Roles of the Circadian and Reward Systems in Alcoholism

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LIST OF ABBREVIATIONS

3V  third ventricle
5-HT  5-Hydroxytryptamine, serotonin
5-HT$_{1A}$  serotonin receptor subtype 1A
5-HT$_7$  serotonin receptor subtype 7
5-HTT  5-Hydroxytryptamine (serotonin) transporter protein
ACSF  artificial cerebral spinal fluid
ADX  adrenalectomized
AMPA  $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptor
ANOVA  analysis of variance
AP  anterior or posterior of bregma, stereotaxic coordinate
[(+): anterior; (-): posterior]
Aq  cerebral aqueduct
AVP  arginine vasopressin
BDNF  brain-derived neurotrophic factor
Bmal1  Aryl hydrocarbon receptor nuclear translocator-like (clock) gene
CCK  Cholecystokinin
c-fos  Fos protein expression
CLOCK  Circadian Locomotor Output Cycles Kaput (clock) gene
CPu  caudate putamen
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<tr>
<th>Abbreviation</th>
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<tr>
<td>DD</td>
<td>constant darkness</td>
</tr>
<tr>
<td>DLG</td>
<td>dorsal lateral geniculate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPAT</td>
<td>(+) 8-hydroxy-(N,N)-dipropyl-2-aminotetralin, 5HT(_{1A,7}) agonist</td>
</tr>
<tr>
<td>EAAT-1</td>
<td>glutamate aspartate transporter</td>
</tr>
<tr>
<td>EPSCs</td>
<td>excitatory post-synaptic currents</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>Fig</td>
<td>Figure</td>
</tr>
<tr>
<td>FR</td>
<td>fasciculus retroflexus</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-Aminobutyric acid</td>
</tr>
<tr>
<td>GRP</td>
<td>gastrin-releasing peptide</td>
</tr>
<tr>
<td>H</td>
<td>depth from dura, stereotaxic coordinate</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IGL</td>
<td>intergeniculate leaflet</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out, knockdown</td>
</tr>
<tr>
<td>L</td>
<td>lateral to midline, stereotaxic coordinate [(+): left; (-): right]</td>
</tr>
<tr>
<td>LD</td>
<td>12 hour light-12 hour dark photocycle</td>
</tr>
</tbody>
</table>

xxiv
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDT</td>
<td>laterodorsal tegmentum</td>
</tr>
<tr>
<td>LL</td>
<td>constant light</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ML</td>
<td>medial lemniscus</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>MM</td>
<td>medial mammillary nucleus</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MPON</td>
<td>medial preoptic nucleus</td>
</tr>
<tr>
<td>n</td>
<td>sample size</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens core</td>
</tr>
<tr>
<td>NAs</td>
<td>nucleus accumbens shell</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-aspartate-D receptor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>non-ethanol preferring rodent</td>
</tr>
<tr>
<td>NR1</td>
<td>N-methyl D-aspartate receptor subtype 1</td>
</tr>
<tr>
<td>NR2B</td>
<td>N-methyl D-aspartate receptor subtype 2B</td>
</tr>
<tr>
<td>NR3a</td>
<td>N-methyl D-aspartate receptor subtype 3a</td>
</tr>
<tr>
<td>NR4b</td>
<td>N-methyl D-aspartate receptor subtype 4b</td>
</tr>
<tr>
<td>OC</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomized</td>
</tr>
<tr>
<td>P</td>
<td>ethanol-preferring rodent</td>
</tr>
</tbody>
</table>

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p  measure of statistical significance
P  micropellet implant
PACAP  pituitary adenylate cyclase-activating peptide
Per1  Period circadian protein homolog 1 (clock) gene
Per2 (mPer2)  Period circadian protein homolog 2 (clock) gene
PPT  penduculopontine tegmentum
PRC  phase response curve
PT  probe tract
REM  rapid eye movement
RHT  retinohypothalamic tract
RN  raphe nuclei
RO15-4513  Ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-1,4-benzodiazepine-3-carboxylate, GABA\textsubscript{A}/\textsubscript{delta} antagonist
SCN  Suprachiasmatic Nucleus
SE/SEM  standard error
SNP  single-nucleotide polymorphism
SYS  subcutaneous, systemic
t1/2 elimination  time of half the maximum plasma concentration of drug
Tmax  time of maximum plasma concentration of drug
UV  ultraviolet
VIP  vasoactive intestinal polypeptide
VTA  ventral tegmental area
WT  wild-type

ZT  zeitgeber time

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ACKNOWLEDGEMENTS

There are numerous individuals in my professional, professional/personal, and familial life that I would like to thank for their invaluable financial and academic support, training, teaching, critiquing, optimism, and patience during my post-baccalaureate education.

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Justin Montemarano: Justin, you’ve been a constant friend and reassurance in my life. When I have a bad experiment or day in the lab, I can always rely on you for moral and professional support. I am grateful to be with someone who can hold his own in his professional life, even if it means going abroad for a few months. I mean, how many couples can admit that their domestic disputes solely reside over recent science and political news?

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**Cassidy McKee:** Cassidy, thank you for being the best friend (personally and professionally) one could ever ask. I am grateful for our weekly phone conversations, most of time which attempt to decompress from the stress of graduate and business school, annual Macedonian convention shenanigans, and the little time I did get to spend with you in Rhode Island, Connecticut, and Charlottesville during graduate school. I can’t wait to see how our futures unfold.

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**Mom, Dad, and Peter:** Thank you for your countless financial and moral support throughout my education. Mom and Dad, words cannot describe how appreciative I am of what you have done for me with the little that we have. Peter, thank you for reminding me that there is more to life than work.
DEDICATION

I’d like to dedicate this dissertation to my former undergraduate advisor, Dr. Mary A. Carskadon. Mary, you spurred (and held) my interest in sleep and circadian rhythms research, and you have invested countless hours into my research training and professional development, all of which I am grateful for. You’ve taught me the importance of conducting honest and accurate science, of investing in the training of future generations, and of fighting gender biases in research.
CHAPTER I
INTRODUCTION

Biological and Circadian Rhythms

The rotation of the Earth on its tilted axis over a 24 hour period drives many rhythmic geophysical phenomena, such as the timing of sunrise and sunset, which establishes fixed photoperiods across 24 hours, and oceanic tides, which alter coastal landscapes and water depths. These rhythmic phenomena, present in all biomes, are characterized as biological rhythms (Aschoff, 1981). Biological rhythms, in turn, drive organism behavior and physiology, and largely influence the likelihood of survival (Aschoff, 1981). A first-hand account of biological rhythms and their influences on organism behavior and physiology has been documented at the Alberto Manuel Brenes Biological Reserve in the neotropical cloud forest of Costa Rica:

Costa Rica’s near equatorial location establishes a relatively constant, alternating schedule of twelve hours of light and dark within a 24 hour period. During light hours, the mating, feeding, and social behaviors of diurnal avian and new world primate species, including the Scarlet Macaw (*Ara macao*), the Cherrie’s Tanager (*Ramphocelus costaricensis*), and howler monkeys (*Alouatta*), are apparent. Near sunset, frugivorous and insectivorous bats (*Pteropodidae*) leave the roost in search of food. Once darkness arrives,
nocturnal crustacean, amphibian, and reptilian species of several folds, including the Hermit Crab (*Paguroidea*), Marine Toad (*Bufo marinus*), and Northern Cat-eyed Snake (*Leptodiera rubricata*) emerge from their cavernous abodes. Meanwhile, the waxing and waning of semidiurnal tides reveal and conceal tidal pools across this 24 hour period, exposing limpets (*Gastropoda*) and damselfish (*Pomacentridae*) that inhabit these intertidal zones to predation by birds, including the Mangrove Black Hawk (*Buteogallus anthracinus subtilis*) during the day. Bioluminescent dinoflagellates (*Dinoflagellata*) illuminate these same tropical waters at night. As both lux intensities and intertidal zone depths rhythmically change across this 24 hour period, the ambient, near deafening acoustics produced from the mating and alerting calls of insects varies as well such as that of male cicadas (*Cicadidae*) across the day, click beetles (*Elateridae*) across the night. Daily rainfall in the afternoon sends grazing animals in search of shelter (Brager 10 April 2009).

In addition to the physical forces that drive photoperiodic, tidal, and meteorologic biological rhythms and subsequent organism behavior and physiology, including sleep/wake, metabolism, and hormone release (reviewed in Pittendrigh, 1981; Dunlap, 1999), organism behavior and physiology are concomitantly influenced by endogenously generated rhythms known as circadian rhythms. The etymology of circadian rhythm manifests from its functional properties of having a rhythm period near, but not exactly 24 hours (Aschoff, 1981). The rhythm period is also species-
specific; the human (*Homo sapiens*) circadian rhythm is ~24.80 hours (Mills, 1964), and the mouse (*Mus musculus*) rhythm is ~23.50 hours (Edgar et al. 1991).

This endogenously driven rhythm, particularly in mammals, can be modulated by photic and nonphotic stimuli from the environment, including light (reviewed in Gillette and Abbott, 2006), food (reviewed in Mistlberger, 2009), mating (Honrado and Mrosovsky, 1989), exercise (Reebs and Mrosovsky, 1989), and pharmacological agents (reviewed in Mistlberger et al. 2000). When these environmental stimuli are presented at an unanticipated time of day, they can change the endogenous period and amplitude of circadian rhythms and subsequent organism behavior and physiology. Routine and time-specific presentations of these environmental stimuli can additionally establish rhythmic entrainment of organism behavior and physiology. Of the many environmental stimuli that drive circadian rhythms, the most potent and universal modulating stimulus is light (Aschoff, 1981). For example, in the presence of a constant, alternating schedule of twelve hours of light and dark (standard laboratory and equatorial photoperiods), the endogenous circadian rhythm is “masked”, causing the period of rhythmic behavior and physiology to be exactly 24.00 hours (Aschoff, 1981). In the absence of a standard laboratory photoperiod and comparable to constant periods of light and darkness experienced during solstices in polar climates (≥ 90°), the endogenous circadian rhythm becomes unmasked (known as “free running”), and the period of overt rhythmic behavior and physiology nears that of the organism’s species-specific endogenous circadian rhythm (Aschoff, 1981). Alternating schedules of light and
dark additionally cue diurnal and nocturnal organisms to be active, respectively (Menaker et al. 1997). Other biological rhythms such as temperature, tides, and seasonality, which affect food availability, fecundity, and predation, can cause organisms to adjust this rhythmic activity accordingly (Hill, 2006).

The adaptive significances of robust rhythms of behavior and physiology are several folds, and include protection, physiological homeostasis, and survival. Evidence for these adaptive significances derives from lesioning and epidemiological studies. For example: 1) Ablation of the brain area mediating circadian rhythmicity in ground squirrels results in a loss of consolidated sleep/wake, and subsequently, increased susceptibility to predation (DeCoursey et al. 1997); 2) Hamsters transfected with mutant genes that stabilize endogenous circadian rhythms have disruptive cardiovascular and renal function and increased mortality (Martino et al., 2008); 3) Exposure to harsh photoperiodic environments in humans, such as that characteristic of rotating shift work and residence in polar latitudes, increases predispositions to sleep and circadian rhythm disorders, including insomnia and advanced/delayed sleep phase syndrome, ulcers, obesity, drug dependence, and cancer (Trinkoff and Storr, 1998; Levine et al., 1994; Spanagel et al., 2005; Sack et al., 2007; Thiele et al., 2003; Davis and Mirick, 2006); and 4) Risks of drug dependence and addiction are similarly potentiated by polymorphisms of circadian clock genes (Spanagel et al., 2005; Brager et al. 2011b,c, in press; McClung et al., 2005, 2007; Ozbourn et al., 2010).
Anatomy of the Mammalian Suprachiasmatic Nucleus

Endogenous regulation of mammalian circadian rhythms arises from the bilateral suprachiasmatic nuclei (SCN) of the anterior hypothalamus. Neuroanatomically, these nuclei rest at the base of the third ventricle and dorsal to the optic chiasm. Each nucleus is comprised of ~10,000 to 12,000 neurons (Moore and Speh, 1993; Van den Pol, 1980), most of which are GABA-expressing neurons (Card and Moore, 1984; Moore and Speh, 1993). In vitro studies have determined that the intensity of neuronal activity within the mammalian SCN pacemaker varies across an entraining 24 hr light/dark schedule (LD), with neuronal activity peaking at midday (Prosser and Gillette, 1991). Critical molecular components of the SCN acting as positive and negative transcription factors, including Bmal1/CLOCK and Per1/2, respectively, influence the endogenous rhythm period, amplitude, and subsequent rhythmic behavioral, hormonal, and metabolic output (Dunlap, 1999). Developmentally, the mammalian SCN arises from the ventral diencephalic germinal epithelium (Moore and Leak, 2001). Throughout embryonic and neonatal development, neurons, synapses, projections, and arising behavioral and physiological outputs mature at different time points. In rodent models, neuron generation and subsequent synaptic connections within the SCN develop on embryonic days 15-16, and continue to increase in volume well after birth. Afferent and efferent projections to and from the SCN develop near birth. On post-natal days 5-10, an established rhythm of sleep/wake, core body temperature, and melatonin appears (reviewed in Moore and Leak, 2001). Evidence supporting that mammalian
circadian rhythms are generated within the SCN has been presented in lesion and transplantation studies; ablating the SCN eradicates corticosterone rhythms (Moore and Eichler, 1972) and the consolidation of sleep/wake (Edgar et al. 1993), while transplanting fetal SCN cells into a previously ablated SCN restores this physiologic rhythmicity (Meyer-Bernstein et al., 1999).

The two major subdivisions of the SCN are the core and shell, which are found in the ventrolateral and dorsomedial region of the SCN, respectively (reviewed in Karatsoreos and Silver, 2007). Cells in the core are less compacted and have larger dendritic arborizations than the more-dense cells in the shell. These differences in cell-types contribute to the differential functions of each subdivision (reviewed in Moore and Leak, 2001). The core, lying directly above the optic chiasm, receives photic input from the retina and integrates photic information within the SCN through glutamatergic and neuropeptidergic signaling (reviewed in Gillette and Abbott, 2006; Karatsoreos and Silver, 2007). The core produces and expresses vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), peptide histidine isoleucine, and neurotensin, which assist in the transmission of photic information and photic phase-resetting of rhythms (Francl et al. 2010a,b; Kallingal et al. 2007; Card et al., 1981, 1988; Mikkelsen et al., 1991). The shell mediates neuroendocrine and autonomic output from the SCN (reviewed in Karatsoreos and Silver, 2007), and expresses arginine vasopression (AVP) and angiotensin II in medial regions and enkephalin and calretinin-immunoreactive neurons in lateral regions (Jacobowitz and Winsky, 1991; Watts et al., 1987; Moore and Leak, 2001).
The core-shell border of the SCN additionally expresses somatostatin and substance P-immunoreactive cells (Mikkelsen and Larsen, 1993; Card et al., 1988). Neuropeptide Y is heterogeneously expressed throughout the SCN, and is critically involved in the regulation of photic and nonphotic phase-resetting of circadian rhythms (Glass et al. 2010; Biello et al. 1994; 1997; reviewed in Moore and Leak, 2001). Studies have found direct projections from the core to the shell, in addition to other hypothalamic projections from each subdivision (reviewed in Moore and Leak, 2001).

**Photic and Nonphotic Signaling within the Mammalian SCN**

As mentioned previously, integration of environmental photic and nonphotic stimuli within the mammalian SCN influences entrainment and/or phase-resetting of the circadian rhythms. The retinohypothalamic tract (RHT), projecting from the retina to the SCN, integrates photic stimuli, including information about its duration and intensity, by means of glutamatergic and pituitary adenylate cyclase-activating peptide (PACAP)-ergic activity (reviewed in Gillette and Abbott, 2006). Lesioning of the RHT and subsequent loss of glutamate signaling to the SCN impairs entrainment to light (Johnson et al., 1988). SCN responsiveness to photic stimuli varies across the 24 hour period in that the presence of photic stimuli during a time of minimal light exposure (i.e. night) can phase-reset circadian rhythms; brief, intermittent presentations of light in the early and late subjective night (active period of nocturnal
animals) phase-delay and advance rhythmic behavior and physiology, respectively (reviewed in Gillette and Abbott, 2006). The presentation of light during any other circadian time has no effect on the phase-resetting of circadian rhythms. Thus, circadian variation in SCN responsiveness to light is characterized in a photic phase response curve (Pittendrigh and Daan, 1976). Applications of glutamate to the SCN slice in the early subjective night in vitro also phase-reset the time of peak SCN neuronal activity, reinforcing that glutamatergic integration within the SCN is critically important for the entrainment of circadian rhythms (Ding et al., 1994).

Nonphotic stimuli, including exercise (Reebs and Mrosovsky, 1989), mating (Honrado and Mrosovsky, 1989), food (reviewed in Mistlberger, 2009), and pharmacological agents (reviewed in Mistlberger et al., 2000) are integrated within the SCN pacemaker via serotonergic projections arising from the raphe nuclei (RN) of the midbrain and NPY-ergic projections arising from the intergeniculate leaflet (IGL) of the thalamus (Dudley et al., 1999; Glass et al., 2003; Glass et al., 2010). RN projections onto the SCN via the IGL additionally influence the integration of nonphotic stimuli within the SCN (Grossman et al., 2004). Recently, a cholinergic pathway, characterized as the “mesopontine cholinergic arousal system,” projecting from the peduculopontine tegmentum (PPT) of the pons onto the SCN via the IGL, has been localized (Vrang et al., 2003; Cain et al., 2004). This arousal system is believed to integrate rewarding and aversive stimuli within the SCN (Cain et al., 2004), suggesting that this system, thus, may possibly influence the underlying neurobiology of drug dependence and abuse. Similar to photic stimuli, the
presentation of nonphotic stimuli, which is typically rewarding, has been shown to elicit phase-resetting of behavioral circadian rhythms at various circadian time points. Nonphotic stimuli elicit phase-advances of behavioral circadian rhythms during the middle of the subjective day—a circadian time in which a nocturnal animal is minimally active—and causes small, phase-delays of behavioral rhythms towards the end of a nocturnal animal’s active period in the late subjective night (reviewed in Mistlberger et al., 2000). Evidence suggesting that serotonergic signaling is necessary for SCN integration of nonphotic stimuli derives from studies showing that: 1) in vitro application of serotonin to the SCN slice phase-resets the time of peak SCN neuronal activity in a dose-dependent manner (Prosser, 2003); and 2) forced wheel running at midday stimulates endogenous release of serotonin in the SCN in a phase-dependent manner (Dudley et al., 1998). GABA neuromodulatory systems additionally mediate photic and nonphotic phase-resetting of behavioral circadian rhythms. The GABA_A agonist muscimol has been shown to attenuate photic phase-resetting in both diurnal and nocturnal rodents (Novak and Albers, 2004; Gillespie et al., 1997). Muscimol has similarly been found to modulate serotonin release and exercise-induced nonphotic phase-resetting within the SCN by decreasing serotonin release within the SCN via the RN (Glass et al., 2003). In vivo and in vitro antagonism of extrasynaptic GABA_Aδ receptors can also modulate photic (glutamatergic) phase-resetting of behavioral rhythms (Ehlen and Paul, 2009; McElroy et al., 2009). Moreover, altering glutamatergic, serotonergic, and
GABAergic neuromodulatory systems and subsequent release of these neurotransmitters onto the SCN has marked effects on the functioning of the circadian timing system.

**Chronobiological Regulation of Alcohol Intake**

Using rodent models, it has been documented that ethanol consumption is influenced by several environmental, physiologic, and pharmacological factors that either rescue or exacerbate ethanol intake. Under LD, the rat (*Rattus norvegicus*), mouse (*Mus musculus*), and Syrian hamster (*Mesocricetus auratus*) show a behavioral circadian pattern of ethanol drinking and ethanol detection within the SCN. In these animals, ethanol intake is greatest during the beginning and end of the animal’s active period (Ruby et al., 2009b; Gauvin et al., 1997; El-Guebaly, 1987; Brager et al., 2010; Brager et al., 2011b, in press). Under constant light and darkness, in which there is a loss entrained behavioral circadian rhythms, rats and mice have been shown to consume more ethanol (Brager et al., in preparation; Gauvin et al., 1997; El-Guebaly et, 1987). Phase-advancing of behavioral circadian rhythms and brief presentations of ethanol during the subjective day that result in acute sleep deprivation also augment ethanol intake (Gauvin et al., 1997; Choi et al., 1990). Withdrawal from chronic ethanol intake and subsequent re-introduction of ethanol additionally potentiate ethanol intake (Spanagel et al., 1996). This
phenomenon has been characterized as an alcohol (ethanol) deprivation effect and is a predictive measure of relapse (Spanagel et al., 1996).

The method of ethanol administration can also affect ethanol intake. Rodent models have been shown to consume more ethanol under forced intake (ethanol alone) versus free-choice (ethanol or water) and if the ethanol is contained within a liquid-sucrose diet versus in accompaniment with food pellets (Freund et al., 1970; Reidelberger et al., 1996). Pairing ethanol with another highly addictive drug, such as morphine, similarly increases ethanol intake, while administrations of the glutamate antagonist and anti-relapse drug, acamprosate, can reduce ethanol intake (Spanagel et al., 1996; Kulkosky et al., 1991). Exogenous administration of melatonin, which has an endogenous circadian pattern of release (Arendt, 1998), can increase ethanol intake (El-Guebaly, 1987, Smith et al., 1980; Geller et al., 1971). Circulating levels of estrogen and corticosterone, which also have an endogenous circadian pattern of release, have been shown to modulate ethanol intake; relative to intact females, both ovariectomized and adrenalectomized females consume more ethanol (Forger and Morin, 1982). A schematic illustrating chronobiological regulation of ethanol intake is presented in Fig. 1.

In humans, environmental and physiologic disturbances similarly potentiate alcohol use, thereby increasing risks of alcoholism. Reports of alcohol use are prominent in working populations of rotating shift workers and residents of polar latitudes where there is potential long-term exposure to constant dark/photic conditions and subsequent disruptions of sleep/wake homeostasis and circadian
physiology (Trinkoff and Storr, 1998; Levine et al., 1994). Polymorphisms of the molecular clock genes *Per2* and *CLOCK*, which are implicated in advanced sleep phase syndrome and other sleep/circadian rhythm disorders, have also been linked to alcohol craving and dependence (Spanagel et al., 2005; Brager et al. 2011b,c, in press; Sjöholm et al., 2010).

**Figure 1:** Concept map depicts many environmental, physiological, and pharmacological factors that mediate the chronobiology of ethanol intake in rodent models, including photoperiod, the circadian time and method of ethanol administration, species, strain, and exogenous hormone and pharmacological injections. Ordinals within the concept map correspond to respective references presented on the left. MORE or LESS within the concept map denotes comparison between two or more treatment groups.
Ethanol Disruption of Brain Systems Related to Circadian Timing

Chronic and acute ethanol intake and subsequent withdrawal markedly disrupt glutamatergic, serotonergic, GABA-ergic, and dopaminergic brain systems, all of which regulate functioning of the circadian pacemaker and subsequent behavioral and physiological outputs. Notably, ethanol blocks glutamate release at the highly ethanol-sensitive NR2B subunit of the N-methyl-aspartate-D (NMDA) receptor, which mediates photic phase-resetting of the SCN circadian clock (Krystal et al., 2003; Dodd et al., 2000; Wang et al., 2008; Mintz et al., 1999). Ethanol has relatively lower binding affinities for the NR1 and NR3a/4b subunits of NMDA receptors, but, nevertheless, blocks glutamate release (Krystal et al., 2003). Ethanol-induced inhibition of glutamate release, particularly under chronic drinking conditions, subsequently increases NMDA receptor density and calcium influxes (Dodd et al., 2000; Snell et al., 1996). This successively potentiates glutamate release during ethanol withdrawal and facilitates physiological manifestations of withdrawal including seizures, cognitive impairments, and necrosis (Floyd et al., 2003; Krystal et al., 2003). Increased glutamate release, NMDA receptor density, and calcium influx related to chronic ethanol drinking additionally increases relapse risk by sensitizing organisms to the rewarding and euphoric properties of ethanol and reducing anxiety during ethanol intoxication (Krystal et al., 2003).

