; p=n.s.</td>
<td>F(2.26)=0.2; p=n.s.</td>
</tr>
<tr>
<td>offset</td>
<td>F(1,10)=0.1; p=n.s.</td>
<td>F(2.20)=11.9; p&lt;0.01*</td>
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<tr>
<td><strong>Post-vapor 3</strong></td>
<td></td>
<td></td>
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<tr>
<td>amp</td>
<td>F(1,13)=28.2; p&lt;0.001*</td>
<td>F(4.52)=17.3; p&lt;0.001*</td>
</tr>
<tr>
<td>onset</td>
<td>F(1,11)=0.03; p=n.s.</td>
<td>F(7.77)=2.0; p=n.s.</td>
</tr>
<tr>
<td>offset</td>
<td>F(1,11)=0.2; p=n.s.</td>
<td>F(7.77)=3.5; p&lt;0.01*</td>
</tr>
</tbody>
</table>
Figure 4.2 Average daily body temperature profiles for Experimental and Control groups for up to 4 days during each biotelemetry recording session. The Y-axis shows the temperature, and days are across the X-axis. Light dark bars across the bottom of the figure indicate times of lights on and off; the vertical dashed lines separate days. A. Baseline temperature profiles. B. Post-vapor 1 temperature profiles. C. Post-vapor 2 temperature profiles. D. Post-vapor 3 temperature profiles.
Figure 4.3  Body temperature amplitude (in degrees C) for Experimental (ethanol vapor exposed mice) and Control (air vapor exposed mice) groups for up to 5 days during each biotelemetry recording session.  A. Baseline temperature amplitude.  B. Post-vapor 1 amplitude.  C. Post-vapor 2 amplitude.  D. post-vapor 3 amplitude.
Figure 4.4 Body temperature entrainment for Experimental and Control groups for up to 7 days during each biotelemetry recording session (on the X-axis). The hour of the day is indicated on the Y-axis and the dashed horizontal lines at 5 and 17 represent the time of lights on and off, respectively. Entrainment values were temperature rise onset and offset. A. Baseline temperature entrainment. B. Post-vapor 1 temperature entrainment. C. Post-vapor 2 temperature entrainment. D. Post-vapor 3 temperature entrainment.
Figure 4.5 Average daily activity profiles for Experimental and Control groups for up to 4 days during each biotelemetry recording session. Days are across the bottom, the Y-axis shows the average number of counts at a particular time. Light dark bars across the bottom of the figure indicate times of lights on and off; the vertical dashed lines separate days. A. Baseline activity profiles. B. Post-vapor 1 activity profiles. C. Post-vapor 2 activity profiles. D. Post-vapor 3 activity profiles.
Figure 4.6 Activity amplitude (in counts) for Experimental (ethanol vapor exposed mice) and Control (air vapor exposed mice) groups for up to 5 days during each biotelemetry recording session. A. Baseline activity amplitudes. B. Post-vapor 1 activity amplitudes. C. Post-vapor 2 activity amplitudes. D. Post-vapor 3 activity amplitudes.
Figure 4.7  Activity entrainment (time of onset and offset each day) for Experimental and Control groups for up to 7 days during each biotelemetry recording session (on the X-axis). The hour of the day is indicated on the Y-axis and the dashed horizontal lines at 5 and 17 represent the time of lights on and off, respectively.  A. Baseline activity entrainment.  B. Post-vapor 1 entrainment.  C. Post-vapor 2 activity entrainment.  D. Post-vapor 3 activity entrainment.
Chapter 5

Summary and Conclusions

Ultimately, understanding the relationship between circadian rhythms and alcohol intake as interacting systems may be beneficial for practical applications ranging from treatment of alcohol use disorders to understanding the influence of prenatal alcohol exposure on developing circadian rhythms. The lines of research in this dissertation took different approaches to address some of these wide ranging questions about interactions of ethanol and the circadian system, specifically 1) How does circadian scheduling affect subsequent voluntary ethanol intake? 2) Is circadian period causally related to ethanol consumption and preference? 3) Do repeated exposures to chronic ethanol vapor create lasting changes to circadian body temperature and activity rhythms?

5.1 Circadian Scheduling Affects Voluntary Ethanol Consumption

In humans alcohol consumption follows predictable temporal patterns as people tend to drink near the end of the day. The mouse may be a convenient model to
understand the relevance of temporal context for addictive behaviors. Chapter 2 showed how establishing a history of ethanol at preferred or non-preferred times of day can influence voluntary ethanol intake for several subsequent weeks in C57BL/6J mice. Mice were exposed to ethanol at a preferred or non-preferred time of day for several weeks. Under 24 hour exposure conditions, mice with a history of drinking during the preferred, night time, consumed significantly more than mice drinking during the day, showing that these histories can induce long-lasting changes in voluntary ethanol intake in an unstructured, un-timed environment. Understanding how circadian rhythms regulate ethanol consumption may be valuable for modifying subsequent intake.

One explanation for why mice with a history of receiving ethanol at a preferred time of day continued to drink higher levels during 24 hour un-timed access is that these mice learned a highly reinforcing pattern of behavior and reward in the brain. In contrast, mice with a history at a non-preferred time did not experience a history of drinking at a most rewarding time of day. Thus, they drank less less than mice with the opposite history when offered 24-hour ethanol despite the fact that they could now ingest it at more rewarding or preferred times of day. It could be that these mice developed a less rewarding memory of ethanol throughout these initial weeks, and so when ethanol was subsequently offered, it was not as reinforcing. A future study could test such a theory at a more biological level. For instance, mice with a preferred or non-preferred ethanol history could be injected with a fixed ethanol dose and then one could compare levels of dopamine release in the nucleus accumbens with microdialysis.

These effects may have translational relevance for understanding the development and the manipulation of human alcohol intake. Specifically, our results show that
the most recent schedule of alcohol exposure controls subsequent intake in an unrestricted environment. Behavioral methods for reducing alcohol intake in humans might be able to change the highly rewarding effects of alcohol through instituting exposure to alcohol at times of day when it is least preferred in alcohol treatment programs. Over time, as is the goal with behavioral cue exposure methods [Glaudier and Drummond, 1994], the physiological response to alcohol may be changed, reducing the rewarding value of alcohol.

5.2 Circadian Period Does Not Directly Influence on Ethanol Intake

Unlike the clear relationship found of circadian timing on current and subsequent ethanol intake, examining how circadian period might mediate ethanol intake produced no clear effects. Chapter 3 examined the relationship of circadian period to ethanol intake in C57BL/6J, HAP and LAP mice. Whereas prior research has showed an association between circadian period and ethanol preferring mice and rats, experimentally testing this idea did not show an influence of period on ethanol intake.

This was despite robust differences on circadian period in a variety of conditions following T cycles (including ethanol allowed while living in T cycles, in DD following T cycles, and in 24 LD following T cycles). HAP2 and LAP2 mice were also allowed to free run in DD to assess differences in period through general locomotor activity, but there were none. These mice did not show period differences in DD; the association between shorter FRP and high ethanol preference was not reserved in this replicate of HAP2 and LAP2 mice as it was in HAP1 and LAP1 mice. Together these lead to the
conclusion that there is no causal relationship between circadian period and ethanol intake or preference in C57 mice or in HAP2 and LAP2 mice. Using mice with low as well as high preference allowed for high sensitivity to detect potential changes in ethanol consumption and preference. Analyses of ethanol intake across days and weeks of exposure also ruled out the possibility of a transient difference in ethanol intake for all experiments.

HAP and LAP mice were also looked at for differences in activity levels. Like previous studies with HAP and LAP mice, HAP2 showed greater levels of activity compared to LAP2 mice. Mentioned above, there were no observed differences in tau in free running conditions between HAP2 and LAP2 mice though one large distinction between the two studies is that our study used general locomotion to assess circadian period whereas the prior study [Hofstetter et al., 2003] used running wheels. High rates of running wheel activity has shown possible period shortening effects [Koteja et al., 2003], which may have contributed to the previous finding.

T cycle manipulations were successful in HAP2, LAP2 and C57 mice. In addition to observed after-effects on period, T cycles have other effects on the body. The body’s master clock, the SCN is responsible for synchronizing peripheral oscillators in the organs, for example. T cycles may interfere with this process, leading to altered phase relationships between the SCN and peripheral body tissues [Molyneaux et al., 2008]. T cycles have been shown to also affect growth (weight gain in prematurely born rats) and behavioral factors such as food intake in rats [Campuzano et al., 1999]. The clock itself is composed of multiple components, which may also be differentially affected in response to T cycles [Campuzano et al., 1998]. Entrainment to periods shorter or longer than 24 hours have far reaching effects on the circadian system. Thus, while the primary use
of T cycles in the current study is to produce changes to entrained period, they create additional effects on the circadian system and provides a good model for disruption to the natural state of the circadian system. As there were no effects of T cycle entrainment on ethanol intake (as measured in T cycles, in DD or in 24LD following T cycles) our evidence suggests that altering fundamental elements of the circadian system does not affect ethanol intake and preference.

Although experimental manipulations of period did not affect levels of ethanol ingestion or preference, past research has shown that clock genes have a relationship with ethanol intake that is mediated through peripheral changes associated with a clock gene mutation. Mammals have several clock genes including 3 period genes (Per1, 2, and 3) and 2 cryptochrome genes. Their resulting proteins regulate the genes' transcription; this process takes approximately 24 hours and creates a negative feedback loop that is the basis for the endogenous circadian rhythm [Kalsbeek et al., 2006]. A mutant of the Per2 gene in humans (hPer2) and in mice (mPer2) is related to increased alcohol consumption [Spanagel et al., 2005]. This is presumably due to down-regulation of a glutamate transporter, leading to a hyper-glutamatergic state that has previously identified in the etiology of alcohol dependence [Pulvirenti and Diana, 2001, Tsai and Coyle, 1998].