The characterization of glutamatergic activity following acute ethanol administration reveals that potentiated glutamate release associated with withdrawal
commences 3 hours and peaks 12 hours post-administration (Rosetti and Carboni, 1995). Upregulation of a metabotropic glutamate receptor, mGluR5, in the nucleus accumbens of the striatum has additionally been shown to be associated with binge drinking in the rat (Besheer et al., 2009). Similarly, mice with homozygous deletions of Per2 have hyperglutamatergic states that potentiate ethanol intake and preference relative to matched wild-types. (Spanagel et al., 2005). Studies elucidating ethanol’s direct effects on glutamatergic regulation of circadian timing show that in vitro application of ethanol to the SCN slice attenuates glutamatergic phase-resetting of the peak time of SCN neuronal activity in a dose-dependent manner (Prosser et al., 2008). Similarly, in vivo acute and chronic ethanol intake in the Syrian hamster and mouse attenuates photic phase-resetting of behavioral circadian rhythms, which is regulated by glutamatergic signaling (Ruby et al., 2009a, b; Brager et al. 2010, 2011a, in press).

Acute and chronic ethanol intake have differential effects on serotonergic tonus; acute ethanol intake augments serotonergic tonus by increasing the retention of serotonin in synapses via reducing reuptake transporter activity, while chronic ethanol intake downregulates serotonin expression (reviewed in Rosenwasser et al., 2001). In vitro application of ethanol to the SCN slice attenuates serotonergic phase-resetting in a dose-dependent manner, while in vivo, an acute, systemic ethanol administration attenuates nonphotic serotonergic phase-resetting of behavioral circadian rhythms in the Syrian hamster. These studies recapitulate ethanol’s direct,
disruptive effects on the SCN circadian pacemaker (Prosser et al., 2009; Ruby et al., 2009a).

Likewise, ethanol alteration of GABA neuromodulatory systems across central nervous system is broad, particularly at GABA_A receptors; ethanol alters chloride ion flux and activates intracellular pathways at GABA_A receptors, which amplifies ethanol actions (Aguayo et al., 2002). Ethanol has the highest binding affinities for the α,β,δ subunits of the GABA_A receptor. These subunits are highly expressed in extrasynaptic areas of the hippocampus, thalamus, and cerebellum (Winsky-Sommerer, 2009). Recently, RO15-4513, a GABA_Aδ receptor antagonist, has been found to reverse ethanol’s attenuating effects on glutamatergic, but not serotonergic phase-resetting of the SCN circadian clock in vitro (Prosser et al., 2009). This indicates that ethanol may disrupt functioning of the mammalian pacemaker through the activation of extrasynaptic GABA_A receptors.

Ethanol additionally amplifies dopaminergic signaling from the ventral tegmental area (VTA) of the pons to the nucleus accumbens via disinhibition of inhibitory medium spiny neurons (Koob and Nestler, 1997). The VTA, nucleus accumbens, and dopaminergic projections to and from these areas are the primary brain centers integrating drug (ethanol) reward (Wise et al., 1996, 1998). Recently, the SCN has been shown to project to this central reward circuit via the medial preoptic nucleus (MPON; Luo and Aston-Jones, 2009). Electrical stimulation of the VTA has additionally been shown to phase-reset behavioral circadian rhythms (Guinn et al., 2010).
Other brain sites projecting directly to the SCN circadian pacemaker that are affected by ethanol include the midbrain raphe nuclei (MRN) and arcuate nucleus. Functioning of the MRN, which is shown to stimulate serotonin release within the SCN (Dudley et al., 1999; Glass et al., 2003), is altered by ethanol withdrawal and subsequently increases anxiety (Knapp et al., 2009). Ethanol absorption in the arcuate nucleus downregulates the circadian expression of peripheral molecular clock genes. Over time, this downregulation alters rhythmic oscillations of the molecular clock genes in the master SCN pacemaker (Chen et al., 2004). Recently, it has been found that disruptions to these clock genes contribute to drug dependence. In human models, polymorphisms of the positive transcription factor CLOCK are linked to alcohol abuse disorders (Sjöholm et al., 2010). Mice with CLOCK mutations similarly show potentiated ethanol intake and preference relative to matched wild-type controls (Ozbourn et al., 2010). Mice with mutations or homozygous deletions of the negative transcription factor, Per2, also have potentiated ethanol intake and preference relative to matched wild-type controls (Spanagel et al., 2005; Brager et al. 2011b,c, in press). In Fig. 2, a schematic illustrating SCN projections to and from brain centers that are affected by ethanol is presented.
Figure 2: Schematic illustrates that the circadian timing system located in the suprachiasmatic nucleus (SCN) is closely and reciprocally tied to brain areas mediating alcohol abuse and reward. The rewarding properties of alcohol are mediated by indirect clock projections to and from the SCN and the central reward circuit of the ventral tegmental area (VTA) of the pons and the nucleus accumbens (NA) of the forebrain via the medial preoptic nucleus (MPON) and the pedunculopontine tegmentum (PPT) and laterodorsal tegmentum (LDT) of the pons, respectively. The raphe nuclei (RAPHE) of the midbrain, shown to directly project to the SCN, mediates anxiety associated with withdrawal, while alcohol-induced disruptions of peripheral circadian clocks within the arcuate nucleus (ARCUATE) of the hypothalamus perturb clock timing within the master circadian clock of the SCN.
Limitations of Pharmaceutical Drugs Available for the Treatment of Alcoholism

Alcohol is the most abused substance in the world (World Health Organization Database). The United States spends $166 billion dollars annually on alcohol treatment and education programs (Substance Abuse and Mental Health Services Administration Database). Within most alcohol treatment programs, pharmaceutical drugs are available to curb craving and prevent relapse. Antibuse®, which blocks the metabolism of ethanol to acetaldehyde and makes alcohol consumers nauseous, is one treatment option. The use of Antibuse® is limited to individuals who want to reduce their drinking, by means of heightening aversion to alcohol, and cannot be used for recovering alcoholics. Additionally, it does not target areas of the central nervous system that are neurochemically altered by ethanol and subsequently drive alcohol craving and addiction. Currently, there are two anti-relapse, pharmaceutical drugs, naltrexone and acamprosate, that act directly within the central nervous system. Naltrexone is a µ-opioid receptor antagonist with moderate efficacy in preventing relapse in abstinent alcoholics (reviewed in Gorwood et al., 2006). It primarily blocks the euphoria experienced during alcohol use and that which is severely craved by abstinent alcoholics (Volpicelli et al., 1995; King et al., 1997). However, the potential for naltrexone to globally treat alcohol abuse is limited because its efficacy varies widely among Caucasian and Asian populations; naltrexone is most efficacious in Asians because this population has a
higher percentage of a particular genetic polymorphism of the opioid \( \mu_1 \) receptor (Oslin et al., 2003; Ramoz et al. 2006).

Aside from naltrexone, acamprosate is a glutamate antagonist and GABA\(_A\) agonist with similar pharmacological properties to ethanol. In animal studies, acamprosate effectively reduces ethanol intake, preference, cue-induced ethanol seeking, and the severity of ethanol withdrawal (Blednov et al., 2008; Bachteler et al., 2005; Gupta et al. 2008; Brager et al. in press). Acamprosate’s potential in fully treating alcohol abuse is illustrated through its broad effects on neuromodulatory systems that drive addiction, including its interactions with ionotrophic and metabotropic glutamate receptors, voltage-gated calcium receptor channels, inhibitory medium spiny neurons on dopaminergic reward centers, and brain sites with serotonergic signaling (Spanagel and Zieglgänsberger, 1997; Littleton, 1995; Bäckström et al., 2004; reviewed in Gorwood et al., 2006). Additional studies have found that acamprosate improves sleep consolidation and sleep architectural transitions across the night, both of which are under circadian control (Kolla et al. 2010). This suggests possible interactions between acamprosate and sleep homeostat and circadian timing systems to suppress drinking and prevent relapse. In humans, acamprosate’s efficacy in reducing drinking and preventing relapse is moderate in that one out of every 7.5 abstinent alcoholics treated with acamprosate does not relapse (Mann et al., 2004). Though no racial population disparities in acamprosate’s efficacy have been identified, it has been observed that little acamprosate crosses biological tissues (reviewed in Zornoza et al. 2003).
Specific Aims and Hypotheses

The following specific aims were designed to investigate:

1) Ethanol disruption of photic and nonphotic (serotonergic) behavioral circadian
entrainment

2) Environmental modulation of ethanol intake and craving

3) Neurobehavioral actions of acamprosate suppression of ethanol intake and
craving

4) Ethanol-specific disruption to behavioral circadian entrainment

Ethanol Disruption of Photic and Nonphotic (Serotonergic) Behavioral

Circadian Entrainment

1) The characterization of ethanol pharmacokinetics within the SCN pacemaker and
systemic tissues under chronic and acute ethanol intake (Experiments 1 and 5).

Hypotheses: Chronic. Mice will demonstrate a robust circadian rhythm of ethanol
intake with high levels of ethanol detected in hypothalamic and systemic tissues
across the nighttime activity period. Acute. Ethanol concentrations detected in
hypothalamic tissue will be dose-dependent and peak 20-40 min post ethanol
administration.

2) The effects of chronic and acute ethanol intake on photic and serotonergic phase-
resetting of behavioral circadian rhythms (Experiments 2-4; 6-7)
**Hypothesis:** *Chronic.* Chronic ethanol intake will dose-dependently attenuate photic and serotonergic phase-resetting of behavioral circadian rhythms. *Acute.* An acute administration of ethanol will dose-dependently attenuate photic phase-resetting and accentuate serotonergic phase-resetting of behavioral circadian rhythms.

3) SCN regulation of ethanol’s disruptive effects on photic and nonphotic entrainment (Experiments 8-9).

**Hypothesis:** SCN perfusion of ethanol will attenuate photic phase-resetting of behavioral circadian rhythms.

4) Ethanol perturbation of neuronal activity within the SCN and projecting reward areas (Experiment 10).

**Hypotheses:** An acute administration of ethanol will not affect basal SCN neuronal activity, but will affect basal mesolimbic neuronal activity.

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**Environmental Modulation of Ethanol Intake and Craving**

1) The characterization of ethanol craving and ethanol relapse potential in C57BL/6J mice (Experiment 11).

**Hypothesis:** Mice will show high ethanol relapse potential in that levels of ethanol intake and preference will be elevated during an ethanol re-introductory period compared to initial exposure.

2) The stimulatory effects of long-term constant light exposure on ethanol intake and craving (Experiment 12).
**Hypothesis:** Constant light exposure will elevate ethanol intake and preference compared to that found under a light-dark photocycle.

**Neurobehavioral Actions of Acamprosate Suppression of Ethanol Intake and Craving**

1) The suppressive effects of a daily regimen of systemic acamprosate on ethanol intake, craving, chronopharmacokinetics, and circadian activity (Experiment 14).

**Hypothesis:** A daily regimen of i.p. acamprosate treatment will reduce ethanol intake and craving and have minimal effects of circadian locomotor and drinking rhythms.

2) The exploration of circadian and reward brain area responses to acamprosate suppression of ethanol intake and craving and modulation of circadian behavior (Experiment 15).

**Hypothesis:** Acamprosate will act in reward and circadian brain areas to suppress ethanol intake and preference and have minimal effects on circadian behavior.

3) The influences of homozygous deletions of the Per2 clock gene on ethanol intake, craving, and acamprosate’s suppressive actions (Experiment 13-15).

**Hypothesis:** At baseline and across acamprosate treatment, levels of ethanol intake and preference will remain elevated in mPer2 mutants compared to wild-types.
Ethanol-Specific Disruption to Behavioral Circadian Entrainment

1) The effects of an acute cocaine administration on photic and serotonergic phase-resetting of behavioral circadian rhythms (Experiments 16-17).

**Hypotheses:** An acute administration of cocaine will attenuate photic phase-resetting and behave as a nonphotic phase-resetting stimulus.

2) SCN regulation of cocaine’s nonphotic phase-resetting properties (Experiment 18).

**Hypothesis:** The nonphotic phase-resetting properties of cocaine are regulated by the SCN circadian clock.

3) The residual effects of an acute cocaine administration on behavioral circadian rhythms (Experiment 19).

**Hypothesis:** Acute cocaine perturbs the temporal structure of circadian activity across the daytime rest and nighttime active periods.
CHAPTER II

Materials and Methods

Animals

In experiments 1-12, male mice (*Mus musculus*) bred on a C57BL/6J background were used (The Jackson Laboratory, Bar Harbor, ME). Male mice homozygous for *Per2* gene deletions (PER2-mutant) and wild-type controls raised from breeder pairs backcrossed to a C57BL background strain (The Jackson Laboratory, Bar Harbor, ME) were used in experiments 13-19. Females were excluded from all experiments due to unstable daily EtOH intake and preference that are possibly due to estrous interactions (see Fig. 3; Forger and Morin, 1982) All mice had aged 8-10 weeks at the time of experimentation. Animals were housed under a 12L:12D photocycle (270 lux; LD) in a temperature-controlled vivarium (23°C). Food (Prolab 3000, PMI Feeds, St. Louis, MO) was provided *ad libitum*. All experiments were performed using the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.*
Figure 3: Wild-type C57 albino male and female mice (n=8, each) were presented with free-choice of a 15% EtOH solution (vol/vol) or tap water under LD. Preference for EtOH (ml daily EtOH/ml daily fluid) was measured at the beginning of the dark-phase. Across eight days of EtOH treatment, daily EtOH preference remained stable in male mice, averaging 45.0±0.7%, while EtOH preference in female was unstable and significantly greater, averaging 65.1±0.8 % ($F_{(1, 14)}=12.70; p<0.01$). Most notably, female mice exhibited a repetitive 4-day cycle of EtOH preference that is possibly estrous-dependent. Means±SE are shown.
Behavioral Analyses

General Circadian Locomotor Activity

General circadian locomotor activity was measured using infrared motion detectors interfaced to a computerized data acquisition system (Clocklab; Coulbourn Instruments, Whitehall, PA). The parameters analyzed included the circadian times of nighttime activity onset and offset and the lengths of the nighttime activity period (alpha) and rhythm periods (tau) under LD or constant darkness/light (DD; LL). Data were collected in 1 min. Activity onset was characterized by an initial period of nighttime activity that: 1) exceeded 10% of the maximum rate for the day; 2) was preceded by at least 4 hr of inactivity; and 3) was followed by at least 60 min of sustained activity. Activity offset was defined as a final period of nighttime activity that: 1) was preceded by at least 60 min of sustained activity and; 2) was followed by at least 4 hr of inactivity. Tau was calculated using a least-squares regression line through a minimum of 7 daily activity onsets. Alpha was the duration of activity between activity onset and offset. Under DD, activity onset was the reference point for the beginning of the subjective night (designated as circadian time [CT] 12).
Phase-Resetting of Behavioral Activity Rhythms

A phase-shift represented the difference between the projected times of activity onsets before and after the administration of a photic or nonphotic treatment as determined by: 1) back extrapolation of the least squares line through activity onsets on days 3-10 after treatment; and 2) extrapolation of the least squares line calculated from activity onset data collected for a minimum of 7 days prior to treatment (see Pittendrigh and Daan, 1976).

Temporal Structure of Circadian Locomotor Activity

An activity bout was defined as being > than/to the mean size of all locomotor activity blocks (separated by at least 10 min of inactivity) averaged separately over the active and rest periods for each animal. Activity bouts for the active period were quantified across alpha. Activity bouts across the rest period were quantified across the subjective day (beta). The number and duration of active and rest period bouts were averaged separately over 3 consecutive day periods of treatment. Frequencies of activity bouts ≤ 60 or > 60 min in duration across the active and rest periods were additionally quantified over 3 consecutive day periods of treatment.
Drinking Analyses

Fluid Intake

Daily intake of a 10% or 15% EtOH solution or tap water alone was measured during the dark phase using plastic graduated 50 ml tubes (Fisher Scientific, Pittsburgh, PA). Daily fluid intake was estimated to the nearest 0.25 ml, and expressed as g/kg of bodyweight.

EtOH Preference

EtOH preference was expressed as a percentage representing the amount of daily EtOH intake (measured in ml) over total daily fluid intake (measured in ml).

Microdialysis Analyses

Probe Construction

Probe construction was undertaken at Kent State University utilizing the same procedure of probe construction outlined in previous studies (Dudley et al., 1998). Briefly, concentrically designed probes were constructed from 26-gauge stainless
steel outer cannula (Small Parts, Miami, FL), into which was inserted 32-gauge fused silica tubing (Phoenix Pharmaceuticals, Phoenix, AZ). Hemicellulose dialysis membrane tubing (12 KDa MW cutoff; 230 µm OD; Spectra-Por, Vernon Hills, IL) was secured to the outer cannula with epoxy glue. The active dialysis probe tip length was 1.0 mm for SCN sampling and 4.0 mm for subcutaneous tissue sampling.

**Probe Efficiency**

To have an *in vitro* estimate of efficiency of EtOH delivery, EtOH was measured in dialysate samples collected from probes bathed in a 50 mM EtOH standard maintained at 35, 37, 39°C. This would determine if fluctuations in body temperature within this range would significantly affect efficiency of EtOH delivery via probes (Hansen et al., 1999). Mean probe efficiency was estimated to be ~10% for SCN brain probes and ~40% for subcutaneous probes. Mean probe efficiency not vary between the tested temperatures (single-classification ANOVA, p<0.05).

**In Vivo Sampling**

Two days before experimentation, the microdialysis probe was stereotaxically aimed at the lateral margin of the SCN (anteroposterior=-0.046 mm from bregma; lateral=+0.02 mm from midline; horizontal=5.5 mm from dura) to avoid extensive damage to the master pacemaker. Animals were anesthetized with pentobarbital.
(Nembutal: 35.0 mg/kg) and pre-treated with Marcaine (0.25% bupivicaine, 0.05 ml) and Atropine (0.09% atropine, 0.10 ml) to manage localized pain and respiratory occlusion, respectively. Probes were secured to the skull with stainless steel screws and dental epoxy. To compare SCN EtOH pharmacokinetics with EtOH pharmacokinetics of neighboring hypothalamic areas and systemic tissue, microdialysis probes were implanted into the medial preoptic nucleus (MPON; AP=+0.02 mm from bregma; L=-0.25 mm from midline; H=-5.0 mm from dura) and subcutaneous scapular region, respectively. The two day recovery period provided optimal time for tissue recovery (blood-brain barrier re-establishment) and probe function. Following recovery, probes of freely-behaving mice were continuously perfused with filtered artificial cerebrospinal fluid (ACSF) during chronic EtOH intake over 24 hrs (Experiments 1 and 12) or 1 hr before and extending 7 hr after an acute, systemic EtOH challenge (Experiment 4). A sampling interval of 20-30 min at a flow rate of 1.0 µl/min began 1 hr. before (baseline period) and extended through the end of the experiment. Samples were immediately stored at -20°C. EtOH concentrations in the dialysate samples were later analyzed in 5 µl aliquots with an Analox AM1, which measures the amount of oxygen needed to convert EtOH to acetaldehyde (Analox Instruments, Lunenburg, MA).
**In Vivo Perfusions**

Following stereotaxic placement of the microdialysis probe into the SCN and subsequent entrainment of general circadian locomotor activity under LD, probes of freely-behaving mice were continuously perfused for 1 hr with filtered ACSF alone or EtOH (500 mM) in ACSF from a syringe pump at a flow rate of 1.0 µl/min. This EtOH concentration was derived from *in vitro* assessments of SCN probe efficiency of ~10%, providing a theoretical outside tissue concentration of ~50 mM.

**Probe Verification and SCN Necrosis**

Brains were stored in 4% buffered paraformaldehyde (pH=7.3) for 24 hrs and a subsequent 30% sucrose solution for another 24 hrs at 4°C in preparation for sectioning at 40 µm-thick. Sections were stained with cresyl violet to verify location of the microdialysis probe tip. Additional SCN sections from mice under forced consumption of a 15% EtOH solution for a minimum of 2 wks were stained with Fluoro- Jade B (Fisher Scientific, Pittsburgh, PA) to detect necrosis associated with chronic ethanol intake.
Micropellet Analyses

Implantation

Two days before experimentation, blank wax pellets or pellets containing calcium acamprosate (300 mg/g of beeswax; dimensions: 1.25 mm x 0.75 mm) were stereotaxically and bilaterally implanted into major reward and circadian areas.

[Reward: 1) ventral tegmental area of the pons (VTA; AP= -3.08 mm from bregma; L= -0.75 mm from midline; H= -4.0 mm from dura); 2) peduculopontine tegmentum of the pons (PPT; AP= -4.72 mm from bregma; L= -0.80 mm from midline; H= -3.00 mm from dura); and 3) nucleus accumbal core/shell border of the forebrain (NA; AP= +1.70 mm from bregma; L= -0.75 mm from midline; H= -4.50 mm from dura).

Circadian: 1) intergeniculate leaflet of the thalamus (IGL; AP= -2.30 mm from bregma; L= -2.40 mm from midline; H= -3.00 mm from dura) and 2) SCN.

Acamprosate micropellets were also stereotaxically targeted at the hippocampal fissure as a control (AP= -2.70 mm from bregma; L= -2.25 mm from midline; H= -2.00 mm from dura). Animals were anesthetized with pentobarbital (Nembutal: 35.0 mg/kg) and pre-treated with Marcaine (0.25% bupivicaine, 0.05 ml) and Atropine (0.09% atropine, 0.10 ml) to manage localized pain and respiratory occlusion, respectively. An incision the length of the skull was made and micropellets were implanted with stainless steel cannula. Following implantation, the skin was sutured.
with stainless steel wound clips, and the animals were given three days of post-operative recovery in order to re-establish blood-brain tissue barriers.

*In Vitro Characterization of Acamprosate Release*

To determine the concentration of daily acamprosate release from an individual acamprosate micropellet, a 1 ml solution consisting of 10 acamprosate micropellets immersed in physiological saline at a constant temperature of 37°C was daily decanted and replenished with new medium over 27 days. Sample concentrations were then measured using UV spectrometry. A Varian Cary 300Bio (Santa Clara, CA) and quartz cuvettes were used to scan absorbance readings between 800nm and 190nm. The detection limit of the technique was investigated by diluting acamprosate standards in physiological saline until the signal at maximum wavelength could no longer be distinguished from the baseline acquired with only physiological saline. The calibration curve was developed using the absorbance scan data at a wavelength maximum of 201nm. The absorbance of samples collected at Day 1 and 2 were outside the linear range of the standard curve, so dilution was necessary.
Animals were deeply anesthetized with Nembutal and intracardially perfused with 100 ml of 4% buffered paraformaldehyde (pH = 7.3). The brains were extracted, and immersion-fixed in 4% paraformaldehyde for 24 h, followed by immersion in 30% sucrose for 24 h at 4°C. Cryostat sections (40µm-thick) were incubated with a rabbit polyclonal IgG antibody (tyrosine hydroxylase for VTA and NAc, choline acetyltransferase for PPT, and glutamate decarboxylase for IGL, hippocampus, and SCN; Santa Cruz Biotechnology; Santa Cruz, CA), and protein expression was visualized using Vectastain Elite ABC kit with 3,3-diaminobenzidine tetrahydrochloride as chromagen (Vector Labs, Burlingame, CA). Sections were mounted with permount (Fisher) and viewed through an Olympus fluorescence microscope.

**Euthansia**

Following experimentation, animals were sacrificed with Euthasol euthanasia solution. This method is recommended by the Panel of Euthansia of the American Veterinary Association.
Statistical Analyses

Multivariate ANOVA and a subsequent Student Newman-Keuls post hoc test were used to assess treatment and dose differences of pharmacokinetics, phase-shifts, rhythm period, activity onset, alpha, solution intake, preference, the quantity and duration of activity bouts across the active and rest periods of the 24 hr circadian day, and c-fos activity. Levels of significance, in all cases, were set at p<0.05. All statistical analyses were undertaken with SPSS 15.0 Software Package (Chicago, Illinois).

Experimental Protocols

Ethanol Disruption of Photic and Nonphotic (Serotonergic) Behavioral Circadian Entrainment

Experiment 1: Concentration-Dependent EtOH Chronopharmacokinetics in SCN and Subcutaneous Tissue during Chronic EtOH Intake.

Microdialysis assessment of EtOH chronopharmacokinetics in the SCN and subcutaneous tissue (peak concentration(s), time of peak concentration(s), half-life of EtOH clearance(s)) was undertaken continuously over LD in freely-behaving mice maintained on EtOH (10% or 15% vol/vol in drinking water; n=7/group) as the sole
source of fluid for a minimum of 2 wk. SCN and subcutaneous levels of EtOH were assessed concomitantly with the animals’ circadian patterns of general locomotor and/or drinking behaviors. Additional microdialysis sampling was conducted in the MPON to compare SCN EtOH chronopharmacokinetics with that of neighboring hypothalamic areas (n=5/group).

Experiments 2 and 3: Concentration-Dependent Effects of Chronic EtOH Intake and Withdrawal on Photic and Serotonergic Phase-Resetting of Behavioral Circadian Rhythms and Daily Activity Patterns.

These experiments examined the effects of chronic EtOH intake and its withdrawal on photic phase-delays (30 min, 25 lux light pulse) and nonphotic phase-advances (systemic (i.p.) administration of the serotonin1A/7 agonist (+)-8-OH-DPAT [10.0 mg/kg]) of behavioral circadian rhythms. Mice were individually caged under LD, and their general circadian activity rhythms were monitored for 2 wk. To determine EtOH effects on photic phase-resetting, animals received water (controls) or 10% or 15% EtOH as their sole source of fluid for 2 wk prior to the presentation of a 30 min light pulse (25 lux) administered at zeitgeber time (ZT) 14 (where ZT 12 is designated as the onset of the dark phase; n=7/group). To determine EtOH effects on nonphotic phase-advances, a separate group of animals received water (controls) or 15% EtOH as their sole source of fluid for 2 wk prior to an i.p. injection of DPAT mixed in dimethyl sulfoxide (DMSO) or DMSO vehicle at ZT 6 (n=7/group). Following
presentation of the light pulse or serotonin agonist, all animals were released to DD with EtOH still provided to assess phase shifting using a modified Aschoff Type II procedure (Daan and Pittendrigh, 1976). Animals in the EtOH withdrawal trial were treated the same way as in the previous trials (n=7/group), except that EtOH was withdrawn for 26 hr after drinking for 2 wks. After this time, they received a photic pulse at ZT 14, and were then released to DD and maintained on tap water. These experimental timelines were derived from chronopharmacokinetic data in Experiment 1 showing high ethanol intake between ZT 12-14 and ZT 22-24.

**Experiment 4: Concentration-Dependent Effects of Chronic EtOH Intake, Withdrawal, and Re-Introduction on Photic Entrainment and Circadian Locomotor Activity.**

Mice entrained to LD were presented with water or a 10% or 15% EtOH solution as their sole source of fluid for 2 wks, and their general circadian activity rhythms were monitored. Thereafter, animals maintained on their respective solutions were placed under a skeleton photoperiod consisting of the daily presentation of a 1 min light pulse (25 lux). Two separate trials were undertaken to determine the effect of chronic EtOH on re-entrainment to a weak photic stimulus (first trial; light pulse delivered at ZT 6 of the original LD cycle; n=7/group; second trial; light pulse delivered at ZT 11 of the original LD cycle; n=10/group). In the trial, EtOH-treated animals were maintained on 10% or 15% EtOH solution (vol/vol) in drinking water
under LD and 4 wks into the skeleton photoperiod. Four weeks after introduction to the skeleton photoperiod, EtOH was replaced with water to assess the effects of EtOH withdrawal on photic entrainment and circadian locomotor activity. After 7 wks of withdrawal, the original EtOH-treated groups were re-introduced to their respective EtOH solutions to assess concentration-dependent effects of EtOH re-introduction on photic entrainment and circadian locomotor activity. In second trial, animals were maintained on the 15% EtOH solution (vol/vol) in drinking water under LD and 4 wks into the skeleton photoperiod, during which time, effects of EtOH on photic entrainment and circadian locomotor activity were assessed.

*Experiment 5: Dose-Dependent EtOH Pharmacokinetics in the SCN following an Acute Systemic EtOH Administration.*

Microdialysis assessment of EtOH pharmacokinetics in the SCN (peak concentration, time of peak concentration, and half-life of EtOH clearance) was conducted in freely-behaving mice entrained to LD following an acute, systemic intraperitoneal (i.p.) EtOH administration of a low (0.5 g/kg of body weight), moderate (1.0 g/kg of body weight), or high (2.0 g/kg of body weight) EtOH dose diluted to 20% in physiological saline (n=3/group). A microdialysis sampling interval began 1 hr before and extended 7 hr after the i.p. EtOH challenge. Additional microdialysis sampling was conducted in the MPON to compare SCN EtOH pharmacokinetics with that of neighboring hypothalamic areas (n=3/group).
Experiments 6 and 7: Dose-Dependent Effects of an Acute Systemic EtOH Administration on Photic and Serotonergic Phase-Resetting of Behavioral Circadian Rhythms

These experiments addressed the effects of a dose-dependent acute systemic EtOH challenge on photic phase-delays and nonphotic phase-advances of behavioral circadian rhythms. Mice under LD were individually housed, and their general circadian activity rhythms were monitored for 2 wk. On the day of the experiment, mice received an acute, systemic (i.p.) administration of a low (0.5 g/kg), moderate (1.0 g/kg), or high (2.0 g/kg) dose of EtOH diluted in 20% physiological saline or saline vehicle 30 min before the presentation of a 30 min light pulse (25 lux) administered at zeitgeber time (ZT) 16 (where ZT 12 is designated as the onset of the dark phase) or the systemic (i.p.) administration of the serotonin1A/7 agonist (+)8-OH-DPAT (10.0 mg/kg) mixed in DMSO or DMSO vehicle at ZT 6 (n=7/group). The animals were then released to DD to assess phase shifting using a modified Aschoff Type II procedure (Daan and Pittendrigh, 1976). This experimental timeline was derived from pharmacokinetic data in Experiment 4 in which EtOH levels in the SCN peaked 20-40 min post-acute EtOH administration.
Experiments 8 and 9: The Direct Effects of an Intra-SCN EtOH Perfusion on Photic and Serotonergic Phase-Resetting of Behavioral Circadian Rhythms

These experiments examined the direct effects of an intra-SCN EtOH perfusion on photic and nonphotic phase-resetting of behavioral circadian rhythms. Mice under LD were individually caged, and their general circadian activity rhythms were monitored for 2 wk. On the day of the experiment, the microdialysis probes of freely-behaving mice were perfused with ACSF or EtOH+ACSF (500 mM) 30 min before mice received a 30 min phase-delaying light pulse (25 lux) or no light pulse (0 lux) administered at ZT 14 (where ZT 12 is designated as the onset of the dark phase; n=4/group) or a systemic (i.p.) administration of (+)-8-OH-DPAT in DMSO (10.0 mg/kg) or DMSO vehicle at ZT 6 (n=4/group). The animals were then released into DD to assess phase shifting using a modified Aschoff Type II procedure (Daan and Pittendrigh, 1976).

Experiment 10: Effects of an Acute Systemic EtOH Administration on Fos Expression in the SCN and VTA

This experiment determined if EtOH affects basal SCN and VTA neural activity at midday. Mice received an i.p. injection of a high EtOH dose (2.0 g/kg) or saline vehicle at ZT 6 (n=5/group). Forty minutes after injection when brain EtOH levels had reached maximal levels, the animals were deeply anesthetized and
transcardially perfused. An additional, separate group of mice (n=5/group) received the EtOH or saline injection one hundred minutes before the animals were deeply anesthetized and transcardially perfused. Fos expression was quantified using ImageJ (National Institutes of Health, Bethesda, MD). Counts of immunostained nuclei were undertaken blindly from within the entire mid-posterior region of the SCN and VTA.

**Environmental Modulation of Ethanol Intake and Craving**

*Experiment 11: Characterization of Ethanol Craving and Alcohol Relapse Potential*

Mice received free-choice of a 10% or 15% EtOH solution or water (n=10) for 4 wks under entrainment to the same skeleton photoperiod used in Experiment 4. Afterwards, mice were either maintained on free-choice EtOH across experimentation or were withdrawn from free-choice EtOH for 3 wks and subsequently re-introduced to free-choice EtOH for another 4 wks to establish a condition of alcohol relapse (n=5/group; see Spanagel et al. 1996). Across EtOH treatment, positions of bottles containing an EtOH solution or water were controlled to determine a side preference (left or right of cage) as documented previously (see Forger and Morin, 1982). General circadian activity was monitored across experimentation.
Experiment 12: Environmental Modulation of Ethanol Intake and Craving and Circadian Activity

Mice were introduced to free-choice of a 15% EtOH solution or water under LD for 2 wks (n=11) followed by release to LL (270 lux) for the remaining 2 wks. Positions of bottles containing an EtOH solution or water were daily flip-flopped to control for a right-sided bottle preference (Brager, unpublished data). General circadian activity was measured across experimentation.

Neurobehavioral Actions of Acamprosate Suppression of Ethanol Intake and Craving

Experiment 13: Assessment of Behavioral and Drinking Chronotypes of Per2-mutant Mice

PER2-mutant and wild-type mice received free-choice of a 15% EtOH solution or water across experimentation. Positions of bottles containing an EtOH solution or water were daily flip-flopped to control for a right-sided bottle preference (Brager, unpublished data). Behavioral and drinking rhythms of individually-caged mice (n=7/group) that had previously entrained to LD were monitored for 4 wks and 1 wk, respectively.
Experiment 14: Characterization of Systemic EtOH Chronopharmacokinetics and Circadian Activity during a Daily Regimen of Systemic Acamprosate Treatment

Systemic EtOH chronopharmacokinetics and EtOH drinking rhythms from lickometers were characterized continuously over LD in PER2-mutant and wild-type mice maintained under free-choice of a 15% EtOH solution or water for a minimum of 2 wks (n=4/group). Positions of bottles containing an EtOH solution or water were daily flip-flopped to control for a right-sided bottle preference (Brager, unpublished data). Pharmacokinetic measurements included peak EtOH concentration (s; mM) and circadian time(s) of peak EtOH concentration(s; T_max). Following baseline measures of EtOH chronopharmacokinetics, mice received a daily, systemic intraperitoneal (i.p.) injection of 300 mg/kg acamprosate diluted in 30% physiological saline or a saline vehicle (~0.5 ml) over 6 days at ZT 10. This experimental timeline was based from actographic data in Experiment 13 showing that the PER2-mutant mice began to heavily consume EtOH around ZT 10. On the last day of acamprosate treatment, all animals underwent secondary 24 hr systemic microdialysis sampling to determine acamprosate effects on systemic EtOH chronopharmacokinetics and drinking rhythms. A second group of PER2-mutant and wild-type mice entrained to LD for 2 wks received a daily regimen of i.p. acamprosate or saline at ZT 6 (n=4/group) and were subsequently released to DD to assess the ability of acamprosate to act as a nonphotic circadian phase-resetting stimulus. General circadian activity was measured across experimentation.
Experiment 15: Effects of Constant- Acamprosate Release in Reward and Circadian Brain Areas on EtOH Intake, Preference, and Circadian Nocturnal Activity

PER2-mutant and wild-type mice (n=12, each genotype) entrained to LD were introduced to free-choice of a 15% ethanol solution or water for 3 wks and subsequently withdrawn from ethanol for 3 wks to establish an experimental protocol of alcohol relapse (see Spanagel et al. 1996). Positions of bottles containing an EtOH solution were daily flip-flopped to control for a right-sided bottle preference (Brager, unpublished data). Four days before ethanol re-introduction, blank wax micropellets or micropellets releasing acamprosate were bilaterally implanted into reward and circadian brain areas of interest (n=6, for each genotype). Five wks after ethanol re-introduction, animals were euthanized and brains were immediately extracted to verify micropellet placements using cresyl violet and immunohistochemical stainings. General circadian activity was measured across experimentation.

Ethanol-Specific Disruption to Behavioral Circadian Entrainment

Experiments 16 and 17: Effects of an Acute Systemic Cocaine Administration on Photic and Serotonergic Phase-Resetting of Behavioral Circadian Rhythms
These experiments addressed the effects of a acute systemic cocaine challenge on photic phase-delays and nonphotic phase-advances of behavioral circadian rhythms. Mice under LD were individually housed, and their general circadian activity rhythms were monitored for 2 wk. On the day of the experiment, mice received an acute, systemic (i.p.) administration of cocaine (20 mg/kg) diluted in 20% physiological saline or saline vehicle 15 min before the presentation of a 30 min light pulse (25 lux) administered at zeitgeber time (ZT) 16 (where ZT 12 is designated as the onset of the dark phase) or at ZT 6 (n=7/group). The animals were then released to DD to assess phase shifting using a modified Aschoff Type II procedure (Daan and Pittendrigh, 1976).

*Experiment 18: The Direct Effects of an Intra-SCN Cocaine Perfusion on Serotonergic Phase-Resetting of Behavioral Circadian Rhythms*

These experiments examined the direct effects of an intra-SCN cocaine perfusion on nonphotic phase-resetting of behavioral circadian rhythms. Mice under LD were individually caged, and their general circadian activity rhythms were monitored for 2 wk. On the day of the experiment, the microdialysis probes of freely-behaving mice were perfused with ACSF or cocaine+ACSF (0.5 mM) at ZT 6 (n=4/group) . The animals were then released into DD to assess phase shifting using a modified Aschoff Type II procedure (Daan and Pittendrigh, 1976).
Experiment 19: Residual Effects of An Acute Cocaine Administration on the Temporal Structure of Circadian Activity

Total activity counts across the subjective daytime and nighttime periods were quantified for up to seven days prior to the acute cocaine administration in Experiment 16 and for up to ten days after the cocaine administration.
CHAPTER III

Results


Ethanol Disruption of Photic and Nonphotic (Serotonergic) Behavioral Circadian Entrainment

*Experiment 1. EtOH chronopharmacokinetics in the SCN during chronic EtOH intake are concentration-dependent.*

Histologically verified microdialysis probe tip locations relative to the SCN are shown in Fig. 4. The 24 h pharmacokinetic profiles of EtOH in the SCN extracellular fluid compartment and composite 24-h pharmacokinetic profiles of EtOH in the SCN extracellular fluid compartment and subcutaneous tissue are presented in Fig. 5.
animals drinking 10% EtOH, the average amount of EtOH consumed was ~20 g/kg/day. The highest levels of EtOH in the SCN occurred just before and intermittently throughout the dark phase, with peak levels averaging ~12 mM. SCN EtOH levels declined to lower levels (0-3 mM) for the remainder of the 24 hr day. Mice drinking 15% EtOH consumed ~22 g/kg/day, and had a temporally similar pattern of EtOH in the SCN, with the highest levels occurring during the dark-phase and immediately after lights-on, (with peak levels averaging 20-30 mM), and the lowest levels (0-3 mM) occurring from midday to lights-off. For both groups, lickometer measurements verified drinking bouts preceding EtOH peaks by approximately 20-40 min. In the MPON, the highest levels of EtOH in mice drinking 10% EtOH (consumption of ~18 g/kg/day) occurred immediately after lights-on and intermittently throughout the dark-phase (with peak levels averaging 20-30 mM). Intra-MPON EtOH levels in mice drinking 15% EtOH and consuming ~23 g/kg/day similarly occurred immediately after lights-on (averaging 25 mM), were relatively low throughout the dark-phase, and precipitously rose again near lights-off (averaging 30-35 mM). Intra-MPON EtOH levels in mice consuming both 10% and 15% EtOH were relatively low (0-5 mM) throughout the light-phase.
Figure 4: Histologically verified microdialysis probe tip locations for measuring EtOH levels in the SCN of animals consuming 10% (*) and 15% (+) EtOH. OC, optic chiasm; 3V, third ventricle.
Figure 5: 24-hour pharmacokinetic profiles of SCN and subcutaneous (SYS) EtOH concentrations superimposed with daily drinking episodes in individual mice consuming 10% (A) and 15% (B) EtOH. Averaged daily SCN EtOH profiles for mice drinking 10% and 15% EtOH are shown in C (n=7/group). Horizontal black bars represent the dark phase of the LD cycle.
Experiment 2. Chronic EtOH intake and withdrawal attenuate photic phase-delays of behavioral circadian rhythms and disrupt daily activity patterns.

Photic phase-resetting. Photic phase-delays of behavioral circadian rhythms under LD were dose-dependently attenuated by chronic forced drinking of 10% and 15% EtOH. In animals maintained on water, a 30 min light pulse delivered at ZT 14 caused phase-delays averaging 1.5±0.2 hr (Fig. 6). This response was inhibited by EtOH, such that groups drinking 10% and 15% EtOH exhibited phase-delay shifts of 1.0±0.1 hr and 0.7±0.1 hr, respectively. These attenuated shifts were significantly different from water controls and from each other (F(2,18)=11.9; p<0.01). Representative actograms of these treatments are presented in Fig. 7. Withdrawal from 10% and 15% EtOH also significantly attenuated the phase-delaying effect of a 30 min light pulse delivered at ZT 14 (0.9±0.1 hr and 0.8±0.1 hr, respectively vs. 1.3±0.2 hr for water (F(2,18)=9.9; p<0.02; Fig. 6).

Daily activity patterns under LD. Chronic forced drinking of 10% EtOH did not affect entrainment to LD; however, there was increased sporadic activity, such that during the active period (dark phase) there were increased activity bouts of reduced duration compared to the water control group (bout quantity, F(1,34)=35.8; p<0.01; bout duration, F(1,34)=8.4; p<0.01; Table 1). This drinking also reduced bout quantity during the rest period (light phase; F(1,34)=8.9; p<0.01). Chronic forced drinking of 15% EtOH had essentially the same effect on increasing activity bouts and
decreasing bout duration during the active period (bout quantity, \( F_{(1,34)}=36.5; p<0.01 \); bout duration, \( F_{(1,34)}=21.8; p<0.01 \)). However, there was no effect on bout quantity during the rest period (Table 1).

<table>
<thead>
<tr>
<th>L:D 12:12</th>
<th>REST</th>
<th>ACTIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WATER</td>
<td>7.10 +/- 0.30\textsuperscript{a}</td>
<td>4.40 +/- 0.20\textsuperscript{a}</td>
</tr>
<tr>
<td>10% EtOH</td>
<td>5.60 +/- 0.40\textsuperscript{b}</td>
<td>7.50 +/- 0.40\textsuperscript{b}</td>
</tr>
<tr>
<td>15% EtOH</td>
<td>7.30 +/- 0.40\textsuperscript{a}</td>
<td>7.20 +/- 0.60\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Table 1. Effects of chronic EtOH on the quantity and duration of locomotor activity bouts throughout the active (dark) and rest (light) phases of the 12L:12D (LD) photocycle. Values are the means\(\pm\)SE. For each phase, values with different letters are significantly different (\(p<0.05\)).
Figure 6: Inhibitory effects of chronic 10% and 15% EtOH drinking (A) or withdrawal (B) on light pulse-induced phase-delays of the circadian locomotor activity rhythm. Bars are the mean ± SE. Within a treatment group, bars with different letters are significantly different (p<0.05; n=7/group).
Figure 7: Representative double-plotted actograms of general locomotor activity showing the effects of chronic EtOH drinking (A, 10%; B, 15%) and withdrawal (D, 10%; E, 15%) vs. respective water controls (C, F) on light pulse-induced phase-delay responses. The "O" denotes the 30 min light pulse delivered at ZT 14.
Experiment 3. Chronic EtOH intake does not attenuate serotonergic phase-advancing of behavioral circadian rhythms.

Serotonergic phase-advances of behavioral circadian rhythms under LD were not affected by chronic forced drinking of 15% EtOH (p<0.05). DPAT induced phase-advance shifts averaged 1.1 ± 0.2 hr (Fig. 8). DMSO vehicle did not elicit phase resetting of behavioral circadian rhythms (0.0±0.0 hr; Fig. 8).
**Figure 8**: 15% EtOH drinking did not affect DPAT-induced phase-advances of the circadian locomotor activity rhythm. Bars are the mean±SE. Within a treatment group, bars with different letters are significantly different (p<0.05; n=7/group).
Experiment 4. Chronic EtOH intake, its withdrawal, and subsequent reintroduction perturb photic entrainment and daily activity patterns and cause SCN necrosis.

Photic entrainment. In the first trial (re-entrainment), animals receiving water, 10% EtOH, or 15% EtOH and exposed to a 6 hr phase-advance in light onset re-entrained at similar rates (21.0 ± 3.4 days, 21.8 +/- 3.2 days, or 21.7 ± 4.1 days, respectively; F(2,18)=0.32; p<0.8), and locked onto the light pulse close to their activity onsets (Fig. 9). In the second trial (re-entrainment), the water and 15% EtOH groups phase-advanced into the 1 hr advanced light pulse at a similar rate (2-3 days; F(1,18)=0.1; p<1.0; Fig. 9). Thereafter, EtOH reduced alpha by ~ 1 hr (F(1,18)=17.6; p<0.01; Table 4) and affected the phase angle of entrainment such that the time of activity onset became progressively delayed compared to water controls (average delay = -1.08 ± 0.56 hr for EtOH vs. +0.28 ± 0.19 for water; F(1,12)=5.0; p<0.04; Fig. 9).