Thus there is evidence of increased ethanol drinking associated with a clock gene mutation but it is mediated through a peripheral mechanism. In addition, phase shifting effects on ethanol intake show unclear effects on ethanol drinking (in high alcohol drinking) rats [Clark et al., 2007]. Combined with our findings that period manipulations did not affect ethanol intake, it appears that fundamental disturbances in the circadian system do not lead to direct, uniform changes in ethanol intake or preference. Although, it
is possible that effects peripheral to these disturbances may influence it. Translationally, our results argue that humans with disturbed circadian systems as a result of endogenous rhythms different from 24 hours or because of travel across time zones (jet lag) do not show direct effects on alcohol ingestion.

5.3 Effects of Ethanol Dependence on Circadian Rhythms

Chapter 4 moved away from circadian manipulations and voluntary ethanol intake to exploring the influence of chronic ethanol dependence and withdrawal on circadian rhythms. Numerous biological rhythms are disrupted during alcohol administration and withdrawal. Humans with a history of alcoholism show particular sleep disruptions which are predictive of relapse [Drummond et al., 1998], and rodents show long term changes to sleep EEG function weeks following ethanol withdrawal [Ehlers and Slawecki, 2000]. Ethanol has also been shown to affect body temperature during ethanol ingestion as well as during withdrawal; however, chronic ethanol effects on circadian body temperature and activity rhythms have not been carefully studied. Thus, we tested for effects of multiple inductions of ethanol dependence on subsequent circadian measures of body temperature and activity rhythms. Results indicate a cumulative effect of vapor exposures on reducing body temperature amplitude during withdrawal. We observed no long term effects of ethanol beyond withdrawal days, suggesting no longer term effects of ethanol vapor on circadian body temperature and activity rhythms in C57 mice.

Ethanol may be affecting the clock during administration, an effect which wears off during withdrawal. There was potentiation of effects on circadian rhythms following cumulative vapor sessions. If ethanol is affecting the clock, its responses are becoming
sensitized to the response. In either case that ethanol is influencing the clock directly or peripheral to the clock, there are not enduring effects on circadian rhythms once ethanol is removed. If prolonged effects were observed, they would have been a strong argument for effects on the clock. Changes only during withdrawal are ambiguous. Other’s research has shown acute ethanol may affect SCN function directly, altering its response to a phase shifting stimulus (Ruby et al., 2009). These effects measured under the influence of ethanol suggest it is at least possible that our results may be due to transient disturbances in clock functioning.

Prior research has found a distal influence of chronic ethanol on entrainment after neonatal ethanol exposure [Allen et al., 2005]. However, we found no differences in entrainment following chronic ethanol exposure, signifying that it did not affect the timing of rhythm phase in our study using adult mice. It might be informative to re-implement this paradigm in DD (constant darkness) to see if chronic ethanol exerts changes to the timing of the rhythm without the influence of light. Further, it would be interesting to explore why ethanol during sensitive developmental periods affects circadian rhythms differently than ethanol in adulthood.

Developmental exposure (prenatal and neonatal) of rodents to ethanol has shown distal effects on phase shifting of circadian rhythms when measured later in early adulthood [Allen et al., 2005, Sei et al., 2003]. Prenatal ethanol exposure in rats also results in distal effects on clock gene expression; Per mRNA expression in the SCN is altered and shifted compared to controls [Chen et al., 2006]. Ethanol exposure during development of circadian rhythms, thus, may have long term effects of circadian function whereas our studies with adult mice do not show sustained impacts of ethanol exposure
on circadian rhythms following withdrawal. While ethanol during development no doubt may play a large role in these effects, research has also found that chronic ethanol can affect long term SCN function in adult rodents [Madeira et al., 1997]. SCN cells showed reduced cell density and weakened mRNA signaling of multiple types of neurons months after withdrawal from chronic ethanol exposure. Chronic ethanol also dampens mRNA rhythms of rPer 1, 2, and 3 in the SCN of the adult rat [Chen et al., 2004].

It remains uncertain why the current study did not find evidence of long term changes to circadian rhythms of body temperature and activity. One possibility is that there were changes to clock function in these mice but they were not powerful enough to cause lasting changes to output rhythms we observed. Alternatively, the temperature differences observed may be peripheral to the clock. That is, they might be caused by withdrawal from ethanol, which has widespread effects on the body. Future studies could use a similar paradigm of repeated withdrawals from ethanol exposure to integrate physiological and behavioral findings with cytochemical or in situ hybridization techniques that look directly at cells and gene expression. Additional behavioral methods i.e., ethanol self-administration post-withdrawal could also be used to investigate possible associations or feedback of these variables on circadian rhythms. Changes to circadian rhythms during withdrawal from ethanol might be useful as an index of withdrawal severity or possibly as a predictor of later voluntary ethanol intake.


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