Circadian locomotor activity. Following entrainment to the 6 hr delay light pulse in the first trial, EtOH reduced the initial 3 hr period of nighttime activity by 40% (EtOH: 154.3 ± 20.0 min; water: 267.7 ± 20.4 min; F(1,12)=494; p<0.05), while its withdrawal (at 10% EtOH concentrations) reduced the mean quantity of nighttime activity bouts compared to the water control group (F(1,34)=13.5; p<0.04; Table 2). There was no
effect EtOH withdrawal at 15% EtOH concentrations on bout quantity or duration (Table 2). Finally, re-introduction to 15% EtOH increased mean nighttime bout quantity ($F_{(1,22)}=10.1; p<0.01$; Table 3) and decreased mean nighttime bout duration ($F_{(1,22)}=26.0; p<0.01$; Table 3) relative to re-introduction to 10% EtOH. Notably, reintroduction to 15% EtOH resulted in a loss of stable entrainment to the skeleton photoperiod, with lengthening of tau (EtOH 15%: $24.10\pm0.03$; EtOH 10%: $24.03\pm0.02$ [$F_{(1,6)}=10.40; p<0.05$]). In a separate experiment, a single 3 wk presentation of 15% EtOH following entrainment to the same skeleton photoperiod also disrupted stable entrainment by progressively delaying nighttime activity onset (average delay = $-2.36\pm0.40$ hr for EtOH vs. $-0.60\pm0.21$ for water; $F_{(1,12)}=50.3; p<0.01$) and increasing sporadic activity during the rest period reflected through an potentiation of mean daytime bout quantity (EtOH: $9.17\pm0.60$; water: $6.58\pm0.36$; $F_{(1,12)}=14.35; p<0.01$). Following entrainment to the skeleton photoperiod in the second trial, forced EtOH intake and its withdrawal reduced the mean duration of an activity bout across the nighttime compared to the water control group (bout duration, $F_{(2,18)}=42.61; p<0.01$; Table 4). Forced EtOH intake and its withdrawal also markedly reduced (by 65%) the initial 3 hr period of intense activity starting at lights off ($F_{(2,18)}=53.7; p<0.01$; Table 4). Forced EtOH intake increased nighttime sporadic activity indicated through a greater mean quantity of activity bouts relative to water controls and EtOH withdrawn mice ($F_{(2,18)}=5.15; p<0.01$; Table 4). Finally, withdrawal from EtOH reduced the mean quantity of activity bouts present during the
active period ($F_{(2,18)}=5.15; p<0.03$; Table 4), illustrating withdrawal-induced depressions in nighttime locomotor activity.

**SCN necrosis.** There were 73.1±13.1 necrotic cells found in the SCN of mice forced to consume a 15% EtOH solution under LD for a minimum of 2 wks (Fig. 10).

<table>
<thead>
<tr>
<th>Withdrawal</th>
<th>Rest</th>
<th>Active</th>
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<tbody>
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<td>Water</td>
<td>9.60 +/- 1.10</td>
<td>11.10 +/- 0.90&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10% EtOH</td>
<td>7.06 +/- 0.80</td>
<td>0.14 +/- 0.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15% EtOH</td>
<td>11.80 +/- 0.90</td>
<td>11.80 +/- 0.50&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

**Table 2.** Effects of EtOH withdrawal on the quantity and duration of locomotor activity bouts throughout the subjective active and rest phases in mice under a skeleton photoperiod consisting of a 1 min light pulse/day delivered at ZT 6. Values are the means ± SE. For each phase, values with different letters are significantly different ($p<0.05$).

<table>
<thead>
<tr>
<th>Re-intro</th>
<th>Rest</th>
<th>Active</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% EtOH</td>
<td>8.20 +/- 0.80</td>
<td>5.90 +/- 1.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15% EtOH</td>
<td>7.30 +/- 1.60</td>
<td>10.70 +/- 1.30&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

**Table 3.** Effects of EtOH re-introduction on the quantity and duration of locomotor activity bouts throughout the subjective active and rest phases in mice under a skeleton photoperiod consisting of a 1 min light pulse/day delivered at ZT 6. Values are the means ± SE. For each phase, values with different letters are significantly different ($p<0.05$).
Table 4: Effects of chronic 15% EtOH drinking on circadian locomotor activity in mice under a skeleton photoperiod consisting of a daily 1 min light pulse/day delivered at ZT 11 of the original LD cycle. Values are the means + SE. For each phase, values with different letters are significantly different (p<0.05).

<table>
<thead>
<tr>
<th>INITIAL PERIOD (min)</th>
<th>BOUT DURATION (MIN)</th>
<th>BOUT QUANTITY</th>
<th>ALPHA (HR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WATER</td>
<td>185.0 +/- 14.7a</td>
<td>17.1 +/- 0.6</td>
<td>56.8 +/- 2.9a</td>
</tr>
<tr>
<td>EtOH</td>
<td>63.0 +/- 9.9b</td>
<td>16.4 +/- 1.4</td>
<td>37.9 +/- 2.6b</td>
</tr>
<tr>
<td>EtOH WITHDRAWAL</td>
<td>50.0 +/- 2.6b</td>
<td>20.2 +/- 1.5</td>
<td>40.2 +/- 1.5b</td>
</tr>
</tbody>
</table>
Figure 9: [Top] A,B: Representative double-plotted actograms showing re-entrainment to a skeleton photoperiod (1 min light pulse designated by the vertical line) delivered daily at ZT 6 of the initial LD photocycle in mice chronically drinking 15% EtOH (shading) or water, respectively (n=7/group). C,D: doubleplotted actograms showing the effect of chronic 15% EtOH or water, respectively on entrainment to the 1 min skeleton photoperiod delivered at ZT 11 of the initial LD photocycle (n=10/group). The horizontal black bars represent the dark phase of the initial LD cycle. [Bottom]. Representative photomicrograph of Fluoro-Jade B stained-necrotic cells (black) in the SCN of a mouse chronically drinking a 15% EtOH solution under LD. OC: optic chiasm; 3V: third ventricle.
Experiment 5. SCN EtOH pharmacokinetics following an acute EtOH injection are dose-dependent.

The pharmacokinetic profiles of EtOH in the SCN and MPON following acute i.p. injections of EtOH (0.5 g/kg to 2.0 g/kg) as assessed in freely-behaving mice by microdialysis are presented in Fig. 10. Peak levels of EtOH in the SCN were independent of dose, and all occurred within 20-40 min post-administration. These levels, estimated using a 10% probe efficiency, were 57.7±5.9 mM, 20.2±3.0 mM, and 8.8±1.5 mM, for the 2.0, 1.0 and 0.5 g/kg doses, respectively. The half-lives of the absorbed EtOH (t_{1/2} elimination) were 1.78±0.2 hr, 1.1±0.5 hr, and 0.6±0.1 hr, for the same doses, respectively. A representative microdialysis probe tract along the lateral margin of the SCN is shown in Fig. 11. In comparison to the SCN, peak levels of EtOH in the MPON were also independent of dose, occurring within 20-40 min post-administration. With an estimated probe efficiency of 10%, peak EtOH levels were 43.4±11.6 mM, 20.3±6.5 mM, and 4.0±1.0 mM, for the 2.0, 1.0, and 0.5 g/kg doses, respectively. The half-lives of the absorbed EtOH (t_{1/2} elimination) were 2.1±0.5 hr, 1.6±0.4 hr, and 0.8±0.2 hr, for the same doses, respectively.
Figure 10. Pharmacokinetic profiles of EtOH in the SCN (top) and MPON (bottom) following i.p. injections of three doses of EtOH. For each dose, peak levels occurred within 20-40 min of injection. Clearance rate was dose-dependent. Time points are the means ± S.E.
Figure 11. Coronal section showing the position of a representative probe tract (PT) targeted to the lateral margin of the SCN. 3V, third ventricular; OC optic chiasm.

Photic phase-delay shifts at ZT 16 were significantly attenuated by i.p. injection of EtOH in a dose-dependent manner (F_{3,24}=3.89; p<0.03; Fig. 12). Vehicle controls receiving i.p. saline injection had phase-delay shifts averaging 1.40±0.20 hr, while animals receiving i.p. injection of EtOH at doses of 0.5, 1.0 and 2.0 g/kg showed reduced phase delays averaging 1.31±0.12 hr, 1.17±0.19 hr, and 0.70±0.20, respectively. The two higher doses significantly inhibited photic phase resetting (both p<0.05 vs. vehicle). Representative actograms for vehicle- and EtOH-treated animals are shown in Fig. 13.
Figure 12. Dose-dependent inhibition of phase-delay responses to a light pulse delivered at ZT 16 by i.p. EtOH injection. Bars with different letters are significantly different (p<0.05). Bars represent means ± S.E.
Figure 13. Representative double-plotted actograms of general locomotor activity showing EtOH attenuation of photic phase-delay responses to a 30m min light pulse delivered at ZT 16. A, EtOH (2 g/kg); B, saline injection. Asterisks denote the time of injection and subsequent light pulse.
Experiment 7. Serotonergic phase-advancing of behavioral circadian rhythms is attenuated by an acute injection of a high EtOH dose.

Serotonergic phase-advance shifts induced by i.p. injection of 8-OH-DPAT at ZT 6 were significantly attenuated by i.p. injection of EtOH in a dose-dependent manner (F_{3,24}=8.89; p<0.01; Fig. 14). Vehicle controls receiving i.p. DMSO injection had phase-advance shifts averaging 0.5±0.2 min, while animals receiving i.p. injection of EtOH at doses of 0.5, 1.0 and 2.0 g/kg showed reduced phase delays averaging 0.4±0.1 hr, 0.3±0.1 hr, and 0.0±0.0 hr, respectively. The highest dose significantly inhibited photic phase resetting (p<0.01 vs. vehicle). Representative actograms for vehicle- and EtOH-treated animals are shown in Fig. 15.
Figure 14. Dose-dependent EtOH inhibition of phase-advance responses to i.p. injection of (+)-8-OH-DPAT delivered during the middle of the light-phase (ZT 6). Bars with different letters are significantly different (p<0.05). Bars represent means ± S.E.
Figure 15. Representative double-plotted actograms of general locomotor activity showing EtOH attenuation of responses to i.p. (+)8-OH-DPAT injection delivered at ZT 6. A, EtOH (2.0 g/kg) + 8-OH-DPAT; B, saline + 8-OH-DPAT. Asterisks denote the time of injections.
Experiment 8. Acute EtOH intra-SCN perfusion directly attenuates photic phase-delays of behavioral circadian rhythms.

Reverse microdialysis perfusion of the SCN with ACSF containing EtOH (50 mM estimated tissue concentration) for 1 hr markedly inhibited photic phase-delay shifting at ZT 14. Controls receiving SCN ACSF perfusion alone had phase delays averaging 2.4±0.5 hr (Fig. 16), whereas mice receiving SCN EtOH perfusion exhibited much smaller phase delays averaging 0.6±0.2 hr (F1,12=16.56; p<0.01). Neither ACSF nor EtOH perfusions had phase-shifting effects in the absence of a light pulse. Representative actograms for ACSF and EtOH perfused mice are shown in Fig. 17.
Figure 16. Inhibition of phase-delay responses to a light pulse delivered at ZT 14 by direct reverse-microdialysis perfusion of EtOH to the SCN. The EtOH and artificial cerebrospinal (ACSF) perfusions had no phase-resetting effect in the absence of a light pulse. For all treatments, bars with different letters are significantly different (p<0.05). Bars represent means ± S.E.
Figure 17. Representative double-plotted actograms of general locomotor activity showing the inhibition by reverse-microdialysis perfusion of EtOH to the SCN of photic phase-delays induced by a light pulse delivered at ZT 14. A, vehicle (artificial cerebrospinal fluid); B, EtOH; C, vehicle/no light pulse; D, EtOH/no light pulse. Double-ringed circles denote onset of the perfusions and subsequent light pulse.
Experiment 9. Acute EtOH intra-SCN perfusion does not alter serotonergic phase-advancing of behavioral rhythms

A reverse microdialysis SCN perfusion of EtOH in ACSF had no effect on the phase-advancing action of 8-OH-DPAT administered at midday. (0.1±0.1 hr [ACSF]; 0.3±0.1 hr [EtOH+ACSF]; F_{1,6}=6.0; p>0.05; Fig. 18). Due to large phase delays ranging from 2.8-4.3 hr in the DMSO-treated controls that resulted from inadequate functioning of the infrared motion detectors in their respective housing chambers, these animals were excluded from analyses. Representative actograms of the EtOH and control 8-OH-DPAT-treated animals are shown in Fig. 19.
Figure 18. Lack of inhibition of phase-advance response to i.p. injection of (+)8-OH-DPAT delivered at ZT 6 by direct reverse-microdialysis perfusion of EtOH to the SCN. For all treatments, bars with different letters are significantly different (p<0.05). Bars represent means ± S.E.
Figure. 19. Representative double-plotted actograms of general locomotor activity showing the lack of inhibition by reverse-microdialysis perfusion of EtOH to the SCN of (+)-8-OH-DPAT-induced phase-advances at ZT 6. A, EtOH; B, vehicle (artificial cerebrospinal fluid).
**Experiment 10. Acute systemic EtOH alters basal VTA Fos expression.**

Forty minutes after acute i.p. injection, EtOH did not affect basal levels of Fos expression in the SCN when compared to saline injected controls (151.3±5.6 cells vs. 180.0±23.0 cells, respectively; F$_{1,4}$=0.8; p>0.05), but acute i.p. injection of EtOH did increase basal levels of Fos expression in the VTA (255.3 ±15.3 cells vs. 1.6 ±0.7 cells; F$_{1,4}$=162.5; p<0.01). One hundred minutes after injection, there were still no differences in basal SCN Fos expression levels between EtOH vs. saline-treated mice (273.6±39.4 cells vs. 279.6±27.8 cells, respectively; p>0.05).

Photomicrographs and a schematic illustrating Fos expression in the SCN and VTA of mice receiving the EtOH and saline treatments forty minutes before the transcaldial perfusion are presented in **Figs. 20 and 21.**
Figure 20. Photomicrographs illustrating acute EtOH (2 mg/kg) elicitation of immunoreactive Fos expression in the VTA (A, EtOH; B, saline) at midday (ZT6) and the lack of an EtOH effect (A, EtOH; B, saline) on immunoreactive Fos expression in the SCN at midday. 3V, third ventricle; OC, optic chiasm; FR, fasciculus retroflexus; ML, medial lemniscus.
Figure 21. Acute EtOH potentiation of immunoreactive Fos expression within the VTA, but not the SCN at midday. Bars represent means ± S.E.
Environmental Modulation of Ethanol Intake and Craving

**Experiment 11: Alcohol relapse potential is concentration-dependent.**

During the first 4 wks of EtOH introduction, EtOH intake and preference were concentration-dependent. Mice introduced to a 15% EtOH solution consumed and preferred significantly more EtOH vs. mice introduced to a 10% EtOH solution (Intake: 9.9±0.7 g/kg vs. 7.4±0.3 g/kg; F(1,18)=18.8; Preference: 21.2±2.0% vs. 12.4±2.0%; F(1,18)=102.3; both, p<0.01). In both treatment groups, there was a side-of-cage preference with EtOH solutions presented on the right being more highly preferred than those on the left (10%: 10.2±1.0% [left]; 13.9±2.0% [right]; 15%; 18.0±2.0% [left]; 24.2±2.0% [right]; F(1,18)=6.6; p<0.05). EtOH intake and preference did not vary in mice presented with free-choice EtOH across the 11 wks of experimentation (p>0.05). Higher values of EtOH intake and preference for mice consuming a 15% EtOH solution compared to 10% EtOH solution were maintained across ethanol exposure (Intake: F(1,18)=7.6; p<0.01; Preference: F(1,18)=58.7; both, p<0.01). Following ethanol re-introduction in ethanol-withdrawn mice, ethanol intake and preference was double that of baseline values (Intake: 17.4±1.6 g/kg [10%]; 19.9±1.0 g/kg [15%]; Preference: 42.5±2.0% [10%]; 41.6±2.0% [15%] all, p<0.01). There was no difference in ethanol intake and preference during the ethanol re-introductory period between mice consuming a 10% or 15% EtOH solution (p>0.05, both). Rhythm period and the circadian time of activity onset did not differ between
treatment groups and phases of the experiment (p>0.05). A schematic of ethanol intake and preference is presented in Fig. 22.
Figure 22: EtOH intake and preference during 4 wks of ethanol introduction (BASELINE) and following 3 wks of ethanol withdrawal (RELAPSE) in mice consuming a 10% or 15% EtOH solution. Significantly different letters represent significant differences between treatment groups (p<0.05).
Experiment 12: Long-term housing in constant light potentiates ethanol intake and craving and disrupts circadian activity rhythms

Mice consumed and preferred significantly more ethanol under LL vs. LD (Intake: 21.3±3.2 g/kg vs. 14.0±2.7 g/kg, respectively; F(1,20)=13.9; Preference: 30.8±2.0% vs. 20.8±1.0%, respectively; F(1,20)=12.2; both, p<0.01). Under LL, the rhythm period was 25.0±0.1 hr compared to a rhythm period of 24.0±0.1 hr under LD (p<0.05). LL increased intermittent, sporadic circadian activity across the 24 hr period as reflected though an increased number of activity bouts reduced in duration compared to that found under LD (Bouts: 21.7±0.8 [LL]; 14.3±0.4 [LD]; Duration: 19.9±1.5 min [LL] ; 52.4±3.4 min [LD]; both, p<0.05). A schematic of ethanol intake and preference and a representative actogram are found in Fig. 23 and 24, respectively.
Figure 23: Daily ethanol intake and preference in mice under LD and LL. Asterisks annotate significant differences in intake and preference between the two photocycles (p<0.05).
**Figure 24**: A representative, double-plotted actogram of general circadian locomotor activity during housing under LD (white region) and LL (shaded region). White and black bars represent the light and dark photoperiods under LD.
Neurobehavioral Actions of Acamprosate Suppression of Ethanol Intake and Craving

*Experiment 13. PER2-mutant mice had an advanced nocturnal activity onset and extended wakefulness concomitant with high EtOH intake.*

Under LD, the nocturnal activity period began 2.2±0.2 hr earlier in *mPer2* mutants compared to WTs (ZT 10.0±0.4 vs. ZT 12.2±0.2, respectively; F<sub>1,10</sub>=34.5; p<0.01). Nocturnal activity duration of the *mPer2* mutants was extended by a similar amount (2.0±0.2 hr; F<sub>1,10</sub>=17.1; p<0.01). Values for total daily ethanol intake, ethanol preference, and total fluid intake were greater for the *mPer2* mutants vs. WTs (*Intake*: 23.9±1.5 g/kg/day vs. 12.9±1.4 g/kg/day, respectively; F<sub>1,10</sub>=104.2; p<0.01; *Preference*: 70.4±3.1% vs. 44.7±2.4%, respectively; F<sub>1,10</sub>=92.9; p<0.01; *Total Fluid*: 309.6±11.7 ml/kg/day vs. 242.6±12.0 ml/kg/day, respectively; F<sub>1,10</sub>=13.6; p<0.01). However, daily water intake did not differ between the two groups (*mPer2* mutants: 131.6±6.1 ml/kg/day; WTs: 138.2±6.6 ml/kg/day; F<sub>1,10</sub>=0.4; p>0.05). The drinkometer measurements revealed that *mPer2* mutants had more ethanol drinking bouts across the entire 24 hr circadian day, including the dark-phase vs. WTs (*24 hr*: 14±2 bouts vs. 8±1, bouts respectively; F<sub>1,10</sub>=108.0; p<0.01; *Dark-phase*: 11±2 bouts vs. 6±1 bouts, respectively; F<sub>1,10</sub>=74.0; p<0.01). Representative actograms/drinkograms and a schematic of EtOH intake, preference, and water intake are presented in Fig. 25 and Fig. 26, respectively.
Figure 25: Representative, double-plotted actograms of general circadian locomotor and drinking activity are shown for a PER2-mutant (A: locomotor; C: drinking) and wild-type mouse (B: locomotor; D: drinking). Black and white bars represent the 12 hrs of lights-off and lights-on periods, respectively.
Figure 26: Left panels: Ethanol (EtOH) intake, ethanol preference, and water intake in mPer2 mutant (PER2) and wild-type (WT) mice provided free-choice ethanol/water plotted every other day for 30 days. Right panels: Same respective values averaged over the 30 day treatment period. Bars represent means+SE. Bars with different letters are significantly different, p<0.05.
Experiment 14. A daily regimen of systemic acamprosate reduces EtOH intake and preference without altering the advanced rise in EtOH intake in PER2-mutant mice.

*Ethanol Intake and Preference:* Baseline values for total daily ethanol intake and preference in the *mPer2* mutants and WTs were comparable to those in Experiment 1 (*Intake*: 24.9±1.8 g/kg vs. 12.5±1.8 g/kg respectively; F$_{1,10}$=119.3; p<0.01; *Preference*: 68.5±2.0% vs. 45.5±1.0%, respectively; F$_{1,14}$=136.2; p<0.01). Following the regimen of daily i.p. acamprosate injections, ethanol intake and preference were reduced in *mPer2* mutants by 73.9±4.1% and 65.0±2.1% and in WTs by 77.4±1.9% and 72.1±1.3%, respectively (all, p<0.01). Higher values for ethanol intake and preference were maintained in the *mPer2* mutants over WTs at the end of the acamprosate treatment regimen (*Intake*: F$_{1,6}$=16.8; *Preference*: F$_{1,6}$=51.4; both, p<0.01). In the saline injection controls the values for ethanol intake and preference were unchanged from baseline levels in both genotypes (*mPer2* mutant; *Intake*: 27.9±2.0 g/kg; *Preference*: 72.3±1.2%; WT; *Intake*: 12.0±1.8 g/kg; *Preference*: 44.0±1.0%; all, p>0.05). Higher values for ethanol intake and preference were also maintained in the *mPer2* mutants over WTs at the end of the saline treatment regimen (*intake*: F$_{1,6}$=462.3; *preference*: F$_{1,6}$=152.9; both p<0.01). Mean values ± SEM of EtOH intake and preference in PER2-mutant and wild-type mice before and after i.p. acamprosate or saline treatment are provided in Table 5 and Fig. 27.
Table 5: A daily regimen of systemic i.p. acamprosate treatment reduced EtOH intake and preference by 68.1±4.3% and 65.0±2.1% in PER2-mutant mice and by 66.5±2.1% and 72.1±1.3% in WT mice, respectively. There was no effect of daily systemic i.p. saline treatment on EtOH intake and preference in either genotype. Means±SE are shown. Different letters across and within treatments are significantly different (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>EtOH INTAKE (G/KG/DAY)</th>
<th>EtOH PREFERENCE (%)</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Acamprosate</td>
</tr>
<tr>
<td>Per2 KO</td>
<td>27.1±1.8a</td>
<td>8.6±1.8b</td>
</tr>
<tr>
<td>WT</td>
<td>14.6±0.8</td>
<td>4.9±0.8c</td>
</tr>
</tbody>
</table>

| Per2 KO     | 68.5±2.0a              | 24.0±2.0b           | 72.3±1.2a |
| WT          | 45.5±1.0               | 12.7±1.0c           | 44.0±1.0  |
Figure 27: Ethanol (EtOH) intake (left) and preference (right) in mPer2 mutant (PER2) and wild-type (WT) mice during the daily regimen of i.p. acamprosate (ACAMP; 300 mg/kg/day) or saline injection. Bars represent means±SE. For each parameter, bars with different letters are significantly different (p<0.05). Asterisks denote strain difference (p<0.05).
Circadian Patterns of Ethanol Intake and Pharmacokinetics. The baseline frequency of daily total ethanol drinking bouts in mPer2 mutants averaged twice that of wild-types (16±2 bouts vs. 8±1 bouts, respectively; F_{1,10}=43.9; p<0.01). Subcutaneous ethanol concentrations following drinking bouts were also significantly greater in mPer2 mutants vs. wild-types (~60 mM vs. ~25 mM, respectively; F_{1,10}=38.2; p<0.01). A drinking bout preceded the appearance of systemic ethanol by 20-40 min.

In both genotypes, daily acamprosate injections reduced the frequency of daily ethanol drinking bouts and subsequent systemic ethanol peaks (mPer2 mutants: 9±1 bouts and ~20 mM, respectively; wild-types: 4±2 bouts and ~15 mM, respectively; all, p<0.05). Higher values for daily ethanol drinking bouts and subcutaneous ethanol peaks were maintained in the mPer2 mutants over wild-types at the end of the acamprosate treatment regimen (Bouts: F_{1,10}=6.5; Ethanol: F_{1,10}=23.2; both, p<0.01).

In mPer2 mutants, acamprosate reduced the frequency of daily ethanol drinking bouts and subsequent systemic ethanol peaks across the nocturnal activity period (Pre-acamprosate: 13±2 bouts and ~60 mM, respectively; Post-acamprosate: 7±1 bouts and ~20 mM, respectively; both, p<0.01). In wild-types, acamprosate also reduced systemic ethanol peaks across their nocturnal activity period (Pre-acamprosate: ~25 mM; Post-acamprosate: ~15 mM; p<0.01), and completely suppressed ethanol drinking and subsequent systemic ethanol peaks across the daytime (Pre-acamprosate: 2±1 bouts and ~10 mM, respectively; Post-acamprosate: 0±0 bouts and ~0 mM, respectively; both, p<0.05). In the saline controls, the
frequency of daily ethanol drinking bouts and subcutaneous ethanol concentrations in mPer2 mutants and wild-types did not differ from baseline measures (all, p>0.05). Higher values for daily total ethanol drinking bouts and subcutaneous ethanol concentrations were maintained in the mPer2 mutants over wild-types at the end of the saline treatment regimen (Bouts: F_{1,10}=31.5; Ethanol: F_{1,10}=46.5; both, p<0.01).

General Circadian Activity. Similar to the experiment above, the nocturnal activity period began 2.2±0.2 hr earlier in mPer2 mutants compared to wild-types F_{(1,12)}=34.8; p<0.01). In both genotypes, daily acamprosate or saline treatments did not alter the circadian time of nocturnal activity onset (p>0.05) or alter the duration of initial nocturnal activity (Baseline: 4.2±1.2 hr [mPer2 mutant] and 3.0±0.8 hr [wild-type]; Treatment: 4.3±1.6 hr [mPer2 mutant] and 4.1±1.0 hr [wild-type]; p>0.05). Also, in both genotypes, a daily regimen of systemic acamprosate or saline at ZT 6 did not phase-advance behavioral circadian rhythms (F_{(1,12)}=0.3; p>0.05). Representative systemic EtOH chronopharmcokinetic profiles and accompanying drinking records of PER2-mutant and wild-type mice before and during daily acamprosate or saline treatment are presented in Figs. 28 and 29. Representative actograms of PER2-mutant and wild-type mice undergoing a daily regimen of i.p. acamprosate treatment are found in Fig. 30.
Figure 28: Mean twenty-four hour pharmacokinetic profiles of systemic ethanol (EtOH) levels measured using subcutaneous microdialysis in mPer2 mutant (PER2) and wild-type (WT) mice. Black bar represents the dark-phase of the 24 hr LD cycle, with lights-off at zeitgeber time 12.
**Figure 29:** Twenty-four hour pharmacokinetic profiles of systemic ethanol (EtOH) levels measured using subcutaneous microdialysis in an \( mPer2 \) mutant (PER2) and a wild-type (WT) mouse superimposed with their ethanol drinking rhythms under free-choice ethanol/water conditions before (PRE-ACAMP) and during (ACAMP) the daily regimen of i.p. acamprosate injection (300 mg/kg/day). Black bars represent the dark-phase of the 24 hr LD cycle, with lights-off at zeitgeber time 12.
Figure 30: Representative, double-plotted actograms of general circadian locomotor activity at baseline and during a daily regimen of i.p acamprosate treatment (300 mg/kg; shaded region; A, acamprosate in WT; B, acamprosate in PER2-mutant; C, saline in WT; D, saline in PER2-mutant). Black and white bars represent the 12 hrs of lights-off and lights-on periods, respectively.
Experiment 15. Acamprosate differentially acts in circadian/reward areas to suppress EtOH intake and preference and increase circadian nighttime activity.

*Characterization of Daily Acamprosate Release.* Stable acamprosate release from the micropellets was achieved after the 6th day of sampling. Daily release of acamprosate was 38.2±5.2 ng/day. Characterization of daily acamprosate release over 4 wks in two separate trials is shown in Fig. 31.
**Figure 31**: The daily rate of *in vitro* acamprosate release over 4wks from acamprosate microimplants incubated in physiological saline was ~50 ng/pellet/day. Means over two separate trials are shown.
Ventral Tegmental Area. Baseline levels of ethanol intake and preference were greater in PER2-mutant vs. wild-type mice (see Table 6; Intake: $F_{1,22}=56.4$; Preference: $F_{1,22}=66.9$; both, $p<0.01$). The circadian time of nocturnal activity onset began $2.1 \pm 0.4$ hr earlier in the PER2-mutant mice compared to wild-types ($ZT_{10.4+0.2}$ vs. $12.5+0.6$, respectively; $F_{1,22}=42.1$; $p<0.01$). Alpha was extended by a similar duration of $1.7 \pm 0.1$ hr in the PER2-mutant mice ($PER2: 14.0 \pm 0.3$ hr; wild-type: $12.3 \pm 0.4$ hr; $F_{1,22}=10.2$; $p<0.01$), and PER2 mutant mice were 1.4 times more active across the nocturnal active period compared to wild-types (see Table 7; Total Activity: $F_{1,22}=16.8$; Bout Duration: $F_{1,22}=48.1$; both, $p<0.01$). In PER2-mutants, acamprosate suppressed ethanol intake and preference from baseline values by $35.6 \pm 5.3$% and $17.5 \pm 4.9$% over $22 \pm 2$ days and $18 \pm 2$ days of ethanol reintroduction, respectively (see Table 6; both, $p<0.01$). In wild-types, acamprosate suppressed ethanol intake and preference from baseline values by $50.2 \pm 10.0$% and $53.0 \pm 9.6$% over $30 \pm 2$ days and $32 \pm 2$ days of ethanol reintroduction, respectively (see Table 7; both, $p<0.01$). Higher values of ethanol intake and preference were maintained in PER2 mutant mice compared to wild-type mice throughout acamprosate treatment (Intake: $F_{1,22}=15.6$; Preference: $F_{1,22}=18.3$; both, $p<0.01$). Blank implants in PER2-mutants and wild-types did not change ethanol intake and preference from baseline values (see Table 6; both, $p>0.05$). In wild-types, acamprosate increased total nighttime activity and bout duration from baseline values (and compared to mice with blank pellets) by $127.1 \pm 2.7$% and $352.3 \pm 17.1$%, respectively, over $30 \pm 4$ days of
ethanol reintroduction before returning to baseline levels (see Table 7; Total Activity: \( F_{1,10}=38.1 \); Bout Duration: \( F_{1,10}=59.0 \); both, \( p<0.01 \)). Total nighttime activities and bout durations did not differ from baseline values in PER2-mutant mice with acamprosate or blank micropellets (see Table 7; both \( p>0.05 \)). Representative actograms and a photomicrograph of VTA micropellet implantation are found in Fig. 33 and 32A, respectively.

### Table 6: Mean±SE of ethanol intake and preference at baseline and following the implantation of acamprosate or blank (wax) micropellets into the respective reward [ventral tegmental, pedunculopontine tegmentum, nucleus accumbens], circadian [suprachiasmatic nucleus, intergeniculate leaflet], and control areas [hippocampus] in PER2-mutant (PER2 KO) and wild-type (WT) mice. Different letters represent
significant differences in ethanol intake and preference between strains and treatment groups (p<0.05).

Table 7: Means+SE of total nighttime activity and nighttime bout duration at baseline and following the implantation of acamprosate or blank (wax) micropellets into respective reward [ventral tegmental, pedunculopontine tegmentum, nucleus accumbens], circadian [suprachiasmatic nucleus, intergeniculate leaflet], and control areas [hippocampus] in PER2-mutant (PER2 KO) and wild-type (WT) mice. Different letters represent significant differences in ethanol intake and preference between strains and treatment groups (p<0.05).

<table>
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<th>BOUT DURATION (MIN)</th>
<th>TOTAL NIGHTTIME ACTIVITY (HR)</th>
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<td>Baseline</td>
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<tr>
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<tr>
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<td>193.8±32.7°</td>
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<tr>
<td>WT</td>
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<td>53.2±13.3</td>
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<tr>
<td><strong>Nucleus Accumbens</strong></td>
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<tr>
<td>Per2 KO</td>
<td>248.6±22.1°</td>
<td>291.5±33.6°</td>
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<tr>
<td>WT</td>
<td>75.8±11.0</td>
<td>206.9±53.7</td>
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<td><strong>Suprachiasmatic Nucleus</strong></td>
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<tr>
<td>Per2 KO</td>
<td>219.0±13.7°</td>
<td>311.6±29.0°</td>
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<td>WT</td>
<td>100.5±28.2</td>
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<tr>
<td>WT</td>
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Figure 32: [Left panel] Representative photomicrographs of micropellet implantation in reward areas: ventral tegmental area [VTA] (A); peduculopontine tegmentum [PPT] (B); and nucleus accumbens [NAc/s] (C). [Right panel] Representative photomicrographs of micropellet implantation in circadian areas: suprachiasmatic nucleus [SCN] (D) and intergeniculate leaflet [IGL] (E). The hippocampal fissure (HP) was targeted as a control brain area. P: micropellet; MM: medial mammillary nucleus; Aq: cerebral aqueduct; DLG: dorsal lateral geniculate; CPu: caudate putamen; OC: optic chiasm; 3V: third ventricle.
Figure 33: Representative actograms of a PER2-mutant (A) and wild-type mouse (C) with acamprosate micropellets and a PER2-mutant (B) and wild-type mouse (D) with blank micropellets in the VTA are shown. White-black bars are indicative of light-dark photoperiods. Surgery represents the preparation and recovery from micropellet implantation.
Pedunculopontine tegmentum. Pedunculopontine tegmentum (PPT). PER2-mutant mice had greater levels of ethanol intake and preference relative to wild-type mice at baseline (see Table 6; Intake: $F_{1,22}=55.1$; Preference: $F_{1,22}=90.6$; both, $p<0.01$). The nocturnal active period of PER2-mutant mice began $1.8\pm 0.1$ hr before that of the wild-type mice ($PER2$: ZT $10.7\pm 0.2$; wild-type: ZT $12.4\pm 0.2$; $F_{1,22}=34.5$; $p<0.01$). Alpha was extended by a similar duration of $2.1\pm 0.2$ hr ($PER2$: 13.9$\pm 0.1$ hr; wild-type: 11.8$\pm 0.3$ hr; $F_{1,22}=19.2$; $p<0.01$). PER2-mutant mice were 1.3 times more active than wild-type mice across the nocturnal active period (see Table 7; Total Activity: $F_{1,22}=34.5$; Bout Duration: $F_{1,22}=47.0$; both, $p<0.01$). In PER2-mutant mice, acamprosate reduced ethanol intake and preference from baseline values by $15.3\pm 6.9\%$ and $14.2\pm 3.4\%$ over $8\pm 4$ days and $6\pm 2$ days of ethanol reintroduction, respectively (see Table 6; both, $p<0.01$). In wild-types, acamprosate decreased ethanol intake and preference from baseline values by $38.7\pm 12.4\%$ and $41.0\pm 3.8\%$ over $22\pm 2$ days and $24\pm 2$ days of ethanol reintroduction, respectively (see Table 6; both, $p<0.01$). Higher values of ethanol intake and preference were maintained in PER2-mutant mice compared to wild-type mice across acamprosate treatment ($Intake: F_{1,22}=22.4$; $Preference: F_{1,22}=17.2$; both, $p<0.01$). Ethanol intake and preference did not differ from baseline values in PER2-mutant and wild-type mice with blank pellets (see Table 6; $p>0.05$). A photomicrograph of PPT micropellet implantation is found in Fig. 32B.
Nucleus Accumbens. At baseline, ethanol intake and preference were greater in PER2-mutant vs. wild-type mice (see Table 6; Intake: $F_{1,22}=64.1$; Preference: $F_{1,22}=65.0$; both, $p<0.01$). The PER2-mutant mice began their nocturnal active period 2.0±0.4 hr earlier than that of the wild-type mice (PER2: ZT 10.5±0.4; wild-type: ZT 12.5±0.4; $F_{1,22}=14.5, p<0.01$). Alpha was extended by a similar duration of 2.2±0.2 hr (PER2: 13.7±0.4 hr; wild-type: 11.5±0.2 hr; $F_{1,22}=14.0, p<0.01$). PER2-mutant mice were 1.7 times more active across the nighttime compared to wild-type mice (see Table 7; Total Activity: $F_{1,22}=24.9$; Bout Duration: $F_{1,22}=17.6$; both, $p<0.01$). In wild-type mice, acamprosate suppressed ethanol intake and preference from baseline values by 23.1±12.4% and 22.0±5.4% over 16±4 days and 20±2 days of ethanol reintroduction, respectively (see Table 6; both, $p<0.01$). Higher values of ethanol intake and preference were maintained in PER2-mutant mice compared to wild-type mice across acamprosate treatment (Intake: $F_{1,22}=16.3$; Preference: $F_{1,22}=18.8$; both, $p<0.01$). Ethanol intake and preference did not differ from baseline measures in PER2-mutant mice with acamprosate or blank pellets or in wild-type mice with blank pellets (see Table 6; all, $p>0.05$). In wild-types, acamprosate increased total nocturnal activity and bout duration from baseline values (and compared to mic with blank pellets) by 131.6±3.6% and 275.5±70.4% over 24±4 days of ethanol reintroduction before returning to baseline levels (see Table 7; Total Activity: $F_{1,10}=9.0$; Bout Duration: $F_{1,22}=18.7$; all, $p<0.01$). Nocturnal activity measures did not differ from baseline values in PER2-mutant mice with acamprosate
or blank pellets or in wild-type mice with blank pellets (all, p>0.05). Representative actograms and a photomicrograph of accumbal micropellet implantation are found in Fig. 34 and Fig. 32C, respectively.
Figure 34: Representative actograms PER2-mutant and WT mice with constant-release acamprosate or blank wax micropellets (A: PER2-mutant acamprosate; B: PER2-mutant wax; C: wild-type acamprosate; D: wild-type wax). White-dark bars represent the light-dark photoperiods. Surgery represents surgical preparation and the subsequent recovery period.
Intergeniculate Leaflet. At baseline, PER2-mutant mice had elevated levels of ethanol intake and preference compared to wild-types (see Table 6; Intake: $F_{1,22}=95.4$; Preference: $F_{1,22}=67.5$; both, $p<0.01$), and the nighttime activity period of PER2-mutant mice began $2.1\pm0.2$ hr before that of the wild-type mice (PER2: ZT $10.0\pm0.4$; wild-type: ZT $12.1\pm0.2$; $F_{1,22}=47.1$; $p<0.01$). Alpha was extended by $1.6\pm0.3$ hr in PER2-mutant mice (PER2: $13.6\pm0.5$ hr; wild-type: $12.0\pm0.2$ hr; $F_{1,22}=55.1$; $p<0.01$). PER2-mutant mice had 1.6 times more activity across their nighttime activity period compared to wild-types (see Table 7; Total Activity: $F_{1,22}=101.2$; Bout Duration: $F_{1,22}=169.7$; both, $p<0.01$). In wild-types, acamprosate suppressed ethanol intake and preference from baseline levels by $38.5\pm18.9$% and $28.8\pm7.8$% over $8\pm2$ days and $16\pm6$ days of ethanol reintroduction, respectively (see Table 6; both, $p<0.05$). Higher values of ethanol intake and preference were maintained in PER2-mutant mice compared to wild-type mice across acamprosate treatment (Intake: $F_{1,22}=22.1$; Preference: $F_{1,22}=16.5$; both, $p<0.01$). Ethanol intake and preference did not differ from baseline values in PER2-mutant mice with acamprosate or blank pellets or in wild-types with blank pellets (all, $p>0.05$). In both strains and treatment groups, IGL implants did not alter nighttime activity measures (see Table 7; all, $p>0.05$). A photomicrograph of IGL micropellet implantation is found in Fig. 32D.
**Suprachiasmatic Nucleus.** At baseline, PER2-mutant mice had greater levels of ethanol intake and preference compared to wild-types (see Table 6; *Intake*: $F_{1,22}=63.4$; *Preference*: $F_{1,22}=71.4$; both, $p<0.01$), and the nighttime activity period of PER2-mutant mice began $2.5\pm0.3$ hr before that of the wild-type mice (*PER2*: ZT $9.5\pm0.6$; *wild-type*: ZT $12.0\pm0.8$; $F_{1,22}=33.9$; $p<0.01$). Alpha was extended by $2.2\pm0.2$ hr in PER2-mutant mice (*PER2*: $13.5\pm0.4$ hr; *wild-type*: $11.2\pm0.4$ hr; $F_{1,22}=36.5$; $p<0.01$). PER2-mutant mice had 2.2 times more activity across their nighttime activity period compared to wild-types (see Table 7; *Total Activity*: $F_{1,22}=74.5$; *Bout Duration*: $F_{1,22}=55.2$; both, $p<0.01$). In PER2-mutant mice, acamprosate reduced ethanol intake and preference from baseline values by $33.3\pm10.9\%$ and $28.8\pm4.7\%$ over $14\pm4$ days and $20\pm2$ days of ethanol reintroduction, respectively (see Table 6; both, $p<0.01$). In wild-type mice, acamprosate reduced ethanol intake and preference from baseline values by $39.1\pm17.3\%$ and $41.1\pm5.9\%$ over $14\pm4$ days and $16\pm2$ days of ethanol reintroduction, respectively (see Table 6; both, $p<0.05$). Higher values of ethanol intake and preference were maintained in PER2-mutant mice compared to wild-type mice across acamprosate treatment (*Intake*: $F_{1,22}=40.0$; *Preference*: $F_{1,22}=9.4$; both, $p<0.01$). Ethanol intake and preference did not differ from baseline values in PER2-mutant or wild-type mice with blank pellets (all, $p>0.05$). During the first three days of calibrated-acamprosate release, both PER2-mutant and wild-type mice were continuously active independent of the dark- or light-phase of LD for $25.2\pm8.3$ hr and $38.8\pm18.0$ hr, respectively ($F_{1,22}=0.4$; $p>0.05$;
p<0.01, compared to baseline values). Thereafter, total nocturnal activity and bout duration in PER2-mutant and wild-type mice were increased from baseline values (and compared to mice with blank pellets) by 142.2±13.2% and 116.8±6.3% and 381.1±35.3% and 231.4±16.7% over 20±4 days and 22±4 days of ethanol reintroduction, respectively, before returning to baseline levels (see Table 7; Total Activity: F_{1,10}=3.7; Bout Duration: F_{1,22}=5.0; all, p<0.01). Representative actograms and photomicrographs of SCN micropellet implantation are found in Fig. 35 and 32E.
Figure 35: Representative actograms PER2-mutant and WT mice with constant-release acamprosate or blank wax micropellets (A: PER2-mutant acamprosate; B: PER2-mutant wax; C: wild-type acamprosate; D: wild-type wax). White-dark bars represent the light-dark photoperiods. Surgery represents surgical preparation and the subsequent recovery period.
**Hippocampal Fissure.** At baseline, levels of ethanol intake and preference were greater in PER2-mutant mice compared to wild-type mice (see Table 6; Intake: F$_{1,22}$=95.4; Preference: F$_{1,22}$=67.5; both, p<0.01). The nighttime activity period of PER2-mutant mice was advanced by 2.3±0.1 hr and (ZT 9.6±0.2 vs. ZT 11.9±0.2, respectively; F$_{1,22}$=33.4; p<0.01) alpha was extended by 2.0±0.1 hr (PER2: 14.6±0.6 hr; wild-type: 12.6±0.5 hr; F$_{1,22}$=53.0; p<0.01). PER2-mutant mice had 1.5 times more activity across the nighttime activity period compared to wild-types (see Table 7; Total Activity: F$_{1,22}$=28.3; Bout Duration: F$_{1,22}$=98.4; both, p<0.01). Acamprosate did not alter ethanol intake, preference, and the intensity of nighttime circadian activity from baseline values in both PER2-mutant and wild-type mice with acamprosate or blank pellets (see Tables 6 and 7; all, p>0.05). Higher values of ethanol intake and preference were maintained across ethanol re-introduction in PER2-mutant compared to wild-type mice (Intake: F$_{1,22}$=95.4; Preference: F$_{1,22}$=67.5; both, p<0.01). A photomicrograph of hippocampal micropellet implantation is found in Fig. 32F. Schematics of brain area responses to acamprosate suppression of ethanol intake and preference are found in Figs. 36-41.
**Figure 36:** Means+SEM of EtOH intake at baseline (days 1-10) and following the implantation of acamprosate-releasing (ACAMP) or blank (WAX) micropellets into the ventral tegmental area [VTA] (A), pedunculopontine tegmentum [PPT] (B), nucleus accumbens [NAc/s] (C), intergeniculate leaflet [IGL] (D), suprachiasmatic nucleus [SCN] (E), and hippocampal fissure (F) in PER2-mutant (PER2 KO) and wild-type (WT) mice with 15% free-choice ethanol access. Different letters represent significant differences in EtOH intake from baseline levels (p<0.05). Arrows represent significant differences in EtOH intake between strains. Hatched/dotted lines represent baseline measures of EtOH intake.
**Figure 37**: Means+SEM of EtOH preference at baseline (days 1-10) and following the implantation of acamprosate-releasing (ACAMP) or blank (WAX) micropellets into the ventral tegmental area (VTA) (A), pedunculopontine tegmentum (PPT) (B), nucleus accumbens (NAc/s) (C), intergeniculate leaflet (IGL) (D), suprachiasmatic nucleus (SCN) (E), and hippocampal fissure (F) in PER2-mutant (PER2 KO) and wild-type (WT) mice with 15% free-choice ethanol access. Different letters represent significant differences in EtOH preference from baseline levels (p<0.05). Arrows represent significant differences in EtOH preference between strains. Hatched/dotted lines represent baseline measures of EtOH preference.
Figure 38: [Left panel] Percent change of ethanol intake + SE from pretreatment levels during the release of acamprosate (ACAMP) or no acamprosate (BLANK) from implants in reward areas (the ventral tegmental area [VTA; top]; pedunculopontine tegmentum [PPT; middle]; nucleus accumbens [NAc; bottom]) of wild-type (WT) and PER2-mutant (PER2 KO) mice under a 15% v/v free choice ethanol relapse paradigm (EtOH INTRO-WITHDRAWAL-EtOH REINTRO). Different letters represent significant differences in EtOH intake from pretreatment levels (p<0.05). Asterisks represent significant differences in EtOH intake between treatment groups (p<0.05). [Right panel] The representative time point of maximal suppression of ethanol intake by acamprosate implants in wild-type (WT) and PER2-mutant (PER2 KO) mice. Asterisk denote significant differences in EtOH intake between strains (p<0.05).
Figure 39: [Left panel] Percent change of ethanol preference±SE from pretreatment levels during the release of acamprosate (ACAMP) or no acamprosate (BLANK) from implants in reward areas (ventral tegmental area [VTA; top]; pedunculopontine tegmentum [PPT; middle]; nucleus accumbens [NAc; bottom]) of wild-type (WT) and PER2-mutant (PER2 KO) mice under a 15% v/v free choice ethanol relapse paradigm (EtOH INTRO-WITHDRAWAL-EtOH REINTRO). Different letters represent significant differences in EtOH preference from pretreatment levels (p<0.05). Asterisks represent significant differences in EtOH preference between treatment groups (p<0.05). [Right panel] The representative time point of maximal suppression of ethanol preference by acamprosate implants in wild-type (WT) and PER2-mutant (PER2 KO) mice. Asterisk denote significant differences in EtOH preference between strains (p<0.05).
Figure 40: [Left panel] Percent change of ethanol intake+SE from pretreatment levels during the release of acamprosate (ACAMP) or no acamprosate (BLANK) from implants in circadian (suprachiasmatic nucleus [SCN; top]; intergeniculate leaflet [IGL; middle]) or control (hippocampus [HP; bottom]) areas of wild-type (WT) and PER2-mutant (PER2 KO) mice under a 15% v/v free choice ethanol relapse paradigm (EtOH INTRO-WITHDRAWAL-EtOH REINTRO). Different letters represent significant differences in EtOH intake from pretreatment levels (p<0.05). Asterisks represent significant differences in EtOH intake between treatment groups (p<0.05). [Right panel] The representative time point of maximal suppression of ethanol intake by acamprosate implants in wild-type (WT) and PER2-mutant (PER2 KO) mice. Asterisk denote significant differences in EtOH intake between strains (p<0.05).
Figure 41: [Left panel] Percent change of ethanol preference+SE from pretreatment levels during the release of acamprosate (ACAMP) or no acamprosate (BLANK) from implants in circadian (suprachiasmatic nucleus [SCN; top]; intergeniculate leaflet [IGL; middle]) or control (hippocampus [HP; bottom]) areas of wild-type (WT) and PER2-mutant (PER2 KO) mice under a 15% v/v free choice ethanol relapse paradigm (EtOH INTRO-WITHDRAWAL-EtOH REINTRO). Different letters represent significant differences in EtOH preference from pretreatment levels (p<0.05). Asterisks represent significant differences in EtOH preference between treatment groups (p<0.05). [Right panel] The representative time point of maximal suppression of ethanol preference by acamprosate implants in wild-type (WT) and PER2-mutant (PER2 KO) mice. Asterisk denote significant differences in EtOH preference between strains (p<0.05).
**Effects of acamprosate treatment and its brain sites of action on water intake**

Pretreatment levels of water intake did not differ between PER2-mutant and WT mice (96.0±1.8 ml/kg/day and 96.2±1.6 ml/kg/day, respectively; F(1,22)= 1.1; p>0.05; **Fig. 7**). Across brain areas of implant and strain, there was a significant effect for treatment such that mice with acamprosate implants consumed more water compared to mice with blank implants (123.1±4.4 ml/kg/day and 103.8±3.5 ml/kg/day, respectively; F(1,22)=46.5; p<0.01; **Fig. 7**). Within acamprosate treatment groups, there was a significant effect for strain such that WT mice consumed more water compared to PER2-mutants (125.9±4.3 ml/kg/day and 112.5±3.3 ml/kg/day, respectively; F(1,10)=66.5; p<0.01) and a significant effect for brain area of implant such that mice with acamprosate implants in reward and circadian brain areas consumed more water compared to mice with acamprosate implants in the hippocampus (132.0±3.1 ml/kg/day and 94.8±3.6 ml/kg/day respectively; F(1,22)=8.5; p<0.05). Water intake did not differ between blank treatment groups (p>0.05). Means+SEM of water intake in PER2-mutant and wild-type mice prior to and during acamprosate treatment are found in **Fig. 42**.
Figure 42: Means+SEM of water intake prior to (left panel) and during calibrated acamprosate release or blank micro-pellet implantation in the ventral tegmental area [VTA], pedunculopontine tegmentum [PPT], nucleus accumbens [NAc/s], intergeniculate leaflet [IGL], suprachiasmatic nucleus [SCN], and hippocampal fissure in PER2-mutant (PER2 KO) and wild-type (WT) mice.
Ethanol-Specific Disruption to Behavioral Circadian Entrainment

Experiment 16: Cocaine Attenuates Photic Phase-Resetting of Behavioral Circadian Rhythms.

There were significant cocaine and strain differences in the phase-delaying response to photic stimulation at ZT 16. In C57 albino mice, vehicle controls receiving i.p. saline injection had light-induced phase-delay shifts averaging 1.50±0.10 hr. Mice receiving pre-treatment with 20 mg/kg cocaine had significantly attenuated phase-delay shifts averaging only 0.60±0.20 hr (F_{1,6} = 15.9; p<0.01; Fig. 43). Cocaine in the absence of a light pulse had no shifting response (0.00±0.00 hr; p=1.00). In a similar trial in PER2 mutants, vehicle controls had light-induced phase-shifts averaging 2.54±0.44 hr, while mutant mice receiving cocaine showed no phase-delay shifting response (0.01±0.37 hr; F_{1,6} = 20.0; p<0.01). Representative actograms for this experiment are shown in Fig. 44.
**Figure 43:** Acute systemic cocaine attenuates photic phase-delay responses to a light pulse at ZT 16 in C57 albino mice (left panel) and completely blocks these responses in PER2-mutant mice (right panel). Bars with different letters are significantly different (p<0.05). Bars represent means ± SE.
Figure 44: Representative double-plotted actograms of general locomotor activity showing cocaine inhibition of photic phase-delay responses to a light pulse delivered at ZT 16. A, B: C57 albino mice; C, D: PER2-mutant mice. Arrows denote time of cocaine injection and subsequent light pulse.
Experiment 17: Cocaine Induces Circadian Phase-Resetting at Midday.

Acute i.p. injection of cocaine at midday (ZT 6) in C57 albino mice induced phase-advance shifts averaging 1.0±0.3 hr, vs. 0.3±0.1 hr and 0.1±0.1 hr hr for saline and uninjected controls, respectively (F<sub>2,7</sub> = 9.8; p<0.01). A similar cocaine response was obtained in C57BL mice, where cocaine induced advance shifts averaging 0.7±0.1 hr vs. 0.2±0.1 hr and 0.0±0.0 hr for saline and uninjected controls, respectively (F<sub>2,12</sub> = 22.3; p<0.01). In contrast, this cocaine treatment in PER2 mutant mice at midday (but not at night [see section above]) produced considerably larger phase-advance shifts, averaging 2.1±0.4 hr, vs. 0.5±0.3 hr and 0.9±0.2 hr for saline and uninjected controls, respectively (F<sub>2,12</sub> = 10.5; p<0.01; Fig. 45). The PER2 mutant response to cocaine was ~2-3-fold greater than for the other strains (p<0.05). Results from the non-injected controls revealed that the PER2 mutants unlike the other strains had a phase-resetting response to the release into DD at midday. Representative actograms of the 3 treatment groups are shown in Fig. 46.
Figure 45: Phase-advance shifting effect of acute systemic cocaine at midday (ZT 6) in C57BL mice (left panel), C57 albino mice (middle panel) and PER2-mutant mice (right panel). Note the greater shifting responses of the PER2-mutant group. Bars with different letters are significantly different (p<0.05). Bars represent means ± SE.
Figure 46: Representative double-plotted actograms of general locomotor activity showing cocaine-induced phase-advance shifts at midday (ZT 6). A-C, C57BL mice; D-F, C57 albino mice; G-I, PER2-mutant mice. Arrows denote time of cocaine injection and release into constant darkness.
Experiment 18: The SCN is a target for cocaine.

Localized perfusion of the SCN region with cocaine (115 µM estimated tissue concentration) at midday in C57 albino mice markedly advanced circadian phase. Controls receiving SCN ACSF perfusion alone had advance shifts averaging 0.3±0.2 hr, whereas mice receiving SCN cocaine perfusion exhibited advance shifts averaging 3.7±0.9 hr (F_{1,12}=16.4; p<0.01; Fig. 47).
Figure 47. Direct reverse microdialysis perfusion of the SCN with cocaine at midday (ZT 6) advances clock phase in C57 albino mice. This effect was significantly different (*p<0.05) from controls perfused with artificial cerebrospinal fluid alone.
Experiment 19: Cocaine disrupts circadian behavioral activity.

Retrospective actogram analysis revealed that a single i.p. cocaine injection at ZT 16 elevated locomotor activity levels for at least 10 days under DD conditions in C57 albinos. Activity (assessed as total counts registered by the infrared sensors over the subjective day [rest period] and subjective night [active period]) prior to treatment did not differ between the cocaine vs. saline groups during the subjective day (F\(_{1,6}\) = 0.1; p>0.1) or subjective night (F\(_{1,6}\) = 0.8; p>0.1; Fig. 48). Activity averaged over the 10 day post-injection period, however, revealed a significant prolonged elevation for the cocaine vs. saline groups during the subjective day (F\(_{1,6}\) = 14.4; p<0.05) and subjective night (F\(_{1,6}\) = 8.9; p<0.05). There was no effect of this cocaine treatment on the free-running period (tau; 23.89±0.07 hr [cocaine]; 23.82±0.03 hr [saline] F\(_{1,6}\)=0.87; p>0.05). There were no effects of the acute cocaine treatment on any of these circadian parameters in the PER2 mutants. Representative actograms for this experiment are presented in Fig. 49.
**Figure 48:** Acute cocaine causes long-term increases in activity measured during the subjective day and night in C57 albino and PER2-mutant released to constant darkness following a light pulse delivered at ZT 16. The right panel shows daily activity averaged over the 10 day interval immediately following cocaine or saline treatment.
Figure 49. Representative double-plotted actograms of general locomotor activity illustrating the long-term effects of acute cocaine. Shaded areas designate time of injection and release to constant darkness.
CHAPTER IV
DISCUSSION

Disruptive Effects of Chronic Ethanol on Murine Photic Entrainment and the Temporal Structure of Circadian Activity.

It is evident from the present results and from previous studies that chronic ethanol intake and withdrawal markedly disrupt circadian photic entrainment and the temporal structure of the daily circadian locomotor activity pattern (Ruby et al., 2009b; Seggio et al., 2009). It is probable that these and related ethanol-induced circadian disruptions contribute to the numerous destructive effects of alcoholism on critical homeostatic functions, such as the sleep-wake cycle (Brower, 2001; Roehrs and Roth, 2001), endocrine secretion (Röjdmark et al., 1993; Kühlwein et al. 2003, Fonzi et al. 1994; Mukai et al., 1998; Schmitz et al., 1996) and body temperature (Wasielewski and Holloway, 2001). Also, as in chronically drinking hamsters (Ruby et al., 2009b), a circadian rhythm of ethanol content in the SCN of drinking mice exists with nighttime extracellular levels (~20-30 mM) high enough to directly attenuate SCN photic phase-resetting as we have reported in hamsters in vivo and mice in vitro (Ruby et al., 2009b; Prosser et al., 2008). Thus, the SCN is a potentially vulnerable target for ethanol’s disruptive effects on circadian timing.

Chronic Ethanol Pharmacokinetics. This study is the first to characterize the 24 hr
profile of brain ethanol levels concomitantly with systemic levels and drinking in freely-behaving mice. The present analyses reveal a daily rhythm in mouse ethanol drinking, and associated SCN ethanol concentrations in mice under LD. For mice drinking 10% and 15% ethanol, peak SCN ethanol concentrations (averaging ~10-20 mM and ~20-30 mM, respectively) were observed during the dark-phase, with smaller sporadic drinking bouts during the day. Notably, each ethanol drinking episode preceded a peak in SCN ethanol concentration by 20-40 min, and individual peak durations ranging from 30-60 min. It is notable that these brain ethanol concentrations: 1) increase during the phase-delaying portion of the photic PRC; and 2) are large enough to potentially inhibit photic phase-resetting as determined in our previous experiments (Ruby et al., 2009a,b). These concentrations and time-course of ethanol metabolism are comparable to those measured by microdialysis within the medial preoptic nucleus (see Figure 10) and in the nucleus accumbens of rats (~16 mM; Nurmi et al., 1999) and mice (12 mM; Griffin et al., 2007) during voluntary limited access drinking. Also, in hamsters drinking 20% ethanol, SCN ethanol concentrations reach ~20 mM across the early subjective night (Ruby et al., 2009b). The present simultaneous measurements of the daily patterns of SCN, subcutaneous ethanol and drinking reveal that systemic ethanol levels are approximately double those in the SCN, while the temporal profiles are essentially identical. This is similar to our previous analyses in the hamster (Ruby et al., 2009b). It is important to note here that subcutaneous, rather than blood sampling of ethanol, was used as it is noninvasive, follows a similar time course (Ruby et al., 2009b), and
offers a more comprehensive picture of the daily profile of systemic ethanol with short (30 min) sampling intervals than that usually obtained from blood sampling.

**Chronic Ethanol and Photic Phase-Resetting.** We and others have shown that chronic ethanol drinking dose-dependently inhibits photic phase-resetting of the circadian locomotor activity rhythm in hamsters and mice (Ruby et al., 2009b; Seggio et al., 2009). Interestingly, in hamsters ethanol attenuates photic phase-advances but not phase-delays, while in mice, ethanol dose-dependently inhibits phase-delays, but not phase-advances (Seggio et al., 2009). Thus, there is an apparent species difference in the inhibitory action of ethanol on the photic PRC.

The neurophysiological basis of this difference is speculative, but could reflect a differential effect of ethanol on the photic signaling cascade downstream from nitric oxide (NO) production, where the pathways mediating phase-advances and phase-delays bifurcate (reviewed in Gillette and Mitchell, 2002). Possible targets for ethanol suggested by recent studies are microRNAs, as they are necessary for photic phase-resetting (Cheng et al., 2007) and their functions are perturbed by ethanol (Sathyan et al., 2007). If true, this would imply that ethanol does not inhibit steps proximal to NO production, including light-induced glutamate release from RHT terminals and subsequent NMDA receptor activation. Conversely, the lack of effect on phase-advances in mice could be due to lower levels of drinking during the late subjective night (the phase-advancing potion of the photic PRC). This is unlikely, however, as our pharmacokinetic analyses reveal that SCN ethanol levels are generally high at this time. A third possibility is that the SCN undergoes rapid
tolerance to the ethanol consumed during the night (Prosser and Glass, 2009),
which could decrease its inhibition of photic phase-advances. Finally, these results
may be explained by species differences in photic PRCs. Hamsters show large
photic phase-advances but smaller phase-delays, while the opposite is true for mice.
Ethanol effects on the on the more modest phase-shifts may exist, but may be too
difficult to detect.

With respect to the attenuation of in vivo photic phase-delays following the ~1
day withdrawal from chronic ethanol seen here in mice, we previously found that
withdrawal enhances photic-phase advances in hamsters 2-3 days (but not 1 day)
after withdrawal (Ruby et al., 2009b). Chronic ethanol suppresses glutamate
(NMDA) receptor-mediated photic signaling (Dodd et al., 2000; Krystal et al., 2003;
Prosser et al., 2008), which leads to an upregulation of NMDA receptors (Floyd et
al., 2003; Iorio et al., 1992). This upregulation could explain the enhanced photic
phase-resetting response seen in hamsters, but not the reduced phase response
seen 1 day after withdrawal in mice. The reason for the dissimilarity between the two
studies is unknown, but could be due to possible differential effects of ethanol
withdrawal on phase-advance vs. phase-delay shifting responses, differences in
concentrations of ethanol used (20% for hamsters and 10 and 15% for mice) or
perhaps species or other differences in the time-course for withdrawal-induced
effects. Interestingly, microdialysis measurements of glutamate output during
ethanol withdrawal in rats have revealed that glutamate release is more than
doubled from 3-12 hrs post-withdrawal and subsides within 36 hrs (Rosetti and
Carboni, 1995). It is thus plausible that within the first day of withdrawal, this enhanced release downregulates SCN glutamate receptors, resulting in the attenuated photic shifts reported here. This, however, would not explain the enhanced photic shifting responses seen in hamsters.

Disturbance of overt circadian behavioral rhythmicity is a hallmark of alcohol abuse in humans (Wasielewski and Holloway, 2001; Brower, 2001). The present results suggest the same for nocturnal animal models, as chronic ethanol consumption in mice and hamsters under LD significantly disrupts the pattern of daily locomotor activity. Specifically, in mice, the number of activity bouts during the night (active period) is increased and the average bout duration is decreased by as much as 40-50% compared to water controls. These quantitative analyses reflect a less consolidated, sporadic pattern of locomotor activity during the active period. In hamsters, chronic ethanol decreased the number of activity bouts during the night, albeit with increased bout duration (Ruby et al., 2009b). Using combined activity and drinking analyses, we have observed that locomotor activity is closely aligned with drinking bouts. Therefore, the decreased activity during the active phase in drinking animals could reflect a direct disruptive action of ethanol on circadian clock pathways regulating behavioral activity. Alternatively, it could reflect a masking (inhibitory) effect of ethanol drinking on activity, possibly related to drinking-induced hypothermia known to suppress locomotor activity (Wasielewski and Holloway, 2001) or the sedative properties of ethanol. This is consistent with the finding that in hamsters under chronic ethanol treatment in free-choice with water daily wheel-
running distance is negatively correlated with daily ethanol consumption (Hammer and Glass, unpublished observations).

*Chronic Ethanol and Photic Entrainment.* Groups of mice maintained on water or forced 15% ethanol were placed under a skeleton photoperiod consisting of a scheduled daily 1 min light pulse delivered at original ZT 6 or ZT 11 to test entrainment-related actions of ethanol under a weak photic zeitgeber with little masking. Each group advanced into the light pulse with activity onsets aligned with the light pulse with a phase angle of approximately -1 hr. Consistent with a previous study on ethanol drinking on re-entrainment in hamsters (Mistlberger and Nadeau, 1992), there was no effect of ethanol on the rate of re-entrainment to a large (6 hr) shift of the LD cycle. With regard to the effects of ethanol on photic entrainment, we and others have observed no effects of ethanol drinking on entrainment of the locomotor activity rhythm in hamsters, rats or mice maintained under standard laboratory photocycles. Notably, we have also seen no disruption in entrainment by chronic ethanol in hamsters maintained under 14L:10D at weak light intensities (0.5-5.0 lux; Glass and Ruby, unpublished data). Given that chronic ethanol markedly inhibits light pulse-induced phase-shifting (present results; Ruby et al., 2009b; Seggio et al., 2009), and alters free-running tau (Mistlberger and Nadeau, 1992; Seggio et al., 2009), it seems reasonable to speculate *a priori* that ethanol would disrupt photic entrainment. However, there are several reasons why previous studies have not demonstrated such an effect. First is the issue of tolerance, which could develop over a short time period and negate the disruptive neurologic effects of
ethanol on photic signaling in the SCN (Prosser and Glass, 2009). Second, the brighter light intensities commonly used for standard LD cycles may produce photic signaling in the SCN at a strength that negates ethanol’s inhibitory effect on this signaling (Ruby et al., 2009b). A third possibility is a masking effect of light on the sleep-activity rhythm, whereby locomotor activity is suppressed by light and stimulated by darkness, producing a seemingly entrained circadian rhythm, irrespective of ethanol-disrupted clock function (Aschoff et al., 1973). This latter possibility is consonant with the present results, in that ethanol disrupted stable photic entrainment under conditions relatively devoid of masking by light, and in particular, attenuated the robust onset of activity associated with the onset of the subjective night. In the context of such masking, it will be important to explore the effects of ethanol consumption on entrainment of non-behavioral rhythms, such as melatonin or corticosterone. This is especially underscored by the findings that the circadian timing of rhythms in blood glucose and cholesterol are markedly altered by daily ethanol administration (Rajakrishnan et al., 1999).

In conclusion, chronic ethanol consumption in mice produces marked disturbances in circadian photic entrainment capacity and circadian locomotor activity patterns. These assessments are relevant to potential disruptive effects of alcohol in individuals engaging in rotating shift work and rapid trans-meridian travel. Alcohol abuse and abstinence may impair adjustment to a physically demanding work schedule and new time zone, creating a vicious cycle of alcohol dependence and chronobiological disturbance. These disruptions may not only impact cognitive
performance and potentially increase workplace accidents, but also underlie the development of other serious physical and mental health problems, including coronary disease and depression.

**Disruptive Effects of Acute EtOH on Murine Photic and Serotonergic Phase-Resetting of Behavioral Rhythms**

Alcohol abuse and subsequent withdrawal are highly disruptive to circadian clock-regulated homeostatic functions, including the sleep-wake cycle, metabolism, and feeding (Chen et al., 2004; Wasielewski and Holloway, 2001; Brower, 2001; Sack et al., 2007; Thiele et al., 2003, Soookoian et al., 2007; Röjdmark et al., 1993; Iranmanesh 1989; Kühlwein et al. 2003, Fonzi et al. 1994; Mukai et al., 1998). Chronic alcohol abuse can promote desynchronization of such rhythms with respect to the LD cycle and to internal SCN clock timing. This lack of internal rhythm coordination together with a loss of control from environmental timekeeping cues ultimately increases the risk of pathologies including diabetes, obesity, ulcers, sleep/circadian rhythm disorders, cardiovascular pathology and cancer (Thiele et al., 2003; Soookoian et al., 2007; Sack et al., 2007; Brower, 2001; Wasielewski and Holloway et al., 2001; Davis and Mirick, 2006). As the temporal regulation of circadian clock-generated rhythms is mediated by photic and non-photic entrainment inputs to the SCN, it is possible that these pathways could be vulnerable to the adverse chronobiological effects of ethanol. This assumption has been borne out by
in vivo studies in hamsters and mice that have characterized ethanol's impairment of photic and non-photic signaling processes (Brager et al., 2010; Ruby et al., 2009a, b; al., Seggio et al., 2007, 2009). The present results extend these findings, showing that acute ethanol strongly inhibits phase-resetting responses of the mouse circadian clock to photic and non-photic (serotonergic) signals. Interestingly, the intra-SCN reverse microdialysis ethanol perfusion trials revealed that ethanol's inhibition of photic, but not serotonergic phase-resetting is manifested within the SCN itself. This latter result strongly implicates the SCN as a target for ethanol's disruption of photic phase-resetting, but that ethanol's inhibition of serotonergic phase resetting involves an extra-SCN site. Collectively, these findings are the first evidence that acute ethanol can affect phase-resetting mechanisms of the circadian clock in mice, and suggest that the SCN represents a critical target for ethanol's disruptive effects on photic but not non-photic phase-resetting mechanisms.

It is apparent that the extent of attenuation of photic shifting associated with the intra-SCN ethanol perfusion was greater than that produced by i.p. injection (~75% vs. ~50%, respectively). This result indicates an increased effectiveness of ethanol when delivered directly to the SCN. The concentrations of ethanol in the SCN (~40-60 mM) were equivalent for both applications, so the reason for this difference is unclear. Possibly it could have been due to the lack of behavioral/whole-body physiological responses to isolated SCN ethanol treatment that are manifest after systemic treatment. Another possibility is the difference in pharmacokinetics of ethanol in the SCN between the two treatment modes. Also,
there may have been an effect of the different times of light+ethanol delivery (ZT 16 and ZT 14, respectively for the systemic and intra-SCN treatments), as control photic phase-delay responses were greater at ZT 14 than at ZT 16. Regardless of the magnitude of effects of the different treatments, however, it is nevertheless clear that intra-SCN ethanol treatment significantly attenuated photic phase-resetting.

**Ethanol disrupts photic phase-resetting.** Treatment with ethanol in hamsters and mice has been shown to strongly suppress photic phase-resetting in a species-specific manner. In hamsters, acute or chronic ethanol administration inhibits phase-advances but not phase-delays, while in mice, chronic ethanol treatment inhibits phase-delays but not phase-advances (Ruby et al., 2009b; Brager et al., 2020; Seggio et al., 2009). Here we show further that photic phase-delays in mice are also inhibited by acute systemic and intra-SCN ethanol treatments. Importantly, this inhibition of photic phase-resetting by intra-SCN perfusion of ethanol in the mouse (present study) and in the hamster (Ruby et al., 2009a) as well as the inhibition of the phase-resetting action of glutamate on spontaneous electrical activity of the isolated mouse SCN slice (Prosser et al., 2008) strongly suggests that the inhibitory action of ethanol occurs within the SCN photic signaling cascade. A plausible target for this action is the NMDA receptor, since ethanol's actions on this receptor are implicated in intoxication, tolerance and addiction (Krystal et al., 2003; Dodd et al., 2000; Floyd et al., 2003). Moreover, the NR2B subunit of the NMDA receptor complex is sensitive to ethanol (Krystal et al., 2003; Dodd et al., 2000), and this
subunit is necessary for mediating the photic phase-resetting effects of glutamate within the SCN (Wang et al., 2008; Mintz et al., 1999). Another possibility is that ethanol could act upstream from the NMDA receptor by inhibiting the action of brain-derived neurotrophic factor (BDNF) that is also a necessary component in the photic phase-resetting pathway. Evidence for such an action are findings that ethanol inhibits BDNF-induced increases in glutamate signaling in the hippocampus (Kolb et al., 2005), and application of BDNF can reverse the inhibitory action of ethanol on glutamate-induced shifts in the mouse SCN slice (Prosser et al., 2008). A third possibility, suggested by recent studies involves microRNAs, which are required for photic phase-resetting (Cheng et al., 2007) and whose functions are perturbed by ethanol (Sathyan et al., 2007; Dippold et al., 2010). Thus, from the foregoing it is likely that ethanol could act at any of multiple steps of the SCN photic signaling cascade to disrupt this phase-resetting.

_Ethanol disrupts serotonergic phase-resetting._ Serotonergic signaling from neurons of the midbrain raphe nuclear complex is implicated in behavioral phase-resetting (Glass et al., 2000, 2003; Grossman et al., 2000; Meyer-Bernstein et al., 1997) as well as modulating photic input to the SCN (Rea et al., 1994; Grossman et al., 2000; Mistlberger and Antle, 1998). The role for 5-HT in non-photic phase-resetting is supported in part by the potent shifting actions of 5-HT agonists, principally 8-OH-DPAT, _in vivo_ (Ehlen et al., 2001; Horikawa and Shibata, 2004) and _in vitro_ (Prosser et al., 1993, 2003; Shibata et al., 1992). This 5-HT_{1A,7} receptor agonist has been used widely to study non-photic mechanisms, and has a phase-response curve
(PRC) similar to behavioral PRCs (Mrosovsky, 1988; Reebs and Mrosovsky, 1989; Grossman et al., 2000; reviewed in Mistlberger et al., 2000). It also produces shifts of similar magnitude as behavioral stimuli (Knoch et al., 2004; reviewed in Mistlberger et al., 2000). Consistent with our previous report in hamsters (Ruby et al., 2009a), acute i.p. ethanol injection in mice inhibits 8-OH-DPAT phase-advance shifting. In hamsters, a dose of 2 g/kg suppressed 8-OH-DPAT shifts by 72%, while in mice this dose completely blocked shifting. The basis of these strong inhibitory effects of ethanol is unclear, but this action seems contrary to observations that acute ethanol enhances serotonergic activity by increasing release and decreasing uptake of 5-HT (reviewed in Rosenwasser, 2001; LeMarquand et al., 1994). A possible explanation for this inhibition of 8-OH-DPAT shifting is that an ethanol-induced enhancement of serotonergic activity causes a rapid down-regulation of 5-HT receptors as reported in vitro (Prosser and Glass, 2009). In our in vivo systemic trials, ethanol was administered 30 min prior to 8-OH-DPAT injection, which (according to the present pharmacokinetic analyses) could have down-regulated 5-HT receptors prior to 8-OH-DPAT entry to the brain, thus attenuating its phase-advancing response. It also must be noted that the inhibition of 8-OH-DPAT-induced phase-advances by i.p. ethanol treatment contrasts with our earlier studies in the mouse SCN slice where ethanol potentiated, rather than inhibited, 8-OH-DPAT phase-advance shifting (Prosser et al., 2008). It was suggested that the potentiated in vitro (~30%) shifting was due to ethanol's inhibition of glutamate signaling, since glutamate agonists inhibit serotonergic phase-resetting in the SCN (Prosser, 2001).
Why such an effect was not evident *in vivo* is not clear, but could relate to the heightened sensitivity/responses characteristic of the deafferented slice preparation.

In addition to assessing the actions of ethanol on non-photic phase-resetting, a trial involving the assessment of Fos expression was undertaken to determine if acute ethanol may affect cellular activity in the SCN during midday when spontaneous neuronal activity is high. Injection of ethanol (2 g/kg) at midday did not significantly affect the number of Fos-immunostained nuclei, indicating that ethanol neither increased nor decreased overall basal levels of neuronal activity in the SCN at this time. This result is in general agreement with those of other studies on ethanol effects on hypothalamic Fos expression, where there was little overall effect of ethanol in anterior and lateral hypothalamic areas, although such effects of ethanol were highly region- and dose-dependent (Bachtell et al., 2000; Ryabinin et al., 2000). With regard to photic phase-resetting effects of ethanol, it will be important in future experiments to determine if light-induced Fos expression in the SCN, used a marker for the activation of the photic signaling cascade (Kornhauser et al., 1990; Glass et al., 1994), is inhibited by ethanol. This would constitute additional evidence for an inhibitory action of ethanol registered in the signaling cascade.

*SCN ethanol pharmacokinetics.* Brain microdialysis assessment of the pharmacokinetic profile of ethanol in the SCN following i.p. injection was undertaken to verify that the timing of phase-resetting treatments coincided with sufficiently raised ethanol levels. It was also of interest to compare the pharmacokinetics of
acute ethanol with that of other species. Similar to our previous measurements in the hamster (Ruby et al., 2009a), peak levels associated with a 2 g/kg dose of ethanol occurred within 20-40 min of injection (hamster, 50 mM; mouse 58 mM). In the mouse, however, the half-life for clearance was shorter than that of the hamster (1.8 hr vs. 3.0 hr, respectively). These measurements confirmed that the administration of the light and 8-OH-DPAT treatments 30 min following ethanol injection would have coincided with near-maximal ethanol levels. Consistent with our previous report (Ruby et al., 2009a), the pharmacokinetic profiles of acute ethanol in the mouse and hamster SCN is similar to that observed in the rat nucleus accumbens (Yan, 1999), striatum (Job et al., 2003) following i.p. injection of 2 g/kg ethanol, with peak levels (~60 mM) occurring 15-30 min post-injection. The half-life for clearance in these reports was 1.5 to 3.0 hrs, which is also similar to the present assessments. It is also notable from the present analyses of multiple doses of ethanol that the estimated SCN concentration was about 3 times higher at 2.0 g/kg compared to that at 1.0 g/kg. We believe that this is attributable to the physiological effects of the different doses of i.p. ethanol. The lower doses (0.5 and 1.0 g/kg) caused behavioral excitation for ~1 hr, while the higher dose (2.0 g/kg) caused sedation and possible hypothermia for ~2 hr. It is thus plausible that the clearance/metabolism of the higher ethanol dose could be slowed by sedation, causing disproportionately higher peak levels and prolonged half-life compared to the lower doses.

In conclusion, the present study is the first to confirm that acute ethanol dose-dependently attenuates photic phase-delay shifts and serotonergic phase-advance
shifts in the mouse. This dual effect theoretically could disrupt photic and non-photic entrainment mechanisms governing circadian clock timing. It is also significant that the SCN clock is a direct target for disruptive effects of ethanol on photic shifting. This suggests that ethanol could impair the activity of multiple transmitter systems and sites involved in circadian timing regulation. Such diversity of action by ethanol could underlie the disruptive effects of alcohol abuse on behavioral, physiological and endocrine rhythms associated with alcoholism.

**Environmental Modulation of Ethanol Intake and Craving**

*Relapse Elevates Ethanol Intake*

It is evident from the present results that ethanol re-introduction following an extended period of ethanol withdrawal in C57-black mice is characterized by exacerbated ethanol intake and craving compared to that found during ethanol introduction. Most notably, elevations in ethanol intake and preference during ethanol re-introduction were most extensive in the C57-black mice consuming the lower (10%) concentrated ethanol solution. Collectively, the relapse-induced increase in ethanol intake and preference at all ethanol concentrations in the present results is in general agreement with the results of Spanagel et al. 1996, wherein this phenomenon was referred to as an ethanol deprivation effect. The present study also found elevated ethanol intake and preference during consumption of the higher 15% concentrated ethanol solution compared to the lower 10% concentrated ethanol
solution across an ethanol introductory period. This effect is also consistent with previous reports (reviewed in Hiller-Sturmhofel and Kulkosky, 2001). Despite these consistencies, it is possible that the ethanol deprivation effect observed in the present results is strain-specific as an ethanol deprivation effect was not found in another wild-type mouse strain (B6-albino bred on a C57BL background) exposed to an identical experimental protocol (Brager et al. 2011, submitted). Neurobiologically, it is thought that elevated ethanol intake and craving following alcohol relapse is manifest from long-term alterations in glutamatergic signaling systems during chronic ethanol intake and its withdrawal (reviewed in Dodd et al. 2000; Krystal et al. 2003). Thus, it is possible that the severity of the ethanol deprivation effect observed in the present results is due to ethanol-induced alterations in glutamatergic signaling cascades that led to elevated ethanol intake and craving.

Surprisingly, free-choice consumption of ethanol and its withdrawal in the present results in C57-black mice had limited effects on (photic) circadian entrainment capacities despite comparable levels of ethanol intake to that reported in the C57-black mice of Brager et al. 2010, wherein forced ethanol intake severely disrupted photic (and nonphotic) circadian entrainment (Brager et al. 2010). This discrepancy warrants further investigation as there may be method-dependent differences in the latency to the development of ethanol tolerance. Though studies of ethanol tolerance effects on in vivo circadian locomotor behavior are sparse, it has been observed in in vitro studies that the SCN circadian pacemaker exhibits
tolerance to the disruptive effects of ethanol on glutamatergic and serotoninergic signaling (Prosser et al. 2009).

Constant Light Exposure Increases Ethanol Intake and Preference

Environmental challenges have modulatory effects on ethanol intake and seeking in rodent models of alcoholism, but the results of these studies are in conflict and are poorly understood (Gauvin et al. 1997; Rosenwasser et al. 2010; Clark et al. 2007; Hammer et al. 2010; Goodwin et al. 1999). In addition, few studies have investigated the effects of transient and long-term photocycle manipulations on the extent of ethanol intake and preference in wild-type and mutant mice, which are useful models of alcoholism due to their moderate and high levels of ethanol intake and preference, respectively (reviewed in Belknap et al. 1993; Crabbe et al. 2006), although one study has found that minor changes to the length of light and dark photocycle phases in wild-type mice has minimal effects on ethanol intake and preference in wild-type mice (Trujillo et al. 2010). Moreover, this study provides the first assessment of the modulatory effects of long-term constant light, which mimics photocycle conditions found in industrial and medical occupations, on ethanol intake and preference. This study also found perturbations in circadian rhythmicity manifest from the photocycle manipulations that likely contributed to altered ethanol intake.

In rat and hamster, it has been observed that long-term housing under constant light and voluntary access to an ethanol solution has a suppressive effect on ethanol intake and preference (Hammer et al. 2010; Goodwin et al. 1999). Hence, the results of the present study are in disagreement with those of previous
studies such that long-term housing under constant light and voluntary access to an ethanol solution had a stimulatory effect on ethanol intake and preference in wild-types. The discrepancy in results may possibly be a consequence of species-specific differences in endogenous circadian timekeeping that have been found in rodents subjected to free-choice ethanol under constant light. For example, in the rat and hamster studies, a decrease in ethanol intake and preference under constant light was concurrent with a biphasic rhythm of sustained locomotor activity across a 24 hr period that is known as “splitting” (Hammer et al. 2010; Goodwin et al. 2009). Splitting has also been documented in rats and hamsters not subjected to free-choice ethanol exposure under long-term constant light (Cheung and McCormack, 1983; Pickard and Turek, 1982). In this study, wild-type mice did not exhibit splitting, but rather had a locomotor rhythm period that was longer than 24 hrs, and similar to that found in previous studies in wild-type mice housed under long-term, constant lighting conditions (see Spoelstra and Daan, 2008). Hence, it is possible that rhythm splitting under constant light offers protection against rampant ethanol intake, and therefore, warrants further investigation.

In the present results, the increase in ethanol intake and preference in wild-type mice under constant light may also be a partial consequence of an LL-induced loss of robust circadian timekeeping across the 24 hr day period reflected through an increase in the number of activity bouts reduced in duration under LL compared to LD. In humans, persistent disruptions to sleep/wake manifest from photic environmental challenges, such as residence in polar latitudes where there is near
year-long exposure to constant conditions, increase self-medication with ethanol (Trinkoff and Storr, 1998; Smart, 1979; Levine et al. 1994; reviewed in Brower, 2001; and Roehrs and Roth, 2001). In rodent models of alcoholism, the stimulatory effects of circadian disruption on ethanol intake and preference are less conclusive (see Rosenwasser et al. 2010; Clark et al. 2007; Gauvin et al. 1997). Despite this gap in knowledge, the stimulatory actions of LL-induced circadian disruption on ethanol intake and preference that were found in the present results lend credence to hypotheses supporting a regulatory influence of behavioral output of the SCN circadian timing system on the extent of ethanol intake and craving. Thus, a functional circadian timing system may limit alcohol craving and dependence, whereas an aberrant circadian timing system may lead to full-blown alcoholism.

**Circadian and Acamprosate Modulation of Elevated Ethanol Intake in mPer2 Deficient Clock Gene Mice**

The neurologic systems involved in alcoholism are reciprocally linked to those regulating circadian clock timing. For example, there is growing evidence that circadian clock genes have substantial regulatory influence over ethanol intake and preference. Notably, disruption of one of these genes, mPer2, exacerbates the risk of alcohol dependence in humans and in rodent models of alcoholism (reviewed in Sack et al. 2007; Spanagel et al. 2005). For these reasons, the mPer2 mutant mouse presents a functional animal model for studying factors contributing to alcohol
abuse, as well as the effects of disrupted circadian behavioral rhythms on ethanol intake. This highly alcohol-preferring mouse also offers a propitious model for researching pharmacologic interventions for the treatment of alcoholism. In the present experiments, the mPer2 mutant mouse was used to examine behavioral aspects of an alcohol-preferring phenotype. This mouse was also used to study the actions of acamprosate, a drug used to prevent relapse in recovering alcoholics, on the circadian pattern and amount of alcohol intake. Results from these experiments confirmed that mPer2 mutants have potentiated ethanol intake relative to wild-types, which was associated with increased numbers of drinking bouts over the 24 hr circadian day, and notably with a 2 hr advance in activity onset associated with the mPer2 phenotype. It was also found that a daily regimen of i.p. acamprosate injections substantially suppressed ethanol intake by reducing the number of daily ethanol drinking bouts in mPer2 mutant and wild-type mice without altering the circadian pattern of drinking.

Circadian drinking profiles of mPer2 mutant and wild-type mice. Studies focusing on the genetic influences on alcohol dependence have revealed that circadian gene mutations profoundly affect ethanol intake. For example, in mice, mutation of the circadian gene Clock enhances ethanol intake and preference (Ozbourn et al. 2010). Likewise, mutation of the mPer2 gene causes marked increases in these parameters, which are linked to alterations in glutamatergic transmission (Spanagel et al. 2005). In drosophila, mutations for Per and Tim each reduce tolerance to
repeated application of ethanol (Pohl et al. 2010). Notably from a clinical perspective, single-nucleotide polymorphisms of \textit{mPer2} and \textit{CLOCK} in humans are associated with increased familial risk of alcoholism (Spanagel et al. 2005; Sjöholm et al. 2010). From these studies it is inferred that a normally functioning circadian timing system limits ethanol use and dependence, and that perturbations of this system promote alcohol abuse. The present demonstrations of potentiated ethanol intake and preference in \textit{mPer2} mutant mice lend credence to this idea, and reinforce the original findings of Spanagel et al. 2005 who reported increased levels of ethanol drinking in \textit{mPer2} mutants vs. wild-type controls. The present circadian behavioral analyses also extend these findings through the demonstration that the enhanced ethanol intake in \textit{mPer2} mutant mice is associated with disturbed circadian behavior: namely a ~2 hr advance in the onset of nocturnal locomotor activity (positive phase-angle of circadian entrainment), with a corresponding increase in duration of the activity/wakefulness period (alpha). Our data confirm that this advanced phase of behavioral activity was associated with an increased number of drinking bouts not exhibited by wild-types. There also was an increased number of drinking bouts during the dark-phase in the \textit{mPer2} mutants, which is the predominate circadian phase of ethanol intake in nocturnal rodents (Brager et al. 2010; Ruby et al. 2009b; reviewed in Hiller-Sturmhöfel and Kulkosky. 2001). The \textit{mPer2} mutants also exhibited more drinking episodes throughout the day compared to wild-types, whose ethanol drinking primarily occurred near light-dark transitions.
Effects of mPer2 mutation and acamprosate on ethanol pharmacokinetics. This study represents the first characterization of the 24 hr profile of systemic ethanol assessed using subcutaneous microdialysis concomitantly with circadian drinkometer measurements in mPer2 mutant mice. Subcutaneous, rather than blood sampling of ethanol was used as it is less invasive, and follows a similar time-course (Engleman et al. 2008). Several points are evident from this experiment. First, ethanol drinking bouts preceded systemic ethanol peaks by ~30min. This relationship is similar to that reported previously in mice and hamsters (Brager et al. 2010; Ruby et al. 2009b). Second, the effect of multiple closely-spaced drinking bouts is cumulative, resulting in prolonged elevations of peripheral levels of ethanol. Third, the profiles of drinking bouts and systemic ethanol peaks differ greatly between mPer2 mutants and wild-types, with the mutants exhibiting a 2-fold greater frequency of drinking and markedly higher systemic peak levels of ethanol. Both parameters are reflective of the mPer2 mutants’ avidity for ethanol.

The six-day daily acamprosate treatment regimen significantly reduced overall daily ethanol intake in mPer2 mutants and wild-types, with saline treatment having no effect. Similar to previous findings, acamprosate treatment reduced mPer2 mutant ethanol intake to that of untreated wild-type mice. Acamprosate also reduced enhanced ethanol intake in C57BL mice subjected to the drinking-in-the-dark protocol (Gupta et al. 2008). Our combined drinkometer and microdialysis assessments revealed that acamprosate reduced daily total drinking bouts and
systemic ethanol peaks without altering the general circadian pattern of ethanol drinking. In agreement with other studies (see Spanagel et al. 2005), we also found that systemic acamprosate treatment suppressed ethanol preference in both mPer2 mutant and wild-type mice, although higher values of ethanol preference were still seen in the mPer2 mutants vs. wild-types at the end of the regimen of acamprosate treatment. It is thought that acamprosate’s attenuation of ethanol craving is due to its suppressive action on hyper-glutamatergic signaling associated with alcohol abuse (reviewed in Spanagel and Kiefer, 2008; Krystal et al. 2003; Dodd et al. 2000). This hyper-glutamatergic state observed in mPer2 mutants is attributable to reduced glutamate reuptake by its transporter (EAAT1; Beaulé et al. 2009). Notably, microdialysis measurements of glutamate levels in the striatum have revealed that a twice daily regimen of i.p. acamprosate injection reduces glutamate levels and subsequent ethanol craving in mPer2 mutant mice to wild-type levels (Spanagel et al. 2005). It was also shown that this treatment likewise reduces glutamate levels and ethanol craving in wild-types.

**Conclusions and Significance.** These experiments represent the first assessments of the behavioral/drinking chronotype of mPer2 mutant mice. These experiments point to a possible causative relationship between mPer2 disruption, impaired circadian behavioral activity, and elevated ethanol intake. Additionally, we have extended previous acamprosate studies in mPer2 mutant mice by showing that acamprosate decreases ethanol intake by reducing the number of daily ethanol
drinking bouts and subsequent systemic tissue ethanol peaks without altering the general daily pattern of ethanol drinking. The mPer2 mutant mouse, studied using conventional circadian behavioral analyses, offers a new approach for understanding how behavioral disruption contributes to alcohol abuse, as well as for exploring the ameliorative actions of ethanol suppressing drugs like acamprosate.

**Brain Targets for Acamprosate Suppression of Ethanol Intake and Preference**

Acamprosate has been used clinically since 1989 as a pharmacologic agent to help promote abstinence in alcohol dependent patients. In the majority of clinical trials, this drug was shown to have a statistically significant effect over placebo in controlling relapse, at least during the initial period of abstinence (reviewed in Kranzler and Gage, 2008). In rodents, acamprosate’s overall suppressive effect on drinking is consistent across studies, despite wide differences in drug and/or ethanol treatment regimens. For example, in C57 mice subjected to the drinking-in-the-dark protocol to enhance nighttime ethanol intake, systemic administration of acamprosate (300 mg/kg, i.p.) suppresses nighttime ethanol intake by 20% (Gupta et al. 2008). Also in C57 mice, acamprosate (300 mg/kg, i.p.) reduces overall daily ethanol intake under free-choice by up to 70% (Brager et al. 2011b, in press). In ethanol-preferring clock gene (PER2) mutant mice, acamprosate (200 mg/kg and 300 mg/kg, i.p.) suppresses free-choice ethanol intake by 60-77% (Spanagel et al. 2005; Brager et al. 2011b, in press). In ethanol-preferring rats, acampsrosate (200
mg/kg, i.p.) suppresses ethanol intake by 20-40% (Chau et al., 2010a; Stromberg et al., 2001). Despite these observations, little is known about the brain site(s) of acamprosate’s suppressive actions on drinking and craving. As shown here, brain stimulation with acamprosate-containing microimplants in selected reward and circadian areas of C57 WT and PER2-mutant mice reduces free-choice ethanol intake during induced relapse by 40-50%. The commonality of effects of acamprosate observed at the behavioral level between humans and rodents suggests that defining the central sites and neurophysiological actions of this drug using basic animal studies has direct implications for understanding the nature of its clinical action in humans.

Acamprosate Implants in Reward Areas Suppresses Ethanol Intake and Preference

The neuroanatomy that modulates repetitious ethanol seeking and intake in humans and animal models has been elucidated, and includes the ventral tegmental area [VTA] and nucleus accumbens [NAc] of the mesolimbic system and the pedunculopontine tegmentum [PPT] of the mesopontine system, which were targeted with acamprosate implants in this study (reviewed in Koob et al. 1998; Wang et al. 2000; Volkow et al. 2008). The ventral striatum and amygdala of the mesolimbic system, which project onto the VTA, NAc, and PPT, also gate ethanol seeking and consumption (reviewed in Koob et al. 1998; Johansson et al. 2000; Hyytiä et al. 1995; Roberts et al. 1996). Further, in rodent models of alcoholism, lesioning or pharmacologic manipulation of the VTA (Gessa et al. 1985; Ericson et al. 1998), the
NAc (Dhafer et al. 2009; Chaudhri et al. 2008), and the PPT (Samson et al. 2001) alter levels of ethanol intake. To date, no brain areas of the mesolimbic and mesopontine reward systems have been targeted with acamprosate to study its effects on ethanol intake and preference.

Hence, these results present the first-time observation of significant and long-term suppression of ethanol intake and preference by constant-release acamprosate implants targeted to multiple reward-processing areas, including the VTA, PPT, NAc, in WT mice. This study also presented the first-time observation of long-term and short-term suppression of ethanol intake and preference to wild-type levels in PER2-mutant mice with acamprosate implants in the VTA and PPT and NAc, respectively. In both WT and PER2-mutant mice, the most pronounced and extensive reward area response to acamprosate’s suppressive actions was found in the ventral tegmental area, in which acamprosate decreased ethanol intake and preference in WT and PER2-mutant mice by 60% over 4 wks and 40% over 3 wks, respectively, from pretreatment levels. Further, the reduction of ethanol intake by 20% from pretreatment levels in WT mice with acamprosate implants in the NAc was considerably less than the suppression observed in the VTA of WT mice. Accumbal acamprosate implants in PER2-mutant mice also suppressed ethanol intake by 20% from pretreatment levels, but this suppression was transient, lasting two days. Finally, acamprosate implants in reward areas of WT and PER2-mutant mice resulted in increased water intake compared to mice with blank implants in reward
areas. Increased water intake in response to intracranial acamprosate treatment suggests that acamprosate, at this particular dose, had limited effects on total fluid intake. Moreover, this response is in agreement with previous studies showing that systemic (i.p.) acamprosate treatment at moderate doses of 300 mg/kg in wild-type mice does not affect total fluid intake (Gupta et al. 2008; Brager and Glass, unpublished results).

Acamprosate Implants: Site Specificity, Release Kinetics and Tolerance

The constant-release micro-implants used here were originally designed to map hypothalamic sites of action of another small molecule, melatonin, in mice (Glass and Lynch, 1981a,b). The in vitro release profile is similar for acamprosate and melatonin, both having a similar molecular mass (181 and 232 g/mol, respectively). The melatonin implants had differential effects within various hypothalamic nuclei over a 7 wk period (Glass and Lynch, 1981a), and autoradiographic analysis of $^3$H-melatonin incorporated into the implants showed that spread of label was ~0.2 mm, indicating site specificity of implant effect. Like melatonin, in vitro release of acamprosate from the implants is asymptotic, with near-constant output of ~50 ng/day/ml incubation medium apparent by day 7. This concentration (0.3 µM) is similar to that in the cerebrospinal fluid of recovering alcoholic patients taking the medication (0.5-1.5 uM; Wilde and Wagstaff, 1997). Given the precise alignment of the profile of acamprosate release measured in vitro with the timeline of ethanol relapse, it is reasonably plausible that stable levels of
acamprosate release from the microimplants in each brain region in vivo coincided with the ethanol re-introduction period, which occurred four days after surgical implantation, and continued over the 4 wks of ethanol re-introduction. Presupposing this profile of acamprosate release, it should also be noted that maximal acamprosate suppression of ethanol intake and preference occurred 2-4 days into the ethanol re-introduction period and from there, gradually increased towards pretreatment levels. Hence, the reduced suppressive effect of acamprosate over time is possibly manifest from tolerance to acamprosate treatment which has been documented previously (Cowen et al. 2005; reviewed Spanagel and Kiefer, 2008).

**Possible Neural Mechanisms of Acamprosate Actions in Reward Areas**

Previous studies have identified the clinically relevant physiologic actions of acamprosate, which, for this study, provide insight on the possible neurobiologic mode(s) of acamprosate action that resulted in the suppression of ethanol intake and craving. First, acamprosate acts on the metabotropic glutamate type 5 receptor (mGlu5; Harris et al. 2002; reviewed in Mann et al. 2008), of which reduces behavioral ethanol withdrawal (Blednov et al. 2008), ameliorates glutamate neurotoxicity, and therefore, can limit the drive to drink (Harris et al. 2002; reviewed in Krystal et al. 2003; Dodd et al. 2000). Acamprosate can also modulate NMDA receptor activity through a weak antagonism and hence, secondarily reduce glutamate (NMDA) neurotoxicity (Naasila et al. 1998; reviewed in Mann et al. 2008).

The density of NMDA receptor expression, however, varies across brain regions
(Allgaier et al. 2000). Further, acamprosate antagonism of NMDA activity is one possible mode of action relevant to this study given that the extent and duration of acamprosate suppression of ethanol intake and preference varied across the targeted reward areas. Thirdly, acamprosate can behave as a GABA\textsubscript{A} receptor agonist and can modulate GABA kinetics in various thalamic and cortical brain areas, including those of the mesolimbic reward circuit (reviewed in Mann et al. 2008; Dauost et al. 1992).

In reward areas, acamprosate has additionally been found to modulate dopamine signaling. For example, previous studies have found that the administration of acamprosate in the NAc increased levels of accumbal extracellular dopamine (Cano-Cebrian et al. 2003; Chau et al. 2010 a,b) and dopamine reuptake transporter (Cowen et al. 2005), and dampened ethanol or NMDA-induced accumbal dopamine release (Cano-Cebrian et al. 2003; Olive, 2002; Spanagel and Weiss, 1999). In the most recent study (Chau et al. 2010b), the acamprosate-induced heightening of accumbal extracellular dopamine levels was concomitant with a 40% reduction in ethanol intake (Chau et al., 2010b). Experimental findings from Chau et al. 2010a have also reported cholinergic modulation of this response. In an animal untreated with cholinergic agents, cholinergic signaling onto the VTA arises from the PPT (Vrang et al. 2003; Morin et al. 2005). Hence, the modulation of dopamine signaling by acamprosate concomitant with cholinergic feedback onto the ventral tegmental area is another possible mechanism through which acamprosate may
have acted in the NAc in this study to suppress ethanol intake and craving. To account for the strain discrepancy in responsiveness to acamprosate implants in the NAc, this could possibly be due to heightened glutamatergic tonus associated with elevated ethanol intake that is found in PER2-mutant mice (Spanagel et al. 2005), which, here, could have attenuated acamprosate suppression of glutamate (NMDA)-induced dopamine release and the subsequent reduction of ethanol intake.

The neurologic actions of acamprosate in the VTA have not been widely studied, but it has been shown that the antagonism of NMDA receptors within the VTA can reduce reward-seeking behaviors (reviewed in Kauer and Malenka, 2007). Thus, this is one possible mode of acamprosate action in the VTA. It is also possible that acamprosate may have acted on GABA-ergic neuronal boutons in the VTA of the WT and PER2-mutant mice. Moreover, acamprosate modulation of GABA signaling in the VTA of PER2-mutant mice would compensate for heightened glutamatergic tonus associated with elevated ethanol intake in PER2-mutants (Spanagel et al. 2005) that possibly could have lead to a null effect of acamprosate implants in the VTA on the long-term suppression of ethanol intake and craving similar to that seen in the NAc.

The neurologic actions of acamprosate in the PPT, which is a brain area enriched with acetylcholine and GABA (Jia et al. 2003), have also not been widely studied. However, given that both WT and PER2-mutant mice with acamprosate implants in the PPT showed a reduction in ethanol intake and craving, it is possible
that acamprosate acted on GABA-ergic expressing neurons in the PPT similar to that which could have taken place in the VTA. Evidence of direct acamprosate modulation of nicotinic cholinergic activity in animal models remains unclear (reviewed in Durbin et al. 1996) although, in humans, acamprosate has been used to treat nicotine cravings (reviewed in Narahashi et al. 2001). Hence, more conclusive evidence showing that acamprosate can modulate cholinergic activity is needed in order to speculate upon acamprosate actions on cholinergic neurons in the PPT and possibly in the VTA as it has recently been shown that the GABA-expressing neuronal boutons of the VTA contain nicotinic receptor subtypes (Yang et al. 2011).

Acamprosate Implants in Circadian Areas Suppresses Ethanol Intake and Preference

In mammals, behavioral and physiologic circadian processes are generated by the SCN of the anterior hypothalamus (reviewed in Moore, 1983; Edgar et al. 1993; DeCoursey et al. 1997), and can be maintained by the integration of environmental stimuli transmitted from neuronal projections of the IGL of the thalamus onto the SCN (Card and Moore, 1982; Johnson et al. 1989; Glass et al. 2003, 2010; Grossman et al. 2004). Recent studies have shown that functioning of the circadian timing system is reciprocally tied to ethanol seeking and consumption. For example, hamster and wild-type mice consume ethanol at specific circadian phases with ethanol intake being the greatest at the beginning and end the nighttime activity period (Brager et al. 2010; 2011b, in press; Ruby et al. 2009b; Dole and
Gentry, 1984; reviewed in Wasielewski and Holloway, 2001). In contrast, mutations of the clock gene PER2 in mice is associated with additional circadian phases of ethanol intake compared to wild-types, which subsequently, increases daily ethanol intake (Brager et al. 2011b, in press). Further, the direct, perfusion of ethanol into the SCN in vivo and the application of ethanol to the SCN slice in vitro perturbs the timing of behavioral and electrophysiologic SCN output, respectively (Brager et al. 2010, 2011a, in press; Ruby et al. 2009a,b; Prosser et al. 2008), while alterations in photocycles, which uncouple the SCN pacemaker from environmental cues, modulate ethanol intake (Hammer et al. 2010; Clark et al. 2007; Brager and Glass, unpublished results). Despite these observations, there is an absence of research showing that direct, pharmacologic manipulation of the circadian system with drugs used to clinically treat alcoholism, such as acamprosate, have an effect on ethanol intake and preference.

Moreover, this study is the first-time observation of the marked and long-term suppressive actions of acamprosate implants in the SCN and IGL on ethanol intake and preference. This study also presented the first-time observation of long-term and short-term suppression of ethanol intake and preference in PER2-mutant mice with acamprosate implants in the SCN and IGL, respectively. Hence, in both WT and PER2-mutant mice with acamprosate implants in the SCN, acamprosate decreased ethanol intake and preference in WT and PER2-mutant mice by ~60% over 3 wks from pretreatment levels. During this time, water intake in WT and PER2-mutant
mice with acamprosate implants in circadian areas was elevated compared to mice
with blank implants in circadian areas, suggesting, again, that acamprosate had
limited effects on total fluid intake.

Most notably, the sole targeted brain area that acamprosate did not act to
suppress drinking and preference in both WT and PER2-mutant mice was the
hippocampus. Previous studies have reported that the hippocampus mediates the
re-instatement of alcohol seeking via operant conditioning and that such seeking can
be prevented by the activation of metabotropic glutamate receptors, of which
acamprosate can bind (Ryabinin et al. 2003), to suppress drinking and craving (Zhao
et al. 2006). Additionally, rhythmic expression of the circadian clock genes PER1
and PER2 has been found in the hippocampus and appears to be important for
determining the circadian phase of activity in diurnal rodents (Ramanathan et al.
2010). Hence, despite these findings of hippocampal regulation of reward- and
circadian-related neurobiology and behavior, further investigation is warranted as to
why acamprosate implants in the hippocampus did not have a suppressive effect on
ethanol intake and preference in this study.

In order to speculate on the possible neurobiologic mode(s) of acamprosate
action in the targeted circadian areas, previous studies have found that GABA
kinetics in the thalamus are significantly altered by acamprosate (Dauost et al. 1992)
and that acamprosate acts on both mGlu5 and NMDA receptors as noted earlier in
the discussion (Harris et al. 2002; Allgaier et al. 2000). Hence, these modes of
Acamprosate's physiologic actions are relevant to this study because both the IGL and SCN contain GABA-immunoreactive neurons (Moore et al. 1993), mGlu5 receptors (Stamp et al. 1997), and NMDA receptors (Blasiak et al. 2007; Hartgraves et al. 1994) and therefore, could have provided for multiple modes of acamprosate action in these circadian brain areas.

**Interactions Between Reward and Circadian Behavioral and Physiologic Brain Systems**

Recent studies have identified critical interactions between reward and circadian neurobiological and behavioral systems. First, the PPT of the mesopontine reward system has a direct, projection onto the circadian timing system at the level of the IGL (Vrang et al. 2003; Morin et al. 2005; Horowitz et al. 2004), and the SCN has an indirect projection onto the VTA of the mesolimbic reward system via the medial preoptic nucleus of the hypothalamus (Luo and Aston-Jones, 2008). Electrostimulation of the VTA and PPT can also adjust behavioral circadian timing in hamsters (Guinn et al. 2010). Third, the seeking of a natural or psychoactive reward at a specific circadian-phase is driven by circadian oscillations in dopamine expression within the mesolimbic reward system (Webb et al., 2009). Psychoactive drugs, such as methamphetamine, opiates, and ethanol, can also modulate circadian rhythms of wheel running (Kosobud et al., 2007; Tartaroglu et al., 2009). Further, mutations of the circadian clock genes, PER2 and CLOCK, are linked to aberrant glutamate and dopamine neurotransmission in the striatum and VTA of the...
mesolimbic reward system, respectively, which, over time, can lead to an increase in the seeking of ethanol (Spanagel et al. 2005) or cocaine (McClung et al. 2005).

Moreover, the results of this study are in agreement with these previous studies that have found crosstalk between reward and circadian neurobiological and behavioral systems, and lend further credence to this developing hypothesis. First, it was observed that acamprosate implants in reward or circadian brain areas resulted in significant and long-term suppression of ethanol intake and preference during ethanol re-introduction. Second, the 1.9- and 1.3-fold greater difference in ethanol intake and preference, respectively, in PER2-mutant mice compared to WT mice prior to and during acamprosate treatment is possibly a partial consequence of an earlier nighttime activity onset of 2.2 hr and subsequent 1.7 hr extension of wakefulness across the subjective night. Further, the differences in ethanol intake, ethanol preference, and circadian behavior found in PER2-mutant mice compared to WT mice in this study were identical to those observed in previous studies (Brager et al. 2011b, in press; Spanagel et al. 2005). In these studies, the behavioral and neurochemical mechanisms underlying elevated ethanol intake in PER2-mutant mice is thought to be a consequence of an advanced phase angle of entrainment that is concomitant with prolonged and high levels of ethanol intake (Brager et al. 2011b, in press) and elevated glutamatergic tonus in the ventral striatum (Spanagel et al. 2005), which can further increase the drive to drink (reviewed in Dodd et al. 2000; Krystal et al. 2003). Although actographic and microdialysis assessments of
ethanol intake and systemic ethanol chronopharmacokinetics were not undertaken in PER2-mutant and WT mice in this study, it is anticipated that a robust circadian pattern of ethanol drinking in the PER2-mutant and WT mice would align with their recorded circadian behavior and would be comparable to that found in Brager et al. 2011.

Finally, in this study, it was shown that acamprosate implants in the VTA and NAc of the mesolimbic reward system significantly increased the amplitude of nighttime activity in WT mice, but had no effect in PER2-mutants. An increase in the amplitude of nighttime activity was also found in WT and PER2-mutant mice during acamprosate release in the SCN. Most notably, this increase in circadian amplitude occurred independently of a lack of an effect of acamprosate on the phase angle of behavioral entrainment and alpha. Implants into reward and circadian areas also did not lead to arrhythmia across any circadian phase, which indicates that the micropellet implants did not damage pacemaker cells driving behavioral circadian rhythmicity. Previous studies of acamprosate’s locomotor stimulating effects have found that systemic acamprosate treatment can increase wheel running activity in rats housed under constant light for up to 3 hrs after injection (Gillman, 2010), but there is little data available on acamprosate’s locomotor modulating effects in WT and PER2-mutant mice. Hence, further investigation is warranted to elucidate the underlying neurobiological and behavioral mechanisms that lead to the brain area-
and strain-specific, acamprosate-induced increase in the amplitude of nighttime circadian activity that was found in this study.

**Conclusion and Significance**

In summary, this study presents the first-time observation of brain sites of acamprosate action that resulted in significant and long-term suppression of ethanol intake and preference. Most notably, this study has delineated critical reward and circadian brain areas sensitive to acamprosate and showed modulation of this response by mutations of the PER2 clock gene. Moreover, targeting of these reward and circadian areas with acamprosate suggests that acamprosate's effects are mediated by affecting reward and/or circadian regulatory areas, both of which are implicated in alcohol dependence. In addition, the more modest and less prolonged suppressive effect of acamprosate implants on ethanol drinking and preference in the PER2-mutants compared to WTs suggests that the mutant’s innate drive to drink makes them less susceptible to acamprosate’s protective action. In alcoholics, this could possibly be a factor in determining the degree of clinical efficacy of acamprosate treatment.
Experiments 1-10 provide clear evidence of the disruptive, inhibitory actions of acute and chronic ethanol use on photic phase-delaying of behavioral rhythms and nocturnal locomotor activity. Using reverse-microdialysis, it was determined that ethanol acts directly within the SCN to exert these inhibitory behavioral effects. The inhibitory actions of ethanol on serotonergic phase-resetting, however, are less conclusive: 1) Only a high ethanol dose (2.0 g/kg) attenuated serotonergic phase-advancing of behavioral circadian rhythms; 2) Chronic ethanol intake had no suppressive effect on serotonergic phase-advancing; and 3) A reverse-microdialysis ethanol perfusion into the SCN did not depress serotonergic phase-advances, suggesting that the observed inhibitory actions of the 2.0 g/kg ethanol dose may involve secondary extra-SCN brain sites.

Circadian environmental and genetic contributions to alcohol dependence and alcoholism were evaluated in Experiments 12-15 by means of implementing long-term exposure to a physiologically-stressful photocycle (constant light) and using mice with homozygous deletions of the oscillating clock gene, Per2, which drives robust SCN pacemaking. The observed potentiation of ethanol intake in these PER2-mutant mice behaviorally manifested from a 2 hr advance in the nocturnal activity period and subsequent extension of wakefulness. This advanced chronotype provided PER2-mutant with additional nocturnal drinking opportunities, as confirmed through actigraphy and systemic ethanol chronopharmacokinetics; ethanol drinking
in the PER2-mutant mice began immediately and continued throughout their extended nocturnal activity period, and was concomitant with high levels of ethanol absorption within subcutaneous tissue. Overall, this nocturnal drinking rhythm in PER2-mutant mice resulted in more ethanol drinking episodes across this nocturnal active period and 24 hr circadian day relative to matched wild-type controls.

The efficacy of acamprosate in rescuing high ethanol intake and preference in PER2-mutant and wild-type in Experiments 14-15 was variable. A daily regimen of systemic acamprosate treatment effectively reduced ethanol intake and preference in both PER2-mutant and wild-type mice without affecting the circadian rhythm of ethanol intake, while the implantation of constant-release acamprosate into circadian and reward brain sites had differential suppressive actions on ethanol intake and preference in PER2-mutant and wild-type mice; acamprosate’s rescuing effects were more prolonged in the wild-types compared to the PER2-mutant mice, and higher levels of ethanol intake and craving were maintained in the PER2-mutant mice across acamprosate treatment.

Moreover, the disruptive, inhibitory actions of ethanol on the murine circadian pacemaker and subsequent behavioral output mirrors reports of ethanol disruption to circadian-controlled physiological processes in humans; these disruptions include increased amplitudes of REM sleep and cortisol release, and a depressed amplitude and premature cessation of melatonin release, all of which largely predict the likelihood of relapse in abstinent alcoholics (reviewed in Roehrs and Roth, 2001; Wasielewski and Holloway, 2001; and Brower, 2001). These residual actions of
ethanol on the mammalian circadian pacemaker and subsequent behavioral output exacerbate alcohol craving, withdrawal, and dependence, and, over time, can lead to alcoholism. This downward spiral towards alcoholism is further potentiated by environmental, metabolic, and genetic circadian disruptions, as demonstrated here in the PER2-mutant mice. Thirdly, while the pharmacological agent, acamprosate, is currently available for the treatment of alcohol dependence and the prevention of relapse, it is only moderately effective in humans due to poor transport across the blood brain barrier and drug tolerance (reviewed in Spanagel et al., 2008). In comparison, the following experiments suggest that the suppressive actions of acamprosate on ethanol intake and craving largely differ between circadian and reward brain sites and strains. Therefore, pharmacological improvements in acamprosate transport across the brain and enhanced screening of genetic polymorphisms may greatly increase the efficacy of acamprosate in curbing alcohol craving and preventing relapse.

*Is in vivo inhibition of behavioral circadian output ethanol-specific?* The *in vivo* experiments and complimentary *in vitro* experiments of Prosser et al. 2008; 2009; McElroy et al. 2009 provide convincing evidence that ethanol acts directly within the mammalian circadian pacemaker to inhibit photic signaling and differentially alter serotonergic signaling. Whether or not other drugs of abuse similarly affect pacemaker activity and subsequent behavioral output is less conclusive. Recently, intracerebroventricular infusions of an cannabinoid agonist comparable to marijuana
has been found to attenuate photic phase-delays in mice. This attenuation was partially reversed by the co-infusion of a cannabinoid antagonist (Acuna-Goycolea et al. 2010). In addition, these researchers observed an abundance of cannabinoid receptors in the dorsolateral regions of the SCN that reduce GABA release onto SCN pacemaker cells (Acuna-Coycolea et al., 2010). Therefore, cannabinoid alteration of presynaptic GABA release in SCN pacemaker cells is the possible underlying mechanism of cannabinoid inhibition of photic phase-delays (Acuna-Coycolea et al., 2010). Likewise, acute systemic administrations of cocaine (20 mg/kg) in wild-type and PER2-mutant mice have been shown to potentiate small, serotonergic phase-advances, attenuate photic phase-delays, and have long-term effects on the temporal structure of circadian activity (Glass, Brager et al. 2011, in preparation). This preliminary evidence of marijuana’s and cocaine’s differential effects on SCN pacemaker activity and photic/serotonergic phase-resetting suggests that in vivo inhibition of behavioral circadian output is not ethanol-specific. Additional in vitro experiments that entail application of cocaine and other drugs of abuse to the SCN slice and in vivo experiments that employ reverse-microdialysis SCN perfusions will confirm this hypothesis.

Future Directions. While there is strong evidence that ethanol acts directly within the mammalian circadian pacemaker to inhibit in vivo photic phase-resetting of behavioral circadian rhythms in mouse and hamster (Brager et al. 2010, 2011, in press; Ruby et al. 2009a,b), the actions of acute and chronic ethanol on serotonergic
phase-advances, and the actions of ethanol tolerance on photic and nonphotic entrainment are less conclusive. To address whether ethanol acts directly within the SCN and/or extra-SCN brain regions to inhibit serotonergic phase-advances, reverse-microdialysis perfusions similar to those executed in Experiment 9 and targeted to extra-SCN brain regions such as the DRN and IGL, both of which are responsive to 8-OH-DPAT-induced phase-advances (Mintz et al., 1999; Challet et al., 1998), can be utilized. To address issues of ethanol tolerance and how it may have masked differences in days to re-entrainment between ethanol or water drinking mice in Experiment 4, the following experiments can be conducted: 1) re-analyzing nocturnal activity intensities presented in Tables 1-4 for charting day-to-day differences between ethanol and water-treated mice, and possibly finding a day where nocturnal activity intensity does not differ between the two groups; 2) gradually introducing the skeleton photoperiod earlier than 2 wks because it may be that ethanol tolerance has already commenced; and 3) similarly, assessing whether an earlier presentation of a 30 min phase-delaying light pulse in the subjective night in Experiment 3 further potentiates ethanol inhibition of photic phase-delays.

To further elucidate circadian contributions to alcohol dependence and alcoholism as studied in Experiments 13-15, environmental and genetic influences need to be considered. That is, PER2-mutant mice and their matched wild-type controls need to undergo short and long-term environmental disruption, such as partial sleep deprivation (carried out by gentle handling and timed air puffs for 3 hrs during the subjective day) and rotating shift work, that is reminiscent of human
populations with high reports of alcohol abuse (Trinkoff and Storr, 2006; Smart, 1979). In addition, to further understand the pharmacological and suppressive actions of acamprosate on ethanol intake, *in vitro* electrophysiological studies with acamprosate application to the targeted brain sites need to be executed. Moreover, these former and future experiments elucidating the reciprocal actions of ethanol on the neural regulation of sleep and circadian timing and the environmental and genetic (circadian) contributions to alcohol dependence will most certainly help reduce rates of alcohol dependence and improve treatment strategies for abstinent alcoholics.


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